REVIEW ARTICLE

STATISTICAL METHOD IN THE FIELD OF BIOLOGICAL ASSAY

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I. General Ideas

Two recent review articles in this journal have told the story of the International Standards for Vitamins D and A (Coward[1], Morton[2]). No careful reader of those articles can avoid seeing the importance of the part which the statistical treatment of biological assays has played in the development of satisfactory ways of measuring vitamin content. The same is true of all those substances for which international standards have been established, and in an even wider field. Here it is proposed to treat the subject in its own right.

A biological assay, strictly speaking, is an attempt to find out from a trial with living creatures of some particular species how much of a given substance is present, per cent., weight or volume, in a preparation containing the substance. For example, from an assay with rats we may make an estimate of the vitamin A content of a cod-liver oil. Biological assays are usually used, originally, when the exact chemical constitution of the substance is unknown, but it is often convenient to go on using them after the constiution has become known, because of the difficulty of actually isolating and measuring the extremely minute quantities which are present.

In a slightly more general sense if two substances each produce a characteristic effect on members of a particular species of living creature, a comparison of the amounts necessary to produce the same effect—amounts which may or may not be in the same ratio at all levels of response—though they usually are in actual applications—may be called a biological assay.

The field of biological assay is thus wider than the field in which standards are available, but standards are so important in pharmacological work that it is desirable to give special emphasis to this part of the subject.

Any intelligent person can understand what is meant by a standard yard or a standard pound, and has no real difficulty in grasping the implied extension of the notion when we speak of a standard for vitamin D or a standard for insulin, namely, a preparation of the substance in question such that the properties and effects of a given amount of it do not change in time and with which the properties of given amounts of more or less similar substances can be compared. This points the way to a definition of potency. The potency of any preparation is the inverse ratio of the amount of it which produces a given effect to the amount of the standard required to produce the same effect. As far as this defi-

nition goes potency might vary with the type of effect under consideration and with its intensity or—which is the same thing—with the amount of standard which produces an effect of that intensity. This is not what we want to happen, but it quite often is what in fact happens.

Once we have a standard a unit may be defined as a given amount of the standard, though even here there may be complications.

Let us illustrate the difficulties by a particular example. We have, say a standard preparation of vitamin A. We are presented with a cod-liver oil, and we want to know how much vitamin A it contains. No question at first sight could seem clearer! We will suppose that for one reason or another a chemical or physical determination is impracticable, so we have to use a biological method. That is the real object of a biological assay, to find out how much of a given drug (the term "drug" is used here in a very general sense) is contained, per unit weight or volume, in a substance under test. This should be emphasised. The biological assay of a drug, we think, should not as such be concerned with the therapeutic effects of the drug in man. That is a different question, and confusion arises unless the two questions are separated conceptually. Compromises in practice, for reasons which will shortly become apparent, will sometimes be necessary.

Suppose that we are in the same position as before the 1949 World Health Organisation Conference on Biological Standardisation. If we are in England we turn to the British Pharmacopœia. We find the following statement: "The standard preparation of vitamin A is a quantity of pure β-carotene. The unit is the same as the international unit. It is the specific activity contained in 0·6 μg. of the standard preparation in use." We do not need to be Socrates to ask "Specific for what?" No clear guidance is given, but as the method of assay suggested is based on the increase of growth in rats, we have to assume that the ratio of the amounts of the cod-liver oil in question and of vitamin A which produce the same effect on the growth of rats (a ratio assumed to be the same at all levels of dosage) remains the same if for the rat test we substitute any other *bona-fide* biological test that might be suggested.

Now there is a rather special difficulty here because β-carotene is not vitamin A, and this has led last year's WHO Conference to recommend the replacement of the β-carotene standard by a preparation of vitamin A acetate. This difficulty has occurred on several occasions, when it has been found that a substance originally assumed, it may be tacitly, to be a pure chemical compound of a particular type was not so in fact. The assay of digitalis is in this position, because digitalis is a mixture of several compounds in unknown proportions; at present therefore the assay of digitalis has to be an assay of "activity" if it is to fulfil as well as possible the practical end of enabling safe and efficacious doses to be prescribed. Here, the ultimate aim should be the ability to state exactly what compounds—and in what proportions—any given preparation contains. Until this is achieved statements about the "activity" of any preparation of digitalis are inevitably to some extent tendentious. The word inevitably is used on purpose, for this is not

meant as a criticism of the efforts of those who carry out assays as well as they can, it is merely a plea for the effort to think out clearly what is being done.

But let us return to vitamin A and suppose we are referring to the new standard—which is what it is intended to be—and see what difficulties remain. Even if the data of the test satisfy the usual statistical criteria (we shall see later what they are), caution is still required. "The vitamin A in the oil" is an ambiguous phrase. It may not and usually will not all be in the form of preformed vitamin A, it may be in the form of β-carotene and be converted into vitamin A in the animal body. If the statistical criteria are satisfied, we know that the total amount of vitamin A utilised bears a constant proportion to the dose of oil given, but this provides in itself no proof that all the β-carotene is converted into vitamin A and that all the vitamin A is used. If this is not the case, a test with a different species of animal might give different results for the vitamin A content of the oil.

This actually happens with vitamin D, which may be a mixture of vitamin D_2 and vitamin D_3. Amounts of vitamin D_2 and vitamin D_3 which are equivalent for rats are far from equivalent for chicks, which can utilise the D_3 and not the D_2. Consequently if a mixture is assayed against D_2 (or against D_3) one obtains different results for rats and chicks.

In the case of vitamin A, fortunately, a check on the biological assay exists. Vitamin A can be assayed spectrophotometrically, and in ordinary practice now is always so assayed; while the value of the conversion factor is implicit in the definition of the new standard. Professor Morton[2] has shown how difficulties about irrelevant absorption can be surmounted, so that it will become possible to state the vitamin A content of an oil in say μg./g. as soon as its spectrophotometric value is known. When this stage is reached a standard will be unnecessary. Sir Henry Dale one said: "The ultimate aim of all progressive work in biological standardisation, as in all progressive medicine, is self-extinction." Vitamin B_1 and vitamin C, being pure substances whose constitution is now known, have already reached this stage. They are controlled by chemical and physical tests and the description of their biological assays has not been included in the *British Pharmacopœia*, 1948. But biological assay will nevertheless remain an indispensable method in pharmacological work for many years to come.

To sum up: If we are given a standard there is no difficulty in defining a unit. The unit is defined as the specific biological activity of a given amount of the standard. It cannot be defined as the given amount itself, because we may want to assay against the standard substances which exhibit the "specific activity" but are not necessarily of the same chemical form. "Specific activity" although somewhat tendentious is an unavoidable phrase. It has as its background a working hypothesis which often has to be abandoned as more is learnt of the drug. A substance which initially has been regarded as though it were a pure chemical compound has often been found to be a mixture of several. The ideal thing is then to enable each of these to be assayed separately, either by

biological or preferably by physical or chemical means. When the constitution of each is known and they can be synthesised we are approaching the stage when the standard will be unnecessary. To make it unnecessary is the ultimate aim of research.

There is no difficulty in defining potency provided we are prepared to admit that it may vary at different levels of dosage or in tests with different species of animals. When this happens the definition is deprived of much of its practical utility, but the results are an indication that more fundamental research is required until the situation is cleared up.

II. Statistical Technique and Design

(*i*) *Technique.* What makes statistical technique necessary in dealing with biological assays is animal variability. No two animals of the same species are exactly alike in their response to any stimulus, and even among litter-mates the variability is usually considerable though less than among unrelated animals. For example the coefficient of variation of increase in weight of 100 female rats of a stock colony between the ages of 5 weeks and 10 weeks was 23 per cent. In a line test of vitamin D with female rats the coefficient of variation of "area of healing" was 23 per cent. for non-litter mates and 14 per cent. for litter-mates.

When it is proposed to introduce a new test for biological assay purposes, after deciding on the response to be observed, the first thing to do is to examine the relation between the average response of a group of animals to the dose of standard given and the dose itself. This is called the dosage-response relation. The dosage is the mathematical function of the dose actually used in calculation. It may be the dose itself, it is commonly the logarithm of the dose but may be some other function. Responses are of two kinds "measurable" and "all or none" or "quantal" as the latter are termed technically. The statistical treatment of assays based on the two kinds of responses are in many respects different. In the former case the dosage-response relation is between the mean response to the dose given and the dose itself, in the latter between the percentage of animals responding positively and the dose. Such a percentage can of course always be regarded as the mean value of a variable which is 100 if the animal responds positively and 0 if it responds negatively.

Dosage-response curves in any satisfactory test are of the same general form for both standard and unknown and retain this form when the test is repeated subsequently. They will of course differ in position according to the relative strength of standard and unknown. If there were no animal variability these curves would be smooth and invariable for one and the same preparation. But this is far from being the case. Statistical technique is enormously simplified if the dosage-response curves are straight lines. In most biological assays for which standards exist and for which the response is measurable, it is found to be linearly related to the logarithm of the dose over a sufficient range for working purposes. This means that in a satisfactory test, the dosage-response relations for the preparation under test and the standard will be repre-

sented by parallel straight lines. The horizontal distance between the two straight lines therefore gives the difference between the logarithm of doses of test and standard which produce the same effects—and this immediately provides the potency ratio of the two preparations. The function of statistical method is to estimate from the data of the test, the position and slope of the dosage-response straight lines—and from this the estimate of potency follows at once—then to estimate the accuracy of the result obtained.

Because the animals are variable the mean values of the responses at the different dose levels will not lie smoothly on straight lines, but will have "sampling errors." Not only is there variation in response to a given dose from animal to animal, but a whole colony of animals may undergo fluctuations in sensitivity over a period of time. In order therefore that the estimates of potency and of accuracy (or "error") may be valid, two conditions must be satisfied. The animals selected for each of the dosage groups of both preparations must be selected at random from the stock, and in every assay there must be a simultaneous comparison of the unknown and standard preparations. At any rate until enough is known of the test for it to become a routine procedure there should be at least three dose levels of each preparation (more are sometimes desirable). Even when the test has become a routine test, there should be two doses of each preparation. These conditions ensure that the slopes and positions of the lines are not biased and enable a check to be kept on variations in slope, and any tendency to depart from straightness.

Many accounts have been given of the statistical procedure necessary in fitting straight lines to the data, and in determining the potency and its error, for instance in papers by Irwin[3], Fieller[4], Finney[5,6] and in the textbooks of Coward[7] and Emmens[8]. It is only necessary to say here that accuracy or "error" is measured by calculating fiducial limits of error. These are limits calculated by a rule such that the true value would lie between them in a specified percentage usually (95 per cent.) or (99 per cent.) of repetitions of the assay under essentially the same conditions.

When responses are quantal the relation between response and log-dose can often be transformed into a straight line. How this is done requires a little explanation. Any individual animal has a tolerance (or minimum individual effective dose) which may be defined as the least dose to which he will respond positively. Sometimes the tolerance may be measured directly. For instance in the cat assay of digitalis, the preparation is injected continuously until the cat dies so that the least amount required to kill is obtained separately for each cat. In this case no elaborate statistical treatment is necessary. If the required number of cats are selected and half, chosen randomly, are put on the standard and half on the test preparation the ratio of the mean tolerances—the tolerance is here the individual lethal dose, or the difference between mean log-tolerances will provide the potency ratio. This only assumes that the tolerances of the same animal to the two preparations are always in a constant ratio. The error is then obtained by the usual elementary statistical methods.

As a rule however the individual tolerances cannot be measured directly, but some function of them (often the logarithm) will have a normal frequency distribution in animals of the type used. In this case the proportion of animals P who respond positively to any dose are those whose tolerances are less than the dose in question. If m and σ are the mean and standard deviation of the distribution of tolerances on the dosage scale used it is known that

$$P = \int_{-\infty}^{Y} \frac{1}{\sqrt{2\pi}} e^{-\frac{1}{2}t^2} dt$$

where $Y = (x - m)/\sigma$ and x is the dosage. Hence if a value of P is given and the corresponding Y obtained from it (many tables exist for the purpose) the relation between Y and x is linear. The curve of Y against x is a straight line whose slope is the reciprocal of σ. Y is called a *normal equivalent deviation* and $Y + 5$ a *probit*.

Hence if groups of animals are put on to a series of doses of the test and standard preparations, if the percentage of positive responses are noted and the corresponding normal equivalent deviations or probits are obtained from them, straight lines may be fitted to the data for the standard and unknown preparations.

Departures of the observed values from the lines fitted should not be more than can be accounted for by the sampling variation of the animals, nor should any departure from parallelism of the two lines. From the actual data of the assay it is possible to test whether this is true, with a sufficient probability. The calculation of the potency ratio and of fiducial limits can then be performed in much the same way in *principle* as for the case when the response is not quantal but measurable. There are complications in detail as regards the weighting of the observations and methods involving successive approximation have to be used. Finney's textbook on probit analysis[6] gives an admirable account of the technique.

