REVIEW ARTICLE

RECENT ADVANCES IN BIOLOGICAL STANDARDISATION

With Particular Reference to the Assays of Digitalis and Posterior Lobe Pituitary Extracts

By G. F. Somers, Ph.D., B.Sc., Ph.C.

Lecturer in Pharmacology, The School of Pharmacy, University of London

General Principles

The most notable advances in the biological standardisation of drugs, hormones and therapeutic substances during the past 20 years have been in the development of experimental procedures and assay techniques suitable for statistical analysis. This has been largely due to the recognition of the growing importance of statistics and to a close collaboration between the assayist and the statistician. The development of bioassays on fundamentally sound principles began with the establishment of a standard reference preparation by Ehrlich as long ago as 1897. However, laboratory workers were slow in adopting this principle and for many years afterwards potency continued to be defined in animal units. Such units were extremely variable and agreement between different laboratories was impossible. Due to the pioneer work of Dale, Hartley, Burn, Gaddum and Trevan bioassays were gradually established on scientifically sound foundations and biological standards adopted on an international basis. Thus a uniformity in the potency of therapeutic substances was ensured throughout the world and, e.g., a diabetic person receiving insulin in the western hemisphere can be assured of receiving the correct dosage in, say, Bombay. Reviews on the establishment, properties and uses of international standards have been published by Dale,[1] by Hartley[2] and by Miles.[3,4]

Much of the progress in biological standardisation has been due to a recognition of the principles of experimental design and a realisation of the need for a strict statistical control. Thus in the 2nd edition of "Biological Standardisation" by Burn *et al.*[5] statistical methods have taken pride of place. This strongly underlines the function of the statistician not only in analysing and assessing the precision of the data obtained but also in the planning of the experimental procedures prior to the assays themselves. Among important contributors to the development of statistical methods are Bliss, De Beer, Knudson and Miller in the United States and Burn, Fieller, Finney, Gaddum, Gridgeman, Irwin, Trevan and Wood in this country. A recent review on the statistical foundation of biological assays has been published by Finney,[6] and books by Emmens,[7,8] Burn *et al.*,[5] Coward[9] and Finney[10] describe the use of statistical methods in bioassay.

The general principles to which any biological standardisation must conform have been defined by Dale,[1] and further characterised by

Gaddum,[11] and by Bliss.[12] These have been summarised by Emmens[13] as follows:—

(1) A standard reference preparation must be used simultaneously with the preparation under test.

(2) The assay must provide a valid, unbiased estimate of the potency of the preparation under test and of the limits of error of this estimate at any required probability.

(3) The assay must provide evidence that the actions of the preparation under test and of the standard preparation do not differ.

(4) The most accurate test method will be that for which the quantity s/b is minimal; where s is the standard deviation of an individual result and b is the slope of the dose-response line.

(5) The living material receiving each dose of the standard and unknown must be as uniformly distributed among such dosage groups as is possible. Potential sources of variation such as differences in response between litter-mates, sexes or strains of animal, must be so allocated to dosage groups that their influence can be isolated and examined in the subsequent statistical analysis.

The Choice of an Assay Method

The choice of an assay method is largely influenced by factors such as the time, cost, materials and labour involved. These are of primary importance in the commercial production of drugs and therapeutic substances. Thus the rabbit assay of insulin is to-day largely obsolete and the mouse method is preferred because of its rapidity and simplicity. The method used should be specific, but when the activity of the standard and test preparations are due to identical active principles, as they should be, it is immaterial whether or no the response measured is the same as the therapeutic effect for which the preparation is to be used. This is not so when the active principles differ or are present as heterogenous mixtures as in digitalis. In such cases the assay method must be carefully defined or results obtained in different laboratories will be conflicting.

The Design of Experiments

In the planning of new experimental techniques a biometrician should be consulted before embarking on the experiments themselves. Good planning can save much tedious arithmetic and considerably improve the precision of the results obtained. The design of the experiment will largely depend on the type of response measured and whether or not the experiment can be repeated on the same test object. This is not always possible. In toxicity tests, which are based on a quantal response, the reaction which is an "all or none effect" can be observed only once, and more complicated and time consuming statistical procedures are necessary for the evaluation[10] of such tests. In assays based on a quantitative response it is often possible to repeat the doses many times on the same test object. By suitable arrangement of the standard and test preparations sources of error such as variation between animals, days and positions

can be eradicated from the final error of the estimate which is dependent on the residual variance. A valuable design is randomisation according to published tables (Latin squares).[14] This has been adopted in the assay of insulin,[15] histamine,[16] adrenaline,[17] curare[18] and posterior lobe pituitary extracts.[19] In some cases it may be difficult to eradicate from the experimental comparisons the effect of changing sensitivity and this may only be partly achieved by the random dose method. Here the experimental design of Vos[20] is preferred, the dose of the standard preparation remaining constant and being alternated with 3 ascending doses of the test preparation. This design is described in the United States Pharmacopeia XIV[21] for the assay of adrenaline and posterior lobe pituitary extracts. It does not give any information on the regression of the standard preparation, however. Other experimental designs include cross-over tests[22] and twin cross-over tests.[23,24,25] Where experimental methods do not permit of statistical evaluation it is often necessary to develop new methods, as in the assay of posterior lobe pituitary extracts. Types of experimental designs used in biological assays have been reviewed by Finney[6,26] and by Emmens,[7,8] and all biological workers will be familiar with the book by Fisher[27] on the design of experiments.

The Assay of Digitalis

The assay of digitalis has long presented the pharmacologist with a problem, and a vast amount of time and energy has been devoted to the investigation of methods which might give a true indication of its activity in man. However, digitalis leaf and galenical preparations consist of a variable mixture of active glycosides and any biological assay will therefore be fundamentally unsound since it will not comply with the first principles of biological standardisation. It is not surprising therefore that results obtained in different species of animals—frogs, cats, guinea-pigs and pigeons—may be conflicting and discrepancies observed with the therapeutic effect in man. Biological standardisation cannot avoid the sources of error associated with impure preparations. Methods should be closely defined and if possible bear a close relationship to the therapeutic effect in man if any reliance is to be placed upon them at all. A major discrepancy in the assay of digitalis lies in the route of administration. In the cat, guinea-pig and pigeon assays toxicities of the test and standard samples are compared by the intravenous route while in man digitalis is administered orally. By mouth even the pure glycosides are not completely absorbed and degradation occurs in the alimentary tract. The effect measured in animals, toxicity, differs from the therapeutic effect in man. While toxicity and therapeutic effectiveness appear to be related, even this is not certain.[28] Attempts have been made to standardise digitalis in man by Gold *et al.*[29] They used two reactions; slowing of the heart and an inversion of the T-wave in the electrocardiogram. In one series, using slowing of the heart rate as their response, the result of a comparison of the standard powder with an unknown in man agreed fairly well with those of the cat assay, while agreement between the human assay and the frog assay was not so close. In another series, however,

using the T-wave test there were marked discrepancies between the human and cat assays.

Therapeutically exact dosage in prolonged treatment with cardiac glycosides is important. This can only be assured by the use of chemically pure crystalline glycosides, the dosage of which can be accurately prescribed. However, while physicians continue to show a preference for the mixed glycosides biological standardisation will remain necessary.

Methods of Assay.

The international standard for digitalis is a sample of dried leaf. It was recently necessary to replace the second standard, which was nearly exhausted, with a new third international standard. One unit is contained in 0·076 g. of this new standard compared with 0·08 g. of the second standard. In establishing the third standard a series of collaborative assays was undertaken in 16 laboratories in different countries. The results and analysis of the different assays have been published by Miles and Perry.[29]

The chief assay methods for digitalis use respectively the frog, guinea-pig, cat and pigeon. Whether or no the assay of digitalis should be restricted to any one method is debatable, but if several alternative procedures are in use it is inevitable that using an heterogenous standard results obtained in different laboratories will be conflicting. Theoretically it would appear desirable that each sample be assayed on several species of animals, as was done in the establishment of the third International Standard,[30] and to take the weighted mean which might be expected to give a result most comparable with man. However, this expensive and time consuming procedure would be impossible on a routine basis. It may be argued that in the collaborative assay in the establishment of the third International Standard agreement between different laboratories and methods was good, but both the second and third standards represented pooled material from different sources and the constitution of the glycosides in the two samples was probably more similar than usual.

The cat method, originally introduced by Hatcher and Brody[31] and perfected by Lind van Wijngaarden,[32] is considered the most satisfactory assay method since it gives almost identical results in the various laboratories,[33] and the results are in general agreement with assays in humans.[29] However, it is difficult to obtain cats in sufficient numbers and in this country the guinea-pig method of Knaffl-Lenz[34] is most widely used. This method has not been popular in the United States, probably because of a lack of agreement with the cat assay.[35] More recently American workers have adopted an intravenous pigeon method first described by Haag and Woodley,[36] who showed that the results obtained agreed favourably with those of the cat method. While the frog method was popular at one time, it has now become largely obsolete in this country and the United States, results obtained in warm blooded animals, which are closer related to man, being preferred. Gold and Cattell[37] reported that frog and man reacted differently to digitalis and the results might be misleading.

In spite of the shortcomings of digitalis assay methods, they should be designed to give the greatest possible precision and the methods should be closely defined if comparative results are to be obtained in different laboratories. It is on these grounds that the methods at present official in the British Pharmacopœia[38] can be criticised. The first frog method described is based on a standard dose response curve originally established by Trevan.[39] It has been shown by Miller[40] and by Miles and Perry[30] that the slope does not remain stable, even in one laboratory, for any length of time, and that the slopes obtained in different laboratories are not homogenous. Admittedly the British Pharmacopœia does suggest a test for slope, but this is impossible with single dosage groups. The alternative frog method is the correct one, the assay being conducted by determining the overnight mortalities in 4 groups of frogs. This is based on a sound statistical foundation enabling the slopes of the standard and test to be compared as an integral part of the assay and the error easily calculated. The method is more adequately described by Miles and Perry.[30]

Both the official cat and guinea-pig methods, in which the diluted tincture is slowly and continually infused intravenously into the anæsthetised animal until the heart is arrested, can also be criticised. In neither case is it necessary to make a simultaneous comparison of the standard and test preparations, the assays being based on the establishment of a standard laboratory figure which is required to be redetermined at a rather indefinite period "from time to time." Bliss[33] has shown that the sensitivity of cats in any one laboratory would only remain constant over a period of 15 days. Similarly in guinea-pigs Jacobsen and Larsen[41] found variations in the sensitivity of the animals to occur and adopted the procedure of always comparing the standard and test samples alongside each other. If such a procedure is adopted it is not logical to use unequal groups, 14 animals on the standard and 6 on the test. Equal numbers should be used in each group and the number determined by the required limits of error, as is done in the U.S. Pharmacopeia.

The British Pharmacopœia assumes that in both cats and guinea-pigs the toxicity of digitalis is related to body-weight, but this is open to question.[5] Heavier cats are relatively more sensitive than lighter ones on a body-weight basis,[30] especially when the continuous infusion method is used. Miles and Perry[30] suggest that this is due to the heavier cats not receiving a lethal dose of a rapidly acting toxic glycoside at as early a stage of the infusion as the lighter cats and they will therefore be exposed for a longer period to the effect of the slowly-acting glycosides and the time of death may thus be relatively earlier than in the lighter cats. It is for this reason that the U.S. Pharmacopœia XIII adopted a procedure of intermittent injections, suggested by Bliss,[33] the injections being made in fixed doses at 5-minute intervals until the heart was arrested. This method allows the slower acting glycosides time for fixation by the tissues. The time for death is standardised by requiring that the average number of doses for any given dilution to produce death should be not less than 13 and not more than 19. The standard and test were required to be tested alongside each other and the assay completed within 15 days.

Similarly in guinea-pigs Miles and Perry[30] showed that the coefficients of correlation between the logarithms of the lethal doses and the body-weights were highly significant and that the heavier were more sensitive than the lighter ones. Miles and Perry emphasise that there is a danger in the use of this method for routine assays the results being biased unless the weights of the guinea-pigs in the two groups are carefully controlled.

The method now official in the United States is the pigeon method of Haag and Woodley[36] as modified by Braun and Lusky.[42] The pigeon is readily obtainable and cheap and the results obtained more consistent than with the cat. It is claimed that only 6 to 8 birds are required in the standard and test groups for a standard error of ±5·7 per cent. Since this method is at present little known in this country it will be described in detail. It should not be confused with the method of Hanzlik[43] which is based on the emetic action of digitalis in pigeons. Adult pigeons are selected for the test so that the weight of the heaviest does not exceed twice the weight of the lightest. They are randomly distributed into groups as nearly alike as possible with respect to breed, sex and weight, so that the average weights of the two groups do not differ by more than 30 per cent. For the assay the pigeons are lightly anæsthetised with ether and an alar vein exposed and cannulated. The diluted tinctures are injected from a small bore burette and made at 5-minute intervals by quickly infusing a volume of the diluted tincture equivalent to 1 ml./kg. of body-weight, until the pigeon dies from cardiac arrest. The test and standard tinctures are previously diluted in such a way that the fatal dose will be diluted to 15 ml. with isotonic saline solution. The requirement is made that the average number of doses required to produce death is not less than 13 or greater than 19. A total of not less than 6 pigeons is used for the standard preparation and 6 for the test, the number being increased if the standard error exceeds 0·08 unit. This method has much to commend it, the technique is simple, animals easily obtained and the end-point —dyspnœa and terminal convulsions—extremely sharp.

Posterior Lobe Pituitary Extract

Posterior lobe pituitary extract, like digitalis, consists of a mixture of active principles. Three of these are used clinically—the oxytocic, vasopressor and antidiuretic hormones. Only two, the oxytocic and vasopressor activities have been almost completely separated. Liquid Extract of Pituitary (Posterior Lobe) was required in the British Pharmacopœia 1932 to contain 10 I.U./ml. and to yield qualitative tests for vasopressor and antidiuretic action. The British Pharmacopœia 1948 contains three preparations:—(*a*) an injection of pituitary standardised to contain 10 I.U. (oxytocic) /ml. and required to be assayed for antidiuretic or pressor activity only if these are stated on the label; (*b*) an injection of oxytocin standardised to contain 10 I.U. (oxytocic) /ml. and not more than 0·5 I.U. (pressor) per ml.; (*c*) an injection of vasopressin standardised to contain 10 I.U. (pressor) per ml.

Standard.

The present international standard is a sample of dried pituitary powder established in 1940 when stocks of the previous standard, established in 1925, were becoming exhausted. Fortunately the new standard had practically the same activity and composition as the previous one, hence it was unnecessary to redefine the unit as was required with digitalis. The unit is defined as the activity of 0·5 mg. of this material for oxytocic, pressor and antidiuretic assays.

Methods of Estimation.

The methods of assay of pituitary (posterior lobe) extracts have been reviewed by Thorp[44] and by Stewart.[45,46]

Oxytocic activity. The method described in the present British Pharmacopœia is the same as that in the B.P. 1932, the assay being performed on the isolated uterus of the virgin guinea-pig. This method was introduced by Dale and Laidlaw[47] and has long presented practical difficulties; suitable uteri are difficult to obtain, many showing an inherent rhythm and a poor differentiation between graded doses of the same extract. The method is based on a scheme of matching doses and is unsuitable for statistical analysis, the experimental error being indeterminable from individual assays but only from separate experiments. Various workers have improved the method.[48,49,50] Some have attempted to improve the assay by modifying the concentrations of magnesium and calcium ions in the Ringer solution,[51,52] thus increasing the sensitivity and preventing spontaneous alternating rhythms. However, there is evidence that vasopressin exerts a considerable degree of oxytocic activity in the presence of magnesium. Attempts to design the guinea-pig uterus method for statistical evaluation have not been very satisfactory due to the difficulty in obtaining a large number of repeatable responses. The virgin guinea-pig method is therefore far from satisfactory and has been largely superseded by the rat uterus method[55,19] and the chicken blood pressure method.[56,57,58]

The rat uterus method employs the isolated uterus of the non-pregnant diœstrous rat. Suitable uteri are easily obtained and since the doses can be repeated at 3- to 4-minute intervals the 4-point assay described by Schild[16] and by Holton[19] can be used, thus enabling the experimental error to be determined for each experiment. An assay can be completed within 4 hours and limits of error ($P = 0{\cdot}95$) within ± 20 per cent. easily obtained.

The chicken depressor method is official in the United States Pharmacopeia XIV and is based on the depressor action of vasopressin on the blood pressure of the cockerel. Blackwell Smith and Vos[58] describe a 4-point assay but changes in sensitivity during the dose schedules increases the error of the assay and, to overcome this, Thompson[59] used the experimental design described by Vos.[20] If the standard and test materials are similar to each other the experimental design of Vos gives the more accurate results, but should the standard and unknown materials be dissimilar then the Schild[16] experimental design should be used when an

analysis of variance is possible and one can determine whether the assay is valid or not.

Pressor activity. The official method for the assay of pressor activity is on the blood pressure of the spinal cat.[47,60] Since it is necessary to allow intervals of one hour between doses a statistical design is impossible, and the potency of the test preparation can only be assessed by bracketing it between doses of the standard extract. With the anæsthetised dog[61] doses can be given at 15-minute intervals and while the discrimination between doses is not as good as with the cat the larger number of doses which can be given results in as great an accuracy.[46]

The anæsthetised rat has been proved a satisfactory preparation for the determination of pressor activity and is extremely sensitive.[62,63,64] The assay is not interfered with by traces of histamine in commercial powders. Landgrebe *et al.*[64] used anæsthetised male rats, the central nervous system of which was pithed caudally from the anterior tip of the pelvic girdle to eliminate fluctuations of the blood pressure. Injections were made into the femoral vein at 15-minute intervals and the blood pressure recorded from the carotid artery. Landgrebe *et al.* used the method of "matching doses" but a randomised block design[16] could be used.

Antidiuretic activity. While numerous methods have been described for the determination of antidiuretic activity few have used modern statistical methods in designing their procedure or in evaluating their results. The vasopressor hormone and antidiuretic hormone are believed to be due to the same active principle,[65] but Heller[66,67] has prepared from the vasopressor fraction an extract containing a high proportion of antidiuretic activity and very little vasopressor activity.

The method most widely used for the determination of the antidiuretic hormone is due to Burn[68] and this is the one described in the British Pharmacopœia. The assay depends on the time for the maximum rate of urine excretion in rats following administration of water by stomach tube and posterior lobe pituitary extract by injection. 8 rats are used on the standard preparation and 8 on the test in a cross-over technique, the test being repeated 2 or 3 days later when the rats which received the standard now receive the test preparation and *vice versa*. The urine excreted is measured at 15-minute intervals from which the time for maximum excretion is determined. The potency is calculated by reference to a standard dose-response curve which should be predetermined for each laboratory and breed of rats. The error of the assay cannot be assessed from a single assay and the Pharmacopœia states that "the data at present available do not permit of a sufficiently accurate determination of the limits of error."

Numerous modifications of Burn's method have been described. Gilman and Goodman[69] obtained a more consistent response by giving a preliminary hydrating dose of water three hours before the commencement of the test, while Silvette[70,71] gave a single dose of 0·2 per cent. saline solution by intraperitoneal injection and measured the total volume of urine excreted during a period of 6 hours. Krieger and Kilvington[72] measured the volumes of urine excreted at intervals of 15 minutes over a period of 6 hours and measured the area of a plotted curve with a

planimeter. Ham and Landis[73] have closely examined the various factors in an antidiuretic assay and conclude that the estimation of chloride excretion is preferable to measurements of the urine volume.

Ginsburg[74] has recently described a method for an assay using rats, which can be readily analysed statistically. This method is based on a regimen of water administration described by Birnie *et al.*[75] Groups of rats are given 2 doses of water by stomach tube with an interval of one hour between doses, followed by a third dose one hour later when the injections of pituitary extract are given. The urine is collected from the rats placed in individual metabolism cages and measured at 60, 90 and 120 minutes from the time of the injections. A 4-point assay procedure is described from which the potency and its error can be determined by standard statistical procedure. A single assay can be completed in 5 hours.

Other methods of determining antidiuretic activity have been described in mice,[76,77] dogs[65,78,79] and rabbits.[80,81,82]

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SCIENCE PAPERS AND DISCUSSIONS

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OBSERVATIONS ON THE PAPER PARTITION CHROMATOGRAM AS APPLIED TO THE DETECTION OF ALKALOIDS

BY D. N. GORE and J. M. ADSHEAD

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INTRODUCTION

A NUMBER of workers have investigated the application of partition chromatography on paper supports to the separation and identification of alkaloids. Foster *et al.*[1] described the separation of ergot alkaloids in butanol-water-acetic acid, using fluorescence under ultra-violet light as a means of detecting position and as a basis for quantitative estimation. Brindle *et al.*[2] investigated the resolution of solanaceous alkaloids and insoluble alkaloids of ergot in a variety of solvents running on buffered papers and suggested a quantitative approach. Munier and Macheboeuf[3] studied the choice of acidified solvents in relation to the dissociation constants of the alkaloids. Schute[4] reported the separation of some solanaceous alkaloids in the form of their bases, using an ammoniacal solvent and studied factors which influence the R_F value.

The present contribution makes some observations on practical features of the paper partition method as applied qualitatively to a number of common medicinal alkaloids. Experience with a number of solvents is reported and three butanol-water systems acidified with formic, acetic or propionic acids are suggested for general use. The practical procedure is typified by a detailed description, of a chromatogram of a series of alkaloids and their salts run in the butanol-acetic solvent and using an aqueous iodine spray for revelation of position. The technique of applying the spray is described, some diagnostic implications are discussed and particular reference is made to the limitations of the R_F value as a criterion of identity in relation to alkaloids.

EXPERIMENTAL

Choice of solvents.—Figure 1 shows a chromatogram of a variety of alkaloids and their salts run simultaneously in adjacent positions on a $22\frac{1}{2} \times 18$ inch Whatman No. 1 paper. The solvent in this instance was that advocated by Partridge[5] for the separation of sugars, namely the saturated upper layer obtained by shaking 40 volumes of *n*-butanol,

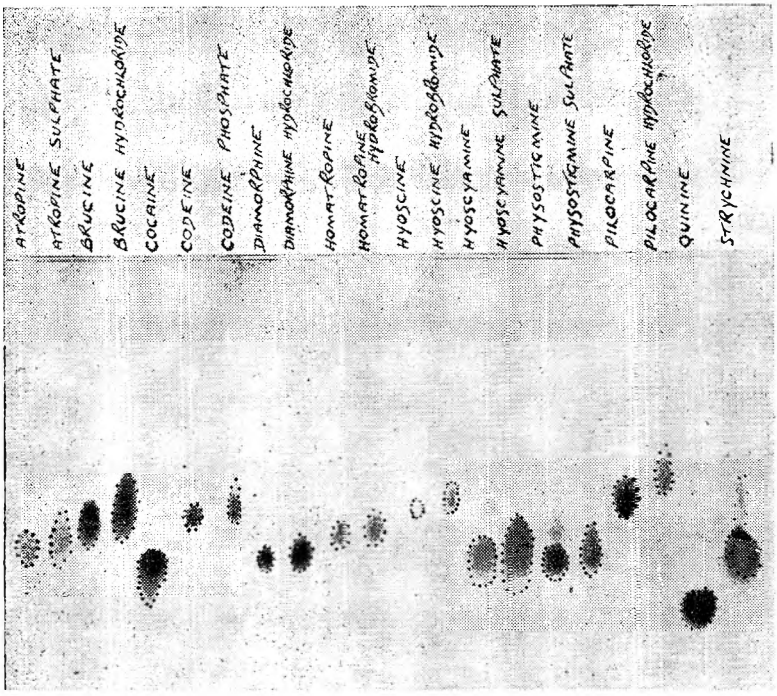

FIG. 1. Paper partition chromatogram of alkaloics.

50 volumes of water and 10 volumes of glacial acetic acid. The water-miscible layer was external to the trough in the usual way, being placed in beakers in the bottom of the tank, the beakers containing a filter paper cylinder to accelerate the attainment of vapour equilibrium. In each case approximately 0·002 ml. of solution (aqueous or organic) containing approximately 100 μg. of alkaloid or salt (i.e., a 5 per cent. w/v solution) was applied to the starting line on the paper. The solvent was run downwards for 16 hours and removed from the paper in an air draught at 80° to 85° C. The positions of the alkaloids or their salts were revealed by spraying the dry paper with aqueous iodine solution which is referred to in greater detail later. Similarly, the same series of alkaloids and their salts run well in the saturated upper layer obtained by shaking 10 volumes of *n*-butanol, 10 volumes of water and 1 volume of formic acid and again, in the solvent obtained by substituting propionic acid for formic acid in the above formula. The use of hydrochloric, citric and tartaric acids in a *n*-butanol-water system was investigated, but on the whole the resolution was less satisfactory than that obtained with the acetic, propionic or formic solvents and attention was concentrated on the use of these latter 3 systems.

Table I records the average R_F values of the selected alkaloids and their salts in the 3 solvents, obtained from a large number of chromatograms prepared in the manner described in connection with Figure 1. Any

apparent inconsistency between the relative positions of alkaloids in Figure 1 and the R_F values in Table I is due to the solvent front at the end of the run of the particular chromatogram illustrated not being parallel with the starting line, a frequent circumstance in downward runs with such broad fronts. The R_F values for codeine phosphate in the butanol-formic and butanol-propionic solvents do not appear in Table I. Certain apparent anomalies occurred here which are referred to in the discussion.

TABLE I

Average R_F VALUES

Alkaloid or salt	*n*-Butanol 40 Acetic acid 10 Water 50	*n*-Butanol 10 Formic acid 1 Water 10	*n*-Butanol 10 Propionic acid 1 Water 10
Atropine	0·73	0·58	0·68
Atropine sulphate	0·72	0·56	0·64
Brucine	0·67	0·44	0·58
Brucine hydrochloride	0·64	0·39	0·53
Cocaine	0·76	0·72	0·79
Cocaine hydrochloride	—	0·66	0·69
Codeine	0·65	0·42	0·51
Codeine phosphate	0·61	—	—
Diamorphine	0·75	0·57	0·65
Diamorphine hydrochloride	0·74	0·55	—
Homatropine	0·68	0·52	0·57
Homatropine hydrobromide	0·66	0·51	0·54
Hyoscine	0·63	0·42	0·51
Hyoscine hydrobromide	0·62	0·41	0·49
Hyoscyamine	0·75	0·57	0·66
Hyoscyamine sulphate	0·73	0·55	0·60
Physostigmine	0·76	0·63	0·68
Physostigmine sulphate	0·74	0·61	0·68
Pilocarpine	0·58	0·36	0·47
Pilocarpine hydrochloride	0·54	0·33	0·40
Quinine	0·84	0·68	0·81
Quinine hydrochloride	0·83	0·69	0·87
Strychnine	0·74	0·58	0·70
Strychnine hydrochloride	0·73	0·56	0·69

Figure 2 shows a chromatogram obtained in precisely the same manner as that illustrated in Figure 1, when extract of jaborandi and tincture of nux vomica were applied to the paper with no preliminary treatment other than a simple evaporation to one-fifth and one-tenth of the original volumes respectively. The spots due to pilocarpine, strychnine and brucine were clearly defined. The use of *n*-butanol or *iso*butanol saturated with water was investigated without success. Many of the alkaloids tended to move in a diffuse manner and, as would be expected on theoretical grounds, the position of the spots was very capricious, particularly in the presence of strong negative radicals; so much so that chromatograms obtained with these solvents appeared to be quite useless for practical purposes. The use of papers previously soaked in buffer solutions had the effect of stabilising the rates of travel, but in general the spots tended to be elongated to an extent which made the measurement of R_F values very indefinite. Water-saturated phenol was considered to have possibilities in relation to the alkaloids, but the results with this solvent with or without the addition of acetic acid or ethanol were disappointing. All the alkaloids and salts in the present series ran in a well-defined manner in these phenolic solvents, but with very high mobilities which brought

them all near the solvent front. Solvents based on phenol appeared to hold very little promise for the purpose of separating alkaloids.

Choice of method.—The descending flow method is well adapted to accommodate the running of large numbers of chromatograms at one time; often a very desirable feature. Also, there is no limitation on the distance the solvent front may be allowed to travel and this is sometimes an advantage for increasing the resolution of substances of low mobility. The tank and troughs used in obtaining the chromatograms typified by Figure 1 were very similar in design to those described by Woiwod.[6] Where these two advantages do not apply, experience in the present investigation confirms the opinion of Brindle *et al.*[2] that the capillary ascent technique originally advocated by Williams and Kirby[7] gives very compact spots with the alkaloids. From experience in this and other investigations the ascending flow method is to be preferred. The solvent front is usually very uniform and generally the reproducibility of the R_F values is superior to that with downward flow methods, at least when these are on the tank scale. The slower attainment of vapour equilibrium in the latter case may be a partial factor.

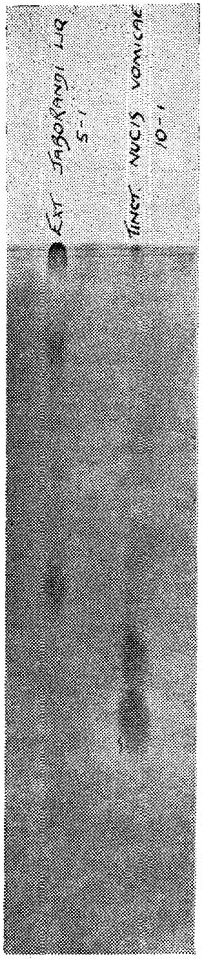

FIG 2.

In addition to the advantages mentioned, the capillary ascent technique has the virtues of being extremely easy to operate and makes no demands for specially designed equipment. The arrangement for upward runs made in the present work was very simple. Samples were applied to a sheet of chromatographic paper 14 × 9 inches on a line parallel with and $1\frac{1}{2}$ inches from one of the short ends. The paper was then bent into the form of a cylinder with the edges just overlapping and secured with paper clips. The cylinder, samples downward, was placed in a petri dish concentric with the rim of the dish and a beaker standing in its centre, the whole standing upon a ground glass plate. The water-immiscible solvent layer was poured into the dish and the water-miscible layer into the beaker which also carried a paper cylinder to accelerate the attainment of vapour equilibrium. A glass cylinder with ground ends was quickly placed in position and closed at the top with a second ground glass plate. A run is most conveniently commenced at the end of the day and allowed to continue overnight, which applies also to the descending flow method.

Choice of paper.—Whatman No. 1 paper gave very satisfactory results. The coarser No. 4 grade supplied also for chromatographic purposes allows faster runs and is sometimes convenient for this reason, but

there is a corresponding loss of precision and definition and the No. 1 grade paper is to be recommended for general purposes.

The iodine spray.—Brindle *et al.*[2] and Schute[4] reported some difficulty in obtaining a satisfactory response to the use of iodine in revealing the positions of alkaloids on the paper. In the present investigation it was found that the success of this reagent is a function of the concentration of iodine used in the solution, which is fairly critical, and, in particular, to the character and manner of application of the spray. A concentration of 0·2 per cent. w/v of iodine obtained by dilution of the B.P. Appendix reagent was found to be just sufficiently strong to give a good reaction with the alkaloids without producing more than a faint transient staining of the rest of the paper, so obtaining a maximum contrast. Early experience showed that in order to obtain a satisfactory result it was absolutely essential to apply the solution fairly forcefully in a fine mist free from coarse droplets, and that to achieve this a well-designed atomising device, operated by a sustained force of compressed air, is required. The hand-operated intermittent puffer type of atomiser was found to be almost useless. Suspending the paper sheet in front of a frosted glass screen illuminated from behind is a considerable aid in observing the course of wetting, thus facilitating the uniform and systematic application of the reagent solution which is essential for success. The photograph from which Figure 1 was reproduced was taken 3 minutes after spraying, during which interval the borders of the spots were permanently outlined by quickly and lightly dotting with ink.

Brindle and co-workers[2] reported that the colour of the iodine stain was brown with hyoscine and tinged with blue in the case of atropine. This is confirmed in the present investigation with the added information that the reaction with hyoscyamine is similar to that of atropine. The colours of the stains from the alkaloids in the present series were otherwise generally brown. The relative rates at which the stains from the various alkaloids faded were very reproducible. It is not suggested that much could be made of this from the diagnostic angle, but it is interesting to note the more extreme cases. The stains from the solanaceous alkaloids fade rapidly, particularly the hyoscine stain, whilst the quinine, brucine and strychnine stains are very persistent and in the case of the latter pair may be observed many months later on papers kept in the dark. Another very reproducible and curious feature concerns the stain from homatropine and salts. As the spots fade a characteristic speckled appearance develops. This and other features mentioned above are probably of some diagnostic value.

Discussion

The methods and techniques described can be used to indicate the presence of the alkaloids mentioned and probably many others, in solutions applied to the paper, provided due regard is paid to their concentration in the sample solution which may be a biological fluid or extract or one which is the result of a preliminary separation and may be aqueous or organic. As a working guide something of the order of 100 μg. of an

alkaloid will need to be present to ensure its detection by the iodine spray. This would apply to the solanaceous group in particular. Some others will be detected in much smaller proportion, e.g., pilocarpine, strychnine and brucine as shown in Figure 2 when as little as 25 μg. were present. It may be that two or more alkaloids are present at very different concentrations and if this possibility has to be taken into account it is desirable to run a number of chromatograms, in adjacent positions on the sheet, of dilutions and concentrations of the sample solution. As an alternative to concentration, two or more applications can be superimposed on the same sample spot.

Following upon an indication of the presence of one or more alkaloids by the methods described a measurement of the R_F value should in theory provide positive evidence of identity. In practice, however, there are a number of factors which influence the mobility of alkaloids in the solvent. Some of these factors apply generally to the paper partition method and were discussed in the original publication by Consen *et al.*[8] It is very doubtful if the reproducibility of an R_F value can be kept within 2·5 per cent. either side of the average of a number of determinations. Superimposed upon this are influences which apply to basic substances such as the alkaloids. The most important point here is the effect of the negative ions present.

It was seen in the present investigation that the alkaloids were much more mobile in saturated butanol than the corresponding salts. This factor was almost but not quite eliminated when an acid was introduced into the solvent as will be seen by reference to Figure 1 and to the average R_F values reported in Table I. The R_F value of the salts are, however, almost without exception consistently less than those of the bases. In natural extracts and the like, the radicals with which an alkaloid may be associated are not known with certainty and its apparent R_F value may therefore be slightly different from that obtained with the pure base or a known salt. For these reasons the determination of an R_F value when the sample solution is a natural extract or where the nature of the negative radical is otherwise unknown, can only be regarded as supplying rough presumptive evidence of the identity of the alkaloid or alkaloids in question. Also, for the same reasons, no more definite information will be obtained by running a chromatogram of the pure alkaloid or a salt alongside that of the test sample as a means of confirmation, although it can be said that the salt will generally provide the more reliable indication. A better procedure is to load the sample solution with the suspected alkaloid. Much more reliable information will of course be obtained by some preliminary separation of any alkaloids present using the usual procedure, when they can be applied either in their basic form in an organic solvent, or in the form of a known salt in aqueous solution. The corresponding reference chromatogram can then be selected accordingly.

A further point arose in the present work in connection with codeine phosphate. In the butanol-acetic solvent one spot was obtained in the normal manner the R_F value of which is indicated in Table I. In the butanol-formic solvent 3 spots (average R_F values 0·25, 0·33, 0·41) and

in the butanol-propionic reagent 2 spots (average R_F values 0·19 and 0·37) were found. This obviously warrants further investigation, but meanwhile draws attention to the false indications which may arise from the presence of phosphate in a sample solution with the two solvents in question.

The foregoing suggests that the R_F value cannot be accepted as a reliable criterion of identity of alkaloids and that some confirmatory evidence should always be sought. As already mentioned, the iodine stains are to some limited extent diagnostic and the use of more specific spray reagents merits investigation. For example, morphine on a chromatogram gives the expected colour reactions when sprayed with 0·1 N sulphuric acid, followed by saturated potassium iodate and ammonia solutions.

Whilst acknowledging the limitations of the paper partition chromatogram it is nevertheless a most valuable expedient to the analyst confronted with the detection of alkaloids in extracts, pharmaceutical preparations and the like. Especially is this so in the exploratory stages of an analysis when information is required as to the presence of alkaloids, their probable number and some indication of identity. The economy of time and labour, the trifling expenditure of sample material and the immense amount of information which can often be obtained overnight on one sheet of paper are the special attributes of this elegant and simple method.

The authors' thanks are due to the Directors of Beecham Research Laboratories, Ltd., for permission to contribute this paper, to Miss E. C. Russell for much of the earlier practical work and to Mr. D. F. Lawson for the photographs from which the two figures were taken and numerous others in connection with this investigation.

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DISCUSSION

The paper was presented by MR. D. N. GORE.

MR. J. E. CARLESS (Manchester) said that the paper would have been more valuable if solvents could have been found to give greater differences between the R_F values of the alkaloids studied. In Table I, corresponding to R_F values between 0·73 and 0·76 of the first solvent, there were at least 5 alkaloids. Identification of the individual alkaloids in that group would be impossible, due to overlapping. The difficulty might be overcome by the use of specific spot tests and smaller spots. The use of buffered filter paper would offer an advantage in those cases where R_F values were close together. Tailing of spots when using buffered filter

paper gave trouble. Tailing of zones on cellulose columns had been overcome by arranging a *p*H gradient down the column. For the identification of alkaloids by paper partition chromatography, the plotting of R_F values against the *p*H might give characteristic curves of diagnostic importance.

DR. W. MITCHELL (London), commenting on the method of applying the iodine, referred to a device known as the "Aerograph Artists' Spray Brush" which he had found to be of value in obtaining a uniform spray.

MR. D. N. GORE, in reply, said that whilst agreeing with Mr. Carless's criticism concerning the solvents, the paper was not intended to convey the impression that the three solvents used would necessarily cover everything. The efficiency of the atomiser was of particular importance where the spots were of a transient nature.

DIGITALIS GLYCOSIDES

The Colorimetric Assay of the Chloroform-Soluble Glycosides of Digitalis

By C. J. Eastland, D. P. Lawday and E. H. B. Sellwood

From The Research Division, Allen and Hanburys, Ltd., Ware, Herts.

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Although there are various reports on the assay of tinctures and of pure glycosides of digitalis, the literature contains little information on the evaluation of mixed glycosides. A number of such products are available commercially. We have assayed, biologically and chemically, samples of mixed chloroform-soluble glycosides as being typical of such preparations and also representing intermediate stages in the isolation of digitoxin. We used, for the chemical assay, the colorimetric procedure based on the Baljet reaction,[1] and we have investigated the relationship between this and the biological assay.

Experimental

The chloroform-soluble glycosides were obtained by extraction of the leaf with ethanol, followed by concentration, clarification with lead subacetate and extraction with chloroform. The concentrated chloroform solution was precipitated with light petroleum. Samples described as "digitoxin" are those which give a strong brown junction by the Keller-Kiliani test and which when chromatographed[2] were found to consist mainly of digitoxin. The results of chemical assays by the sodium picrate method and of biological assays, and the ratio of chemical assay to biological potency are shown in Table I, the results being arranged in order of increasing biological assay. The figures for 2 samples of *D. lanata* glycosides are included.

The colorimetric assays were carried out with a reagent consisting of a fresh mixture of 95 ml. of 1 per cent. trinitrophenol solution in water and 5 ml. of 10 per cent. sodium hydroxide solution. 5 to 10 mg. of the sample, according to the expected potency, accurately weighed, was dissolved in 25 ml. of ethanol (70 per cent.). 5-ml. portions were mixed with 5 ml. of reagent and the optical density measured in the E.E.L. Portable Colorimeter after standing for exactly 20 minutes at 20° C. The general purpose green filter No. 404, which has its maximum transmission at about 530 mμ, was used. We did not make use of the lower wavelength suggested in the recent literature[3,4,5,6] because many of our results were already available when these papers were published. The instrument was calibrated in terms of units of activity with digitoxin of known biological potency (950 I.U./g.).

The biological assays were carried out by Dr. H. O. J. Collier using intravenous infusion, from a burette, of a saline preparation of the glycoside. In each assay 5 or more guinea-pigs were used and the standard errors of the determinations were not greater than 8 per cent.

Discussion

With mixed chloroform-soluble glycosides there is no simple relationship between the chemical and biological assays; the chemical assay may be as much as 3 times that of the biological. As the potency increases, with increased digitoxin content, this ratio falls (Table I) and for digitoxin of B.P.C. potency the ratio is close to unity.

It would be desirable to be able to predict the biological potency from the chemical assay but we have found that this cannot be done with any accuracy. Knowing the biological potency, however, it is

TABLE I

Sample	Description	Chemical assay I.U./g.	Biological assay I.U./g.	Assay Ratio chemical/ biological
1	Chloroform-soluble glycosides	467	168	2·78
2	"	505	197	2·56
3	"	780	273	2·86
4	"	746	280	2·68
5	"	478	304	1·57
6	"	550	305	1·82
7	"	765	358	2·14
8	"	721	358	2·01
9	"	555	386	1·44
10	"	690	483	1·43
11	"	790	526	1·50
12	"	863	562	1·53
13	"Digitoxin"	955	679	1·40
14	"	927	681*	1·36
15	"	982	683	1·44
16	"	940	690	1·36
17	"	966	693	1·40
18	"	890	711	1·25
19	"	960	760	1·26
20	"	1026	768*	1·34
21	"	1050	788	1·33
22	"	1000	805	1·24
23	"	908	924*	0·97
24	"	1074	1073*	1·00
25	"	1022	1298*	0·78
26	*D. lanata* glycosides	700	493	1·42
27	"	520	684	0·76

* These figures were obtained by infusion from two joined burettes, one containing an ethanolic solution of the glycoside and the other containing saline solution.

possible to estimate the expected chemical activity with more certainty. It is unfortunate that these estimates are more accurate in the less useful direction, i.e., from biological to chemical. The chemical assay is most useful when considered in conjunction with information as to the origin, colour, solubility, physical constants and chromatographic behaviour of the sample.

We have confirmed that when the aglycones are assayed the colour intensities are twice those produced by equal weights of their respective glycosides. It will be recalled that the molecular weights of digitoxigenin and gitoxigenin are approximately half those of the corresponding glycosides. Thus the presence of aglycone in a mixture will increase the optical density. Conversely, any primary (initial) glycosides will assay low by the colour reaction. Sample 27, for example, was found by chromatography to contain a high proportion of the primary glycosides and in this case the colorimetric assay is lower than the biological. Thus the degree of complexity of the glycoside mixture, namely the relative

proportions of primary and secondary glycosides and aglycones, has an important influence on the chemical assay. The presence of physiologically inactive material giving the Baljet reaction will obviously favour a high chemical assay. With the isolation of digitoxin in mind, the lowering of the chemical/biological ratio will indicate increasing purity of the sample.

Summary

1. It has been shown experimentally that when mixtures of chloroform-soluble glycosides of digitalis are assayed by the Baljet reaction figures are obtained which may be 3 times those by biological assay.

2. This ratio falls with an increase in potency and for digitoxin of B.P.C. potency the ratio is close to unity.

3. It is not possible from the chemical assay of mixed glycosides to estimate the biological activity with any certainty but the assay is valuable when combined with knowledge of the origin, chromatography and physical constants of the material under examination.

We are indebted to Dr. Collier for carrying out the biological assays. Thanks are also due to the Directors of Allen and Hanburys, Ltd., for granting permission to publish these results.

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STUDIES IN THE GENUS *DIGITALIS*

PART I. THE COLORIMETRIC ESTIMATION OF DIGITOXIN AND OF PREPARATIONS OF *DIGITALIS PURPUREA*

By J. M. ROWSON

From the Museum of the Pharmaceutical Society of Great Britain

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IN recent years a number of different methods have been proposed for the quantitative estimation by colorimetric methods of either digitoxin or the mixture of glycosides obtained by extraction from the leaves of *Digitalis purpurea*. These processes, which have been reviewed in two recent publications,[1,2] depend upon the interaction of either the aglycone portion of the glycoside molecule, essentially the butenolide group, or of the digitoxose sugar part of the molecule with suitable colour-producing reagents. Alkaline picrate or several different dinitro aromatic compounds have been employed for interaction with the butenolide group of the glycoside molecule; such processes are more or less specific for this linkage but do not distinguish between the various closely related glycosides or their aglycones which may be present in the leaf of *D. purpurea*. The Keller-Kiliani reagent has been used for the quantitative estimation of digitoxose. It is not specific, but several workers have claimed that in a partially hydrolysed glycoside mixture the unhydrolysed digitalis glycosides along with aglycones may be separated from free digitoxose by solvent extraction; when the solvent is removed the sugar moiety of the unhydrolysed glycoside only is estimated by the Keller-Kiliani reagent. The intensity of colour produced in each of these processes has been measured spectrophotometrically, but in a number of instances the time allowed for colour development, the selection of a blank for comparison in the spectrophotometer and the choice of wavelength of light for observations have not been critically examined.

For the quantitative evaluations of powdered digitalis leaf by means of these colorimetric processes different methods of leaf extraction by means of ethanol or water have been employed, also different methods for the preliminary decolorisation of these extracts have been recommended by the different workers. The results obtained by these processes have, in the majority of cases, been compared with those obtained by one or other of the biological methods of assay but no worker has attempted to prove the efficiency of each process, and no critical comparison of the different proposed processes of extraction of leaf, decolorisation of extracts and of methods of colorimetric estimation, have been made. Fuchs and his co-workers[2] have examined leaf samples from 8 different species of *Digitalis* by different colorimetric and biological methods of estimation but using the same process of extraction and decolorisation for all samples; and their method of extraction will be shown in this paper to be inaccurate.

The work to be reported in this paper includes a critical examination both of the methods for colorimetric estimation of digitoxin and of the efficiency of processes for the decolorisation of digitalis leaf tinctures or infusions, prior to applying the colorimetric methods of estimation.

1. The Colorimetric Estimation of Digitoxin

A commercial sample of digitoxin containing 1735 I.U./g. has been employed in this work. All spectrophotometric readings were made by means of the "Unicam" quartz spectrophotometer and in every estimation the 1 cm. cells were used.

(*a*) *The Aglycone* (*Butenolide*) *Estimations*

The alkaline picrate method has been very extensively examined by Bell and Krantz[3] and it is now official in the United States Pharmacopeia XIV for the quantitative estimation of digitoxin in tablets. The process depends upon the interaction of the glycosides in ethanolic solution with a freshly prepared mixture of aqueous or ethanolic solution of trinitrophenol and sodium hydroxide; an orange colour develops after standing, the intensity of which is measured at wavelength 525 mμ in a spectrophotometer using a dilution of the reagent as a blank. Bell and Krantz prepared standard graphs for the relation of colour intensity to digitoxin content up to 15 mg. per cent., and found that these were not straight lines and that the Beer-Lambert law was not obeyed.

For the following work a solution of digitoxin in ethanol (50 per cent.) and aqueous sodium picrate reagent were used. A volume of solution equivalent to from 0·1 to 0·4 mg. of digitoxin was measured into a stoppered 10 ml. cylinder, adjusted to 5 ml. with ethanol (50 per cent.), and 5 ml. of fresh picrate reagent added [this reagent is a mixture of 95 ml. of 1 per cent. aqueous trinitrophenol with 5 ml. of 10 per cent. sodium hydroxide solution]. At the same time a blank consisting of 5 ml. of ethanol (50 per cent.) and 5 ml. of picrate reagent was also prepared. Colour densities of these solutions reached a maximum some 10 minutes after mixing and remained constant for a further 20 minutes before fading commenced. Thus future readings were taken 20 minutes after mixing. Colour densities of 3 different digitoxin concentrations of 1, 2 and 3 mg. per cent. were determined at wavelengths between 400 and 600 mμ. These are shown in Figure 1, from which it is seen that maximum colour measurement was obtained at wavelengths of 490 to 500 mμ. Finally the colour densities of a range of digitoxin levels from 0·5 to 4 mg. per cent. were determined at wavelength 495 mμ. The graph obtained by plotting these values of colour densities against digitoxin concentration between 1 and 3·5 mg. per cent. was a straight line corresponding to k (1 mg. per cent. digitoxin) = 0·190. Thus between these concentrations the reaction obeys the Beer-Lambert law.

The suggestion by Bell and Krantz[4] that a more intense colour was developed if tetraethylammonium hydroxide in high concentration replaced sodium hydroxide in the reaction, was not confirmed. For the same concentration of digitoxin in final reaction mixture the use of this

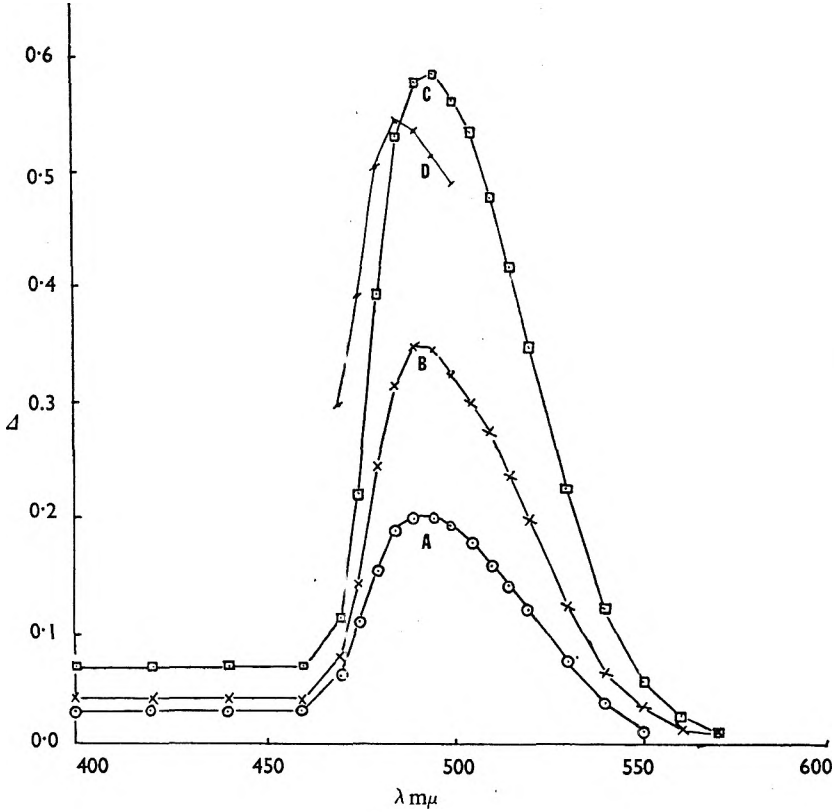

FIG. 1. Alkaline picrate reagent.

A. Digitoxin 1 mg. per cent.
B. Digitoxin 2 mg. per cent.
C. Digitoxin 3 mg. per cent.
D. Tincture of digitalis 3 units per cent.

quaternary hydroxide gave a weaker colour, but with its peak at the same wavelength of 495 mμ; k (1 mg. per cent. digitoxin) = 0·134.

The metadinitrobenzene process (the Raymond process) has been used by Canbäck for the estimation of digitoxin and other digitalis glycosides, but has not been applied to the estimation of digitalis tinctures. The process depends upon the production of a blue colour when a mixed ethanolic solution of digitoxin and *m*-dinitrobenzene is rendered strongly alkaline with sodium hydroxide. The blue colour fades rapidly but logarithmically in relation to time: thus colour densities of the reaction mixture are determined at exact half-minute intervals, from mixing, at a wavelength of 620 mμ: and by graphic extrapolation the colour density at zero time is calculated.

Repeatable results by this process have been obtained for a number of estimations of the same solution of digitoxin. Colour density readings at different wavelengths were plotted, giving a curve with a well defined

broad peak at 600 to 650 mμ, thus confirming Canbäck's choice of a wavelength of 620 mμ. At this wavelength digitoxin concentrations of 1 to 4 mg. per cent. were shown to obey the Beer-Lambert law, k (1 mg. per cent. digitoxin) = 0·192.

The dinitrobenzoic acid process (*Kedde*). This method is described by Canbäck[1] as a modification of the Raymond process which should be efficient. Kedde[6] added a 2 per cent. solution of 3:5 dinitrobenzoic acid in ethanol to a decolorised tincture of digitalis, more ethanol was added followed by normal sodium hydroxide and adjusted to volume with water. The brown colour was measured at wavelength 530 mμ after standing for 1 hour and using as blank a dilution of the decolorised tincture. This choice of blank is wrong, for the dinitrobenzoic acid solution is lemon yellow, gradually becoming brown after adding alkali. The method was investigated by using as test solution a mixture of 0·1 to 0·8 mg. of digitoxin in ethanol (50 per cent.) with 2 ml. of a 2 per cent. ethanolic solution of dinitrobenzoic acid and ethanol (50 per cent.) to 9 ml., followed by 1 ml. of N sodium hydroxide, and, as blank, this solution without digitoxin. Observations at 530 mμ showed that the colour density increased up to 6 minutes after mixing, remained constant up to 12 minutes and faded progressively up to 75 minutes, when the experiment was stopped. Hence Kedde's choice of measurement 1 hour after mixing is wrong. Colour densities of 3 different concentrations of digitoxin were determined at different wavelengths; the peak values were between 525 and 550 mμ with a shallow maximum at 535 mμ as shown in Figure 2 and this wavelength was used in subsequent work. Concentrations of 1 to 8 mg. per cent. of digitoxin were then examined and it was found that the reaction obeyed the Beer-Lambert law.

This process was easy to handle, the reagents are stable, no difficulties were experienced in making any observations, the concentration range of 2 to 8 mg. per cent. is convenient, also the process is rapid.

(*b*) *The Digitoxose Estimations*

The Keller-Kiliani process. This qualitative test has been modified by James and his co-workers,[7] also by Soos,[8] as a quantitative process. Water must be excluded from the reaction mixture. Soos dissolved dried digitoxin equivalent to 10 to 20 mg. per cent. in a freshly prepared reagent of strong acetic acid, ferric chloride and strong sulphuric acid: after standing for 5 hours colour densities were measured at wavelength 570 mμ every half-hour until maximum values were obtained.

Preliminary experiments have been carried out using as reagent a mixture of 60 ml. of glacial acetic acid, 2 ml. of a 5 per cent. solution of ferric chloride and 5 ml. of strong sulphuric acid. A solution of digitoxin in ethanol was evaporated to dryness and dissolved in freshly prepared reagent to give a final volume of 10 ml., a blank of reagent only was employed. At 570 mμ it was found that colour densities increased for 15 minutes after mixing, remained constant for a further 15 or 20 minutes, then faded slowly up to 90 minutes. Taking readings in this constant period between 15 and 30 minutes, the colour densities of 2 different

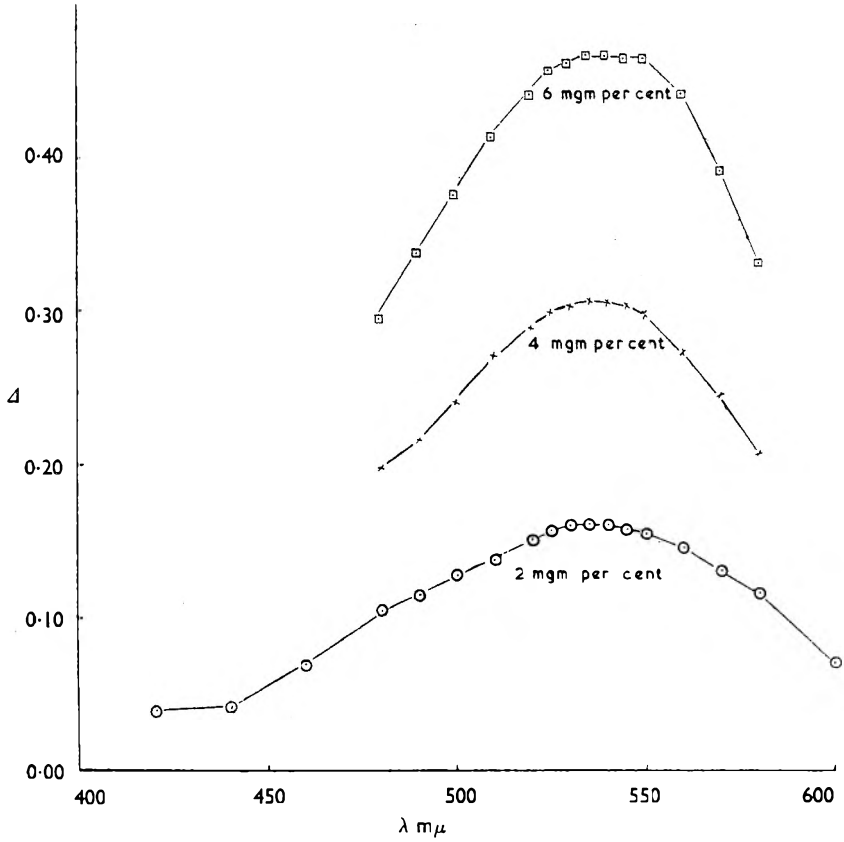

FIG. 2. 3:5-Dinitrobenzoic acid reagent and digitoxin.

digitoxin contents were determined at different wavelengths. These are plotted in Figure 3 where a sharp peak was observed at 590 mμ, and not at 570 mμ as used by Soos. A second peak was found at 470 mμ; but repeatable results were obtained at 590 mμ with greater ease than at 470 mμ and the higher wavelength was chosen for future use. Quantities of 1 to 6 mg. per cent. of digitoxin were found to obey the Beer-Lambert law under these conditions, and in general the process was considered satisfactory.

The influence of water upon the Keller-Kiliani reaction was investigated by preparing a series of batches of reagent containing from 0·5 to 5·0 per cent. of added water; a quantity of 4 mg. per cent. of digitoxin was then estimated by means of each reagent. The results are shown in Table I, from which it is seen that the colour density for the same amount of digitoxin decreases with increase in water content of the reagent, whilst the period of time required to develop the maximum colour is correspondingly increased.

From this group of investigations it was concluded that the picrate,

TABLE I

COLOUR DENSITY OF DIGITOXIN WITH KELLER-KILIANI REAGENT IN THE PRESENCE OF ADDED WATER $\lambda = 590$ mμ

k (1 mg. per cent. of digitoxin)

	Water added to reagent per cent.							
	0·0	0·5	1·0	1·5	2·0	2·5	3·5	5·0
k 1 mg. per cent. of digitoxin	0·118	0·112	0·112	0·112	0·109	0·106	0·101	No constant colour
Time for maximum colour development (minutes) ..	15	15	20	25	25	30	40	60

dinitrobenzoate and Keller-Kiliani processes for the estimation of digitalis glycosides should be further employed in the second stage of this work.

2. THE DECOLORISATION AND ESTIMATION OF TINCTURES OR LEAF EXTRACTS

Previous workers have extracted the leaves of *Digitalis purpurea* with water, either cold or hot, or with ethanol; from these extracts the pigments have been precipitated by the addition of either lead acetate or lead sub-

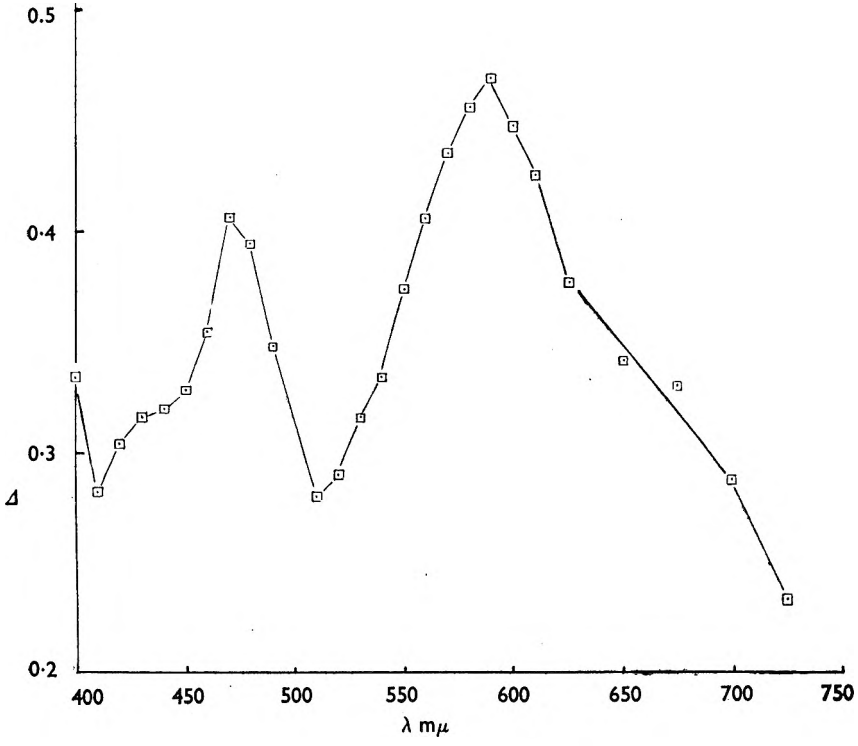

FIG. 3. Keller-Kiliani reagent and digitoxin 4 mg. per cent.

acetate solution, excess of lead being removed by means of disodium hydrogen phosphate; to the final clear filtrate representing an aqueous dilution of the original extract one or other of the chemical estimation processes have been applied. In order to compare the efficiencies of these different processes of decolorisation and to compare the results obtained by different methods of colorimetric estimation for each decolorisation process, a commercial sample of tincture of digitalis containing 1 I.U./ml. was employed for the following work.

(*a*) *The influence of ethanol on alkaline picrate reagent.* A preliminary examination was made of the method proposed by Bell and Krantz[3] in which lead acetate, followed by disodium hydrogen phosphate, was used to decolorise the tincture. Quantities of 2 ml. of final clear filtrate, being a 1 in 10 dilution of the tincture, were diluted to 5 ml. with distilled water and were mixed with 5 ml. of freshly prepared sodium picrate reagent; as blank a mixture of sodium picrate reagent with an equal volume of water was used. Colour densities were measured spectrophotometrically at 495 mμ every 5 minutes until a constant value was obtained. A constancy of colour development, corresponding to k (1 ml. tincture) = 2·607, was obtained 35 minutes after mixing and was maintained for a further 30 minutes. This prolonged period of colour development was in contrast to the 10 minutes required when digitoxin was estimated by means of the same reagent. A further estimation of the decolorised tincture carried out with the addition of 2 ml. of ethanol (95 per cent.) to both test solution and blank required 1 hour to attain a maximum colour development corresponding to k (1 ml. tincture) = 1·782. The presence of ethanol in the blank thus exerted a marked influence upon the colour of that solution, and the general process of estimation by the alkaline picrate reagent was also influenced by the amount of ethanol in the reaction mixture.

To determine the extent of this influence of ethanol upon aqueous sodium picrate reagent, a blank containing the reagent diluted with an equal volume of water was compared with similar dilutions of reagent containing from 1·4 to 20 per cent. of ethanol in the final reaction mixtures. Colour densities measured at a wavelength of 495 mμ are shown in Figure 4,A, from which it is seen that a linear relation exists between ethanol content up to 10 per cent., and colour density produced in the picrate reagent. In other words this reagent gives a direct measure of the ethanol content of an aqueous-ethanolic mixture. It thus follows that if alkaline picrate reagent is to be used for the colorimetric estimation of digitalis glycosides the ethanol content of both test and blank solutions must be the same for each estimation.

To show the extent of the influence of ethanol within the reaction mixture a series of solutions were prepared containing 2 ml. of decolorised tincture (1 in 10 dilution as described above, ethanol content 7 per cent.) mixed with 5 ml. of fresh picrate reagent and sufficient diluted ethanol to produce 10 ml. of reaction mixture containing 1·4 to 25 per cent. of ethanol. Maximum colour densities were measured against blanks containing picrate reagent with the same amounts of ethanol. The

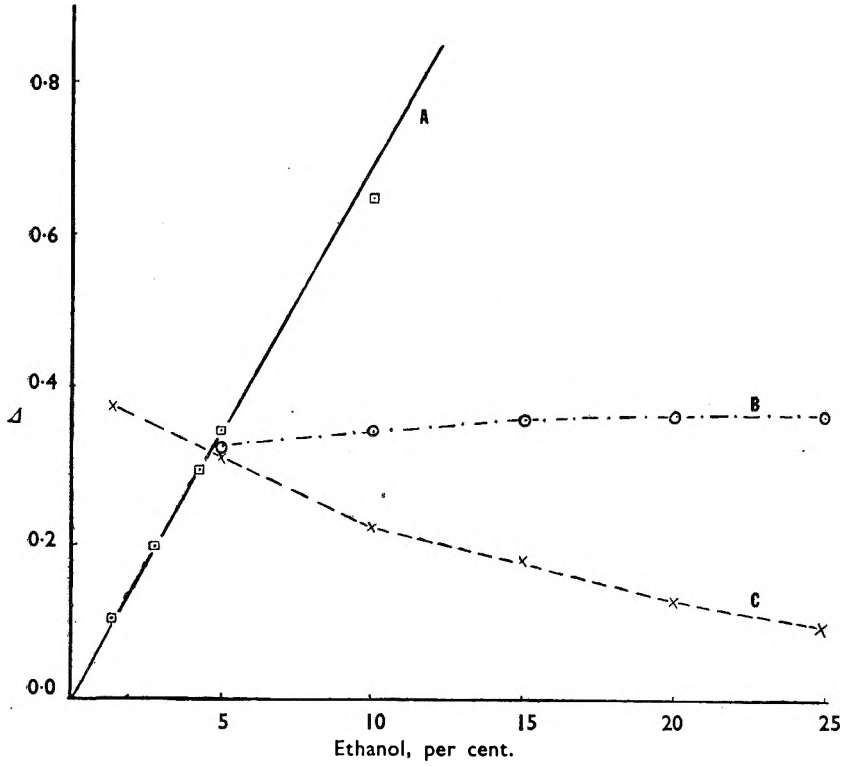

FIG. 4. Alkaline picrate reagent and ethanol.