In a rather exceptional class of cases in which some microbiological assays are included response is linearly related to the dose itself. In that case the potency ratios will be given by the ratio of the slopes of the two lines. The lines may be estimated by the usual statistical technique of regression, and fiducial limits for the potency ratio may be calculated. It is interesting to note that whether the response is linearly related to the logarithm of the dose or to the dose itself, the problem of estimating error reduces to the statistical problem of calculating fiducial limits for a ratio.

(*ii*) *Design.* The need for consultation with a statistician over the design of an assay is now generally recognised. The amount of information that a biological experiment or test of any kind will provide, for a given number of animals used, depends largely on the design. If the latter does not satisfy certain criteria, it will be impossible to obtain a valid estimate of the accuracy of the result.

The necessity of randomisation has already been mentioned. A definite randomisation procedure is necessary so that each animal has an equal chance of being allotted to every dosage group. This is greatly facilitated by the existence of tables of random numbers such as that given in Fisher and Yates Tables[9]. An excellent passage from Emmens' textbook may be quoted here. "Many workers have been under the impression that such a procedure as taking the first 20 animals that come to hand from a cage and allotting them to the first dosage group, taking the next 20 and allotting them to the second dosage group and so on constitutes random selection. This is most definitely not the case, the first 20 animals that come to hand will often be the tamest animals. They may be the biggest animals and they will quite rarely be representative of the group as a whole. A striking instance of this occurred when an assistant was requested to select groups of mice at random, and it was afterwards possible to demonstrate a highly significant correlation between the order in which the animals were taken from the cage, and the weight of the animals. Similarly it is not random selection to allot the top-rack in an animal room to one dose, the second rack to another and so on, because the position of the animals in the room will sometimes affect their response in the tests. The top of the room may be lighter than the bottom; one wall may be warmer than another; and animals in the one position may receive more food than those in another if assistants feed them in a set routine, and these are factors likely to affect the results of a large number of tests. The order in which doses are administered may also affect results; this is particularly likely to happen when the response is measured within a short time after administration or if the drug is given at a certain period after preparation of the test object. Thus whenever such factors are even remotely likely to affect results, the order of administration of doses should also be determined by a process of randomisation. It should be noted also that attempts to adjust groups of animals so that their mean weights shall be approximately the same are open to criticism. Methods of making such adjustments and of allowing for differences which may be found to exist, which are more statistically acceptable, will be described later on." The last two sentences are a reference to the statistical technique of analysis of covariance, which is the best way of allowing for uncontrolled concomitant variation.

The advantage of using litter-mates has also been stressed. If for example we have two dosage groups of the standard preparation and two of the unknown, and litter-mates of four (preferably of the same sex) are available, one member of each litter may be placed on each dose.

Comparisons between the two preparations are then unaffected by litter differences, and the error of the assay is reduced. The correct error may be estimated by use of what are now well-known analysis of variance procedures. Here we have an example of randomisation subject to a simple restriction. More complicated restrictions are often useful.

For example, an assay of insulin using rabbits with percentage blood sugar reduction or final blood sugar as a response might have a Latin

Square design. Six rabbits might each be tested on 6 days and 3 dosage levels used for both the standard and unknown preparations. The arrangement might be as follows: —

Days	1	2	3	4	5	6
Rabbits :—						
I	S_1	S_2	U_2	U_1	U_3	S_3
II	U_3	U_2	S_1	S_3	U_1	S_2
III	S_2	S_1	U_1	U_3	S_3	U_2
IV	S_3	U_3	S_2	S_1	U_2	U_1
V	U_1	S_3	U_3	U_2	S_2	S_1
VI	U_2	U_1	S_3	S_2	S_1	U_3

The Latin Square is one chosen at random from the possible 6×6 Latin Squares. Each rabbit has every dose once, and each dose is given once on each day. Thus day to day and rabbit to rabbit variations in sensitivity are eliminated from the comparisons, and may, by statistical analysis, be eliminated from the estimate of error. The analysis of variance procedure necessary is now a standard technique and needs no elaboration here. If say 24 rabbits were available, 4 groups of 6 rabbits with 4 separately randomised Latin Squares could be used.

Many variations in design are possible to meet differing experimental circumstances; but all designs must satisfy the requirement of adequate randomisation and replication. Some useful examples are given in the textbooks of Finney and Emmens. R. A. Fisher's "Design of Experiments"[10] lays down the principles necessary in the wider field of biological experimentation which includes that discussed here; the recent textbook of Cochran and Cox, "Experimental Designs,"[11] describes and gives examples of all the types of design hitherto suggested.

III. History

An excellent account of the history of biological standards was given by Sir Percival Hartley[12] in his Dixon lecture of 1945. As regards the development and application of statistical methods, the reviewer's 1937 paper[3] gave a not unreasonable account of what had been done up to that time; he would now only regard it as a datum line from which to reckon advances made by others. A very fine bibliography was published by Bliss and Cattell[13] in 1943; Bliss[14] also summarised the work done on fiducial (or confidence) limits, in the first volume of Biometrics in 1945. In 1946 Finney gave the Research Section of the Royal Statistical Society an account of progress since 1937, particularly mentioning Fieller's work published in 1941. His textbook and that of Emmens have already been mentioned.

As Bliss and Cattell say, few references antedate the textbooks by Burn and Coward; very little was done in the twenties if we except Trevan's important paper[15] in the Proceedings of the Royal Society for 1927. Trevan really inspired Gaddum who is the real inventor of the modern statistical technique of treating quantal responses in biological assay.

It is interesting to note, however, that the statistical ideas behind the

quantal response technique goes back to the work of the psychophysicists Fechner[16] and Müller[17] in the last century, and that of Urban[18] and Thomson[19] about 1910. Fechner seems to have been the real discoverer of the method, which he used in discussing the distribution of just perceptible differences in weight.

The most important advances in statistical methodology since 1937 have been advances in design. Bliss and Marks[20] led the way with their now famous work on insulin and in a very long series of papers, many fertile suggestions have come from the former. Next advances in methods of stating errors must be mentioned, the use of confidence or fiducial limits for ratios. Fieller, Bliss, Finney and the reviewer have all played their part in this work. The statistical techniques necessary for dealing with slope-ratio assays where the response is linearly related to the actual dose given have been developed by Finney and Wood[21]. Recently Irwin has reconsidered the problem of the combination of results from different assays which Fieller was the first to deal with in any exact way; Armitage[22] and Irwin[23] have compared the results obtained from the alternative assumptions of logistic and normal tolerance distributions in the quantal case, and Irwin has examined the adequancy of the usual χ^2 test for the satisfactory fit of linear probit-dosage response curves. The remaining advances since 1937 have been in the nature of particular applications of general advances in statistical technique, such as the use of covariance to allow for concomitant variation and the transformation of dosage-response scales (other than the probit transformation which came earlier) to effect linearity or equalise variance.

The development of methods suitable for biological assay has been an outstanding example of the value of scientific co-operation. Previous review articles in this Journal by Coward and by Morton have elaborated particular instances of how this co-operation developed and shown to what useful results it led. The whole subject was fully discussed at the First International Conference of the Biometric Society at Geneva in 1949. The writer of this article is a statistician and the names of the leading statistical contributors to the subject have been mentioned in its course. He would like to conclude by emphasising his own personal admiration for the magnificent achievement of the pioneers who succeeded in getting standards established, people like Dale, Gautier, Gaddum, Hartley and Trevan, thereby enabling many of the newer discoveries of medicine to be utilised on a comparable basis throughout the world to the immense advantage of thousands of sufferers.

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RESEARCH PAPERS

THE ARSENIC LIMIT TESTS OF THE BRITISH PHARMACOPŒIA

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Limit tests for arsenic were first introduced into the British Pharmacopœia, 1914. In the Pharmacopœias of 1932 and 1948 the application of these tests was much extended and the limits were made more stringent, but the general method has remained substantially unchanged, as have the several procedures for preparing solutions suitable for testing. The methods appear to have been based primarily on the work of Hill and Collins[1]. They advocated the use of hydrochloric acid containing a small amount of stannous chloride to ensure reduction of the arsenic, and also to promote a steady evolution of hydrogen, in the Gutzeit test. For tests of materials containing iron or other metals which interfere with the test, they recommended distillation with hydrochloric acid and a slight excess of stannous chloride as a means of obtaining the arsenic in a suitable solution.

In those cases where preparation of the test solution involves exposure to oxidising agents, it is to be expected that the arsenic will be converted into the pentavalent form, and numerous workers have stated that the official test then fails to give correct results. Thus, Bird[2] tested equivalent quantities of arsenic in the forms of arsenite and arsenate and found the stain from the latter to develop much more slowly, and to be of a different colour from that produced from arsenite. Gotthelf[3] confirmed Bird's findings and recommended pre-reduction of pentavalent arsenic by warming the solution with sulphurous acid before applying the test. Sanger and Black[4] also studied the matter, and showed only 40 to 50 per cent. of arsenic, originally present in the pentavalent form, to be detected after 30 minutes, even when the reaction mixture was maintained at 90°C. They found the addition of stannous chloride, or of potassium iodide, to effect no improvement, and, like Gotthelf, recommended pre-reduction with sulphurous acid.

More recently, the Association of Official Agricultural Chemists[5] has recommended, for those cases where the test solution is prepared by wet oxidation, the addition of small amounts of stannous chloride and of potassium iodide to the cold solution before subjecting it to the test. Davies and Maltby[6] have also confirmed that pentavalent arsenic was

incompletely reduced in the Gutzeit test; that stannous chloride in the cold, or at water-bath temperature, did not improve the results; and claimed that the A.O.A.C. method was entirely successful. However, these authors preferred pre-reduction with sulphurous acid. They also discussed the possible effect of variation in the "sensitivity" of the zinc used, and suggested that such variation could be eliminated by conducting the test at 40° to 60°C. Stainsby and Taylor[7] also have recommended the use of stannated hydrochloric acid containing potassium iodide when dealing with solutions containing pentavalent arsenic, and use of zinc "activated" by a preliminary treatment with hydrochloric acid. On the other hand, Taylor and Hamence[8] have claimed that use of zinc containing 0·3 per cent. of copper eliminated all need for any special pre-treatment of the test solution.

Electrolytic methods, eliminating any possible variations arising from the use of zinc, have also been employed. Thorpe[9] devised an apparatus having platinum electrodes, but found that the method failed to reduce pentavalent arsenic. This was confirmed by Trotman[10] who recommended zinc electrodes, and by Sand and Hackford[11] who recommended lead, zinc, or iron electrodes, as capable of effecting complete reduction. More recently, Roche Lynch[12] has claimed satisfactory results with platinum electrodes, provided that pre-reduction with sulphurous acid was applied. Monier Williams[13] found lead electrodes to be satisfactory, and claimed that solutions containing iron, which normally interferes with the reduction, could be tested directly if organic matter was present or added. It was found that lead electrodes became insensitive after use and gave erratic results. Callan[14] investigated this phenomenon and claimed that it could be avoided by appropriate treatment and care of the electrodes. These methods had been employed for the Marsh-Berzelius test, but Evers[15], applying the recommendations of Callan and others, devised an electrolytic Gutzeit apparatus with lead electrodes which was claimed to give quantitative recovery of arsenic, even where the solution contained iron, or had been exposed to oxidising conditions. He added cadmium sulphate to the solutions as a catalyst.

Although, in the above references, there appears to be general agreement that the Gutzeit test fails in those cases where pentavalent arsenic is present, and that special treatment is then necessary, there is no agreement on what this treatment should be; nor does it appear that stannous chloride, on which the B.P. tests are based, is capable of ensuring reduction of pentavalent arsenic in the Gutzeit test. There must be few analysts who have not encountered anomalous and disturbing results in applying the official tests in such cases. It is therefore rather surprising, especially in view of the numerous publications quoted above, that the official tests have remained virtually unchanged during nearly 40 years.

Our interest in this matter arose from the use of ferric chloride as a source of ferric hydroxide for the manufacture of iron scale preparations. These latter were found, on occasion, to contain excessive amounts of arsenic, although all the deliveries of ferric chloride used had been tested by the B.P. test (as for solution of ferric chloride) and apparently con-

tained little or no arsenic; this applied also to the other materials used in the process. Since the ferric chloride seemed to be the most likely source of the trouble, it was decided to investigate the official test for solution of ferric chloride. The B.P. 1948 describes the preparation of the test solution as follows: "Heat 4 g. in a porcelain dish with 1 ml. of *sulphuric acid AsT.*, until white fumes are given off; cool, add an equal volume of *water*, and again heat, until white fumes are given off; cool, and dissolve the residue in 10 ml. of *water* and 15 ml. of *hydrochloric acid AsT.*; transfer to a small flask, add *solution of stannous chloride AsT.*, until the yellow colour disappears, connect to a condenser, and distil 20 ml.; to the distillate add a few drops of *solution of bromine AsT* in order to oxidise any sulphurous acid, remove the excess of bromine by a few drops of *solution of stannous chloride AsT.*, and add 40 ml. of *water*." Tested by this method, solution of ferric chloride is required to contain not more than 2·5 p.p.m. of arsenic*.

Two samples of ferric chloride were selected. The first (1), which had given satisfactory scale preparations, was believed to be practically arsenic-free; whereas the other (2), from the arsenic content of products made from it, was calculated to contain 5 p.p.m. of arsenic. Each was used to make two samples (A and B) of solution of ferric chloride B.P. In each case, 5 p.p.m. of arsenic (as dilute solution of arsenic AsT.) was added to sample B. The four samples were each tested several times by the B.P. test; the results, given in Table I, confirmed that the test was unsatisfactory.

TABLE I

ARSENIC LIMIT TESTS ON SOLUTION OF FERRIC CHLORIDE B.P.

Sample	Arsenic content(p.p.m.)	
	Expected	Found
1A	nil	nil
1B	5	0 to 2
2A	5	0 to 2
2B	10	0 to 3

Solution of ferric chloride, if made by nitric acid oxidation of ferrous chloride solution, is liable to contain dissolved oxides of nitrogen. It is presumably to expel these latter that the preliminary evaporation with sulphuric acid is prescribed. Since the samples tested were unlikely to contain oxides of nitrogen, it seemed remotely possible that the arsenic might be present in the arsenious form and be lost during the evaporation process. Accordingly, the evaporation was conducted in a small flask attached to a condenser; the distillate showed no trace of arsenic on subjection to a Gutzeit test. In addition, 5 per cent. v/v of nitric acid

* Throughout this paper, and following the practice of the B.P., the term "arsenic" refers to arsenious oxide, As_2O_3.

B.P. was added to each sample and the tests repeated; the results agreed with those in Table I.

On the assumption that the trouble lay solely in the evaporation procedure, it was at this stage decided to examine the B.P. test for potassium nitrate. This includes the evaporation stage, but not the distillation and subsequent bromine treatment applied to solution of ferric chloride:—

" Heat 5 g. in a porcelain dish with 5 ml. of *sulphuric acid AsT.* and 5 ml. of *water*, until white fumes are given off; cool, add 5 ml. of *water*, and again heat, until white fumes are given off; cool, and add 50 ml. of *water* and 5 ml. of *stannated hydrochloric acid AsT.*"

Tests were conducted on potassium nitrate A.R., to which 2 p.p.m. of arsenic (as dilute solution of arsenic AsT.) had been added; the results varied from nil to less than 1 p.p.m. It was noted that the evolution of hydrogen and the development of the stain were much slower than in a normal test; after 40 minutes there was usually no stain, and to obtain results even of the above low order it was necessary to allow the reaction to proceed for at least 2 hours. Evidently the arsenic had been oxidised to the pentavalent state. Further tests were carried out, incorporating the various procedures recommended to deal with such a case, and the results are shown in Table II.

TABLE II

ARSENIC LIMIT TESTS ON POTASSIUM NITRATE CONTAINING 2 P.P.M. OF ARSENIC

No.	Details of test	Arsenic found
		(p.p.m.)
1	B.P. test	nil to 0·5
2	B.P. test, but using a zinc-copper couple	nil to 0·3
3	B.P. method for preparation of test solution ; this tested in Evers's[15] electrolytic apparatus	nil to 0·4
4	B.P. test, but adding potassium iodide (1g.) to prepared test solution at room temperature	2
5	B.P. test, but prepared solution diluted with water to 120 ml., sodium sulphite (1g.) added, and the mixture digested on a boiling water-bath for 30 minutes. Finally, the solution was boiled until all sulphur dioxide was expelled, and the volume was reduced to about 60 ml. ; cooled	2
6	Heated with sulphuric acid according to the B.P. test, and diluted with water (10 ml.) ; added hydrochloric acid AsT. (15 ml.) and solution of stannous chloride AsT. (5 drops). Distilled 20 ml., and added water (40 ml.), and solution of stannous chloride AsT. (5 drops)	1
7	As No. 6, but added solution of stannous chloride AsT. (1 ml.) before distillation	2
8	As No. 6, but added solution of stannous chloride AsT. (0·5 ml.) before distillation	2

Similar tests were also applied to dilute solution of arsenic AsT. In each case, 1 ml., after the pre-treatment described, was subjected to the test as for a standard 1 ml. stain; the results (Table III) are expressed as percentage recoveries by comparison with normal standard stains.

The results given in Tables II and III confirmed that arsenic was oxidised (presumably to the pentavalent state) on heating in the presence

of nitric acid; and that the B.P. test failed to detect more than part of such material. Recovery of the arsenic was not improved by using a zinc-copper couple in the test; and was even poorer using Evers's electrolytic apparatus. Of the other treatments previously recommended, the addition of potassium iodide[5,6,7] to the prepared solution at room temperature, or

TABLE III

ARSENIC LIMIT TESTS ON 1 ML. PORTIONS OF DILUTE SOLUTION OF ARSENIC AsT.

No.	Details of test	Arsenic recovered (per cent.)
9	Diluted with water (5 ml.) and nitric acid AsT. (1 ml.); evaporated to dryness. Residue dissolved in water (50 ml.) and stannated hydrochloric acid AsT. (10 ml.)	30 to 40
10	Diluted with water (5 ml.), sulphuric acid AsT. (1 ml.) and nitric acid AsT. (1 ml.); proceeded as for potassium nitrate by B.P. test, but adding stannated hydrochloric acid AsT. (10 ml.) finally	30 to 40
11	As No. 10, but using a zinc-copper couple	30 to 40
12	As No. 10, but solution tested in Evers's electrolytic apparatus ...	10 to 30
13	As No. 10, but omitting the nitric acid	100
14	As No. 10, but adding potassium iodide (1g.) to prepared test solution at room temperature	100
15	As No. 10, but prepared solution diluted with water to 120 ml., sodium sulphite (1g.) added, and the mixture digested on a boiling water-bath for 30 minutes. Finally, the solution was boiled until all sulphur dioxide was expelled, and the volume was reduced to about 60 ml.; cooled	100
16	As No. 10, but prepared solution treated with solution of stannous chloride AsT. (0·2 ml.), and left at room temperature for 30 minutes. (Similar experiments, using larger volumes of solution of stannous chloride AsT., failed owing to the formation in the Gutzeit test of a spongy mass of tin which coated the zinc and stopped the evolution of hydrogen)	30 to 40
17	Initial evaporation procedure as for No. 10. Cooled residue diluted with water (10 ml.); added hydrochloric acid AsT. (15 ml.) and solution of stannous chloride AsT. (5 drops). Distilled 20 ml., and added water (40 ml.), and solution of stannous chloride AsT. (5 drops)	40 to 50
18	As No. 17, but added solution of stannous chloride AsT. (1 ml.) before distillation	100
19	As No. 17, but added solution of stannous chloride AsT. (0·5 ml.) before distillation	100

heating the prepared solution with sulphurous acid[3,4,6] and finally boiling off excess of sulphur dioxide were entirely satisfactory in securing complete recovery of arsenic in the B.P. test; the potassium iodide method commended itself on the grounds of simplicity. Stannous chloride in the cold appeared quite ineffective, but distillation in the presence of a sufficient excess also secured complete recovery of arsenic in the B.P. test. The significance of this in regard to those B.P. tests incorporating such a distillation is referred to later in this paper.

At this stage, it seemed likely that application of one or other of these procedures would suffice to remove any error from those B.P. arsenic limit tests, relatively small in number, involving heating with oxides of nitrogen in the preparation of the test solution. In the case of solution of ferric chloride, distillation with a sufficient excess of stannous chloride was the obvious choice, representing only a small modification of the present

official procedure. This method was therefore applied to the samples mentioned in Table I; the results are given in Table IV.

TABLE IV

ARSENIC LIMIT TESTS ON SOLUTION OF FERRIC CHLORIDE B.P. BY THE OFFICIAL TEST, BUT USING 1 ML. OF SOLUTION OF STANNOUS CHLORIDE AsT. IN EXCESS OF THAT REQUIRED TO DECOLORISE THE SOLUTION BEFORE DISTILLATION

Sample	Arsenic content (p.p.m.)	
	Expected	Found
1A	nil	nil
1B	5	1 to 3
2A	5	1 to 3
2B	10	2 to 4

These results were most disappointing, and it seemed clear that another source of error must be involved. This seemed the more likely since, as already mentioned, these samples were unlikely to contain nitric acid, and hence the added arsenic was unlikely to have been oxidised during the heating with sulphuric acid.

It had already been noted that bromine oxidation of the distillate was peculiar to this and one or two similar tests where the presence of sulphur dioxide was likely. It was therefore decided to repeat the tests as described in Table IV, but omitting bromine treatment of the distillate. Instead, the latter was diluted with water to 120 ml., and concentrated by boiling to a volume of about 60 ml. (in order to remove any sulphur dioxide) and finally treated with solution of stannous chloride AsT. (5 drops). In all four cases, the expected results were obtained, recovery of the arsenic present being quantitative.

It thus appeared that bromine, even in the cold, was capable of oxidising arsenic to a form in which it was not fully recovered in the B.P. test. This important possibility, which does not appear to have been considered before, if correct would obviously introduce errors into a much larger number of B.P. arsenic limit tests, and clearly required full investigation. Accordingly, a series of tests was performed on dilute solution of arsenic AsT. Each test was performed on a 1 ml. quantity and the results (Table V) are expressed as percentage recoveries by comparison with normal standard stains.

The results in Table V confirmed that bromine, in the cold, seriously interfered with the accuracy of the test. It was observed that evolution of hydrogen was very slow in such cases, and it was necessary to allow the reaction to proceed for at least 2 hours to obtain even the partial recoveries indicated above. The results also showed that the same treatments effective after nitric acid oxidation were successful in this case also, the use of potassium iodide again commending itself as the simplest procedure.

It was now apparent that a much larger number of B.P. arsenic limit tests were likely to give inaccurate results; the very considerable number

involving the use of bromine at any stage required consideration along with those incorporating any other oxidation procedure in the prepara-

TABLE V

ARSENIC LIMIT TESTS ON 1 ML. QUANTITIES OF DILUTE SOLUTION OF ARSENIC AsT.

No.	Details of test	Arsenic recovered (per cent.)
20	Diluted with water (50 ml.); treated with hydrochloric acid AsT. (10 ml.) and solution of bromine AsT. (5 drops); excess of bromine removed by adding solution of stannous chloride AsT. (5 drops)	30 to 40
21	As No. 20, but adding potassium iodide (1g.) to the prepared test solution at room temperature	100
22	As No. 20, but prepared solution diluted with water to 120 ml., sodium sulphite (1g.) added, and the mixture digested on a boiling water-bath for 30 minutes. Finally, the solution was boiled until all sulphur dioxide was expelled, and the volume was reduced to about 60 ml.; cooled	100
23	As No. 20, but adding solution of stannous chloride AsT. (0·3 ml.) and leaving at room temperature for 30 minutes	30 to 40
24	Diluted with water (10 ml.); added hydrochloric acid AsT. (15 ml.) and solution of bromine AsT. (5 drops); excess of bromine removed by adding solution of stannous chloride AsT. (5 drops plus 0·5 ml. in excess). Distilled 20 ml., and added water (40 ml.) and solution of stannous chloride AsT. (5 drops)	100

tion of the test solution. A review of the B.P. tests showed that no fewer than 76 merited such consideration. They are tabulated in the following groups based on the procedure involved:—

Group A: Those involving direct treatment with brominated hydrochloric acid AsT., excess of bromine being removed by adding slight excess of solution of stannous chloride AsT. in the cold (30 tests involved):—

Ammonii Bicarbonas
Calcii Carbonas
Calcii Hydroxidum
Creta
Extractum Malti
Gelatinum
Glucosum Liquidum
Liquor Ammoniæ Dilutus
Liquor Ammoniæ Fortis
Liquor Magnesii Bicarbonatis
Magnesii Carbonas Levis
Magnesii Carbonas Ponderosus
Magnesii Oxidum Leve
Magnesii Oxidum Ponderosum
Mistura Magnesii Hydroxidi
Potassii Bicarbonas
Potassii Hydroxidum
Potassii Tartras Acidus
Sodii Benzoas*
Sodii Bicarbonas
Sodii Carbonas
Sodii Carbonas Exsiccatus
Sodii Hydroxidum
Sodii Salicylas*
Sulphur Praecipitatum†
Sulphur Sublimatum†
Theobromina et Sodii Salicylas
Theophyllina et Sodii Acetas
Zinci Oxidum
Bromine Reagent†

* first carbonized and residue used for test.
† special procedure, *q.v.*

Group B: Those involving direct treatment with brominated hydrochloric acid AsT., excess of bromine being removed by adding slight

excess of solution of stannous chloride AsT., and distillation of the mixture. (2 tests involved):

Bismuthi Carbonas — Bismuthi Oxychloridum

Group C: Those involving ignition with calcium hydroxide AsT. and dissolution of the residue in brominated hydrochloric acid AsT., excess of bromine being removed by adding slight excess of solution of stannous chloride AsT. in the cold. (18 tests involved):

Acidum Acetylsalicylicum
Acidum Benzoicum
Acidum Mandelicum
Acidum Nicotinicum
Acidum Salicylicum
Mersalylum
Methylthiouracilum
Nicotinamidum
Saccharinum
Saccharinum Sodium
Succinylsulphathiazolum
Sulphacetamidum
Sulphacetamidum Sodium
Sulphadiazina
Sulphadiazina Sodium
Sulphathiazolum
Sulphathiazolum Sodium
Thiouracilum.