A. Ethanol and picrate reagent; aqueous picrate blank.
B. Digitoxin 2 mg. per cent.; ethanol in test and in blank.
C. Tincture of digitalis 2 units per cent.; ethanol in test and in blank.

values are shown in Figure 4,C, from which it is seen that there is a progressive decrease in colour density for the same volume of tincture, as the ethanol level of both reaction mixture and blank rise from 1·4 to 25 per cent. The calculated values for the tincture are k (1 ml.) = 1·895 in the presence of 1·4 per cent. of ethanol, and k (1 ml.) = 0·495 in the presence of 25 per cent. of ethanol. Digitoxin in quantities of 0·2 mg. was also estimated under similar variations of ethanol content from 5 to 25 per cent. Colour density values, shown in Figure 4,B, increase significantly with ethanol levels between 5 and 15 per cent. but only to a smaller extent at ethanol levels up to 25 per cent. [k (1 mg. per cent. digitoxin) = 0·165 in the presence of 5 per cent. of ethanol; = 0·184 in the presence of 15 per cent. of ethanol; = 0·187 in the presence of 25 per cent. of ethanol].

The further usefulness of the alkaline picrate process for the estimation of digitalis extracts depended upon the possibility of obtaining reproducible results with it under carefully controlled conditions of ethanol content in both reaction mixtures and blanks. Since curve C, Figure 4, shows continued change in colour density with variation in ethanol level, replicate estimations of the same tinctures were carried out as described

above at ethanol levels of both 3·5 per cent. and 20 per cent. Reproducible values, in agreement with those of Figure 4,C, were obtained at each ethanol level. A 3·5 per cent. ethanol content of reaction mixture was chosen for future work, since this is obtained when equal volumes of alkaline picrate reagent and decolorised filtrate, prepared as described above, from tincture of digitalis are mixed. If smaller proportions of filtrate were used, ethanol (7 per cent.) was added as a diluent. Under these conditions the wavelength of maximum absorption was redetermined and was found to be 485 mμ, as shown in Figure 1. At this wavelength of observation different volumes of filtrates were estimated and the colour densities were found to obey the Beer-Lambert law when volumes equivalent to from 0·05 to 0·3 ml. of tincture of digitalis were employed, giving an average value k (1 ml. tincture) = 1·93. When volumes of filtrate equivalent to 0·4 ml. of tincture were estimated the values obtained showed a 9 per cent. deviation from the Beer-Lambert law.

(*b*) *A comparison of decolorisation processes.* It has been shown above that the process for decolorisation of tincture of digitalis by means of lead acetate, followed by alkaline picrate estimations under carefully controlled conditions, may be used to obtain repeatable results. Kedde[6] has employed a 4-stage decolorisation of digitalis tincture, tannin being removed by ferric chloride, excess of ferric chloride by sodium hydroxide, pigments being precipitated with lead subacetate solution and excess of lead removed with disodium hydrogen phosphate; the final filtrate was a 5-fold dilution of the tincture and was estimated by the dinitrobenzoate process described above. This complete process was carried out upon the stock tincture and found to give reproducible values for colour density corresponding to k (1 ml. tincture) = 0·240. The first 2 stages of this decolorisation are superfluous since tannin would be removed by lead subacetate employed in the later stage; they were thus omitted and values for colour density corresponding to k (1 ml. tincture) = 0·285 were obtained. At the same time as the dinitrobenzoate estimations were made on these 4-stage and 2-stage decolorisations employing lead subacetate, the picrate estimations at ethanol level of 3·5 per cent. were also carried out upon each filtrate. Colour density values equivalent to k (1 ml. tincture) = 1·19 and 1·47 were obtained respectively and the alkaline picrate process appeared to work as smoothly upon lead subacetate decolorisations as upon the neutral lead acetate decolorisations. The percentage loss of colour density in the first 2 stages of the Kedde process were found to be of the same order when either the dinitrobenzoate or the picrate processes of estimation were employed; but the colour density equivalent to 1 ml. of tincture using the picrate method and the last 2 stages of the Kedde (lead subacetate) process (k = 1·47) is lower than when the picrate method follows the lead acetate decolorisation (k = 1·93), as shown in Table II.

When attempts were made to carry out the dinitrobenzoate estimations upon digitalis tincture decolorisations prepared by means of lead acetate, no results were obtained. Instead of a maximum colour developing some 6 minutes after mixing the filtrate and reagent, a continuously fading

colour was produced. This was overcome by reducing the amount of disodium hydrogen phosphate in the decolorisation process, until only just sufficient was added to precipitate the excess of lead, and at the same time, in the estimation process, the mixture of filtrate and ethanolic solution of 3:5 dinitrobenzoic acid was allowed to stand for up to 30 minutes before rendering alkaline. Constant colours over the period of 2 to 6 minutes from the time of rendering the mixture alkaline were obtained giving a colour density equivalent to k (1 ml. tincture) = 0·465, but the method was somewhat inflexible. A more simple procedure was to employ just sufficient sodium sulphate to remove excess of lead acetate in the decolorisation process in place of disodium hydrogen phosphate; the unmodified dinitrobenzoate process of estimation worked smoothly on this filtrate giving colour densities equivalent to k (1 ml. tincture) = 0·473. The picrate process of estimation at 3·5 per cent. ethanol level also yielded reproducible results upon this decolorisation filtrate, as shown in Table II.

TABLE II

COLOUR DENSITY EQUIVALENT TO 1 ML. OF DIGITALIS TINCTURE (IN 10 ML. OF REACTION MIXTURE)

Process of decolorisation	Picrate estimation (3·5 per cent. of ethanol)	Dinitrobenzoate estimation
Lead acetate and sodium phosphate (Bell and Krantz)	1·93	0·465 (prolonged nitration)
Lead acetate and sodium sulphate	1·73	0·473
Lead subacetate and sodium phosphate (Kedde: stages 3 and 4)	1·47	0·285

Table II summarises the colour density values obtained by 2 different processes of estimation when applied to the same tincture of digitalis decolorised by 3 different methods; from which it is seen that the results by the picrate process are not paralleled by those of the dinitrobenzoate method. This may be due to some loss of glycosides in, as well as to interference with the estimation methods by, the different precipitation processes. The processes reported in Table II are based on those employed by previous workers and differ in volume of tincture, ratio of lead to tincture and to total volume as well as to the nature of the lead salts used. A series of decolorisations of 10 ml. quantities of the same tincture were prepared using either lead acetate or lead subacetate in the ratios of 20, 30, 40, and 50 mg. Pb. to 1 ml. of tincture, excess of lead was removed by adding sodium sulphate equivalent to total lead added and the final filtrates represented a 4-fold dilution of tincture. Each filtrate was estimated both by the dinitrobenzoate and by the controlled picrate processes. Results are shown in Figure 5, from which it was concluded that results for the same volume of tincture varied with the lead level of decolorisation when neutral lead acetate was employed (Figure 5, A and C). When lead subacetate was employed as the pigment precipitant, estimations of the same amount of tincture were identical at different lead levels, as shown by the dinitrobenzoate estimation process (Figure 5, D);

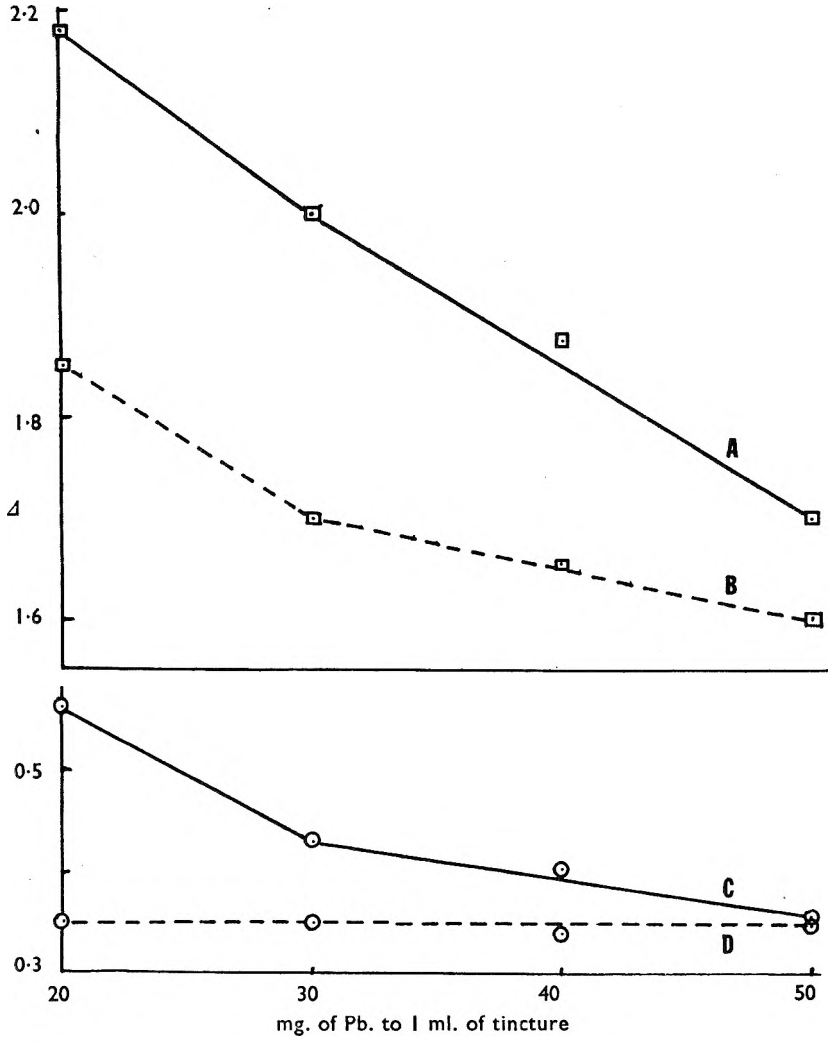

FIG. 5. Tincture of digitalis estimations; decolorisation at varying lead levels. k (1 ml. tincture).

A. Lead acetate decolorisation, picrate estimation.
B. Lead subacetate decolorisation, picrate estimation.
C. Lead acetate decolorisation, dinitrobenzoate estimation.
D. Lead subacetate decolorisation, dinitrobenzoate estimation.

estimations by the picrate process upon these same filtrates showed some variation in values (Fig. 5,B).

Figure 5 may indicate that a progressive precipitation of glycosides is occurring with increased amounts of lead added to decolorise the tincture as suggested also by Table II. However, if this were the only cause, there should be a parallelism between graphs A and C and between B and D

which does not occur; hence side reactions must also be interfering with the processes. To investigate these points, parallel experiments to the above were set up for the estimation of (*a*) digitoxin; (*b*) digitoxin and a 1 in 10 tincture of grass meal in ethanol (70 per cent.); (*c*) a second sample of tincture of digitalis containing added digitoxin. It was found that the decolorisation by either lead acetate or lead subacetate of digitoxin solution, alone or in the presence of tincture of grass, resulted in a constant loss of about 15 per cent. of glycoside as shown by both estimation processes and concordant results between the different processes were obtained; thus differing from Figure 5. Similar concordance of results was obtained for the estimations of mixed tincture of digitalis and digitoxin. These values are expressed in Table III and also the colour density values determined for the same quantities of digitoxin and of the tincture of digitalis when estimated separately under similar conditions.

From these results it is apparent that fictitiously high values were obtained for tincture of digitalis when decolorised by means of lead acetate followed by dinitrobenzoate estimation; consistency of deviations in the last line of Table III for the other 3 methods is a measure of the losses involved in the decolorisation processes.

TABLE III

COLOUR DENSITIES PRODUCED BY A MIXTURE OF DIGITOXIN AND TINCTURE OF DIGITALIS UNDER DIFFERENT CONDITIONS OF DECOLORISATION AND ESTIMATION

	Dinitrobenzoate estimation of 0·5 ml. of tincture + 0·7 mg. of digitoxin		Picrate estimation of 0·15 ml. of tincture + 0·21 mg. of digitoxin	
Decolorisation (20 mg. of Pb per ml. of tincture) by	Lead subacetate	Lead acetate	Lead subacetate	Lead acetate
Colour density of mixture	0·693	0·654	0·587	0·623
Colour due to digitoxin present	0·572	0·554	0·356	0·365
Calculated colour density due to tincture of digitalis	0·121	0·100	0·231	0·258
Results found for tincture of digitalis alone ..	0·150	0·271	0·300	0·317
Difference between calculated and observed values for tincture per cent.	19	63	23	19

The general conclusion to be drawn from this work is that decolorisation of digitalis tincture by means of lead subacetate followed by sodium sulphate and estimation by means of the dinitrobenzoate process, gives concordant results not subject to variations in lead levels or to changes in the ethanol content, within the range of 20 to 50 per cent., of the reaction mixture. The recommended process for estimation is as follows. Mix 10 ml. of tincture of digitalis with about 7 ml. of water, add 1 ml. of strong solution of lead subacetate B.P.C., adjust the volume to 20 ml., shake and filter. To 10 ml. of clear filtrate add 2 ml. of a 6·3 per cent. solution of sodium sulphate (Na_2SO_4 $1OH_2O$), adjust the volume to 20 ml., shake and filter through a fine filter paper (Whatman No. 42). 1 ml. of filtrate is equivalent to 0·25 ml. of tincture and has ethanol content

17·5 per cent. [This decolorisation employs 25 mg. of Pb. to 1 ml. of tincture; if it is desired to employ a ratio of 50 mg. of Pb. to 1 ml. of tincture, the quantities of strong solution of lead subacetate and of sodium sulphate solution should be doubled.] Measure a suitable volume of this filtrate (usually 2 to 4 ml.) into a 10-ml. stoppered measuring cylinder, adjust to 4 ml. by the addition of ethanol (17·5 per cent.), add 5 ml. of a freshly prepared 0·8 per cent. solution of 3:5-dinitrobenzoic acid in ethanol (95 per cent.), mix and add 1 ml. of N sodium hydroxide, at the same time prepare a blank solution as above but omitting the decolorised filtrate. Transfer both solutions to a spectrophotometer and measure the colour density of the test solution relative to the blank at wavelength 535 mμ every minute until a maximum value is obtained.

The process proposed by Soos[8] for the extraction of digitalis leaf, followed by decolorisation and estimation, employing the Keller-Kiliani method, was investigated in 2 stages. Soos extracted powdered digitalis leaf by shaking with water for 1 hour; this solution was decolorised by adding lead subacetate and filtering; the filtrate was shaken out with 3 quantities of chloroform which leaves any free digitoxose in the aqueous layer but extracts the total glycoside and any aglycone; in this mixture the Keller-Kiliani process estimates glycoside only. To test the possibility of totally extracting all glycoside and aglycone with chloroform from an aqueous solution, a bulk of tincture was decolorised by lead subacetate and, as a control, an aliquot part was estimated by the dinitrobenzoate and the Keller-Kiliani processes. The bulk of filtrate was then diluted with water and extracted with successive 25-ml. quantities of chloroform, from the separated chloroform layers the solvent was removed and the extracted glycosides determined. It was found that 3 quantities of chloroform extracted about 73 per cent. of the glycosides present, 6 quantities extracted 78 to 82 to 86 per cent. and 12 quantities of solvent extracted less than 90 per cent. of glycosides. Thus the process did not seem very promising and the Soos claim of extraction with 3 quantities of chloroform is wrong. The aqueous extraction of a powdered leaf sample was then attempted by the Soos method; the aqueous filtrate being extracted with 6 quantities of chloroform. Since the efficiency of the aqueous extraction alone was under test, the dinitrobenzoate estimation process was used. 3 repeats of the process gave values of 45 to 47 to 55 per cent., of those obtained when the same sample of digitalis leaf was estimated independently by the recommended process for estimation described above. If we assume the chloroform extractions to have an 82 per cent. efficiency this means that the aqueous extraction yields 60 per cent. of the activity present in the sample. Thus both stages of this process are considered inefficient as at present proposed.

Discussion of Results

Alkaline picrate reagent has been extensively used for the quantitative estimation of either digitoxin or decolorised preparations from digitalis tinctures by many different workers and has been hotly criticised as non-specific and untrustworthy by other workers. The present investigations

have shown that the ethanol level of the reaction mixture is the most important factor to be controlled when this reagent is employed. Figure 4,A, shows that sodium picrate reagent may be employed for the colorimetric estimation of ethanol present in aqueous dilutions and that the reaction follows the Beer-Lambert law with a steeply rising graph of colour density plotted against ethanol content. It thus follows that when this reagent is used for quantitative estimations by spectrophotometric methods employing a control solution as blank, that control solution must contain exactly the same amount of ethanol as is present in the reaction mixture in order to balance, in both solutions, the colour produced when solvent and reagent interact. By a careful observance of these conditions the picrate reagent has been shown to yield concordant results for the estimation of either digitoxin or preparations from powdered digitalis leaves. These reactions have been shown in each instance to follow the Beer-Lambert law over a reasonably wide range of concentrations which may be employed in such quantitative estimations. An examination of the published work dealing with the use of this reagent in the investigation of digitalis glycosides suggests that this significance of ethanol levels has not been appreciated. Some workers, perhaps fortuitously, have employed the same concentrations of ethanol in both blank and reaction mixtures, whilst others have not done so and in these instances such lack of balance may explain why the reaction did not follow the Beer-Lambert law.

Under a number of conditions of estimation the behaviour of tincture preparations have not paralleled those of solutions of digitoxin. This is seen in the slower development of maximum colour density of the former with sodium picrate reagent, and this colour density reaches a maximum at wavelength 485 mμ for tincture preparations, but the maximum is at wavelength 495 mμ for the samples of digitoxin examined, as shown in Figure 1. Moreover, the behaviour of digitoxin and tincture preparations differ when estimated by sodium picrate reagent in different concentrations of ethanol, as shown in Figure 4,B and C, the colour density of the former increasing somewhat with increase in ethanol concentration of both test and blank for the same concentration of digitoxin; the latter showing considerable decrease in colour density under the same conditions. It thus follows that colour density values for tincture preparations cannot be equated to those of digitoxin under these conditions of estimation with sodium picrate with the object of expressing the results for tinctures as an equivalent digitoxin content.

No explanations are offered in the present paper for the differences in behaviour of digitoxin and of preparations of tincture of digitalis discussed above and also reported in Figure 5 and Tables II and III. It was however concluded that sodium picrate reagent under very carefully controlled conditions might be used for the estimation of digitalis preparations. The control of ethanol levels in the extraction and estimation of powdered leaves is practicable but the examination of galenical preparations would necessitate the determination of their ethanol contents. The choice of the correct wavelength for observation is somewhat critical

involving the use of a suitable spectrophotometer as shown by the shape of the curve peaks in Figure 1. Figure 1 also supports the work of Abrams[9] in criticising the choice of wavelength 525 mμ by Bell and Krantz and employed also in the United States Pharmacopoeia XIV for the reaction. Wavelength 495 mμ should be used for digitoxin estimations and 485 mμ for digitalis tincture estimations.

The use of 3:5-dinitrobenzoic acid in alkaline solution for these estimations was smooth and rapid. It was not susceptible to variations between 20 to 50 per cent. of ethanol present in the final reaction mixture, hence the control of conditions in which this reagent may be employed is not so stringent as for the picrate reagent. The dinitrobenzoate reagent develops a maximum colour density with either digitoxin or tincture preparations in about 6 minutes after mixing, when measured at wavelength 535 mμ and compared with a blank of diluted reagent. These conditions result in the reaction following the Beer-Lambert law. They differ from those proposed by Kedde[6] who measured colour densities 1 hour after mixing and used as a blank a dilution of decolorised tincture only. The shape of the curves in Figure 2 suggests that the wavelength of observation need not be so critically observed as for the picrate reagent; Kedde has employed wavelength 530 mμ for making his observations. Canbäck[1] has stated that the modifications of the Raymond process using *m*-dinitrobenzene or dinitrobenzoic acid are to be preferred to the use of picrate for the estimation of digitoxin; throughout the work reported in this paper the dinitrobenzoic acid reagent has also been found satisfactory and flexible when employed as the recommended process for estimation.

Canbäck's process for the estimation of digitoxin and other isolated glycosides by means of *m*-dinitrobenzene in ethanol (95 per cent.) was found to be satisfactory in operation but did not offer any obvious advantage over the dinitrobenzoic acid method. It has not been applied to tincture preparations and the need to maintain an ethanol level of at least 40 per cent. in the final reaction mixture would present some difficulties in the handling of such preparations. Also the method of obtaining colour density values at zero time by graphic extrapolation demands a critical accuracy in timing the observations to be extrapolated. In the first 2 minutes after mixing the colour fade is very rapid at room temperatures and readings must be made at exact half-minute intervals; this is difficult to secure with a spectrophotometer circuit that must be balanced against a colour-changing blank for each observation. With these considerations in mind the dinitrobenzene process has not been explored further.

The results obtained in the series of experiments based upon the use of either lead acetate or lead subacetate for the decolorisation of tinctures of digitalis have been discussed above. The deductions which were made have led to the recommended process for decolorisation and estimation. It was found that digitoxin either alone or in the presence of a chlorophyll solution in ethanol (70 per cent.), extracted from grass meal, could be submitted to the decolorisation process using different amounts of either

lead acetate or lead subacetate. Under these variable conditions a constant estimation figure for the same amount of glycoside was obtained, showing a loss of about 15 per cent. of glycoside in the process by either the dinitrobenzoate or controlled picrate estimations. Similar results, shown in Table III, were obtained for a mixture of digitoxin and tincture of digitalis, showing a loss in the process of about 20 per cent. except for the decolorisation using lead acetate, followed by dinitrobenzoate estimations where an abnormally high assay figure was obtained. This is in agreement with the values shown in Figure 5 and in consequence lead subacetate was preferred for decolorisation of tinctures. The lack of parallelism between the picrate and dinitrobenzoate estimations of the same series of solutions shown in Figure 5,B and D, is of interest and it suggests that the picrate process is more susceptible to interference.

The process employed by Soos[8] for the extraction of powdered digitalis leaf by shaking with cold water for 1 hour has been shown to extract only 60 per cent. of the activity yielded to ethanol (70 per cent.) after a 48 hours' maceration. Also it has been found that the glycosides present in such an aqueous extract cannot be conveniently extracted by means of chloroform. In consequence this method for the estimation of the unhydrolysed glycosides of digitalis leaf by means of the Keller-Kiliani reaction has not, as yet, been investigated further.

Summary and Conclusions

1. Colorimetric processes have been examined for the estimation of digitoxin by means of alkaline picrate, alkaline *m*-dinitrobenzene, alkaline 3:5-dinitrobenzoic acid or the Keller-Kiliani reagents. The conditions under which these reactions obey the Beer-Lambert law, when measured spectrophotometrically, have been investigated.

2. A process is recommended for the decolorisation of digitalis tinctures and their estimation by means of alkaline solution of 3:5-dinitrobenzoic acid.

3. Lead subacetate is recommended for the decolorisation of tinctures of digitalis in preference to lead acetate.

4. Alkaline picrate reagent produces a colour with ethanol which interferes with the use of this reagent for the estimation of digitalis glycosides, unless the ethanol contents of test solution and blank are identical.

5. The colour density produced by decolorised tincture of digitalis and alkaline picrate reagent decreases with increase in ethanol content of reaction mixture; digitoxin under similar conditions gives increases in colour densities.

6. Alkaline picrate reagent may be used for the quantitative estimation of digitalis glycosides when the ethanol levels are controlled, but the reaction is sensitive to changes in concentration levels of reagents employed for the decolorisation of tinctures.

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STUDIES IN THE GENUS *DIGITALIS*

PART II. A COMPARISON OF THE COLORIMETRIC AND BIOLOGICAL METHODS FOR THE EVALUATION OF *DIGITALIS PURPUREA*

By J. M. ROWSON and F. J. DYER

From the Museum of the Pharmaceutical Society of Great Britain and The Laboratory Animals Bureau, Medical Research Council, Hampstead

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IN a previous paper[1] of this series a number of colorimetric methods for the estimation of digitalis glycosides have been investigated and conditions under which they may be applied to the examination of digitalis preparations were determined. This present paper reports the results obtained when 16 different powdered leaf samples of *Digitalis purpurea* were examined by these colorimetric processes and also by biological methods.

EXPERIMENTAL

Chemical. All leaf samples were extracted by mixing 1 part by weight of powdered leaf with 10 parts by volume of ethanol (70 per cent.) and allowing to macerate with gentle, continuous agitation for 48 hours. This process has been shown to extract the total glycosides present and has been employed regularly by one of the present authors (F.J.D.). An apparatus was designed for the agitation of a number of samples; 4 galleries each holding ten 60-ml. screw-capped bottles were built onto a wheel which was rotated by means of a low-speed electric motor provided with a variable resistance for control of speed of rotation. The efficiency of the newly designed apparatus for extraction was checked upon a commercial sample of Digitalis Pulverata containing 10 I.U./g. Tinctures were prepared by agitation for 48 hours and also for 72 hours; each was then estimated colorimetrically by the recommended process for estimation.[1] Colour densities for tinctures prepared by the two periods of maceration were the same.

It has been shown in a previous paper[1] that the behaviour of different volumes of one decolorised and diluted tincture of digitalis in the dinitrobenzoate process of estimation was in accordance with the Beer-Lambert law. A more detailed investigation of the application of this law to different extracts from the sample of Digitalis Pulverata was designed. The recommended process for estimation was modified by:—(*a*) tinctures containing between 0·5 and 1·75 I.U./ml. were prepared by using different weights of leaf sample and 10 ml. quantities were used for decolorisation; (*b*) a bulk tincture containing 1·75 I.U./ml. was prepared, aliquot volumes being used along with added ethanol (70 per cent.) for decolorisation to give the same range of concentrations as those prepared in (*a*) above; (*c*) a volume of 10 ml. of the bulk tincture containing 1·75 I.U./ml. was decolorised and different volumes of clear filtrate were employed for estimation. All solutions were estimated by means of the dinitrobenzoate process and concordant values were obtained at each concentration level

for each of the 3 types of leaf extract (*a*), (*b*) and (*c*) above. Over the range of concentrations of 5 to 15 I.U. per cent. for reaction mixture, the value of k (10 I.U. per cent.) was 0·274 to **0·286** to 0·301. At the level of 17·5 I.U. per cent. k (10 I.U. per cent.) = 0·261; thus decolorised tinctures when estimated by the dinitrobenzoate process obey the Beer-Lambert law if between 5 and 15 I.U. of activity are present in each 100 ml. of final reaction mixture. The picrate estimation at 3·5 per cent. ethanol level was also applied to each of the filtrates and similar concordant results were obtained over the range of 7·5 to 17·5 I.U. for tinctures, k (10 I.U. per cent.) = 1·07 to **1·11** to 1·15. Thus the recommended method of extraction of digitalis leaf powder may be conveniently applied to a range of samples differing widely in the amounts of glycosides present, and proportionate differences in colour densities will be obtained in their colorimetric estimations.

The conversion into equivalent digitoxin contents of figures obtained by the picrate estimations of tinctures of digitalis have been shown[1] to be of little value, hence it seemed desirable that all estimations of leaf samples should be expressed in terms of international units of activity. A sample of Standard Preparation of Prepared Digitalis supplied by the Medical Research Council and containing one I.U. in 76 mg. was examined. 4 tinctures of I in 10 concentration were prepared as described above and were examined by the recommended process of estimation, values obtained were k (10 I.U. per cent.) = 0·265, 0·275, 0·289, 0·294, average = 0·281. Decolorisations were also carried out at the higher lead level of 50 mg. of Pb. to each ml. of tincture and also all solutions were estimated by the picrate process at 3·5 per cent. ethanol level. These results are shown in Table I.

TABLE I

STANDARD PREPARATION OF PREPARED DIGITALIS

Colour densities k (10 I.U. per cent.)

	Lead level employed in decolorisation (mg. of Pb per ml. of tincture)	
	25	50
Dinitrobenzoate estimation ..	0·281	0·270
Picrate estimation in 3·5 per cent. ethanol	1·138	0·985

10 samples of the powdered leaf of *Digitalis purpurea* were estimated by these processes. The samples were harvested in September, 1951, from first year plants grown in the Museum Experimental Grounds from seeds of this species collected in different geographical areas. All leaf samples were rapidly dried at 55° C. immediately after collection, were reduced to powder, were further dried and securely bottled. Each wide-mouthed bottle for storage was fitted with an aluminium container, holding silica gel, rivetted onto an aluminium disc which acted as a washer to the screw-capped closure; the perforated lower half of the silica gel container was lined with a layer of filter paper thus preventing the contamination of the

leaf sample. Moisture contents of the 10 samples after 6 months' storage under these conditions, determined by drying at 105° C. for 5 hours, were 3·8 to 5·3 per cent. Tinctures were prepared in duplicate by maceration with agitation for 48 hours, they were decolorised by the recommended process described previously,[1] using lead subacetate both at the 25 mg and 50 mg. levels of Pb to each ml. of tincture. The decolorised filtrates were then estimated by both the picrate process in 3·5 per cent. ethanol and the dinitrobenzoate process. In each estimation 2 different quantities of decolorised filtrate were taken (e.g., 2 ml. and 3 ml. for dinitrobenzoate estimation) to ensure that in every reaction mixture the Beer-Lambert law was operating. Results were calculated in terms of I.U./g. of powdered leaf by using the values of Table I. Every preparation followed the Beer-Lambert law and concordant results between duplicate tinctures from each sample of leaf were obtained. Results are given in Table III. The total solids present in each tincture was estimated by the evaporation

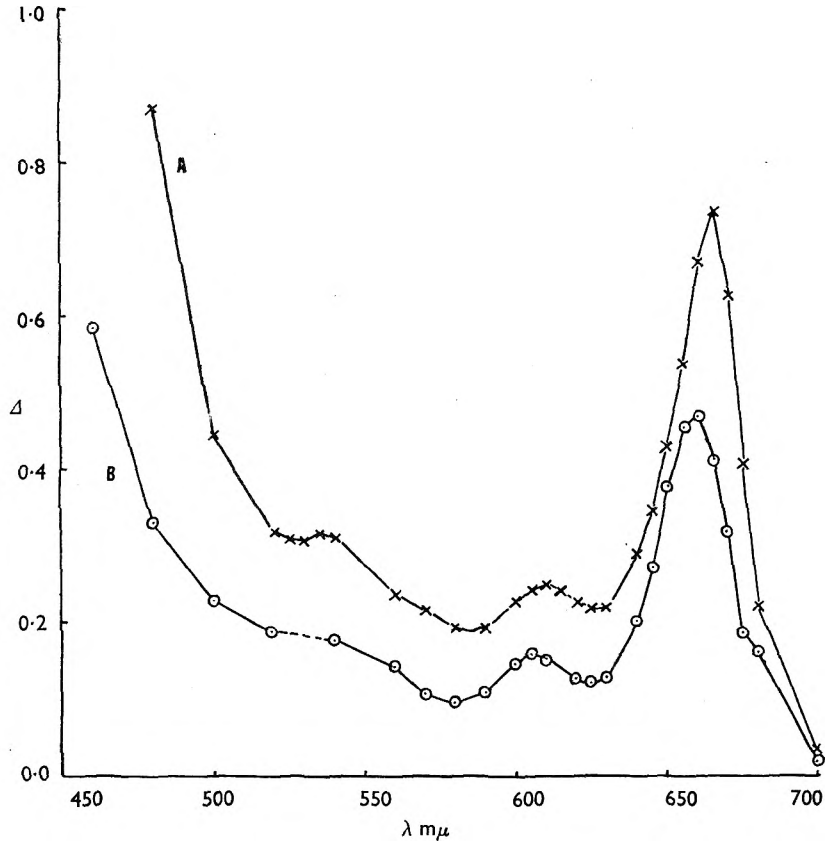

FIG. 1. Colour density of tincture of digitalis (diluted 1 in 10).
A. Dilution of tincture from leaf sample A.
B. Dilution of tincture of digitalis B.P.

of 5 ml. quantities and drying at 105° C. for 18 hours. Results for duplicate tinctures from each sample of leaf were in agreement and values for each sample are also given in Table III.

A subsidary investigation was made of the changes occurring in the decolorised filtrates upon storage at laboratory temperatures. The values reported in Table III were based upon estimations made within 4 hours of the decolorisation of the tinctures. These 40 filtrates were re-examined after 24, 48 and 72 hours' storage and it was found that results varied between +1·1 to **0·23** to 1·8 per cent. from those obtained initially, which is within the order of experimental error.

During the systematic biological estimation of many digitalis leaf samples one of the present authors (F.J.D.) formed an impression that a poor assay figure is often associated with an inferior colour of the tincture prepared for estimation, but as a result of this present work the impression has not been sustained. Preliminary experiments showed that a 10-fold dilution of tincture of digitalis with ethanol (70 per cent.) could be examined in the spectrophotometer. Colour densities at wavelengths between 400 and 700 mμ were determined for commercial tincture of digitalis and also for a 1 in 10 tincture prepared from sample A, using ethanol (70 per cent.) as a blank. Values are plotted in Figure 1, from which it is seen that good agreement between the 2 samples was obtained. 2 maximum colour density peaks were found at 610 mμ and, most pronouncedly, between 650 and 670 mμ. A subsidiary peak also exists at

TABLE II

MAXIMUM COLOUR DENSITIES AND WAVELENGTHS OF MEASUREMENTS FOR TINCTURES OF DIGITALIS DILUTED 10-FOLD

Sample	λ of observation mμ	Colour density
A to K	665	0·526–0·910 (See Table III)
Digitalis Pulverata (comme cial)	665	0·317
Tincture of digitalis B.P. No. 1 ..	660	0·472
Tincture of digitalis B.P. No. 2 ..	655 and 660	0·440

TABLE III

ESTIMATION OF DIGITALIS LEAF POWDERS

Sample \ Decolorisation at lead level per ml. of tincture	Dinitrobenzoate estimation I.U./g.		Picrate estimation in ethanol (3·5 per cent.) I.U./g.		1 in 10 tincture	
	25 mg	50 mg.	25 mg.	50 mg.	Total solids per cent.	Colour density of 1 in 10 dilution at 665 mμ
A	13·1	12·6	12·8	14·3	3·87	0·728
B	12·9	13·6	14·0	15·3	3·51	0·526
C	11·7	12·8	13·1	14·3	3·80	0·799
D	14·4	13·8	15·8	14·4	3·92	0·752
E	12·5	13·3	12·8	13·9	3·88	0·777
F	12·0	13·5	14·1	13·9	3·65	0·572
G	15·0	14·8	14·7	14·9	3·89	0·910
H	11·9	11·5	13·9	15·1	3·79	0·767
J	13·7	13·7	15·3	14·7	3·80	0·728
K	15·6	15·6	14·8	16·4	3·74	0·893

535 mμ. The peak between 650 and 670 mμ was steep and this peak wavelength differed for the 2 tinctures but it was chosen for the examination of each of the 10 leaf samples. Colour densities were determined at wavelengths differing by 5 mμ intervals between 650 and 670 mμ and the value for maximum colour density was employed. These values are expressed in Tables II and III: figures for samples A to K are averages of readings obtained from duplicate tinctures and each pair showed good agreement.

The samples of which the estimations are given in Tables III and V were all grown, dried and stored under similar conditions and at the same time; also their activities show them to be average to good samples. It was thus desired to examine commercially produced samples which were average to poor in activities. These were decolorised at the 25 mg. lead level and estimated by the recommended process for estimation; picrate estimations were also carried out. The results are shown in Table IV.

TABLE IV

ESTIMATION OF COMMERCIAL LEAF SAMPLES (AVERAGE TO POOR)

Sample	Dinitrobenzoate estimation I.U./g.	Picrate estimation in ethanol (3·5 per cent.) I.U./g.
I	11·3	13·2
II	14·0	14·4
III	7·9	7·2
IV	13·5	13·7
V	11·7	16·6
VI	15·3	15·8

Biological. In a previous study upon the content of active principle in strains of digitalis (Mather and Dyer)[2] frogs were used to assess the potency. The British Pharmacopœia, 1948, permits the use of cats, guinea-pigs and frogs, and at the same time indicates that any other biological method fulfilling the basic principles of biological standardisation, viz., the use of standard and test preparations simultaneously, may be employed. In the present series of assays, guinea-pigs were used, the method followed being that originally described by Knaffl-Lenz,[3] as modified by Gage.[4] When this method was employed as a routine procedure in the Pharmacological Laboratory of the Pharmaceutical Society, and later in the School of Pharmacy of the University of London, the error of the test when 14 animals were used for the standard and not less than 6 for each test preparation was calculated by Emmens[5] to be ± 14 per cent. ($P = 95$). This calculation of accuracy was based on the procedure where the animal is allowed to respire unaided, i.e., without tracheal tube attached to a respiratory pump. In the present series some of the experiments have been done using this procedure, whilst in a few others tracheal tube respiration was used. Consequently, for some of the experiments, a laboratory standard figure of 1·28 I.U./kg. of body weight is used, and for the others (artificially respired) a figure of 2·25 I.U./kg. is employed.

It is, of course, understood that the potency of a "test" preparation is always determined against the International Standard, or a Laboratory sub-standard checked periodically against the International Standard. Although the chief purpose of this series of bioassays is to provide a "yard-stick" to measure the validity of the chemical methods described by Rowson,[1] there is also a subsidiary purpose. Some of the animals employed were purchased from breeders or dealers and may be described as mixed breed: others were obtained from a closed colony and the animals are, therefore, more genetically homozygous than the very heterozygous animals designated as mixed breed. From the results of assays based upon these 2 types of animals an indication was sought whether the closed colony cavies would give a greater degree of accuracy than the mixed breed animals. In this respect the present series can only be regarded as preliminary.

In brief the method of bioassay employed was as follows:—A freshly prepared tincture, representing 10 per cent. w/v of digitalis leaf in ethanol (70 per cent.), was diluted 10-fold with physiological saline solution and slowly infused intravenously into guinea-pigs previously anæsthetised by giving urethane 1·25 g./kg. by the intra-peritoneal route. The rate of infusion was regulated so that cessation of heart-beat occurred not sooner than 20 minutes nor later than 50 minutes, the animals being maintained at uniform temperature throughout the experiment. The lethal dose calculated in terms of ml. of the test tincture (or I.U. of Standard)/kg. of body weight of guinea-pigs, was recorded for at least 4 animals per "unknown" tincture, and for at least 10 animals on the standard tincture. The results of a series of assays made on 10 cultivated leaves and upon several commercial samples are shown in Tables V and VI.

Discussion of Results

Before referring to the intrinsic merits of the chemical sorting test for digitalis, a few observations on the bioassay may be appropriate. In the course of these assays there were used guinea-pigs from 2 main sources, the

TABLE V

Bioassays of digitalis leaf powders

Digitalis leaf	Number of guinea-pigs used	Potency values: I.U./g.	
		Range of results	Mean
International standard	15	11·0 to 19·1	[13·2]
A	8	11·7 „ 16·2	14·1
B	8	9·3 „ 15·3	12·4
C	4	10·8 „ 14·4	12·0
D	4	10·6 „ 14·9	12·7
E	4	10·8 „ 12·3	11·5
F	4	11·4 „ 14·8	13·1
G	8	11·7 „ 13·0	12·2
H	4	11·7 „ 13·6	12·7
J	4	10·9 „ 14·0	12·7
K	4	15·1 „ 16·7	15·9
International standard	10	11·8 „ 15·3	[13·2]

Note: Using 10 animals on Standard and 4 animals on a "Test" preparation, the error of the test may be expressed 100 ± 22 (p = 0·95).

TABLE VI

BIOASSAYS OF COMMERCIAL LEAF SAMPLES

Commercial digitalis leaf	Number of guinea-pigs used	Potency values: I.U./g.
International standard	10	(13·2)
I	6	7·9
II	6	5·2
III	5	4·9
IV	4	13·1
V	8	10·6
VI	8	12·0

TABLE VII

COMPARISON OF RESULTS

Cultivated samples			Commercial samples		
Sample	Recommended colorimetric estimation	Bioassay	Sample	Recommended colorimetric estimation	Bioassay
A	13·1	14·1	I	11·3	7·9
B	12·9	12·4	II	14·0	5·2
C	11·7	12·0	III	7·9	4·9
D	14·4	12·7	IV	13·5	13·1
E	12·5	11·5	V	11·7	10·6
F	12·0	13·1	VI	15·3	12·0
G	15·0	12·2			
H	11·9	12·7			
J	13·7	12·7			
K	15·6	15·9			

first being commercially bred, and the second closed-colony reared animals. It has emerged, during this investigation, that the lethal dose (I.U./kg.) for the International Standard varies from 1·28 to 1·66 according to their source of supply. Again, experience in 2 different laboratories, employing slightly varying technique, gives figures differing as widely as 1·28 and 2·25 I.U./kg. There is some evidence that, within limits, light-weight animals (190 to 260 g.) show a higher lethal value than others belonging to a heavier weight-group (350 to 520 g.). This merely emphasises the necessity for carrying out simultaneous determinations of T and S—or at least of frequently redetermining any "accepted" laboratory value of S—in every estimation of potency. Although the main object of this investigation is to compare 2 different types of sorting test (bearing in mind the limits of error of each), an ancilliary purpose was to collect data so as to decide whether closed-colony cavies give statistically more accurate results than mixed breed animals. Preliminary indications are in favour of the animals from the closed-colony.

Previous work has indicated[1] that the colorimetric estimation of digitalis preparations by means of dinitrobenzoic acid is more accurate and less susceptible to interference than when the alkaline picrate process is employed. The present investigation has confirmed these findings. In Table III both processes of estimation have been applied to preparations decolorised by 2 different amounts of lead subacetate; results for which are the same by the dinitrobenzoate method but are different by the picrate

process. Moreover, when results obtained by biological estimations are compared with those yielded by these 2 colorimetric processes it is seen that a closer approach is achieved by the dinitrobenzoate method. All these findings confirm the preferential choice of the dinitrobenzoate process for estimation, and although the absolute loss of glycoside in the decolorisation process has been found to be 15 to 20 per cent., this would appear to be a constant figure; hence it is cancelled out when the estimations of unknown samples are compared with those for the Standard Preparation of Prepared Digitalis.

A critical comparison of the results obtained by methods for the chemical estimation of 16 samples of digitalis leaf with those obtained by biological assay is obtained by examining Tables III to VII. A deviation in value of ± 10 per cent. may be considered reasonable in the values for chemical estimation, although a smaller error is probable. For two samples, II and VI, there is no good parallelism between either colorimetric estimation and those obtained biologically; for one sample, A, the picrate estimation is in closer agreement with the biological assay but for the remaining 13 samples the dinitrobenzoate estimations show a closer agreement with the biological assays than do the values by the picrate process. The results expressed in Table III for dinitrobenzoate estimations at the 25 mg. Pb level of decolorisation of tinctures prepared from samples A to F and H to K are in agreement, within the limits of experimental error, with those for biological estimations. Thus, if this colorimetric estimation were used as a sorting test, these 9 leaf samples would have been passed as of pharmacopœial quality with values ranging to only a limited extent from that of the Standard Preparation of Prepared Digitalis (13·2 I.U./g.). Sample K possesses the highest activity as shown by both estimations. There is a marked high assessment of sample G by colorimetric estimation as compared with the biological assay, although some difficulties have been experienced with the examination of this sample and it is still the subject of investigation.

The results of Table IV are a more critical test of the colorimetric method as at present proposed, for the samples are derived from different commercial sources and were deliberately chosen for the inferior quality of some of them. Sample I was known to be of continental origin. When the dinitrobenzoate assay is used as a sorting test, sample III would be rejected as poor, sample I might be suspect and samples IV to VI would be passed as satisfactory, thus agreeing with the biological assay although over-valuing samples I and VI. The results for sample II are, however, violently in conflict and the sample is being further examined.

It is apparent that the colorimetric process for the estimation of digitalis is of use, in association with biological methods. It is fully appreciated that, as used at present, this method estimates total glycosides plus aglycones and this may account for the occasional falsely high assessment obtained of a poor drug such as sample II. Such a faulty assessment is probably coupled with bad conditions of preparing the drug for the market by which hydrolysis of glycosides may occur. If the conditions of harvesting, drying and storage of digitalis leaf samples are carefully controlled,

as for those of Table III, then the colorimetric process, as at present proposed, may be used with some confidence as a sorting test.

Fuchs, Soos and Kabert[6] have compared the colorimetric and biological assessments of a number of *Digitalis* species. They found some agreement between biological values and those obtained by the dinitrobenzoate or other butenolide-estimating processes, but a closer parallel was obtained with the Keller-Kiliani process. All of these estimations both chemical and biological, are based upon aqueous extraction of the leaf, precipitation with lead subacetate and extraction of glycosides with three quantities of chloroform. Such extractions are subject to considerable losses of glycosides and so the value of the comparisons is somewhat weakened. That loss in glycosides had occurred in the Fuchs work is seen by the equivalent digitoxin yields calculated, which are of the order of 0·1 to 0·2 per cent. for leaf samples of *D. purpurea* and similar values are quoted by Langejan.[7] If values obtained for samples A to K in Table III are calculated on the basis of k (1 mg. per cent. of digitoxin) = 0·192 (see Rowson,[1] Figure 2) then digitoxin contents of the order 0·5 per cent. for these leaf samples of *D. purpurea* are obtained. It is thus concluded that the present work gives a more faithful comparison between the estimations of leaves of this species by biological methods and by certain colorimetric processes. These investigations, including the examination of deviations reported above, are being continued.

Summary and Conclusions

1. The choice of alkaline solution of 3:5-dinitrobenzoic acid for the colorimetric estimation of preparations of *Digitalis purpurea* is preferred to that of sodium picrate reagent.

2. Results of the bioassays, in which guinea-pigs from 2 distinct sources were used, provide evidence in favour of a slightly greater degree of accuracy in the closed colony animals.

3. Slight differences in weight of animals, or in details of technique, may seriously affect the lethal doses of digitalis per kg. of guinea-pig. Hence simultaneous determinations of the "laboratory standard value" is essential, whenever a bioassay of digitalis is made.

4. Estimations have been made of the potency of 10 samples of *D. purpurea* leaves grown and dried under good conditions. They show a very close correlation for 9 samples between results for bioassays and for colorimetric estimations using the dinitrobenzoate process. The tenth sample shows a deviation of 19 per cent. between the two processes.

5. 6 leaf samples, chosen because they were average to very poor, were examined by chemical and biological methods. A fair agreement between the 2 methods was found for 4 samples and marked disagreement for 1 sample. This was a very deliberate "weighting" of the test by selecting bad samples and it cannot be regarded as a random sampling of commercial specimens of *Digitalis purpurea*.

6. It is concluded that the recommended process for colorimetric estimation may be used as a sorting test for leaf samples which have been prepared for the market in accordance with pharmacopœial instructions.

We wish to thank Miss M. E. Cammiade and Mr. H. J. Fearn for technical assistance with bioassays. One author (J.M.R.) is also indebted to the Agricultural Research Council for a grant towards the cost of the drug drying plant employed in the Museum Experimental Grounds.

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DISCUSSION

The three papers on Digitalis were discussed together. The paper on Digitalis Glycosides was presented by Mr. C. J. Eastland, and the other two by Dr. J. M. Rowson.

The CHAIRMAN referred to Picot's work with *Rana esculenta* and *Rana temporaria*. Marked differences in effect were observed with similar doses in the two species. No species variation was observed with a preparation made with hot water. There appeared to be no explanation or contradiction of that work.

MR. C. J. EASTLAND commented on the high degree of colour reported by Dr. Rowson in the Baljet reaction, involving the use of alkaline picrate with alcohol. He confirmed the intense coloration when industrial alcohol was used, but with Analar ethanol the amount of colour produced was very small. Blank readings, however, had been carried out with the alcohol actually used. All results were obtained on samples of mixed chloroform-soluble glycosides, and it was possible that the discrepancy between the colorimetric and biological assays might be greater when dealing with a mixture of relatively pure glycosides than when much cruder extracts were being tested. The latter preparations contained, in addition to the chloroform-soluble glycosides, the water-soluble glycosides.

DR. J. M. ROWSON agreed that he used industrial spirit, but his work had been of an introductory nature and primarily concerned with proving his methods. Mr. Eastland had dealt with the very important point of colour produced by ethanol, but had he investigated the effect illustrated in Figure 4, which showed that with the leaf preparations the colour density was markedly affected by variation in the ethanol levels? A 1 per cent. change in ethanol level, for example from 25 to 24 per cent., brought about a 12 per cent. change in the glycosidal content as shown in the assay. He pointed out that Mr. Eastland was dealing with a different type of material and was separating it fractionally. Therefore different results were understandable. He did not like the picrate process very much. He noted that Mr. Eastland stressed that 20 minutes should be allowed for colour development and asked whether he had plotted

the colour development against time? In his experience with preparations of the leaf, the maximum depth of colour did not develop until 40 to 45 minutes had elapsed.

Mr. Eastland had criticised the "chemical assay" on the basis of the results he had obtained, his criticism should have been directed to the particular chemical assay procedure which he had used. He asked whether Mr. Eastland could say anything about the quality of the digitoxin used as a standard; it was stated in the paper to have a potency of 1000 I.U./g. which indicated that it was only about 50 per cent. pure. Pure digitoxin had a potency of 1750 I.U./g. but was not available in this country.

DR. G. E. FOSTER (Dartford) said that no attempt should be made to determine colour visually; a photo-electric instrument should always be used. It was a pity that pharmacologists did not record the actual dosage at which the guinea-pig heart began to slow and the dosage at which it stopped, because there was a difference between the cardiotoxic and cardiotonic effects and the ratio was not the same for different batches. In a paper which was to be published shortly it was shown that if less alkali were used in the dinitrobenzene reagent the colour obtained was red and the test was useful in the determination of individual glycosides. Some progress had been made in determining individual glycosides by using paper chromatography. If a glycosidal mixture were run on paper using a mixture of chloroform, methanol and water, then dried off and heated to 100° C. spots of different glycosides would be seen clearly.

DR. W. MITCHELL (London) said that Pratt, in a recent paper in *Analytical Chemistry*, had described a method which was similar to Dr. Rowson's procedure using 3:5-dinitrobenzoic acid in alcohol but, instead of sodium hydroxide, he used benzyltrimethylammonium hydroxide, claiming that hydrolysis was thereby diminished. Pratt did not allow time for the development of colour and read at 550 mμ, a bluish red colour.

MR. A. F. CALDWELL (Singapore) said that in a colorimetric assay of morphine tablets he had overcome a difficulty due to the effect of alcohol on the colour by distilling it over quicklime immediately before use. A method of detecting deterioration in digitalis galenicals quickly was needed in the tropics.

DR. J. G. DARE (Kippax) said that the problem of higher lethal doses for light-weight animals than for heavier animals had been investigated by two independent groups of workers, whose results were in agreement with those of Dr. Rowson. The observations of one of the two groups, an American team, were that if the number of mg. of powdered digitalis were divided by the number of hundreds of g. of guinea-pig body-weight to the power of 0·64, a linear relationship would be found between the weight and the toxic dose from which the adjustment could be made.

DR. N. EVERS (Hertford) said it would have been useful if Mr. Eastland had included limits of error for his biological results. If they had been the same as those of Dr. Dyer their results might have been the same.

Professor H. BRINDLE (Manchester) asked Mr. Eastland whether his samples 1 to 12 were prepared in the same way as samples 13 to 25,

which were described as digitoxin. Did not samples 1 to 12 give a Keller-Kiliani test or give it only very weakly? If prepared from reasonable leaf samples a good Keller-Kiliani test should be obtained. It would appear that Mr. Eastland used the Ulrix chromatographic process for separating the glycosides. He had tried that method, and had obtained only a partial qualitative separation. He agreed that for a complex mixture of primary and secondary glycosides and aglycones the colorimetric test would not give as good a measure of activity as the biological test. For example, he found that gitoxin, which gave a high colour, had very little effect upon frogs so that digitoxin containing gitoxin gave a good colour but gave a low result in the biological test.

The biological test was not the last word. The action of the glycosides on the normal heart was not the same as on the diseased heart and both these actions differed from that causing death in animals. Biological standardisation did not therefore necessarily give a correct idea of therapeutic value. Estimation of the separate glycosides should be attempted. He supported the suggestion that the trouble experienced by Dr. Rowson was due to impurities present in the industrial spirit used. The speaker had experienced no trouble when using pure ethanol distilled over caustic potash.

MR. R. L. STEPHENS (Brighton) asked Dr. Rowson whether he had considered drying the leaves by radio frequency dielectric heating by which drying could be completed in 1 to 1½ minutes.

DR. T. WALLIS (London) pointed out that two sets of specimens had been used by Dr. Rowson, one supplied commercially and one grown by himself. This might account for the variation in the results. At Dr. Rowson's request he had examined one sample giving a very divergent result and had found pollen grains present which were not from digitalis flowers. It was possible that the presence of weeds in the sample of digitalis affected the results.

MR. C. J. EASTLAND, in reply, said that as glycosides were used it was possible to have a constant volume of alcohol so that the question of alcohol level using the Baljet reaction did not arise. He had not studied the effect of varying the alcohol strength of the tincture. He confirmed that with comparatively pure glycosides the maximum colour was produced in about 10 to 12 minutes; in all cases it was obtained in 15 minutes, and a standard time of 20 minutes had been adopted. The weight yields of the chloroform-soluble glycosides varied enormously, from 0·38 g. to 1·2 g. per lb. of dry leaf. Digitoxin of only medium potency was adopted as standard because it was anticipated that samples of prepared glycosides with activity of between 800 and 1200 I.U./g. would be obtained. He agreed that it would be interesting to compare the results of the current methods of assay of digitalis preparations with therapeutic efficiency. In answer to Dr. Evers, the standard errors were not greater than 8 per cent. and were given in the paper. In regard to the difference between samples 1 to 12 and 13 to 26, samples 1 to 12 were all prepared in the same way using the same volume of chloroform for each extraction and some of the series 13 to 17 were also prepared

in that way, showing that the latter set of leaf samples did contain a much higher proportion of digitoxin. Ulrix's method using chloroform and methanol had been tried in chromatographic work. It gave some idea of the different glycosides present. The method had shown that many commercial samples of digitoxin of high activity contained as much as 5 per cent. of gitoxin in addition to a small percentage of primary glycosides.

DR. J. M. ROWSON, in reply, said that Dr. Dyer had certain reservations concerning artificially respired guinea-pigs, which seemed to die smoothly, whereas with ordinary conditions of respiration the cardiotonic effect was observed. In reply to Dr. Mitchell, Figure 2 showed that the wavelength effects were not pronounced, and the curve, which was flat topped, could be used over a wide range; thus, Pratt's use of 550 mμ seemed reasonable. Professor Brindle had referred to the bioassay using frogs. When the assay was carried out using guinea-pigs, gitoxin had the same order of activity as digitoxin. It was gratifying to learn that the American work mentioned by Mr. Dare was in agreement with that now reported by Dr. Dyer and himself. He had not yet tried drying by radio frequency heating.

SOME FURTHER STUDIES ON TUBERCULOSTATIC COMPOUNDS

By E. M. Bavin, D. J. Drain, M. Seiler and D. E. Seymour

From the Research Department, Herts Pharmaceuticals, Ltd., Welwyn Garden City

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Introduction

Continuing our examination of the tuberculostatic activity of substances related to *p*-aminosalicylic acid, we report on some further derivatives of this substance and on the activity of some heterocyclic compounds.

Table I lists the compounds which have been examined together with their inhibitory concentrations against a standard inoculum of 0·001 mg./ml. of *M. tuberculosis* H37Rv strain. The culture medium and technique used for the determination of *in vitro* activity was similar to that described previously.[1] Table II lists the acute toxicity and the *in vivo* activity of some of the compounds, the latter being determined by the mouse corneal test of Rees and Robson.[2] The corneal test was carried out on groups of 10 animals and a positive sign in the column marked "activity" indicates that more than 50 per cent. of the animals under test were protected from the development of corneal lesions after a period of 30 days treatment. A more detailed estimate of the degree of protection was obtained in some cases by microscopical examination of the eyes and the figure in parenthesis indicates the percentage of eyes protected. In some instances, a number of animals were eliminated from the test owing to the presence of non-tuberculous lesions.

Results

(*a*) *p-Aminosalicylic acid derivatives.* Earlier work with esters of *p*-aminosalicylic acid had indicated to us that they were not sufficiently active to justify extended trial. However, reports by Freire *et al.*[3,4,5] that the phenyl ester (phenyl-4-aminosalicylate) has an *in vitro* and *in vivo* tuberculostatic activity many times greater than *p*-aminosalicylic acid and at least equal to that of streptomycin, prompted us to re-examine this compound and a series of related aryl esters. The results as given in the tables indicate that the aryl esters have an *in vitro* activity of the same order as *p*-aminosalicylic acid; this activity being maintained *in vivo* with 3 typical members of the group (Compounds No. 77, 82 and 83). No marked difference in *in vivo* activity was observed according to whether the substance was administered by oral or subcutaneous route. The poor protective power of the butyl ester confirms reports by others[6] that the alkyl esters have little *in vivo* activity, and although the aromatic esters are probably the most useful members of the group, they do not appear to offer the advantages over *p*-aminosalicylic acid reported by the French workers. Compound 108 is of interest in so far as it can be regarded as a conjugate of 2 molecules of *p*-aminosalicylic acid, this

TABLE I

TUBERCULOSTATIC ACTIVITIES *in vitro*

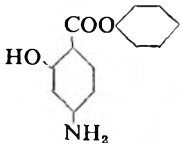
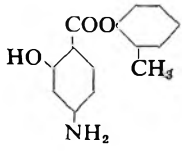
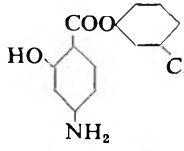
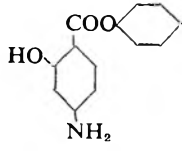
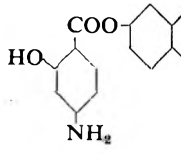
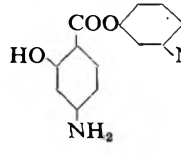
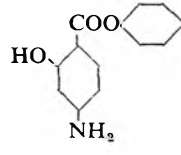
No.	Name	Formula	*In vitro* activity mg./100 ml.
	(a) *Derivatives of* p-*Aminosalicylic acid*		
77	Phenyl-4-aminosalicylate		0·0487–0·0243
84	*o*-Cresyl-4-aminosalicylate		0·0243–0·0121
85	*m*-Cresyl-4-aminosalicylate		0·0243–0·0121
82	*p*-Cresyl-4-aminosalicylate		0·0243–0·0121
83	β-Naphthyl-4-aminosalicylate		0·0121–0·006
88	*m*-Aminophenyl-4-aminosalicylate		0·0243–0·0121
98	*p*-Aminophenyl-4-aminosalicylate		0·0487–0·0243

TABLE I (*continued*)

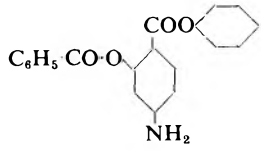
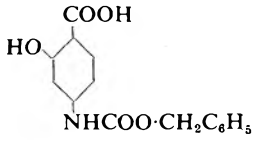
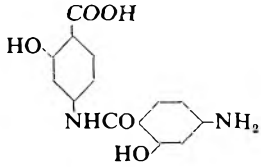
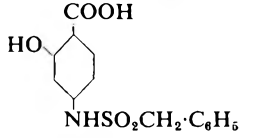
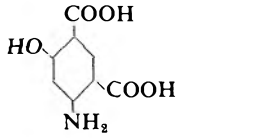
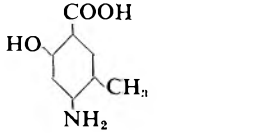
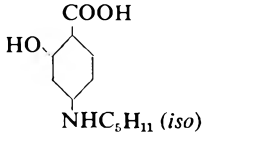
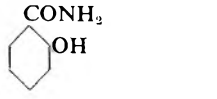
No.	Name	Formula	*In vitro* activity mg./100 ml.
80	Phenyl-4-amino-2-benzoyloxybenzoate		0·195–0·0975
106	4-Carbobenzyloxy-aminosalicylic acid		0·39–0·195
108	*4-(4′-Amino-2′-*hydroxybenzamido)-salicylic acid		*0·0975–0·0487*
123	4-Benzylsulphonamido-salicylic acid		0·39–0·195
102	4-Amino-6-hydroxy-*iso*phthalic acid		0·0121–0·006
72	4-Amino-5-methyl-salicylic acid		0·0975–0·0487
120	4-*iso*Amylaminosalicylic acid		0·0487–0·0243
	(b) *Amides and Thio-amides:*		
34	2-Hydroxybenzamide (salicylamide)		>25

TABLE I (*continued*)

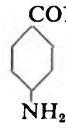
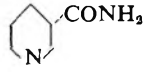
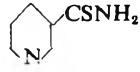
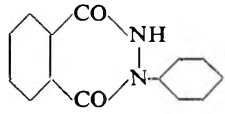
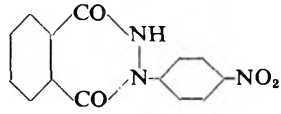
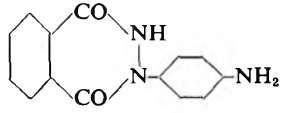
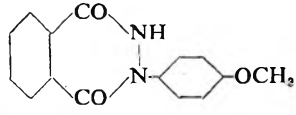
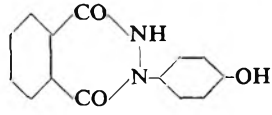
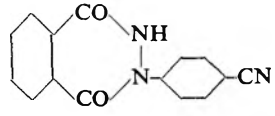
No.	Name	Formula	*In vitro* activity mg./100 ml.
74	4-Aminobenzamide		0·78–0·39
50	4-Aminobenzthioamide		0·0975–0·0487
79	Nicotinamide		>12·5
78	Nicotinthioamide		3·125–1·56
	(c) *Phthalazine Derivatives:*		
127	1:4-Diketo-3-phenyl-tetrahydrophthalazine		0·0243–0·0121
233	1:4-Diketo-3-(*p*-nitrophenyl)-tetrahydrophthalazine		0·0243–0·0121
256	1:4-Diketo-3-(*p*-aminophenyl)-tetrahydrophthalazine		0·78–0·39
257	1:4-Diketo-3-(*p*-methoxyphenyl)-tetrahydrophthalazine		1·56–0·78
261	1:4-Diketo-3-(*p*-hydroxyphenyl)-tetrahydrophthalazine		0·78–0·39
263	1:4-Diketo-3-(*p*-cyanophenyl)-tetrahydrophthalazine		1·56–0·78

TABLE I (*continued*)

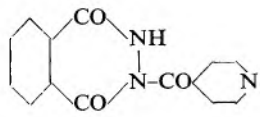
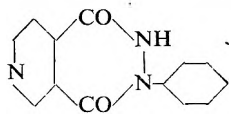
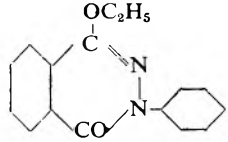
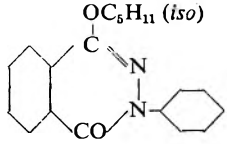
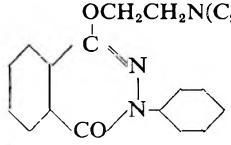
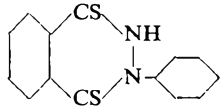
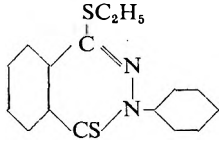
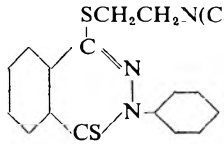
No.	Name	Formula	*In vitro* activity mg./100 ml.
238	1:4-Diketo-3-*iso*nicotinyltetrahydrophthalazine		0·0243–0·0121
232	6-Aza-1:4-diketo-3-phenyltetrahydrophthalazine		3·125–1·56
152	1-Ethoxy-4-keto-3-phenyl-3:4-dihydrophthalazine		3·125–1·56
153	1-*iso*Amyloxy-4-keto-3-phenyl-3:4-dihydrophthalazine		3·125–1·56
207	1-β-Diethylaminoethoxy-4-keto-3-phenyl-3:4-dihydrophthalazine hydrochloride		1·56–0·78
129	1:4-Dithio-3-phenyltetrahydrophthalazine		0·39–0·195
223	1-Ethylthio-3-phenyl-4-thio-3:4-dihydrophthalazine		0·0975–0·0487
224	1-Diethylaminoethylthio-3-phenyl-4-thio-3:4-dihydrophthalazine hydrochloride		1·56–0·78

TABLE I (*continued*)

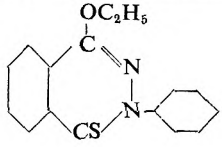
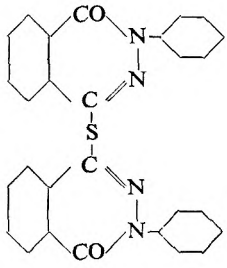
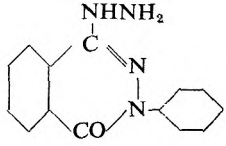
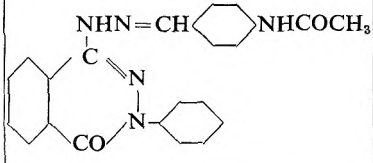
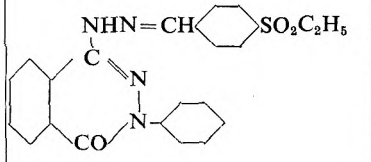
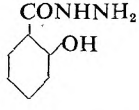
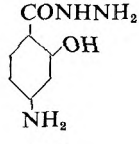
No.	Name	Formula	*In vitro* activity mg./100 ml.
227	1-Ethoxy-4-thio-3-phenyl-3 : 4-dihydrophthalazine		0·0243–0·0121
225	1 : 1′-Bis-(4-keto-3-phenyl-3 : 4-dihydrophthalazinyl)-sulphide		0·0975–0·0487
228	1-Hydrazino-4-keto-3-phenyl-3 : 4-dihydrophthalazine		3·125–1·56
230	1-(*p*-Acetamidobenzalhydrazino)-4-keto-3-phenyl-3 : 4-hydrophthalazine		0·0975–0·0487
231	1-(*p*-Ethylsulphonylbenzalhydrazino)-4-keto-3-phenyl-3 : 4-dihydrophthalazine		0·0975–0·0487
	(d) *Acid Hydrazides:*		
185	2-Hydroxybenzhydrazide		0·78–0·39
116	4-Amino-2-hydroxybenzhydrazide		0·0975–0·0487

TABLE I (*continued*)

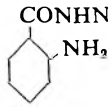
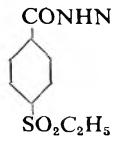
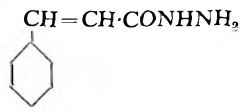
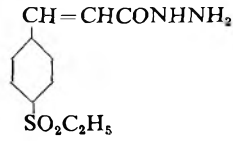
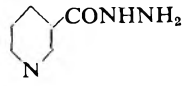
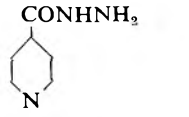
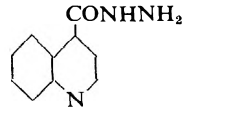
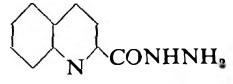
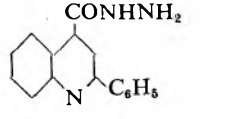
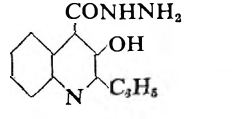
No.	Name	Formula	*In vitro* activity mg./100 ml.
186	2-Aminobenzhydrazide		>12·5
191	4-Ethylsulphonylbenzhydrazine		3·125–1·56
184	*Cinnamic acid hydrazide*		>12·5
188	4-Ethylsulphonylcinnamic acid hydrazide		6·25–3·125
187	Nicotinyl hydrazide		1·56–0·78
181	*iso*Nicotinyl hydrazide		0·0008–0·0004
195	Cinchoninyl hydrazide*		6·25–3·125
252	Quinoline-2-carboxyhydrazide		0·39–0·195
194	2-Phenylcinchoninyl hydrazide		0·39–0·195
189	3-Hydroxy-2-phenylcinchoninyl hydrazide		1·56–0·78

TABLE I (*continued*)

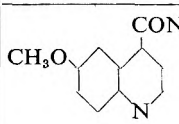
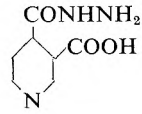
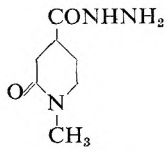
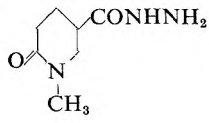
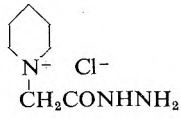
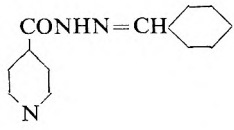
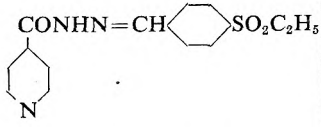
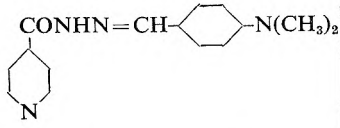
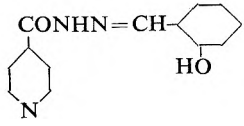
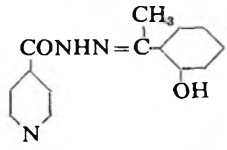
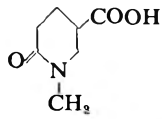
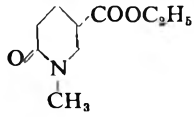
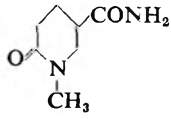
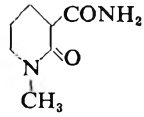
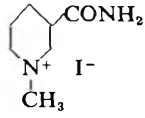
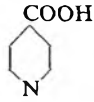
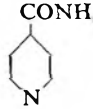
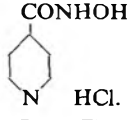
No.	Name	Formula	*In vitro* activity mg./100 ml.
196	Quininic acid hydrazide*		1·56–0·78
197	3-Carboxy-*iso*nicotinyl hydrazide		>12·5
190	1-Methyl-2-pyridone-4-carboxyhydrazide		0·0487–0·0243
204	1-Methyl-2-pyridone-5-carboxyhydrazide		0·78–0·39
222	Pyridinium acethydrazide chloride		>12·5
250	Benzal-*iso*nicotinyl-hydrazone		0·0002–0·0001
192	4-Ethylsulphonylbenzal-*iso*nicotinyl hydrazone		0·006–0·003
212	4-Dimethylaminobenzal-*iso*nicotinyl hydrazone		0·003–0·0017
213	2-Hydroxybenzal-*iso*-nicotinyl hydrazone		0·003–0·0017

TABLE I (*continued*)

No.	Name	Formula	*In vitro* activity mg./100 ml.
214	2-Hydroxyacetophenone-*iso*nicotinyl hydrazone		0·0121–0·006
	(e) *Miscellaneous Pyridine Compounds:*		
136	1-Methyl-2-pyridone-5-carboxylic acid		>12·5
137	Ethyl-1-methyl-2-pyridone 5-carboxylate		3·125–1·56
135	1-Methyl-2-pyridone-5-carboxyamide		0·78–0·39
144	1-Methyl-2-pyridone-3-carboxyamide		0·39–0·195
138	Nicotinamide methiodide		6·25–3·125
198	*iso*Nicotinic acid		3·125–1·56
203	*iso*Nicotinamide		>12·5
210	*iso*Nicotin-hydroxamic acid hydrochloride		>12·5

* We wish to thank Professor F. S. Spring, of the Royal Technical College, Glasgow, for kindly supplying compounds 195 and 196.