Group D: Those involving ignition with calcium hydroxide AsT., dissolution of the residue in brominated hydrochloric acid AsT., excess of bromine being removed by adding slight excess of solution of stannous chloride AsT., and distillation of the mixture. (6 tests involved):

Bismuthi et Sodii Tartras
Bismuthi Salicylas
Bismuthi Subgallas
Ferri Carbonas Saccharatus
Ferri et Ammonii Citras
Iron Citrate Reagent.

Group E: Those involving distillation with stannated hydrochloric acid AsT., the distillate being treated with slight excess of solution of bromine AsT., and excess of bromine removed by adding slight excess of solution of stannous chloride AsT. in the cold. (4 tests involved):

Cupri Sulphas
Ferri Sulphas
Ferri Sulphas Exsiccatus
Liq. Ferri Perchloridi.*

* After preliminary evaporation with sulphuric acid AsT.

Group F: Those involving heating with sulphuric acid AsT., and/or nitric acid AsT., followed by treatment with slight excess of solution of stannous chloride AsT. in the cold. (5 tests involved):

Acidum Nitricum
Injectio Diodoni
Iodoxylum
Potassii Nitras

Sodii Nitris.

Group G: Those involving heating with sulphuric acid AsT. and nitric acid AsT., and distillation of the mixture with stannated hydrochloric acid AsT. (5 tests involved):

Methylthioninæ Chloridum
Phenolphthaleinum
Suraminum
Viola Crystallina

Viride Nitens.

Group H: Those involving treatment with hydrochloric acid AsT. and

potassium chlorate AsT., excess of chlorine being removed by gentle warming followed by treatment with slight excess of solution of stannous chloride AsT. in the cold. (4 tests involved):

Acidum Hypophosphorosum Dilutum	Sodii Metabisulphis
Potassii Chloras	Sodium Thiosulphate Reagent.

Group I: Those involving treatment with hydrochloric acid AsT. and potassium chlorate AsT., excess of chlorine being removed by gentle warming followed by treatment with slight excess of solution of stannous chloride AsT., and distillation of the mixture. (2 tests involved):

Bismuthum Præcipitatum	Ferrum

On the basis of the work already described, the following modifications to the official methods of preparing the test solutions are suggested:—

Group A: Add potassium iodide (1 g.) to the test solution at room temperature.

Group B: Add solution of stannous chloride AsT. (1 ml., in excess of that required to remove the excess of bromine) prior to distillation. As an additional safeguard, add potassium iodide (1 g.) to the final test solution at room temperature.

Note: In applying the present official method, some analysts may unwittingly have complied with this requirement; whereas others, interpreting strictly such directions as "a few drops," "sufficient to decolorise the solution," etc., would not. Discrepancies between laboratories would thus be explained, since a sufficient excess of stannous chloride is essential in order to secure correct results.

Group C: As for Group A.

Group D: As for Group B.

Group E: As for Group B. In addition, since traces of sulphur dioxide may be present in the distillate, add slight excess of solution of bromine AsT., remove the excess of bromine by adding a few drops of solution of stannous chloride AsT., and add potassium iodide (1 g.) to the final test solution.

Note: It is worth recording that direct oxidation of sulphur dioxide in the distillate with hydrogen peroxide, in place of bromine, was found also to cause low results, unless potassium iodide was added.

Group F: As for Group A.

Group G: As for Group E.

Group H: As for Group A.

Group I: As for Group B.

Substances from each group were tested both by the B.P. method and by the suggested modified method. In each case, the substances were tested (a) as received, and (b) after adding (as dilute solution of arsenic

AsT.) the maximum quantity of arsenic permitted by the B.P. limit. In these laboratories it has been the practice for many years to add the test solution at room temperature to the Gutzeit apparatus, and to stand the latter on a warm surface so that the liquid temperature at the end of the reaction period is between 65° and 70°C. This procedure, which probably counteracts any variations in the "activity" of the zinc used (cf. Davies and Maltby[6]), was applied throughout this series of tests, the results of which are given in Table VI:—

TABLE VI

COMPARATIVE ARSENIC LIMIT TESTS BY THE B.P. METHODS AND BY THE MODIFIED METHODS

Group	Material	Arsenic added (p.p.m.)	Arsenic detected by	
			B.P. method (p.p.m.)	Modified method (p.p.m.)
A	Calcii carbonas	nil	nil	nil
,,	,, ,, ...	5·0	2·0	5·0
,,	Calcii hydroxidum	nil	1·5	3·0
,,	,, ,, ...	5·0	4·0	8·0
,,	Gelatinum*	nil	nil	nil
,,	,, ...	1·4	0·7	1·4
,,	Sodii benzoas	nil	nil	nil
,,	,, ,, ...	2·0	0·4	2·0
,,	Sulphur sublimatum	nil	nil	1·5
,,	,, ,, ...	5·0	3·0	6·5
B	Bismuthi carbonas	nil	nil	nil
,,	,, ,, ...	2·0	1·6	2·0
C	Acidum acetylsalicylicum	nil	nil	nil
,,	,, ,, ...	2·0	0·8	2·0
,,	Saccharinum	nil	nil	nil
,,	,, ...	5·0	1·5	5·0
D	Ferri et ammonii citras	nil	nil	0·5
,,	,, ,, ,, ...	5·0	3·5	5·5
E	Ferri sulphas	nil	nil	trace
,,	,, ,, ...	2·0	0·6	2·0
,,	Liquor ferri perchloridi	nil	1·0	5·0
,,	,, ,, ,, ...	2·5	2·0	7·5
F	Potassii nitras	nil	nil	nil
,,	,, ,, ...	2·0	0·8	2·0
G	Methylthioninae chloridum	nil	nil	trace
,,	,, ,, ...	10·0	7·0	10·0
H	Sodii metabisulphis	nil	nil	nil
,,	,, ,, ...	5·0	2·5	5·0
I	Ferrum	nil	nil	4·0
,,	,, ...	200·0	140·0	200·0

* The general method proposed for Group A substances did not give entirely satisfactory results for gelatin. The results recorded above were obtained by the following special method which is recommended for gelatin:—Heat 7 g. on a boiling water-bath for 15 minutes with 5 ml. of hydrochloric acid AsT. and 3 ml. of solution of bromine AsT.; cool. Add 10 ml. of water and 15 ml. of hydrochloric acid AsT.; transfer to a small flask, add 1·5 ml. of solution of stannous chloride AsT., connect to a condenser, and distil 20 ml.; to the distillate add a slight excess of solution of bromine AsT. in order to oxidise any sulphurous acid, remove the excess of bromine by a few drops of solution of stannous chloride AsT.; add 40 ml. of water and 1g. of potassium iodide AsT.

The results given in Table VI confirm that the B.P. arsenic limit tests give very low results in those cases where preparation of the test solution involves exposure to oxidising conditions at any stage. On the other hand, they show that the simple modifications to these tests, proposed above, give entirely satisfactory results. The need for revision

of the B.P. tests is emphasised. Adoption of the modified tests proposed would require the specification of an additional reagent, potassium iodide AsT., to be defined as material which, when exposed to the B.P. test, gives no visible stain. The material used in the present work complied with this requirement.

The purpose of the lead acetate paper in the Gutzeit test is, of course, to trap any traces of hydrogen sulphide which may be generated. Provided care is taken to avoid the presence of sulphurous acid in the test solution, no appreciable amount of hydrogen sulphide is likely to be generated; in the present work this has applied. However, an open cylinder of lead acetate paper does not appear to be the best device for "scrubbing" a gas stream; and the use of plumbised cotton wool, as recommended by Evers[15], would appear to be more efficient. Both devices are in use in these laboratories, though the official lead acetate paper has been used in the work described. It is recommended that the official apparatus be modified to require the use of plumbised cotton wool.

It has not been possible, in the time available, to apply the comparative methods to more than the small selection of suspected tests listed in Table VI, but there is good reason to suppose that the results obtained are typical. However, it is possible that other substances may, like gelatin, require special treatment. It is hoped, therefore, similarly to examine all the other substances listed in groups A to I, and to report the results at a later date. Although the B.P. arsenic limit tests applied to substances other than those in these groups appear to be satisfactory, it seems possible that in cases where the materials have been manufactured by methods involving oxidising conditions, the arsenic present might be in a form not fully detectable by the official tests. It is therefore recommended that in all the B.P. arsenic limit tests the modification of adding 1 g. of potassium iodide AsT. to the final test solution at room temperature be adopted.

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DISCUSSION

In the unavoidable absence of Dr. Mitchell the paper was read in abstract by Mr. H. Deane.

THE CHAIRMAN (Mr. A. D. Powell) said that certain oxidising substances required excess of stannous chloride, usually followed by distillation, in order to obtain correct results. He also had used potassium iodide in the manner described by the authors, and could confirm the efficiency of the method. The possibility of re-oxidation by bromine water was a valuable point brought out by the paper.

DR. E. F. HERSANT (Dagenham) said that many arguments he had had over arsenic limits had been due to the incomplete reduction of the arsenic when present in the pentavalent state. He would emphasise the advantages of using plumbised cotton wool instead of lead acetate paper as had been observed in testing potassium iodide. The more carefully the lead acetate paper was inserted, so that it was in a roll, the more easily the top paper was contaminated by minute splashes of hydriodic acid, but with plumbised cotton wool the droplets of hydriodic acid were entirely eliminated and the results were much more satisfactory.

DR. D. C. GARRATT (Nottingham) said he thought that most analysts were agreed that the B.P. arsenic tests were inadequate, and that higher results were obtained by other techniques. The standard technique of the Society of Public Analysts, which he thought was recognised as being good when applied to organic materials, gave erratic results in some cases, for example, gelatin, and they had sanctioned the use of sulphite as reducing agent. He advocated the use of sulphite rather than iodide. It was simple to use, there was very little chance of the further complication of blanks and also it was cheaper.

DR. R. E. STUCKEY (London) said that one should not be too critical of some of the arsenic limit tests, as they worked well in the hands of someone who knew the technique. Up to a few years ago he had been very critical of the B.P. technique and had advocated a wholly electrolytic procedure. He had been surprised by the accuracy and reproducibility of the B.P. method in the hands of an experienced worker who was familiar with the technique. Many people did not adhere to the exact detail which was necessary. For example, the exact amount of hydrochloric acid and water specified must be strictly adhered to, or one would get the hydrochloric acid too concentrated and start to lose arsenic before distillation had commenced.

DR. G. E. FOSTER (Dartford) supported what Dr. Stuckey had said with regard to hydrochloric acid. Many years ago they had had a dispute with a German firm about arsenic in a preparation. The discrepancies in their results were found to be due to the fact that the Germans were not using constant-boiling hydrochloric acid, but hydrochloric acid which fumed, and they had lost all the arsenic before it had started to distil over.

THE BEHAVIOUR OF SOME THIOSEMICARBAZONES TOWARDS SILVER NITRATE AND A GRAVIMETRIC ESTIMATION OF 4-ACETAMIDOBENZALDEHYDE THIOSEMICARBAZONE

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Following reports by Domagk[1,2,3] on the tuberculostatic activity of 4-acetamidobenzaldehyde thiosemicarbazone, CH_3CONH—C$_6$H$_4$—$CH{=}NNHCSNH_2$, which have since led to clinical trials in this country, the analytical behaviour of several aromatic thiosemicarbazones has been investigated in order to establish a satisfactory method of assay. A review of the literature shows that little work has been carried out on the analysis of this type of compound, although several methods are described for the estimation of semicarbazones. These methods[4,5,6] based on the quantitative estimation of the ammonia formed during hydrolysis of the compound have given very unsatisfactory results when applied to the analysis of 4-acetamidobenzaldehyde thiosemicarbazone. The variations appear to be due to the partial hydrolysis of the acetamido group to give free ammonia under the conditions necessary for the complete hydrolysis of the thioamide group. Using the Kjeldahl method of determining nitrogen, the ammonia liberated corresponded to only 2 of the 4 nitrogen atoms. Furthermore, in the presence of sugar or starch the method gave abnormally high values for the nitrogen content, probably as a result of some reduction of the hydrazine formed in the reaction[5]. Thus this method could not be applied to the estimation of the compound in the form of tablets containing carbohydrates, and alternative methods of assay were therefore investigated.

In an attempt to find an improved procedure for the isolation of aldehydes and ketones, Neuberg and Neimann[7] examined the metal derivatives of several thiosemicarbazones. Of these, the silver derivatives appeared to be the most useful and the authors found that the complexes obtained corresponded to the general formula $RR'C{=}NN{=}C(SAg)NH_2$. In all cases, the Volhard estimation of silver showed that one silver atom combined with one "thiosemicarbazide residue." Later, Harlay[8,9] re-examined these complexes and found that, in addition to the Neuberg form, other combinations of thiosemicarbazones and silver nitrate could be obtained in which more than one thiosemicarbazone group was associated with each silver atom. For example, by using $\frac{2}{3}$ of the theoretical volume of 0·1N silver nitrate solution, acetone thiosemicarbazone yielded a crystalline precipitate consisting of a combination of 3 molecules of the thiosemicarbazone and 2 molecules of silver nitrate. In the present investigation we have precipitated the silver derivative from a solution of 4-acetamidobenzaldehyde thiosemicarbazone in methyl alcohol

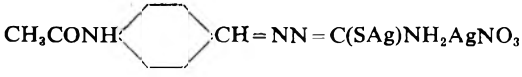
using excess of a saturated methyl alcoholic solution of silver nitrate. Using aqueous silver nitrate the precipitate tended to be gelatinous but in methyl alcohol and particularly in the presence of a few drops of nitric acid the precipitate was denser, more crystalline in appearance and easily filtered. The silver derivatives of several other aromatic thiosemicarbazones have been prepared and the silver content determined by Volhard's method. The stability of the silver derivatives varied considerably and many were not sufficiently stable towards light and heat to permit quantitative estimation of the silver. Those complexes which were examined are given in Table I and show that in each case more than

TABLE I

THE SILVER DERIVATIVES OF SOME THIOSEMICARBAZONES

Thiosemicarbazone	Mol. Wt.	M. Pt.	Characteristics of silver derivatives		
			Silver content per cent.	Thiosemicarbazone per cent.	No. of silver atoms/mol. of thiosemicarbazone
Benzaldehyde	179·2	159 to 160°C.	46·34 46·50	39·60 39·66	1·94 1·90
4-Nitrobenzaldehyde	208·2	250°C.(d)	38·98 38·91	52·39 50·84	1·44 1·48
Salicylaldehyde	195·2	220°C.	40·48 40·44	49·23 49·09	1·49 1·49
4-Ethylsulphonyl-benzaldehyde...	269·3	235°C.(d)	35·61 35·12	55·89 56·59	1·59 1·55
4-Acetamidosalicylaldehyde ...	251·3	252°C.(d)	34·65 34·32	54·94 55·55	1·47 1·44

one silver atom is associated with each thiosemicarbazone molecule. By far the most stable complex was that obtained from 4-acetamidobenzaldehyde thiosemicarbazone, and a complete examination of the derivative was possible. Elemental analysis indicated that it had the formula $C_{10}H_{11}O_4N_5SAg_2$ while determination of the nitrate group with nitron suggested a possible structure:—



The derivative, which is stable at 100° to 105°C., remains unchanged after boiling with water, but treatment with boiling N nitric acid, results in the elimination of one silver atom. The compound can also be estimated in the form of tablets by extracting with methyl alcohol, filtering off the excipient (e.g. starch) and precipitating the silver derivative quantitatively from the filtrate. These results together with the assay figures for several samples of 4-acetamidobenzaldehyde thiosemicarbazone are summarised in Table II.

During the course of our investigation it was found necessary to obtain

a simple and rapid method of assaying thiosemicarbazide. Again a review of the literature has shown that few details are given on the estimation of this compound[8,11] but semicarbazide has received more

TABLE II

THE GRAVIMETRIC ESTIMATION OF 4-ACETAMIDOBENZALDEHYDE THIOSEMICARBAZONE

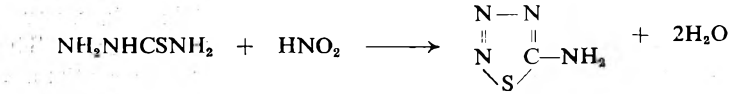
Sample	Melting Point*	Assay per cent.
1	226 to 227°C. (d)	99·63
2	228 to 229°C. (d)	100·0
3	227 to 228°C. (d)	99·82 99·86
4	226 to 227°C. (d)	99·51 99·79

THE ASSAY OF TABLETS (NOMINAL ACTIVE CONTENT 50 MG.)

Sample	Assay—per cent.	Wt. of drug/tablet
1	33·45	49·2 mg.
2	33·49	49·8 mg.
3	32·04 32·09	49·9 mg.

*M.pt. 230°C. (d) Behnische[1c].

thorough investigation[12,13,14]. We have evolved a novel method for the estimation of thiosemicarbazide using the known reaction with nitrous acid to form 5-amino-1,2,3,4-thiotriazole[15]



$$NH_2NHCSNH_2 + HNO_2 \longrightarrow \begin{matrix} N-N \\ \| \quad \| \\ N \quad C-NH_2 \\ \diagdown S \diagup \end{matrix} + 2H_2O$$

The reaction is quantitative and proceeds rapidly at room temperature to give a sharp end-point with starch-iodide indicator. We have compared this method with that referred to by Harlay[8] in which the silver derivative was precipitated from acid solution. The results, which are given in Table III, show that the volumetric assay gives more consistent

TABLE III

THE ESTIMATION OF THIOSEMICARBAZIDE

Sample	Melting point*	Assay as silver complex per cent.	Sodium nitrate assay per cent.
1	176 to 178°C.	98·86 99·82	98·88 98·83
2	182 to 183°C.	99·74 99·42	100·1 99·93
3	182·5 to 183°C.	99·97 99·86	100·0 99·93

* M.pt. 181° to 183°C. Freund and Imgart[1g].

figures and is unaffected by the presence of hydrochloride in the thiosemicarbazide.

EXPERIMENTAL

Silver derivative of 4-Acetamidobenzaldehyde thiosemicarbazone. 0·2 g. of 4-acetamidobenzaldehyde thiosemicarbazone, accurately weighed, was dissolved in 60 ml. of aldehyde-free methyl alcohol, warmed on a steam bath to about 60°C. and treated with excess of a hot saturated methyl alcoholic solution of silver nitrate. The white colloidal precipitate which formed, rapidly became crystalline and separated readily. After cooling, the precipitate was collected on a No. 3 sintered glass filter, washed with methyl alcohol until free from silver and dried to constant weight at 100° to 105°C. Found: C, 23·5; H, 2·15; N, 13·6; S, 6·18; Ag, 42·1; NO_3, 11·3. $C_{10}H_{11}ON_4SAg.AgNO_3$ requires C, 23·4; H, 2·16; N, 13·65; S, 6·25; Ag, 42·1, NO_3, 12·1 per cent.

Estimation of content of 4-Acetamidobenzaldehyde thiosemicarbazone in tablets. 10 tablets were powdered as finely as possible and 0·3 g. of the powder (equivalent to 0·1 g. active material), accurately weighed, was extracted with 50 ml. of methyl alcohol by warming on the steam bath for 2 hours. The insoluble residue was filtered off, washed 5 to 6 times with small portions of methyl alcohol to give a total volume of 70 to 80 ml. An excess of methyl alcoholic silver nitrate was added to the hot filtrate and the precipitate which formed was filtered, washed free from silver with methyl alcohol and dried to constant weight at 100° to 105°C. 1 g. of residue = 0·4606 g. of $C_{10}H_{12}ON_4S$.

Estimation of Thiosemicarbazide. (a) *Sodium nitrite titration.* A standard solution of thiosemicarbazide was prepared by dissolving 1 g. in 100 ml. of 0·25N hydrochloric acid. 25 ml. of this solution was titrated with 0·1N sodium nitrite solution at room temperature. The nitrite was added fairly rapidly with vigorous shaking until near the end point, then drop by drop until the solution gave an immediate reaction with starch-iodide paper. 1 ml. of 0·1N sodium nitrite = 0·009106 g. of CH_5N_3S.

(b) *Precipitation of silver complex.* 0·3 g. of thiosemicarbazide, accurately weighed, was dissolved in 40 ml. of 0·5N nitric acid. 12·5 ml. of 5 per cent. aqueous silver nitrate solution was added slowly in the cold to give an excess of about 10 per cent. The crystalline precipitate was allowed to settle, filtered through a No. 3 sintered glass crucible and washed free from silver with 0·5N nitric acid. The precipitate was dried to constant weight at 45°C for 18 hours. 1 g. of residue = 0·3489 g. of CH_5N_3S.

SUMMARY

(1) A gravimetric method for the estimation of 4-acetamidobenzaldehyde thiosemicarbazone as its silver derivative is described.

(2) The silver complexes of the thiosemicarbazones of benzaldehyde, 4-nitrobenzaldehyde, salicylaldehyde, 4-ethylsulphonylbenzaldehyde and 4-acetamidosalicylaldehyde have been examined. Estimation of their

silver contents showed in each case that the ratio of silver to thiosemicarbazone was greater than 1.

(3) A rapid volumetric method of estimating thiosemicarbazide is described.

The authors wish to thank Mr. D. E. Seymour for his advice and criticism in preparing this paper and the Directors of Herts Pharmaceuticals Limited, for permission to publish these results.

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DISCUSSION

MR. B. W. MITCHELL read an abstract of the paper.

DR. E. F. HERSANT (Dagenham) said that the authors stated that in the sodium nitrite titration they titrated until the solution gave an immediate reaction with starch-iodide paper. In most cases one then obtained a false, early end-point. The B.P., in all the other similar titrations, advised that the titration be continued until the blue colour was given after allowing the solution to stand for one minute. He thought that this was the usual practice in all sodium nitrite titrations.

MR. G. F. HALL (Nottingham) agreed that the assay of thiacetazone was very troublesome from the nitrogen aspect, and said that since receiving the paper they had done only a few determinations by the silver precipitation method, but so far it looked very promising. They had used the sulphur determination, and had generally found it preferable to the nitrogen assay.

MR. B. W. MITCHELL in reply, said that it was correct to say that the end-point with starch-iodide paper was taken after 2 or 3 minutes. Actually, it was found, and was stated in the paper, that a very sharp end-point was obtained, i.e., the first indication of a blue colour was very sudden and seemed to be repeatable after 2 or 3 minutes. He did not know whether there was any reason for that, but comparing it with the titration of sulphonamide they had found it much easier. He did one or two sulphur determinations at the beginning, but did not get very good results.

THE TUBERCULOSTATIC ACTIVITY OF SOME THIOSEMICARBAZONES

By E. M. Bavin, R. J. W. Rees*, J. M. Robson, M. Seiler, D. E. Seymour and D. Suddaby

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During the past five years, there has been a marked increase in the search for substances of possible value for the treatment of tuberculosis. The work has resulted in a large number of publications describing a number of compounds which show activity *in vitro*, in animals and, in some instances, in humans.

The discovery by Lehmann[1], and confirmation by others[2,3,4] that *para*-aminosalicylic acid possesses marked activity in the experimental animal and in humans prompted a detailed examination of a relatively large number of related substances. Reports which have been made[5,6,7,8] suggest that the particular configuration displayed by *para*-aminosalicylic acid is specific for activity.

Reports from Germany that certain benzaldehyde thiosemicarbazones, particularly those which are substituted in the aromatic nucleus in the 4-position, possess a high degree of activity both in the animal and in the human[9], suggested an investigation of a group of benzaldehyde thiosemicarbazones bearing some chemical relationship to *para*-aminosalicylic acid. The German workers have chosen from a number of thiosemicarbazones, 4-acetamidobenzaldehyde thiosemicarbazone (Thiacetazone, T.B.1, Conteben) as being the most useful substance.

The compounds which prompted the present study were thiosemicarbazones of derivatives of salicylaldehyde. The high *in vitro* activity of 4-aminosalicylaldehyde thiosemicarbazone reported by some of us in an earlier paper[8] stimulated a detailed investigation of a series of related substances.