TABLE II

TUBERCULOSTATIC ACTIVITIES *in vivo*
(Mouse Corneal Test)

No.	Name	LD50 mg./g.	*In vivo* activity. Mouse corneal test. Dosage mg./g.	Route	Activity
	(a) *Derivatives of* p-*Amino-salicylic acid:*				
77	Phenyl-4-aminosalicylate	>5 (oral)	1·5	Gastric tube	+
		>3 (subcutaneous)	1·5	Subcutaneous	+
82	*p*-Cresyl-4-aminosalicylate	5 (subcutaneous)	2·0	In diet	+
			2·0	Subcutaneous	+
			1·0	In diet	+
83	β-Naphthyl-4-aminosalicylate	>5 (oral)	2·0	In diet	+
		>5 (subcutaneous)	1·5	Subcutaneous	+
			1·0	In diet	+
115	*n*-Butyl-4-aminosalicylate	5 (oral)	2·0	In diet	−
		5 (subcutaneous)	1·5	Subcutaneous	−
108	4(4'-Amino-2'-hydroxybenzamido) salicylic acid	—	2·0	In diet	+
			1·0	In diet	+
123	4-Benzylsulphonamidosalicylic acid	4 (oral)	—		
		4·5 (subcutaneous)	2·0	In diet	+
125	3:-Di-iodo-4-aminosalicylic acid*	0·5 (subcutaneous)	0·4	In diet	+
	(b) *Amides:*				
79	Nicotinamide	2·5 (oral)	—	Oral	−†
		2·75 (subcutaneous)			
	(c) *Phthalazine Derivatives:*				
	1:4-Diketo-3-phenyltetrahydrophthalazine	—	0·5	In diet	+
	(d) *Acid Hydrazides:*				
116	4-Amino-2-hydroxybenzhydrazide	0·5 (oral)			
		0·35 (subcutaneous)	0·2	In diet	−
181	*iso*Nicotinyl hydrazide	0·1 (oral)	0·008	In diet	+ (100)
		0·1 (subcutaneous)	0·004	In diet	+ (100)
			0·002	In diet	+ (55)
190	1-Methyl-2-pyridone-4-carboxy-hydrazide	—	0·008	In diet	+ (75)
			0·004	In diet	− (40)
192	4-Ethylsulphonylbenzal *iso*nicotinyl hydrazone	—	0·008	In diet	+ (57)
			0·004	In diet	− (30)
213	2-Hydroxybenzal-*iso*nicotinyl hydrazone	>10 (oral)	1·0	In diet	+ (100)
	(e) *Miscellaneous Pyridine Compounds:*				
137	Ethyl-1-methyl-2-pyridone-5-carboxylate	—	0·5	In diet	−
135	1-Methyl-2-pyridone-5-carboxamide	—	0·5	In diet	−

* We have previously reported the *in vitro* activity of this compound.[8]
† Rees and Robson—personal communication.

"dimer" being an attempt to produce a condensed molecule with the aim of maintaining *in vivo* activity with a reduced dose. Further studies on these lines are continuing and will be reported later. Nuclear substitution in the 5-position of the *p*-aminosalicylic acid molecule does not markedly reduce the tuberculostatic activity as is shown by compounds 72 and 102, the latter being a known by-product in some of the commercial methods of synthesis of *p*-aminosalicylic acid.[7]

We have previously reported[8] the *in vitro* activity of the hydrazide of *p*-aminosalicylic acid (Compound No. 116). The low *in vivo* activity of this substance and that of the other aromatic hydrazides examined (Compounds 185, 186 and 191) confirms the reports by others[9,10] on the low activity of the hydrazides of aromatic acids.

(*b*) *Nicotinamide.* Earlier work by one of us[11] confirmed that nicotinamide is active by the mouse survival test at a dose level of 0·9 mg./g. The inactivity of the substance *in vitro* and by the corneal test is the only instance we have encountered to date where the 2 *in vivo* tests do not correlate. Preliminary results of clinical trial with nicotinamide indicate that the substance, although displaying activity, is not so effective as *d*-aminosalicylic acid or streptomycin.[12]

(*c*) *Phthalazine derivatives.* Following a report by Buu-Hoi *et al.*[13] that the *iso*amyl ether of 1:4-diketo-3-phenyltetrahydrophthalazine is more active than streptomycin in a mouse survival test, we synthesised this compound (No. 153) and other related ethers (152 and 207), and found them all to exhibit low *in vitro* activity. The high *in vitro* activity of the 1:4-diketo-3-phenyltetrahydrophthalazine (No. 127) itself, coupled with its promising behaviour in the mouse corneal test, led us to synthesise further compounds of this series, the *in vitro* results of which are given in Table I.

(*d*) *Acid hydrazides and derivatives.* Following the recent reports[14,15] from the United States that *iso*nicotinyl hydrazide possesses an outstandingly high antitubercular activity *in vitro*, in animals and in man, we directed our attention to a further series of hydrazides and other derivatives of this compound. We confirm the high *in vitro* activity of *iso*nicotinyl hydrazide (Compound 181) and also show that the substance exhibits remarkable protective power in the mouse corneal test. From the series of derivatives reported in this paper, and from other published work,[10] it is apparent that *iso*nicotinyl hydrazide is another example of a substance displaying specificity for tuberculostatic activity. The substituted aldehyde and ketone *iso*nicotinyl hydrazones (No. 250, 192, 212, 213 and 214) however, show comparable *in vitro* tuberculostatic activity, but it is conceivable that these derivatives may owe their activity to breakdown to *iso*nicotinyl hydrazide.

The authors wish to thank Professor Knox and Professor J. M. Robson of Guy's Hospital, London, for their interest and advice, and the Directors of Herts Pharmaceuticals Ltd., for permission to publish these results.

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2. Rees and Robson, *Brit. J. Pharmacol.*, 1950, **5**, 77.
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11. Bavin. *Unpublished observations.*
12. Nagley. *Personal communication.*
13. Bui-Hoi, Dechamps, Nguyen-Hoan, Le Bihan, Ratsimamanga and Binon, *C.R. Acad. Sci., Paris*, 1949, **228**, 2037.
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DISCUSSION

The paper was presented by MR. D. E. SEYMOUR.

DR. F. HARTLEY (London) commented on the statement that the substituted aldehyde and ketone *iso*nicotinyl hydrazones might owe their activity to breakdown to *iso*nicotinyl hydrazide. Hydrazones, generally speaking, would be expected to be very stable compounds. He suggested that if Mr. Seymour would measure the stability of those hydrazones to oxidising agents and to acid hydrolysis he would probably find them to be stable. If that were so the evaluation of one or other of the four compounds should be further pursued.

MR. D. E. SEYMOUR, in reply, said he thought that the compounds might break down. Their stability was being studied in detail. Since the paper was written, further work had been done with a few of the compounds, in particular the benzaldehyde derivative, which was more active than isoniazid *in vitro* but not so active *in vivo*. American workers had reported similar results with some of the derivatives.

FURTHER ASPECTS OF THE PHARMACOLOGY OF *PARA*-AMINOSALICYLIC ACID

By E. M. Bavin and Barbara James

From the Research Department, Herts Pharmaceuticals Ltd., Welwyn Garden City

Received July 16, 1252.

The place of *p*-aminosalicylic acid as a therapeutic agent against tuberculosis is now well established and recent reports[1] have amply confirmed the earlier findings that it has a marked effect in delaying the emergence of streptomycin resistance. In contrast to the large number of papers which have appeared upon the tuberculostatic effect of this drug, there appear to have been very few dealing with the more general pharmacology of the substance and this present series of experiments is an extension of the work described in a previous communication.[2]

It has been generally accepted that aqueous solutions of *p*-aminosalicylic acid should not be used clinically more than about 24 to 48 hours after preparation on account of the rapid darkening in colour which occurs at room temperature. So far as we are aware, however, no pharmacological investigation of these old solutions has been carried out and it appeared to be of interest to determine in what way, if any, these solutions differed in their properties from freshly prepared solutions.

In addition, recent work,[3,4,5,6] suggesting the possibility of a stimulant effect of salicylates on the anterior pituitary gland, particularly a report[7] that *p*-aminosalicylic acid produced a marked eosinopenia in rats, was of considerable interest and it seemed desirable to repeat this work to obtain more data on this important point. A report had also appeared[8] that *p*-aminosalicylic acid had a trypanocidal action in mice and rats and here again it was felt that further evidence would be helpful.

Experimental Methods

(1) *Investigation of Old Solutions*

(*a*) *Long term feeding experiments.* 20 per cent. w/v aqueous solutions of sodium *p*-aminosalicylate were prepared and allowed to stand at room temperature, exposed to light, for 1 week, 1 month and 6 months respectively. Fresh volumes of these solutions were prepared at weekly intervals during the entire course of the experiment so that the period of ageing was relatively constant throughout the course of the work. These solutions were administered at a dose of 1·0 mg. of sodium *p*-aminosalicylate/g. by stomach tube daily to adult male Wistar rats. The animals were fed on rat cakes (a mixture of Thompsons diet and Parkes diet 41), bread and milk daily, with cooked liver and green food once weekly. Some control groups received fresh 20 per cent. solution and others normal saline solution. The animals were weighed twice a week and growth curves constructed. Blood counts were carried out at intervals of 28 days. During and at the end of the experiments, histological specimens were prepared of the thyroid glands. The tissues were fixed in Susa and

stained with hæmatoxylin and eosin. The thyroid glands were weighed after fixation.

(*b*) *Effect on blood pressure and respiration.* Experiments to determine the effect of solutions on blood pressure and respiration were carried out on rabbits and cats anæsthetised with chloralose and ether. Blood pressure was recorded from the carotid artery and respiration by a tambour from the tracheal cannula. Experiments on the perfused rabbit heart were carried out by the usual Langendorff technique, using the apparatus described by Baker.[9]

(*c*) *Blood levels.* Blood levels in rats were estimated by the method of Newhouse and Klyne.[10]

(*d*) *Therapeutic effect.* The therapeutic activity of fresh and old solutions was determined by the mouse survival test.[2]

(II) *Eosinopenic effect.*

Swiss albino mice, males, weighing from 18 to 24 g. were used. From the start of the experiment each animal was housed separately at a room temperature of 27° C. Blood was obtained by amputating the tail at 0·5 cm. from the distal end. A fairly rapid flow of blood was thus obtained. Eosinophils were enumerated by the method of Speirs and Meyer[11] using Teepol as detergent in place of Alconox. Blood was taken from each mouse prior to subcutaneous and intraperitoneal injection of the drug and normal saline solution. At 2 and 4 hour intervals further samples were taken. Most workers have found considerable experimental variation in eosinophil counts and, to ascertain the accuracy of our own counts, 3 different samples of blood were taken from each of 10 mice and 4 hæmocytometer chambers counted from each sample, that is 12 counts were made on blood from each mouse. The results obtained are shown in Table I.

TABLE I

EOSINOPHIL COUNTS

Mouse	Mean hæmocytometer count and standard error (mean of 12 values)
1	40·6 ± 2·46
2	70·3 ± 7·69
3	48·0 ± 6·17
4	32·2 ± 2·18
5	129·4 ± 10·34
6	33·4 ± 3·21
7	44·4 ± 3·26
8	51·0 ± 6·04
9	50·6 ± 5·66
10	78·3 ± 7·96
	Average 57·8 ± 5·49

The figures in Table I should be multiplied by 6·25 to give eosinophils/cu. mm.

It will be seen that, under these conditions, the standard error is about 10 per cent., but as it was not practicable to carry out 12 counts on each mouse as routine, only one count was made on each blood sample and the test was used as a quantal response. Consideration of the work of Spiers and Meyers (*loc. cit.*) and Mushett[12] suggests that this method is capable of being used as a variable-response type of assay and work is in hand on this problem.

(III) *Trypanocidal activity.*

Swiss albino mice and Wistar albino rats were infected with *Trypanosoma equiperdum* by intraperitoneal injection of suspensions

of the organism in glucose saline solution. These suspensions were obtained by decapitating a mouse heavily infected with *T. equiperdum* and allowing it to bleed into glucose saline. Chemotherapy was commenced either immediately following infection or after the infection had developed for 2 days. The effect of treatment was judged by blood smears and survival time of the animals compared with controls.

Results

(I) *Chronic Toxicity of Old Solutions*

The results of typical experiments are shown in Figures 1 and 2, from which it will be seen that, after a period of 2 to 3 months, all the groups of rats receiving the drug either fresh or aged, were growing less rapidly than the controls. This retardation of growth becomes quite marked after a further period of 1 to 2 months and suggested the effect which is obtained by giving small doses of thiouracil or other anti-thyroid drug to rats. Consequently some of the experimental animals were killed and the thyroid glands examined histologically. There was a marked hyperplasia of the gland shown by almost complete loss of colloid material and by some thickening of the epithelium (Fig. 3).

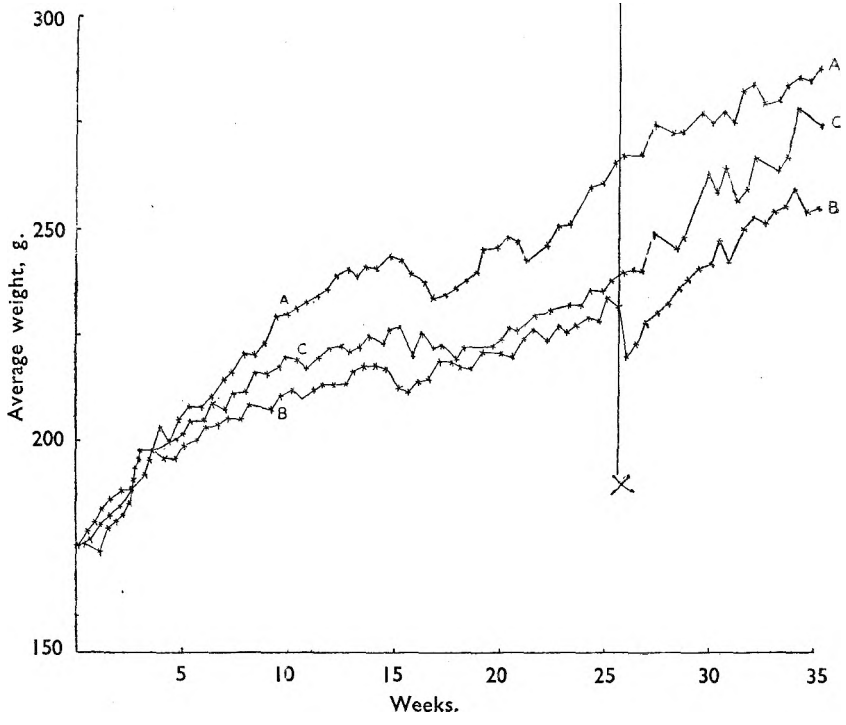

FIG. 1. The effect of sodium *p*-aminosalicylate in a dose of 1 mg./g./day on growth of rats (10 animals per group). A, Control group. B, Fresh solution of sodium *p*-aminosalicylate. C, 1 month old solution of sodium *p*-aminosalicylate. X, Sodium *p*-aminosalicylate solution replaced by normal saline solution.

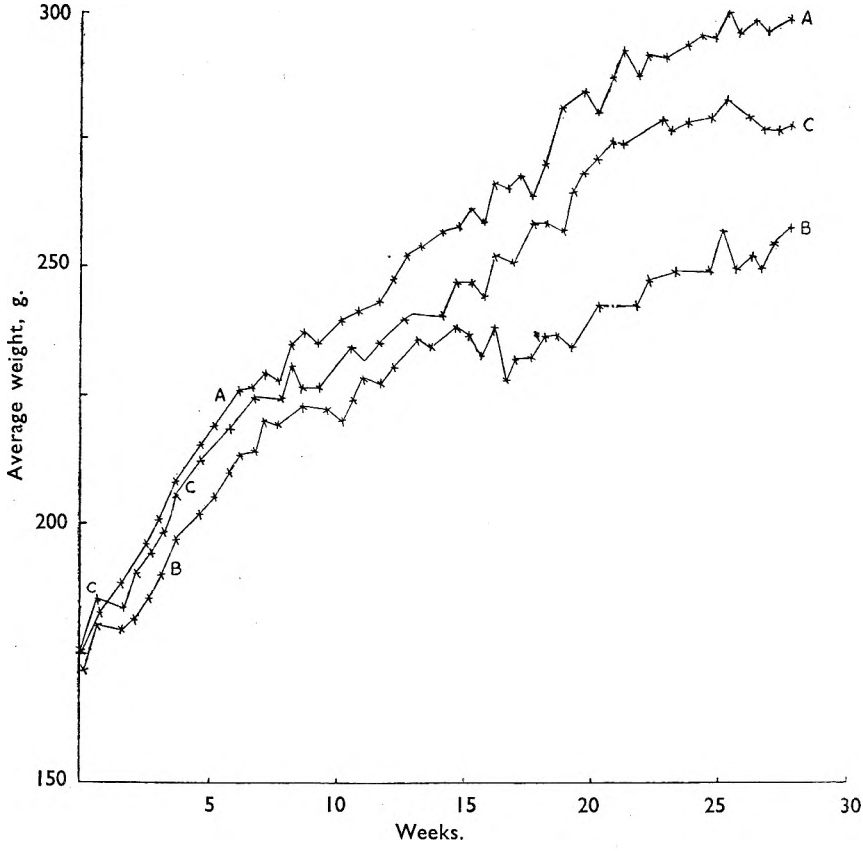

FIG. 2. The effect of sodium *p*-aminosalicylate on growth of rats (10 animals per group). A, Control group. B, Fresh solution, 1 mg./g./day. C, 6-months old solution, 1 mg./g./day.

p-Aminosalicylic acid treatment of the remaining animals was then replaced by treatment with normal saline solution and an immediate rapid increase in rate of growth occurred. This is shown in Figure 1 where the vertical line indicates cessation of treatment. This result suggests therefore that, as in the case of thiouracil, the action on the growth of rats is easily reversible.

Repetition of the above experiments with larger groups of rats confirmed the hyperplastic effect on the thyroid gland. The weight of the thyroid gland was not increased following administration of sodium *p*-aminosalicylate and typical values are shown in Table II.

From all our experiments there seems little evidence that the solutions kept for periods up to 6 months have any greater effect than the freshly prepared solution. This suggests that the thyroid hyperplasia observed is due solely to the sodium *p*-aminosalicylate present. Nevertheless, it was felt desirable to test the action of *m*-aminophenol which is the degradation product of *p*-aminosalicylic acid most likely to be present in these old

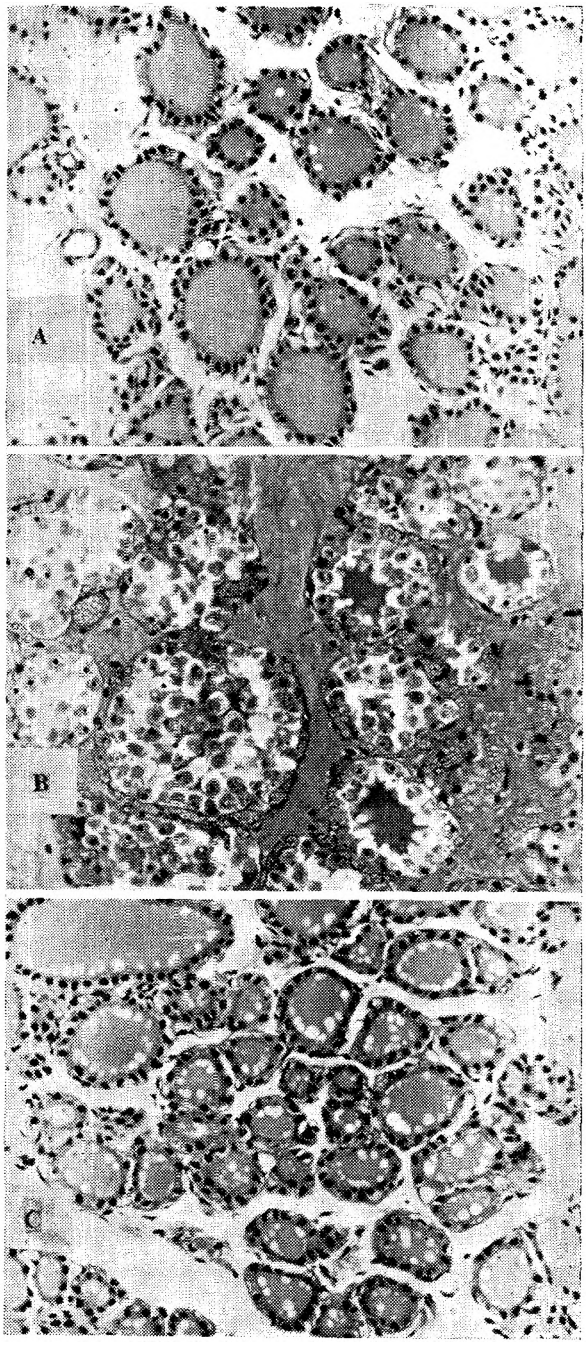

FIG. 3. Thyroid gland of rats (× 300). A, Normal. B, After receiving fresh solution of sodium *p*-aminosalicylate, 1 mg./g./day for 22 weeks. C, After receiving *m*-aminophenol, 0·15 mg./g./day for 20 weeks.

TABLE II

THYROID WEIGHTS

Rat	Dose (oral)	Weight of thyroid mg./100 g. of body weight	Mean thyroid weight mg./100 g. of body weight
42L	1 mg./g. of fresh *p*-amino-salicylic acid solution for 14 weeks	5·73	7·58
42R		6·29	
52L		10·54	
52R		11·92	
4P		5·15	
41L		6·00	
41R		7·30	
41LIR		7·7	
82R	1 mg./g. of 1 month old solution for 14 weeks	8·59	8·67
91LIR		7·53	
92L		10·68	
92R		8·41	
7P		8·6	
72R		9·8	
71LIR		7·0	
12R	Nil	9·50	8·83
22R	Nil	5·29	
32L	Nil	12·67	
32R	Nil	8·57	
IP	Nil	6·83	
IIL	Nil	10·80	
11R	Nil	11·00	
11LIR	Nil	6·00	

solutions. Analytical examination of these solutions showed that they contained the following percentage of *m*-aminophenol:—6 months old solution—0·6 per cent., 3 months-old—0·3 per cent., 1 month old—0·2 per cent., and 1 week old—0·1 per cent. of *m*-aminophenol compared with 0·06 per cent. in a fresh sodium *p*-aminosalicylate solution. For these determinations we are indebted to Mr. B. W. Mitchell, using methods described by Seaman *et al.*[13] and Pesez.[14]

Some preliminary acute toxicity tests showed that the subcutaneous LD50 of *m*-aminophenol to mice was approximately 0·2 mg./g. and a long term feeding experiment in rats was therefore carried out using oral doses of 0·075 and 0·15 mg./g. daily for 20 weeks. At the end of this period, histological examination showed the rats to have completely normal thyroid glands (Fig. 3) and the growth curve on the lower dose showed no departure from the control curve. However, on the higher dose some retardation of growth was apparent. It seems clear therefore, that the action on the thyroid gland is not due to contamination with *m*-aminophenol.

To avoid the uncertainty regarding the composition of supplements to the diet in the foregoing experiments, it was decided to carry out another experiment in which the animals were fed on Thompson cubes only. It was somewhat surprising to find in this experiment no evidence of growth retardation (Fig. 4) but moderate thyroid hyperplasia. This latter effect, however, was definitely less marked than in the previous experiments. One explanation of this result may be the possible presence of thyroxine in the fish-meal constituent of the diet in sufficient amount to inhibit the growth retarding effect of *p*-aminosalicylic acid but insufficient completely to inhibit the hyperplastic effect on the thyroid. A somewhat similar suggestion has been advanced by Lawson and Searle.[15] A second possible

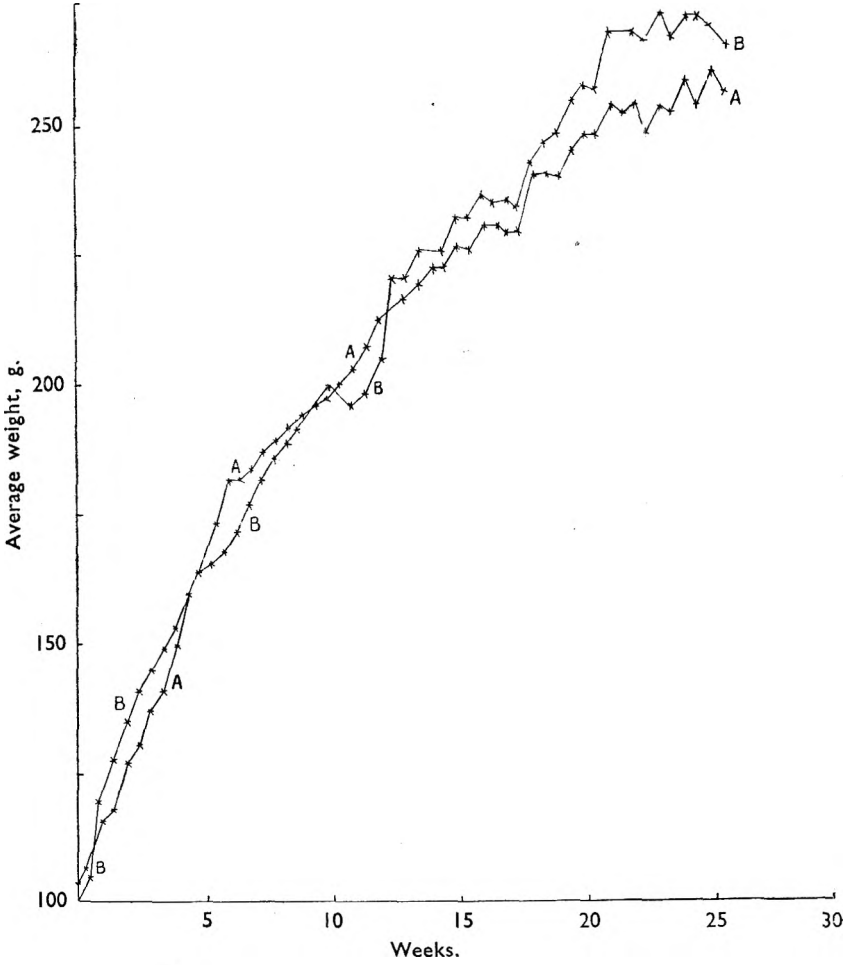

FIG. 4. The effect of fresh sodium *p*-aminosalicylate solution on growth of rats fed on Thompson's cube diet only. A, Controls. B, Fresh solution, 1 mg./g./day.

explanation of the result of this experiment may be that the supplements of cabbage, liver, bread and milk, etc., given in the previous experiments contain an anti-thyroid factor, which increases the effect of *p*-aminosalicylic acid. This suggestion is supported to a certain extent by the work of Chesney *et al.*[16] and Kennedy[17] on the anti-thyroid factor present in Brassicas.

This effect of sodium *p*-aminosalicylate solutions on the thyroid gland of rats is now the subject of further investigations, in which the oxygen consumption and radio-iodine uptake of the animals is being measured. The results will form the substance of a future communication.

(II) *Effect on Blood Pressure and Respiration*

(*a*) *Blood pressure.* A 6-months old 20 per cent. solution of sodium

p-aminosalicylate in a dose of 100 mg./kg. produced a definite increase in blood pressure in both rabbits and cats. This pressor effect was maintained for about 20 to 30 minutes. A similar dose of freshly prepared sodium *p*-aminosalicylate produced only a slight and transient increase in pressure (Fig. 5). This pressor effect was thought to be due to the *m*-aminophenol present in the old solution, but a dose of *m*-aminophenol equal to that present in the old solution did not produce this pressure increase. The injection of a solution containing the same dose of *m*-aminophenol together with fresh sodium *p*-aminosalicylate (100 mg./kg.) also produced no pressor effect (Fig. 4). These results, therefore, appear to exclude *m*-aminophenol as the pressor agent present in old solutions. The mechanism of this pressor effect was further investigated. The adrenolytic agents, benzylimidazoline (priscol) was given in a dose of 7·8 mg./kg. This reversed the normal pressor response to adrenaline but had no effect on the pressor action of sodium *p*-aminosalicylate (Figs. 6 (*a*) and 6 (*b*)). This result suggests that the observed effect of old solutions is not due to a sympathomimetic action.

In the perfusion of the isolated rabbit heart, both freshly prepared and old solutions of sodium *p*-aminosalicylate increased the amplitude of the heart beat without noticeably increasing the rate (Fig. 7). In some experiments, the old solutions appeared to have a more lasting effect than the fresh solutions but this was not an invariable finding. Hence, it appears that the increased pressor effect of old solutions may be partly but not entirely due to their cardiac effect. It is interesting to note that in a few of the heart perfusion experiments some initial decrease in amplitude was noticed with *p*-aminosalicylic acid solutions both fresh and old. This may correlate with the occasional slight initial fall in blood pressure observed in the anæsthetised rabbit and cat.

(*b*) *Respiration.* When 15 mg./kg. of *m*-aminophenol is injected intravenously into the chloralosed cat there is an increase in respiration rate from 31 to 49 inspirations per minute. With the 6 months old sodium *p*-aminosalicylate solution, injection of a dose containing the same amount of *m*-aminophenol produces a decrease in the depth but no change in the rate of respiration. The effect of *p*-aminosalicylic acid on the respiration is not marked and appears not to be due to the presence of *m*-aminophenol.

(III) *Effect of Sodium* p-*Aminosalicylate on Circulating Eosinophils in Mice*

Following subcutaneous injection of fresh sodium *p*-aminosalicylate in a dose of 1·0 mg./g., no decrease in the eosinophil level was observed at two and four hours after administration. Van Cauwenberg (*loc. cit.*) reported that in an intraperitoneal dose of 0·05 mg./g., *p*-aminosalicylic acid produced a significant eosinopenia in rats. We repeated our experiments in mice using this dose and route of injection. In this instance, the results showed an increase in eosinophils at 2 hours and a decrease at 4 hours; this latter effect was not significant. Administration of adrenocorticotrophic hormone in a dose of 5 μg./g. produced a very marked eosinopenia (Table III).

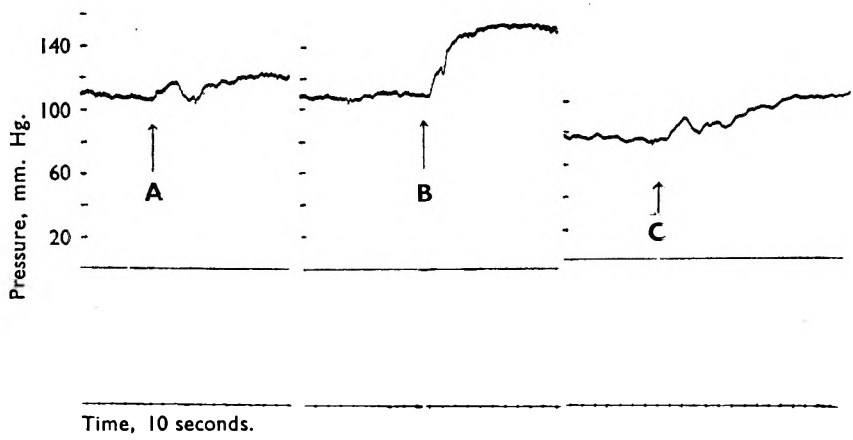

FIG. 5. Effect of fresh and 6 months old sodium *p*-aminosalicylate solution and *m*-aminophenol on the carotid blood pressure of the anæsthetised rabbit. A, Fresh sodium *p*-aminosalicylate solution, 200 mg./kg. (2·5 ml.). B, 6 months old sodium *p*-aminosalicylate solution, 100 mg./kg. (1·25 ml.). C, Equivalent of 6 months old sodium *p*-aminosalicylate solution (15 mg. of *m*-aminophenol and fresh sodium *p*-aminosalicylate solution).

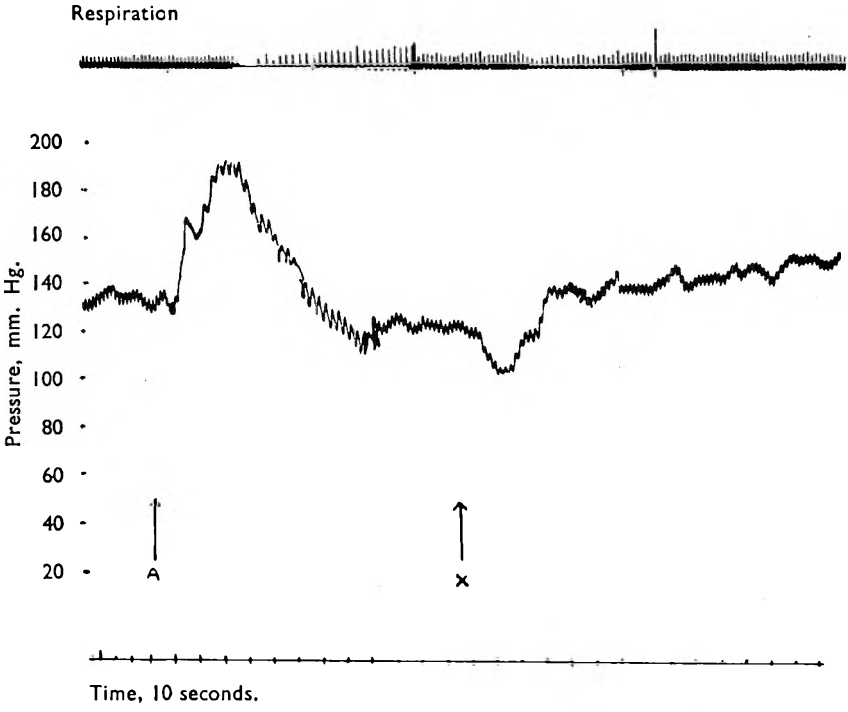

FIG. 6a. The effect of priscol on the pressor activity of a 6 months old solution of sodium *p*-aminosalicylate (cat under chloralose and ether). A, 10 μg. of adrenaline. X, 100 mg./kg. of 6 months old solution of sodium *p*-aminosalicylate. (See Fig. 6b.)

Under the conditions of our experiments, therefore, we were not able to observe any eosinopenic effect of sodium *p*-aminosalicylate in normal mice, suggesting that it has no adrenocorticotrophic hormone-like action. This finding agrees with a recent report by Cronheim *et al.*[18] that *p*-aminosalicylic and *p*-hydroxysalicylic acids were the only compounds out of a number of salicylic acid derivatives which failed to produce a decrease in the adrenal ascorbic acid level in normal rats.

TABLE III

EFFECT OF SODIUM *p*-AMINOSALICYLATE ON EOSINOPHIL COUNT

Number of mice	Compound administered	Dose	Hours after administration	Eosinophils per cu. mm.			
				Initial	Final	Percentage difference	*t*
10	Sodium *p*-aminosalicylate	1·0 mg./g. subcutaneously	2	690	641	− 6·1	1·1
10	,, ,,	1·0 mg./g. subcutaneously	4	468	882	+17·5	—
10	,, ,,	0·05 mg./g. intraperitoneally	2	449	561	+27·8	—
10	,, ,,	0·05 mg./g. intraperitoneally	4	638	569	−10·8	1·5
10	Adrenocorticotrophic hormone	5 μg./g. subcutaneously	2	241	71·3	−70·4	3·46

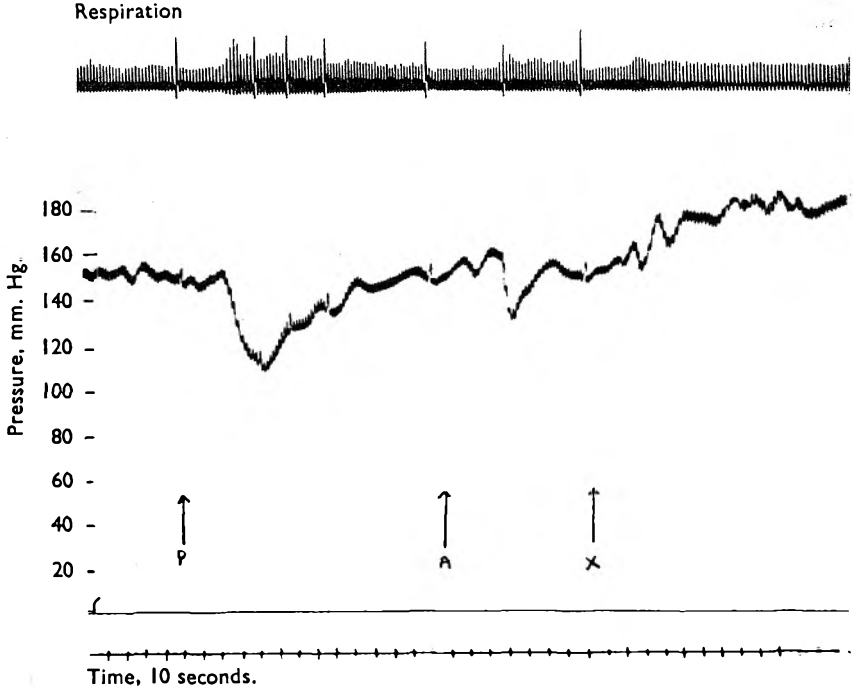

FIG. 6b. (Continuation of Fig. 6a.) The effect of priscol on the pressor activity of a 6 months old solution of sodium *p*-aminosalicylate (cat under chloralose and ether). P, priscol (5 mg./kg.). A, 10 μg. of adrenaline. X, 100 mg./kg. of 6 months old solution of sodium *p*-aminosalicylate.

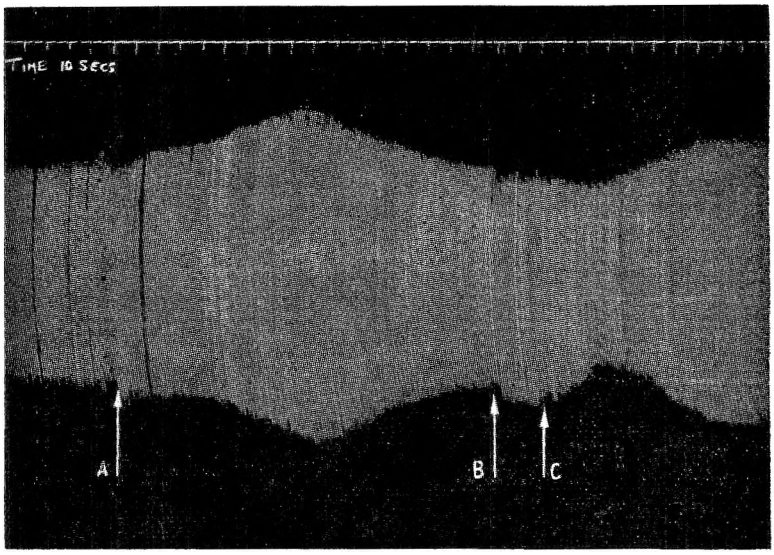

FIG. 7. Effect of fresh and 6 months old sodium *p*-aminosalicylate solutions on the perfused rabbit heart. A, 0·25 ml. of 20 per cent. solution of sodium *p*-aminosalicylate, 6 months old. B, Stop. C, 0·25 ml. of 20 per cent. solution of sodium *p*-aminosalicylate, freshly made.

(IV) *Trypanocidal Activity*

Subcutaneous and intraperitoneal injection of maximal doses of the sodium salt into mice and rats infected with *T. equiperdum* afforded no evidence for any trypanocidal activity. The survival times of treated and control groups were identical. An experiment in which drug was given after the development of infection was equally ineffective. A positive control group of mice treated with a subcutaneous dose of 0·04 mg./g. of sulpharsphenamine showed a very significantly increased survival time.

The possibility existed that the trypanocidal effect reported by Pick (*loc. cit.*) was due to impurities in the drug used. We therefore investigated the trypanocidal action of possible impurities, and, to this end,

TABLE IV

TRYPANOCIDAL ACTIVITY (*Trypanosoma equiperdum*)

Compound administered	Number of animals used	Daily dose mg./g.	Route	Survival time days
Sodium *p*-aminosalicylate	10 mice	1·0	Intraperitoneal	4·0
"	8 rats	1·0	Subcutaneous	4·75
m-Aminophenol	5 mice	0·03	Intraperitoneal	4·6
"	5 mice	0·05	Intraperitoneal	5·4
o-Aminophenol	5 mice	0·03	Intraperitoneal	5·3
"	5 mice	0·05	Intraperitoneal	5·2
5-Aminosalicylic acid	10 mice	0·5	Intraperitoneal	5·4
Sulpharsphenamine	10 mice	0·04	Subcutaneous	>10·0
Nil	10 mice	—	—	4·0
Nil	4 rats	—	—	4·0

maximal doses of *m*-aminophenol, *o*-aminophenol, and 5-aminosalicylic acid[19] were each used. No effect was obtained with any of these compounds and our results are summarised in Table IV.

(V) *Toxicity, Blood Levels and Therapeutic Activity*

(*a*) The acute toxicity of 1 month- and 6 months-old sodium *p*-aminosalicylate solutions was compared with that of fresh solution by intravenous injection into albino mice. Results were observed over a period of 3 days. The results are given in Table V. A graphical determination of the approximate LD50 gave the following results: fresh solution, 3·8 mg./g.; 1 month-old solution, 2·6 mg./g.; 6 months-old solution, 2·3 mg./g. These results suggest that a fairly rapid increase in toxicity takes place during the first months of keeping, followed by little or no increase in toxicity during the succeeding 5 months.

TABLE V

ACUTE TOXICITY

Intravenous dose mg./g.	Number of mice dead		
	Fresh solution	1 month old solution	6 months old solution
2·0			3/10
2·3			3/5
2·4			5/10
2·5		5/10	15/20
2·8			9/15
3·0	0/5	8/10	4/5
3·5	0/5	6/10	5/5
3·8	9/15		
4·0	12/20		
4·2	7/10		

(*b*) It was felt to be of interest to determine the blood levels in some of the rats which had been receiving 1 mg./g. of the sodium salt daily for 14 weeks since these levels might indicate whether any storage took place under these rather extreme conditions. Some preliminary results are given in Table VI, the set of figures for the respective solutions being in each case the average values from a group of 3 rats at the stated intervals after dosage. A similar set of figures is included for a similar group of rats which had received

TABLE VI

BLOOD LEVELS

Number of rats	Daily dose (oral) mg./g.	Period of dosage	Solution used	Mean blood levels µg./ml. (Hours after dosage)		
				17 hours	20 hours	23 hours
3	1·0 mg./g.	14 weeks	Fresh	28·7	3·7	8·0
3	1·0 mg./g.	14 weeks	One month old	23·9	8·3	8·1
3	1·0 mg./g.	One day	Fresh	1·9	—	—

only one dose of 1 mg./g. of sodium *p*-aminosalicylate. It will be seen that, in the group of rats receiving prolonged treatment, there is a moderate level in the blood 17 hours after the daily dose but this rapidly decreases during the next few hours to a negligible level. After a single dose, the amount present after 17 hours is negligible. Hence, it appears that even after prolonged administration, excretion occurs fairly normally but there is some evidence of accumulation in the tissues.

(*c*) A therapeutic test carried out on 1 month old and 6 month old solutions, using 12 mice per group in comparison with a similar size group treated with freshly prepared solutions showed that there was very little difference between the tuberculostatic activities of any of the solutions. A similar size group of infected untreated mice showed the usual high mortality during the test period.

Discussion

Probably the most interesting result of the present series of experiments has been the demonstration that *p*-aminosalicylic acid has a marked effect on the thyroid gland, causing disappearance of the colloid material and some increase in the epithelial tissue. This effect has been referred to as "hyperplasia" on account of its partial resemblance to the effect produced by anti-thyroid agents, such as thiouracil, but with *p*-aminosalicylic acid there is none of the increase in thyroid weight which occurs with thiouracil. Indeed the histological picture more closely resembles that produced by thiocyanate and it seems possible that part of the effect at least may be similar to that of thiocyanate in blocking the synthesis of thyroid hormone. Further work now being carried out with I^{131} and estimation of oxygen consumption may help to elucidate further this action. While the present work was in progress, two references appeared to the effect of *p*-aminosalicylic acid on the thyroid gland, viz., a reference given by Suter[20] and a report by Kjerulf-Jensen and Wolffbrandt.[21] The former merely mentions *p*-aminosalicylic acid as having a weak anti-thyroid action (1/100th that of thiouracil) but the latter workers have obtained marked hyperplasia and increased weight in the rat thyroid after only 10 days treatment. He also obtains similar results with *m*-aminophenol. These effects of *p*-aminosalicylic acid are reversed by thyroxine but not by sodium iodide. Although our own results confirm qualitatively those of Kjerulf-Jensen and Wolffbrandt with *p*-aminosalicylic acid, we find a prolonged test period is required to demonstrate the anti-thyroid effect. Also, we have been quite unable to demonstrate any anti-thyroid effect with *m*-aminophenol. At present, we have no explanation to suggest for these differences in results.

It seems probable that this effect of *p*-aminosalicylic acid on the thyroid may explain the occurrence of enlarged thyroid and mild myxœdema which has been reported during its clinical use.[22,23,24]

There appears to be no evidence in our experiments of any marked difference between the effects of fresh and old solutions so far as the effect on the thyroid gland of rats is concerned. Some difference between these solutions is noticeable as regards their toxicity which appears to increase somewhat rapidly during the first few weeks after preparation and then to increase only very slowly during the succeeding months. In this connection it is perhaps of interest to note that decomposition to *m*-aminophenol is not very extensive even after 6 months keeping, in spite of the fact that the colour of these solutions becomes increasingly darker with age. Indeed the colour of the 6 months solution is almost black. In addition, the increase in toxicity does not run parallel to and cannot be

accounted for quantitatively by the slow but steady increase in *m*-aminophenol. Hence, it appears that neither the increase in colour nor toxicity is due to the formation of *m*-aminophenol. These conclusions are supported by Koelzer and Giesen,[25] who appear to be the only other workers who have examined the properties of old solutions. The figures given by these workers for the toxicity of *p*-aminosalicylic acid and *m*-aminophenol agree almost exactly with our own, but they state that they observed no change in the histological picture after administration of *p*-aminosalicylic acid for up to 30 days. However, they do not state specifically that they examined specimens from the thyroid glands.

Old solutions appear to differ markedly from freshly prepared solutions by reason of the pressor effect of the former. This pressor effect is not blocked by priscol and therefore does not appear to be of sympathetic origin; some part of the effect may be due to a direct cardiac stimulation but there appears to be some other component, possibly a vaso-constrictor action, which we have not yet recognised.

Although there appears to be little diminution in the therapeutic activity of old solutions, the increase in toxicity and pressor effect which takes place on keeping, suggests that the general aversion to using old solutions is well-founded.

We have been unable to confirm earlier reports that *p*-aminosalicylic acid has an eosinopenic and a trypanocidal effect.

SUMMARY

1. Prolonged administration of solutions of sodium *p*-aminosalicylate, both fresh and old (varying in age from 1 week to 6 months), produces retardation in the growth of rats and hyperplasia of the thyroid glands. This effect is easily reversible by cessation of the drug.

2. The toxicity of the solutions to mice increases fairly rapidly during the first month of keeping and then more slowly.

3. Old solutions have a marked pressor effect in rabbits and cats, not reversed by adrenolytic agents. This pressor effect is almost negligible with fresh solutions.

4. The presence of *m*-aminophenol in the old solutions does not appear to be responsible for the increase in toxicity and development of a pressor effect.

5. After daily administration to rats for 14 weeks, there is little evidence that the normal rapid excretion of the drug is affected.

6. No confirmation has been obtained of earlier reports that it has an eosinopenic and trypanocidal effect.

We are indebted to Dr. J. Beattie for much helpful advice, for a great deal of the histological work, and for the photomicrographs. Our thanks are due to Mrs. E. Painter for valuable assistance and to Dr. M. Seiler for the therapeutic experiments. We would also like to thank the Directors of Herts Pharmaceuticals Ltd., for permission to publish this work.

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DISCUSSION

The paper was presented by MR. E. M. BAVIN.

DR. F. HARTLEY (London) asked whether the authors could give any information about the possible difference in toxicity between salts having different degrees of hydration. Arguments had been advanced as to the relative merits of the anhydrous calcium salt and the trihydrate. Would the toxicity of the anhydrous salt increase in the course of time as the result of absorption of water? Had the authors made any measurements on the calcium salts? Did they consider it likely that such salts would exhibit any difference in toxicity?

MR. J. JACOBS (Sunderland) asked whether the authors could give any reason for the instability of the solutions. Had any toxic effects been observed clinically when old solutions were used?

MR. T. D. WHITTET (London) referred to the fact that one firm issued *p*-aminosalicylic acid in the form of a solution of the sodium salt. This was preferred to a freshly made solution, about which the patients complained but, after the latter had been kept for several weeks, it had been reported as satisfactory by the patients.

DR. E. I. SHORT (London) asked whether the authors had measured the tissue distribution of the drug in the chronic toxicity test. After a single dose she had observed an accumulation of the salt in the liver. Was there any information on liver damage in the chronic toxicity test?

MR. E. M. BAVIN, in reply, said that he had no information as to the stability of the sodium or calcium salts in various degrees of hydration. All materials examined were in solution, and the periods of time referred

to in the paper were the ages of the solutions and not the age of the solid material from which those solutions were made. Attempts were being made to identify the toxic principle, and using the blood pressure effect as a criterion it appeared that the toxic principle could be extracted by butanol or ethanol. On the question of toxic effects, in clinical practice the well known effects of nausea, vomiting, dizziness and so forth were used, but the toxicity they were assessing was measured by the mortality in animals. On the distribution of the salt in the tissues, some Scandinavian workers, using the fluorescence test, had shown that there was a concentration of the salt in the lung and in the liver. They had not observed any significant liver damage in their animals.

THE ANALGESIC AND ANTIPYRETIC PROPERTIES OF SOME DERIVATIVES OF SALICYLAMIDE

By E. M. Bavin, F. June Macrae, D. E. Seymour and Pamela D. Waterhouse

From the Research Department, Herts Pharmaceuticals Ltd., Welwyn Garden City

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Introduction

The increase in the use of salicylamide (2-hydroxy benzamide) as an analgesic in recent years and its introduction into clinical use in this country prompted us to investigate the pharmacology of the substance and some of its derivatives. Other workers[1,2] have reported that salicylamide is a more powerful and less toxic analgesic than acetylsalicylic acid. As a part of a study of the chemistry of salicylamide, a series of alkyl ethers was prepared, and the analgesic and antipyretic activities of some of these compounds has been compared with those of salicylamide.

Experimental

(i) *Chemistry*

The preparation of Alkyl Ethers of Salicylamide. The ethers were prepared by treatment of salicylamide (1 mole) with the corresponding alkyl bromide (1 mole) in boiling ethanol in the presence of sodium ethoxide (1 mole). In the case of the methyl and amyl ethers, the iodide was found to work as satisfactorily as the bromide. The mixture, after refluxing for 6 hours, was freed of ethanol by distillation and the alkyl ether of salicylamide precipitated with water. The 2-*n*-dodecyl ether (compound 221) was recrystallised from ethanol and the hexadecyl (compound No. 268) from light petroleum (b.pt. 80° to 100° C.). All the other compounds were recrystallised from aqueous ethanol (50 per cent.). The yields in most cases were between 70 and 80 per cent. of a theoretical yield. With the exception of methyl,[(a)] ethyl[(b)] and *iso*propyl ethers,[(c)] all the compounds synthesised have not previously been reported.

(ii) *Pharmacology*

(*a*) *Toxicity Tests.* The acute intraperitoneal LD50's to mice were approximately determined, using up to 5 mice in groups separated by logarithmic dosage intervals. Animals which died on the same day were examined for unabsorbed drug and for any abnormalities due to the injection. The following day, and at the end of the 4-day test period, survivors were sacrificed and examined for unabsorbed drug. This gave an idea of the absorption of these compounds.

The acute oral LD50's to mice of the first 5 compounds in the series were determined on 10 to 20 mice in groups separated by logarithmic dosage intervals. The drugs were administered by stomach tube in the form of suspensions in 10 per cent. mucilage of acacia. Owing to poor absorption, the toxicities of the higher members were only roughly

TABLE I

Compound number	Name	Melting point °C.	Analysis		
			C per cent.	H per cent.	N per cent.
H.P. 209	2-Methoxybenzamide	127	—	—	—
„ 208	2-Ethoxybenzamide	130	—	—	—
„ 206	2-*n*-Propoxybenzamide	98 to 99	Found: 67·00	7·35	7·90
			Required: 67·00	7·30	7·80
„ 215	2-*iso*Propoxybenzamide	67 to 68	Found: 67·35	7·50	7·60
			Required: 67·00	7·30	7·80
„ 165	2-*n*-Butoxybenzamide	75 to 76	Found: 68·30	7·80	7·35
			Required: 68·35	7·80	7·25
„ 211	2-*n*-Amyloxybenzamide	86	Found: 69·15	8·10	6·95
			Required: 69·50	8·30	6·75
„ 216	2-*n*-Hexyloxybenzamide	71	Found: 70·60	8·50	6·45
			Required: 70·50	8·65	6·35
„ 217	2-*n*-Heptyloxybenzamide	56 to 57	Found: 71·40	8·80	5·70
			Required: 71·40	9·00	5·95
„ 218	2-*n*-Octyloxybenzamide	58 to 59	Found: 72·15	9·30	5·80
			Required: 72·25	9·30	5·60
„ 219	2 (3'-5'-5'-trimethylhexyloxy) benzamide	87 to 88	Found: 72·80	9·45	5·40
			Required: 72·95	9·60	5·30
„ 221	2-*n*-Dodecyloxybenzamide	83 to 84	Found: 74·80	10·25	4·75
			Required: 74·70	10·25	4·60
„ 268	2-*n*-Hexadecyloxybenzamide	79 to 80	Found: 76·00	11·15	4·10
			Required: 76·40	10·90	3·90

Analyses by Drs. Weiler and Strauss. m.pts. uncorrected.

(*a*) Sachs and Harold (*Ber. dtsch. chem. Ges.*, 1907, **40**, 2724) report m.pt. 127°
(*b*) Remsen and Reid (*J. Amer. chem. Soc.*, 1899, **21**, 290) report m.pt. 132·1°
(*c*) Kraut (*Liebigs Ann.*, 1869, **150**, 8) prepared 2-*iso*propoxybenzamide, but no melting point is reported.

determined. In each test, salicylamide was administered to 3 groups of mice as a standard and the figure for this compound is expressed as the mean and standard error of these 5 results.

White male mice weighing from 18 to 25 g. were used for both types of test and for both they were starved for 18 hours prior to injection. The experimental period was 4 days. The LD50's with their standard errors were calculated by the graphical method of Miller and Tainter.[3]

The chronic oral toxicity of salicylamide was determined on female white rats. 30 rats weighing from 115 to 130 g. were divided into 3 groups of 10 to receive daily:—

(*a*) 0·005 ml./g. of 10 per cent. of gum acacia in normal saline solution.
(*b*) 0·005 ml./g. of 5 per cent. of salicylamide in 10 per cent. mucilage of gum acacia = 0·25 mg./g.
(*c*) 0·005 ml./g. of 10 per cent. of salicylamide in 10 per cent. mucilage of gum acacia = 0·5 mg./g.

Food was removed from the cages in the morning until doses had been administered at the end of the afternoon. The animals were weighed twice weekly. The experiment was continued for 19 weeks during which time 4 animals were killed by the catheter entering the trachea instead of the œsophagus. Total red and white cell counts were made at intervals, and at the end of the experiment. Also at the end of the experimental period, plasma prothrombin times were determined on samples of blood, using the technique of Quick.[4] The animals were examined for macroscopic abnormalities, especially gastric ulcers.

(*b*) *Antipyretic Effect.* Rats were used for this work. Pyrexia was produced by injecting a dose of 1·0 ml. of 20 per cent. suspension of dried yeast B.P.C. subcutaneously at 5.0 p.m. on the afternoon previous to the actual test.[5,6] The next morning, using clinical thermometers, 3 readings of rectal temperature were made at 20-minute intervals. The rats were sorted into groups of 4 with the same average temperature. The drugs were usually administered in 10 per cent. mucilage of acacia intraperitoneally to facilitate rapid administration and absorption. A few experiments were performed to compare the effect of intraperitoneal and oral injections of the butyl ether (2-*n*-butoxybenzamide H.P. 165) the ethyl ether (2-ethoxybenzamide, H.P. 208) and acetylsalicylic acid. In every experiment two control groups were used, one was injected with 0·01 ml./g. of 10 per cent. gum acacia in normal saline solution and the other with 0·05 mg./g. of acetylsalicylic acid.

(*c*) *Analgesic Effect.* A modification of the Hardy-Wolff-Goodell apparatus was used, as described by Thorp,[7] in which the tail of the rat is exposed to the heat from a strong beam of light. We have measured the time in seconds for the tail to be withdrawn, using a constant intensity of light. The average normal response was about 5 seconds. The rats were kept in tubular cages for the duration of the experiment. The tails were blackened in order that they should absorb the heat and that the reflected light should not dazzle the operator. To avoid any risk of actual burning, the tails were exposed for no longer than 12 seconds. When no response was obtained, it was recorded as 12 seconds. Rats of both sexes, weighing about 200 g., were used.

The drugs were administered intraperitoneally in the same form as used in the antipyretic tests with the exception of acetylsalicylic acid which, on account of the high toxicity of the free acid at the dose required, was dissolved in the minimum amount of sodium hydroxide to give a soluble sodium salt.

In agreement with other workers,[7,8] we found that the rats require a training period before they react regularly to the stimulus. Readings on each rat were taken at 20-minute intervals throughout the experiments, i.e., at 20, 40, 60, 80, 100 and 120 minutes after the administration of the drug. 3 readings were taken before the administration of the drugs to provide normal reaction times. The increase in seconds of the reaction time of a group after the administration of a drug over the normal reaction time is called the response. This was estimated at each time interval. When these responses were plotted against the logarithm of the dose, we found that the effect after 20 minutes gave the best discrimination.

Our rats are obtained from dealers, and we find that, while the normal reaction times of the animals are remarkably constant, the sensitivity of the rats to analgesic drugs varies to a considerable extent from batch to batch. For instance, the analgesic activity of salicylamide could be detected on one batch of rats in a dose of 0·05 mg./g. but at the other extreme, another batch of animals was not sensitive to 0·2 mg./g. For this reason, we did not think it desirable to express our results, as the AD50 of Ross Hart,[1] but to give them in comparison with a compound

of recognised analgesic value. Salicylamide was used as this standard and a further check was made by including some tests on the widely used acetylsalicylic acid. In screening tests 2 doses of salicylamide and 2 doses of the new compound were used with 4 rats in each group. For more detailed studies latin square tests were devised where the same 16 rats were used for each point.

Results

Toxicity Test

All these compounds except the higher members of the series exhibited a sedative effect similar to that of salicylamide. Although an efficient analgesic, the sedative effect of this compound is very weak in man, and it might be supposed that its derivatives should behave similarly. The results of the acute toxicity tests to mice are expressed in Table II. The decrease in toxicity above the amyl derivative may be due to poor absorption and this view is confirmed by the finding of appreciable quantities of the nonyl and dodecyl derivatives in the intraperitoneal cavity twenty four hours after administration.

TABLE II

Acute toxicity to mice g./kg.

Compound number	Compound	Oral LD50 S.E.	Intra-peritoneal LD50 S.E.	Analgesic ratio compound salicylamide
H.P. 34	2-Hydroxybenzamide (Salicylamide)	1·59 ± 0·05	0·89 ± 0·029	1
„ 208	2-Methoxybenzamide	1·2 ± 0·116	0·9	1 – 2
„ 209	2-Ethoxybenzamide	1·76 ± 0·256	0·4	2 – 4 *2·3*
„ 206	2-*n*-Propoxybenzamide	1·26 ± 0·01	0·51	2 – 4
„ 165	2-*n*-Butoxybenzamide	1·3 ± 0·086	0·36 ± 0·019	2 – 4 *3*
„ 211	2-*n*-Amyloxybenzamide	>4·7	0·9	2 – 4
„ 216	2-*n*-Hexyloxybenzamide	>4·7	1·0	2
„ 217	2-*n*-Heptyloxybenzamide	>4·7	1·7	1 – 2
„ 218	2-*n*-Octyloxybenzamide	>4·7	>4·7	<1
„ 219	2-, (3'-5'-5'-Trimethylhexyloxy) benzamide	>4·7	>4·7	<1
„ 221	2-*n*-Dodecyloxybenzamide	>4·7	>4·7	<1
	Acetylsalicylic Acid	1·3 ± 0·06	0·28 ± 0·015	*0·3*

In the chronic toxicity test on salicylamide no rats died due to daily doses of 0·5 mg./g. and 0·25 mg./g. and all the 3 groups gained weight evenly. No difference between the treated and control groups was observed in total red and white cell counts, nor in prothrombin times. On post-mortem no macroscopic abnormalities were observed, in particular, no ulcers were detected in the stomachs.