Table I lists the substances studied. Compounds 11, 12 and *para*-aminosalicylic acid were included for purposes of comparison as Nos. 11 and 12 had previously been the subject of work by Domagk and his collaborators[9,10].

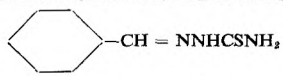
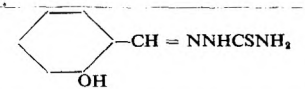
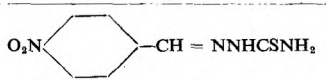
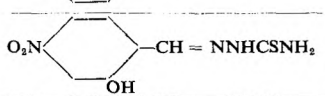
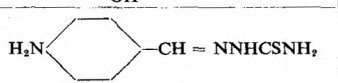
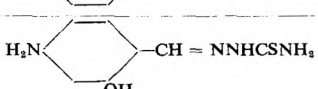
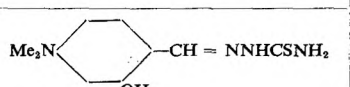
Experimental

(a) *Tuberculostatic Activity.* The *in vitro* activities of the compounds were determined by the technique previously described[6,7].

The *in vivo* activities were determined by two methods, firstly, the method of Rees and Robson[11], based on the effect of the drug on the development of tuberculous lesions in the mouse cornea, referred to in Table II as the "corneal test." The compounds were administered orally

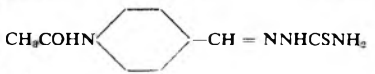
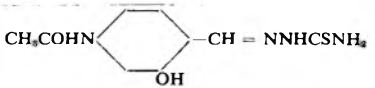
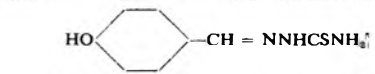
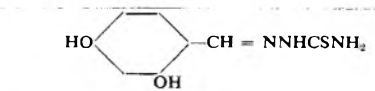
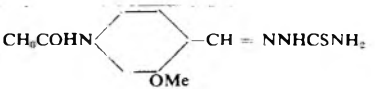
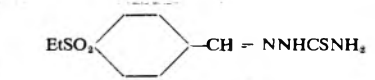
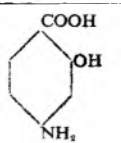
*At present on the scientific staff of the Medical Research Council.

TABLE I

Compound No.	Name	Formula	Activity *in vitro* against *Mycobacterium tuberculosis* H37RV (Dubos type medium; inoculum 0·001 mg./ml.) Inhibitory concentration mg./100 ml.		Acute toxicity (Mouse LD50, g./kg.)	Chronic toxicity, maximum tolerated oral daily dose for 28 days g./kg.
			In absence of serum	In presence of 10 per cent. of human serum		
15	Benzaldehyde Thiosemicarbazone		0·0121—0·0060	0·195—0·0975	0·1—0·2 (oral)	———
10	Salicylaldehyde Thiosemicarbazone		0·195—0·0975	1·56—0·78	4 (subcutaneous) >2·5 (oral)	0·125
32	4-Nitrobenzaldehyde Thiosemicarbazone		0·39—0·195	1·56—0·78	2 (oral)	0·125
30	4-Nitrosalicylaldehyde Thiosemicarbazone		0·195—0·0975	0·78—0·39	>10 (oral)	———
24	4-Aminobenzaldehyde Thiosemicarbazone		0·195—0·0975	0·78—0·39	0·5—0·6 (oral)	0·0025
5	4-Aminosalicylaldehyde Thiosemicarbazone *		0·0121—0·006†	0·39—0·195	3 (oral)	0·15
7	4-Dimethylamino-salicylaldehyde Thiosemicarbazone		0·0121—0·006	1·56—0·78	>10 (oral)	0·5

* The inhibitory concentration of this compound was reported in a previous paper as being 0·003 to 0·0015. We have not been able during the course of this present study to give a satisfactory explanation for this discrepancy. † Hoggarth, *et. el. Brit. J. Pharmacol*, *1949*, 4, 248.

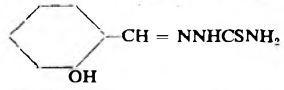
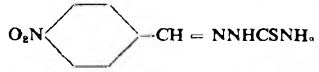
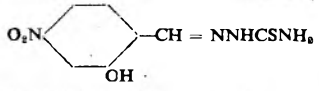
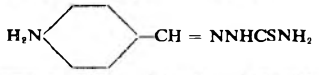
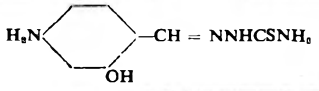
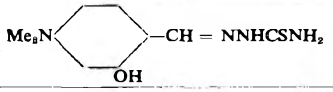
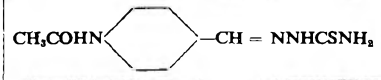
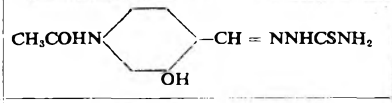
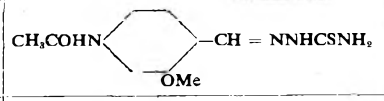
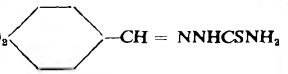
TABLE I (continued)

Compound No.	Name	Formula	Activity *in vitro* against *Mycobacterium tuberculosis* H37RV (Dubos type medium; inoculum 0·001 mg./ml.) Inhibitory concentration mg./100 ml.		Acute toxicity (Mouse LD50, g./kg.)	Chronic toxicity, maximum tolerated oral daily dose for 28 days g./kg.
			In absence of serum	In presence of 10 per cent. of human serum		
11	4-Acetamidobenzaldehyde Thiosemicarbazone (Thiacetozone; Domagk's T.B.1 Conteben)		0·0121—0·006	0·048—0·024	1—2 (subcutaneous) >20 (oral)	0·20
6	4-Acetamidosalicylaldehyde Thiosemicarbazone		1·56—0·78	1·56—0·78	>10 (oral)	0·5
49	4-Hydroxybenzaldehyde Thiosemicarbazone		>0·006	0·39—0·195	4—5 (oral)	>0·25
9	4-Hydroxysalicylaldehyde Thiosemicarbazone		0·39—0·195	——	>3 (oral)	0·125
51	4-Acetamido-2-methoxybenzaldehyde Thiosemicarbazone		0·78—0·39	3·125—1·56	>5 (oral)	——
12	4-Ethylsulphonylbenzaldehyde Thiosemicarbazone (Domagk's T.B.3, 8338†)		0·0976—0·0487	——	>10 (oral)	0·20
	para-Aminosalicylic Acid		0·0487—0·0243	0·0975—0·0487	4 (subcutaneous) 4 (oral)	1·25

Hoggarth *et. al. Brit. J. Pharmacol.*, 1949, **4**, 248.

in admixture with the diet and a compound shown as " active " in Table II is one which after 28 days' administration prevented the development of a corneal lesion, as compared with the controls which all developed tuberculous lesions by the 14th day.

TABLE II

Compound Number	Formula	Survival test activity (a) (b)	Corneal test activity (a)
10		——	Inactive
32		Active	Active
30		——	Inactive
24		Active	Active
5		Inactive	Inactive
7		Inactive	Inactive
11		Active	Active
6		——	Inactive
51		——	Inactive
12		Active	Active

(a) The compounds were administered at approximately the maximum tolerated dose.
(b) Administered by gastric tube in suspension in gum acacia.

The second test employed was the mouse survival test described by several authors[12,13,14] which is based on the effect of the drug on the survival time of mice after intravenous injection with *Mycobacterium tuberculosis*. By the " survival test " compounds in Table II are listed as " active," if they give a significant increase in the survival time of a group of 10 mice over a group of controls.

(b) *Toxicity*. The approximate acute LD50 of each compound was determined in the usual way, using the oral route in all cases, supplemented by subcutaneous administration in a few instances. Chronic toxicities were determined by daily oral doses given over a period of 28 days.

(c) *Synthesis*. The syntheses of the compounds not previously described are herewith reported:—

4-Nitrosalicylaldehyde Thiosemicarbazone (*4-nitro-2-hydroxybenzaldehyde thiosemicarbazone—Compound No. 30*). 4-Nitro-2-acetoxytoluene (40 g.) was dissolved in a mixture of acetic anhydride (600 ml.) and acetic acid (600 ml.). The solution was cooled to 0°C. and sulphuric acid (96 ml.) was added with stirring. To the cold acid solution chromium trioxide (112 g.) was added in portions over 30 minutes. The mixture was finally stirred for a further 3 hours, maintaining the temperature between 5° and 10°C. On pouring into iced water (5 l.) the precipitate which separated was filtered, dried and crystallised from benzene-ligroin to yield 4-nitro-2-acetoxybenzal diacetate (20·5 g.) as prisms, m.pt. 79°C. Found: C, 50·3; H, 4·2; N, 4·8. $C_{13}H_{13}O_8N$ requires: C, 50·2; H, 4·2; N, 4·5 per cent.

The above compound (1 g.) was hydrolysed by boiling with aqueous ethyl alcohol (50 per cent.) (4 ml.) containing sulphuric acid (0·25 ml.) for 30 minutes. On cooling to 0°C. 4-nitrosalicylaldehyde (0·5 g.) separated and was recrystallised from aqueous ethyl alcohol from which it was obtained as pale yellow plates, m.pt. 133° to 134°C. undepressed by a specimen prepared by Seggesor and Calom[15]. Found C, 50·5; H, 3·1; N, 8·0. $C_7H_5O_4N$ requires: C, 50·3; H, 3·0; N, 8·4 per cent.

The theoretical yield of the thiosemicarbazone was obtained by treating the above aldehyde in alcohol with an aqueous solution of thiosemicarbazide. 4-Nitrosalicylaldehyde thiosemicarbazone was obtained as yellow needles, m.pt. 230°C. (decomp.) from aqueous dioxan. Found: C, 40·0; H, 3·35; N, 23·0; S, 13·45. $C_8H_8O_3N_4S$ requires: C, 40·0; H, 3·33; N, 23·3; S, 13·33 per cent.

4-Aminosalicylaldehyde Thiosemicarbazone (*4-amino-2-hydroxybenzaldehyde thiosemicarbazone—Compound No. 5*). 4-Nitro-2-acetoxybenzal diacetate (2·5 g.) was reduced in ethyl acetate solution (20 ml.) by means of hydrogen in the presence of Adam's catalyst. After filtration and removal of the solvent *in vacuo* 4-amino-2-acetoxybenzal diacetate (2·1 g.) was obtained, which on crystallisation from benzene-ligroin separated as pale yellow prisms, m.pt. 101°C. (decomp.). Found: C. 55·8; H, 5·25; N, 5·2. $C_{13}H_{15}O_6N$ requires C, 55·5; H, 5·33; N, 5·0 per cent.

The above compound (8 g.) on adding to a boiling solution of thiosemicarbazide (8 g.) in water (130 ml.) containing ethyl alcohol (10 ml.) and 2N hydrochloric acid (10 ml.) was converted to the crude thiosemicarbazone. 4-Aminosalicylaldehyde thiosemicarbazone was obtained as yellow needles, m.pt. 217°C. (decomp.), from aqueous propylene glycol.

Found: C, 45·7; H. 4·7; N, 26·7; S, 15·4. $C_8H_{10}ON_4S$ requires: C, 45·7; H, 4·8; N, 26·7; S, 15·2 per cent.

4-Acetamidosalicylaldehyde Thiosemicarbazone. (4-acetamido-2-hydroxy benzaldehyde thiosemicarbazone—Compound No. 6). 4-Nitro-2-acetoxybenzal diacetate was reduced in ethyl acetate solution by means of hydrogen in the presence of Adams catalyst. The resultant 4-amino-2-hydroxybenzal diacetate was acetylated without purification by means of acetic anhydride. The resultant tetracetate was isolated by precipitation with a large volume of water. 4-Acetamido-2-acetoxybenzal diacetate separated in lustrous plates from ethyl acetate-ligroin, m.pt. 135°C. Found: C, 55·7; H. 5·38; N, 4·40. $C_{15}H_{17}O_7N$ requires C, 55·7; H. 5·26; N, 4·35 per cent.

4-Acetamido-2-acetoxybenzal diacetate (1 g.) was dissolved in ethyl alcohol (5 ml.) and 20 per cent. sodium hydroxide solution (5 ml.) and allowed to stand for 2 hours at room temperature. Acidification with 5N sulphuric acid gave crude aldehyde (0·4 g.). 4-Acetamidosalicylaldehyde separated with 1 molecule of water as pale yellow needles from aqueous alcohol, m.pt. 186°C. (decomp.). Found: C, 54·7; H, 5·56; N, 7·11. $C_9H_9O_3N,H_2O$ requires C, 54·8; H, 5·58; N, 7·10 per cent.