Antipyretic Effect. The results are shown in Figure 1 where all drugs were administered intraperitoneally in doses of 0·05 g./kg. These results were subjected to a "t" test. At 20 minutes salicylamide is not significantly different from acetylsalicylic acid, but its effect wears off rapidly. The effect of the methyl ether (2-methoxy benzamide H.P. 209) lasts much longer. There is then a remarkable rise in activity and the ethyl derivative has a very powerful and enduring effect. Above this, however, the activity decreases so that the propyl and butyl derivatives are not significantly different from each other, but are more active than

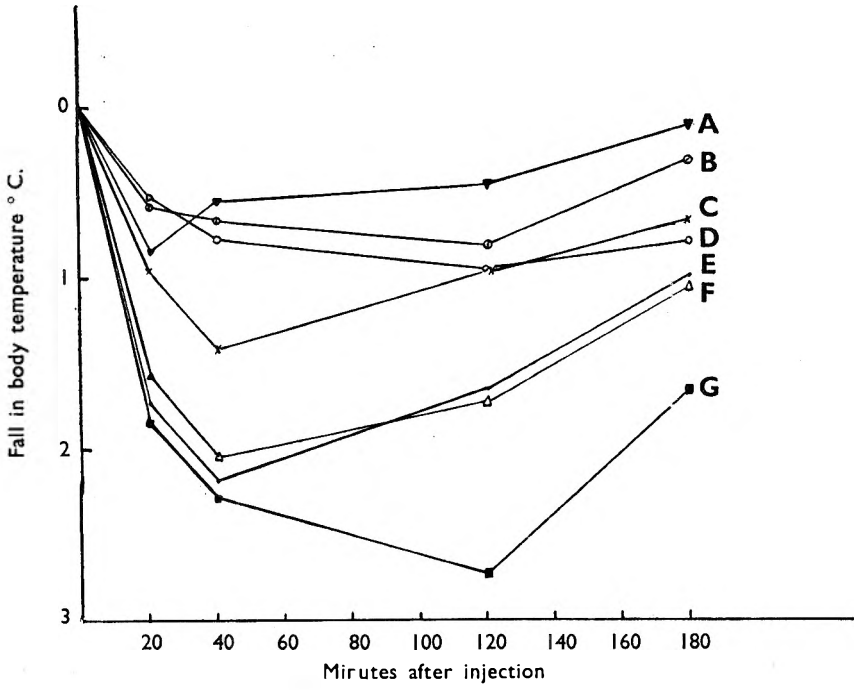

FIG. 1. Comparative antipyretic effect in rats of a series of benzamide derivatives. Dose for each compound, 0·05 g./kg.

A. 2-Hydroxybenzamide (salicylamide). B. 2-Methoxybenzamide.
C. 2-*n*-Amyloxybenzamide. D. Acetylsalicylic acid.
E. 2-*n*-Butoxybenzamide. F. 2-*n*-Propoxybenzamide.
G. 2-Ethoxybenzamide.

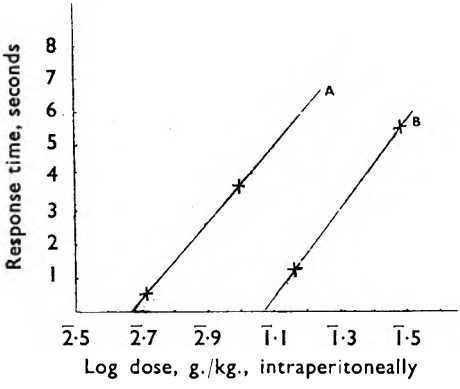

FIG. 2. Comparative analgesic effect in rats of 2-ethoxybenzamide and 2-hydroxybenzamide (salicylamide).

A. 2-Ethoxybenzamide.
B. 2-Hydroxybenzamide.

the amyl ether (2-*n*-amyloxybenzamide H.P. 211). The amyl ether only maintains its superiority to acetylsalicyclic acid for forty minutes.

When oral and intraperitoneal doses of acetylsalicylic acid, ethyl and propyl ethers were compared, it was found that the oral doses were about half as effective as the intraperitoneal. The ethyl, propyl and butyl derivatives exert a remarkable antipyretic effect and experiments have shown that they are capable of lowering the normal temperature of the rat without any permanent toxic symptoms.

Analgesic Effect. These results are expressed in Table II, those in italics representing results from latin square tests. The results for a typical latin square test are given in Figure 2, from which it can be calculated graphically that ethoxybenzamide possesses 2·3 times the analgesic activity of salicylamide. Statistical analysis of the results confirms that the two dose response lines may be regarded as parallel and provides an estimate of 2·25 (fiducial limits 80 per cent. to 133 per cent. P = 0·95) for the ratio of analgesic activity of ethoxybenzamide to salicylamide. We are indebted to Mr. E. C. Feiller for the statistical examination of these results. It can be seen that the propyl, butyl and amyl ethers are better analgesics than salicylamide and a considerable improvement on acetylsalicylic acid.

Discussion

We have confirmed that salicylamide is a considerably better analgesic but a poorer antipyretic than acetylsalicylic acid. We have confirmed that it is less toxic than the latter, especially on repeated doses, as the rats used throughout our chronic toxicity test showed remarkable tolerance to the drug and a complete absence of macroscopic signs of damage to the stomach.

Contrary to Litter *et al*[9] who claim that salicylamide shortens the prothrombin time in human subjects, but in agreement with Ichniowski and Heuper,[10] we did not find the drug to have any significant effect on the prothrombin time of rats fed on a normal diet.

Some of the derivatives made in these Laboratories are, without seriously increasing the toxicity, a considerable improvement on salicylamide. The best of these, for example, may have 10 to 12 times the analgesic activity of acetylsalicylic acid. Some, notably the ethyl derivative, have remarkable antipyretic properties, including the ability to lower the normal temperature without permanently damaging the animal. The activities of the higher members are probably limited by poor absorption.

Summary

1. The toxicity and analgesic and antipyretic properties of salicylamide have been studied. A long term toxicity test using rats has shown it to be remarkably well tolerated.

2. A series of salicylamide derivatives has been investigated, and some members have shown better analgesic activity than salicylamide and better antipyretic activity than acetylsalicylic acid.

Our thanks are due to Joan Sargerson and R. A. Webster for technical assistance. We are indebted to the Directors of Herts Pharmaceuticals for permission to publish this work.

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DISCUSSION

The paper was presented by Mrs. P. D. WATERHOUSE.

DR. F. HARTLEY (London) said it was stated that at post-mortem on rats no macroscopic damage was found. He was not certain how long it would take for an ulcer to develop in a rat's stomach, and asked whether any histological examination had been made. It might have been wiser to use a larger animal than the rat in the investigation of possible gastric damage.

MR. D. N. GORE (Dorking) asked whether there was any clinical evidence of the comparative value of salicylamide and salicylates as antirheumatic agents.

MRS. P. D. WATERHOUSE, in reply, said that as far as the histological examination of the stomach was concerned there were no obvious signs of tissue damage. It might perhaps be worth while carrying out small scale toxicity tests on larger animals. Salicylamide had been used on the Continent for many years and there were a number of reports on its use in the treatment of rheumatism. It had been reported that there were fewer side effects with salicylamide than with salicylates.

NEOVITAMIN A AND VITAMIN A ALCOHOL IN COMMERCIAL FISH-LIVER OILS AND VITAMIN A CONCENTRATES

By Edmund Hayes and Margaret A. Petitpierre

From The Vitamin Oils Department, Crookes Laboratories, Ltd., Park Royal, London

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The spectroscopic method described in the Addendum, 1951, to the British Pharmacopœia, 1948, for the estimation of vitamin A in "Halibut-liver Oil, Concentrated Solution of Vitamin A, Concentrated Solution of Vitamins A and D and Products of Similar Properties" requires a determination of the absorption curve of the whole oil in *cyclo*hexane between 320 and 350 mμ. The value of $E^{1\%}_{1\,cm.\ 327{\cdot}5\,m\mu}$ can then be converted directly to I.U./g., provided two conditions are satisfied: first, the absorption maximum must be within the range 325 to 328 mμ, and secondly, intensities of absorption in the region 320 to 350 mμ expressed as decimal fractions of the maximum must not differ by more than 0·02 from similar ratios determined on the international standard with a compensating solution of the diluent oil as blank. If the second of these requirements is not met, the geometric correction of Morton and Stubbs[1,2,3,4] is applied and the corrected value of $E^{1\%}_{1\,cm.\ 327{\cdot}5\,m\mu}$ calculated from the formula—

$$E_{327{\cdot}5\,m\mu}\ (\text{corr.}) = 7(E_{327{\cdot}5\,m\mu} - 0{\cdot}405\ E_{312{\cdot}5\,m\mu} - 0{\cdot}595\ E_{337{\cdot}7\,m\mu}),$$

is taken instead of the gross.

Both these procedures are based on the assumption that the vitamin A is present in a form identical, spectroscopically over the material range of wavelength and biologically, with the all-*trans* vitamin A acetate of the international standard. No allowance is made for the presence of free vitamin A alcohol or of neovitamin A.

Vitamin A occurs in fish-liver oils in the form of esters of the higher fatty acids, only small amounts of the alcohol being present.[5,6,7,8,9,10,11] Concentrates prepared from fish-liver oils by molecular distillation or by the Solexol process[12] also contain little or no free alcohol. The esters of all-*trans* vitamin A have the same molar biological activity as the alcohol[13,14]; the Subcommittee on Fat Soluble Vitamins of the World Health Organisation[15] accepts the acetate and the alcohol as being identical in this respect. It is also generally believed that the spectroscopic properties of the higher esters are identical with those of the acetate, though published evidence on this point is lacking. The spectroscopic properties of the alcohol, however, are different; consequently, a different standard curve and a different correction formula must be used when a preparation containing all its vitamin A in the alcohol form is being dealt with. But the pharmacopœial monographs for concentrated solution of vitamin A and concentrated solution of vitamins A and D allow the use of vitamin A concentrates prepared by the now almost obsolete processes of saponification and partial saponification, and

vitamin A alcohol could legitimately form the whole or any proportion of the total vitamin in these preparations. Obviously, the presence of both alcohol and ester forms would complicate the method of estimation.

A further complication arises from the presence of *trans-cis* or neovitamin A which differs biologically and spectroscopically from the all-*trans* isomer. Neovitamin A was first isolated by Robeson and Baxter,[16,17] and they developed a method for estimating it in mixtures of the two isomers, making use of the difference in their rates of reaction with maleic anhydride. The results of estimations made on various fish-liver oils and concentrates by Robeson and Baxter and by other workers using their method are shown in Table I; the table includes figures for synthetic concentrates of uncrystallised material made by two different processes. Robeson and Baxter also showed that the anthraquinone carboxylate of either isomer could be converted to an equilibrium mixture containing 30 per cent. of the neo form. It is of interest that most of the samples in Table I contain the isomers in proportions not far removed from those found in the equilibrium mixture; in 27 of the 33 samples the neo isomer constitutes between 20 and 40 per cent of the total.

The spectroscopic properties of neovitamin A have been described by Robeson and Baxter[17] and, in greater detail, by Chatain and Debodard.[18] A concentrate containing about 50 per cent. of neovitamin A, as natural esters with not more than 5 per cent. of all-*trans*, has been prepared by Dalvi and Morton.[19] The absorption curve of neovitamin A is displaced towards the long-wave side relative to all-*trans* and its molar extinction coefficient is lower. Moreover, Harris, Ames and Brinkman[20] found that neovitamin A has only 80 per cent. of the biological potency, as measured by rat-growth assays, of the all-*trans* form. Because of these differences, the presence of significant amounts of neovitamin A in oils and concentrates disturbs the spectroscopic determination of potency. If the ratio of the isomers in oils and concentrates were constant or nearly so, its presence could be allowed for by altering the requirement for the "purity" of the curve and adjusting the correction formula. The general problem is discussed by Cama, Collins and Morton.[1]

The present paper reports the results of neovitamin A and vitamin A alcohol estimations on 26 samples of fish-liver oils and natural vitamin A concentrates which passed through our hands in the autumn of 1951. These samples were drawn from large commercial batches and they represent a total of well over 3 million million I.U. of vitamin A. Our aim was to obtain information about the types of oils and concentrates at present used for pharmaceutical and veterinary purposes, and we were particularly interested in finding how the proportions of the isomers varied.

Experimental

Estimation of neovitamin A. We used the method of Robeson and Baxter[17]; a Hilger biochem absorptiometer with a vitamin A filter having maximum transmission at 605 mμ was used to measure the intensity of the blue colour produced by the reaction of vitamin A with antimony trichloride. The blue colour was measured after the maleic anhydride

had been allowed to react for 16 hours at 25° C. A blank solution without maleic anhydride was also tested. The percentage of neovitamin A was calculated from the formula:

$$\text{Per cent. neovitamin A} = \frac{100\,R - 5}{85} \times 100$$

where R = ratio of blue colour in the test solution to blue colour in the blank. Several experiments showed that the vitamin A solutions in benzene remained stable during the period of the reaction. It was also found that the presence of maleic anhydride had no effect on the blue colour when this was measured immediately after the anhydride had been added to the vitamin A solution in benzene.

Estimation of vitamin A alcohol. We used the method of Reed, Wise and Frundt.[8] A test of this method on three cottonseed-oil solutions containing known amounts of pure vitamin A alcohol and palmitate gave the following results for the percentage of total vitamin A present as alcohol: found 7·9, 18·1, 2·5; calculated 8·2, 19·1, 2·2. These solutions contained about 1 per cent. of total vitamin A.

The results of the estimations are given in Table II. Sample No. 13, one of 2 tunny-liver oils which had what appeared to be an abnormally low proportion of neovitamin A, was subjected to a more detailed examination; the rate of addition of maleic anhydride was measured at intervals over 16 hours on this sample and at the same time, for comparison, on a Solexol concentrate and on pure vitamin A acetate. To check the stability of the vitamin A, the corresponding blank solutions without maleic anhydride were also tested. The results are shown in Figure 1, which clearly indicates the difference between the 3 samples.

The average difference between duplicate neovitamin A estimations, done at different times, was 1·5 per cent. and the maximum 3·5 per cent.: both percentages are based on the total vitamin A.

Discussion

Neovitamin A. It will be noted that the proportion of total vitamin A as neovitamin A in 22 of the 26 samples is within the range 28 to 40 per cent., that is, fairly close to the equilibrium proportion. In 4 samples of fish-liver oil, however, the proportion is significantly lower. The low values for 2 tunny-liver oils are interesting, especially as the third tunny-liver oil we examined gave a figure of 37 per cent., and the only other figures for tunny-liver oils reported in the literature, those of Meunier and Jouanneteau,[21] are 55 and 46 per cent. It therefore seems that commercial batches of fish-liver oil may vary in the proportions of isomers they contain, though proportions of about 2 parts of all-*trans* to 1 of neo are the most common.

This variability makes the development of a standard spectroscopic method of vitamin A estimation applicable to all oils and concentrates a matter of difficulty, if the necessity of a neovitamin A determination on each sample is to be avoided. The B.P. method could not safely be altered in any case on the basis of our present knowledge of the properties

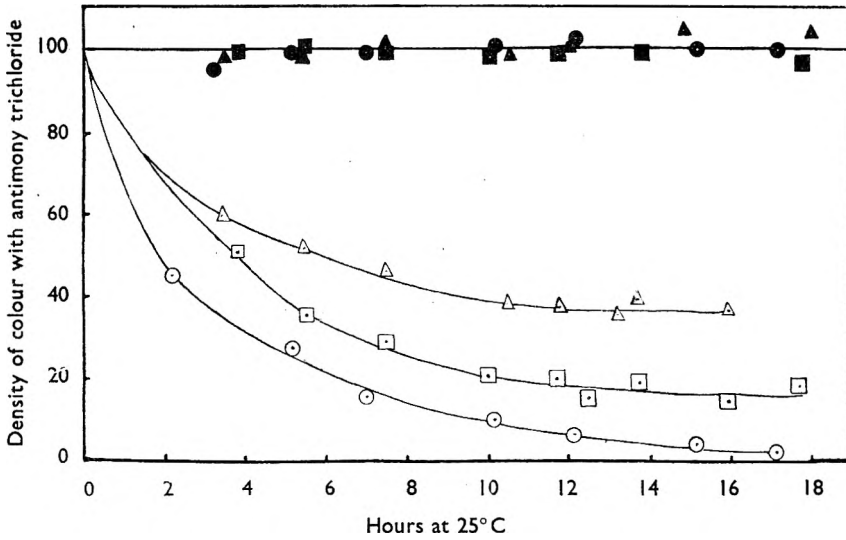

FIG. 1. Reaction of vitamin A with maleic anhydride

△ Solexol concentrate (25).
⊡ Tunny-liver oil (13).
⊙ All-*trans* vitamin A acetate.
●■▲ Controls.

of neovitamin A, and it has perhaps the merit that it tends to under- rather than over-estimate the potency of many oils. There is something to be said, however, for the removal of the "tolerance" clause which allows the direct use of the 1900 factor for oils with curves close to that of the international standard; occasionally concentrates are encountered having curves which just come within the tolerance even though they contain nearly one-third of their vitamin A as the neo-isomer and consequently do not merit the 1900 factor.

We are only concerned here with the ratios of the isomers as found in commercial oils. It does not follow that the isomers occurred in the same proportions in the fish livers from which the oils were prepared. In the preparation of large batches of fish-liver oil, differences due to age, sex, size or other biologically important conditions of the fish tend to be obliterated and only an average product is obtained. Methods of extraction, too, may result in some isomerisation. Any investigation into the biological significance of the difference in the proportions of isomers would need to be done on fresh individual livers.

Because neovitamin A gives a more intense blue colour than the all-*trans* form,[22] results obtained by the method of Robeson and Baxter, in which no allowance is made for this difference, are somewhat higher than the true values; the true proportion is related to that found by the method as follows:—

$$x = \frac{100\,r}{100\,y - ry + r}$$

where x = true percentage of neovitamin A

r = percentage found by Robeson and Baxter's method

$$y = \frac{\text{Intensity of blue colour of neo}}{\text{Intensity of blue colour of all-}trans}$$

In the absence of a sample of pure neovitamin A the ratio, y, is difficult to determine, but comparison of a number of high-potency oils and concentrates with all-*trans* vitamin A acetate suggests that, for our absorptiometer and filter, it is in the region of 1·4; this means that an apparent value of 30 per cent. would correspond to a true value of about 23 per cent. We have not attempted to correct the results in Table II to allow for this difference; they are therefore too high, but they are directly comparable with the figures in Table I. All the figures may be regarded as upper limits.

TABLE I

NEOVITAMIN A IN FISH-LIVER OILS AND CONCENTRATES

(Data from the literature)

Reference	Type of oil or concentrate	Neovitamin A as percentage of total vitamin A
Robeson and Baxter[17]	U.S.P. Reference cod-liver oil	39
	Distilled vitamin A concentrates	39, 35
	Distillate from dogfish-liver oil	39
	Dogfish-liver oil	36
	Soupfin shark-liver oil	37
	Distillate from soupfin shark-liver oil	33
	Halibut-liver oil	32
	California Jewfish-liver oil	34
Karnovsky *et al.*[20]	Soupfin shark-liver oils:	
	Mixed oils	31
	Fat females	34
	Thin females	24
	Males	29
Meunier and Jouanneteau[21]	Shark-liver oil	18
	Argentine shark-liver oil	42
	Dakar shark-liver oil	42
	Spanish red-tunny-liver oil	55
	Red-tunny distillate	46
	Portuguese red-tunny-liver oil	46
Chatain and Debodard[18]	Molecular distillates	33, 31
Cama *et al.*[1]	High-potency oils	25, 24
Dalvi and Morton[19]	Solexol concentrates	23, 29, 24, 23
Cawley *et al.*[30]	Synthetic concentrates	40, 33
Schwarzkopf *et al.*[31]	Synthetic concentrates	37, 38, 41, 40

It may be noted here that we have little information about the other possible isomers of vitamin A, the *cis-cis* and the *cis-trans*. The possibility of their occurrence in fish-liver oils should not be overlooked, and if they were present, they might conceivably interfere with the Robeson-Baxter estimation. Little is known, too, of the effect on the estimation of vitamin A congeners and inhibitors of the blue colour.

Vitamin A alcohol. Table II shows that most of the samples contain little free vitamin A alcohol. 3 of the halibut-liver oils contain 7 or more per cent. of their total vitamin A as alcohol, but these are exceptional; it is quite likely that hydrolysis of some of the vitamin A esters occurred during the extraction of these oils. By careful extraction of the livers

TABLE II

NEOVITAMIN A AND VITAMIN A ALCOHOL IN SAMPLES

No.	Type of oil	$E^{1\ per\ cent.}_{1\ cm.}$ at 327·5 mμ	Percentage of total vitamin A	
			as vitamin A alcohol	as neovitamin A
1	Halibut-liver oil	55	2·1	37
2	" " "	17	2·3	35
3	" " "	23	10·8	31
4	" " "	34	7·0	18
5	" " "	15	7·0	32
6	" " "	13	0·2	32
7	" " "	15	1·3	33
8	" " "	25	1·5	39
9	" " "	13	3·0	28
10	" " "	50	4·0	31
11	" " "	24	2·2	18
12	" " "	52	2·4	35
13	Tunny-liver oil	301	1·1	12
14	" " "	340	1·3	11
15	" " "	348	0	37
16	Shark-liver oil	13	0	37
17	Fish-liver oil blend	6	1·3	33
18	" " " "	13	0	35
19	Molecular distillate	30	0	37
20	" "	130	0	29
21	" "	33	0	37
22	" "	129	1·5	33
23	" "	32	0	34
24	Solexol concentrate	31	1·2	36
25	" "	125	0	38
26	" "	130	0·9	37

it should be possible to avoid this hydrolysis and so produce oils containing not more than, say, 7 per cent. of total vitamin A as alcohol. We suggest that the B.P. monographs for halibut-liver oil, concentrated solution of vitamin A and concentrated solution of vitamins A and D should include such a limit test for vitamin A alcohol. It would, of course, be necessary to withdraw recognition from concentrates prepared by saponification and partial saponification as a source of vitamin A for the concentrated solutions.

This would ensure the removal of one likely source of error in the estimation of the vitamin A potency of these preparations. Moreover, it has been shown by various workers[14,23,24,25,26,27,28] that vitamin A alcohol is less stable than the esters. This is an additional reason for keeping the proportion of vitamin A alcohol as low as possible

SUMMARY

1. Examination of 26 samples of commercial fish-liver oils and natural vitamin A concentrates showed that the proportion of total vitamin A present as neovitamin A varied between 11 and 39 per cent when measured by the Robeson-Baxter method.

2. Because of the difference in the intensity of the blue colours produced with antimony trichloride by all-*trans* and neovitamin A, the Robeson-Baxter method gives results somewhat higher than the true values.

3. Most of the samples contained little or none of their vitamin A in the form of the free alcohol.

4. It is proposed that the B.P. monographs for halibut-liver oil, concentrated solution of vitamin A and concentrated solution of vitamins A and D should include a limit test for vitamin A alcohol.

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SOME OBSERVATIONS ON THE DETERMINATION OF VITAMIN A IN COD-LIVER OIL

By R. V. SWANN

From the Research Division, Allen and Hanburys Ltd

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IT has long been known that naturally occurring vitamin A esters which are present in fish liver oils exhibit selective absorption of ultra-violet radiation of wavelength of about 328 mμ. For many years the value obtained by multiplying $E_{328\ m\mu}$* obtained on the unsaponifiable matter by the factor declared by the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations was regarded as indicating the potency of cod-liver oil. This was the procedure from 1936 until 1948, and a factor of 1600 was used. The B.P. 1948 laid down that the factor 1600 should be used to convert spectrophotometric data into biological units per g. The Addendum 1951 to the B.P. 1948 stipulates that cod liver oil shall be saponified, the unsaponifiable matter dissolved in *cyclo*hexane and optical density values determined at 3 stated wavelengths. A formula is then applied to the results and a corrected $E_{326\cdot5\ m\mu}$ obtained, which is then multiplied by a factor of 1900 to indicate the potency. This departure from the procedure indicated in the B.P. 1948, arose largely as a result of the work of Morton and his colleagues[1] who developed a geometrical formula which would allow for the contribution of irrelevant material to the absorption at the wavelength of maximum absorption. It is a prerequisite of the application of this formula that the irrelevant absorption should be linear at the specified wavelengths. It is also assumed that no impurity or artefact has a maximum very close to that of vitamin A. The development of this method was made possible by the development of the photoelectric spectrophotometer, whereby optical densities may be taken with ease and speed at different wavelengths, and by the production of synthetic all-*trans* vitamin A alcohol and vitamin A acetate. Morton *et al*[2] have published data giving the ratio of optical densities at $E_{max.}$ to that at other stated wavelengths in specified solvents, and have also given correctional formulæ for the determination of vitamin A in these solvents. Corrected values ($E_{max.}$ (corr.)) may be multiplied directly by the factor 1900. It should be mentioned that a factor of 1900 is employed to convert to biological units $E_{max.}$ values determined on pure all-*trans* vitamin A alcohol and pure all-*trans* vitamin A acetate when dissolved in *cyclo*hexane. The U.S.P. up to the 14th revision specified that vitamin A assays should be carried out biologically, and no physical method of assay was given. The U.S.P. XIV specifies that vitamin A shall be determined on the unsaponifiable matter followed by the application of a formula which corrects for irrelevant absorption. Materials other than vitamin A which occur in cod-liver oils and which may tend to vitiate the results

* Throughout this paper $Ex_{m\mu}$ is used to indicate $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ at wavelength x mμ.

obtained by a correctional formula are anhydro vitamin A, vitamin A_2, neo-vitamin A, kitol, oxidation products and polyene acids.

Vitamin A_2 shows selective absorption at 351 mμ ($E = 1460$) with a subsidiary peak at 287 mμ ($E = 820$), and has a biological potency of about 40 per cent. of that of all-*trans* vitamin A. Vitamin A_2 reacts with a solution of antimony trichloride in chloroform giving a blue colour with maximum absorption at 693 mμ. Vitamin A gives a similar colour with maximum absorption at 620 mμ.[3] From readings taken at 620 mμ and at 693 mμ of the colour developed with antimony trichloride by the unsaponifiable matter of a cod-liver oil, the absorption at 693 mμ due to vitamin A_2 may be calculated. The contribution of the vitamin A_2 absorption at 328 mμ may then be estimated using data given by Morton for vitamin A and by Shantz for vitamin A_2.[3]

Neo-vitamin A has an absorption curve which is similar to that of all-*trans* vitamin A, but the wavelength of maximum absorption is moved 3 mμ towards the visible range.[4] Its absorption at the wavelength of maximum absorption is 10 per cent. lower than that of all-*trans* vitamin A at its wavelength of maximum absorption. In the region 280 to 330 mμ differences between the absorption spectrum of neo-vitamin A and all-*trans* vitamin A are small. From 330 to 390 mμ the neo-form shows higher absorption. It is stated by Morton that neo-vitamin A esters are more difficult to saponify than all-*trans* vitamin A esters.

The problem of arriving at a satisfactory estimate of potency is therefore one of considerable difficulty. Unsaturated acids exhibiting absorption maxima at 270 mμ, 315 mμ and 320 mμ, may be removed by a saponification process, but saponification cannot remove either vitamin A_2 or kitol. In the present investigation we are considering only fresh cod-liver oils, and therefore, expect to find only a very small amount of oxidation products. Further, by a chromatographic method, details of which will be given later, the presence of only very small amounts of anhydro-vitamin A could be demonstrated.

A number of cod-liver oils when assayed by the B.P. Addendum 1951 method in this laboratory gave considerably lower results for the value $E_{326\cdot5\ m\mu}$ (corr.) $\times$ 1900 than for the uncorrected value $E_{326\cdot5\ m\mu}$ (gross) $\times$ 1600. It was decided to investigate the reason for this discrepancy. The Addendum specifies that vitamin A in cod-liver oils shall be determined on the unsaponifiable matter obtained by the method given by the B.P. 1948. The instructions are as follows: "Boil 1 g. of the cod-liver oil with 10 ml. of freshly prepared *N/2 alcoholic solution of potassium hydroxide* for five minutes or until clear. . . ."

The U.S.P. XIV specifies a half-hour saponification period. In our experience the solution of cod-liver oil in ethanolic potassium hydroxide solution becomes clear in a much shorter time than 5 minutes. The solution is apparently clear as soon as sufficient heat has been applied to the flask to effect solution of the oil. This occurs within 1 minute of placing the flask on the hot plate. We have saponified several cod-liver oils for periods of 5 minutes and longer. The saponification time has been accurately observed and has been taken to start from the condensation

of the first drop of ethanol from the end of the condenser, the flask being placed on a previously heated hot plate. The flask was removed from the hot plate at the end of the 5-minute period. The following figures illustrate the change in shape of the absorption curve of sample A with increase in the time of saponification.

TABLE I

ABSORPTION SPECTRUM OF THE UNSAPONIFIABLE MATTER OF COD-LIVER OIL, A, AFTER 5 MINUTES, AND AFTER 30 MINUTES, SAPONIFICATION

mμ	5 Minutes saponification	30 Minutes saponification
	E_1^1 per cent. cm.	E_1^1 per cent. cm.
300	0·333	0·289
305	0·359	—
310	0·389	0·357
312	0·412	0·371
312·5	0·416	0·372
313	0·423	—
314	0·426	0·379
315	0·427	—
316	0·424	0·387
317	0·423	—
318	—	0·390
320	0·416	0·398
322	0·416	0·404
326·5	0·423	0·414
328	0·422	0·414
329	0·418	—
330	0·415	0·409
335	0·384	0·382
336·7	0·372	0·372
340	0·346	0·346
Ratio $\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$	0·985	0·899
Ratio $\frac{E_{336\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$	0·879	0·899

The absorption spectrum of the extracted material after 5 minutes saponification exhibits two maxima, one at 315 mμ and another at 326 mμ. The absorption maximum at 315 mμ which is due to unsaturated acids is removed by a 30-minutes saponification period. A 30-minutes saponification period removes additional amounts of irrelevant absorbing material over that removed by a 5 minutes saponification period, as shown in Table I by the reduction of the ratio $\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$ from 0·985 to 0·899.

The results shown in Table II were obtained on the oil and on the unsaponifiable matter.

The above figures indicate that a period of saponification longer than 5 minutes has had little effect on the E (gross), but quite a large effect on the value of E (corr.). The application of the correctional formula to the unsaponifiable matter obtained by a 5-minute period of saponification

TABLE II

	$E_{max.}$ (gross)	$E_{max.}$ (gross) ×1600	E (corr.)	E (corr.) ×1900
On the whole oil	0·456	730	0·385	730
After 5 minutes saponification ..	0·422	675	0·224	425
After 30 minutes saponification ..	0·415	665	0·301	570

has in the above case led to erroneously low results. Tests carried out on further samples indicate the inadequacy of the current instructions for the determination of vitamin A.

TABLE III

ABSORPTION SPECTRUM OF THE UNSAPONIFIABLE MATTER OF COD-LIVER OIL, B, AFTER 5 MINUTES AND 30 MINUTES SAPONIFICATION

mμ	5 Minutes	30 Minutes
	$E_1^{1\ \text{per cent.}}_{1\ \text{cm.}}$	$E_1^{1\ \text{per cent.}}_{1\ \text{cm.}}$
310	0·980	0·905
312	1·040	0·939
313	1·066	—
314	1·072	0·965
315	1·082	0·974
316	1·080	0·983
317	1·075	0·990
318	—	0·994
320	1·072	1·010
326·5	1·113	1·048
Ratio $\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$	0·940	0·896
Ratio $\frac{E_{326\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$	0·890	0·896

Sample B	$E_{max.}$ (gross)	$E_{max.}$ (gross) × 1600	E (corr.)	E (corr.) × 1900
On the whole oil	1·212	1940	1·008	1910
After 5 minutes saponification ..	1·113	1780	0·686	1300
After 30 minutes saponification ..	1·048	1680	0·777	1480

Table III gives the values on sample B obtained by taking the $E_{max.}$ (gross) and $E_{max.}$ (corr.) and multiplying by the factors 1600 and 1900 respectively. Figures are also given for the whole oil.

Table IV gives similar results for samples C and D.

TABLE IV

Sample C	E (gross)	$E_{max.}$ (gross) × 1600	E (corr.)	E (corr.) × 1900
After 5 minutes saponification ..	0·658	1050	0·497	945
After 5 minutes saponification ..	0·682	1090	0·399	760
After 5 minutes saponification ..	0·651	1040	0·539	1020
After 30 minutes saponification ..	0·661	1060	0·511	970
	$\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ E\mu}}$		$\frac{E_{336\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$	
After 5 minutes saponification ..	0·872		0·910	
After 5 minutes saponification* ..	0·946		0·893	
After 5 minutes saponification ..	0·870		0·891	
After 30 minutes saponification ..	0·870		0·904	

Sample D	$E_{max.}$ (gross)	$E_{max.}$ (gross) × 1600	E (corr.)	E (corr.) × 1900
After 5 minutes saponification ..	0·474	760	0·385	730
After 5 minutes saponification ..	0·486	780	0·350	665
After 30 minutes saponification ..	0·475	760	0·364	690
	$\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$		$\frac{E_{336\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$	
After 5 minutes saponification ..	0·876		0·890	
After 5 minutes saponification ..	0·893		0·906	
After 30 minutes saponification ..	0·883		0·893	

* The absorption spectrum of this extract showed a subsidiary maximum at 315 mμ.

Discussion of results obtained on the foregoing samples

Examination of the above samples indicates that a 5 minute saponification period is not sufficient to remove the non-linear irrelevant material. In general, improvement in the shape of the absorption curve is obtained by increasing the saponification time from 5 minutes to 30 minutes. The ratios $\frac{E_{312\cdot5\ m\mu}}{E_{326\cdot5\ m\mu}}$ and $\frac{E_{336\cdot7\ m\mu}}{E_{326\cdot5\ m\mu}}$ are of value in indicating the efficiency of a given saponification. The variable nature of the results obtained after 5 minutes saponification is not due to faulty technique since only a few oils exhibit this variation. The variation may be attributed to the presence of matter which is more difficult to saponify than that which is generally found in medicinal cod-liver oils.

Table V indicates the repeatability of $E_{max.}$ (gross) and $E_{max.}$ (corr.) as determined on the unsaponifiable matter of several samples after a 30-minutes saponification period. It will be seen that the values of $E_{max.}$ (gross) are reproducible to within about 2 per cent. and that E (corr.) is reproducible to within about 5 per cent.

TABLE V

REPRODUCIBILITY OF $E_{max.}$ (GROSS) AND $E_{max.}$ (CORR.) AFTER 30 MINUTES SAPONIFICATION

Sample	$E_{max.}$ (gross)	$E_{max.}$ (corr.) Formula			E_{max} (gross) × 1600	$E_{max.}$ (corr.) × 1900 Formula		
		(a)	(b)	(c)		(a)	(b)	(c)
6	0·525	0 385	0·376	0·390	840	730	715	740
6	0·535	0 399	0·402	0·399	855	760	765	760
6	0·535	0 399	—	—	855	760	—	—
7	1·000	0 805	—	—	1600	1530	—	—
7	1·000	0 784	0·757	0·799	1600	1490	1440	1520
8	1·135	0 924	—	—	1820	1750	—	—
8	1·143	0 875	0·852	0·905	1830	1660	1620	1720
10	1·050	0·826	0·826	0·872	1680	1570	1570	1660
10	1·067	0·847	—	—	1710	1610	—	—
12	0·477	0·343	0·334	0·350	765	650	635	665
12	0·475	0·336	—	—	760	640	—	—

(*a*) (*b*) and (*c*) are formulæ given by Morton[2] which correct the E (gross) for irrelevant linear absorption.

It should be noted that good agreement is obtained between E (gross) values as carried out in duplicate, and also good agreement between E (corr.) results when determined using *one* given formula (*a*), (*b*) or (c). However, inter-agreement is not always good between results obtained using (*a*), (*b*) and (*c*). Poor inter-agreement tends to indicate that the irrelevant absorption is non-linear.

It was next decided to obtain a comparison of E (max.) values, both gross and corrected on the whole oil, on the unsaponifiable matter and by chromatography on alumina, containing 5 per cent. of added water. These values are given in Table VI.

All E values given in Table VI, which have not been determined in

*cyclo*hexane, have been calculated to those which would have been obtained in *cyclo*hexane.

Table VI illustrates good agreement between potencies obtained by fully correcting chromatographic and unsaponifiable matter E (gross) values for the presence of 10 per cent. of vitamin A_2 and the presence of 25 per cent. of neo-vitamin A,* and those obtained by multiplying the gross absorption of the unsaponifiable fraction by the factor 1600. These results are marked by an asterisk. Results obtained by applying the correctional formula given in the B.P. Addendum 1951, and multiplying the corrected E value by a factor of 1900 are given in the column marked with two asterisks and on the whole, give low results for the vitamin A potency.

The details of the chromatographic procedure are as follows, and are, in the main, similar to those of Gridgeman for the determination of vitamin A in whale liver oil.[5] The alumina used for chromatography was that of Peter Spence Grade O "activated alumina for chromatography." The alumina was heated for 2 hours before use in a furnace at 800° C. and then allowed to cool to room temperature. The chromatographic column consisted of a glass tube about 45 cm. long, and 7 mm. internal diameter. It was plugged with cotton wool at the lower end. A wider tube, forming a reservoir, 50 cm. long and of 12 mm. internal diameter was sealed to the top end of the narrower tube. 10 ml. of activated alumina, as measured in a 25 ml. cylinder of diameter $\frac{3}{4}$ inch were covered with light petroleum (b.pt. 40° to 60° C.) in a mortar, 0·5 ml. of water was added in drops from a pipette with a fine orifice, the pipette being moved about above the surface of the light petroleum during the addition. The moistened alumina was then ground and mixed under the surface of the light petroleum. The column was then set vertically in a stand, the lower end of the column was closed by means of a cork, and the narrow part of the column half filled with light petroleum. The moistened alumina from the mortar was then washed into the wide reservoir and allowed to settle, the tube being rotated during the period of settling. · An air pressure of 40 cm. of mercury was applied to the top of the column, the pressure being released when the solvent surface had sunk to within 1 or 2 cm. above the surface of the material in the column. 1 g. of cod-liver oil, dissolved in 20 ml. of light petroleum, was then washed into the reservoir, the air pressure re-applied, and the portion of the eluate which fluoresced yellowish-green when examined in ultra-violet light, was collected separately. It was found to be necessary to add 20 ml. of light petroleum to the reservoir, when practically all the cod-liver oil solution had left, in order to remove all traces of vitamin A from the column. The pressure was then re-applied and the yellowish-green fluorescent fraction added to that portion previously collected. The whole was then made up to volume with light petroleum and suitably diluted for spectrophotometry.

In the early experiments the course of the chromatography was followed by means of the antimony trichloride reagent, but observation of the fluorescence in the ultra-violet light proved to be less troublesome and

* Hereafter referred to as fully corrected chromatographic and fully corrected unsaponifiable E values.

TABLE VI

COMPARISON OF $E_{max.}$ AND ($E_{max.}$ × FACTOR) FOR A NUMBER OF COD-LIVER OILS

Sample	E (gross)			E (corr.)			E (gross) × 1600			E (corr.) × 1900			(Fully corrected) ×1825		$\lambda_{max.}$ mμ oil	$\lambda_{max.}$ mμ chromatography
	Whole oil	Unsaponifiable matter	Chromatography on oil	Whole oil	Unsaponifiable matter	Chromatography on oil	Whole oil	Unsaponifiable matter*	Chromatography on oil	Whole oil	Unsaponifiable matter**	Chromatography on oil	Unsaponifiable matter*	Chromatography on oil*	Whole oil	Chromatography on oil
1	1·305	1·175	1·084	1·09	0·94	1·020	2080	1880	1730	2070	1780	1940	1940	1790	—	325
2	0·814	0·758	0·718	0·679	0·588	0·650	1300	1210	1150	1310	1120	1230	1260	1180	—	—
3	0·743	0·701	0·694	0·623	0·567	0·610	1190	1120	1110	1180	1080	1160	1150	1140	—	—
4	0·597	0·522	0·533	0·526	0·406	0·464	955	835	850	1000	770	880	860	875	—	319
5	0·865	0·822	0·827	0·728	0·665	0·710	1380	1310	1320	1380	1260	1360	1350	1360	—	325
6	0·576	0·535	0·560	0·476	0·399	0·451	920	855	895	905	760	855	880	920	323	322
7	1·013	1·000	0·990	0·858	0·805	0·902	1620	1600	1580	1630	1530	1710	1640	1640	327·5	325
8	1·217	1·135	1·182	1·050	0·924	1·063	1950	1820	1890	1995	1750	2020	1860	1940	327·5	325
9	1·277	1·204	1·260	1·068	0·994	1·141	2040	1930	2020	2030	1890	2170	1980	2070	327·5	325
10	1·147	1·067	1·100	0·980	0·847	0·935	1830	1710	1760	1860	1610	1780	1760	1810	324·5	320
11	0·540	0·467	0·476	0·434	0·357	0·424	860	745	760	825	680	805	770	780	321·5	320
12	0·547	0·475	0·480	0·439	0·335	0·431	875	760	770	835	640	820	780	790	321	320
13	0·450	0·415	0·425	0·363	0·301	0·378	720	665	680	690	570	720	680	700	323	322
14	1·212	1·093	1·070	1·008	0·651	0·990	1940	1750	1710	1920	1640	1880	1770	1760	—	325
15	0·716	0·661	0·660	0·632	0·511	0·597	1130	1060	1060	1200	970	1130	1085	1085	324	325
16	0·522	0·475	0·484	0·426	0·364	0·426	835	760	775	810	690	810	790	780	322·5	322
17	0·582	0·514	0·482	0·504	0·371	0·437	930	820	770	960	705	830	845	790	—	—
18	0·848	0·725	—	0·713	0·553	—	1335	1160	—	1355	1050	—	1190	—	—	—
19	0·456	0·415	0·425	0·385	0·301	0·378	730	665	680	730	570	715	680	695	—	320

much quicker. 4 chromatograms could be carried out in the course of 4 hours by this means. The equations of Morton, quoted below, correcting for irrelevant absorption, were applied to density readings obtained by chromatography.[2]

$$E \text{ (corr.)} = 7\ (E_{325}-0{\cdot}399\ E_{310}-0{\cdot}601\ E_{334{\cdot}9}) \quad .. \quad .. \quad (a)$$
$$\text{and } E \text{ (corr.)} = 3{\cdot}5\ (2E_{325}-E_{313}-E_{337}) \quad .. \quad .. \quad .. \quad .. \quad (b)$$

The corrected results appearing in the "oil" columns of Table VII were obtained by using the equations,

$$E \text{ (corr.)} = 7\ (E_{327{\cdot}5}-0{\cdot}405\ E_{312{\cdot}5}-0{\cdot}595\ E_{337{\cdot}7}) \quad .. \quad .. \quad (a)$$
$$E \text{ (corr.)} = 6{\cdot}58\ (E_{328}-0{\cdot}412\ E_{313}-0{\cdot}588\ E_{338{\cdot}5}) \quad .. \quad .. \quad (b)$$
$$\text{and } E \text{ (corr.)} = 3{\cdot}52\ (E_{328}-E_{316}-E_{340}) \quad .. \quad .. \quad .. \quad .. \quad (c)$$

The equations:

$$E \text{ (corr.)} = 7\ (E_{326{\cdot}5}-0{\cdot}422\ E_{312{\cdot}5}-0{\cdot}578\ E_{336{\cdot}7}) \quad .. \quad .. \quad (a)$$
$$E \text{ (corr.)} = 6{\cdot}82\ (E_{326}-0{\cdot}4\ E_{311}-0{\cdot}6\ E_{336}) \quad .. \quad .. \quad .. \quad (b)$$
$$\text{and } E \text{ (corr.)} = 3{\cdot}36\ (2E_{327}-E_{315}-E_{339}) \quad .. \quad .. \quad .. \quad .. \quad (c)$$

were applied to results obtained on the unsaponifiable matter.

Agreement within 5 per cent. between the values obtained by the application of given sets of correctional formulæ indicates that the irrelevant absorption is linear. It will be seen from an examination of the above data, that, although in some cases the agreement indicates that the irrelevant absorption is linear, in other cases the indication suggests non-linearity. In cases where a single result is given, only equations (*a*) have been applied.

The *E* values in the chromatographic experiments were determined using light petroleum as a solvent, but the values given in Tables V and VI are calculated to those which would be obtained using *cyclo*hexane as solvent, in order that the *E* values may be comparable.

The chromatographic results are rather variable. In some cases *E* values were obtained which, when expressed as a ratio $E_{max.}$, agreed with the published data with $\pm$ 2 per cent. over the range 310 to 340 mμ. In cases where appreciable amounts of unsaturated acids were present, chromatography failed to remove them completely, and maxima were found at wavelengths less than 325 mμ.

The agreement between the wavelengths of maximum absorption of the chromatographic fraction and that as determined on the oil is very good. In the case of those oils which contain unsaturated acids, displacement of the wavelength of maximum absorption towards the 320 mμ region is noted, and a similar displacement is found in the case of the chromatographic fraction.

The results appearing in the fully corrected unsaponifiable matter column are results compensated for the presence of 25 per cent. of neo-vitamin A of 80 per cent. of the activity of the all-*trans* variety and also for the presence of a 10 per cent. contribution by vitamin A_2 to the $E_{max.}$ (gross) value. In the course of a number of determinations of vitamin A_2 a contribution to the $E_{max.}$ (gross) by vitamin A_2 of not more than 10 per cent. of the value of $E_{max.}$ (gross) has been found. The factor 1825 was

calculated using the values $E_{max.}$ for all-*trans* vitamin A alcohol in *cyclo*hexane = 1760 and $E_{max.}$ for neo-vitamin A alcohol in *cyclo*hexane = 1650.

The potency of all-*trans* vitamin A alcohol is taken as $3{\cdot}33 \times 10^6$ I.U./g., and it has been stated[6] that the potency of neo-vitamin A is about 80 per cent. of the all-*trans* variety. On this basis the potency of neo-vitamin A is $2{\cdot}664 \times 10^6$ I.U./g. Hence the $E_{max.}$ (gross) values when corrected for vitamin A_2 may be multiplied by the factor 1825 to convert to biological units.

The percentage increase of E fully corrected unsaponifiable matter over the value of E (gross) unsaponifiable matter $\times$ 1600 is given in Table VIII.

From a consideration of Tables VI, VII and VIII the following facts emerge:

(1) There is fair agreement between the $E_{max.}$ (gross) as determined on the unsaponifiable matter and by chromatography on the whole oil. However, the chromatographic values corrected by Morton's formula tend to be greater than the unsaponifiable matter values, and are not considered to be reliable unless the shape of the absorption curve in the region 310 to 325 mμ indicates the absence of subsidiary maxima or a tendency to maxima. The absorption curves of samples 1, 5, 7, 8, 9, 14 and 15 do not indicate the presence of unsaturated acids.

(2) Chromatography of samples 1, 5, 7, 8, 9, 14 and 15 yields fractions agreeing with $\pm$ 2 per cent. with the published data for vitamin A acetate within the range 310 to 335 mμ. Outside this range, deviation occurs due to vitamin A_2. The column E (fully corrected) $\times$ 1825 chromatography must, therefore, represent a fairly true estimate of the potency of these samples. With the exception of sample 1, good agreement is obtained between these potencies and those of E (gross) $\times$ 1600 as obtained on the unsaponifiable matter.

TABLE VII

COMPARISON OF POTENCIES AND $E_{max.}$ VALUES OBTAINED BY THE USE OF CORRECTIONAL FORMULÆ

Sample	Oil E (corr.)			Unsaponifiable matter E (corr.)			Chromatographic E (corr.)		Oil E (corr.) $\times$ 1900			Unsaponifiable matter E (corr.) $\times$ 1900			Chromatographic E (corr.) $\times$ 1900	
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)
6	0·476	0·446	0·442	0·385	0·376	0·374	0·451	0·428	905	850	840	730	715	710	855	810
7	0·858	0·842	0·842	0·784	0·757	0·799	0·902	0·885	1630	1600	1600	1490	1440	1520	1710	1680
8	1·050	1·032	1·068	0·875	0·852	0·905	1·063	1·067	1995	1960	2030	1660	1620	1720	2020	2030
9	1·068	1·063	1·097	—	—	—	1·141	1·137	2030	2020	2080	—	—	—	2170	2160
10	0·979	0·947	0·942	0·826	0·826	0·872	0·935	0·965	1860	1800	1790	*1570*	*1570*	*1660*	*1780*	*1830*
11	0·434	0·416	0·384	—	—	—	0·424	0·394	825	790	730	—	—	—	805	750
12	0·439	0·395	0·345	0·343	0·334	0·350	0·431	0·405	835	750	655	650	635	665	820	770
13	0·363	0·347	0·347				0·378	0·345	690	660	660				720	655
15	0·632	0·658	0·638				0·597	0·568	1250	1250	1210				1130	1080
16	0·426	0·390	0·358				0·426	0·397	810	740	680				810	755
18	0·713	0·592	0·561				—	—	1355	1215	1065				—	—

TABLE VIII

THE INCREASE OF FULLY CORRECTED RESULTS OVER THOSE CALCULATED FROM E (GROSS) × 1600

UNSAPONIFIABLE MATTER

Sample	E (fully corrected) × 1825 I.U./g.	E (gross) × 1600 I.U./g.	Difference	Percentage
1	1940	1880	60	3·1
2	1260	1210	50	3·9
3	1150	1120	30	2·6
4	860	835	25	2·9
5	1350	1310	40	2·9
6	880	850	30	2·7
7	1640	1600	40	2·6
8	1860	1820	40	2·1
9	1980	1930	50	2·5
10	1760	1710	50	2·8
11	770	745	25	3·2
12	780	760	20	2·6
13	680	665	15	2·2
15	1085	1060	25	2·3
16	790	760	30	3·8
17	845	820	25	2·4
18	1190	1160	30	2·5
				Mean 2·7

(3) The unsaponifiable matter curves are more inclined to follow all-*trans* acetate curves above 330 mμ. This is supposedly due to the presence of, and the more difficult saponification of, neo-vitamin A.

(4) The E (gross) values as expressed on the whole oil are the greatest. This is due to the presence of irrelevant absorbing material.

(5) There is fair agreement between E (gross) × 1600 and E (corr.) × 1900 as determined on the whole oil.

(6) Good agreement may sometimes be obtained between E (corr.) values on the whole oil as calculated using formulæ (*a*) (*b*) and (*c*). Samples 7, 8, 13 and 15 show particularly good agreement between the sets of $E_{max.}$ values. This is strong indication that the irrelevant absorption is linear over the range 312·5 to 316 mμ, 327·5 to 328 mμ and 337·7 to 340 mμ. In the case of certain of the other samples the indication is that the irrelevant absorption is non-linear, and that the correctional formulæ should not be applied.

(7) There is good correlation between E (corr.) values as determined by the different formulæ on the whole oil, and on the chromatographic fraction.

(8) The absorption spectra of many of the whole oils show a displaced maximum towards the 320 mμ region, indicating the presence of unsaturated acids.

(9) Good agreement is obtained between the results which are thought to be the most accurate in the table, viz. the unsaponifiable matter E (fully corrected) × 1825 and the "unsaponifiable matter E (gross) × 1600."

(10) Corrected values on the unsaponifiable matter tend to be of the order of 10 per cent. low.

(11) Table VIII indicates from a comparison of "unsaponifiable matter E (fully corrected × 1825" and "unsaponifiable matter E (gross) × 1600" that the factor 1600 for conversion to biological potencies is not likely

to be greatly in error, assuming the presence of a 10 per cent. vitamin A_2 contribution to $E_{max.}$ and the presence of 25 per cent. of neo-vitamin A.

(12) A good agreement is shown between potencies in the column unsaponifiable matter *E (fully corrected) × 1825 and chromatography* E (fully corrected) × 1825. The absorption spectra of the unsaponifiable fractions of the oils do not indicate the presence of unsaturated acids, whilst the spectra of eluates of certain of the chromatographed oils indicate the presence of these acids. The good agreement between the results in the two columns indicates that the effect of the presence of *conjugated acids on the E (gross)* value of the chromatographic fraction would not appear to be very great.

General Conclusions

1. Corrected E values as determined on the unsaponifiable matter are lower than those which may be obtained by any other method and are considered to undervalue a given oil.

2. It is considered that a fair estimate of the potency may be obtained by saponifying the oil for a period of 30 minutes followed by a determination of the $E_{326{\cdot}5\ m\mu}$ (gross). Optical density readings should also be taken over the range 310 to 360 mμ, those from 310 to 320 mμ being at intervals of 1 mμ, the others at 5 mμ intervals. The plotted optical densities between 310 and 360 mμ should present a smooth curve indicating the absence of oxidation products and of unsaturated acids with absorption maxima at 315 and 320 mμ. If maxima are found indicating the presence of unsaturated acids, the oil shall be saponified for a longer period. The $E_{326{\cdot}5\ m\mu}$ (gross) value may then be multiplied by the factor 1600 to obtain the potency in units per g.

3. The factor 1600 to convert E (gross) of the unsaponifiable matter to biological units is fairly reliable.

4. It has been shown that a 30 minutes saponification period as specified by the U.S.P. XIV is desirable, since a 5 minutes saponification period is sometimes insufficient to remove unsaturated acids. A symptom of incomplete saponification is a tendency to emulsification during separations.

5. Chromatography, using alumina containing 5 per cent. of added water, in some cases gives results in accordance with those obtained on the unsaponifiable matter but in other cases the results are unreliable. It is possible that use of alumina containing a smaller amount of added water might result in improved removal of unsaturated acids. Care would have to be taken to prevent de-esterification of the vitamin A esters, and/or subsequent loss of vitamin A on the column. Use of stoppered cells should be of value in preventing the evaporation of the light petroleum solvent. Chromatography possess the advantage over the saponification method in that there can be no doubt as to whether the natural esters present in a cod-liver oil are hydrolysed or not.

6. It is not justifiable to determine vitamin A on the whole oil by application of Morton's three point correctional formulæ when the wavelength of maximum absorption is displaced from 327·5 mμ. When

displacement is shown, a preliminary treatment, such as saponification, is necessary.

This investigation was carried out in part on a Beckman Quartz Spectrophotometer and in part on a Unicam Spectrophotometer, both instruments being calibrated using potassium dichromate solution and pure synthetic all-*trans* vitamin A acetate. Ratios of the optical density at a given wavelength, to that of the optical density at the maximum obtained using synthetic all-*trans* vitamin A acetate dissolved in *cyclo*hexane agreed with those of Morton.[2]

The spectrophotomers were calibrated for wavelength using the mercury lines of wavelength 2967Å, 3022Å, 3126Å, 3341Å, 3650Å, 4047Å and 5461Å. Wavelength settings on both instruments were found to be correct or not to differ from the correct values by more than 2Å.

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THE STABILITY OF VITAMIN A ALCOHOL IN AQUEOUS AND OILY MEDIA

By C. L. J. Coles and D. F. W. Thomas

From Glaxo Laboratories, Ltd.

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There have been numerous investigations[1,2,3,4,5,6,7,8,9,10] into the stability of vitamin A, generally in oil solution. However, research over the past 10 years into the development of synthetic non-ionic surface-active agents has now made it possible to prepare aqueous dispersions of vitamin A; to the naked eye they look like true solutions. As a consequence, interest now centres upon the comparative stability of this vitamin in aqueous dispersions and in oily solutions.

The preparation of aqueous dispersions is the subject of numerous patents. In particular, British Patent 588,298 (1947) has received considerable attention. It describes the use of polyoxyethylene-sorbitan monolaurate and mono-oleate as dispersing agents. The stabilities of vitamin A alcohol in aqueous dispersions containing these agents and in cottonseed oil have been studied by Kern and Antoshkiw.[11] In accelerated temperature tests they showed that vitamin A alcohol in aqueous dispersions containing polyoxyethylene sorbitan monolaurate is significantly more stable than when in solution in cottonseed oil.

Considerable search and experiment have gone to discover other non-ionic surface-active agents that will permit preparation of stable aqueous vitamin A dispersions, and several have been found suitable by us. These substances are condensation products of polymerised ethylene oxide with higher fatty alcohols. Two of them are commercially available —"Brij 35" (Atlas Powder Co.), which is described as polyoxyethylene lauryl alcohol, and "Lubrol W" (I.C.I.), which is described as consisting substantially of polyoxyethylene cetyl-stearyl alcohol. The aqueous dispersion of vitamin A alcohol that is the subject of this paper was prepared with "Lubrol W."

Experimental

We have found that the amount of "Lubrol W" required to prepare aqueous dispersions of vitamin A is a direct function of the weight of the substance being dispersed and is of the order of 10 times its amount. This is in agreement with British Patent 588,298 (1947). However, incorporating glycerol to the extent of 30 per cent. of the final volume of the dispersion makes it possible to halve the amount of dispersing agent. Accordingly, our aqueous preparations have been made at a potency of approximately 12,000 I.U. of vitamin A in each g. by using 5·0 per cent. w/v of "Lubrol W" and 30·0 per cent. v/v of glycerol.

The vitamin A alcohol used for this work was prepared by saponification of a rich concentrate of vitamin A acetate. The potency of the resultant

vitamin A alcohol was $1{\cdot}6 \times 10^6$ I.U./g. determined spectrophotometrically. The B.P. arachis oil used in preparing the oily solution of vitamin A alcohol had a peroxide value of less than 1·0.

To evaluate the stability of vitamin A alcohol in aqueous dispersion and in oily solution, we have made tests of two kinds. First, the stability has been determined over a long period under conditions approximating to commercial storage. The test samples were stored for alternate periods of 14 days at room temperature (15° to 22° C.) and at 37° C. The samples of the oily solution and the aqueous dispersion were sealed in ampoules under air, the free air space in the ampoule occupying approximately 75 per cent. of the ampoule capacity. Initially and intermittently over a period of 20 months one ampoule has been withdrawn and the vitamin A content determined spectrophotometrically. The initial potency of the vitamin A in both of the test preparations was approximately 12,000 I.U./g.

Secondly, accelerated heat tests have been performed on identical preparations. In a similar manner an aqueous dispersion or an oily

TABLE I

VITAMIN A ALCOHOL IN ARACHIS OIL

Initial potency—12,000 I.U./g.

Exposed in ampoules for alternate 14 days at room temperature (15° to 22° C.) and 37° C.

	Age of sample in months					
	0	1	3	11	15	20
Percentage of vitamin A remaining	100	87	68	67	53	34

TABLE II

VITAMIN A ALCOHOL IN AQUEOUS DISPERSION

Initial potency—12,000 I.U./g.

Exposed in ampoules for alternate 14 days at room temperature (15° to 22° C.) and 37° C.

	Age of sample in months						
	0	1½	4	5	7	9	20
Percentage of vitamin A remaining	100	102	98	101	96	96	92

TABLE III

VITAMIN A ALCOHOL IN AQUEOUS DISPERSION AND OILY SOLUTION

(Accelerated test)

Initial potency—12,000 I.U./g.

Samples exposed in ampoules at 100° C.

	Time of exposure in hours			
	0	2	4	8
Vitamin A alcohols in arachis oil	100	87	70	57
Vitamin A alcohol in aqueous dispersion	100	94	86	70

NOTE: Figures are for percentages of Vitamin A remaining.

solution of vitamin A alcohol was sealed in ampoules under air, with a free air space equal to 75 per cent. of the ampoule capacity. The samples were assayed initially and then exposed to a temperature of 100° C. ± 1° in a thermostatically-controlled oven for a period of 8 hours. At the end of 2, 4 and 8 hours ampoule contents were assayed spectrophotometrically.

Results are recorded in Tables I and II (long-term tests) and Table III (accelerated tests).

Discussion

It is apparent from these results that vitamin A alcohol is substantially more stable in an aqueous dispersion than in an arachis oil solution. These results are in agreement with the conclusion recorded by Kern and Antoshkiw.[11] From their results it might have seemed that polyoxyethylene sorbitan monolaurate was exerting a specific stabilising effect upon the vitamin. However, taken into consideration along with the present work, the superior stability of the aqueous dispersions over the oily solutions would appear to be a function of the physical state in which the vitamin A is present. The solubilisation of hydrophobic lipoid substances requires the presence of micelles. These micelles are of colloidal dimensions and are believed to consist of loose spherical aggregates of the hydrocarbon chain ions, with the polar heads turned towards the aqueous phase. It is considered that lipophilic substances such as vitamin A alcohol are taken up into the interior of the micelles. It is postulated by Kern and Antoshkiw that such micelles are impermeable to oxygen and their evidence supports this view. The greater stability exhibited by aqueous dispersions may also be explained by the instability of peroxides. In oily solutions the formation of peroxides is an important preliminary step in the destruction of vitamin A. In an aqueous medium at elevated temperatures, however, one would not expect peroxides to be stable, and any peroxides that might be present in the vitamin A concentrate would subsequently be destroyed.

Summary

1. The stability of vitamin A alcohol in aqueous dispersion with "Lubrol W" as the dispersing agent, and in oily solution have been compared over a period of 20 months.

2. Accelerated stability tests have been performed on an aqueous dispersion of vitamin A alcohol and an oily solution.

3. It was found that an aqueous dispersion of vitamin A alcohol, when "Lubrol W" was used as the dispersing agent, exhibited greater stability than a solution in arachis oil.

Our thanks are due to Miss R. P. Russell, B.Sc., of the Analytical Department, for carrying out the assays.

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DISCUSSION

The three papers on vitamin A were discussed together. The first was presented by MR. E. HAYES, the second by DR. N. EVERS, and the third by MR. C. L. J. COLES.

DR. R. E. STUCKEY (London) said that results obtained by molecular distillation indicated that the amount of vitamin A alcohol in fish-liver oils was negligible. He asked to what percentage the authors would suggest limiting the amount of vitamin A alcohol in the more concentrated products such as halibut-liver oil. The estimation of vitamin A was becoming more and more complex. The 3-point correction procedure was rarely satisfactory with raw cod-liver oil. He did not entirely agree with Mr. Swann's work. Cod-liver oils fell into two categories. In the first the oil could readily be saponified, the curve obtained was fairly free from irrelevant absorption, and it was possible to use the 3-point correction procedure. In the second, which unfortunately included many B.P. oils, the curve even after 30 minutes' saponification was unlike that of pure vitamin A. In his view it was not satisfactory either to use the factor of 1600 or to apply the 3-point correction procedure, the magnitude of the correction being too large. A satisfactory procedure for such oils was to carry out a chromatographic separation after saponification. It was surprising to note in the paper by Coles and Thomas the lack of stability of a solution in oil of vitamin A alcohol stored under room conditions. He asked whether they had any comparative figures for other forms of vitamin A or natural oils.

MR. J. H. OAKLEY (London) said the use of the agents described by Coles and Thomas for dispersing oils seemed to have a wide application. It had been shown in papers relating to soaps that various materials affected the micellar structure. Had the authors any information on the effect of glycerol and ethanol on the micellar structure? Did the use of those substances enable the proportion of dispersing agent to be further reduced? Had the authors tried the addition of antioxidants, e.g., ascorbic acid, in the aqueous phase, and what effect had they had on the stability of the vitamin A?

MR. D. N. GORE (Dorking) asked Mr. Coles about the effect of dilution on the stability of the aqueous dispersion? Was there any critical concentration above or below which the stability fell?

DR. G. E. FOSTER (Dartford) supported Mr Swann in his preference for the gross absorption in the assay of cod-liver oil. Until something better was available, in his view the conversion factor of 1600 was the

best for ensuring agreement between laboratories. It would be interesting if the authors could give some information concerning the assay of vitamin A in malt preparations.

DR. R. M. SAVAGE (Barnet) asked Mr. Coles if there was any correlation between the biological and spectrophotometric results on the aqueous dispersion. If the micellar structure turned out to be so stable in retaining vitamin A, the assay results by physical methods might not be related to the biological results.

MR. C. J. EASTLAND (London) asked whether there was any information regarding the chronic toxicity of the dispersing agent.

DR. F. WOKES (King's Langley) observed that Mr. Swann used for detection of the end-point the fluorescence in ultra-violet light, and asked how that compared with antimony trichloride for sensitivity. It would be useful if some indication could be given of the proportion of irrelevant absorption occurring in the various preparations to which the Morton and Stubbs correction was applied.

MR. E. HAYES (London) said that it was very difficult to get a true estimation of vitamin A potency in cod-liver oil. Probably everybody in the industry would like to see a reversion to the 1600 times gross *E* factor. The difficulty about the Morton and Stubbs correction was the assumption that the irrelevant absorption was linear at three fixation points. Many people did three estimations and put the solutions in the spectrophotometer at the same time. That did not provide a good estimate of reproducibility, and he had found that differences between laboratories of 10 per cent. were common, and differences of 5 per cent. very common. Demands for precision of 2 per cent. were, in his opinion, impracticable. The biological assay for cod-liver oil appeared to have fallen into disuse, and he was of the opinion that it was a little too early for that to have happened. The World Health Organisation produced an international standard in 1949 which was still being issued. However, it was difficult to see the purpose of the standard because it had been shown that it was not up to specification; the vitamin A percentage was a little low. Spectrophotometry was not specific. It was possible to get many materials which had an absorption curve close to that of vitamin A but which had no biological activity. Much work on the biological side was necessary before estimating the true potency of preparations.

DR. N. EVERS (Hertford) asked Mr. Hayes why he desired to limit the amount of vitamin A alcohol in fish oils. Was vitamin A alcohol inactive or harmful?

MR. C. L. J. COLES, in reply, said that the alcohols were solubilised more easily than esters, and in an aqueous dispersion the vitamin A alcohol was more stable than the ester. Antioxidants had been used and tocopherol had been found to be the most satisfactory. Vitamin A in aqueous dispersion was utilised two or three times more efficiently than vitamin A from an oily vehicle, and if it were to be assayed biologically there was the difficulty of defining a standard. On dilution with water or milk the product was stable for 4 to 5 hours. He did not think that Lubrol W had previously been used in medicine. Each batch of the

substance should be examined individually for toxicity. By the addition of glycerol it was possible to reduce the amount of Lubrol, and by the introduction of alcohol it could be reduced still further.

MR. E. HAYES (London), in reply, said that in high potency preparations a limit of 7 per cent. for the vitamin A alcohol content would be adequate. There were two reasons for desiring the exclusion of vitamin A alcohol. First, it interfered with the Morton and Stubbs correction which was based on the assumption that vitamin A was present as an ester, and if there were two forms with two different absorption spectra it complicated matters. Second, it was generally assumed that vitamin A alcohol was less stable than vitamin A esters.

DR. N. EVERS, in reply, said he thought that Mr. Swann would agree that on the whole the chromatographic separation gave the most reliable results. The B.P. 1948 method was empirical in their view, but it did give results comparable with the vitamin A present. With malt preparations no difficulty was experienced with the absorption method. On the question of detecting vitamin A in the eluate by the fluorescence method, Mr. Swann would probably say that it was more sensitive than the antimony trichloride test and could be observed continuously. He did not think that Mr. Swann stated that the *E* correction multiplied by 1600 gave results comparable with biological figures.

THE ASSAY OF PENICILLIN USING PENICILLINASE

By A. Royce, C. Bowler and G. Sykes

From the Microbiology Division, Standards Department, Boots Pure Drug Co., Ltd., Nottingham

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The hydrolysis of the penicillins by penicillinase to penicilloic acid is a specific enzyme reaction. The acid produced is easily titratable with strong alkali, but so far no simple method has been described for regular and routine estimations by this means. As a result of recent investigations, we have now developed a technique which, in our hands, gives results in a much shorter time and is more accurate and more economical than the normal ring-plate or serial dilution methods of assay. It is also at least as accurate and more easily carried out than the chemical methods of assay in the fields where these are practicable.

The principle of estimating penicillin by treating with penicillinase and then titrating potentiometrically the penicilloic acid produced was first adopted by Murtaugh and Levy in 1945.[1] Later Henry and Housewright[2] used the same principle for estimating both penicillin and penicillinase activities but employed a manometric procedure with a Warburg apparatus. The potentiometric and manometric methods were also used by Wise and Twigg,[3] and by Pollock[4] for investigating some of the fundamental properties of penicillinase, but none appears to have considered a simple alkalimetric method using indicators, presumably because they were concerned largely with the dynamics of the reaction. We have also used these methods, but find that for quantitative estimations the indicator method gives equally satisfactory results.

Among the requirements for the successful operation of this method are (*a*) the penicillin-penicillinase reaction must be specific, (*b*) the penicillinase must be reasonably stable, (*c*) it must be highly active, and (*d*) the results must be reproducible.

Properties of Penicillinase

The penicillin-penicillinase reaction. Henry and Housewright[2] examined the action of penicillinase prepared from *Bacillus cereus* on many substances of penicillin-like structure and on various degradation products of penicillin, and in no case did they record any reaction. They also calculated the molecular weights of the penicillins, on the assumption that one titratable carboxyl group was produced from each molecule of penicillin attacked. The value for benzylpenicillin (G) and *n*-heptylpenicillin (K) agreed closely with those already found and accepted, but those for the Δ^2-pentenyl- (F) and *p*-hydroxybenzyl-(X) compounds differed considerably. The discrepancies were thought to be due to the limited number of experiments which could be performed on the very small amount of material available. However, we have recently obtained some confirmation of this discrepancy with a sample of Δ^2-pentenylpenicillin. In our hands this had a potency of 1056 I.U./mg. by

the cylinder-plate assay, but only 550 I.U./mg. by the penicillinase method.

Since the penicillin now produced is practically all benzyl-penicillin, these discrepancies have virtually no significance in normal assays, but such considerations may well have detracted from earlier attempts to adapt the penicillinase method for routine use. Another factor of possibly greater significance may have been the low activity of the penicillinase then available.

Penicillinase is produced by a large number of organisms, mainly of the Gram-negative non-sporing or the Gram-positive aerobic sporing types, but many preparations from these are quite unstable. We have perhaps been fortunate in that our work has been carried out with a strain of *Bacillus subtilis*, which yields a very stable penicillinase. The strain was originally obtained from the National Collection of Type Cultures, but has since been subjected to careful selection towards greater penicillinase production. Highly potent material is obtained by methods based on the continued addition of penicillin to the growing culture, as advocated by Duthie.[5] Batches are obtained regularly of which 1 ml. is capable of inactivating 200,000 to 400,000 I.U. of penicillin per hour at *p*H 7·5 and normal room temperature. This potency is adequate for normal purposes but it can be further increased, if desired, by adsorption and elution methods. The material is very stable and does not lose more than 10 per cent. of its activity on storage for periods of 3 to 6 months at either 4° C. or at room temperature.

Rate of inactivation and effect of temperature and pH value. Provided there is excess of substrate (penicillin) present, the rate of reaction at any given *p*H and temperature remains constant and is dependent only on the enzyme (penicillinase) concentration. When the substrate is nearing exhaustion there is a retarding of the rate of reaction proportional to the concentration of the residual substrate at any moment. Pollock[6] has found with two penicillinase preparations that the enzyme continues to function at its maximum activity (at *p*H 7·2 and 30° C.) at penicillin concentration down to about 100 I.U./ml. Under our conditions of assay (see later), in which the reaction is allowed to proceed with relatively high concentrations of substrate and enzyme for 30 minutes, the process appears to go practically to completion. If the solution is back titrated continuously during the process of inactivation, the reaction continues at a uniform rate, then rapidly slows down and finally ceases abruptly. Beyond this point no further acid production is detectable. This is illustrated in Table I in which the course of inactivation of 50 mg. of benzylpenicillin (sodium salt) with 1 ml. of penicillinase was followed by titrating the acid produced during consecutive periods of 3 minutes each.

TABLE I

RATE OF ACID PRODUCTION DURING INACTIVATION OF PENICILLIN

Period (minutes)	Titration 0·01N sodium hydroxide ml.
0 to 3	2·25
3 „ 6	2·35
6 „ 9	2·35
9 „ 12	2·35
12 „ 15	2·25
15 „ 18	1·45
18 „ 21	0
21 „ 24	0
24 „ 27	0
27 „ 30	0

TABLE II

RATE OF INACTIVATION OF PENICILLIN BY PENICILLINASE AT DIFFERENT TEMPERATURES

Temperature °C.	Relative rate of inactivation
1 to 2	30
12 „ 13	60
24	100
36 „ 38	150
46 „ 47	190
55 „ 60	220
65	enzyme destroyed

(Rate at 24° arbitrarily taken as 100.)

The rate of inactivation also depends very largely on temperature, and a short examination was made of this relationship under otherwise constant conditions. Details are given in Table II, from which it is seen that activity increases regularly, but not steeply, with temperature up to the point at which the enzyme is destroyed. Although there is some advantage to be obtained by working at elevated temperatures the reaction proceeds quite satisfactorily at room temperature, and, since it is also more convenient, this was adopted for all subsequent tests.

Henry and Housewright[2] investigated the rate of reaction in relation to *p*H value and found that the optimum for a temperature of 36° C. was at *p*H 7·2. Wise and Twigg[3] obtained an optimum at 25° C. at *p*H 7·8. Using approximately 50 mg. amounts of benzylpenicillin (potassium salt) dissolved in 10 ml. of water with 1 ml. of penicillinase added, and keeping the *p*H value constant to within 0·1 unit we found that maximum activity at 22° to 25° C. occurred at *p*H 7·5. Table III illustrates the relative rates of reaction at different *p*H values, the rate at *p*H 7·5 being arbitrarily fixed at 100. It is notable that the rate declines more slowly on the alkaline side than on the acid side, and that alkaline hydrolysis becomes increasingly significant with increase in *p*H value beyond about 9·0. Considerable buffering of the reacting solution also occurs due to the penicilloic acid formed preventing the *p*H value from falling below about 5·5.

TABLE III

RATE OF INACTIVATION OF PENICILLIN BY PENICILLINASE AT DIFFERENT *p*H VALUES

*p*H value	5·0	5·5	6·0	6·5	7·0	7·5	8·0	8·5	9·0	9·5
Relative rate of inactivation	23	36	45	82	86	100	95	91	75	61

METHODS OF ASSAY OF PENICILLIN PREPARATIONS AND RESULTS

Penicillin salts. The following is a procedure which is particularly suitable for the assay of the salts of penicillin, but which, by adjusting the amount of sample, can be used with many of the pharmaceutical preparations of penicillin.

Adjust the penicillinase to *p*H 7·5 using phenol red as indicator. Prepare a colour control by mixing 1 ml. of this with 10 ml of distilled water containing 0·2 ml. of phenol red indicator. Weigh accurately 50 mg. of the penicillin sample, dissolve in 10 ml. of water also containing 0·2 ml. of phenol red indicator, and adjust the *p*H value to match that of the control. Add 1 ml. of penicillinase and allow to stand at room temperature for 30 minutes. Titrate with 0·01N sodium hydroxide until the colour of the solution again matches that of the control. Allow to stand

for some minutes longer to ensure that the reaction is completed, and titrate further if necessary. Calculate the potency of the preparation on the basis that each 1 ml. of 0·01N sodium hydroxide is equivalent to 6023 I.U. of penicillin. This value is obtained from the facts that the British Working Standard of Penicillin (ASC 2) has an agreed potency of 1690 I.U./mg., and that each molecule of benzylpenicillin gives rise to one of penicilloic acid. It is very close to the values of 6015 and 6021 found experimentally.

It is obvious that the time taken to complete the inactivation will be influenced by the activity of the penicillinase, weaker preparations requiring longer inactivation periods. It is not advisable, however, to use material of considerably weaker activity as the reaction time then becomes inconveniently long and the results less accurate. The accuracy of the assay can be increased by using larger amounts of penicillin, but results of quite a high order of accuracy can be obtained from amounts as low as 5 or 6 mg.

To demonstrate the reliability of the method, assays were carried out on a large number of production batches of the different penicillin salts and compared with cylinder-plate and iodimetric results. In each case the normal routine procedures were used. The iodimetric assays, and some of the penicillinase assays, were carried out by a colleague, Mr. A. S. Middleton of our Chemical Division, Standards Department. Table IV shows the values obtained on several of these batches by the 3 methods and illustrates their general concordancy. Replicate penicillinase values are included to show the reproducibility of the method. Of the 28 batches of sodium, potassium and calcium salt examined, there is a mean difference between the penicillinase and cylinder plate results of less than 0·1 per cent., and between the penicillinase and iodimetric results of 0·9 per cent. With the procaine salt the mean differences are 3·2 per cent. and 1·7 per cent. respectively, both values being positive. This is a reflection of our experience that cylinder plate assays on procaine benzylpenicillin are frequently rather higher than theory. The penicillinase method has also been applied successfully to other compounds of penicillin which are difficult to assay by the iodimetric method.

Pharmaceutical preparations. Solution tablets and oral tablets are readily assayed by this method, although they may occasionally be formulated with buffer substances which are liable to impair the accuracy of the estimations. Since, however, they are usually of high potency the inaccuracies tend to be minimised. Determinations in lozenges present difficulties owing to the presence of only small quantities of penicillin in a large amount of base. However, difficulties have also been reported by other workers[7,8] using the plate assay due to the sugars in the preparations tending to yield abnormally high values. Assays on penicillin mixtures with insoluble substances, e.g., sulphonamide powders and snuffs, can be carried out on the mixture directly by simple suspension in water, but generally it is advisable to separate the insoluble matter and complete the assay on an aliquot of the clarified aqueous extract.

The various preparations of penicillin in oily bases, oil-wax suspension,

TABLE IV

COMPARATIVE ASSAYS OF PENICILLIN SALTS

Salt and batch number		Assay values (I.U./mg.)			Percentage difference between penicillinase and	
		Penicillinase	Cylinder plate	Iodimetric	Cylinder plate	Iodimetric
Sodium	786	1576:1575	1627		+3·2	
	780	1634:1629	1616		−1·0	
	2285	1648:1631	1624	1648	−1·0	+0·5
	2286	1642:1635	1604	1671	−2·1	+2·0
	2287	1646:1651	1565	1628	−5·0	−1·2
	10918	1569:1566	1603		+2·2	
			Mean difference, per cent.		−0·6	+0·4
Potassium	1144	1559	1579	1544	+1·3	−1·0
	1145	1576	1551	1584	−1·6	−0·8
	1146	1558	1583	1555	+1·6	−0·2
	1147	1578	1545	1553	−2·1	−1·6
	1148	1557	1518	1540	−2·5	−1·1
	1149	1547	1533	1547	−1·0	0·0
	1205	1549:1541	1520	1537	−1·6	−0·5
	1206	1571:1560	1529	1596	−2·0	+2·0
	1208	1568:1581	1563	1553	−0·8	−1·4
	1221	1570:1579	1592		+1·4	
	1222	(1590:1577) (1578:1579)	1530	1554	−3·2	−1·7
	1229	1603:1607	1599	1562	−0·4	−2·7
	1230	1607:1604	1616	1578	+0·7	−1·7
	1231	1602:1601	1627		+1·6	
	1468	1602:1602	1649	1555	−2·9	−2·9
	1469	1605:1611	1658	1547	+3·1	−3·8
			Mean difference, per cent.		−0·2	−1·2
Calcium	771	1477:1477	1557		−5·3	
	1309	1525:1519	1592		−4·6	
	1320	1533:1543	1512		−1·7	
	1347	1568:1552	1587		−1·7	
	1348	1546:1537	1501		−2·7	
	1387	1517:1532	1498		−1·7	
			Mean difference, per cent.		−0·7	
Procaine	1487	1031:1025	1048		−2·0	
	2231	1006:992	1037	1024	−3·7	+2·4
	2238	993:1005	1042	1025	−4·4	+2·7
	2239	983:1008	1075	1007	−7·5	+0·7
	2240	997:1017	1000	1025	−0·7	+1·8
	2241	1007:1007	1015	1021	+0·7	+1·4
			Mean difference, per cent.		+3·1	+1·7

ointments, etc., can also be assayed without prior extraction of the penicillin. For these preparations, a weighed quantity (depending on its potency) is suspended in water with indicator and an equal volume of chloroform or other suitable organic solvent is added. The mixture is shaken gently, adjusted to *p*H 7·5 and 1 ml. of penicillinase added. It must be titrated continuously during the inactivation period to keep the penicillin in the aqueous phase. If excess acidity is allowed to develop the free penicillin will migrate into the organic solvent phase, and thus retard the process of inactivation. The colour control in this case is the adjusted mixture of reagents without penicillinase. This procedure yields very satisfactory results when high potency oil-wax suspensions are assayed, but with ointments the accuracy is lowered owing to the initial low potency of the preparation. Penicillin fermentation liquors cannot be assayed directly by this method due to the buffering capacity and high

colour of the solution. It is the frequent practice to assay these liquors by chemical or physico-chemical methods, usually after extraction with an organic solvent; the penicillinase method can be introduced at this stage giving results of the same order of accuracy.

Summary

1. A simple routine method is described of carrying out accurate assays of penicillin and its preparations, by inactivating with penicillinase and titrating the acid formed. The method is readily applicable for use in pharmacies, hospital dispensaries or small laboratories.

2. The accuracy of the method depends on (*a*) the use of a potent penicillinase, (*b*) the absence of buffering substances, and (*c*) the amount of penicillin present to be estimated.

3. Comparative assays of the penicillinase with the cylinder plate and iodimetric methods are given.

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DISCUSSION

The paper was presented by MR. A. ROYCE.

MR. G. F. HALL (Nottingham) said that, unlike benzylpenicillin, for procaine benzylpenicillin the iodimetric method was not entirely satisfactory. In the U.S.A. the Federal Food and Drug Administration specified the bioassay with the iodimetric method as an alternative. The British Pharmacopœia used the biological assay. He himself had suggested an extraction method which did not find favour. A further suggestion in which the procaine was precipitated with silicotungstic acid had not been favourably received.

DR. K. BULLOCK (Manchester) asked whether the control was just a colour standard. Was there no non-enzymic hydrolysis?

MR. A. ROYCE, in reply, said that there were difficulties with the iodimetric method and also with the ring plate method. It was found in routine determinations of procaine penicillin that results tended to be a little too high. The control tube contained no substrate, only water and enzyme being present. The *p*H and the buffering capacity varied with different batches of penicillinase. When penicillin broke down some acid was produced, but none was produced within the time limit of the test. Although the reaction was stated to be over in 30 minutes in the paper, in practice in most cases the reaction was concluded in 10 minutes. There would be no change in that time in the colour of the indicator added to the solution, but if it were left a change was detected later in the day.

The CHAIRMAN remarked that no one had suggested that Mr. Royce should have a standard strain of *B. subtilis*.

MR. A. ROYCE replied that there was a great deal of literature on penicillinase and many organisms were used. The organism he used was originally a N.C.T.C. culture of *B. subtilis*, but it had been in the laboratory for a number of years and had been selectively cultured with a view to greater penicillinase production. It was to be doubted whether the same strain would be available in the N.C.T.C. at the present time. He would be happy to supply the culture to anyone interested.

PRELIMINARY OBSERVATIONS ON THE STABILISATION OF PENICILLIN SOLUTIONS WITH HEXAMINE

By R. J. Hobbs, J. L. Livingstone, J. Reece and W. A. Woodard

From Glaxo Laboratories, Ltd., Greenford, Middlesex

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The rapid decomposition of penicillin salts in aqueous solution has attracted considerable attention. The introduction of crystalline highly purified penicillin salts led to an increase in dose levels; it therefore became more important to find ways of prolonging the effective life of solutions, because strong solutions are less stable than weak ones, and the less pure amorphous yellow material had usually a longer life than the crystalline compounds. This is believed to have been due to impurities exerting a buffering effect. Apart from ensuring that penicillinase-producing organisms are excluded or inactivated, there are two main approaches to the problem. An obvious step is to incorporate a suitable buffer. Alternatively, substances must be found that have a direct stabilising action on the penicillin molecule.

Various workers have reported their findings with buffers. For example, Johnson and Lerrigo,[1] and Paul, Gaillot and Baget[2] found phosphate buffers were useful. Pratt[3] and several others showed that phosphates had a stabilising action not entirely accounted for by their buffering action. The amounts needed to give the best effect varied with the purity of the penicillin and, according to Pedersen-Bjergaard and Tønnesen,[4] with the concentration of the penicillin solution. The latter workers state that for optimal effect

$$\frac{\text{Mol. conc. Na benzylpenicillin}}{\text{Mol. conc. phosphate}} = 1{\cdot}25.$$

To-day, citrate buffers are commonly used; in our experience they are superior to phosphates and several other salts, although some of these are better buffers over the relevant *p*H range. Ulex[5] has also shown that citrates are superior to phosphates. Sodium citrate creates an effective buffering system once a small amount of breakdown has occurred. This has been shown by Clapham,[6] Hadgraft, Hopper and Short[7] and Carr and Wing.[8] Potassium and sodium salts of penicillin blended with 4·5 per cent. w/w of anhydrous sodium citrate have been commercially available for several years.

Among substances known to have a stabilising action, other than buffers, is sodium hexametaphosphate. Lester Smith[9] considered this effect to be due to its ability to sequester small amounts of heavy metals present among the impurities in the penicillin. More recently, 2:3-dimercaptopropanol and similar compounds have been the subject of a British patent.[10] At the 1951 British Pharmaceutical Conference, Coulthard,

Fawcett, Lewis and Sykes[11] showed that soil extracts had some stabilising power. Stabilisation of the earlier impure penicillin salts was reported by Ramon and Richou[12] and by Fleury *et al.*,[13] who used formaldehyde, though the latter authors could not reproduce their results with pure crystalline penicillin. We selected some 40 compounds for trial with pure penicillin, but hexamine alone proved to be suitable.

Experimental

Normal production batches of sodium benzylpenicillin having a potency of not less than 1600 I.U./mg. were used throughout this work. Anhydrous sodium citrate, passing the B.P. tests for purity, was used. All buffered solutions contained 4·5 per cent. w/w of sodium citrate based on the dry weight of the penicillin, except for those shown in Table V. For the experiments tabulated in Table V the penicillin was dissolved in a solution containing hexamine, 0·5 per cent; sodium citrate, 0·3 per cent.; and phenylmercuric nitrate, 0·001 per cent. This was an attempt to find a suitable solution for use in, say, hospitals, where the penicillin is dissolved in a sterile vehicle before being sent to the wards or out-patient departments. The hexamine was of B.P.C. standard and the solvent consisted of water for injection B.P. Except when phenylmercuric nitrate was added, all solutions were prepared aseptically, aliquot parts for assay being withdrawn under aseptic conditions. Containers and closures were of the normal type used for soluble penicillin salts, that is, sulphured soda glass vials with red rubber composition caps. Assays were carried out by the iodimetric method described in the Addendum 1951 to the B.P. 1948. The colour values illustrated in Figure 1 were obtained by examining the solutions in the 4-cm. cell of a Lovibond tintometer.

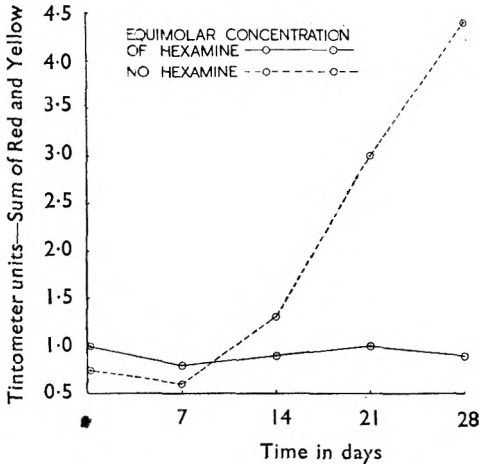

Fig. 1. Buffered solutions of sodium benzyl penicillin with or without hexamine. Tintometer readings (Lovibond units). Initial potency 100,000 units/ml. Storage 22° C. Batch 2963.

TABLE I

BUFFERED SOLUTIONS (*p*H 7·0) OF SODIUM BENZYLPENICILLIN WITH VARIOUS CONCENTRATIONS OF HEXAMINE

Room storage (22° C.). Initial potency 100,000 I.U./ml. Batch 2597.

Hexamine per cent. w/v in solution	Approximate molar proportion hexamine to penicillin	Percentage of initial potency remaining after				
		3 days	7 days	14 days	21 days	28 days
Nil	—	95	85	33	trace	blank
0·05	0·025	93	89	29	trace	blank
0·2	0·10	98	91	83	69	47
0·5	0·25	101	92	89	83	63
2·0	1·0	99	103	94	88	72
4·0	2·0	95	94	91	89	70
6·0	3·0	99	93	88	86	74

TABLE II

UNBUFFERED SOLUTIONS OF SODIUM BENZYLPENICILLIN WITH EQUIMOLAR CONCENTRATIONS OF HEXAMINE

Room storage (22° C.) Initial potency 100,000 I.U./ml.

Batch No.	Percentage of initial potency remaining after				
	7 days	14 days	21 days	28 days	35 days
2676	82	83	81	59	51
3020	88	87	75	64	43
2946	85	82	76	59	39
2975	84	79	74	57	39
Mean	85	83	77	60	43

TABLE III

BUFFERED SOLUTIONS (*p*H 7·0) OF SODIUM BENZYLPENICILLIN WITH EQUIMOLAR CONCENTRATIONS OF HEXAMINE OR WITHOUT HEXAMINE

Room storage (22° C.) Initial potency 100,000 I.U./ml.

Batch No.	Whether hexamine added	Percentage of initial potency remaining after				
		7 days	14 days	21 days	28 days	35 days
2676	+	91	92	88	72	59
2676	+	94	87	84	73	48
3020	+	97	84	79	67	55
3020	+	99	89	88	61	32
2946	+	97	89	91	68	55
2946	+	95	94	83	69	51
Mean		96	89	86	68	50
2676	—	89	38	trace	blank	—
3020	—	77	41	trace	blank	—
2946	—	79	39	18	blank	—
Mean		82	39	6	—	—

DISCUSSION

Any substance used for stabilising penicillin must be of low toxicity, easily available pure, pharmacologically inert in the amounts likely to be used, relatively cheap and easily sterilised. Hexamine fulfils these requirements. The amount injected with the penicillin is unlikely to exceed a quarter of the normal intravenous dose of 2 g. It may be sterilised by dry heat at 160° C. Its use lessens the risk of an injection

TABLE IV

BUFFERED SOLUTIONS OF SODIUM BENZYLPENICILLIN WITH EQUIMOLAR CONCENTRATIONS OF HEXAMINE OR WITHOUT HEXAMINE

Refrigerator storage (+4° C.). Initial potency 100,000 I.U./ml.

Batch No.	Whether hexamine added	Percentage of initial potency remaining after					
		21 days	35 days	52 days	65 days	80 days	107 days
2963	+	91	111	98	94	94	75
2963	+	98	100	96	93	94	78
2975	+	109	109	99	96	90	87
2975	+	91	94	103	95	95	97
3000	+	89	96	108	98	100	91
3000	+	96	97	99	101	94	90
Mean		96	101	100	96	95	86
2963	−	94	84	79	63	43	31
2975	−	93	89	77	68	51	19
3000	−	96	88	76	74	57	28
Mean		94	87	77	68	50	26

TABLE V

BUFFERED SOLUTIONS OF SODIUM BENZYLPENICILLIN WITH 0·001 PER CENT. OF PHENYLMERCURIC NITRATE AND 0·5 PER CENT. OF HEXAMINE OR NO HEXAMINE

Refrigerator storage (+4° C.)

Initial potency I.U./ml.	Batch No.	Whether hexamine added	Percentage of initial potency remaining after						
			14 days	28 days	42 days	56 days	70 days	84 days	98 days
200,000	3048	+	98	94	89	86	83	81	87
200,000	3020	+	111	105	100	100	99	102	91
200,000	2946	+	101	98	92	90	85	80	80
200,000	2938	+	102	98	93	90	86	85	94
200,000	Mean		103	99	94	92	88	87	88
100,000	3048	+	93	86	82	79	76	78	74
100,000	3020	+	102	95	93	92	84	89	84
100,000	2946	+	102	96	92	89	87	89	82
100,000	2938	+	107	97	97	93	91	90	85
100,000	Mean		101	94	91	88	85	87	81
200,000	3048	−	102	91	83	74	44	38	trace
200,000	3020	−	99	86	75	72	42	22	trace
200,000	Mean		101	89	79	73	43	30	—
100,000	3048	−	103	89	92	85	63	59	28
100,000	3020	−	102	94	87	60	33	38	trace
100,000	Mean		103	92	90	73	48	49	16

of penicillin being given after losing most of its potency owing to improper storage. Moreover, it should ease the work of hospital pharmacists involved in frequent renewal of such solutions and the supervision of their storage in wards. Hexamine also considerably improves the type of product containing dry procaine benzylpenicillin together with the sodium salt for the following reasons. Multi-dose containers are commonly used for this form of penicillin; the length of time the supension may be kept after addition of the aqueous vehicle is limited by the life of the highly soluble sodium penicillin. Also, if such products are presented in silicone-treated vials to produce a drain-clear effect, thus lessening the surplus required and improving the appearance of the suspension,

the degradation of a very small amount of the soluble penicillin then spoils the elegant appearance by producing a greasy film on the glass. The inclusion of hexamine doubles the period for which such suspensions may be kept after water is added, delays the onset of the yellow colour that normally appears within a day or so and retards unsightly filming in silicone-treated vials.

It is not yet known why the hexamine behaves as it does; further work is in progress to find this out. Throughout the effective life of solutions the *p*H remains well above 6·0, so the liberation of more than a trace of formaldehyde is unlikely. The fact that hexamine retards filming of the fortified preparation in silicone-treated vials suggest that it may modify one of the breakdown products of the soluble sodium salt. The most vulnerable part of the penicillin molecule is the β-lactam ring. Hexamine may stabilise this in several ways. For example, there may be a direct attachment to the ring, although this appears unlikely if the hexamine operates as an entity. Alternatively, there may be an attachment to some other part of the penicillin molecule, producing the necessary electronic drift to stabilise the ring, or there may be a similar attachment that prevents by steric hindrance any other substance from reacting with the ring. A further possibility is that, since hexamine is known to form compounds with certain acids (e.g., salicylic, benzoic and boric acids) and metallic salts such as sodium acetate, it may form a similar compound with sodium penicillin.

Summary

1. Stability tests have been carried out with solutions of sodium benzylpenicillin to which hexamine has been added.

2. Hexamine stabilises both buffered and non-buffered solutions. The optimum effect appears to be produced in the presence of a citrate buffer, when the penicillin and hexamine are present in equimolar proportions.

3. Hexamine prevents discoloration of solutions during their effective life. It also retards the appearance of unsightly greasy films on the surface of silicone-treated vials. The latter property is particularly valuable for products containing procaine benzylpenicillin together with the sodium salt.

4. Hexamine, when added at optimum concentrations to buffered solutions containing 100,000 I.U./ml. of penicillin, enables them to be kept for more than twice as long at room temperature (22° C.), and 3 times as long in the refrigerator (4° C.), without significant loss of potency.

5. When solutions at 100,000 I.U./ml. and 200,000 I.U./ml. are prepared in a vehicle containing 0·5 per cent. w/v of hexamine, 0·3 per cent. w/v of sodium citrate, and 0·001 per cent. w/v of phenylmercuric nitrate, they may be stored in a refrigerator for approximately twice as long as normal buffered solutions.

Our thanks are due to Mrs. M. J. Williams, Ph.C., of the Analytical Department, for carrying out the assays and to Mr. D. S. Thipthorpe for technical assistance.

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9. Smith, *Quart. J. Pharm. Pharmacol.*, 1946, **19**, 309.
10. British Patent, No. 638,535.
11. Coulthard, Fawcett, Lewis and Sykes, *J. Pharm. Pharmacol.*, 1951, **3**, 748.
12. Ramon and Richou, *Presse Med.*, 1947, **61**, 693.
13. Fleury, Schuster, Dessus, Roux-Delmal and Morel, *Ann. Pharm. franc.*, 1949, 7, 529.

DISCUSSION

The paper was presented by MR. J. L. LIVINGSTONE.

DR. G. E. FOSTER (Dartford) asked whether any toxicity tests on the actual preparations containing hexamine had been carried out.

MR. T. D. WHITTET (London) asked whether the authors had made any clinical tests. Did the inclusion of about 0·5 per cent. of hexamine cause pain on administration?

DR. F. HARTLEY (London) said that there was no evidence in the paper that hexamine itself was not affected during the assay procedure. Had the authors established that the presence of hexamine did not interfere with the iodimetric determination?

MR. P. CLAPHAM (Speke) asked for information on the stablising effect of hexamine at concentrations somewhat higher than those mentioned.

MR. J. L. LIVINGSTONE, in reply, said that the results of toxicity tests carried out both with injections of penicillin containing hexamine and with hexamine alone were satisfactory. Animal and clinical trials had been carried out without any noticeable increase in pain or adverse result. Iodimetric assays were used because they were generally more reliable. Bioassays were carried out at the beginning of each run on each solution, and occasionally during that time as a check on the iodimetric assay. Results were closely related, and it did not appear that the hexamine had any untoward effect on the iodimetric assay. At 200,000 I.U./ml. the stabilising effect of hexamine appeared to be practically the same as at 100,000 I.U./ml. At 500,000 I.U./ml. the solution was not so stable, but the ratio of stability of normal buffered solutions to solutions containing hexamine was similar.

BACTERIAL SURVIVAL IN SYSTEMS OF LOW MOISTURE CONTENT

PART IV. THE EFFECTS OF INCREASING MOISTURE CONTENT ON HEAT RESISTANCE, VIABILITY AND GROWTH OF SPORES OF *B. subtilis*

BY KENNETH BULLOCK and ALAN TALLENTIRE

From the Department of Pharmacy of the University of Manchester

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INTRODUCTION

PREVIOUS papers[1,2] in this series have shown that the spores of *B. subtilis* will remain viable in dry spray-dried powders for long periods. This is true for peptone powders, yet if such powders are dissolved in a suitable quantity of water they form an excellent medium for bacterial growth; the spores germinate, and the resultant vegetative cells rapidly multiply. Some intermediate proportion of water must be just sufficient to cause the spores to initiate the germination process. It was the purpose of this present work to determine this critical water content and to investigate the fate of the spores. Obviously they will lose their heat resistance at some point, but, the interesting question is, "will the resultant vegetative cells multiply or will they die off due to unfavourable environmental conditions?" Such questions have an intrinsic interest of their own, but also it might be possible that advantage could be taken of the facts to devise conditions under which sterility would be attained with or without the use of minimal quantities of heat or antiseptics. Methods might then be elaborated which could have direct pharmaceutical applications in the preparation of sterile powders.

Previous work related to this subject seems to have been of a qualitative rather than a quantitative nature. Thus Tompkins[3] found that the range of humidities, over which germination of certain species of mould spores is possible, varies with the temperature; the further the temperature was removed from the optimum for growth, the narrower the range of humidity over which germination occurred.

When a spore germinates a series of changes is initiated. (*a*) The spore-case cracks or is absorbed or is gelatinised and then dissolved.[4] (*b*) The spore contents enlarge, a germination tube may grow out through the envelope, or the cell may simply enlarge and divide, giving rise directly to vegetative multiplication. (*c*) The resistance to adverse environmental conditions, such as heat and harmful chemical substances falls.[4] All these changes have been used as criteria of germination. Observation of (*a*) and (*b*) involves direct microscopical examination of the individual spores. This can easily be carried out in the case of moulds where the spores are relatively large. This method was employed by Tompkins.[3] Bacterial spores are approximately one-tenth the size of mould spores and morphological changes on germination are indistinct and difficult to observe. On the other hand, as pointed out by Wynne and Foster,[5]

the heat resistance of unaltered spores compared that that of vegetative cells or germinating spores can form a very definite criterion of germination, giving reproducible results under standard conditions. This criterion has been adopted in the present work.

It is likely that the interrelationship of spore germination and moisture content will be modified by the nature of the substrate. The *B. subtilis* spores have, therefore, been examined dispersed in 4 types of powder: (1) peptone, (2) lactose, (3) equal parts of lactose and peptone and (4) kaolin. The peptone, on sufficient dilution with water, gives an excellent nutrient medium. The lactose powder does not constitute a source of organic nitrogen, while kaolin is simply an inert powder.

Materials and Methods

Except where indicated, the materials and methods used in this work were those previously described.[2] A suspension of spores of *B. subtilis* (Marburg No. 3610) prepared and stored as usual constituted the source of contamination in all experiments.

In order to obtain a sufficient bulk of spray-dried product for protracted experiments, the concentration of solids in the solutions and suspensions before spray-drying was increased to 10 per cent. and the degree of contamination was arranged to give an expected count of $2{\cdot}5 \times 10^4$ viable spores per ml. of suspension. In the case of peptone 2 batches of powder were mixed by milling. The analysis of variance of quintuplicate platings of 10 samples of the resultant powder, given in Table I, shows that the spores were evenly distributed. A powder of equal parts of peptone and lactose was prepared by drying a contaminated solution containing 5 per cent. of peptone and 5 per cent. of lactose.

TABLE I

Analysis of variance of quintuplicate platings of 10 samples of the mixed spray-dried peptone powder

Source of variance	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples	1221·7	9	135·7	1·538	>0·2
Difference between individuals	8348·8	40	208·7		
Total	9570·5	49			

A kaolin powder was prepared by spray-drying a contaminated 10 per cent. suspension of light kaolin B.P. in distilled water. The feed bottle containing the suspension was shaken continuously throughout the drying. It has previously been shown that an even distribution of spores in a powder can be obtained by spray-drying a solution containing a suspension of spores. In the case of the kaolin suspension in spite of agitation during the drying, the spores were unevenly distributed in the resultant powder. Evenness of distribution was obtained by milling as previously found necessary with *Bact. lactis aerogenes* in peptone.[2]

Table II shows that during the drying of the 4 types of powder used, the spores did not suffer any significant mortality. The low mortality

TABLE II

PERCENTAGE OF *B. subtilis* SPORES KILLED BY SPRAY-DRYING ON THE VARIOUS SUBSTRATES

Substrate	Inlet temperature (° C.)	Mortality per cent.
Peptone, 10 per cent.	175	3·8
Peptone, 10 per cent.	175	0·0
Peptone, 5 per cent.; Lactose, 5 per cent.	80	6·4
Lactose, 10 per cent.	80	0·0
Kaolin, 10 per cent.	185	11·2

is consistent with the view that the spores in the powder were not a selected heat-resistant variety.

THE METHOD FOR THE DETERMINATION OF LOSS OF RESISTANCE TO HEAT

Loss of heat resistance was determined by placing test-tubes containing the culture, or a suitable dilution of it, in a water bath at 80° C. for 5 minutes and subsequently cooling the tubes under the cold water tap. Quintuplicate platings were performed before and after heating. In the work described below the count obtained before heating will be referred to as the total viable count, that obtained after heating as the viable spore count, while the differences between the two will be considered to represent the number of viable heat-labile organisms. A 24-hour culture containing more than 3000 viable *B. subtilis* vegetative organisms per ml. was found to be sterile after heating for 1 minute in a water bath at 80° C. Table III shows that heating at this temperature for 3½ hours had no significant effect on the viable count of *B. subtilis* spores.

The difficulty of counting vegetative cells of *B. subtilis* on account of chain formation is fully appreciated but the experiments described in this paper were terminated whenever rapid multiplication occurred, so that the viable heat-labile organisms were almost certainly spores which had lost their heat resistance in the process of germination but which had not yet given rise to typical rapidly multiplying vegetative cells.

TABLE III

EFFECT OF HEATING AT 80° C. ON THE COUNT OF A SUSPENSION OF *B. subtilis* SPORES

Time in minutes	0	30	90	120	150	180	210	240	270	300
Mean count of 5 tubes ..	162	161	171	152	153	154	151	139	133	120

CONTROL OF MOISTURE UPTAKE BY THE POWDERS

The spray-dried powders were, in the first place, further desiccated by storage over phosphorus pentoxide. For each experiment a number of approximately 0·5-g. quantities of powder were weighed accurately into sterile aluminium-capped glass tubes 7 cm. long and 2·5 cm. diameter. After removal of the caps the tubes and caps were placed in desiccators in which the relative humidity was controlled by saturated salt solutions[6] placed in the lower compartment. At intervals tubes were capped and

weighed and the contents dissolved or suspended in water (in the proportion of 9 ml. of water for 0·5 g. of dry powder, but allowing for the quantity of water known to have been taken up from the atmosphere in the desiccator). Viable spore counts and total viable counts were made on the resulting mixture.

To avoid having, at all stages, to make allowance for the increase in weight of the powders due to increasing moisture contents all viable counts were expressed as the number of organisms or spores per g. of the original phosphorus pentoxide-dried powder. For the same reason the moisture content of powders was expressed not as a percentage of the powder as it stood but as a percentage moisture uptake calculated on the original phosphorus pentoxide—dried powder. It was assumed that this original powder could be considered to be "dry." To avoid lengthy tables, graphs have been constructed from the results showing the relationships between the moisture content of the powder and the viable spore counts and total viable counts on a time basis at the various degrees of relative humidity. At high moisture contents rapid multiplication of the bacteria sometimes occurred; in such cases the graph terminates in an ascending arrow.

The humidity controls at least two factors, (*a*) the rate of moisture uptake by the powder, and (*b*) the maximum water content finally attained by the powder. Broughton and Mather[7] have reported that in desiccators containing only the saturated salt solution, the vapour and liquid phases acquire 97 per cent. equilibrium within 1 hour, but they point out that the rate of uptake of moisture by any materials exposed in the desiccator "is dependent rather upon their own (the materials') rate of attainment of equilibrium with the air than upon the rate of humidification of the latter by the salt solution." Table IV shows the saturated salt solution used and the corresponding relative humidities.

TABLE IV

RELATIVE HUMIDITY IN DESICCATORS CONTAINING SATURATED SALT SOLUTION AT 20° C.

Solid phase	Relative humidity per cent.
Disodium hydrogen phosphate, Na_2HPO_4, $12H_2O$ ..	95·0
Zinc sulphate, $ZnSO_4$, $7H_2O$	90·0
Sodium thiosulphate, $Na_2S_2O_3$, $5H_2O$	78·0
Sodium nitrite, $NaNO_2$	66·0
Potassium acetate, CH_3COOK	20·0

RESULTS

Storage of contaminated peptone powders in atmospheres of various relative humidities. The fact that the spores of *B. subtilis* will remain viable and heat-resistant, i.e., apparently unchanged in dry peptone powder for long periods of time has again been confirmed. The bulk peptone powder which formed the starting material for the various experiments described in this section has now been stored over phosphorus pentoxide for over 2 years. Viable spore counts and total viable counts have been performed

on it at various intervals and a statistical analysis of the results obtained shows that the variation in the counts does not exceed that to be expected by random sampling. The viable spore counts and total viable counts remained sensibly constant and equal at 270×10^3 per g.

When the dry peptone powder was exposed to an atmosphere of 66 per cent. relative humidity the moisture content increased in 20 days to approximately 29 per cent. and remained constant at this figure during the rest of the 8 months experimental period. Statistical analysis of the counts performed during the same period showed that the variations in viable spore counts and total viable counts did not exceed the errors of random sampling and that viable spore counts and total viable counts were the same. Thus, in peptone powders containing up to 30 per cent. of moisture the spores remain viable and heat resistant.

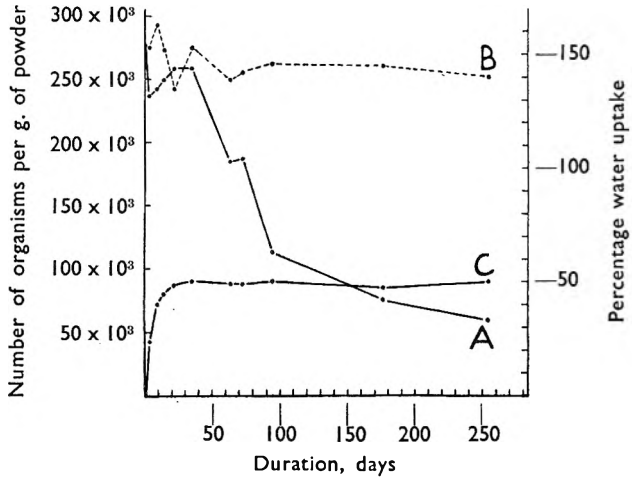

FIG. 1. Storage of infected peptone powder in an atmosphere of 78 per cent. relative humidity at 20° C.

A, Viable spore count. B, Total viable count. C, Water uptake. ● Experimental determination.

When exposed to 78 per cent. relative humidity (Fig. 1) the moisture uptake of the peptone powder was 50 per cent. in the first month and it remained constant at approximately that figure during the remainder of the 8 months. Although after 3 days (at a moisture uptake of a little over 30 per cent.) the powder became a paste, the total viable count remained constant at about 270×10^3 organisms per g. throughout the experiment. On the other hand, a little after the first month the viable spore count commenced to fall, at first rapidly, then more slowly to 60×10^3 organisms per g. after 8 months. It is clear that with a moisture uptake of 50 per cent. the majority of the spores lose their resistance to heat but remain viable.

Exposed to 90 per cent. humidity (Fig. 2) the moisture uptake of the dry peptone powder was at first rapid and, although the rate fell off subsequently, the moisture content was increasing during the whole

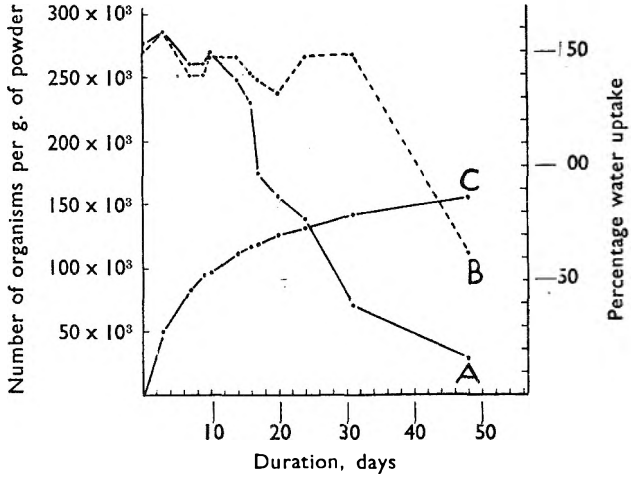

FIG. 2. Storage of infected peptone powder in an atmosphere of 90 per cent. relative humidity at 20° C.

A, Viable spore count. B, Total viable count. C, Water uptake. ● Experimental determination.

48 days of the experimental period. When the moisture uptake had risen to 50 per cent. the viable spore count began to fall while the total viable count remained constant. After 32 days at a moisture uptake of 80 per cent. with the viable spore count still decreasing the total viable count commenced a rapid fall which continued to the end of the experiment. This suggests that the spores which lose their heat resistance at a moisture uptake of 50 per cent. begin to die off when the moisture uptake has risen to 80 per cent.

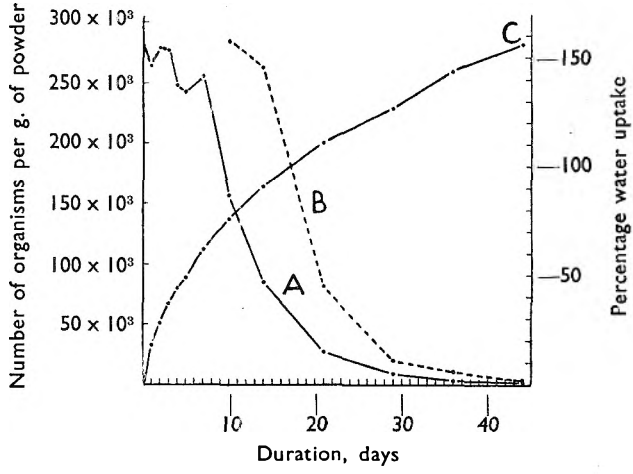

FIG. 3. Storage of infected peptone powder in an atmosphere of 100 per cent. relative humidity at 20° C.

A, Viable spore count. B, Total viable count. C, Water uptake. ● Experimental determination.

At 100 per cent. relative humidity the same picture developed a little further (Fig. 3). The moisture content rose at first rapidly then more slowly throughout the 44 days of the experiment. At 50 per cent. moisture uptake the viable spore count commenced to fall while the total viable count remained constant. At 80 per cent. moisture uptake the total viable count commenced to fall. After 44 days the viable spore count had fallen to 18×10^2 while the total viable count was approximately twice that figure although at 14 days the total viable count had been about 3 times the viable spore count.

When the 100 per cent. humidity experiment was carried out at 36° C. yet another variation occurred. Moisture uptake was rapid all the time and when it reached over 50 per cent. the viable spore count fell rapidly. No marked fall in the total viable count was however detected. This was doubtless because when the moisture uptake reached a value over 160 per cent. a rapid multiplication of the organisms commenced. The time taken for the moisture uptake to pass from just over 80 per cent. at which a fall in total viable count might have been expected to 150 per cent., the lower limit for rapid multiplication, was only about 8 days.

To confirm the above findings instead of exposing the dry peptone powder to various humidities so that the moisture uptake increased during the experiment a few experiments were carried out in which the moisture was added all at once as a weighed quantity of sterile distilled water. In this way powders with moisture uptakes of 40 per cent., 60 per cent., 80 per cent. and 100 per cent. were prepared. 2 samples at each moisture level were stored in firmly stoppered containers at room temperature. At 2 and 23 days respectively viable spore counts and total viable counts were performed on the samples. The results obtained are shown in Figures 4, 5 and 6. No loss of viability or heat resistance occurred in the spores in the powder having a 40 per cent. moisture uptake during the 23 days storage. In the samples containing more moisture, heat sensitisation without loss of viability of some of the spores occurred after 2 days. There was no fall in total viable count except, after 23 days, in the powders having 80 per cent. and 100 per cent. moisture uptakes.

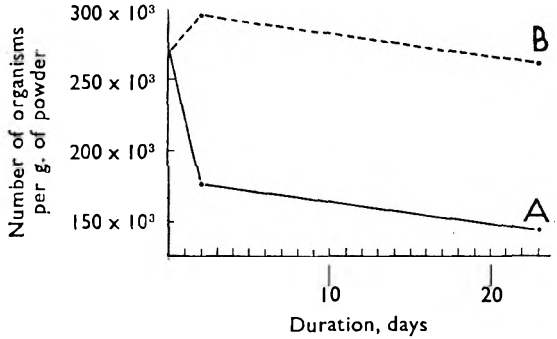

FIG. 4. Storage of infected peptone powder with an equivalent of 60 per cent. water uptake at 20° C.
A, Viable spore count. B, Total viable count. ● Experimental determination.

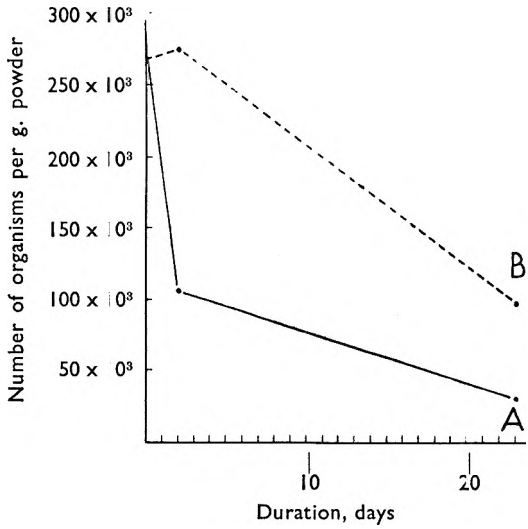

FIG. 5. Storage of infected peptone powder with an equivalent of 80 per cent. water uptake at 20° C.

A, Viable spore count. B, Total viable count. ● Experimental determination.

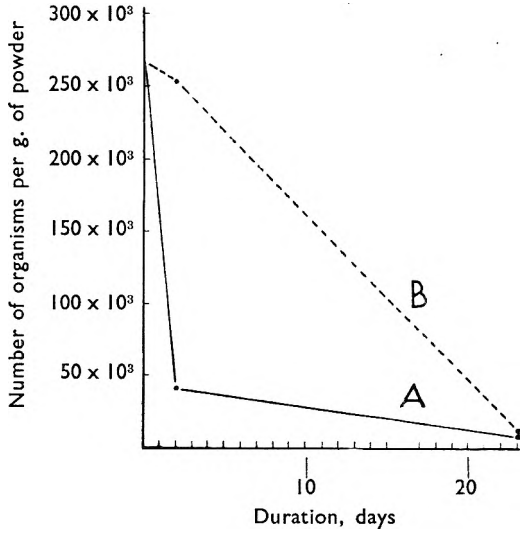

FIG. 6. Storage of infected peptone powder with an equivalent of 100 per cent. water uptake at 20° C.

A, Viable spore count. B, Total viable count. ● Experimental determination.

Storage of contaminated lactose powder in an atmosphere of 100 *per cent. relative humidity.* The results obtained with spores of *B. subtilis* in lactose powder were relatively simple (Fig. 7). The moisture content rose in about 9 hours to 9 per cent. At this point the viable spore and total viable counts began to fall rapidly for 3 or 4 days and then steadily

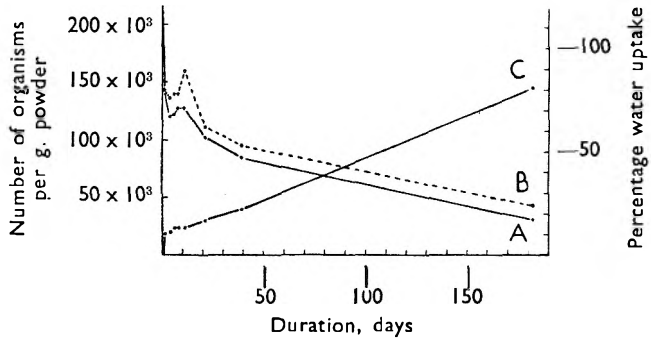

FIG. 7. Storage of infected lactose powder in an atmosphere of 100 per cent. humidity at 20° C.

A, Viable spore count. B, Total viable count. C, Water uptake. ● Experimental determination.

throughout the remainder of the 6 months period of the experiment and this, in spite of the fact that the moisture uptake increased steadily to 80 per cent. It is obvious that the organisms were losing their viability at about the same rate as the spores were losing their heat resistance.

It should be mentioned that even with high moisture uptake the lactose powders never became pastes. The powder at first becomes more coherent or "ropy" and then hard and brittle.

Storage of the contaminated lactose-peptone powder in an atmosphere of 100 per cent. relative humidity. Figure 8 shows that the results obtained with this mixed powder were intermediate between those obtained with the peptone powder and those obtained with the lactose powder. Counts remained steady at 250 × 10³ until the moisture content reached about 35 per cent. The counts then commenced to fall, the viable spore count

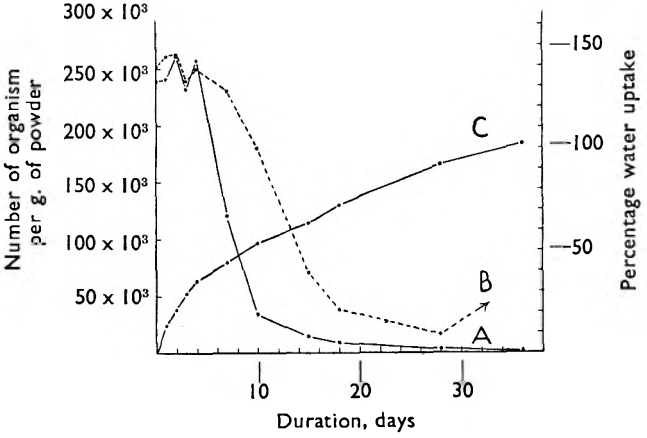

FIG. 8. Storage of infected peptone and lactose powder in an atmosphere of 100 per cent. relative humidity at 20° C.

A, Viable spore count. B, Total viable count. C, Water uptake. ● Experimental determination.

more rapidly then the total viable count, showing that the spores were losing their heat resistance at a greater rate than the organisms were losing their viability. At a moisture uptake of about 90 per cent., when the total viable count had fallen to about 20×10^3 there was a rapid multiplication of the vegetative organisms. At high moisture content this powder became first a paste and then a solution.

Storage of the contaminated kaolin powders in atmospheres of various relative humidities. The results of exposing the kaolin powders (previously dried over phosphorus pentoxide) to 20, 78 and 90 per cent. relative humidities respectively, establish the fact that under these conditions, with not more than a 2 per cent. moisture uptake, the spores remain heat resistant and viable for at least 3 months.

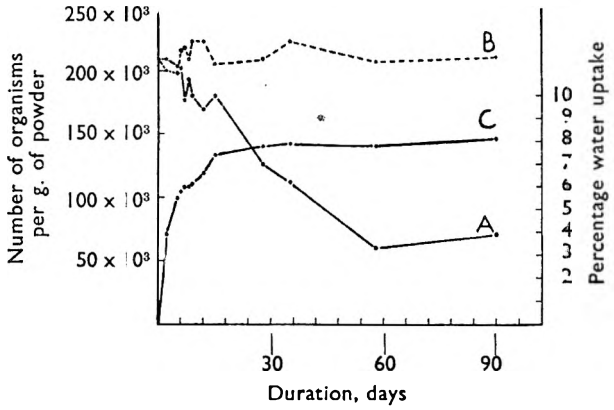

FIG. 9. Storage of infected kaolin powder in an atmosphere of 95 per cent. relative humidity at 20° C.

A, Viable spore count. B, Total viable count. C, Water uptake. ● Experimental determination.

Figure 9 (95 per cent. relative humidity) shows that, as with the peptone and lactose powders, the first observable change is a loss of resistance to heat by the spores, this time at a moisture uptake of about 6 per cent. The heat-sensitive organisms nevertheless remain viable as shown by the fact that the total viable count remained sensibly constant throughout the 3 months period. Figure 10, a record of events taking place in an atmosphere of 100 per cent. relative humidity, shows a feature found only in the case of the kaolin powders. The spores first lost their heat resistance at 6 per cent. moisture uptake, but subsequently heat resistance was regained, the viable spore count becoming, after 48 days, identical with the total viable count which had not changed throughout the experiment. There is a hint in the previous Figure 9 that recovery of heat resistance by the spores might be commencing after 60 days' exposure to 95 per cent. relative humidity.

When the exposure of the kaolin powder to an atmosphere of 100 per cent. relative humidity was repeated at 36° C. the same sequence of events occurred but more rapidly. At first the viable spore count

fell and then rose while the total viable count remained constant, indicating loss of heat resistance followed by recovery of such resistance. The spore count never regained its original value, however, because before this could occur the moisture uptake had risen to over 20 per cent., a higher value than that attained in the previous experiments and this high moisture content apparently caused death of the organisms since both viable spore counts and total viable counts began to fall and

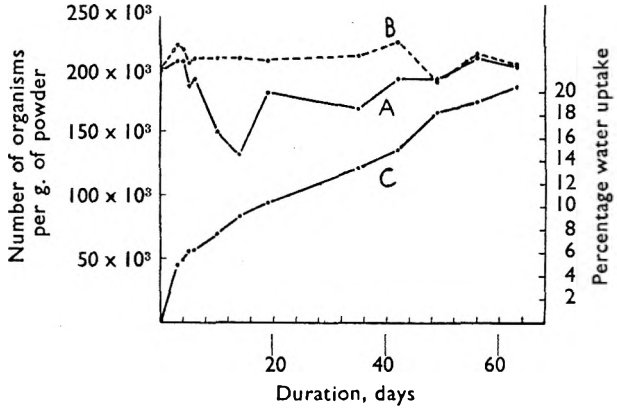

FIG. 10. Storage of infected kaolin powder in an atmosphere of 100 per cent. relative humidity at 20° C.
A, Viable spore count. B, Total viable count. C, Water uptake. ● Experimental determination.

to approach each other in value. In none of the kaolin experiments did rapid multiplication of the organisms occur. This is understandable since in such powders there is no source of either energy or organic nitrogenous food material.

DISCUSSION

When spray-dried powders containing spores of *B. subtilis* are exposed to conditions causing increasing moisture uptake 2 points may be observed irrespective of the nature of the supporting powder, (*a*) below a certain moisture uptake the spores do not appear to undergo change, since they remain for a long period of time viable and heat resistant. The value of this critical moisture uptake varies with the nature of the supporting powder, being approximately 50 per cent. for peptone, 10 per cent. for lactose, 35 per cent. for peptone and lactose and 6 per cent. for kaolin. It will be noted that for the powders containing peptone, the critical moisture uptake is considerably higher than for the lactose and kaolin powders. A possible explanation of this difference may be found in the work of Hills,[8] who has shown that, taking loss of heat resistance as the criterion of germination, inhibition of the germination process is affected by critical concentrations of certain D-amino-acids. These or other inhibitory substances may be present in the peptone and require dilution beyond their active concentration before germination can proceed.

(*b*) There is a range of moisture content in which the spores lose their heat resistance but remain viable. This range is wider in the case of the peptone powders than in the case of the lactose powders; intermediate in the case of the peptone and lactose powders. It is possible that this range is to some extent conditioned by the presence or absence of food substances, and anti-multiplication factors in the peptone. Inhibitory factors have been shown to be present in hydrolytic products of proteins,[9] but that the presence of these does not offer a full explanation is indicated by the fact that this range does occur with the inert kaolin powders. Other points dependent, however, on the nature of the supporting powder are: (*c*) above a certain moisture uptake germination of the spores and rapid multiplication of the resultant vegetative organisms occurs if suitable nutrient material is present, e.g., in the case of the peptone powders. (*d*) In the case of the kaolin powders, between that moisture uptake below which the spores are unchanged and that at which death of the spores occurs, there is a range of moisture uptake over which the spores at first lose and then regain their heat resistance. It is proposed to study this behaviour further. Unfortunately, it is not possible to ascertain the moisture content of the spores themselves in such a powder. It may be that the behaviour of the spore is relatively uninfluenced by the supporting powder, i.e., that the moisture content at which it loses its heat resistance, dies, or germinates may be always the same, the differences in the powders being explained by, for example, the greater moisture binding power of peptone compared with that of kaolin. Thus the spores in a peptone powder with an 80 per cent. moisture uptake may only contain as much moisture as those in a kaolin powder with a 2 per cent. moisture uptake. On the other hand, it is likely that multiplication and germination may be limited by the presence or absence of food materials and the presence or absence of "anti" factors as well as by the degree of availability of moisture. Perhaps the most important observation from a pharmaceutical point of view is that at a certain moisture uptake spores in a powder lose their resistance without gaining the capacity to multiply. Experiments are being undertaken with a view to determining the reaction of such spores to minimal concentrations of antiseptics.

It seems obvious that, in all powders examined, between the low moisture content at which the spores remain unchanged and the relatively high moisture content at which the spores germinate and multiplication occurs there is an intermediate zone where the spores become sensitive to adverse conditions such as heat and in fact tend to die if no further moisture uptake occurs. It will be of considerable interest to explore this zone further and to see if application can be made of it to sterilise powders for pharmaceutical use. An obvious difficulty from this point of view is the change in the physical characters of some powders to give pastes (peptone) or coherent masses (lactose).

It should be emphasised that the facts have turned out to be more complex than was anticipated. The original object of this work was to determine the one critical moisture content at which spores germinate

and to ascertain the behaviour of the spores at this point. Actually, 3 critical moisture contents have been found; there is a lower critical content below which no change occurs in the spores but above which the spores lose their heat resistance while remaining viable for up to three months at least. A second critical point has been found below which the spores lose heat resistance but remain viable and above which they tend to die off. A third upper critical point also exists, below which the spores die off and above which they give rise rapidly multiplying vegetative cells. So far as we can ascertain, the zones of loss of heat resistance with retained viability and loss of heat resistance with loss of viability have not before been reported in the literature.

It will be noticed that so far in this discussion the behaviour of the spores has been interpreted in terms of moisture uptake although it appears probable that a time factor might play some part. Thus, at first sight, the graphs of Figure 2 might be interpreted by postulating that in an atmosphere of 90 per cent. relative humidity the spores required about 15 days to lose their heat resistance and under the unfavourable conditions a further 15 days to die off. This would be an attempt to explain all the phenomena on a time basis ignoring the effects of increasing moisture uptake by the powder. It is quite probable that time factors play a part, but that they are not dominant can be seen by a comparison of the results of various experiments. It has been shown above that the spores do not lose their heat resistance for at least 3 months if the moisture content is below the critical value while curves A and B of Figures 1 and 9 show that spores which have lost their heat resistance can remain viable for more than 3 months. However, a careful examination of Figures 4, 5 and 6, where the moisture was added all at once, does clearly indicate the operation of time factors.

Certain positive conclusions of pharmaceutical interest can be drawn from this work. It is obvious that if powders containing spores are stored in a dry state there will be no germination of the spores and no increase in the degree of contamination, although the spores may retain their heat resistance and viability over long periods of time. Even if the powders are not initially dry, or if they are stored badly under conditions where moisture uptake can occur, the first change will be loss of heat resistance of the spores followed by a decrease in the degree of infection due to death of the now more sensitive spores. If exposed to conditions causing excessive moisture uptake the spores in the powders may germinate and the resultant organisms may multiply, but only if suitable nutrient materials are present. The moisture uptake necessary for this type of change would usually result in obvious changes in the physical conditions of the powder.

These conclusions have been reached using a variety of substrates, namely nitrogenous (peptone) carbohydrate (lactose), a mixture of the two and finally an inert powder (kaolin). Only one type of spore has been used, namely that of *B. subtilis* and it would of course be desirable to confirm the work using the spores of other organisms.

SUMMARY

1. When powders containing the spores of *B. subtilis* are exposed to atmospheres of increasing humidity the following sequence of events has been found to occur: (*a*) below a certain moisture content the spores remain viable and heat resistant. (*b*) Over a certain range of moisture uptake the spores lose their heat resistance while retaining their viability. On kaolin powder the heat resistance may subsequently be recovered. (*c*) At a still higher moisture content both heat resistance and viability are lost. (*d*) Above a certain moisture content in the presence of nutrient materials the spores germinate and the organisms multiply.

2. Conclusions of pharmaceutical interest are that: (*a*) Spores in a dry powder undergo no change over long periods, although they remain viable and heat resistant, there is no germination or multiplication. (*b*) If the powders are not kept in a dry condition the first change is a loss by the spores of their resistance to heat. This is followed by death of many of the organisms. Thus, far from increasing, the degree of contamination of the powder decreased. (*c*) If exposed to conditions causing excessive moisture uptake, the spores in the powders germinate and the resultant organisms may rapidly multiply in the presence of nutrient materials. The moisture uptake necessary for these changes might be expected to result in obvious changes in the physical condition of the powder.

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DISCUSSION

The paper was presented by MR. A. TALLENTIRE.