Drying at 100°C. 1 mm. gave the anhydrous aldehyde. Found: C, 60·1; H, 5·00; N, 7·8. $C_9H_9O_3N$ requires C, 60·3; H, 5·03; N, 7·8 per cent.

4-Acetamidosalicylaldehyde (0·1 g.) in ethyl alcohol (3 ml.) was added to a hot solution of thiosemicarbazide (0·06 g.) in water (5 ml.) and two drops of acetic acid and boiled for 1 minute. 4-Acetamido-salicyaldehyde thiosemicarbazone separated as golden plates, m.pt. 252°C. (decomp.). Found: C, 47·8; H, 4·9; N, 21·9; S, 12·7. $C_{10}H_{12}O_2N_4S$ requires: C, 47 6; H, 4·8; N, 22·2; S, 12·7 per cent.

4-Dimethylaminosalicylaldehyde Thiosemicarbazone (4-dimethylamino-2-hydroxybenzaldehyde thiosemicarbazone—Compound No. 7), 4-Dimethylaminosalicylaldehyde (4·0 g.) was dissolved in ethyl alcohol and added to a boiling solution of thiosemicarbazide (2·6 g.) in water (50 ml.) containing acetic acid (2 ml.). After refluxing for 5 minutes 4-dimethylaminosalicylaldehyde thiosemicarbazone separated and was purified by recrystallisation from a mixture of dioxan and alcohol, from which it was obtained as yellow plates m.pt. 235°C. (decomp.). Found: C, 50·60; H, 5·95; N, 23·5; S. 13·4. $C_{10}H_{14}ON_4S$ requires: C, 50·42; H, 5·88; N, 23·5; S, 13·4 per cent.

4-Hydroxysalicylaldehyde Thiosemicarbazone. (2:4-Dihydroxybenzaldehyde thiosemicarbazone—Compound No. 9). 2:4-Dihydroxybenzaldehyde (2 g.) was dissolved in hot water (10 ml. and added to a solution of thiosemicarbazide (1·6 g.) dissolved in hot water (20 ml.) containing acetic acid (1 ml.). The thiosemicarbazone, which separated almost immediately, was purified by crystallisation from aqueous alcohol whereby 4-hydroxysalicylaldehyde thiosemicarbazone was obtained as needles,

m.pt. 235°C. Found: C, 45·7; H. 4·25; N, 19·7; S, 15·16. $C_8H_9O_2N_3S$ requires: C, 45·5; H, 4·26; N, 20·0; S, 15·2 per cent.

4-Acetamido-2-methoxybenzaldehyde Thiosemicarbazone (*Compound No. 51*). 4-Amino-2-methoxybenzaldehyde prepared by a modification of the method of Hodgson[16] was acetylated by refluxing with acetic anhydride. 4-Acetamido-2-methoxybenzaldehyde was isolated by pouring the reaction mixture into iced water and, when recrystallised from hot water, it separated as needles, m.pt. 145° to 146°C. 4-Acetamido-2-methoxybenzaldehyde (25 g.) dissolved in boiling water (250 ml.) was added to a solution of thiosemicarbazide (12·3 g.) in boiling water (100 ml.) and 2 drops of acetic acid added. The thiosemicarbazone separated as a pale yellow solid which was filtered and washed with hot water, (36 g.). 4-Acetamido-2-methoxybenzaldehyde thiosemicarbazone crystallises from aqueous alcohol as pale yellow needles, m.pt. 232°C. (decomp.). Found: N, 20·6. $C_{11}H_{14}O_2N_4S$ requires N, 21·05 per cent.

Results

From Table I, it will be seen that most of the compounds possess *in vitro* activities of a high order, which (in those tested) is not markedly affected by the presence of human serum. From the same table, it is also clear that substitution of an hydroxyl group in the 2-position results, generally, in a decreased toxicity.

Table II shows that the 2-hydroxyl substitution results in a complete loss of *in vivo* activity and the same table shows a satisfactory degree of correlation between the two tests for tuberculostatic activity.

Discussion

The above results illustrate once again that the incorporation of the chemical characteristics of two active compounds into one molecule cannot be relied upon to produce an active resultant. Compounds Nos. 5, 6 and 7 are examples of substances containing characteristics of both thiacetazone (TB1) and para-aminosalicylic acid and are themselves inactive *in vivo*.

It was considered possible that this loss in activity might arise from a change in properties resulting from hydrogen bonding involving the 2-hydroxyl group. Supporting evidence for this postulation was supplied from the fact that the 2-hydroxylated compounds were invariably less soluble than their parent compound in water and alcohol. To test this suggestion, Compound No. 51, which contains a methoxyl group in the 2-position in place of a hydroxyl group, was synthesised. This compound, however, proved to be inactive and it appears, therefore, that an alternative explanation must be sought for the inactivity of the 2-hydroxylated thiosemicarbazones. This inactivity is probably a further illustration of the necessity for 4-substitution only for this type of compound, a fact which was pointed out by Behnisch, Mietzsch and Schmidt[17] and more recently by Hamre, Bernstein and Donovick[18]. It is noteworthy, however, that the former workers mention 3-substituted com-

pounds which were very active although they do not state whether they refer to *in vitro* or *in vivo* activity. Hoggarth, Martin, Storey and Young[19] also comment on the necessity for a 4-substituent for highest activity but report a few substituents in the 3-position confer activity.

A comparison of the *in vitro* and *in vivo* results affords another illustration of the fact, reported by other workers, that *in vitro* activity, even in the presence of serum, is no criterion of *in vivo* activity. It might be concluded that compounds active *in vivo* would, at least, show *in vitro* activity but this is not necessarily true since nicotinamide, admittedly a compound of a different type, has a marked effect *in vivo* by the survival test in high dosage but no *in vitro* activity.

The results obtained with the survival test appear to correlate well with those obtained by the mouse corneal test with the compounds studied. Further work is being carried out to determine whether this degree of correlation is applicable to other types of tuberculostatic substances.

Summary

1. Of a series of thiosemicarbazones substituted in the aromatic nucleus by a 2-hydroxyl group, several showed a high degree of *in vitro* activity coupled with a lower toxicity than the corresponding thiosemicarbazone un-substituted in the 2-position. The only compounds showing significant *in vivo* activity, however, were those substituted solely in the 4-position. The results confirm the findings of others that the ethylsulphonyl compound (Compound No. 12) is somewhat more active than the 4-acetamido compound.

2. There is no relationship between the *in vivo* and *in vitro* tuberculostatic activity within this series of substances.

3. A good correlation exists between the results obtained in this series of compounds by the mouse survival method and the mouse corneal method.

The authors wish to thank Mr. C. R. B. Williamson for considerable help in part of the pharmacological work, Mr. D. J. Drain for contributions to the chemical part of the work and the directors of Herts Pharmaceuticals Ltd., and Fisons Ltd., for permission to publish the results.

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DISCUSSION

The paper was read in abstract by MR. D. E. SEYMOUR.

MR. C. E. COULTHARD (Nottingham) said that he was very interested to see that the Rees and Robson test was now being applied to practical estimations in the laboratory. The guinea-pig test used a lot of material and took a long time. The egg test had not proved very successful in their laboratory and they had not tried Glover's aerosol test because it seemed dangerous. The administration of the tubercle bacilli intravenously, intraperitoneally and by other routes had been tried but variations in the bacterial strain and in the mice made it difficult to get standard results. More recently they had tried Youman's method in which the tubercle bacilli are injected intra-cerebrally. He asked whether the authors had had any difficulty in applying the corneal test (Rees and Robson) on the scale on which they had used it from the point of view of the susceptibility of the strains of mice. If one was going to use any of the other types of tuberculous infection of mice it was necessary to select suitable mice in order to get reproducible results.

PROFESSOR SPRING (Glasgow) said that the fact that the *ortho*methoxy group did not produce any effect must have been a great disappointment, but it was only one more example of the fact that one could not look at a formula and predict the result which would be obtained in these matters. The fact that the *ortho*methoxy derivative was not active was not of very great significance, unless it were established that the methyl ether of *para*-amino-salicylic acid was inactive also.

MR. D. E. SEYMOUR, in reply, said that he was sorry that Professor Robson, who carried out all the work on the corneal test, was not able to be present. Since the work described had been completed they had been doing the test themselves. With regard to the inactivity of compound No. 51 (the methyl ether), the methyl ether of *para*-aminosalicylic acid had been synthesised, though it had not been tested in animals, so that they could not come to any conclusion about it.

MR. E. M. BAVIN, dealing with the question of the variability of mice of different strains, said it was quite definite that there was this variation. He had started with a strain of Swiss mice which he had kept throughout, and had found, using the intravenous inoculation, that in 80 per cent. of the tests the survival time of the mice was about 20 to 25 days. Sometimes the mice lived for 40 or even 50 days and he did not know why that happened. There was some American work which showed that an increase of fat in the diet of the mice tended to increase their sensitivity to the bacteria. His own experience did not substantiate this.

STUDIES IN THE CHROMATOGRAPHY OF SENNA AND RELATED COMPOUNDS

By George H. Macmorran

From the Department of Chemistry, University of Edinburgh

Received June 30, 1950

The chromatographic examination of vegetable drugs is only now beginning to receive detailed study, and so there are few references to this method as applied to senna or other anthraquinone-containing drugs. Ernst and Weiner[1] used a column of magnesia, and stated that, because of its alkalinity, it had the advantage of causing the emodin-containing layer to be coloured red. Gibson and Schwarting[2] in a paper on the chromatographic isolation of the trihydroxymethylanthraquinones of cascara sagrada used a mixture of 3 parts of celite and 1 part of magnesia as the adsorbing agent. They had difficulty in differentiating the anthraquinone zones, and therefore carried out comparative experiments using dilute chloroform solutions of the pure anthraquinones which they anticipated would be present. On chromatographing a chloroform solution containing the three pure substances, emodin, aloe-emodin and isoemodin, continued washing of the column with chloroform over a period of a week was required before differentiation of the initial red layer was evident. Cropper[3] recommended heavy magnesium carbonate as an adsorbent for hydroxyanthraquinone derivatives. No work appears to have been published concerning the behaviour of simple anthrones on chromatographic columns.

Experimental

Chromatography of Senna Extracts

Chloroform Extract. 10 g. of powdered Tinnevelly senna leaves was completely extracted with chloroform in a Soxhlet apparatus. The extract (about 20 ml.) was chromatographed on a column of alumina 45 cm. long and 2 cm. diameter. The result is shown in Figure 1. Development was accomplished by addition of more chloroform, the lowest layer passing completely into the filtrate. After extrusion, the different zones were treated as follows.

Zone 1—Red. After many trial experiments, continuous extraction with ether was found to be necessary to elute the adsorbed substance. The resulting yellow solution was concentrated and allowed to evaporate. A yellow oily residue (orange in ultra-violet light) remained. This residue gave a negative reaction for glycosides by Molisch's test, and for free anthraquinones by Fairbairn's modification of Borntträger's reaction[4].

Zone 2—Yellow. Easily eluted with alcohol. The solution was concentrated and allowed to evaporate spontaneously. The yellowish residue gave negative reactions for glycosides and anthraquinones.

Zone 3—Green in daylight and red in ultra-violet light. These colours are characteristic of chlorophyll, so this zone was not further examined.

Zone 4—Yellow. Negative reactions obtained for glycosides and anthraquinones.

Alcohol Extract. An alcoholic extract, prepared similarly to the chloroform extract, was chromatographed on a column of alumina 25 cm. × 2 cm. The result is shown in Figure 2. On developing with alcohol, the yellow layer filtered through completely, but the three upper zones remained strongly adsorbed. Chloroform was no more successful. On

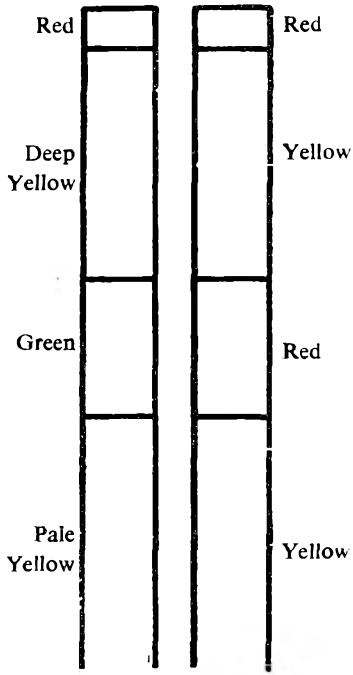


Fig. 1. Chromatograms of chloroformic extract of senna on alumina. Left, in daylight; right, in ultra violet light.