DR. K. BULLOCK (Manchester) said that he was puzzled as to why, at certain moisture levels, spores should germinate and then die off. Was that due to osmotic effects, to the fact that nutritional requirements could not diffuse to and from the organism readily, or to the presence of some anti-growth factor in the powders? Again, it was not possible at present to explain why, at a particular moisture level in the kaolin powders, the spores should first lose and then regain their resistance. The aim was to repeat the work with non-sporing organisms, but the difficulty was that non-sporing organisms died off fairly rapidly even in dry powders.

DR. K. R. CAPPER (London) said he understood that young spores were often much more resistant than older spores. There was no indication that that was related to the moisture content of the substrate.

He believed that an anti-growth factor for staphylococcus had been found in peptone, also that kaolin might contain very small amounts of nutrient material. How was dormancy related to the changes occurring in the spores? Did it occur only under dry conditions or if the spores had reduced resistance? In pharmacy the most troublesome cases were the powders which must be dissolved before injection and which could not be sterilised by heat. Very few did not provide nutrient solutions, but there would be factors such as osmosis which would affect the life of the spores which they contained.

Dr. R. M. SAVAGE (Barnet) referred to the survival of bacterial spores in surgical dressings, and said that during sterilisation there was a danger of the atmosphere not being completely saturated with steam. An extension of the investigations might throw some light on what happened at much higher temperatures than room temperature.

DR. F. WOKES (King's Langley) said he assumed that the moisture content was determined by drying in vacuo over phosphorus pentoxide. Had this been checked by the Karl Fischer method?

MR. A. MARSH (Brighton) asked whether there was any relationship between the minimum water content necessary for the germination of dry spores and the water sharing which would occur in a mixture of spores and diluent powder.

MR. D. N. GORE (Dorking) said that in the substrate there was a difference between the mobile and the total moisture, so far as the organism was concerned.

MR. P. LAWRIE (Edinburgh) said that in his experience with surgical catgut the Karl Fischer reagent gave high results due to combination of the iodine with protein.

DR. K. BULLOCK (Manchester) said that the authors had used powders dried over phosphorus pentoxide and reported the moisture uptake. That was why the phrase "moisture uptake" was used.

MR. A. TALLENTIRE, in reply, said that *Streptococcus fæcalis* was found to be the best non-sporing organism for spray-drying purposes. A culture of spores all of one age was used; consequently comparative resistance of young and old spores did not enter into the work. Anti-growth factors had been found in peptone and work might be carried out on separating them later. One or two spores remained dormant up to 12 months, and it had not been possible to get them to germinate by increasing the moisture content. The method used for the determination of moisture was the difference in weight, and moisture uptake, not moisture content, was recorded.

THE APPLICATION OF INFRA-RED HEATING TO PHARMACEUTICAL PRODUCTS

PART I. PRELIMINARY INVESTIGATION

BY H. W. FOWLER

From the School of Pharmacy, Leicester College of Technology

Received June 30, 1952

INTRODUCTION

THE manufacture of many pharmaceutical preparations involves heating processes and in the past these have largely been carried out by conduction or convection methods. In both cases there are disadvantages. In the former the substance is in contact with a hot surface so that overheating may occur, while in the latter hot air is used and heat losses are considerable.

The use of radiation as the method of heat transmission has the advantage that the heat is transmitted directly from the emitter to the substance, without the losses due to an intermediate stage.

THEORY

Radiant heat is emitted by all bodies, the amount and the quality of the energy depending on the temperature of the body. By the Stefan-Boltzmann law, the total energy radiated by a black body (which is a perfect emitter) is proportional to the fourth power of the absolute temperature.[1] Radiant energy is distributed over a range of wavelengths above the red of the visible spectrum, hence the commonly used title of "infra-red" to describe this radiation. The infra-red band ranges from the "near infra-red" with a wavelength of less than 1 μ to the "far infra-red" with a wavelength of more than 100 μ.

The emission of energy at any temperature is not evenly distributed

TABLE I

WAVELENGTH, TEMPERATURE, ENERGY, AND TYPE OF GENERATOR

Wavelength of peak emission in Ångstrom units	Temperature of generator °C.	Energy per sq. ft. of generator surface in B.Th.U./hour	Type of generator
10,000	2,700	1,300,000	Electric lamp
14,000	2,000	450,000	Electric lamp
20,000	1,200	80,000	Electric lamp
21,000	1,100	62,000	Electrically heated rods
25,000	875	28,800	Gas panels and electric rods
30,000	700	14,400	Gas panels and electric rods
35,000	550	7,500	Gas panels, electric rods and discs
47,000	340	2,400	Large surfaces gas heated vapour and liquid heated tubes
50,000	300	1,800	Ditto
55,000	250	1,350	Ditto

over the infra-red band, but there is a maximum or peak wavelength, the position of which depends again on the temperature. Wien's law states that the wavelength of the peak is inversely proportional to the absolute temperature.[2] Wien also found that the intensity of the radiation at the peak wavelength is related to the temperature, being proportional to the fifth power of the absolute temperature.[2]

Hence if intensity of radiation is plotted against wavelength, curves of the form shown in Figure 1 are obtained.

Thus, by varying the temperature of the generator, it is possible to choose approximately a particular peak wavelength, which, therefore, gives control of the intensity of the emission and hence, of the heating effect. Table I shows the relation between wavelengths, generator temperature, energy emitted and the type of generator producing these wavelengths.[3]

The heating effect in any substance on which radiation falls will depend partially on the intensity of radiation as shown and partially on the absorptivity of the substance. This varies according to the wavelength of the radiation, the colour of the surface and the nature of the surface.

The mechanism of drying can occur in two ways according to circumstances. Stout *et al.*[4] have investigated the mechanism of drying in beds of solids up to 1 inch thick and find that it does not differ substantially from atmospheric and vacuum drying when using sand, soap and magnesium stearate. The penetration of the radiation did not appear to exceed $\frac{1}{16}$ inch.

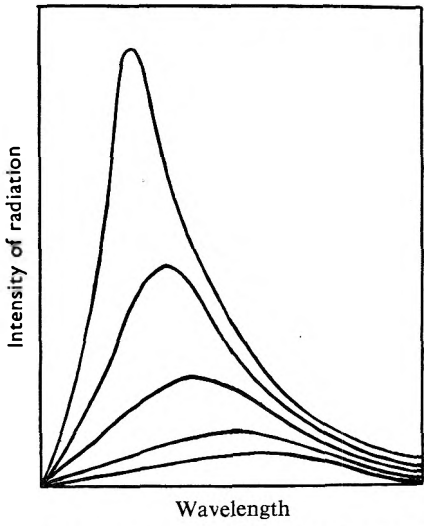


FIG. 1. Typical curves showing peak wavelength varying with temperature.

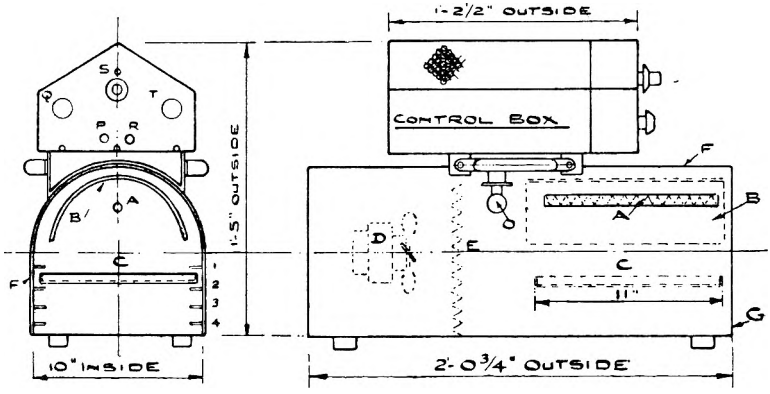
In the case of paint finishes, which would be of less than this thickness, McCloud[5] concludes that the drying is from the inside outwards, the radiation penetrating the paint, heating the metal and hence, the inner paint layers. The theory is supported by the difficulty of infra-red drying paint finishes on woods. The drying of films, such as scale preparations, would occur in this way.

Much has been published with regard to heating and drying by infra-red radiation, but this has been mainly confined to the drying of paint finishes and other industrial processes, such as drying textiles, heating plastics, etc. Publications referring specifically to pharmaceutical applications are surprisingly few in number. Youngken and Hassan,[6] while carrying out pharmacognostical and chemical studies of Indian belladonna, dried berries of *Atropa acuminata* and *A. belladonna* using infra-red lamps. A

number of samples were dried for 1 hour using 2 lamps, one 10 inches above and the other 10 inches below the berries. In these cases a temperature of 145° C. was obtained and considerable loss of alkaloid occurred. Further samples were dried for 2 hours using one lamp 24 inches above the fruits giving temperatures of 55° C. The alkaloidal content of these samples was comparable with results obtained on berries which had been dried spontaneously at room temperature over a period of 6 to 8 weeks. Patel *et al.*[7] have carried out an investigation of the effect of infra-red radiation on a number of substances commonly used in tablets. Their work will be referred to in more detail in Part II of this paper. Déribéré has given descriptions of a number of infra-red lamp dryers but unfortunately no useful performance figures are given. The dryers described include drum dryers[8] of two types for syrups and liquids. The first combines steam and infra-red, the liquid in the feed tank being preheated by lamps and the drum itself heated internally by steam and externally by further lamps. The second is heated externally by infra-red lamps only. An account has been given of a number of smaller dryers.[9] A lamp may be simply used in a universally jointed stand and placed at a suitable height over the work, or cabinet dryers used, with adjustable grille shelves and heated by one or three lamps according to size. Also a vacuum dryer is described consisting of two bell-shaped portions fitting together and each containing a lamp, so that one is above and the other below the work. This is intended for moisture tests and it is claimed that a sample of 5 to 10 g. can be dried by 2 × 250 watt lamps in 5 to 8 minutes at 65° C. Déribéré also describes two commercial installations.[10] The first, a cabinet dryer intended for drying vegetable extracts, is about 6 feet in height and has 7 or 8 perforated shelves, the top shelf only being radiated by infra-red lamps. The remainder of the shelves do not receive radiation and are used for preheating, so that drying capacity would be low in relation to the surface area in the cabinet. The door has an inspection window but this is mounted too low to see the top shelf where the actual drying process takes place. The second is a tunnel dryer heated by 60 × 250 watt lamps, and trays are passed through from end to end. Radiant heat has been used by Zamzow and Marshall[11] to supply the heat of sublimation in the freeze-drying process and it was found that the rate of drying was increased compared with conduction heating. An infra-red lamp has been used as the heating unit of a moisture tester suitable for pharmaceutical products.[12] A 5-g. sample is placed on the pan and by means of a continuous weighing device a pointer indicates the percentage moisture content directly on a scale.

With the exception of Zamzow and Marshall's work,[11] in which they use wavelengths of 2 to 4 μ, all the papers previously quoted have referred to infra-red lamps which operate at a peak wavelength of about 1·2 μ in the case of tungsten filament lamps and 1·4 μ if the filament is of carbon. McCloud[5] has stated that "of the various ways of applying heat to an object, radiation is recognised as the most efficient, provided the path that the radiant energy has to travel can be

kept short." This is not possible for pharmaceutical purposes with lamps, since the greater heating effect of the short peak wavelength causes overheating, and in the cases already referred to it was necessary to place the lamps at distances of 1 to 2 feet to avoid this. Furthermore, the greater operating distance means that any unevenness of reflection will be of more importance so that "hot spots" may occur with corresponding irregularity of heating. Hence, this investigation has been confined to the use of lower powered generators emitting radiations with peak wavelengths of 3·2 and 3·6 μ which permitted the reduction of the operating



A	INFRA RED GENERATOR
B	REFLECTOR
C	TRAY
D	FAN
E	CONVECTION HEATER
F	MAIN CASING
G	DOOR
O	LAMP
P	I.R GENERATOR SWITCH
Q	I R GENERATOR WAVE LENGTH CONTROL
R	AIR HEATER SWITCH
S	AIR HEATER CONTROL
T	MAIN SWITCH

CONTROL PANEL

FIG. 2. Diagram of laboratory infra-red dryer.

distance to approximately 10 cm. There is a further advantage to the use of these wavelengths since both water and ethanol show a great increase in absorption between 3 and 3·5 μ.[3] This means that at about these wavelengths there will be a considerable improvement in heat transfer to water or ethanol with correspondingly increased drying rates.

As a preliminary, various pharmaceutical substances and preparations have been dried with a view to finding whether the use of infra-red is physically a suitable method. In some cases comparisons have been made with other types of dryers. Generally, no attempt has been made to record temperature or to check for deterioration other than anything visually obvious.

EXPERIMENTAL

Apparatus.—A standard laboratory infra-red dryer, a diagram of which is shown in Figure 2 was used for this work.

The dryer, manufactured by the Kestner Evaporator and Engineering Co., Ltd., uses an electric rod generator made by winding resistance wire on a ceramic former. This is placed at the focus (A) of a parabolic reflector (B) so that a theoretically parallel beam of radiation falls on

TABLE II

DRYING RESULTS

Substance	Conditions of drying	Conclusion
Fresh drug (hyoscyamus).	Approximately 1 hour at 3·6 μ.	Shrivelled, but colour better than normal commercial specimens.
Extracts (cascara).	3·6 μ, the time depending upon the depth. 1 litre of percolate, in a layer initially 2 cm. deep, containing 3 per cent. of solids dried in 5 hours. Maximum recorded temperature, 70° C. Thin films dried in 15 to 20 minutes.	The product is hard and not so satisfactory as vacuum dried extracts. The surface tends to harden, while the bulk is still moist unless the layers are very thin.
Scale preparations (iron and ammonium citrate).	15 to 20 minutes at 3·6 μ.	Probably the best method for this type of preparation.
Precipitates (saccharated iron carbonate).	Time variable according to the thickness of the layer. Sample in layer 0·2 cm. deep dried in 20 minutes and in layer 1 cm. deep in 3 hours, both at 3·6 μ. Sample in 1 cm. layer in steam oven required 4½ hours. Ferrous carbonate content in both cases about 60 per cent.	Satisfactory. Quicker, especially in shallow layer.
Granules.	Time varies from 20 minutes for a layer 0·3 cm. thick to 2 hours for a layer 1 cm. deep. Depends also upon the initial moisture content, which was 30 to 40 per cent. in the above cases.	Very satisfactory. Much quicker than the usual methods. See Part II of this paper.
Pastilles (glycogelatin base, B.P.C.). (25 grain, 2 cm. dia. and 0·5 cm. thick.)	3·6 μ. Drying not completed.	Even low temperatures soften the base. Would probably be satisfactory for bases with higher melting points.
Lozenges. (Approximately 20 grains. Oval, 1·75 cm. × 1·25 cm. and 0·25 cm. thick.)	20 to 30 minutes at 3·6 μ.	Satisfactory provided temperatures are kept low. Otherwise lozenges swell and burst due to the outside hardening and shrinking before the inside is dry.
Lamellae.	Approximately 10 to 15 minutes at 3·6 μ.	Satisfactory.
Tablet triturates. (1 grain 0·5 cm. diameter and 0·25 cm. thick.)	Approximately 10 to 15 minutes at 3·6 μ.	Satisfactory.

the drying tray (C). Resistances can be switched into the circuit so that the generator can operate at peak wavelengths of 2·4, 2·8, 3·2 and 3·6 μ. In addition, a current of air can be directed across the tray by an axial flow fan (D), the air being heated to any temperature up to about 60° C. by the spiral heating element (E). This allows the use of the apparatus for comparisons with convection drying and the combination of this method with infra-red.

Procedure.—Infra-red radiation was used to dry representative drugs and various preparations which were made in the usual manner. The results are summarised in Table II.

Conclusions

Drying by infra-red radiation appears to be satisfactory for certain types of pharmaceutical preparations, when using a peak wavelength of about 3·6 μ. In all these cases the drying times are shorter than those obtained by conventional methods. The method appears to be useful for materials which may be dried in films or thin layers, for granular preparations in shallow layers and for small solids such as lozenges. The degree of usefulness will vary with the individual substance being treated, since it will depend upon the properties of that substance, with regard to reflection, transmission or absorption of the appropriate wavelength. If reflection is high, then the heating effect is reduced, while if absorption is great the surface will be heated excessively and transmission take place to the remainder by conduction or convection. It is therefore, important when considering the application of infra-red drying, to assess at what point the energy will be transformed from radiant energy to heat energy. Some transmission of the radiation in the substance is desirable so that the transformation to heat takes place within the substance and not on the surface. The investigation of this property for pharmaceutical substances is necessary. The infra-red dryer was not found suitable for extracts in its present form since a vacuum-dried extract is much more convenient in handling and use. It is possible that a dryer for operation under reduced pressure could be designed.

Summary

1. An account of the theory of infra-red radiation is given and the literature on the pharmaceutical uses of such radiation is reviewed.

2. The advantages of using a radiation having a longer peak wavelength than that emitted by infra-red lamps are stated.

3. A laboratory infra-red dryer is described.

4. The method has been used successfully, with shorter drying times, for fresh drugs, scale preparations, granular preparations, wet precipitates, lozenges, lamellæ and tablet triturates.

5. The method was not found to be suitable at present for extracts and for soft pastille masses.

Part II. Drying of Granules

Introduction

As indicated in Part I of this communication, infra-red radiation is a satisfactory means of drying granular preparations at a rate greater than that obtained by the usual methods. The drying process in the preparation of tablet granules is of great importance, if good quality tablets are to be produced. This is especially true if the tablets are coloured, as

improperly dried granules can produce unevenly tinted tablets.[13] Patel *et al*[7] have investigated the effect of radiation from infra-red lamps on a variety of substances used in tablets. 67 chemicals were heated by lamps of various wattages and at various distances and any decomposition noted. In the majority of cases there was little or no change. The work was extended to tablet granules of 55 different formulæ, of which 22 were of inorganic compounds, 19 of organic compounds and 14 were compound formulæ. No difficulties were experienced in 90 per cent. of the cases, the only unsuccessful formulæ being those containing yellow mercurous iodide, sodium acid phosphate, tannic acid, dextrose and buchu and atropine compound. It would, therefore, appear that the infra-red drying of tablet granules merits further investigation, and the object of this work was to prepare a standardised granule and to ascertain the effect on the drying rate, of the peak wavelength and of passing a current of air over the drying surface.

Experimental

Apparatus.—The infra-red dryer described in Part I was used for the experimental work with a modification to allow the sample to be weighed without removal from the dryer, so that drying conditions were not disturbed. The drying tray was supported in the correct position on two cantilevers which projected into the dryer from the front. These were attached to vertical members which were in turn fastened to two other cantilevers mounted on a balance underneath the dryer. The proportions were arranged so that the centre of gravity of the structure together with the loaded drying tray was exactly over the centre of the balance pan. A lock was provided so that the drying tray remained at the same distance from the infra-red generator except during the actual weighing process. Theoretically, slight variations of this distance should have no effect as the parabolic reflector should give a parallel beam. An attempt was made to indicate approximately the temperature during drying by drilling a hole in the front of the drying tray close to the bottom and inserting a mercury thermometer.

Procedure.—It was necessary that the substance used for the granules should be inert and heat stable so that at first kaolin was tried but this was found to be unsuitable as it returned to the clay state on moistening. Finally, light magnesium oxide B.P. with 2 per cent. of acacia B.P. as a binding agent, moistened with distilled water and passed through a No. 16 sieve was found to give a satisfactory granule.

The granules were evenly spread in the drying tray and placed in the dryer. The weight of the sample and the thermometer reading were recorded at intervals of five minutes during drying.

When equilibrium conditions were achieved, (i.e., when the weight remained constant over 15 minutes), the heating was increased until the completely dry state was reached. Using this dry weight, the weight of moisture at each time increment was obtained, from which the percentage moisture content was calculated. This is expressed in the accepted manner as "percentage moisture (dry basis)" by which is meant the

number of parts by weight of moisture per 100 parts by weight of dry material.

Results

The results of selected drying runs are shown in Figure 1, in which the percentage moisture (dry basis) is plotted against the time in minutes. In each case the time was calculated from 25 per cent. moisture content. The drying runs are numbered and refer to the runs enumerated in Table I.

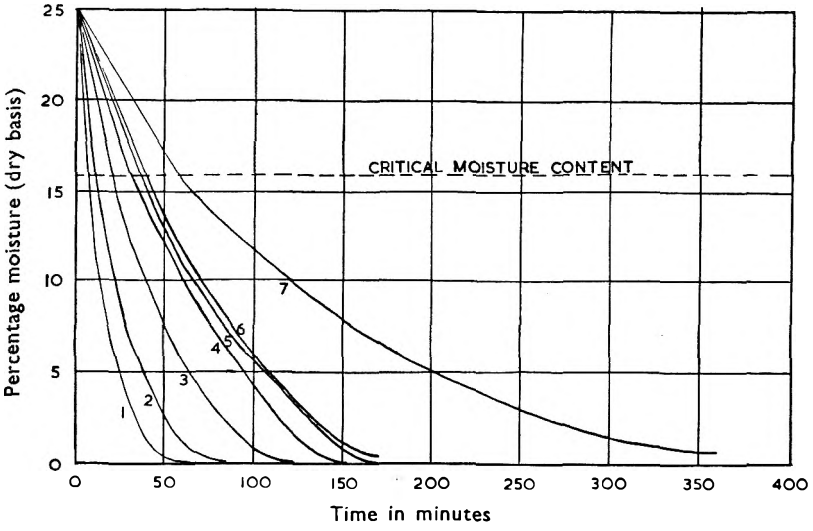


Fig. 1. Drying curves.

In Table I further data are given showing the conditions of drying during each run, the constant drying rate, the critical moisture content (i.e., when the constant drying rate ended), the temperatures during the constant rate and at completion, and the times taken.

TABLE I

Result of drying of granules

Run No.	Wave-length μ	Fan	Air temper-ature °C.	Constant drying rate g./hr./sq. cm.	Temper-ature during constant rate period °C.	Critical moisture content per cent. (dry basis)	Time to reach critical moisture content from 25 per cent. minutes	Time to reach equili-brium from 25 per cent. minutes	Temper-ature at equili-brium °C.
1	3·2	Off	—	0·34	80	13·6	10	75	170
2	3·6	On	55	0·26	68	16·25	10	85	105
3	3·6	Off	—	0·18	65	15·8	20	125	105
4	3·6	On	40	0·107	55	17·7	25	140	75
5	3·2	On	15	0·099	55	16·6	40	160	89
6	3·6	On	15	0·091	50	12·4	55	160	72
7	Off	On	55	0·048	35	15·7	60	360	52

Depth of bed, 1 cm.; drying area, 375 sq. cm.; volume of air stream, approximately 50 cu. ft./minute; distance of drying surface from generator, 12 cm.; depth of centre of thermometer below surface, 0·5 cm.

Discussion of Results

Effect of wavelength.—The use of the shorter wavelength (3·2 μ) gave increased drying rates but, as would be expected, correspondingly higher temperatures, of the order of 80° C. during the constant rate period and rising to 170° C. at the end. It was felt that such temperatures were high in view of the thermolabile nature of many pharmaceutical substances and hence the investigation was confined mainly to the use of a peak wavelength of 3·6 μ. This gave satisfactory drying rates, but considerably lower temperatures.

Effect of air stream.—A current of air at 15° C. was found to give decreased drying rates and lower temperatures due to the heat losses caused by the greater air movement. This is in agreement with the work of Stout *et al.*[4] who observed a similar effect when drying sand with infra-red lamps. The heat losses are considerable, so that radiations of 3·2 and 3·6 μ with unheated air give similar drying curves. It is possible that the stream of cold air will slightly alter the peak wavelength of the radiation, since the air stream passes round the generator, the temperature of emission would be lowered slightly and this would in turn increase the peak wavelength and so decrease the heating effect.

A stream of air warmed to 40° C. was found to have a similar effect in decreasing the drying rate. No increase of drying rate was obtained until the air temperature was raised to 55° C., but this was combined with higher temperatures so that the improved drying rate was probably due only to the greater heating effect. As well as giving further heat to the surface receiving radiation, the hot air would raise the temperature of the underside of the drying tray, whereas cold air would have a cooling effect on the latter. In addition, raising the temperature of the air increases the vapour pressure gradient and so enhances the chances of evaporation.

Temperature during the constant rate period.—The temperature quoted in Table I is only an approximate value as it tended to rise during this period. The temperature reached by such drying solids is of great importance for pharmaceutical products and it is proposed to investigate this in detail using temperature measurement methods of greater accuracy.

Critical moisture content.—The critical moisture content appears to be fairly constant for this granule at 16 per cent. The value given for run 1 is low, probably due to the fact that the 5-minute intervals were too long for the greater drying rate and hence the figure is not accurate. The results for runs 4 and 6 also seem to be inconsistent, but both of these runs used a radiation of 3·6 μ and air streams which showed a cooling effect. The drying curves for these runs fluctuated as shown in Figure 1, probably due to this same effect.

Time.—As would be expected the time increases as drying rate decreases. The only result not in agreement with this, is the time to reach the critical moisture content in run 6. The latter value is low and hence a longer time was required to reach that point.

Temperature at equilibrium.—In all runs there was a rise in temperature

at the end of the constant rate period, in some cases quite marked. The rate of drying had decreased and hence the amount of heat used as latent heat of vaporisation was less. More, therefore, becomes apparent as sensible heat and showed a temperature rise. In some cases the rise was great, for example, in run 1, the temperature at equilibrium conditions reached 170° C. and was continuing to rise.

Conclusions

The use of infra-red radiation appears to offer a means of drying tablet granules rapidly. Granulations may be taken from 25 per cent. moisture content to equilibrium conditions (which is some cases represent actual dryness) in times varying from 75 to 160 minutes according to drying conditions. The use of conditions which give rapid drying in the early stages, tends to cause excessive temperature increase in the later stages. It is possible that a satisfactory procedure would be the use of a shorter wavelength (or a longer wavelength with hot air), until the end of the constant rate period when the heating effect could be decreased by increasing the wavelength (or lowering the air temperature). The time required to reach the end of the constant rate period could be estimated from drying curves such as those shown in Figure 1, provided the initial moisture content is known. It appears that an air stream does not assist in speeding the rate of drying, unless the air is heated, when it increases the rate by transferring heat to the underside. Normally, using radiation, only the upper layers are heated and the lower layers rely on conduction from the upper. This could be overcome by using thinner layers and by agitation. The effect of the latter is, at present, being investigated by other workers. A consideration of temperature is of great importance and it is hoped to investigate this aspect, especially with regard to temperature gradients through the bed of solid, since there is risk of overheating the upper layers before the lower layers are dry.

Summary

1. An account is given of work which suggests that the use of infra-red radiation does not harm the majority of substances commonly used in tablets.

2. A modification of a laboratory infra-red dryer, to enable the sample to be weighed *in situ*, is described.

3. The method is described, and the results given, for experiments to investigate the effect on the drying rate of granules of variations of the peak wavelength of radiation. The effect of an air stream, in addition to radiation is considered.

4. It is concluded that it might be possible to use the high heating effect of a short wavelength during the constant rate period, followed by a reduction in the intensity of the radiation to minimise the subsequent temperature rise.

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DISCUSSION

The paper was presented by Mr. H. W. Fowler.

The Chairman observed that many pharmaceutical products were not brought completely to dryness but merely concentrated.

Mr. R. L. Stephens (Brighton) said that the paper brought out the fact that all drying, even in the conventional convection oven, depended upon infra-red radiation, and tests which he had carried out had shown that probably 50 per cent. of the heating at 100° C. in ovens was due to infra-red radiation from the walls and top of the ovens. The heat transfer in a vacuum oven was very largely due to infra-red radiation and to conduction from the plates. The disadvantage of heating with infra-red lamps had been illustrated in the paper by the fact that the granules dried rose in temperature to 170° C. Had the author carried out any work on the use of radio frequency heating in the drying of pharmaceutical preparations? He had tried high frequency heating in which the material to be heated or dried became the dielectric in a condenser, and a radio frequency impulse was imposed upon that condenser. The heating effect depended on the dielectric loss of the material in question. The dielectric constant of a wet material was high and fell off rapidly as the moisture evaporated.

Dr. W. Mitchell (London) gave a brief account of the results obtained in routine control work with an infra-red moisture tester incorporating a continuous wave device. Most air-dried vegetable materials could be dried in 2 to 3 minutes, and fresh plant material of heavy moisture content in up to 20 minutes. Woody materials took a little longer. Extracts could be dried successfully in 20 minutes provided they were spread in thin layers, the resultant texture being similar to that of vacuum-dried material. It was difficult to measure temperature accurately

on a continuous band machine, but even at maximum energy the temperature at completion of drying did not usually exceed 130° C., and it was obvious that for control work a machine such as that had great advantages. It had speed, accuracy comparable with ordinary methods, and no risk of rehydration of the material while allowing it to cool. Any industrial drying method to be satisfactory should be continuous. Therefore he considered that the only way in which to utilise the infra-red drying technique efficiently was to work it on a conveyor belt system.

MR. B. A. BULL (Nottingham) asked the author for some information with regard to the economics of drying by infra-red radiation. In his view the orthodox methods were less costly, although in special circumstances there might be a use in pharmacy for that type of drying.

MR. J. H. OAKLEY (London) stated that for the method to be effective it was necessary to have a thin layer, and that frequently led to difficulties because of the greater area involved. He asked for information on the drying of iron ammonium citrate scales by infra-red heating.

MR. C. L. J. COLES (London) asked for information concerning the difficulties in determining the temperature in the middle of the powder.

MR. H. W. FOWLER, in reply, said that the method was more advantageous in drying that in the concentration of liquids. With the infra-red drier the vapour produced went into the atmosphere. He had used with success a climbing film type of evaporator with infra-red heating instead of the usual steam jacket. Radiation was, of course, a very important point in ordinary ovens; but the trouble with the ordinary oven was that there was conduction, and where there was contact with the hot surface there was risk of local overheating. He had not done any work on radio frequency drying; the apparatus was far too elaborate and expensive. It was very much better to use infra-red driers continuously. He had not gone into the economics of infra-red drying, although there was the point that drying time was so much less that very often smaller plant occupying less floor space could handle the output of the larger plant required for other methods. He had obtained very quick drying of iron ammonium citrate. The question of temperature in the middle of the powders was one of the great difficulties of infra-red work. He hoped to continue the work using a number of thermocouples at various levels and obtaining temperature gradients through the material during drying.

THE WATER RETENTION COEFFICIENT OF SURGICAL DRESSINGS

By R. Maxwell Savage, D. Maxwell Bryce and J. R. Elliott

From the Laboratories of S. Maw Son and Sons, Ltd., New Barnet, and St. Bartholemew's Hospital

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Introduction

One of the most important properties of an absorbent surgical dressing is its ability to absorb water, medicaments or secretions. There are two factors which contribute to the successful function of the dressing for this purpose. One is the speed with which the liquid soaks into the dressing, for if this is too slow, the liquid may fail to penetrate in the comparatively limited time which may be all that is available. This is the property usually called "absorbency" and is measured, for example, by the sinking test of the British Pharmaceutical Codex. It is, without doubt, dependent on the physical chemistry of the surface of the fibres, and has been studied by one of us (Savage[1]). The other property, with which this paper is alone concerned, is often confused with absorbency, but it is in reality quite different, for it determines, not the speed at which the liquid is absorbed, but the maximum quantity which it can absorb. It is important because on its magnitude depends, for example, the time for which a dressing of a certain size absorbing a secretion can be left in position, or the quantity of blood which can be taken up by a swab of a given size, or the quantity of a medicinal liquid which can be applied by a swab which is soaked in it. It is widely known, in a vague way, that sphagnum moss possesses this property in high degree (Martindale[2]), and that cotton wool is better than gauze for this particular purpose. An attempt was made by the British Government to specify the property in sanitary towels for the women's forces during the war, and the Belgian Pharmacopœia 4th Edition, 1st Supplement, 1940, p. 76, includes a test for it in the monograph on cotton wool, but we believe that a detailed examination of a number of dressings under a range of pressures has not been published before, and that, although many of our conclusions confirm informed opinions on this subject, some will be found to be new.

Theoretical

Although a dressing used to absorb secretion is often renewed before it is saturated, it seems likely that even in this case, there is a rough proportionality between the amount absorbed at the time when it is deemed advisable to renew the dressing and the amount required completely to saturate it. In other cases the dressing is actually allowed to become fully saturated before it is discarded. It seems therefore that it is the maximum quantity of liquid absorbed that is of interest. When dressings are wetted, they always collapse to a certain extent. Under conditions of practical use, there is often a further decrease of volume,

because there is some external pressure, such as that of a bandage, or the pressure exerted by packing into a body cavity. No dressing can absorb more liquid than its own volume in this final condition minus the volume of the fibres themselves. The volume of the fibres is usually very small in comparison with the volume of the dressing, so that we can state the principle, at the risk of inviting criticism for making an obvious statement, that the maximum absorbing capacity of a dressing is its own final volume under the working pressure. Unless the working pressure be defined, the volume is indeterminate, and comparisons are almost impossible. This is the weakness of methods of measurement depending on soaking a dressing and then allowing it to drain for a certain length of time. Not only is the end-point difficult to define, but the load is the minimum likely to be experienced in practice (although it does apply to swabs) and the results may be very different when practical loads greater than these are applied. Mention must be made of another method of determining a property which at first sight seems related to that under discussion. The British Cotton Industry Research Association developed a test for another purpose, in which weighed quantities of material were wetted, and then spun in a centrifuge. The quantity of water left on the fibres was measured. At the end of this experiment the water is retained on the fibres as a thin film, the main spaces between the fibres being filled with air. This is not at all what happens in a saturated dressing under pressure, where the liquid fills all, or almost all the spaces between the fibres, and we think that the centrifuge test is usually inapplicable as a measure of the efficiency of a surgical dressing.

It is interesting to consider the forces involved in the absorption and expulsion of the liquid. Liquids enter such materials by capillarity. The laws involved in this are well known, and have been discussed in their application to this particular problem in the paper already cited (Savage[1]). Clearly the expulsion of water may involve, under certain conditions, a mere reversal of this process, and when dressings drain under gravity, suction or a centrifugal force, capillarity may be the dominant factor. But the changes in dimensions of the dressings are not considered on this simple theory.

When water soaks into a dry dressing the latter tends to collapse. The forces involved in this process appear to be: (*a*) at the beginning, the surface tension of the films of water extending from one fibre to another; and (*b*) at the end, the surface tension of the whole exterior water surface, when the completely saturated dressing is considered as a mere support for the volume of water retained by the dressing; and (*c*) the weight of the water, hanging, so to speak, from the upper surface of the dressing. Opposing these forces is the mechanical rigidity of the fibres, and equilibrium is obtained when this balances the sum of the other three.

We have considered whether viscosity plays any important part in the process. At very high pressures there can be little doubt that, since the passage of liquid through narrow spaces is involved, viscosity must enter into the total conditions, but a characteristic of the expulsion of water from dressings under pressure either in practice or in our apparatus,

is that the expulsion is rapid but ceases almost at once when the pressure is held constant. This is not the way a liquid behaves when its flow is mainly determined by its viscosity. It would then be expected that compression would be strongly resisted by any sudden application of force, but if the force were maintained at a steady value, the liquid would continue to flow until the dressing had completely collapsed. We think therefore that viscosity cannot be involved to any large extent. It can now be seen that the elastic properties of the fibres and their arrangement in the dressing are important features, for it is the elasticity of the fibres which resists the complete collapse of the dressing under its load.

It will be shown in a later section that our results can be expressed by a simple mathematical transformation into the form of a rectilinear equation. In spite of what might thus appear to be a manifestation of a simple physical law, we consider that it is merely an empirical relation, and that the whole process is complicated, with the various forces exerting influences which are not in the same proportions at various phases of the process. For example, when pressure is first applied to a dressing the fibres are well separated and it seems reasonable to suppose that they act as an assembly of springs, and so Hooke's law, that strain is proportional to stress, should be followed. As pressure is applied, the fibres pack more tightly, and friction effects enter into the process. It would be expected therefore that the stress required to produce a given strain would increase. Later still, the fibres may come into closer contact, and perhaps lock with one another and a further increase in the force required to compress the dressing is to be expected. Since none of these additional effects begin suddenly at any particular pressure, it would be expected that on plotting stress against strain, a curve would be obtained in which the slope of the portion representing the events at low pressure is that due to Hooke's law and that as pressure increases the slope would decrease gradually. This is just what we find.

It must be noted that the processes are not reversible—a wetted dressing, particularly of cotton wool and similar materials, does not regain its original volume after the water has been expelled by pressure and the pressure has been removed, or even when it is dried. It can easily be understood that the expansion of a wet dressing in air would involve the extension of films of water between one fibre and another. The energy needed for this expansion is no doubt one cause of the failure of the wet dressing to expand, but the failure of the dressing to expand when it is dried must presumably be due to an irreversible rearrangement of fibre orientation—the fibres may perhaps coil round each other in a new way when wetted, and not be able to uncoil when dried, until mechanical work, such as teasing or carding, is done on the material.

We have not carried our theoretical analysis further than this brief account, but we feel that the interpretation of the practical experiments described below may be helped by providing a simple physical picture, however incomplete, of the events which probably occur when a dressing is in use. We have replaced the term "absorbing capacity" by the term "water retention coefficient" in order to avoid confusion with

"absorbency." We define water retention coefficient (W.R.C.) as the number of g. of water absorbed per g. of dressing.

Before the laboratory work on this subject commenced it seemed essential that the pressures likely to be encountered in practice should be investigated, and J. R. Elliott undertook to carry out tests. In Part I of this paper the laboratory experiments are described, and are mainly by two of us (R.M.S. and D.M.B.), although certain results have been taken into this part from Mr. Elliott's work. Part II of the paper describes the methods and results of the experiments on the actual pressure found in bandaging. The two parts are therefore complementary, and it was very convenient to present these as a single paper.

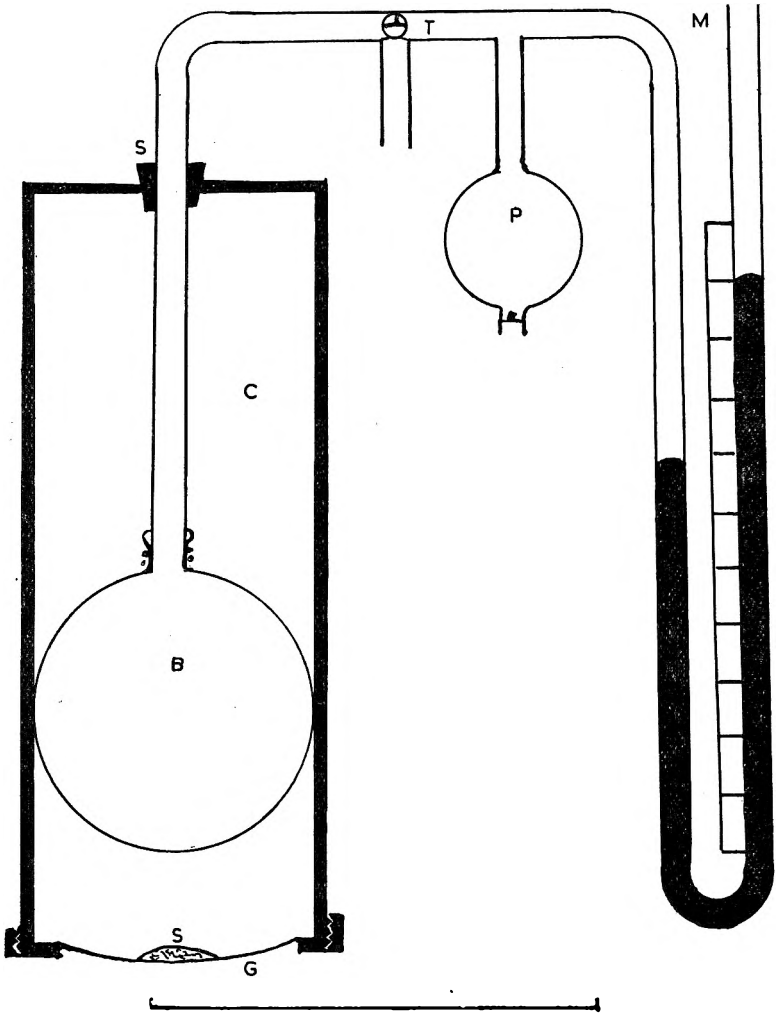

FIG. 1

Part I Laboratory Experiments

Experimental.—The nature of the apparatus used will be evident from Figure 1. Essentially, uniform pressure is applied to a sample of the dressing S supported on bandage cloth G, by inflation of a rubber balloon B enclosed in an aluminium cylinder C. The pressure in the balloon is measured by a mercury manometer M. Determinations were made as follows. The weighed sample was placed in position on its support and both sample and bandage cloth were thoroughly saturated with water. It was then placed in position at the base of the cylinder and the balloon was inflated with a hand pump P to the required pressure.

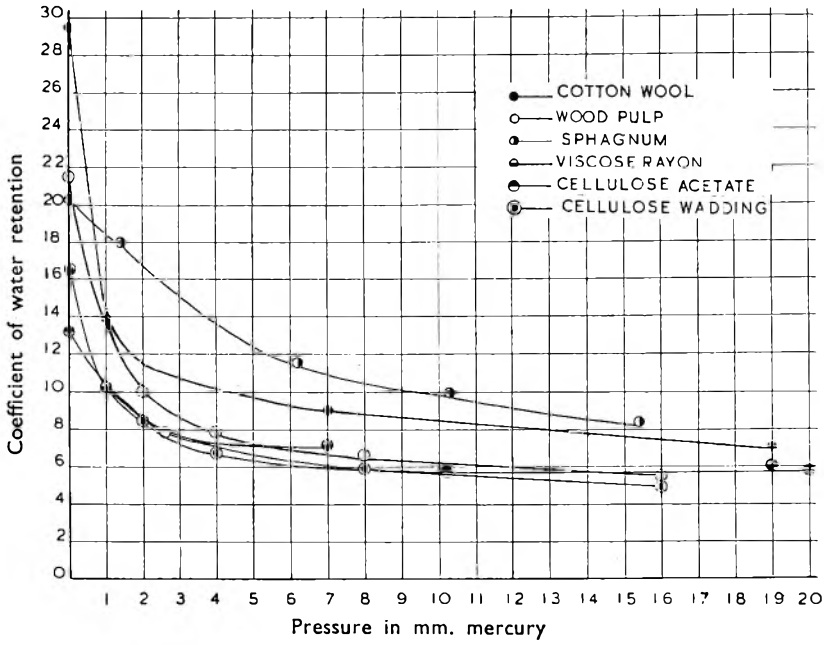

Fig. 2. Non-woven dressings.

Water ran out of the dressing until the maximum pressure was reached and fractional drops hanging beneath the support were adroitly removed with filter paper. The air pressure was then released and the sample removed and weighed. Experiments were made to determine the pressure required to inflate the balloon to the point where good contact was made between balloon and the supporting gauze. This pressure was considered the zero value, and the actual manometer readings were corrected by subtracting this value. This procedure would not be correct if the volume changes in the balloon were large after the zero value had been reached, but the pieces of dressing used were quite small (about 0·25 g.) and we consider that the much larger balloon was so little altered in size during the experiments that this procedure was justifiable.

The apparatus gave sharp readings—there was no delayed drip and the results were reproducible. It also appears to be reasonably close

imitation of practical conditions for dressings are often compressed under bandages against yielding tissues. We considered that methods depending on the use of rigid compression devices were undesirable, for in such cases, the load might be carried largely by an accidentally dense part of the dressing and might then give rise to an abnormally high water retention coefficient. The results are shown in Tables I, II and III and in Figures 2, 3 and 4.

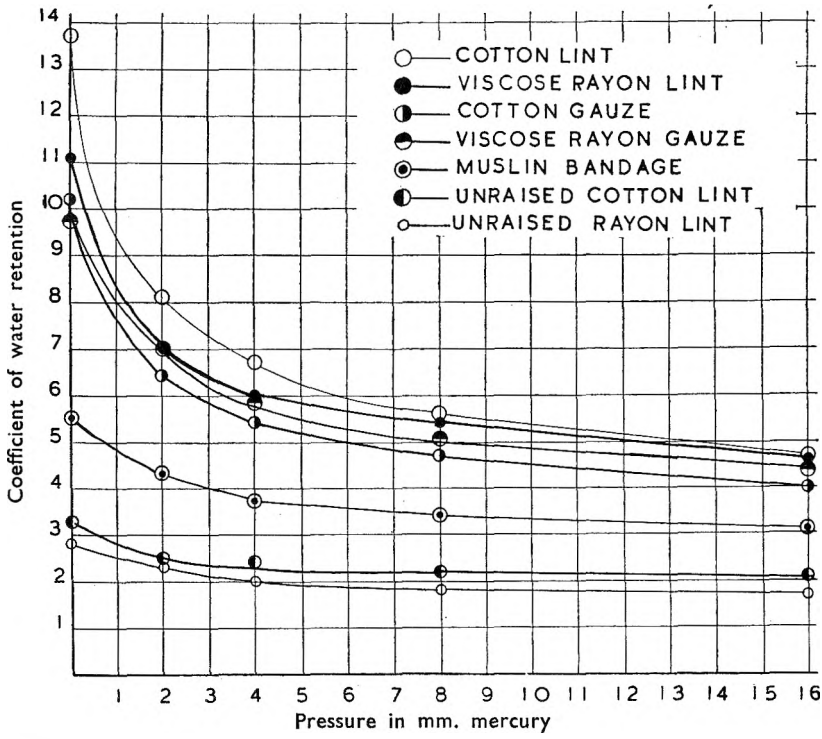

FIG. 3. Woven dressings.

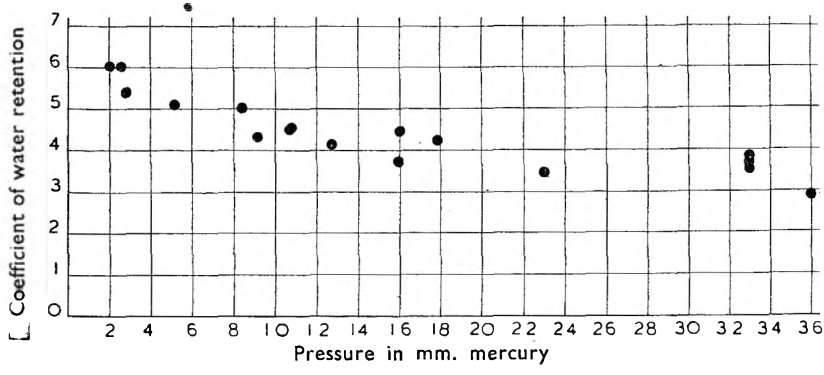

FIG. 4. Lint, scatter diagram.

TABLE I

PRESSURE (CM. HG)

Material	0	1	2	4	7	8	16	19	Regression‡
Gauze, 19 × 10*	9·7		6·3	5·4		4·6	4·0		0·87–0·22x
„ B.P.C.*	10·2		6·4	5·4		4·7	4·0		0·87–0·23x
„ 12 × 8*	10·8		7·4	6·1		5·3	4·5		0·94–0·24x
„ viscose rayon 19 × 15*	9·9		7·0	5·9		5·1	4·4		0·92–0·23x
Muslin Bandage B.P.C.†	5·5		4·3	3·7		3·4	3·1		0·67–0·16x
Lint, B.P.C.	13·7		8·1	6·7		5·6	4·7		0·99–0·27x
„ viscose rayon	11·1		7·0	6·0		5·4	4·6		0·90–0·20x
„ cotton, unraised	3·3		2·5	2·4		2·2	2·1		0·42–0·08x
„ viscose rayon, unraised	2·8		2·3	2·0		1·8	1·7		0·39–0·13x
Cotton wool, hospital	33·0		12·5	9·9		8·5	7·4		1·15–0·24x
„ „ B.P.C. *a*	33·0		12·4	9·8		8·3	7·2		1·16–0·25x
„ „ B.P.C. *b*	29·5	13·9			9·0			7·0	1·14–0·23x
Wool, viscose rayon	16·5	10·6			7·7			6·2	1·02–0·18x
„ towel	24·0		10·9	8·5		7·3	6·3		1·11–0·26x
Wadding, cellulose	16·5		8·4	6·7		5·8	4·9		1·00–0·26x
Wood-pulp, sulphite	21·7		10·0	7·8		6·6	5·5		1·08–0·28x

* 16 Layers. † 8 Layers.

‡ Log W.R.C. = a – bx where a and b are the constants shown in this column, and x is log pressure in cm. of Hg.

In all cases the points represent the means of at least 4 independent readings. In Figure 3 the range of individual readings in the course of a single experiment is shown for lint B.P.C.

The experiments in Table II, however, carried out by J. R. Elliott, were performed in a rigid apparatus, and produced similar results. It is possible that the depth of cotton used in his experiments was sufficient to produce an averaging effect, so that the effective pressure was more uniform than would show in a thin layer. His method is as follows: 2 g. of the cotton wool was packed into a cylindrical tube with a perforated base plate of about 2·5 cm. diameter, so that it occupied a volume of about 15 ml. It was thoroughly saturated with water and then subjected to a given load, applied from above by means of brass weights. After 5 minutes, no further water was being squeezed out from the sample, and it was then carefully removed from the tube and weighed. Further samples were subjected to different loads, and the whole series repeated using a rayon wool (see Table II).

Discussion.—The wide difference between different kinds of dressings is at once evident. Cotton and rayon wool are, on this basis in which equal weights of dressing are compared, much better than the woven

TABLE II

SHOWING THE QUANTITY OF WATER RETAINED BY 2 G. OF COTTON WOOL B.P.C. AND RAYON WOOL UNDER DIFFERENT PRESSURES

Load applied in g. per sq. cm.	Water retained by 2 g. of sample		Approx. ratio of water retained cotton/rayon
	B.P.C. cotton wool	100 per cent. rayon wool	
15·8	27·9, 26·5	22·4, 22·9	1·20
18·8	Not taken	21·1, 22·0	—
24·0	24·2, 24·5, 24·0	20·4, 20·1	1·19
31·6	22·8, 22·5, 22·8	19·1, 19·3, 20·2, 19·3	1·15
39·2	21·4	18·3	1·17
46·8	20·3, 20·5, 21·8	17·3, 17·0, 17·5	1·20
64·0	18·0	15·0	1·20

form of surgical dressing, although sphagnum is still better (Table III). Then follow paper pulp and cellulose wadding.

Lint comes next, followed by the open gauzes, B.P.C. and hospital qualities, that are commonly used in Britain. The finer gauzes, in use in most other countries are not so good.

This is an interesting point because since these open gauzes are almost always used in portions several layers thick, it could easily have been supposed that fewer layers of a closer cloth would be as good or even better. The British practice, however, is now seen to be the best.

TABLE III

SPHAGNUM (CAPITULA)

Pressure (cm. Hg)	0	1·4	6·2	10·3	15·4	25·5	36·0
Water retention coefficient ..	22	18·0	11·5	9·9	8·3	7·1	6·4

In cotton wool we have not found that quality makes any difference to the water retention coefficient. B.P.C. cotton wool behaves in the same way as a sample so poor in other respects that it could not have been used as a dressing. It is different with lint. Poorly raised material has a lower water retention coefficient, and unraised is much inferior, although the cloth is otherwise identical. Lint is another dressing almost peculiar to British countries, and it is interesting to find that no other woven fabric equals it in water retention coefficient. We have considered the reason for these results, and suggest that a high water retention coefficient is associated with disorder in the arrangement of the fibres. In cotton wool the fibres are not parallel and are curled. In rayon wool the fibres are straighter, and the degree of order is higher. Woven fabrics have the fibres much more parallel. In lint, disorder is deliberately introduced by the raising process and with the increase in disorder goes an increase in water retention coefficient, for unraised lint is considerably inferior to raised lint, poorly raised lint is intermediate. Paper pulp, the disintegrated and disorientated raw material of cellulose wadding, has a higher water retention coefficient than cellulose wadding, in which the fibres are laid layer on layer in a more orderly manner. A multiple layer of coarsely woven gauze is a more disorderly arrangement than a single layer of a closely woven fabric, and the water retention coefficient is higher in the multiple layer dressing.

So far, we have considered the results calculated on a weight/weight basis. This is the only way in which precise figures can be stated, for the volume of a dressing is an indefinite quantity, and depends on the load upon it and upon its previous history. But some consideration must be given to the efficiency of dressings judged by the capacity of a given *volume* of dressing to absorb liquid for there are important uses where the quantity of dressing which can be used is limited by the volume. For example, a cavity or space in or on the body may limit the amount of dressing which can be applied, or a soldier's pocket or a first-aid outfit may be of limited size, and we must know what the most efficient

kind of dressing would be under these conditions. Clearly the required figure can be obtained by multiplying the water retent on coefficient by the apparent density of the dressing, and in Table IV we show some results. The difficulty in presenting these results has been to choose a figure for the apparent density. We have taken that which is found in commercial packages. We think that this is as good a guide as any, but it must be realised that to get a true value for any particular case, the actual apparent density should be determined under working conditions, and used in the calculation.

TABLE IV

	Water retention coefficient at 2 cm. Hg.	Apparent density g./ml.	Volumetric water retention coefficient
Cotton wool	12·5	0·16	2·0
B.P.C. cotton gauze	6·4	0·26	1·7
Cotton lint	8·1	0·31	2·5

Table IV shows that in these representative kinds of dressing when volumes are considered, gauze gains considerably in its efficiency when compared with cotton wool, but is still not so efficient. Lint, however, from being second to cotton wool when compared on a weight/weight basis takes first place on a volumetric basis.

Surface-active agents.—Some experiments were made in which a commercial detergent was added to the water. The results (Table V) show that the water retention coefficient is diminished; this is to be expected for there can be no doubt that the surface tension of the liquid is the factor retaining it in the dressing. The particular interest, however, lies in the clear demonstration of the independence of the water retention coefficient and absorbency, for measures which would increase the rate of wetting decrease the quantity retained by a fully saturated dressing.

TABLE V

EFFECT OF SURFACE-ACTIVE AGENT ON WATER RETENTION COEFFICIENT OF VISCOSE RAYON WOOL

Pressure (cm. Hg)	2·6	10·2	25·5
Water retention coefficient	9·7	6·7	5·3
Water retention coefficient in presence of surface-active agent	8·0	5·4	4·1

The absorption of blood.—The use of a complex fluid such as blood containing suspended solids introduces complications, and although the relation between the water retention coefficient and the corresponding values obtained by using blood or pathological fluids would be of much interest, the matter was felt to be too large for inclusion in a study which is not exhaustive. One series of experiments was made, using sulphated blood and showed that blood is retained by a dressing in larger quantities than is water under similar pressures.

Mathematical considerations.—By plotting the logarithm of the pressure against the logarithm of the water retention co-efficient, it was found that the points fell approximately on straight lines. Regression equations were calculated (Table I) for the materials. Tests for rectilinearity were applied and in many cases it was found that there was no significant departure from the expected values, but in other cases there were signs that the points followed a slightly sigmoid curve. It is unlikely that the equations indicate any fundamental mathematical law in our results, but there are two advantages in this method of expression—it is sufficient to determine the water retention coefficient at only two pressures in order to characterise the dressing completely either graphically by drawing in the line between the points or numerically by the two constants of the equation, whose physical meanings are (*a*) the quantity of water retained under unit load and (*b*) the rate at which this quantity diminishes as the load is increased. These constants could be incorporated in any description of a dressing, or used in a specification.

PART II

The pressure exerted by bandages on absorbent dressings.—A limited series of experiments was undertaken in the first instance to investigate the variation in pressure obtained when one worker bandaged a forearm, on a number of occasions, using a fast-edge, open wove, bandage, 2 inches wide, under as nearly the same conditions as possible to produce a comfortable dressing.

The apparatus used consisted of a water manometer, graduated at 1-cm. intervals, to one limb of which was attached a length of pressure tubing, and at the end of this tubing a soft rubber bag was attached by means of rubber solution. The end of the tube with the rubber bag was then loosely tied along the limb to the bandages and the bandage applied in such a way that it commenced about 2 inches below and finished about 2 inches beyond the bag.

TABLE VI

PRESSURES EXERTED ON A BARE FOREARM, BY A WHITE OPEN WOVE (FAST EDGE) 2 INCH BANDAGE, THE ARM BEING RESTED AND RELAXED

Number of bandage	1	2	3	4	5	6	7	8	9	10	11	12	Average
Pressure when applied ..	39½	37½	33½	28	37	29½	35½	25½	33	32	26½	23	31½ g./sq.cm.
Pressure after 10 minutes	34	33	30	24	33	24	32	22	28½	25	24	20½	27½ ,,
Fall in pressure	5½	4½	3½	4	4	5½	3½	3½	4½	7	2½	2½	4 ,,

It was found that during the first few minutes after application of the bandage the pressure dropped to a noticeable extent (Table VI) presumably due to the fabric settling down around the limb, and so, in subsequent experiments, pressures were not compared until the bandage had been in place for 10 minutes.

In order to obtain as much information as possible, after the bandage was applied the subject was instructed to hold his arm in 4 different positions, namely: (1) seated, with arm loosely supported at the wrist

by a second person; (2) standing, with arm held loosely by the side; (3) standing, with arm held horizontal as in a sling; (4) arm extended and fingers widely stretched apart. 4 sets of readings were taken in each position and averaged for each bandage (Table VII).

TABLE VII

PRESSURES EXERTED ON BARE FOREARM, BY A 2 INCH WHITE OPEN WOVE (FAST EDGE) BANDAGE, AFTER BEING ALLOWED TO SETTLE FOR TEN MINUTES

Each figure represents an average of four readings
All readings are cm. of water

No. of bandage	1	2	3	4	5	6	7	8	9	10	11	12	Average
Seated, arm rested ..	34	32½	29	24½	32	24	31¼	21½	28¼	25½	21½	20	27 g./sq. cm.
Standing, arm by side ..	39¼	35	25	25½	36½	27	36½	26½	31¾	29½	20¾	19½	30 ,,
Standing, arm as in sling	33½	28	28½	22	30½	25	31¼	23¾	29	27½	19¾	19¾	26½ ,,
Arm extended, fingers apart	53	47½	46	49½	57	49½	62¼	49¼	59¼	54¾	42	42½	51 ,,

The above experiment was then repeated using a pad of absorbent cotton wool, weighing ½ ounce, beneath the bandage, and once again a series of readings was taken after allowing the dressing to settle down (Table VIII). From this it is seen that the arm was not so tightly compressed as it was when no wool was used.

TABLE VIII

PRESSURES EXERTED ON FOREARM COVERED WITH ½ OUNCE OF COTTON WOOL, AND BANDAGED WITH A 2 INCH WHITE OPEN WOVE (FAST EDGE) BANDAGE, AFTER BEING ALLOWED TO SETTLE FOR 10 MINUTES

Bandages 1 to 6 were applied over hospital quality cotton wool, and bandages 7 to 12 over B.P.C. quality cotton wool
Each figure represents an average of 4 readings.
All readings are in cm. of water

Number of bandage	1	2	3	4	5	6	7	8	9	10	11	12	Average
Seated, arm rested ..	15¾	14¼	15	12	16¼	17	19¾	15¼	17	18¼	10¾	17¼	15¾ g./sq. cm.
Standing, arm by side ..	15½	16¼	17¼	12¼	19¼	19	22¼	17¾	21¼	22¼	17¼	22½	18¾ ,,
Standing, arm as in sling	16	13¾	16¼	11¾	19¾	17¼	20	14	17	16½	12	16	15¾ ,,
Arm extended, fingers apart	23	34¼	27¾	29¾	27	39	42½	26	37½	35½	27	32	31¼ ,,

A further set of experiments was then carried out using a 3-inch crêpe bandage B.P.C. to retain the wool in position, because such a dressing has been recommended to dress burns, on the grounds that the bandage will not stretch or slip (Table IX).

TABLE IX

PRESSURES EXERTED ON FOREARM COVERED WITH ½ OUNCE OF COTTON WOOL, B.P.C. AND BANDAGED WITH A 3 INCH CRÊPE BANDAGE B.P.C. AFTER BEING ALLOWED TO SETTLE FOR 10 MINUTES

Each figure represents an average of 4 readings
All readings are in cm. of water

Number of bandage	1	2	3	4	5	6	7	8	9	10	11	12	Average
Seated, arm rested ..	19¾	21¼	20¼	22¼	27½	26½	26	23¼	37¾	23¼	28¾	25¼	25½ g./sq. cm.
Standing, arm by side ..	20½	23¾	22½	25	29¼	26½	30¼	25½	45	32¼	33	31¼	28¾ ,,
Standing, arm as in sling	19	21½	20	20½	24¼	22½	24¾	21¼	36	26¼	26½	24¾	24 ,,
Arm extended, fingers apart	27¼	37½	29½	33½	38¼	34½	39½	35¾	53½	38¼	37½	39	37 ,,

Tables X, XI and XII show examples of the actual readings from which the figures shown in Tables VII, VIII and IX were respectively obtained. Readings could only be made to the nearest 0·5 cm. on each limb of the manometer as the colnmn of liquid never settled to complete rest.

The lowest pressure recorded on the manometer was taken as the reading for each of the first 3 positions, and the highest recorded for the fourth, as it was felt that this probably represented the maximum pressure which would be exerted beneath the bandage under normal circumstances.

Table XIII shows the fall in pressure in cm. of water from time of applying to time when "arm-resting" pressure was recorded in Tables VII, VIII and IX.

TABLE X

Bandage number	1				5				12			
Arm rested ..	34	34	34	33½	33	32	32½	30½	20½	20½	19½	19½
Standing	40	38½	40	40	36	37½	36½	36½	20½	18½	19½	19½
Arm "slung" ..	34	33	34	33½	30½	30½	30½	30½	20	19½	19½	18½
Arm extended ..	53½	53	53½	53	59	57½	58	54½	43	43½	43	41

TABLE XI

Bandage number	1				7				11			
Arm rested ..	16	16	15	16	19½	20½	19½	19½	12	10	11	10
Standing	16	16	15½	15	24½	22	22	21½	18	18	17½	16
Arm "slung" ..	16	16	16	16	21	20	20	19	12	12	12	12
Arm extended ..	23	23	23	23	42	44	43	41	27	27	27	27

TABLE XII

Bandage number	1				6				9			
Arm rested ..	20	20	20	19	27½	27	26	26	38½	38½	37	37
Standing	20	20	21	21	26½	26	27	27	45	45	45	45½
Arm "slung" ..	19½	19	19	19	23	22½	22½	22	35½	36	36½	36
Arm extended ..	27	27½	27½	27	34	34	34	36	54	54	53	53

TABLE XIII

Fall in pressure in cm. of water, from time of applying, to time when "arm resting" pressure was recorded in Tables VII, VIII and IX

Bandage number	1	2	3	4	5	6	7	8	9	10	11	12	Average fall
Table VII	5½	4½	2½	4	4	5½	3½	3½	4½	7	2½	2½	4
Table VIII	5	4½	4½	4	3½	3	3½	2	2	3	2½	4½	3½
Table IX	3	3	4½	3	7½	1	4½	3	3½	5½	5	2½	3½

Additional information shown in Table XIV was obtained by once bandaging a 40-ounce bottle with a 2 inch white open wove bandage over a pad of cotton wool.

TABLE XIV

Time in minutes from application of the bandage	0	10	30	65	85	120	660
Pressure in cm. of water	66½	65	64	63	63	62½	57½

This gives an indication of the way in which a bandage "settles down" over a period of time, when there is no movement of muscles to assist its loosening.

Having determined the variations of pressure experienced when one individual bandaged a limb on a number of occasions, a series of volunteers then applied a similar set of bandages in order to find the range of pressures which might be met with in routine work.

The volunteers included sister tutors, trained nurses and other persons experienced in bandaging. In this series only the minimum pressure recorded as soon as the bandage was completed and when the arm was maintained in a slung position was determined (Table XV).

TABLE XV

VARIATION IN PRESSURES OF BANDAGING AS SHOWN BY DIFFERENT WORKERS. PRESSURE RECORDED AS SOON AS BANDAGE WAS APPLIED, AND TAKEN WITH THE "ARM IN A SLING" POSITION

Worker number	1	2	3	4	5	6	7
White open wove 2 inch bandage over bare forearm	24	26 31	30 20	22 18	24 30	31 35	34 30
White open wove 2 inch bandage over cotton wool	28	19	17	—	23 20	19 21	20 24
Crêpe 3 inch bandage over cotton wool	38 40	60 67	28 28	—	35 29	31 36	44 35

DISCUSSION

From these preliminary experiments it is seen that one worker, using a white open wove bandage over a bare arm held as if in a sling was able to produce minimum pressures falling between 19 and 33·5 g./sq.cm. after the bandage had been in place for 10 minutes. When the arm was extended with the muscles tensed the pressure beneath the bandage was approximately doubled. When a pad of cotton wool was introduced between the skin and bandage the minimum pressures ranged from 12 to 20 g./sq.cm., and again when the muscles were tensed the pressure was approximately doubled. However, when a crêpe bandage was used to retain the wool in position, the average minimum pressure reached 24 g./sq. cm. and was thus of the same order as for a white open wove bandage over the bare arm, but on exerting the muscles as on previous occasions, the maximum pressure only rose by about 50 per cent. due to the elasticity of the bandage. It appears therefore that cotton wool beneath a crêpe bandage may be subjected to a greater pressure than when beneath an open wove bandage when the arm is supported, but that the difference is much less marked when the patient uses his muscles.

When different workers applied open wove bandages, with or without cotton wool in the manner described above, it was found that the variation in pressures between one worker and another fell approximately within the range of the original experiments, but that when a crêpe bandage was used a greater variation was found. This may have been due to a certain difference of opinion between the workers as to the correct tension to use when applying this type of bandage.

It would be of interest to pursue this investigation further in order to determine the differences in pressure which result from the use of different bandaging materials, and different dressings, when used on various parts of the body. Stanton, Wilkins and their colleagues[4,5] have recently reported on the pressures required beneath elastic stockings, and suggest that when a knee-length stocking is used a pressure of from 10 to 15 mm. of mercury (i.e., about 13 to 20 cm. of water) will accelerate deep venous blood-flow in the limb and that the effect is greatest in people with dilated deep veins. Such work is not, however, comparable with this present investigation, which was undertaken with the main purpose of determining the pressure exerted by a bandage over a dressing used to absorb exudate from a burn or wound.

Summary

1. Absorbency and absorbing capacity (or water retention coefficient) are two entirely distinct properties of surgical dressings.

2. The water retention coefficient is greatly dependent on the actual working pressure on the dressing. This must be defined before any figure can be considered valid.

3. The water retention coefficient appears to vary with the degree of disorganisation of fibre arrangement in the dressing. The most regular structures, such as finely woven gauze, have the lowest water retention coefficient and the most irregular, such as cotton wool, the highest.

4. The order of efficiency of a number of dressings, when judged by the weights of liquid retained by a given weight of dressings is not necessarily the same as when the order is decided by measuring the weights of liquid absorbed by given volumes of dressings.

5. Except in the case of lint, the water retention coefficient is not apparently affected by the quality of a dressing. It is primarily a character of a particular kind of dressing.

6. The relation between working pressure and water retention coefficient is curvilinear. By taking logarithms of both variables, fair approximamations to straight lines result, and the whole behaviour of a dressing in respect of water retention coefficient can be expressed by two constants.

7. The pressures under bandages have been measured, using white open wove with and without a cotton wool pad, and a crêpe bandage. It was found that a cotton wool pad reduces the pressure on the dressing, and that the pressure under a crêpe bandage falls within a smaller range of values than under a white open wove bandage when the muscles are flexed and tensed.

References

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2. *Extra Pharmacopœia*, 22nd Edition, Vol. 1, Pharmaceutical Press, London, 1941, p. 564.
3. Stanton, Fries and Wilkins, *J. Clin. Invest.*, 1949, **28**, 553.
4. Wilkins, Mixter, Stanton and Littler, *New England J. Med.*, 1952, **246**, 360.

DISCUSSION

The paper was presented by DR. R. M. SAVAGE.

MR. W. R. THOMPSON (London) asked for information regarding the effects of sterilisation on the water retention coefficient.

DR. G. E. FOSTER (Dartford) asked concerning the difference in water retention coefficient of a dressing before and after compression.

DR. K. R. CAPPER (London) referred to the authors' statement that, in the case of lint, the "well raised" was an important factor in water retention, and said that "well raised" might be interpreted in various ways. It appeared that the water retention test would control the way in which the lint nap had been raised.

MR. T. D. WHITTET (London) said he was not sure how the water retention coefficient could be correlated with clinical efficiency. For example, as between rayon and cotton dressings the cotton seemed to have a better water retention coefficient, yet in quite an extensive series of tests on rayon lint the difference clinically was undetectable, and in one or two cases the rayon was considered to be preferable to cotton.

DR. K. R. CAPPER (London) said that there had been adverse comment from hospitals that rayon lint did not appear to take up exudate to the same extent as cotton.

MR. T. D. WHITTET asked Dr. Capper whether his comment referred both to glossy and matt rayon lint.

DR. K. R. CAPPER relied that he was unable to say which kind of lint was supplied to the hospitals.

MR. A. MARSH (Brighton) asked whether there was any difference between water and body fluids from the point of view of absorption.

DR. R. M. SAVAGE, in reply, said that he could give no information as to whether the water retention coefficient increased or decreased on sterilisation. A compressed cotton dressing increased in size when moistened without external pressure being applied. The important difference was that between volumetric and gravimetric water retention coefficient. For lint, the water retention coefficient did seem to provide an opportunity for giving a quantitative measure to the qualitative statement "well raised." The difference between rayon and cotton dressings was not very great. A few tests had been carried out on sulphated blood, but the field of biological fluids was so wide that it could not be incorporated in the paper.

THE USE OF SURFACE ACTIVE AGENTS IN PHARMACEUTICAL PREPARATIONS:

THE EVALUATION OF EMULSIFYING POWER

BY J. R. COCKTON and J. B. WYNN

From Imperial Chemical Industries Limited, Pharmaceutical Research and Service Laboratories, Hexagon House, Blackley, Manchester, 9

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DURING the past twenty years economic and other circumstances have led to an increasing use of synthetic surface active agents in pharmaceutical products. At present their use is primarily as alternatives to excipients of natural origin, such as gums and waxes, but there are indications that certain of their properties may be used advantageously to modify drug action by increasing the solubility of sparingly soluble substances, aiding skin penetration and so on. It has also been shown that some surface active agents have bactericidal power and hence may be used as independent therapeutic agents. Several factors have tended to limit the use of these synthetic materials to specialised pharmaceutical manufacture and to delay their introduction into normal dispensing practice. In the first place, relatively few surface active agents have been made specifically for use in pharmacy, with the result that no accepted quality standards exist for many otherwise satisfactory products. Secondly, much remains to be done in investigating the pharmacology of the new materials to ascertain their particular suitability to the various routes of administration. Thirdly, the efficiency of surface active agents in various formulations has not yet been fully established, chiefly due to the absence of satisfactory methods of evaluation; hence, their advantages and disadvantages relative to one another and to corresponding natural products are not clearly understood. This latter situation is particularly true of emulsifying power and as an initial attack on the general problem of assessing the pharmaceutical potentialities of surface active agents it was decided, in the work described here, to seek a method of measuring the efficiency of these substances as emulgents. Since it was already known that their emulsifying power was approximately of the same order as that of soaps, it seemed clear that the method of evaluation should be capable of detecting relatively small differences.

The term "efficiency," when applied to emulgents, may be interpreted in various ways. One of the most useful of these links the "efficiency" of an emulgent with the stability of the emulsions which it will produce under standard conditions. For this reason, the method selected for the present work was based essentially on:—(i) preparation of standard oil/water emulsions; (ii) homogenisation; (iii) globule counts at time intervals during storage.

Standard Emulsions. It is recognised that the relative efficiencies of two emulgents may depend on the concentration and chemical nature of the disperse phase. Two oils of different type commonly encountered

in pharmaceutical practice were selected and emulsions were prepared at an arbitrary concentration of 15 per cent.

Homogenisation. As part of the standardisation of an emulsifying process, it is necessary to fix the amount of mechanical work. This is best done by controlled homogenisation which can be set at a fixed quantity, in comparison with which the amount of work done in preliminary mixing becomes negligibly small. It might be argued that this simplifies unduly the rôle of the emulgent but it should be borne in mind that homogenisation is now common manufacturing practice.

Globule Count. It is generally accepted that the most accurate representation of the state of an emulsion at any given time is provided by a size frequency analysis, i.e., an examination of globule size based on the counting and measuring of a large number of globules. Moreover, it has been shown by King *et al.*[1] that when interfacial areas (calculated from size frequency analysis) are plotted against storage life a linear relationship is obtained. Similarly, Jellinek and Anson[2] have derived various other functions from size frequency analyses and have shown that certain of these are also linearly related with time. Size frequency analyses, however, are inherently tedious, some methods necessitating the counting and measuring of approximately 2000 globules in order to obtain statistically reliable results. A simpler method proposed by Smith and Grinling,[3] requiring a direct count of a substantially smaller number of globules and eliminating the necessity for measurement, appeared to provide a suitably accurate alternative. In preliminary tests, the method, with slight modifications in technique, was found to give reproducible results, and was therefore chosen for the present purpose.

Storage. The inherent stability of homogenised emulsions prepared with surface active agents is such that significant changes can only be detected after long storage. It was therefore necessary to apply a standard artificial breakdown stress, and centrifugal force was chosen for this purpose, although it is not suggested that any simple relationship exists between the behaviour of an emulsion in a centrifuge and under normal storage conditions. It was found that spinning at 20,000 r.p.m. in a Sharples Supercentrifuge for periods of 5, 10, 15, 20 and 25 minutes produced a suitable degree of progressive deterioration.

In a typical experiment, therefore, a standard emulsion was prepared and a preliminary dilution made in order to ascertain the final dilution necessary for a satisfactory globule count. This count was then made on separate dilutions, after which the original emulsion was centrifuged for a series of time intervals and further counts made after each centrifuging. By suitable mathematical treatment, the results were interpreted so as to provide an expression of the stability of the emulsion.

EXPERIMENTAL

(1) *Materials*

(*a*) *Surface Active Agents.* The surface active agents were selected so as to provide examples of different chemical types (anionic, cationic

and non-ionic). The following were used, each at concentrations of 0·1, 0·5 and 1 per cent. w/v.

(1) Cetyltrimethylammonium bromide (Cetrimide B.P.).
(2) Polyoxyethylene sorbitan mono-oleate (Polysorbate 80 U.S.P.).
(3) Stearyl alcohol/ethylene oxide condensate.
(4) Alkyl phenol/ethylene oxide condensate ("Lissapol" NX).
(5) Cetyl alcohol/ethylene oxide condensate ("Lubrol" W).
(6) Sodium oleate.

The selection was made chiefly from non-ionic substances (2), (3), (4), (5) since these appear to offer the most promise as excipients, due to absence of chemical incompatibility. Sodium oleate was selected partly as a convenient anionic agent and partly as a control since the satisfactory properties of emulsions prepared with it are already well known in pharmacy. Lubrol W and lissapol NX are not commercially available as pharmaceutical excipients but were, nevertheless, included since they provide useful examples of certain types of ethylene oxide condensates.

(*b*) *Disperse Phase.* Separate emulsions were made of arachis oil B.P. and of liquid paraffin B.P., using the same samples throughout. The concentration of the disperse phase was arbitrarily fixed at 15 per cent. v/v for all experiments.

(2) *Preparation of Emulsions* (*Quantities of* 600 *ml.*)

(*a*) *Equipment.*

(i) *Moritz Turbo-Emulsifier:* This comprises a rotating impeller surrounded by an emulsifying crown. The latter has a large number of pins which finely divide the liquid veins centrifugally thrown by the impeller. The speed of rotation is controlled by means of a variable resistance.

(ii) *Weir Junior Homogeniser:* This consists of a motor driven, single cylinder pump designed to operate at pressures up to 3500 lb./sq. in. The pump forces the premixed emulsion through a fine orifice in the discharge valve. To permit recirculation of the emulsion through the homogeniser the outlet was connected to the inlet *via* a reservoir of approximately 1 l. capacity.

(*b*) *Method:* In all cases except stearyl alcohol/ethylene oxide condensate and sodium oleate, the required weight of surface active agent was dissolved in the calculated volume of water and added to the oil. In the case of stearyl alcohol/ethylene oxide condensate, the required weight was dissolved in the oil with the aid of gentle heat and added to the calculated volume of water heated to the same temperature. The sodium oleate was made *in situ* by dissolving the calculated quantities of sodium hydroxide and oleic acid in the water and oil respectively. The liquids were premixed by the turbo-emulsifier at approximately 3000 r.p.m. for 5 minutes, and the emulsion so formed was then re-circulated through the homogeniser at maximum pressure for 5 minutes. All emulsions were introduced into the homogeniser at 20° to 23° C.,

the rise in temperature during homogenisation being approximately 5° C. for all emulsions.

(3) *Accelerated Breakdown of Emulsions*

(*a*) *Equipment. Sharples Centrifuge:* This consists essentially of an accurately balanced hollow rotor or "bowl" capable of being rotated about a vertical axis at speeds up to 28,000 r.p.m. The centrifuge was used in conjunction with a sensitive tachometer driven directly from the spindle, and a Variac resistance.

(*b*) *Method:* A 100-ml. sample was placed in the centrifuge and the speed adjusted by means of the Variac resistance. The time of centrifuging was taken from the moment of attaining a speed of 20,000 r.p.m. until the centrifuge was switched off. The contents of the bowl were thoroughly mixed before a sample was withdrawn.

(4) *Examination of Emulsions*

(*a*) *Equipment. Helber Counting Chamber:* This consists of a microscope slide having a central portion sunk 0·02 mm. below the surface of the slide and ruled into 16 blocks of 16 small squares, each of area 0·0025 sq. mm. The sunken portion is surrounded by an annular well into which the superfluous liquid can overflow. A Helber counting chamber was preferred to a hæmocytometer since its shallower chamber permits thorough searching of the ruled area with minimum focussing.

(*b*) *Method:* The emulsion was first diluted to such an extent that when a drop was placed on the central portion of the slide, and covered with a specially thick and optically plane cover-glass, a countable number of globules (10 to 40) was contained in each small square, when observed through a microscope fitted with a 1/6th in. objective and a × 10 eyepiece. In practice, the degree of dilution varied between 1 in 100 and 1 in 400. The dilution was made by thoroughly mixing a pipetted volume of the emulsion with a sufficient quantity of 80 per cent. aqueous glycerin to produce half the final volume of the dilution, after which a 10 per cent. aqueous solution of nigrosin was added with constant stirring to produce the final volume. In order to ensure that the globules remain evenly dispersed in the dilution, Smith and Grinling employed a 25 per cent. acacia mucilage as the diluting fluid. In the method described here, the aqueous glycerin adequately fulfils the function, and in the present authors' opinion is preferable to acacia mucilage. In addition to possessing the advantages of chemical stability and a standard viscosity, aqueous glycerin has no intrinsic emulsifying action and thus eliminates risk of additional emulsification of the oil. It was also shown experimentally that the converse risk, i.e., of break-down of emulsion on dilution, was absent. The inclusion of nigrosin makes the globules more conspicuous since by suitable adjustment of substage illumination they can be made to appear as bright circles against a blue background. The initial dilution of the emulsion with aqueous glycerin was found to yield a more uniform dispersion of the globules than was obtained by adding the emulsion to a premixed glycerin/water/nigrosin solution. When

the counting chamber containing the dilution of appropriate strength had been prepared, the ruled area was examined to ensure that the distribution of the globules was uniform: if not, the chamber was cleaned and refilled. When the distribution was satisfactory, the globules contained in 20 small squares selected at random were counted, the entire depth of each square being well searched for small globules. In order to avoid counting the same globule twice, the count for each square included all those globules which lay on or touched the top and left hand side, and excluded all those which lay on or touched the bottom right hand side. After each complete count the chamber slide and cover slip were thoroughly washed with soapy water, rinsed with warm water and distilled water, and dried with a clean cloth. A final polish was given with a lens tissue and before re-use it was examined under the microscope to ensure absence of débris.

Results

(1) *Methods of Calculation.* From the total number of globules counted in 20 squares the following values can be calculated.

(*a*) "H," which expresses the number of millions of globules into which 1 cu. mm. of oil has been subdivided.

Volume under 20 small squares in the Helber chamber = (20 × 1/50 × 1/20 × 1/20) = 1/1000 cu. mm.

If N is the number of globules counted in this volume of a 1 in Z dilution of an emulsion containing 15 per cent. v/v. of oil then,

$$\text{"H"} = N \times 1000 \times Z \times \frac{100}{15} \times \frac{1}{10^6}$$
$$= N \times 1000 \times Z \times 6{\cdot}67 \times 10^{-6}$$

(*b*) "D," the "root mean cube diameter," which is derived from "H" and represents the diameter (in microns) the globules would have if they were uniform in volume and the same in number as in the emulsion examined.

$$\text{Volume of a globule (assumed spherical)} = 4/3\,\pi r^3 = \frac{\pi d^3}{6} = \frac{1}{H \times 10^6}$$

$$\text{therefore d (in mm.)} = \sqrt[3]{\frac{6}{H \times 10^6 \times \pi}}$$

$$\text{and "D" (in microns)} = 10 \times \sqrt[3]{\frac{6}{\pi H}}$$

It is emphasised that "D" is not the arithmetic mean diameter because it is derived from the mean volume. It is probably a more useful measure of the average globule size than the former quantity. From the rate of change of either "H" or "D" separate but equivalent expressions of stability can be derived.

(*c*) *Rate of increase of "D":* In preliminary work it was found that the relationship between log. "D" and time of centrifuging was sensibly linear. Figure 1 shows this relationship for the system arachis oil/lubrol

W 0·5 per cent. The slope of a line chosen to fit the points obtained was considered a satisfactory measure of the stability of the emulsion tested. This slope was calculated by the method of least squares.[4] If Figure 2 represents a typical result, then the slope, i.e., the increase in log. "D" per 5 minute interval, is given by the following expression, which is applicable to six results at equal 5 minute intervals:—

$$\frac{-5D_1 - 3D_2 - D_3 + D_4 + 3D_5 + 5D_6}{35}$$

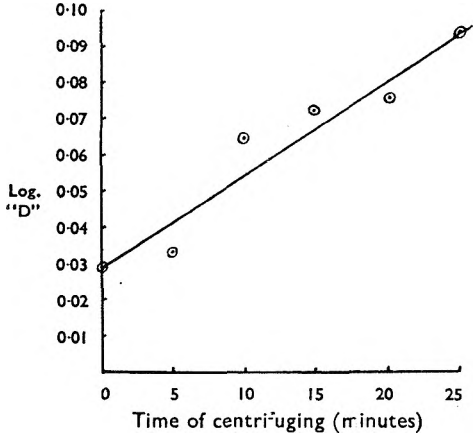

FIG. 1. Relationship of log. "D" with time of centrifuging (arachis oil emulsion prepared with 0·5 per cent. lubrol W).

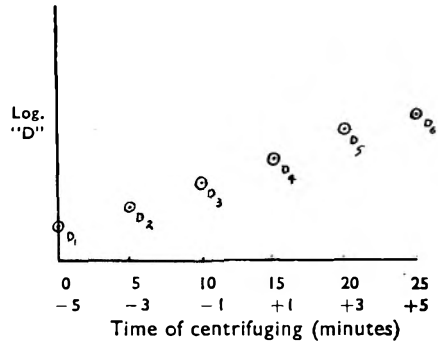

FIG. 2. Application of formula to calculate slope.

For convenience of expression the numerical value obtained was multiplied by 10,000. This value is, of course, not a constant but relates to the particular experimental conditions adopted here. The higher the value, the less stable the emulsion and *vice versa*.

(*d*) *Rate of decrease of* "H": The decrease in "H" per 5 minute interval ($H_1 - H_2$) may also be used as an index of stability and is most conveniently expressed as a percentage, i.e., $\left(1 - \frac{H_2}{H_1}\right) 100$. The actual values may be obtained either by first calculating the slope of log. "H" (by the method of least squares) or by derivation from the slope of log. "D." In the case of arachis oil/0·1 per cent. polysorbate 80, for example, the latter method may be applied as follows:—

Slope of log. "D"/5 minute interval = 0·0395

therefore $\log. \frac{H_2}{H_1} = -3 \times 0{\cdot}0395 = -{\cdot}1184$ or $\bar{1}{\cdot}8816$

therefore $\frac{H_2}{H_1}$ = antilog. $\bar{1}{\cdot}8816$ = 0·7614

and $\left(1 - \frac{H_2}{H_1}\right) 100 = 23{\cdot}8$ per cent.

Clearly an increased percentage indicates lower stability.

(2) *Limits of Error:* The reproducibility of the method is clearly dependent on (*a*) the accuracy of preparation and homogenisation of the emulsion, and (*b*) the accuracy of sampling, diluting and counting. In the case of cetrimide and lubrol W, two batches of each test emulsion were prepared and two samples from each batch diluted and counted. The results obtained showed that the experimental error was almost exclusively associated with (*a*) above. It was therefore decided that, in the case of polysorbate 80, stearyl alcohol ethylene oxide condensate, lissapol NX and sodium oleate, it was sufficient to prepare the emulsions in duplicate and to examine single samples from each batch. From the results obtained using the latter technique, a 95 per cent. limit of error, having a numerical value of ± 0·0034, has been calculated for the slope of log. "D" per 5 minute interval. Thus, the value of 395 reported for the emulsion prepared using 0·1 per cent. of polysorbate 80 with arachis oil would lie within the range 361 to 429 in 95 per cent. of determinations. This limit of error is only applicable to emulsions which withstood 25 minutes in the centrifuge without separation of oil, thus enabling

TABLE I

EMULSION OF ARACHIS OIL WITH 0·5 PER CENT. OF LUBROL W. GLOBULE COUNTS ON A 1 : 400 DILUTION

Sq. No.	Sample A						Sample B					
	Time in Minutes						Time in Minutes					
	0	5	10	15	20	25	0	5	10	15	20	25
1	35	28	23	23	19	18	29	27	25	20	22	19
2	30	27	24	20	19	20	30	26	20	19	20	17
3	30	32	24	22	25	19	32	26	22	26	21	18
4	28	24	22	23	22	24	34	26	22	20	18	17
5	26	27	23	20	20	19	36	31	23	21	18	16
6	32	25	22	19	22	18	29	29	24	18	18	17
7	26	30	20	21	21	21	28	27	26	22	20	22
8	25	28	25	18	19	18	30	24	21	24	22	19
9	24	28	23	24	19	19	26	30	19	18	24	19
10	32	29	21	22	26	17	26	28	27	20	19	15
11	31	30	24	23	22	22	28	28	26	22	19	20
12	31	28	25	19	22	20	28	27	25	24	20	16
13	29	26	23	20	25	16	29	26	24	22	21	19
14	28	26	25	26	20	18	30	28	23	19	17	16
15	26	26	23	24	18	19	28	21	20	24	21	17
16	28	27	25	26	19	18	27	25	22	24	20	18
17	29	28	26	20	23	16	28	22	25	18	25	17
18	30	28	22	19	20	17	30	28	26	22	20	18
19	31	27	23	21	24	18	28	27	26	25	22	16
20	30	26	26	25	22	17	28	28	21	25	20	18
TOTAL ..	581	550	469	435	427	374	584	534	467	433	407	354

TABLE II

EVALUATION OF LUBROL W

Strength of agent per cent.	Time of centrifuging (minutes)	Arachis Oil								Liquid Paraffin							
		"H"				"D"				"H"				"D"			
		Emulsion 1		Emulsion 2		Emulsion 1		Emulsion 2		Emulsion 1		Emulsion 2		Emulsion 1		Emulsion 2	
		Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B	Sample A	Sample B
0·1	0	369	351	368	372	1·73	1·76	1·73	1·73	564	564	540	523	1·50	1·50	1·52	1·54
	5	336	332	328	332	1·78	1·79	1·80	1·79	483	478	463	455	1·58	1·59	1·60	1·61
	10	306	296	306	317	1·84	1·86	1·84	1·82	313	314	311	289	1·83	1·82	1·83	1·88
	15	280	284	294	294	1·90	1·89	1·87	1·87	Oil separated after centrifuging							
	20	281	278	262	259	1·90	1·90	1·94	1·95								
	25	245	243	241	239	1·98	1·99	1·99	2·00								
0·5	0	1550	1558	1622	1630	1·07	1·07	1·06	1·05	855	851	851	841	1·31	1·31	1·31	1·31
	5	1468	1424	1558	1516	1·09	1·10	1·07	1·08	766	767	767	734	1·36	1·36	1·36	1·38
	10	1251	1246	1414	1409	1·15	1·15	1·11	1·11	723	724	710	715	1·38	1·38	1·39	1·39
	15	1161	1155	1169	1280	1·18	1·18	1·18	1·14	599	606	615	603	1·47	1·47	1·46	1·47
	20	1139	1086	1056	1070	1·19	1·21	1·22	1·21	564	560	562	534	1·50	1·51	1·50	1·53
	25	998	944	977	966	1·24	1·27	1·25	1·26	478	475	464	479	1·59	1·59	1·60	1·59
1·0	0	1702	1686	1516	1470	1·04	1·04	1·08	1·09	1280	1319	1276	1286	1·14	1·13	1·14	1·14
	5	1443	1491	1464	1505	1·10	1·09	1·09	1·08	987	1010	1002	997	1·25	1·24	1·24	1·24
	10	1446	1462	1468	1369	1·10	1·09	1·09	1·12	923	934	916	930	1·28	1·27	1·28	1·27
	15	1478	1464	1403	1428	1·09	1·09	1·11	1·10	694	682	678	700	1·40	1·41	1·41	1·40
	20	1310	1331	1348	1304	1·13	1·13	1·12	1·14	706	676	692	710	1·39	1·41	1·40	1·40
	25	1169	1150	1139	1206	1·18	1·18	1·19	1·17	520	586	540	531	1·54	1·48	1·52	1·53

the slope to be derived from 6 values. In those cases where separation of oil was observed after 10 or 15 minutes centrifuging, thus permitting only 2 or 3 examinations respectively, the emulsions were considered too unstable to be evaluated under the experimental conditions described.

(3) *Tabulated Data:* A typical series of globule counts is given in Table I, using emulsions prepared from arachis oil and lubrol W as the example. The full examination of a single surface active agent is illustrated

TABLE III

SUMMARISED RESULTS FOR ALL SURFACE ACTIVE AGENTS

Agent	Concentration per cent.	Initial "H" value		Slope of log. "D" per 5 minute interval × 10^4		Percentage decrease in number of globules per 5 minute interval	
		Arachis oil	Liquid paraffin	Arachis oil	Liquid paraffin	Arachis oil	Liquid paraffin
Cetrimide*	0·1	342	255	[5]	[5]	[5]	[5]
	0·5	440	400	247	253	15·7	16·0
	1·0	1079	446	103	220	5·9	14·1
Lubrol W*	0·1	365	548	113	[15]	7·5	[15]
	0·5	1590	850	147	167	9·7	11·0
	1·0	1593	1290	78	227	5·2	14·5
Lissapol NX†	0·1	429	266	550	[10]	31·6	[10]
	0·5	1094	532	107	577	7·1	32·8
	1·0	1763	548	71	164	4·8	10·7
Polysorbate 80†	0·1	624	472	395	402	23·8	24·2
	0·5	1584	750	163	380	10·6	23·1
	1·0	1861	804	85	202	5·7	13·0
Stearyl alcohol/ethylene oxide condensate†	0·1	[ ]	[ ]	[ ]	[ ]	[ ]	[ ]
	0·5	412	346	218	270	14·0	17·0
	1·0	657	416	89	237	5·9	15·1
Sodium oleate†	0·1	532	339	223	[10]	14·3	[10]
	0·5	875	241	126	280	8·3	17·6
	1·0	1633	467	90	110	6·0	7·3

[ ] Oil incompletely emulsified using this concentration.
[5] Separation of oil after 5 minutes centrifuging.
[10] Separation of oil after 10 minutes centrifuging.
[15] Separation of oil after 15 minutes centrifuging.

* Average of four results. † Average of two results.

in Table II, where the results are expressed as "H" and "D" values. Similar data were obtained for the remaining 5 surface active agents and these are summarised in Table III, results being expressed as initial "H" values together with rates of change of both "H" and "D."

Discussion

The problem of evaluating emulsifying power can be approached in various ways. An approximate assessment, meeting many of the requirements of the dispensing counter, can be obtained rapidly and simply by

shaking or trituration of the materials under test followed by visual (macroscopic) examination of the emulsion produced. Such a procedure is obviously subject to severe limitations, since it provides no information on the internal state of the emulsion and is only capable of detecting relatively large differences in behaviour. There are many circumstances which demand a more scientific approach yielding accurate quantitative data. For example, during the testing of new potential emulgents prepared synthetically, it is necessary to record relatively small differences in efficiency so as to decide on what are the most promising compounds.

The work described here in no sense approaches a full assessment of even a limited number of surface active agents. It is simply an attempt to provide a technique of evaluating emulsifying power with a sufficient degree of accuracy to make it possible to assess materials of this type. It is hoped that this technique, or modifications of it, will prove of value to other workers in this field. We have found the method to be reliable and reproducible, and, in contrast to methods involving size-frequency analysis followed by "shelf" storage, it is reasonably straightforward and more rapid in application. There is, of course, no necessity to employ the particular homogeniser and centrifuge described. The basic requirement is that the homogenising procedure should be adequate to produce an emulsion capable of being suitably degraded by a convenient time of centrifuging. The technique with appropriate modifications could probably be extended to water/oil emulsions and to emulsions of organic liquids, other than oils.

The following appear to be the most significant facts which can be deduced from the experimental results.

(1) The technique is capable of detecting small differences in emulsions, both in regard to their initial physical state and to their storage life. It should, therefore, provide a useful method of assessing emulgents, particularly from the viewpoint of their use in pharmaceutical manufacture. Subsequent "shelf" storage tests have so far confirmed the results obtained.

(2) The relationship between either log. "D" or log. "H" and the time of centrifuging is sensibly linear for all the emulsions examined. The existence of a linear relationship has not previously been shown for an artificial breakdown stress.

(3) Although it might have been expected that an initial small globule size would be indicative of emulsion stability (and *vice versa*), the results do not confirm this.

(4) Under the conditions of the test, all the surface active agents examined were approximately of the same order of efficiency as sodium oleate.

(5) In all cases, an increase in the concentration of surface active agent produced a larger initial "H" value and, in most cases, an increased stability. At the lowest concentration studied (0·1 per cent.) there was a marked difference in the behaviour of the various emulgents. At 1·0 per cent. this difference was almost insignificant, particularly in emulsions of arachis oil. It is possible, of course, that a change in the

conditions of homogenisation and centrifuging would reveal differences in behaviour, even at higher concentrations.

(6) It was not possible to arrange the surface active agents in any single order of efficiency applicable to all concentrations, although certain trends could be observed, e.g., cetrimide was the least satisfactory emulgent for arachis oil at all concentrations.

(7) In accordance with the normal behaviour of emulgents, the materials under test were less effective for liquid paraffin than for arachis oil.

SUMMARY

A method is described for the evaluation of emulsifying power of surface active agents, based on the measurement of stability of the emulsions they yield.

We are indebted to Dr. O. L. Davies for the mathematical treatment of the results and to Mr. A. G. Fishburn for help in the preparation of the paper.

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DISCUSSION

The paper was presented by Mr. J. R. Cockton.

The CHAIRMAN pointed out that it was sometimes desirable clinically that an emulsion should not be too stable.

MR. H. LEVIUS (Dagenham) said that the authors referred to size frequency analyses, and claimed that the method of globule counts was quicker. That method was also more accurate in order to determine "H" and "D." Artificial ageing would give a better picture of emulsion stability than centrifuging; it more closely approximated storage tests and, although not exactly analogous, it gave useful information. King, in the paper quoted by the authors, tried to show a linear relationship between storage time and decrease in specific interface. Was there any reason why the present authors had determined "D" in preference to area? It appeared that the authors had assumed a direct relation between the efficiency of an emulsifying agent and the stability of the emulsion produced.

DR. W. MITCHELL (London) asked whether the authors had found much variation in the emulsifying power of different batches of proprietary emulsifying agents. In practice he found such a gross variation that it was often necessary to reformulate either the quantities of material or the method of making an emulsion for each batch of emulsifying agent received. He also asked whether their method, and in particular the mathematical treatment of the results, applied equally well when the emulsifying agent was dissolved in either phase or present as solid.

MR. E. ADAMS (Plymouth) said that the authors assumed that the reduction in the number of globules of oil was proportional to the reduction in total area of the interface. If the globules of an oil dispersed in water were uniform in diameter that would seem logical, but in practice the diameter varied quite considerably.

MR. D. E. SEYMOUR (Welwyn) asked whether the authors had considered interfacial tension measurement as a means of assessing emulsifying power. He agreed that size frequency analysis was a tedious method, and in his experience did not correlate so directly with stability as the paper implied. A good way of assessing the stability of an emulsion was to store it at about 40° C., rather than at room temperature; most emulsions broke down in a short time. With water-in-oil emulsions oxidation of the emulgent could be a serious factor in breakdown, for there was a high degree of surface distribution. Metallic ions tended to increase the degree of oxidation. He confirmed that initial globule size was not connected with ultimate stability.

MR. T. D. WHITTET (London) said that little was known about the pharmacology of the large number of emulgents coming into use. Intravenously-fed emulsions were beginning to be used, and it would be useful to know the toxicity or otherwise of many of the substances referred to. The stability of an emulsion was not necessarily related to its clinical efficiency. The work of Fraser on the absorption of fats had shown that if an emulsion of liquid paraffin had a small enough particle size, the paraffin was absorbed. Emulgents were not necessarily inert pharmacologically. Polysorbate 80 and sodium cetyl sulphate were given orally in capsules, the former to aid the emulsification of fats, the latter to inactivate gastric lipase.

MR. D. N. GORE (Dorking) thought that unless there was some overriding therapeutic reason for presenting a drug in the form of an emulsion it should never be done.

MR. A. G. FISHBURN (Manchester) suggested that the answer to the unsatisfactory position with regard to emulsifying agents might be found in synthetic materials.

MR. A. E. DAVIS (Nottingham) pointed out that the statement was made that all surface active agents examined were approximately of the same order of efficiency as sodium oleate, and asked whether that did not show a serious defficiency in the methods used to determine the various results. The non-ionic agents would be more stable over a wider range of *p*H. He asked whether it was possible to relate centrifuging time to shelf storage time, and why they had used concentrations of 0·1, 0·5 and 1 per cent. for their comparisons. They showed a big difference in stabilising properties of the emulsifiers, but the concentrations were not the strengths used in practice.

MR. A. F. CALDWELL (Singapore) said that there was always the possibility of bacterial or fungal infection causing decomposition in emulsions and it was important to know that there were no bacteria or fungi present in the emulsifying agents.

MR. J. R. COCKTON, in reply, said that the calculation of interfacial

area was considered, but it was felt that accuracy would be in doubt owing to the admittedly approximate nature of the calculation. There was a batch-to-batch variation in surface-active agents, but they had had the advantage of working with a specially purified set. The effect of electrolytes was not known. In reply to Mr. Adams, homogenisation was employed, as it tended to make the globule size more homogeneous. Visual observation methods had been tried. The emulsions were very similar in appearance when prepared, and even after standing for 6 months there was very little visible deterioration. Storage had been carried out at high temperature and at room temperature, approximately 20° to 25° C. Oxidation had to be considered, but the main purpose of the work had been to investigate the differences between the surface-active agents. It was hoped that toxicity tests on the emulsifying agents would be carried out shortly. The concentrations of surface-active agents were purposely selected in order to emphasise the differences.

THE ASSAY OF BACTERIAL PYROGENS

By M. Dawson and J. P. Todd

From The School of Pharmacy of Glasgow Royal Technical College

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The methods based on temperature response at present in use in the testing of solutions for pyrogen are useful as limit tests for fever-producing effect, but for estimation of effect involving comparison of preparations which do not differ markedly they are of little value for two reasons—the lack of a stable reference standard and the variation in the temperature responses of the rabbit. This work describes the preparation of a standard and its use in investigating variations in rabbit responses.

The Preparation of a Standard

As a source of standard we chose first *Escherichia coli* because it had been shown to produce pyrogen copiously,[1] to grow well in simple media of known chemical composition and to be relatively non-pathogenic. The pyrogenic supernatant liquid from cultures of this organism was, however, found to be unstable to even mild degrees of heat, whether the liquid was heated at the *p*H value of the growth, 4·7 to 4·9, or whether it was adjusted to *p*H 7 before attempting to concentrate by heating. The results of heating are shown in Table I.

TABLE I

Loss of pyrogenic effect from *Escherichia coli* provisional standard on heating under reduced pressure

Dilution required that response might fall within the quantitative range	Time of heating (minutes)	Temperature ° C.	Average rise in temperature in groups of 5 rabbits	
			Solution before heating	Solution after heating
0·2 per cent.	120	55	0·49	0·37
0·2 ,, ,,	45	50	1·13	0·47
0·2 ,, ,,	20	40	1·19	0·47
0·1 ,, ,, *	20	40	0·94	0·58

* Solution adjusted to *p*H7 before heating.

The pyrogenic effect also decreased on storage (Table II).

Attempts were made to store this pyrogen in the dry state by adsorbing it on asbestos pads and storing these in a desiccator. Complete adsorption of pyrogen on to a 3·6-cm. asbestos pad took place from 100 ml. of solution of pyrogen at *p*H 4·7 to 4·9, which was the normal *p*H value of a 4-days' growth of *E. coli*. Complete elution took place at *p*H 9 to 12. The dried pad retained the activity but the eluate soon decomposed. This method of storing pyrogen was soon abandoned as it was not convenient to elute the pyrogen and free the solution from asbestos fibres before each experiment. Table III compares the residual activity after storing the pyrogen on the pad and in the eluted form.

TABLE II

LOSS OF PYROGENIC EFFECT FROM *Escherichia coli* PROVISIONAL STANDARD ON STORAGE

Dilution	Period of storage days	Average rise in temperature in groups of 5 rabbits	
		Solution before storage ° C.	Solution after storage ° C.
1·0 per cent.	6	0·65	0·16
0·2 ,, ,,	7	*1·56	0·83
0·2 ,, ,,	12	*1·56	0·35
0·2 ,, ,,	9	0·67	0·14
1·0 ,, ,,	40	0·85	0·38

* Same solution.

TABLE III

COMPARISON OF THE LOSS OF PYROGEN IN THE ADSORBED AND ELUTED STATES ON STORAGE FOR 5 DAYS

Average rise in temperature in groups of 5 rabbits due to		
Pyrogen adsorbed, immediately eluted and immediately injected	Pyrogen stored on a pad for 5 days, eluted and immediately injected	Pyrogen adsorbed, immediately eluted and eluate stored for 5 days
0·77	0·80	0·31
0·95	0·75	0·49

No attempt was made to freeze-dry this preparation on account of its lack of stability.

E. coli was now discarded as a source of pyrogen and a standard prepared from *Proteus vulgaris*. The organism was grown in simple medium and separated from the liquid by continuous, high-speed centrifuge. The liquid was filtered through sterile, unglazed porcelain candles into sterile freeze-drying tubes and spin-freeze-dried. After the secondary drying under vacuum and with phosphorus pentoxide the ampoules were sealed by fusion of the glass and tested for faulty sealing by a high-frequency, glow-discharge tester. No loss of pyrogen occurred in the freeze-drying process and the material suffered no obvious storage loss during the 20 months it was used as the standard for the temperature response experiments. In carrying out these experiments the supply of this standard was exhausted, the amount prepared being limited by the capacity of the freeze-drying unit.

Freeze-drying of eluate from pads in an attempt to prepare a purer standard was not a success, as shown in Table IV. Neutralisation of the eluate before freeze-drying did not prevent loss of pyrogen.

A new standard was prepared from *P. vulgaris*. The culture was centrifuged and the supernatant liquid filtered as before. In order to obtain a purer product the filtrate was dialysed through cellophane to free it from inorganic salts. It was then re-sterilised by filtration and freeze-dried. No pyrogen was lost during drying and no obvious storage loss occurred while this standard was in use for the leucocyte response experiments.

TABLE IV

LOSS OF PYROGEN DURING FREEZE-DRYING OF ELUATE

*p*H value of solution before drying	Average rise in temperature in groups of 5 rabbits	
	Eluate before drying	Eluate dried and reconstituted
9·9	0·86	0·34
10·4	*1·33	0·69
6·7	*1·33	0·75

* Same solution.

RABBIT TEMPERATURE RESPONSE TO PYROGEN STANDARD

Animals. 25 rabbits, adult, either sex, weighing about 2·5 kg.

Method. The animals were placed in boxes adjustable for size and held lightly and comfortably in a normal sitting position and the temperatures were read by thermocouple junctions balanced against a junction in a water bath of known temperature, as described by us elsewhere.[1]

When the rabbit basic temperature was reached it was noted and the pyrogenic solution then injected, *via* the marginal ear vein, at 37° C. and diluted to a volume of 2 ml./kg. of body weight. Temperatures were read half an hour after injection and then at 10-minute intervals until they had risen to a peak and had begun to show a definite fall. The rabbits were kept awake throughout the experiment. Each of 25 rabbits was injected 4 times with each of 3 dose levels of pyrogen standard, the doses being 0·2 ml./kg., 0·06324 ml./kg. and 0·02 ml./kg., the middle

TABLE V

TEMPERATURE INCREASES DUE TO INJECTIONS OF PYROGEN STANDARD

Dose	0·02 ml./kg.	0·06324 ml./kg.	0·2 ml./kg.
Mean of 100 responses	0·90	1·14	1·21
Standard deviation	0·36	0·34	0·39
Mean of 25 mean responses ..	0·90	1·14	1·20
Standard deviation	0·24	0·26	0·30

dose being chosen so that its logarithm was equidistant from that of the other two.

Results. The temperature increases due to these injections are shown in Table V along with their standard deviations. In every case this is a large fraction of the response and it is questionable whether a test showing a deviation of this magnitude can be regarded as of value except for limit tests, as used in the B.P. It is not sufficiently accurate for systematic work involving comparisons of solutions of approximately the same concentration.

Investigation of Results. Increased response or decreased variance would lessen the error. Seibert[2] showed that the response could not be increased beyond a maximum by further increase in dose and Wylie and Todd[1] found that maximum under the present conditions of experiment to be 1·3° C. For this reason causes of variance were sought in

order to lessen the error by their elimination. The possibilities considered were (*a*) variance within rabbits and variance between rabbits, (*b*) variance due to breed, sex, weight and colour.

(*a*) Analysis of variance of temperature response within and between rabbits showed the latter to be the greater. Comparison of an unknown sample of pyrogen with a standard would therefore be more accurate if carried out on the same rabbits.

(*b*) Dutch and Blue Fox rabbits were the predominating breeds in the population and no difference between the responses of the 2 groups was brought to light by t-tests which showed 30 to 40 per cent. probability of the 2 samples coming from the same population. Similar results were obtained from a comparison of the responses of bucks and does (40 to 50 per cent.) and those of dark-eared rabbits and light-eared rabbits which it was thought might radiate differently (70 to 80 per cent.). Pearson's correlation coefficient was also calculated to see if the following pairs of measurements were related, basic temperature and rise in temperature, weight and basic temperature, and weight and rise in temperature. The results were inconclusive.

Methods of measuring the response other than by simple rise in temperature were now considered. These were the use of only the maximum temperature attained as opposed to the use of the difference between this and the temperature at injection, and a measure taking into account not only the height of the rise but also the time taken to reach it. Emmens[3] says that the measure of the response after test is as useful as a comparison of the before-test and after-test states where the first is variable and the two are correlated. Applying this to pyrogen, the results were noted for the maximum temperature attained after each injection. These results (Table VI) show that the maximum temperature gives no real information and that the basic temperature must be taken into account.

300 graphs were drawn plotting rise against time until the maximum temperature was attained. From this point a perpendicular was dropped to the time axis and the area enclosed measured by planimeter. The average area and its standard deviation for each of the 3 dose levels was calculated and found to be as variable as height of rise alone and therefore of little use in a quantitative assay. The magnitude of the standard deviation in the results of all the above experiments led us to believe that temperature response in the rabbit is not an accurate method to use for the quantitative assay of pyrogen.

TABLE VI

MAXIMUM TEMPERATURES ATTAINED AFTER INJECTION OF PYROGEN STANDARD

Dose ml./kg.	Mean of 100 maxima °C.
0·02	39·44
0·06324	39·72
0·2	39·47

PRELIMINARY REPORT ON RABBIT LEUCOCYTE RESPONSE TO INJECTED PYROGEN

Pyrogen has several pharmacological properties, the main properties being an effect on the white blood cell picture,[4,5,6,7,8,9,10,11] inhibition of thermal panting in dogs,[12] ulcer inhibiting action,[13] an effect on peripheral

circulation,[14] reduction of gastric acid secretion[15] and reactions of tissues to the administration of pyrogen.[16] Of these it was decided that changes in the white cell picture as the basis of a method of assay warranted investigation. No quantitative examination seems to have been carried out on the changes in the relative numbers of the different types of white cells due to pyrogen.

Animals. 25 rabbits, adult, either sex, weighing about 2·5 kg. Some had been members of the population used in the first part of this work. Others were new, replacing those whose ear veins had become occluded due to repeated injection.

Standard pyrogen. The standard pyrogen used for these experiments was the dialysed standard previously described.

Methods. Some preliminary work on differential white cell counts was done. This established that (*a*) the error in repeated readings of the same smear was less than the difference between smears from the same rabbit on successive days and that (*b*) this in turn was less than the difference between smears before and after injection of pyrogen. The white cell count did not, of course, fluctuate as rapidly as temperature, and the departure from normal was greatest about 3 hours after injection.

In the main investigation the population of 24 was given 4 injections each, at weekly intervals, of 0·2 ml./kg. of standard. The temperature responses were measured as before and, at the same time, differential white cell counts were made from drops of blood from the marginal ear veins, the cells being stained with Leishman's stain and examined at a magnification of 600. Smears were made before injection and 3 hours after injection. In the differential counts the cells counted were classed as large lymphocytes, small lymphocytes, monocytes, eosinophils, basophils and neutrophils.

The usual number of cells counted in differential white counts is 300. Error may be introduced by the tendency of small lymphocytes to stay at the beginning of the smear or to be drawn along the centre and for granulocytes to be drawn to the end of the smear or to lie along the edges. To avoid this error strips across each end and the middle of the smear were counted and, if by then a total of 300 had not been attained, 2 intermediate strips between the centre and each end were added. This gave various totals of more than 300 for each smear. To make the results more readily comparable, all the individual cell counts were expressed as percentage of the total number counted, thus giving figures for the percentage of large lymphocytes, etc.

Results. Normally small lymphocytes predominate. After injection a fall in the percentage of small lymphocytes and a rise in the percentage of neutrophils occurred. In the other less numerous types there were no significant differences. We considered from the general appearance of the smears that there was probably an absolute as well as the measured relative increase in the neutrophils but the present work is restricted to differential counts and their use as an index of pyrogenic effect. Total counts were not carried out.

The changes in percentage of small lymphocytes were first considered.

The differences between the percentages of small lymphocytes before and after injection were extremely variable from one rabbit to another. It was considered that this was because the percentage before injection was itself a variable. To overcome this difficulty the differences were expressed as percentages of the small lymphocyte percentage before injection. These results are referred to as the percentage falls in small lymphocyte count. The mean percentage fall in small lymphocytes in the 96 responses was 75 per cent. with a standard deviation of 15. When the average for each rabbit was calculated from its 4 results and the 24 averages considered, the mean fall was still 75 per cent. with a smaller standard deviation, in this case 9.

Similar calculations were done for "total mononuclears," i.e., large and small lymphocytes and monocytes. The mean of the 96 percentage falls was 75 per cent. with a standard deviation of 14. The mean of the 24 was 75 and the standard deviation 9. The temperature results obtained at the same time as the white blood cell counts were comparable with those in Table V, the population mean being 1·18° C. and the standard deviation 0·37° C. considering mean rises, 0·45° C. considering individual rises. It was established that there was no correlation between temperature rise and white blood cell change, i.e., a rabbit sensitive to pyrogen by one response was not necessarily sensitive by the other.

Discussion

In this preliminary investigation these figures seem to indicate that small lymphocyte count is a more accurate method of assay of pyrogen than temperature measurement. The standard deviation of the temperature responses is a larger fraction of the response than in the case of white cell responses giving an assay with wider limits of error. Work is in progress on the effect on the differential count of different dose levels of pyrogen and of pyrogen from different organisms.

Summary

1. The preparation of a provisonal standard pyrogen has been described.

2. Temperature rises and white blood cell changes in the rabbit in response to this pyrogen standard have been investigated.

3. A smaller variance was found in white blood cell changes than in temperature rises.

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DISCUSSION

The paper was read by MISS M. DAWSON.

The CHAIRMAN observed that it was interesting to see that the standard pyrogen was sufficiently stable to permit it to be freeze dried, and there should be no difficulty in reproducing it.

DR. J. C. DARE (Kippax) drew attention to the change in the situation during the past twelve months in relation to the determination of bacterial pyrogens. Pyrogen preparations were being used in the United States, and it was now becoming urgent that an adequate quantitative method of calculating the potency of pyrogen preparations should be developed as distinct from a test for their absence. The authors suggested that temperature measuring methods were not of much use for preparations which did not differ markedly. It depended on what was meant by the word "markedly." There were at least three teams of workers in this country who had been studying the question of a standard, and all had arrived at the conclusion that a dried preparation of *Proteus vulgaris* was the most promising. The question of whether temperature measurements were going to be adequate or whether some other method, as had been suggested, was to be used, was rather an open one. He felt that much more information was needed about differences of blood counts before accepting that method as being superior to the temperature test. With the temperature measuring equipment which would shortly become available it would be possible, for all practical purposes, to make an error-free determination of temperature, but that could not be said about cell counts. In a recent paper it was shown that the standard deviation of the differential count was of the order of $\pm$ 7 per cent. if the cells in which one was interested constituted 50 per cent. of the white cells. If the proportion of cells in which one was interested was only 10 per cent. of the white cell count, then a standard deviation of about $\pm$ 21 per cent. was obtained. The lymphocyte count of the rabbit was usually between 50 and 60 per cent. of the total white cell count. If that were reduced by 75 per cent. it was getting down to about 12·5 to 15 per cent.

of the total. In other words, there would be a standard deviation and error in the counting in the initial stages of ± 7 per cent. which would be correspondingly larger after the depression. The full potentialities of the temperature-measuring methods had not yet been investigated. By appropriate design of experiments to allow for individual animal variations the standard deviation could be improved. Limits of error would be somewhere between 50 and 200 per cent. of the true value. Those were wide limits, and he asked the authors what they meant by "markedly," because for human beings it was necessary to increase the dose threefold in order to obtain a significant change in response.

It had previously been shown that the lymphocyte count falls after a stress stimulus, becoming minimal 3 hours after the stimulus. Had the authors any evidence to show that the reduction in the proportion of lymphocytes which they observed 3 hours after an injection was, in fact, due to pyrogen and not to the stimulus given by the process of injecting the solution?

DR. K. BULLOCK (Manchester) said that the complex filtrate used raised the temperature and altered the white cell count. There was no correlation between those effects, yet the authors assumed that the pyrogen effect could be assayed on white cells. Why did they assume that one substance caused both effects?

MR. K. L. SMITH (Nottingham) said that the main use of the pyrogen test was qualitative. The authors had detected the activity of their standard pyrogen by means of a quantitative test. He had attempted to establish a response curve, and his slope was about as good as that of the authors, namely, a tenfold change in dilution gave a 0·3° C. change in temperature. The authors' standard deviation was greater than his. The changes of dose should have been carried out earlier because it was the relation of the standard deviation to the slope which gave the accuracy of the assay.

MR. T. D. WHITTET (London) said he was convinced that there was more than one pyrogen. He wondered whether the absence of correlation between the two factors was general and whether the pyrogen from one organism was consistently more effective in giving one response than the other.

MISS M. DAWSON, in reply, said that the standard deviation of the temperature response could be reduced considerably by appropriate grouping of the rabbits once an idea of the individual response was obtained. This was, however, an inconvenient method to use, depending as it did on the continued presence of the same rabbits in the population. With regard to the phrase "markedly different", to find how close in pyrogen content two samples may be and yet be distinguishable depends on the number of rabbits used in the test. The number of rabbits required in the sample to show a given difference in response with a given probability might be ascertained, as is well known, by applying the t-test in reverse. The lack of correlation between the temperature response and the white cell count in any one rabbit might be a reflection on the observed instability of the rabbit's temperature-regulating mechanism. The question of comparing pyrogens from different organisms had not been investigated in the present work.

A SIMPLE APPARATUS FOR THE DETERMINATION OF BLOOD CLOTTING-TIMES AND ITS APPLICATION TO THE ASSAY OF HEPARIN

By D. Maxwell Bryce

From the Laboratories of S. Maw Son and Sons, Ltd., New Barnet

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Introduction

Determination of the clotting time of suitable fibrinogen-containing systems forms the basis of most assays of blood coagulants and anti-coagulants. The purpose of this paper is to describe a simple apparatus whereby the precision of such determinations may be much improved. In particular, we have applied it to the method of assay which Adams[1] developed for heparin solutions. We have found this method very satisfactory and have used it also for thrombin assay. He observes the clotting process in standard glass tubes and takes for end-point the time when the clot formed remains adherent to the walls of the tube on inversion. We have not found this end-point entirely satisfactory; it is subject to considerable variation, and we do not find it convenient to examine a series of tubes in this manner according to a strict time schedule. Further, since premature inversion permanently alters the clotting system and leads to the abandonment not only of the individual result, but also of the whole series (for this is the most satisfactory way of dealing with the contingency), operators tend to be rather tardy in inverting, so that a bias creeps into the assay. For these reasons it seemed necessary to find a suitable end-point indicator, a problem which has received much attention from workers in the past.

There are two main approaches to the problem; one is to detect fibrin-fibril formation; this, in long-clotting-time systems may occur a considerable time before formation of a reasonably firm clot; the other is to detect the increase in viscosity of an incipient-clotting system.

In the very substantial literature of the subject are to be found many and ingenious devices for detecting one or other end-point. For example, of the first type, Wright[2] observes dispersion of the system in water, Trought and Riddoch[3] observe fibril formation when a capillary tube containing the system is momentarily brought into contact with a rough surface, and there is a widely-used method, probably also due to Wright, in which fibrin is detected in a micro-system by dredging with a fine glass rod. A macroscopic modification of this is described by Quick.[4] To the second type belong the Brodie-Russell-Bogg, and Dale and Laidlaw[5] coagulometers, which consist respectively of a spinning drop observed under the microscope, and a glass tube containing a metal sphere whose fall under gravitation through the liquid is observed. Measurements based on viscosity-change are capable of considerable precision. Hartert,[6] using a rotating cylinder viscometer, has shown that it is possible to determine clotting times with an "average error" of <3 per cent. Randall[7] has

described a similar type of apparatus which has the further advantage of being automatic. He quotes replicate times of 5·1, 4·8, 4·9 and 4·8 minutes for a particular sulphated-blood/ox-brain extract/heparin system. The coefficient of variation is therefore 2·9 per cent. which confirms the accuracy claimed by Hartert.

We could find no apparatus already described which seemed adaptable to the assay, chiefly because none would lend itself to multiple determinations, and so the following simple apparatus was devised. It depends on the viscosity change and yields reproducible results.

Apparatus

Description. The apparatus consists of a wooden block, drilled to hold 6 test tubes ($3 \times \frac{1}{2}$ inch), which slides vertically up and down 4 guides set in a base-plate. A metal plate is screwed to the top of the guides to maintain them in correct position, and this bears 6 holes, each lying on the axis of one of the test tubes (Fig. 1). The block is moved up and down by means of a lever. Indicators are hung from the top plate, each of which consists of a glass bead set on the end of a suitable length of wire, whose last cm. of length is bent through approximately 100°. Thus when an indicator is lowered through a hole in the upper plate into a test tube, its weight is carried by the terminal portions of the wire which is resting on the plate. Glass indicators prepared by allowing molten glass to elongate under gravitation were originally used, but were too fragile to permit ready cleaning. Clearly the detail of the apparatus is of little importance, all that is necessary being a block to hold the required number of tubes and impart to them a vertical motion of about 1 cm. together with some form of superstructure from which to hang the indicators.

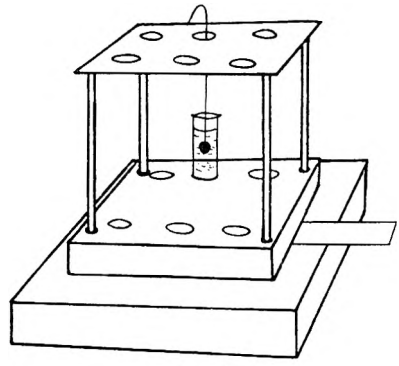

Fig. 1

Method of operation. The reagents are introduced into the tubes and are mixed by inverting, the mouth of the tube being closed with a waxed cork. The cork is removed and the tube is then placed in position in the block and an indicator lowered into it. At 10-second intervals the lever is gently depressed. As long as the system remains fluid, the indicator remains still, but on attaining a critical viscosity, the vertical motion of the tube is transmitted to the indicator which makes a distinct movement. This end-point is quite distinct and has been used to record clotting-times to the nearest 5 seconds, but 10 seconds is preferable since it allows time for recording results and is a convenient time unit for computational purposes. The critical viscosity depends on the weight and dimensions of the indicator used. For example, an indicator weighing 0·38 g.

prepared from a 4 mm. diameter glass bead is suitable for detecting the formation of a soft clot.

Accuracy. The accuracy of the apparatus may be gauged from the following experiment which was designed to detect any significant difference in the end-points shown by 6 similar indicators. The system used was heparin 4 units in 2 ml. of water, ox-brain extract 0·5 ml., sulphated blood (containing 3·6 per cent. of Na_2SO_4 10 H_2O) 2 ml., the ingredients being added in that order at 3-minute intervals, and mixed immediately after the addition of blood. The experiments were carried out in 3 randomised blocks. The indicators were coated with paraffin wax. Times are recorded in 10 second units (see Table I).

TABLE I

	Indicator number						
Blocks	I	II	III	IV	V	VI	Totals
1	34	34	35	34	36	34	207
2	38	34	36	37	37	34	216
3	40	39	39	41	38	40	237
Totals	112	107	110	112	111	108	660

Mean clotting time 36·7 seconds/10.

TABLE II

ANALYSIS OF VARIANCE

Item	Sum of squares	Degrees of freedom	Mean square	Variance ratio	Probability
Series	79·0	2	39·5	25·2	<0·1 per cent.
Indicators	7·3	5	1·47		
Residual	15·7	10	1·57		
Total	102·0	17			

It is evident that the above data give no ground for supposing that there is any significant difference in the performance of the different indicators. The highly significant series mean square is attributable to the warming-up of the reagents which had been stored in a refrigerator. It is important to bear this effect in mind when conducting an assay in which the observations in each series are not randomised.

The total variance within samples is $23/15 = 1{\cdot}53$.

$$\therefore \text{coefficient of variation} = 100\sqrt{\frac{1{\cdot}53}{36{\cdot}7}} = 3{\cdot}4 \text{ per cent.}$$

which compares very favourably with that obtained with the more elaborate apparatus previously mentioned. A fully mechanised model is at present under construction and it is hoped that with this the precision will be further increased

A further experiment was carried out to determine the effect on clotting-time of an increase in the liquid/glass interface, and of varying the order of mixing the reagents. The system used was the same as that in the above experiment, but reagents were added at 2-minute intervals (see Table III).

TABLE III

	Order of adding reagents				
	Heparin—Thromboplastin—Blood		Heparin—Blood—Thromboplastin		
Series No.	With bead	Without	With bead	Without	Totals
1	42	41	39	39	161
2	36	40	36	34	146
3	35	37	33	34	139
4	37	36	35	38	146
5	38	40	33	37	148
6	38	39	36	36	149
	226	233	212	218	889

In 10-second units.

The experiment consisted in observing the clotting time of the 4 possible combinations of order of adding reagents and presence or absence of a 4 mm. diameter soda-glass bead. The experiments of each series were conducted in random order.

TABLE IV

ANALYSIS OF VARIANCE

Item	Sum of squares	Degrees of freedom	Mean square	t	Probability
Series	64·7417	5	12·94833		
Order of addition ..	35·0417	1	35·04167	4·19	<0·1 per cent.
Bead	7·0417	1	7·04167	1·87	5 to 10 per cent.
Inter-action	0·0417	1	0·04167		
Residual	30·0917	15	2·00611		
Total ..	136·9583	23			

We may therefore assert that addition of reagents in the order: heparin, blood, thromboplastin, produces a more rapidly clotting system, so that the more desirable order of mixing is heparin, thromboplastin, blood. The analysis also suggests that the presence of the additional glass surface accelerates clotting, but the level of significance is not high and the experiment is not conclusive in this respect. It does however point to the desirability of having solutions of standard and unknown of as near as possible the same potency in the assay.

The interaction term is subnormal and remarkably small but no significance is to be attached to this as the probability of such a value lies between 0·1 and 0·2.

The coefficient of variation in this data

$$= 100 \times \sqrt{\frac{2{\cdot}0061}{37{\cdot}042}} = 3{\cdot}8 \text{ per cent.}$$

In order to effect a comparison with the data published by Adams, the data was transformed into logs of clotting time in minutes and the residual variance found.

Adams data	Variance 0·0015
Above data	,, 0·000273

so that the precision of his method of assay is much improved by use of this indicator.

SUMMARY

1. An apparatus is described for the simultaneous determination of several clotting times.

2. The accuracy is compared with that obtainable by other methods.

3. Its application to the assay of heparin described by Adams is suggested.

4. The effect of glass-liquid interface and order of mixing in the assay of heparin is investigated.

The author thanks Dr. R. Maxwell Savage for many stimulating discussions and suggestions.

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DISCUSSION

The paper was presented by MR. D. M. BRYCE.

MR. K. L. SMITH (Nottingham) stated that he used the Adams method of determining end-points, but automatic methods were under consideration.

MR. J. M. MYERS (Bradford) pointed out that if the glass surface had been treated with silicones to start with, it might have prevented clotting.

MR. D. M. BRYCE, in reply, said that the real purpose of designing the apparatus was to overcome the personal factor of operators, and in its present form it gave satisfactory results. The effect of surface coating was very important. An endeavour had been made to make comparisons at the same potency level, in order to remove any error, because the variation caused by different types of surfaces was such as sometimes to prevent clotting altogether. He hoped to try the silicone covered materials.

THE COMBINATION OF MERCURIAL DIURETICS WITH DIMERCAPROL (2:3-DIMERCAPTOPROPANOL); THE EFFECT ON DIURETIC ACTIVITY AND TOXICITY

By EILEEN I. SHORT

From the Wellcome Research Laboratories (Biological Division), Beckenham, Kent

With a

NOTE ON THE PREPARATION OF THE COMPOUNDS OF MERCURIAL DIURETICS WITH 2:3-DIMERCAPTOPROPANOL (DIMERCAPROL)

By THOMAS M. SHARP

From the Wellcome Laboratories of Tropical Medicine, London

Received July 16, 1952

RECORDS of the diuretic action of mercury compounds go back as far as the sixteenth century. Paracelsus reported the use of mercurous chloride as a diuretic and there are many references to the combined use of digitalis and mercurous chloride, but because of the occurrence of untoward toxic effects, the use of inorganic mercury compounds tended to fall into disrepute. The accidental discovery of the diuretic properties of novarsarol (a double salt of sodium mercurichlorophenyl oxyacetate with diethylbarbituric acid) introduced as an antisiphylitic agent, led to an extensive search for less toxic organic mercurials. As a result, a number of organic derivatives of mercury have been introduced as diuretics and are used in cases of œdema due to heart, kidney and liver disease, although, because of the risk of toxic effects on the cardio-vascular system, preliminary tests of the susceptibility of the patient and caution in their use are recommended. Also, because of their selective action on the kidney, these compounds cannot be used in cases of advanced chronic nephritis and acute renal disease. One of the organic mercurials introduced recently in the United States by Lehman is thiomerin, the disodium salt of *N*(γ-carboxymethylmercaptomercuri-β-methoxy)-propyl-camphoramic acid. This compound differs in that the organic mercurial has been combined with a monothiol derivative and it has been shown in animal experiments to be considerably less toxic to the heart than the commonly used mercurial diuretics (Lehman[1]). Clinical trials showed it to be well tolerated, producing much less local irritation at the injection site than other organic mercury compounds and to be well absorbed from subcutaneous injection, the diuretic response after administration in this way being similar to that produced by equivalent doses of other mercurial diuretics given intravenously. The nephrotoxicity of the mercury is not, however, eliminated and the use of thiomerin is still contra-indicated in cases of advanced chronic nephritis and acute renal disease.

In view of these results with the combination of an organic mercurial with a monothiol, it was thought to be of interest to study similar products

of the combination with a dithiol derivative, such as dimercaprol to see if the reduction in toxicity is maintained or even further reduced, and also whether some reduction of the renal toxicity could be achieved.

Two such compounds have been prepared by Mr. Sharp of the Wellcome Laboratories of Tropical Medicine. They are the product of combination of two molecules of mersalyl and one molecule of dimercaprol, which has been given the name "balmersal" and the disodium salt of 2:3-bis-(3'-camphoramido-2'-methoxypropylmercurimercapto)-propan-1-ol or "balmercamph," the product of dimercaprol and the organic mercurial used in thiomerin.

In spite of a large amount of work on the subject, evidence on how the mercury compounds produce their diuretic effect is conflicting. The two fundamentally different points of view, one, that the diuretic effect is primarily extra-renal, the mercury producing a dilution of the blood which acts as the stimulus for diuresis (Jendrássik,[2,3] Saxl and Heilig[4,5]), and two, that the effect is a direct one due to the action of the mercurial on the kidney (Govaerts,[6] Bryan, Evans, Fulton and Stead,[7] Gremels[8]) have still to be reconciled, and studies of the behaviour of the mercurials with dimercaprol have been made in the hope of contributing to this problem.

Experimental

Diuretic activity in rats. A qualitative comparison of the diuretic activity of the compounds was made in rats. Cross-over tests were carried out on groups of 4 rats, each weighing about 200 g., the animals being selected so that the total weights of the groups were as nearly equal as possible. The animals were starved overnight and moderate hydration with 5 ml./100 g. of warm water preceded treatment. The compounds were given by intramuscular injection in doses equivalent to 8 mg. of Hg./kg., balmersal and balmercamph being given as solutions in distilled water and mersalyl and thiomerin as suitable dilutions of the commercially prepared injections with distilled water. A control group of animals for each test group was injected with a similar volume of saline and after 1 week's rest the control and test groups were reversed. The volume of urine excreted every 15 minutes from the appearance of the first drop was noted for the first 6 hours and also the total volume excreted in 24 hours. The animals were kept at a temperature of 18° to 22° C. to minimise as far as possible variations in renal flow due to temperature. Figure 1, giving the mean results of 4 such cross-over tests, shows that combination with dimercaprol does not affect the diuretic activity of mersalyl, balmersal being at least as active as mersalyl at this dose. Although there was some increase in urinary excretion in the first 6 hours in most of the groups, particularly from 4 to 6 hours, these differences were variable and the significant increase constituting the real diuretic effect occurred between 6 and 24 hours. These results are similar to those of Dicker[9] and Lipschitz, Hadidian and Kerpscar,[10] who reported a diuretic effect in rats about 10 hours after intramuscular injection of the mercurial compounds. Under the conditions of the test neither thiomerin nor balmercamph show definite diuretic activity.

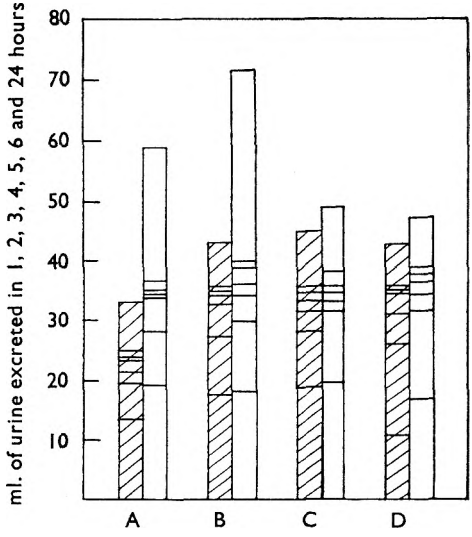

FIG. 1. Comparison of the diuretic activity of mersalyl, balmersal, thiomerin and balmercamph in rats. The results are the mean of 4 cross-over test with each compound. Cross hatch, control; open, test period.

Dose:—Equivalent of 8 mg. of Hg/kg. by intramuscular injection.

A. Mersalyl. B. Balmersal. C. Thiomerin. D. Balmercamph.

The dose range over which mercury compounds show diuretic activity in rats is a narrow one and in order to determine the effect of combination with dimercaprol on this range and to obtain a more quantitative comparison of the activity of the compounds, a modification of the method of bioassay of diuretics described by Lipschitz *et al*[10] was employed. Groups of 8 rats, of approximately equal weight were used, and hydrated with 2·5 ml./100 g. of saline solution instead of water. The volume of urine excreted in 24 hours after treatment was noted and the excretion expressed as a percentage of the liquid administered. The excretion was compared with that produced by a standard dose of urea. The dose of 25 millimols/kg., was selected since it produced an approximately similar diuretic effect. The "diuretic activity" was calculated as the difference between the logs of the excretion and the urea excretion. The curve relating the log dose to diuretic activity is shown in Figure 2. Diuretic activity increases with increasing dosage of mersalyl to a maximum at the dose corresponding to 8 mg. of Hg./kg. above which it rapidly decreases. With the dose 32 mg. of Hg./kg. complete oliguria occurred in some cases and all the animals treated with this dose died within a few days. The diuretic activity of balmersal is similar to that of mersalyl up to a dose equivalent to 8 mg. of Hg./kg. but the maximum activity occurs at 16 mg. of Hg./kg. and there is still activity at 32 mg. of Hg./kg. None of the animals treated with the latter dose died. The curve for thiomerin is similar, though the activity is lower than that shown by the other two compounds, the maximum activity shown by the dose corresponding to 16 mg. of Hg./kg. being less than the maximum for both mersalyl and balmersal. Diuretic

activity disappears at the dose corresponding to 32 mg. of Hg./kg. This dose was also toxic, all the animals dying in 3 days. No significant diuretic activity was obtained with balmercamph.

Diuretic activity in dogs. The activity of mercurials is potentiated by certain other drugs, particularly acidifying salts such as ammonium chloride (Keith, Barrier and Whelan[11]). The effect of the combination of the mercurials with dimercaprol on this property was studied in dogs.

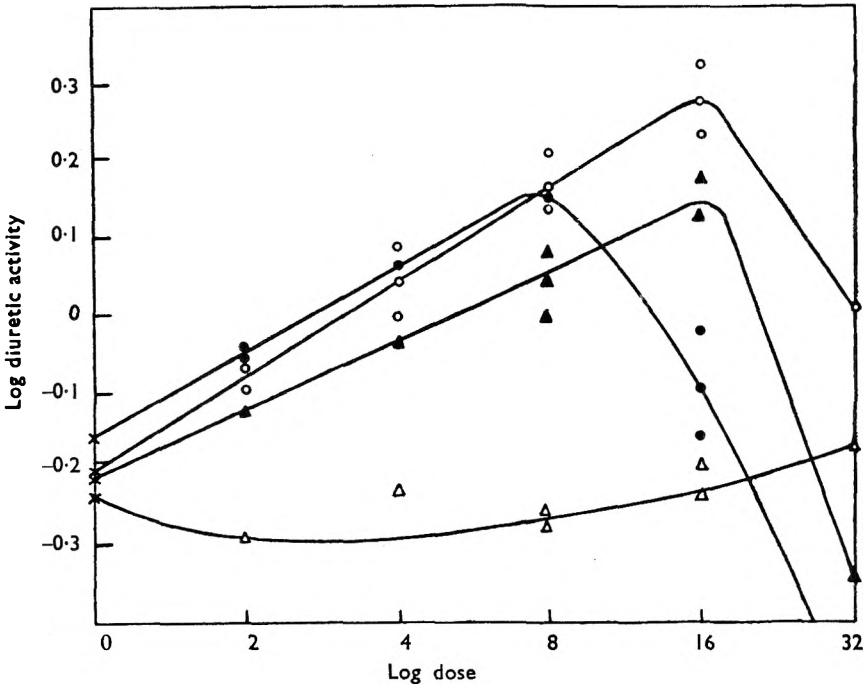

FIG. 2. Dose—response curves for diuretic activity of the 4 compounds in rats. The diuretic activity is measured as the difference between the logs of the excretion and the excretion after the standard dose of urea. × controls, ●—● mersalyl, ○——○ balmersal, ▲——▲ thiomerin, △——△ balmercamph.

The mercurial diuretics have been shown to be active in normal (Schloss[12]) and sometimes even in dehydrated dogs (Roby and Pfeiffer[13]). To ensure standard conditions the animals were deprived of food and water for 18 hours and then hydrated immediately before treatment with approximately 50 ml./kg. of milk and water. Bitches weighing approximately 15 kg. were used and they were catheterised immediately before, and at 2, 4, 6, and 24 hours after hydration, the volume of urine obtained at each period being noted. The excretion was expressed as a percentage of the volume of liquid administered. This constituted a control excretion period for each dog. After 2 days rest, the experiment was repeated, an intramuscular injection of the mercurial compound in a dose equivalent to 1·2 mg. of Hg./kg. being given immediately after hydration. After a further 3 days rest, the control and test periods were repeated, ammonium

chloride, 100 mg./kg., being given orally at the same time as the milk and water and also during the 2 days rest between the two periods. The results are given in Figures 3 and 4 and the diuretic effect calculated as the difference in percentage excretion between the test and the control period is given in Table I. A definite diuretic response was obtained with mersalyl, the onset of diuresis occurring between 2 and 4 hours and still

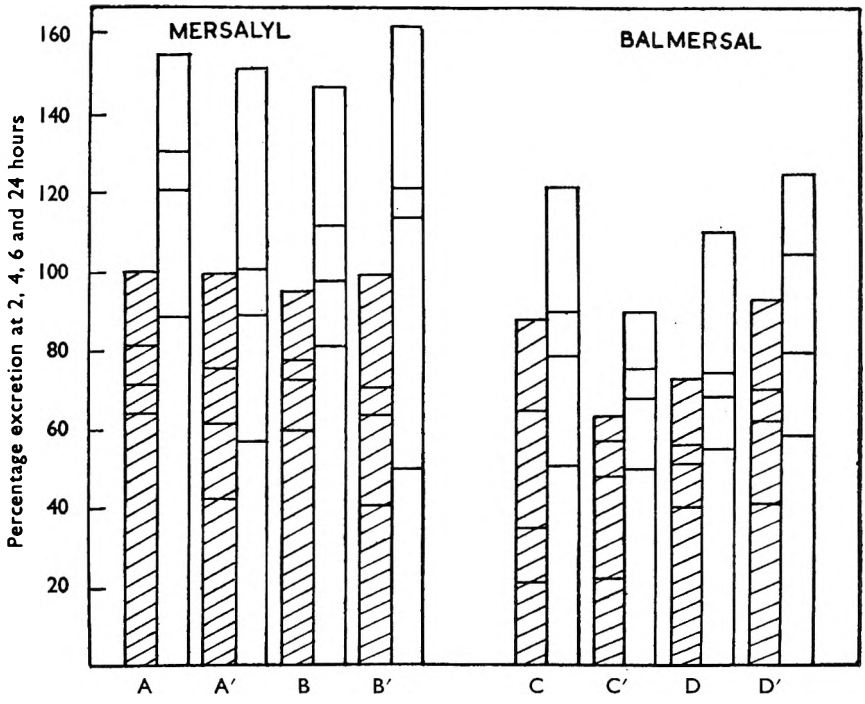

FIG. 3. Diuretic effect of mersalyl and balmersal in dogs, with a dose equivalent to 1·2 mg. of Hg./kg. given alone and after premedication with ammonium chloride. The excretion measured as the percentage of the liquid administered, is shown at 2, 4, 6 and 24 hours. Cross hatch, control; open, test period.

A. Dog 16. Mersalyl alone.
A′. ,, Mersalyl and ammonium chloride.
B. Dog. 14. Mersalyl alone.
B′. ,, Mersalyl and ammonium chloride.
C. Dog 16. Balmersal alone.
C′. ,, Balmersal and ammonium chloride.
D. Dog 30. Balmersal alone.
D′. ,, Balmersal and ammonium chloride.

being well marked in the 6 to 24 hour period. An overall increased effect was obtained when treatment was accompanied by premedication with ammonium chloride. The diuretic response with balmersal was similar although somewhat smaller than that obtained with mersalyl. The effect does not appear to be potentiated by simultaneous treatment with ammonium chloride. In one case (dog 43), a definite diuretic response was obtained with thiomerin, similar in magnitude to that obtained with an equivalent dose of mersalyl and the effect was increased to about the

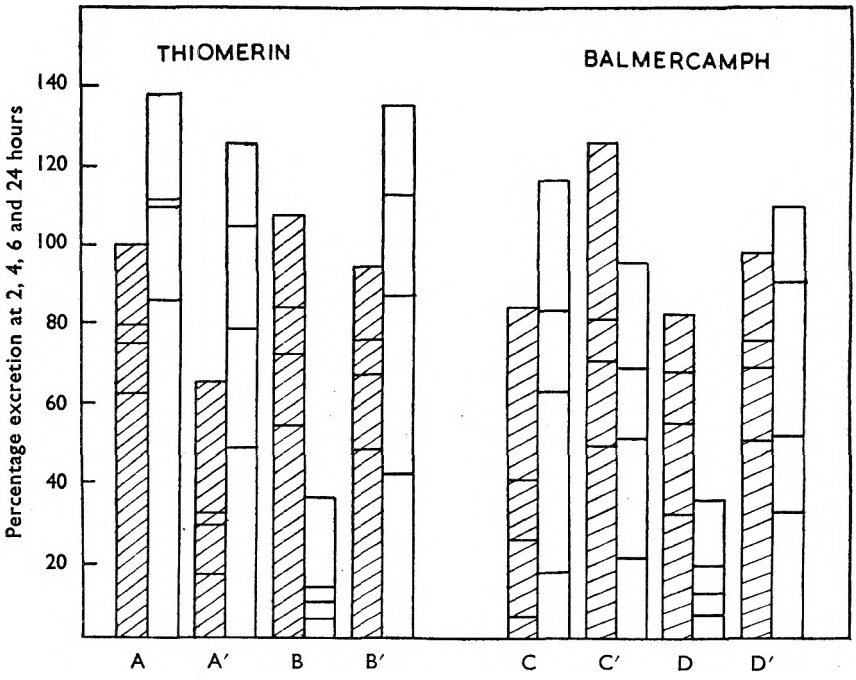

FIG. 4. Diuretic effect of thiomerin and balmercamph in dogs with a dose equivalent to 1·2 mg. of Hg./kg. given alone and after premedication with ammonium chloride. The percentage excretion at 2, 4, 6 and 24 hours is shown. Cross hatch, control; open, test period.

A. Dog 43. Thiomerin alone.
A′. ,, Thiomerin and ammonium chloride.
B. Dog 45. Thiomerin alone.
B′. ,, Thiomerin and ammonium chloride.
C. Dog 14 Balmercamph alone.
C′. ,, Balmercamph and ammonium chloride.
D. Dog 45. Balmercamph alone.
D′. ,, Balmercamph and ammonium chloride.

TABLE I

DIURETIC EFFECT IN DOGS AFTER A DOSE CORRESPONDING TO 1·2 MG. OF HG/KG. BY INTRAMUSCULAR INJECTION

Compound	Dog number	Diuretic effect expressed as difference in percentage excretion for control and test period	
		Drug alone	Drug + ammonium chloride
Mersalyl	16	49	66
	14	36	68
Balmersal	16	34	26
	30	37	32
Thiomerin ..	43	41	60
	45	− 71	40
Balmercamph ..	14	32	− 29
	45	− 46	12

same extent by previous and simultaneous treatment with ammonium chloride. In dog 45, a reduced urinary excretion was shown when the animal was first treated with the drug, similar to the effect obtained in rats treated with high doses of the compound, although the dose employed in

the test lies within the therapeutic range. The response when the test was repeated with ammonium chloride was normal. The slight increase in urinary excretion in dog 14 is probably not significant and the results with balmercamph confirm those obtained in rats, that the diuretic effect of the organic mercurial is lost by combination with dimercaprol.

Effect on electrolyte excretion. In addition to their diuretic effect, organic mercurials have a marked chloruretic effect (Kourilsky, Corre, Delcambre and Scordel,[14] Keith *et al.*[11]) and inhibit sodium tubular reabsorption causing as a result an increased sodium excretion (Farah,

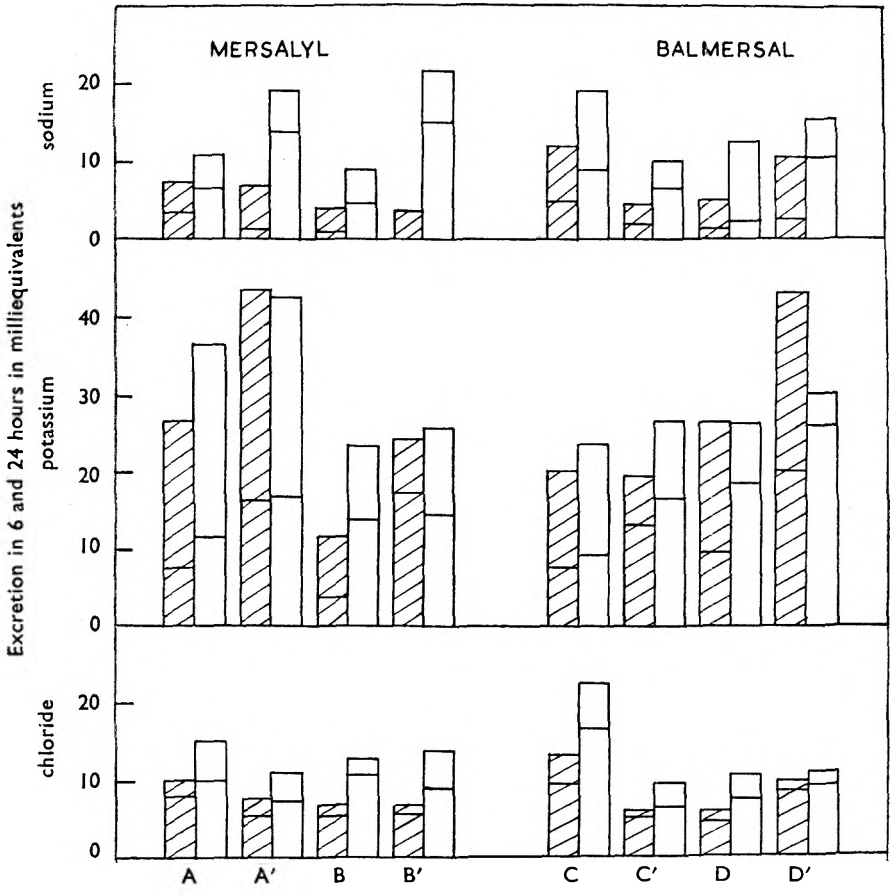

FIG. 5. Electrolyte excretion in dogs after mersalyl and balmersal, equivalent to 1·2 mg. of Hg./kg. intramuscularly, alone and with ammonium chloride. The total weight excreted, expressed as milliequivalents of Na, K and NaCl in 6 and 24 hours is shown. Cross hatch, control; open, test period.

A. Dog 16. Mersalyl alone.
A'. ,, Mersalyl and ammonium chloride.
B. Dog 14. Mersalyl alone.
B'. ,, Mersalyl and ammonium chloride.
C. Dog 16. Balmersal alone.
C'. ,, Balmersal and ammonium chloride.
D. Dog 30. Balmersal alone.
D'. ,, Balmersal and ammonium chloride.

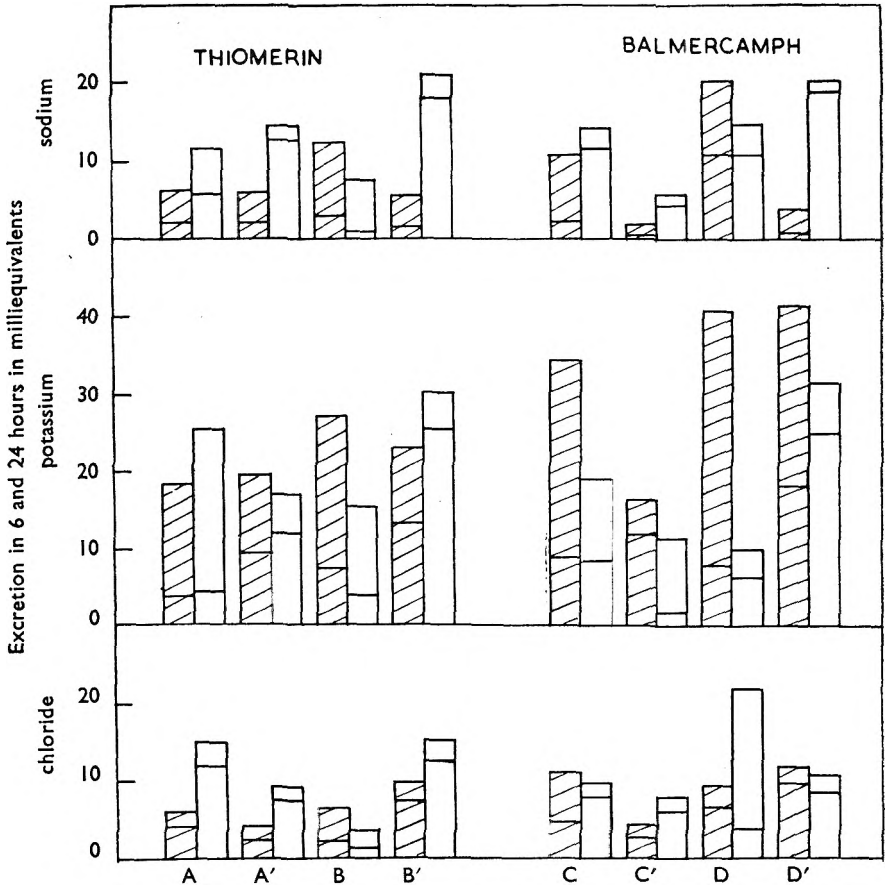

FIG. 6. Electrolyte excretion in dogs after thiomerin and balmercamph, equivalent to 1·2 mg. of Hg./kg. intramuscularly, alone and with ammonium chloride. The total weight excreted expressed as milliequivalents of Na, K and NaCl in 6 and 4 hours is shown. Cross hatch, control; open, test period.

A. Dog 43. Thiomerin alone.
A'. ,, Thiomerin and ammonium chloride.
B. Dog 45. Thiomerin alone.
B'. ,, Thiomerin and ammonium chloride.
C. Dog 14. Balmercamph alone.
C'. ,, Balmercamph and ammonium chloride.
D. Dog 45. Balmercamph alone.
D'. ,, Balmercamph and ammonium chloride.

Cobbey and Mook[15]). Blumgart, Gilligan, Levy, Brown and Volk[16] in their studies on the action of mercurial diuretics, also reported increased potassium and magnesium excretion but little or no effect on the excretion of phosphates and sulphates. Comparison of the effect of the compounds on electrolyte excretion is made in Figures 5 and 6. Balmersal, mersalyl and thiomerin all produce an increase in chloride and sodium excretion, the effect being similar whether the drug is given alone or with ammonium chloride. The effect on the potassium excretion is variable but there is no indication of a general tendency for the potassium excretion to increase.

Similarly no general increase of electrolyte excretion was found with balmercamph. Thus the variation in electrolyte excretion runs roughly parallel with the diuretic effect, the greatest increase being found where the diuretic effect is greatest.

Toxicity. In the course of the experiments on rats with varying doses of the compounds, indications were obtained that combination with dimercaprol had considerably reduced the acute toxicity of the mercurial derivatives. With the dose corresponding to 32 mg. of Hg./kg. all of the animals treated with mersalyl or thiomerin died while all those treated with balmersal and balmercamph survived. Two dogs were given balmersal in a dose equivalent to 4 mg. of Hg./kg. 3 times weekly for 6 weeks with very little general toxic effect, while a similar dose of mersalyl has been reported to cause acute nephrosis terminating in death within 2 weeks (Minatoya and Hoppe[17]). These results were confirmed quantitatively by determination of the LD50 intravenously in mice (Table II). In balmersal, the toxicity has been reduced to approximately one fifth that of mersalyl and that of balmercamph to about one sixth that of thiomerin.

TABLE II

ACUTE INTRAVENOUS TOXICITIES IN MICE

Compound	LD50 mg./kg.
Mersalyl	99
Balmersal	475
Thiomerin	79
Balmercamph	512

Renal toxicity. After absorption of mercury compounds, the mercury tends to become more concentrated in certain organs, particularly the kidney. Single large doses of mercury or smaller doses acting over a period result in necrotic changes in the tubular epithelium of the kidney (Burmeister and McNally[18]). The comparative renal toxicity after a single large dose and repeated smaller doses of the compounds was studied in rats, by determination of the amount of proteinuria produced and by histological examination of the kidneys at varying periods of time after treatment. The resultant proteinuria in groups of 4 rats after a single injection of the compounds equivalent to 20 mg. of Hg./kg. and after two 5-day periods of treatment with a dose equivalent to 4 mg. of Hg./kg. once daily is shown in Table III. A single high dose of thiomerin caused a very marked proteinuria, a 15- to 20-fold increase in the normal protein excretion being produced in the first 4 days. The protein excretion then gradually decreased and had returned to normal in about 14 days. No significant increase in protein excretion was observed with balmercamph. Except on the first day, only a 4- to 5-fold increase in protein excretion was obtained with balmersal and approximately normal values from the twelfth day onwards. The dose for mersalyl was beyond the diuretic range and almost complete anuria resulted. Although insufficient urine was obtained for quantitative analysis, qualitative tests indicated that a very high protein concentration was present. The results with repeated smaller doses are similar, a 2- to 3-fold increase was obtained with thiomerin, the effect being somewhat less with balmersal and greater with mersalyl. Protein excretion returned to normal when treatment was stopped. No significant increase in proteinuria was obtained with balmercamph.

TABLE III

PROTEIN EXCRETION (mg./100 g. rat) IN RATS AFTER TREATMENT WITH MERCURIAL DIURETICS BY INTRAMUSCULAR INJECTION

Day	Single dose equivalent to 20 mg. of Hg/kg.				4 mg. of Hg/kg. for 2 × 5 day periods				Control
	Mersalyl	Balmersal	Thiomerin	Balmercamph	Mersalyl	Balmersal	Thiomerin	Balmercamph	
− 3 to 0 (mean)	1·09	0·71	0·92	1·45	0·80	1·20	1·30	1·12	1·18
1	Anuria	7·23	15·67	4·1	2·87	1·84	—	1·11	0·75
2	,,	3·34	22·0	0·94	4·88	2·4	2·7	0·88	0·45
3	,,	2·34	27·75	1·22	6·3	1·84	3·3	0·6	1·73
4	All dead	4·68	15·28	0·61	1·13	1·52	1·15	1·06	0·93
5	—	3·06	5·12	0·52	2·63	0·61	2·03	2·71	0·95
6	—	2·0	5·65	0·89	4·32	1·72	2·03	0·99	1·1
7	—	2·0	5·6	0·88	—	1·85	—	1·08	0·86
8	—	3·3	5·43	1·26	6·49	1·29	1·1	0·85	0·34
9	—	Insufficient	2·19	2·11	—	1·72	1·47	1·03	0·72
10	—	,,	5·13	1·22	2·11	0·97	1·31	0·81	0·71
11	—	1·73	4·11	0·92	—	0·74	2·5	0·67	0·66
12	—	Insufficient	2·35	1·08	1·91	—	1·13	0·57	0·37
13	—	1·19	Insufficient	0·51	1·15	0·92	0·52	0·57	0·78
14	—	—	—	—	—	—	—	—	—
15	—	Insufficient	2·69	0·62	0·81	1·2	1·2	0·25	0·98
16	—	,,	1·49	1·10	0·87	1·78	1·73	0·77	0·96
17	—	,,	Insufficient	2·75	1·0	0·74	0·93	0·36	0·99
18	—	,,	,,	0·75	2·8	0·98	1·62	1·49	0·39
19	—	0·48	0·25	0·40	1·65	0·56	1·28	0·55	0·68
20	—	0·42	Insufficient	0·56	0·65	0·53	1·71	1·26	0·79
21	—	0·19	0·69	0·81	0·66	0·68	1·29	1·31	0·47
22	—	0·97	1·03	0·41	1·66	1·45	1·56	0·59	0·65
23	—	0·48	0·46	0·26	0·28	2·07	1·64	0·67	1·03
24	—	—	—	—	1·48	1·75	1·56	1·44	2·13

The progressive effect of the renal lesions was followed histologically and confirmed the observations made from the protein excretion. The kidneys were examined 24 hours, 1 week and 4 weeks after the single injection, 24 hours after the 5 and 10 repeated injections, and 1 and 4 weeks after the 10 injections. 24 hours after the single injection equivalent to 20 mg. of Hg./kg., severe acute nephrosis with damage to the cortical tubules and production of hyaline and granular casts, was seen in the animals given mersalyl, balmersal and thiomerin. There was some localised severe damage with balmercamph, but nothing like that produced with the other three compounds. The animals dying on the fifth day after treatment with mersalyl still showed similar severe damage. After 1 week the kidneys from the animal receiving thiomerin still showed generalised dilation of the cortical tubules with many granular and hyaline casts. There was a similar moderate generalised dilatation in the case of balmersal but the damage was less severe. In the case of balmercamph, no gross changes were observed. After 4 weeks, recovery was complete in all cases and the kidneys appeared normal, except for occasional patchy plasma cell infiltration and a slight generalised œdema.

5 daily injections of mersalyl, equivalent to 1 mg. of Hg./kg., produced acute cortical degeneration with large irregular nuclei in the damaged tubules. Similar areas of disintegration of the cells of the convoluted tubules forming granular cases were found with thiomerin. The damage

with balmersal was somewhat similar but less severe, fewer than 10 per cent. of the tubules being affected. 5 injections of balmercamph appeared to have no effect on the kidney, but after a further 5, occasional single cortical tubules showed a swelling of the epithelium and pyknosis of the nuclei. 1 week after the end of treatment, granular casts were still seen in the convoluted tubules of the animal treated with mersalyl and disintegration of the cells and nuclei from those treated with thiomerin but

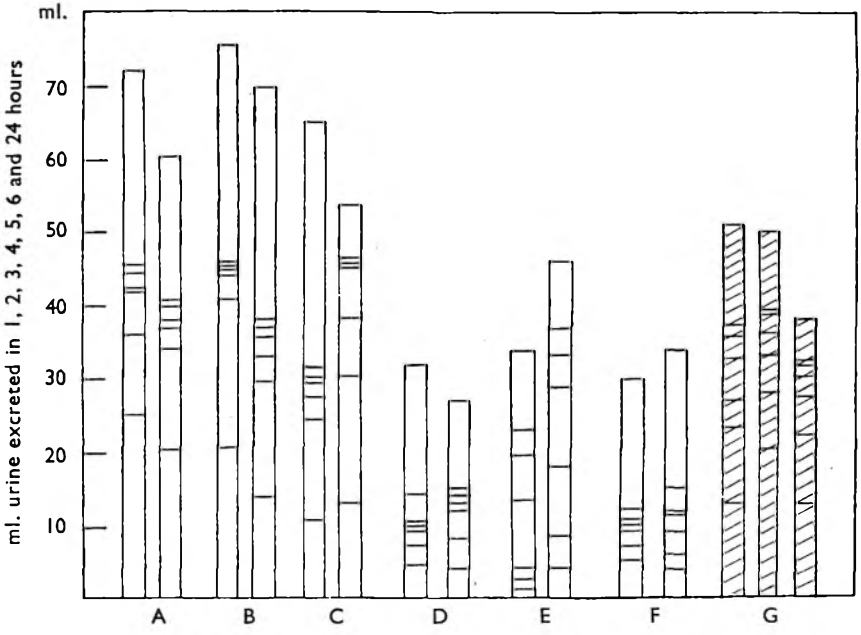

FIG. 7. Diuretic effect of mersalyl, 8 mg. of Hg./kg. administered intramuscularly with 1, 2, 5, 10 and 50 times the molecular equivalent of dimercaprol.

A. Mersalyl.
B. Mersalyl and dimercaprol 1 : 1.
C. ,, ,, 1 : 2.
D. Mersalyl and dimercaprol 1 : 5.
E. ,, ,, 1 : 10.
F. ,, ,, 1 : 50.
G. Controls.

those given balmersal and balmercamph were more or less normal. After 4 weeks, recovery was complete and the kidneys were normal in all cases.

Local toxic action. Mercury exerts a local toxic effect at the site of contact with the tissues, and the local irritant effect of mercurial diuretics when given intramuscularly or subcutaneously is well known. One of the advantages of thiomerin is that it shows a reduced local toxic effect and appears to be satisfactorily absorbed from subcutaneous tissue. The relative local irritant action of the compounds was compared in rats. The sites after 3 consecutive daily subcutaneous injections into a shaved area of approximately 1 sq. in. was examined. Balmersal and balmercamph showed an irritant effect similar to that of mersalyl; with all 3 compounds it was more marked than with thiomerin.

Mechanism of diuretic action. Simultaneous administration of mercurial diuretic and dimercaprol has been reported to eliminate the diuretic effect (Farah and Maresh[19]). In view of the well maintained diuretic activity of balmersal the effect of simultaneous administration of mersalyl and dimercaprol in the test conditions described, was examined. Mersalyl, equivalent to 8 mg. of Hg./kg., was given by intramuscular injection simultaneously with doses of dimercaprol corresponding to 1, 2, 5, 10 and 50 times the molecular equivalent. The results of the qualitative comparison are given in Figure 7. Mersalyl and dimercaprol when given in equivalent amounts showed a diuretic effect similar to that of mersalyl itself. This effect was abolished completely when 10 times the equivalent of dimercaprol was given and intermediate results were obtained in the other molecular proportion. The time of diuresis was also similar, the bulk of the increased urinary excretion occuring between 5 and 24 hours. Quantitative measurement of the diuretic action by comparison with urea, confirmed these results (Fig. 8).

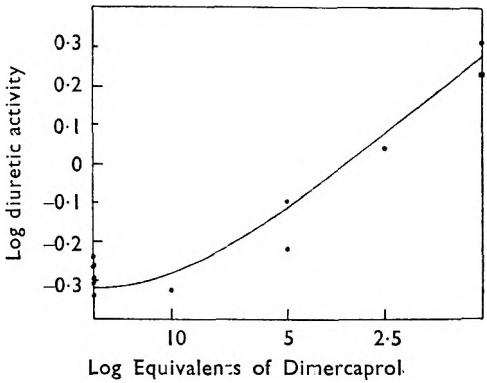

FIG. 8. Dose-response curve for diuretic action of mersalyl, 8 mg. of Hg./kg., in rats, administered with varying molecular proportions of dimercaprol.

It has been suggested that the diuretic effect of the mercurials may be due to inactivation of one or more enzyme systems. Hatta[20] showed that mercuric chloride inhibited many proteolytic enzymes and that the inhibition could be reversed by substances such as potassium sulphide which precipitated the mercury, and studies with the radio-active metal have confirmed that protein binding of the mercury occurs in the body. The findings of Fawaz and Fawaz[21] that mersalyl in therapeutic doses has no effect on the succinic oxidase activity of cortical homogenates, indicate that mercurials may react with proteins other than through the ·SH groupings. The results with dimercaprol suggest that when only equivalent amounts are administered, the mercury reacts preferentially with the protein, producing the usual diuretic effect, but when increasing amounts are given, a mass action effect is obtained and the dimercaprol binds the mercury more firmly than do the tissues. Attempts to find evidence for an extra-renal action of the compounds by determination of the effect on the concentrations of electrolytes in the blood gave inconclusive results. Measurements of the concentration of sodium potassium and chloride ions showed no significant alteration after injection of any of the compounds.

Discussion and Conclusions

From the standpoint of practical value in replacing present mercurial diuretics with less toxic drugs, the compounds with dimercaprol do not appear to provide the complete answer. Because of the greatly reduced acute toxicity and some modification of the renal toxicity with maintenance of an equivalent diuretic effect, balmersal may be of some value. The effects on cardiovascular responses have still to be determined. From the theoretical point of view, the experiments on renal toxicity lend some support to the theory that the mercurials owe their diuretic activity at least in part to a mild tubular irritation which inhibits tubular reabsorption. In the case of balmercamph where combination with dimercaprol has practically eliminated renal toxicity, little or no diuretic effect is observed, while with balmersal where some nephrotoxic action is still maintained, diuretic activity is still present.

The equivalence of the diuretic effect of mersalyl and balmersal suggests that the reaction with mersalyl and dimercaprol is a reversible one and that balmersal is slowly decomposed in the body leaving the mercury free to exert its diuretic effect. The results with mersalyl and varying equivalents of dimercaprol confirm this, since the diuretic effect is gradually abolished when increasing amounts of dimercaprol are administered. These preliminary results also suggest that mercury may react with proteins other than through the SH-groupings but further work with varying doses of mercury and dimercaprol and observations on other enzyme systems and specific inhibitors is necessary to confirm this.

Summary

1. The two compounds formed by combination of 1 molecule of the dithiol, 2:3-dimercaptopropanol (dimercaprol) with 2 molecules of the organic mercury compounds, mersalyl and γ-hydroxymercuri-β-methoxypropylcamphoramide (mercurophyllin U.S.P.) are called respectively balmersal and balmercamph.

2. The diuretic effect of these compounds is compared in rats and dogs with that of mersalyl and thiomerin, a monothiol derivative of mercurophylline and thioglycollic acid. The diuretic activity of balmersal is at least equal to that of mersalyl, but the introduction of the second thiol group in balmercamph abolishes the diuretic effect.

3. The effect of the compounds on electrolyte excretion is studied in dogs.

4. Measurements of the intravenous LD50 in rats, show that the acute toxicity is considerably reduced by combination of the organic mercurials with dimercaprol. Some reduction of the renal toxicity also occurs.

5. Administration of increasing equivalents of dimercaprol to rats reduces the diuretic activity of mersalyl, suggesting that the combination of mersalyl and dimercaprol *in vivo* is a reversible reaction.

6. The significance of these results in the interpretation of the mechanism of the diuretic action of organic mercury derivatives is discussed.

I wish to thank Mr. Sharp of the Wellcome Laboratories of Tropical Medicine for his co-operation and the preparation of the compounds with dimercaprol, Dr. David Trevan for the preparation and examination of the histological preparations of the kidneys and Miss A. Facey for valuable technical assistance.

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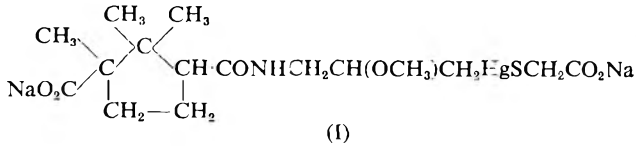
A NOTE ON THE PREPARATION OF COMPOUNDS OF MERCURIAL DIURETICS WITH 2:3-DIMERCAPTOPROPANOL (DIMERCAPROL)

By T. M. Sharp

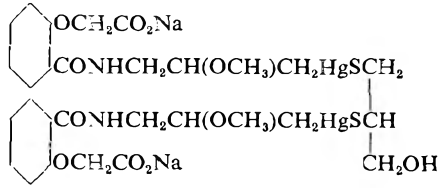
Lehman[1] has shown that the acute toxicity of mercurial diuretics is greatly reduced by combining the mercury with monothiols such as thioglycollic acid. One of these is now in clinical use in the United States of America under the names thiomerin and mercaptomerin.[1] It was thought that a similar or greater reduction in toxicity might be attained by combining



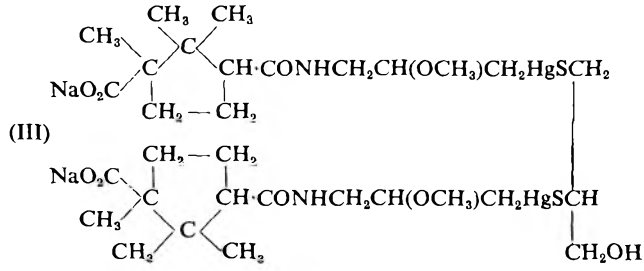
(I)

known mercurial diuretics with dimercaprol, 2:3-dimercaptopropanol, which is a well established antidote for heavy metal poisoning. It is now shown that 2 mols. of mersalyl will combine readily in alkaline solution

(II)



with 1 mol. of dimercaprol to form the disodium salt of 2:3-bis(3′-*o*-carboxymethoxybenzamido-2′-methoxypropylmercurimercapto)-propane-1-ol (II) for which the trivial name balmersal is suggested. Similarly γ-hydroxymercuri-β-methoxypropylcamphoramide (mercurophyllin, U.S.P.) yields 2:3-bis(3′-camphoramido-2′-methoxy mercurimercapto)-propane-1-ol (III) (balmercamph).



(III)

Experimental

Balmersal (II). Salicyl(γ-hydroxymercuri-β-methoxypropylamide)-*O*-acetic acid (19·35 g., 2 mols.) was dissolved in an equivalent amount of N sodium hydroxide. To the strongly alkaline solution 2:3-dimercaptopropanol (2·486 g., 1 mol.) was added slowly with stirring. A slight rise in temperature took place with drop in *p*H to about 8·0. After standing for 1 hour the disodium salt of 2:3-bis-(3′-*o*-carboxymethoxybenzamido-2′-methoxypropylmercurimercapto)-propane-1-ol could be precipitated as a hygroscopic powder by pouring into a large volume of acetone, but it was found preferable to isolate the salt by freeze-drying, when it was obtained as a colourless powder with no definite m.pt.

Found: Hg, 35·9; S, 5·9; $C_{29}H_{36}O_{11}N_2S_2Hg_2Na_2$ requires Hg, 36·5; S, 5·8 per cent.

Balmercamph (III). γ-Hydroxymercuri-β-methoxypropylcamphoramide (4·86 g., 2 mols.) was treated as above with N sodium hydroxide and 2:3-dimercaptopropanol (0·62 g., 1 mol.). The solution was diluted with 4 vol. of water and left in the dark for about a week during which time it slowly deposited a small amount of white insoluble material which was not investigated further. After filtration the solution was freeze-dried to yield the disodium salt of 2:3-bis-(3′-camphoramido-2′-methoxymercurimercapto)-propane-1-ol as a colourless powder with no definite m.pt.

Found: Hg, 35·0; S, 5·92; $C_{31}H_{50}O_9N_2S_2Hg_2Na_2$ requires Hg, 36·3; S, 5·8 per cent.

Mercury was estimated by the U.S.P. method for mercurophylline, and sulphur by the Carius method.

The author thanks Messrs. K. H. Pratley and A. G. Turner for technical assistance.

Summary

Mersalyl and mercurophylline combine with 2:3-dimercaptopropanol in the proportion of 2 mols. of the mercury compound to one of the dithiol.

Reference

1. Lehman, *Proc. Soc. exp. Biol., N.Y.*, 1947, **64**, 428. U.S.Pat. 2,576,349–49.

DISCUSSION

The paper was presented by DR. E. I. SHORT.

MR. A. F. CALDWELL (Singapore) said that as mercury diuretics were frequently used in cases involving kidney damage it would be of great value if the risk of further harm to the kidney could be reduced without affecting the diuretic effect.

VOTE OF THANKS

The Chairman's proposition that a hearty vote of thanks be accorded to all the authors of the papers was carried with acclamation, as was a vote of thanks to the Editor of the *Journal of Pharmacy and Pharmacology* for advance proofs of the papers.

Correction.

THE PURITY OF VITAMIN B_{12}

BY J. G. HEATHCOTE.

This Journal, 1952, **4**, 643.

TABLE I

For *Cyanocobalamin* (*Vitamin* B_{12b}) read *Cyanocobalamin* (*Vitamin* B_{12}).
For *Hydroxycobalamin* (*Vitamin* B_{12d}) read *Hydroxycobalamin* (*Vitamin* B_{12b}).

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Aspirin, Phenacetin and Caffeine, Analysis by Partition Chromatography. T. Higouchi and K. P. Patel. (*J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 171.) A solvent combination of water and a nonpolar solvent was used, since caffeine has a definite affinity for water and considerably less affinity for nonpolar solvents such as ethers, whereas the contrary is true for phenacetin. Water was used as the stationary phase and the elution curve showed that it was possible to obtain a clean separation of the two components. Silica gel was used for the column and a chloroform solution containing phenacetin and caffeine was added. Elution with a 75:25 di*iso*propylether-chloroform mixture removed the phenacetin, the caffeine component which remained on the column being then stripped out by elution with chloroform. Recovery data for both constituents were satisfactory. If aspirin was present in the samples it was removed first by extraction of the chloroform solution with a mildly alkaline solution, the aspirin being determined separately. Typical analyses of commercial preparations are reported. R. E. S.

Atropine, Photometric Determination of. A. Romeike. (*Pharm. Zentralh.*, 1952, **91**, 80.) The vegetable material is dried at 60° to 70° C., and powdered. A weighed portion of the powder is moistened with ammonia solution (5 per cent.), transferred to a Soxhlet thimble, and extracted for several hours with chloroform. The extract is concentrated to about 1.5 ml. and shaken out twice with 5 ml. quantities of hydrochloric acid (0·4 per cent.). If the amount of alkaloid expected is very small, the amount of acid should be reduced to one-half. The alkaloid is precipitated with 3 ml. of 1·7 per cent. solution of sodium silicomolybdate, allowed to stand for 24 hours, filtered on a sintered glass filter, and washed with small quantities of 0·5 per cent. hydrochloric acid containing 1 per cent. of sodium chloride. The residue is dissolved in 10 ml. of reduction solution (glycine, 0·5; sodium sulphite, 1·5; water 15; 5 per cent., ammonia, 83) and made up to a definite volume. The absorption is then determined using filter S72. The standardisation graph is straight between 61 and 40 per cent. absorption, and the minimum concentration of atropine at which a satisfactory determination is possible is 7·7 mg./100 ml. Seeds should be defatted with petroleum ether, after drying and powdering. G. M.

Heavy Metals in Pharmaceutical Chemicals. F. N. Stewart and C. W. Strode, Jr. (*J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 242.) Heavy metals are determined as sulphides by colour measurements under standardised conditions with compensation for sample colour and adjustment of the *p*H to 3 to 4 before addition of hydrogen sulphide, acetone being used to decrease the solubility of metal sulphides, while increasing that of some of the organic compounds to be tested. 50 ml. of the test solution is placed in each of 2 graduated cylinders, 25 ml. of acetone is added and the *p*H adjusted to 3·5. To one cylinder is added 20 ml. of a saturated solution of hydrogen sulphide, and the volume in each cylinder is adjusted to 100 ml. by the addition of water. After 10 minutes, the

light absorption of the solutions is compared, using a photoelectric colorimeter and a 420 mμ broad band filter. The content of heavy metals, calculated as lead, is found from a curve prepared by similarly treating quantities of U.S.P. standard lead solution and plotting the light absorption against lead content. For weak acids, the test solution is prepared by dissolving a sample in 50 ml. of acetone and adding sufficient water to produce 100 ml. Water-soluble neutral compounds are dissolved in 40 ml. of water, 8 ml. of acetic acid (6 per cent.) and 50 ml. of acetone added, and made up to 100 ml. with water. Salts of weak acids are treated with a few ml. of water and an equivalent of hydrochloric acid, and acetone and water added. A wet-ashing process is recommended for organic compounds which are deeply coloured or which react with hydrogen sulphide. Recovery of added lead in the experiments was accurate to within 2 p.p.m. in tests on a variety of pharmaceutical chemicals. Experimental results with and without compensation for colour of sample are compared. G. B.

Histamine, Gravimetric Determination of. M.Schmall, E. G. Wollish and J. Galender. (*J. Amer. pharm. Ass. Sci. Ed.*, 1952, **41**, 138.) The following method is recommended. Dissolve about 100 mg. of histamine dihydrochloride or 150 mg. of histamine phosphate in 10 ml. of water and add slowly, while stirring, 10 ml. of a 3·5 per cent. solution of nitranilic acid in ethanol (95 per cent.). Allow to stand for 15 minutes, add 10 ml. of ethanol (95 per cent.), leave in a refrigerator for 3 hours, filter, wash the precipitate with cold ethanol followed by ether, dry at 120° C. for 2 hours and weigh. Each g. of precipitate is equivalent to 0·5395 g. of the dihydrochloride or 0·9009 g. of the phosphate. Ointments may be warmed with chloroform-light petroleum mixture and extracted twice with water, the extracts being separated by centrifuge and assayed. The precision is about ± 1·0 per cent. on the parent substances and ± 2·0 per cent. on pharmaceutical preparations. During the assay of histamine phosphate the proportion of ethanol should not exceed 60 per cent., or histamine phosphate may be precipitated. Glycine and histidine give precipitates with nitranilic acid and consequently their absence should be confirmed by the ninhydrin reaction. G. B.

Papaverine and Phenobarbitone in Compound Preparations Potentiometric Titration of. P. Roland. (*J. Pharm. Belg.*, 1952, **7**, 86.) Titrations were performed in ethanol (75 per cent.) to avoid the formation of precipitates, the ethanol concentration being adjusted to 75 per cent. at the end of the titration so as to obtain a sharper end-point for phenobarbitone. Nitrogen was used to stir the solutions and prevent absorption of carbon dioxide by alkaline solutions. For the assay of pills, a sample containing 0·1 to 0·2 g. of papaverine hydrochloride and 0·15 to 0·25 g. of phenobarbitone was triturated with calcium carbonate and water to a paste, dried with anhydrous sodium sulphate, powdered and extracted with chloroform. The residue after drying the chloroform extract was dissolved in ethanol and titrated with 0·1N hydrochloric acid (one end-point due to papaverine). The solution was back-titrated with 0·1N sodium hydroxide (first end-point due to papaverine and second to phenobarbitone). The papaverine content was calculated from the mean of the two titrations. If the chloroform extract was coloured owing to the presence of liquorice as an excipient, a correction was applied to the phenobarbitone titration. Satisfactory results were obtained on the pure substances and tablets, without the preliminary extraction treatment. The method was extended to a determination of mixtures of phenobarbitone and methylphenobarbitone, based on the reaction of these substances with 2 and 1 molecules of silver nitrate respectively. G. B.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Insulin, Maximum Molecular Weight of. J. M. Creech. (*Nature, Lond.*, 1952, **170**, 210). The sedimentation and diffusion constants of the untreated crystalline protein have been redetermined. In phosphate buffer, *p*H 7·4, the mean of 7 determinations in the Spinco ultracentrifuge gave $S_{20,w} = 3{\cdot}12 \pm 0{\cdot}02$ (S.D.) Svedberg units. This value was independent of concentrations in the range 0·4 to 0·8 per cent. of temperature in the range 17° to 26° C., and of ionic strength in the range 0·10 to 0·30. A table is given of diffusion coefficients, determined with the Gouy diffusiometer at *p*H 7·4 and ionic strength 0·10 together with notes on the method of calculation used; calculation of D*m* for the most concentrated solution gave 8·16. This work provides qualitative confirmation for the dissociation phenomena reported from osmotic measurements by Gutfreund (*Biochem. J.*, 1948, **42**, 156 and 544), for concentrations lower than about 0·3 per cent., but gives a different value for the molecular weight in the stability range. Thus, using the value s = 3·12, D = 8·2, application of the Svedberg equation gives M = 34·800 or 36,700 according to the value of $\bar{v}$ used. The value of 35,000 to 36,000 refers to the maximum molecular weight of insulin as it exists in solution (investigations at *p*H 7·0 and 7·2 gave similar results) agreeing with the solid molecular weight (from X-ray determination) of 36,000. R. E. S.

Radioactive Penicillin, Preparation of. E. L. Smith and D. J. D. Hockenhull. (*J. app. Chem.*, 1952, **2**, 287.) Details of the preparation biosynthetically of crystalline radioactive penicillin with a specific activity of over 1 c./g. are given. The medium was a modification of that recommended by Jarvis and Johnson (*J. Amer. chem. Soc.*, 1947, **69**, 3010), the sulphur content being reduced to 0·1 mg. of sulphur per ml., and 0·1 per cent. of phenylethylamine hydrochloride being included as a benzylpenicillin precursor. One preparation gave 2·2 mg. of crystalline penicillin at 0·74 μc. per unit (1·23 c./g.); it was purified by 2 steps of solvent extraction, followed by crystallisation as the *N*-ethylpiperidine or *cyclo*hexylamine salt. R. E. S.

BIOCHEMICAL ANALYSIS

Acetone Bodies, Estimation of. C. Thin and A. Robertson. (*Biochem J.*, 1952, **51**, 218.) The method depends on the development of an orange to red coloration when an alkaline solution of salicylic aldehyde is left in the presence of acetone, the depth of colour formed being directly proportional to the amount of acetone present; the test can be used to estimate free acetone, the acetone formed by hydrolysis of acetoacetic acid, and that formed by chromic acid oxidation of β-hydroxybutyric acid and of *iso*propanol. Details of experimental procedure are given together with standard calibration curves, and a description of the method as applied to biological materials such as blood, milk, urine and rumen liquor. Recovery experiments using ethyl acetoacetate purified by distillation under reduced pressure gave results within ±4 per cent. of the theoretical amounts. Acetic acid, lactic acid, sodium chloride, cholesterol, urea, and formaldehyde did not interfere with the estimation, but acetaldehyde in concentrations as low as 3 mg./100 ml. reacted with the colour reagent to give a slightly opaque orange solution, which was sufficient to prevent an accurate determination even of total ketones. R. E. S.

CHEMOTHERAPY

Mersalyl Analogues, Synthesis of. W. O. Foyre, H. M. Kotak and J. J. Hefferren. (*J. Amer. pharm. Ass., Sci. Ed.*, 1952, **41**, 273.) The following analogues of mersalyl, in which naphthalene or thiophene is substituted for the benzene nucleus and in which sulphonamide linkages sometimes replace the carbonamide type, were prepared:—(I) 2-oxyacetic acid 3-[*N*-(γ-acetoxymercuri-β-ethoxypropyl)] naphthoylamide, (II) 2-[*N*-(γ-acetoxymercuri-β-methoxypropyl)] thiophenesulphonamide, (III) 2-carbethoxy-5-[*N*-γ-(acetoxymercuri-β-methoxypropyl)] thiophenesulphonamide and (IV) 2-carboxy-5-[*N*-(γ-hydroxymercuri-β-methoxypropyl)] thenoylamide. Mersalyl was prepared by treating allyl*iso*cyanate with salicylic acid to form *N*-allylsalicylamide, reaction with chloracetic acid and subsequent treatment with mercuric acetate. The 2-hydroxy-3-naphthoic acid analogue was similarly prepared but using the acid chloride to form the amide, as a better yield was obtained than with the free acid. Compound (III) was prepared from the allylamide of 2-thiophenesulphonic acid. This compound was not soluble in alkali, and α-mercuration of the ring took place at elevated temperatures. Compound (III) was prepared because of the difficulties experienced with compound (II); it was soluble in dilute alkali, but it was not possible to hydrolyse the ester without affecting the mercury linkage. Compound (IV) was obtained from the half-ester of dicarboxythiophene, formation of the allylamide and treatment to give the mercury derivative. The ester was readily hydrolysed to the free acid which contained a solubilising group and was soluble in dilute sodium carbonate solution. The compounds exhibited diuretic activity but only compound (I) was sufficiently soluble to be administered by intravenous injection. G. B.

PHARMACY

NOTES AND FORMULAE

Ethyl Biscoumacetate (Tromexan Ethyl Acetate). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1952, **149**, 277.) Ethyl biscoumacetate is 3:3′-carboxymethylene bis-(4-hydroxycoumarin) ethyl ester, and occurs as a white, odourless, bitter, crystalline solid, m.pt. 177° to 182°, or 154° to 157°; soluble in acetone and benzene, slightly soluble in ethanol and ether, and insoluble in water. When warmed with sulphuric acid, an orange colour forms which is unaffected by ammonia but turns yellow on the addition of sodium hydroxide. A solution in ethanol becomes reddish-brown on the addition of ferric chloride. When treated with sodium hydroxide and allowed to stand for an hour, the odour of iodoform is produced on the addition of iodine. On double extraction with ammonia, it leaves not more than 1·4 per cent. of a residue of 3:3′-carboxymethylene bis-(4-epoxycoumarin) ethyl acetate, after washing with water and drying at 105° for 2 hours. The difference between 100 and the sum of the percentage of this impurity and the percentage of ethyl biscoumacetate found in the assay is the amount of 3:3′-carboxymethylene bis-(4-hydroxycoumarin) present. Ethyl biscoumacetate loses not more than 0·3 per cent. of its weight when dried at 105° for 4 hours, and leaves not more than 0·1 per cent. of residue on ignition. It contains 96·0 to 100·0 per cent. of ethyl biscoumacetate and is assayed by distilling a solution in aqueous sodium carbonate with strong sodium hydroxide, treating the distillate with potassium dichromate and sulphuric acid and estimating the excess of potassium dichromate iodometrically. Ethyl biscoumacetate is an anticoagulant. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

o-Aminophenol in Experimental Tuberculosis. B. Croshaw. (*Nature, Lond.*, 1952, **169**, 966.) Using an *in vitro* floating pellicle method with *Mycobacterium tuberculosis* var. *hominis H37Rv* and a modified Long's medium with 10 per cent. of ox serum, a dilution of 1 in 729,000 of *o*-aminophenol inhibited growth for 4 weeks. Mice were treated with 2 or 4 mg./20 g. mouse/day for 2 to 23 days after infection with Revenel strain bovine tubercle bacilli and the macroscopic extent of lung lesions examined in survivors. Guinea-pigs infected with H418 strain of human tubercle bacilli were treated with 50 mg. of *o*-aminophenol/500 g. guinea-pig/day starting 21 days after infection and continuing for 60 days. The drug did not retard tuberculosis *in vivo*, but caused tissue damage at the site of injection, which did not occur with streptomycin, used as an active drug control. G. B.

Chloramphenicol, Aplastic Anæmia Following Prolonged Administration. L. E. Wilson, M. S. Harris, H. H. Henstell, O. O. Witherbee and J. Kahn. (*J. Amer. med. Ass.*, 1952, **149**, 231.) In a study of the effect of chloramphenicol in the control of chronic bronchopulmonary suppuration, 62 patients ranging in age from 1 year to 72 years were given doses of 1 g. twice or 3 or more times weekly for periods of from 1 month to 14 months, after an initial period of more intensive antibiotic therapy, which included penicillin and streptomycin. Two of the patients, both female, developed aplastic anæmia. One of these, who subsequently died, received a total of 56 g. of chloramphenicol over a period of 7 months; the other received 52 g. over $5\frac{1}{2}$ months. Early untoward symptoms included heavy epistaxis, which occurred after 5 months and 3 months respectively, profuse menstruation, and purpuric manifestations. During the administration of chloramphenicol, blood cell counts, including studies of stained smears, should be carried out twice weekly. The drug should be discontinued when the level of granulocytes, erythrocytes, or thrombocytes falls below normal. G. R. K.

Digitalis, Comparison of the Biological Activity of Leaves of Various Species. P. Duquenois. (*Ann. pharm. franç.*, 1952, **10**, 177.) Plants were grown on chalky soil of *p*H 7·2 to 7·3, without manure. Leaves of all the species were collected at the time of flowering, at the same time of day and under identical weather conditions. Drying (24 hours at 45° to 50° C.) commenced 1 hour after collection. The biological activity, determined in guinea-pigs, was compared with that of the international standard preparation of digitalis and with an average sample of wild *Digitalis purpurea* collected in the Vosges in the same year. The activity of *D. purpurea* varied from 73 per cent. of that of the international standard in 1950 to 90 per cent. in 1948, being about the same for wild and cultivated plants. *D. ambigua* and *D. lutea* had a similar activity when grown under the same conditions. The yield of leaves from *D. purpurea* was poor in calcareous soil, but was improved by manures and fertilisers. *D. lanata* gave a greater yield of material of higher activity. *D. ferruginea* yielded material twice as active as *D. purpurea* under the same conditions. This species appeared suitable for cultivation in the calcareous soils of Alsace. The yield of leaves was increased by manuring. G. B.

Nicotinic Acid Ester, Skin Response to Local Application in Rheumatoid Arthritis. J. R. Nassim and H. Banner. (*Lancet*, 1952, **1**, 699.) Trafuril, (5 per cent. tetrahydrofurfuryl nicotinic acid ester in a water miscible-base),

when rubbed into the flexor surface of the forearm in normal individuals produces an area of erythema with a sensation of tingling and heat. This effect is not produced in patients with rheumatoid arthritis, but occurs when symptomatic relief is obtained with cortisone or adrenocorticotrophic hormone. Normal responses have been observed in a few cases of osteo-arthritis and tuberculous arthritis. It is suggested that the reaction may be valuable in elucidating the action of adrenocorticotrophic hormone and cortisone and in studying abnormal peripheral circulation in rheumatoid arthritis and allied conditions. G. B.

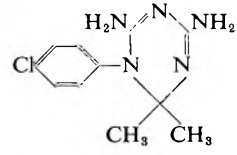
Nitrogen Mustard Treatment of Rheumatoid Arthritis. C. J. Díaz, E. L. García, A. Merchante and J. Perianes. (*J. Amer. med. Ass.*, 1951, **147**, 1418.) Since a relationship can be observed between the effects produced by cortisone and corticotrophin (ACTH) and those of nitrogen mustard, two patients with rheumatoid arthritis were treated by injections of 6 mg. of nitrogen mustard. No details are given of the form, frequency or route of the injections or of the duration of the treatment. In each case after the second injection the patients became free from pain; there was considerable reduction in joint swellings, and movement of limbs, where it was not prevented by bony ankylosis, was restored. The improvement had been retained 1 month and 2 months respectively after treatment had been stopped. Subsequently 7 further cases were treated. There was complete disappearance of pain and recovery of joint movements in 4. In 2 others improvement was less obvious owing to bony ankylosis or irreversible muscular retraction, and the remaining case showed improvement in that pain disappeared and the exudative condition regressed. The treatment causes a drop in the eosinophil count from 100–200 to 20–50 by about the third injection. The sedimentation rate at first increases and then falls but does not become normal. Elimination of 17-ketosteroids is increased. Nitrogen mustard was also tried in 2 patients with prolonged status asthmaticus, the dyspnœa disappearing after the first injection. H. T. B.

Noradrenaline, Adrenaline and the Human Circulation. H. J. C. Swan. (*Brit. med. J.*, 1952, **1**, 1003.) The author reviews published work on the action of adrenaline and noradrenaline in the circulation and on their part in normal and abnormal physiology. Owing to the variable absorption time after intramuscular or subcutaneous injection, responses obtained during intravascular infusion are regarded as of most value and most of the results reviewed were obtained by using this route. The output of the heart is increased by adrenaline and either unaltered or decreased by noradrenaline. The increased output is due in part to changes in the heart rate and in part to alterations in stroke volume. Both substances increase the stroke volume. Adrenaline causes an increase in heart rate, an initial increase to 90 to 120 beats per minute being followed after 30 to 45 seconds by a decrease in the rate to 5 to 30 beats per minute above the resting value. Noradrenaline produces a bradycardia, both this and the secondary slowing of adrenaline being due to vagal influences. Adrenaline increases the systolic pressure and the pulse pressure but its effect on the diastolic pressure is variable. Noradrenaline increases systolic, diastolic, and mean pressures, the pulse pressure remaining unaltered. Adrenaline decreases the total peripheral resistance, measured by the ratio of mean blood pressure to cardiac output, while noradrenaline increases it. The action of adrenaline on the blood flow in muscles is complex. When infused into the main artery of a limb an initial dilatation lasting 45 to 60 seconds is followed, with a small dose, by a return of the blood flow to its resting level or, if the dose is

large, by constriction of the blood vessels. The initial dilatation is so transient that its physiological significance is doubtful. Intravenously the transient dilatation is followed by a sustained increase in blood flow. Noradrenaline infused into the femoral artery does not produce the transient dilatation and the dose required to produce restriction is only one-tenth to one-twentieth of the dose of adrenaline required to produce the same effect; given intravenously it does not produce sustained dilatation. Noradrenaline is now accepted as the predominant transmitter substance at sympathetic nerve endings. Only small amounts leak away into the general circulation from neurovascular junctions and they cause no significant effect on the general circulation. The nerves to the skin and the cardiac sympathetic nerves have been cited as possible exceptions but their effect could equally well be mediated by liberation of noradrenaline.

Support for the view that adrenaline functions in an emergency is given by the reports of more than 60 patients that intravenous adrenaline produces symptoms similar to what they experienced during times of alarm, while the milder symptoms caused by noradrenaline were always unfamiliar to the subjects. Experience suggests that during stress, adrenaline enters the circulation at the rate of rather less than 10 μg./minute. Noradrenaline may well play a causative role in human hypertension and it has been suggested that essential hypertension is due to a failure of methylation of noradrenaline to adrenaline. Another possibility is that hypertensive vessels may be sensitive to noradrenaline. There is no conclusive evidence that either of the compounds is concerned with the vasospastic diseases. In phæochromocytoma there is usually a tumour either in the gland itself or in the para-aortic region and the physiology of the gland changes. The total amount of pressor hormone secreted is greatly in excess of normal and it may be liberated from the gland continuously over long periods; the mixture of hormones liberated may contain a high proportion of noradrenaline so that the effect of the latter is more marked than that of adrenaline. Noradrenaline has been used therapeutically for its pressor action in doses of 5 to 40 μg. per minute, but is not yet proved superior to other pressor drugs; it would appear to be the most rational agent in the treatment of the hypertensive state due to peripheral collapse. H. T. B.

Proguanil (Paludrine), a Metabolite of, with High Antimalarial Activity. H. C. Carrington, A. F. Crowther, D. G. Davey, A. A. Levi and F. L. Rose. (*Nature, Lond.*, 1951, **168**, 1080.) A basic substance, isolated first as the picrate and then as the free base, which crystallised from a mixture of moist chloroform and ether in colourless prisms, was obtained from the urine of rabbits receiving daily doses of proguanil hydrochloride (50 mg./kg.). The same product was also isolated from the fæces of rabbits, and from the urine of human volunteers receiving proguanil. This metabolite is about 10 times as active as the parent drug against infections of *P. gallinaceum* in chicks. Analysis of the picrate and the free base showed the empirical formula to be $C_{11}H_{14}N_5Cl$. Heating the base alone or in alkaline aqueous solution gave a ready conversion to an inactive isomeric substance. The chemical evidence indicated that this active metabolite is 2:4-diamino-1-*p*-chlorophenyl-1:6-dihydro-6:6-dimethyl-1:3:5-triazine (I), and this structure has been confirmed by X-ray crystallographic analysis. An easy synthesis of this substance has been achieved.



A. H. B.

LETTER TO THE EDITOR

The Suprarenal Cortex and its Relation to the Biosynthesis of Adrenaline

SIR.—The exact method of formation of adrenaline in the body is still an open question, although it is possible that the primary amine, noradrenaline, is one of its precursors. Embryonic tissue in the suprarenal glands of man, cat, rabbit, guinea-pig and dog contains a high proportion of noradrenaline and a very small amount of adrenaline.[1] Large amounts of noradrenaline have also been found in the Organs of Zuckerkandl of children aged less than 70 days[2] and in the retroperitoneal tissue of many young animals[3]; this abdominal accessory chromaffin tissue lacks connection with the suprarenal cortical cells. In adult animals, however, methylation of noradrenaline is almost complete in the suprarenal glands when the cortex is large relative to the medulla (e.g., rabbit and guinea-pig), but when the medulla is relatively large (e.g., whale and fowl) methylation of noradrenaline occurs to a very small degree. It was suggested[1] therefore that the ratio of cortical size to medullary size may be related to the proportion of noradrenaline present in the suprarenal gland. This hypothesis has now been tested in lower vertebrates in which structures homologous to the adrenal of higher vertebrates remain separated throughout life.

In the dogfish, an unpaired inter-renal body representing the suprarenal cortex is quite separate from the chromaffin bodies (the rudimentary suprarenal medulla), and it was of interest therefore to identify by chromatographic and biological methods[1] the pressor amines present in these suprarenal analogues. Both adrenaline (0·9 mg./g.) and noradrenaline (2·4 mg./g.) were found in extracts of the chromaffin bodies, but no dihydroxyphenylalanine or hydroxytyramine were detected. This result leaves no doubt that methylation of noradrenaline does not require cortical tissue. A recent finding that the Organs of Zuckerkandl in children aged more than one year contain both adrenaline and noradrenaline[4] supports this conclusion.

D. M. SHEPHERD.
G. B. WEST.

Department of Pharmacology and Therapeutics,
University of St. Andrews Medical School,
Dundee.

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