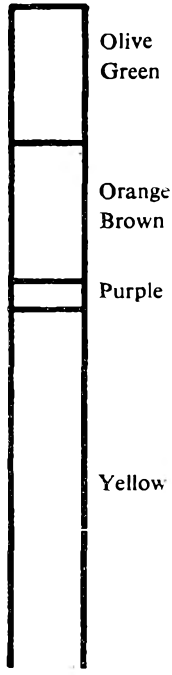


Fig. 2. Alcoholic extract of senna on alumina in daylight.

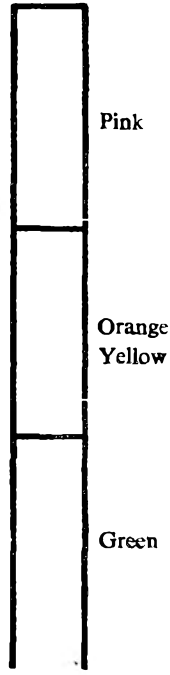


Fig. 3. Chloroformic extract on heavy magnesium carbonate and potato starch, in daylight.

adding water, a yellow zone washed through from the upper zones and the purple zone disappeared, leaving three zones—1 green, 2 orange, 3 yellow. After extrusion the zones were examined.

Zone 1—Green. Continuous extraction with hot alcohol was necessary for elution. After evaporation of the solvent an oily residue remained, which gave a positive reaction for glycosides and also for both free and combined anthraquinones.

Zone 2—Orange. Eluted similarly to Zone 1, giving a yellowish oily residue. This gave a positive reaction for glycosides but negative for anthraquinones.

Zone 3—Yellow. When similarly treated negative reactions were obtained for both glycosides and anthraquinones.

Search for less powerful adsorbents. Chromatographic columns were prepared, using magnesium carbonate, magnesium oxide, calcium hydroxide, calcium carbonate, prepared chalk, and kieselguhr, but all were in such a fine powder that it was only with the greatest difficulty that the liquid percolated. Dilution of these substances with lactose or potato starch effected some improvement, but was not entirely satisfactory.

A chloroform extract of Tinnevelly leaf, prepared as formerly, was chromatographed on alumina as before. The red zone was eluted with hot chloroform. The eluate was chromatographed on a column composed of heavy magnesium carbonate 1 part and potato starch 3 parts, and developed with methyl alcohol. Figure 3 shows the result. The bottom green zone filtered through.

Zone 1—Pink. The magnesium carbonate was dissolved in dilute sulphuric acid and the liberated adsorbate extracted by shaking with chloroform in a separator. The chloroform solution gave a positive reaction for anthraquinones.

Zone 2—Orange Yellow. Similar treatment showed the presence of anthraquinones.

Zone 3.—Green filtrate. Anthraquinones absent.

The use of neutral alumina. It was thought that the slightly acidic nature of the hydroxyanthraquinones present in senna might result in combination with free alkali present in the alumina, and might be, at least partly, the cause of the firm adsorption. Before use, therefore, the alumina was washed with water containing a little hydrochloric acid, and then with water until the washings were neutral. In order to reduce still further the adsorptive power of the alumina, the wet material was washed several times with methyl alcohol, drained well, and air dried, as described by Williams[5]. This material was used for the following chromatogram.

30 g. of powdered Tinnevelly leaf, which had previously been completely extracted with chloroform (and therefore contained no free anthraquinones) were extracted with alcohol in a Soxhlet apparatus. The resulting extract was chromatographed on a column of alkali free alumina measuring 40 cm. × 2 cm. with the result shown in Figure 4. On addition of more alcohol, partial development took place, and the lowest zone filtered through. Elution of the other zones was difficult, and all required continuous extraction with methyl alcohol. The following reactions were given by the eluates.

	Zone					
Test	1	2	3	4	5	6
Bornträger's	+	+	+	−	−	−
Molisch's	−	+	+	+	+	−

"Blocking" of hydroxyl groups by acetylation. Although the use of the neutral alumina gave separation into a larger number of zones than

the other adsorbents, the elution of these zones still presented considerable difficulty. Many hours' continuous extraction in a Soxhlet apparatus was necessary. In order to try and reduce this great adsorbency, it was decided to acetylate the material before chromatographing.

An alcoholic extract was prepared from 20 g. of powdered Tinnevelly leaf, and the alcohol distilled off. A soft extract weighing about 6 to 7 g.

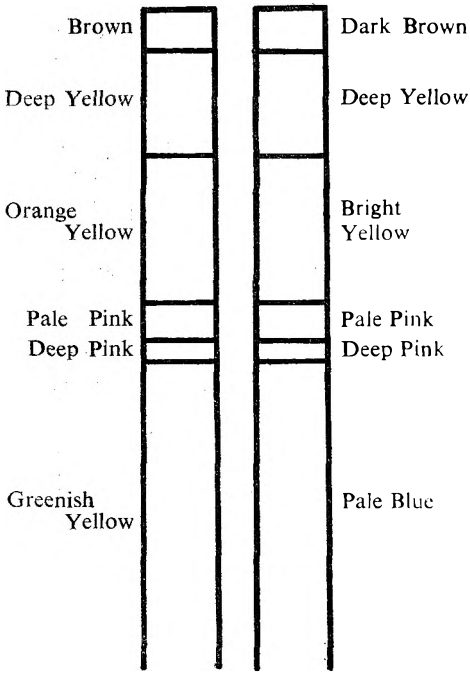


Fig. 4. Alcoholic extract of leaf exhausted with chloroform, on alumina. Left, in daylight; right, in ultra violet light.

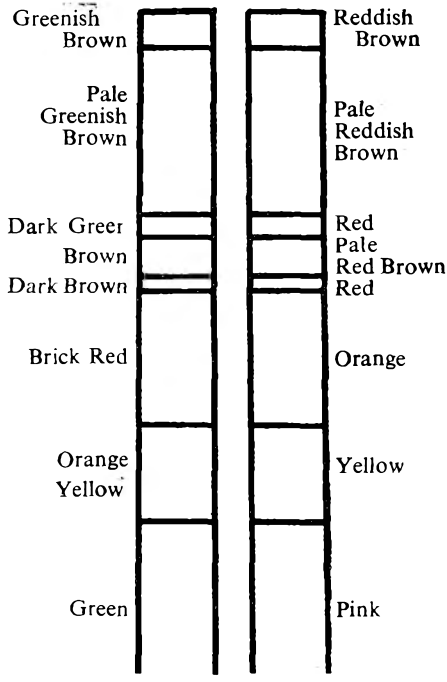


Fig. 5. Acetylated extract on neutral alumina. Left, in daylight; right, in ultra violet light.

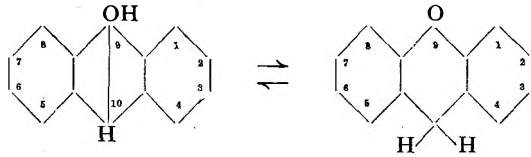
remained. To this was added 14 ml. of acetic anhydride containing 2 drops of concentrated sulphuric acid, and the mixture was heated on a water-bath for 30 minutes. It was poured into water, and the resulting precipitate filtered, washed and dried.

The acetylated extract was found to be incompletely soluble in light petroleum, ether and alcohol. Almost complete solution was obtained in acetone, so an acetone solution was prepared, filtered, and chromatographed on neutral alumina (column 40 cm. × 2 cm.), the appearance of the column being shown in Figure 5. On addition of more acetone, the lowest (green) zone filtered through. Elution was found to be very difficult, the usual solvents being unsuccessful. It was found necessary to separate the zones after extrusion and extract repeatedly with warm acetic anhydride. The solution was concentrated under reduced pressure, poured into water, and the resulting precipitate filtered off, washed with

water, and dried. Zones 1 to 4 all yielded substances which gave a positive reaction for anthraquinones.

Chromatography of Known Related Substances

Owing to the great difficulty experienced in development and elution of the adsorbed substances present in the various extracts of senna leaves tested, it was decided to carry out chromatographic experiments on known substances of a similar constitution to those present in senna leaves.



Anthranol Anthrone

1 : 8-dihydroxyanthranol. 10 ml. of a 1 per cent. solution of 1:8-dihydroxyanthranol in benzene was chromatographed on a column of neutral alumina 15 cm. × 12 mm. with the result shown in Figure 6. On development with benzene the bottom yellow layer passed into the filtrate. The main orange zone was unaffected by methyl alcohol, ethyl alcohol, chloroform or pyridine, even when hot. The use of heavy magnesium carbonate as adsorbent gave a chromatogram of similar appearance. Elution was no easier when the usual organic solvents were used.

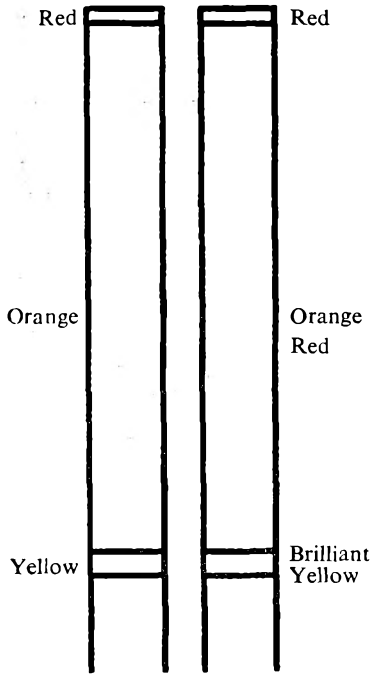


Fig. 6. 1 : 8. Dihydroxyanthranol in benzene, on neutral alumina. Left, in daylight ; right, in ultra violet light.

Di-acetate of 1 : 8-dihydroxyanthranol. 1 g. of 1:8-dihydroxyanthranol was refluxed for 30 minutes with 2 ml. of acetic anhydride containing 1 drop of concentrated sulphuric acid. The mixture was poured into water, filtered and crystallised from glacial acetic acid. M.pt. 208° to 209°C. A 1 per cent. solution of the di-acetate in acetone was chromatographed on neutral alumina. The usual solvents were inefficient eluants,

but acetic anhydride was a little more active. A 1 per cent. solution in chloroform was chromatographed on heavy magnesium carbonate. There was a narrow yellowish green zone at the top of the column; the remainder of the column was yellow. On developing with chloroform the yellow zone passed into the filtrate, which had an intensely brilliant yellow fluorescence in ultra-violet light. On evaporation of the solvent, the residue had melting-point 208° to 210°C. Mixed melting-point with the original di-acetate showed no depression.

Aloin. According to Rosenthaler[6], aloin is a compound of arabinose with aloe-emodin-anthranol. It is thus very similar to the senna glycosides described by Straub and Gebhardt[7]. The aglycone may be identical, but the sugar present in the latter is stated to be dextrose. The similarity between the compounds, however, would suggest that their adsorptive properties should be very similar.

5 ml. of a 1 per cent. solution of aloin in methyl alcohol was chromatographed on neutral alumina (15 cm. × 12 mm.). The main part of the column was orange yellow, but there was a narrow greenish yellow zone at the top, and a yellow zone of the same size at the bottom. Elution was found to be very slow, but after allowing methyl alcohol to flow through the column for several hours the filtrate became yellow, and aloin was identified in it.

10 ml. of a similar solution of aloin was chromatographed on a column of magnesium carbonate of the same size. A narrow zone of pink formed at the top, the remainder of the column being yellow. On further addition of methyl alcohol, the lower zone filtered through. The filtrate, on concentration and spontaneous evaporation, gave a reddish brown residue with a melting-point above 300°C. An amyl alcohol solution of aloin was chromatographed similarly, with the same results. This suggested that decomposition of the aloin took place on the alkaline adsorbent.

Tri-acetate of Aloin. 1 g. of aloin + 2 ml. of acetate anhydride + 1 drop of concentrated sulphuric acid were boiled under a reflux condenser for 30 minutes. The product was poured into water, filtered, washed with warm water, and dried. Greenish yellow aloin tri-acetate was obtained, m.pt. 93° to 94°C. (lit. m.pt. 95° to 96°C.), yield 1·2 g.

10 ml. of a 2 per cent. solution of this in chloroform was chromatographed on neutral alumina (20 cm. × 12 mm.), with the result shown in Figure 7. On further addition of chloroform the lowest zone filtered through as a yellow liquid with a blue fluorescence in ultra-violet light. On evaporation of the filtrate a pale yellow powder was obtained, m.pt. 93° to 94°C. The mixed m.pt. with the original aloin acetate was unchanged. A similar result was obtained when the aloin acetate was chromatographed on heavy magnesium carbonate. The main yellow zone filtered through the column when eluted with chloroform, and the original substance was identified in the filtrate.

Aglycone of Aloin. 10 g. of aloin + 10 g. of borax + 100 ml. of water were boiled for 30 minutes, cooled, and acidified with hydrochloric

acid. The orange red precipitate was recrystallised from toluene and gave m.pt. 195° to 200°C. It complied with the tests given by Cahn and Simonsen[8] for the aglycone of aloin. An acetone solution was

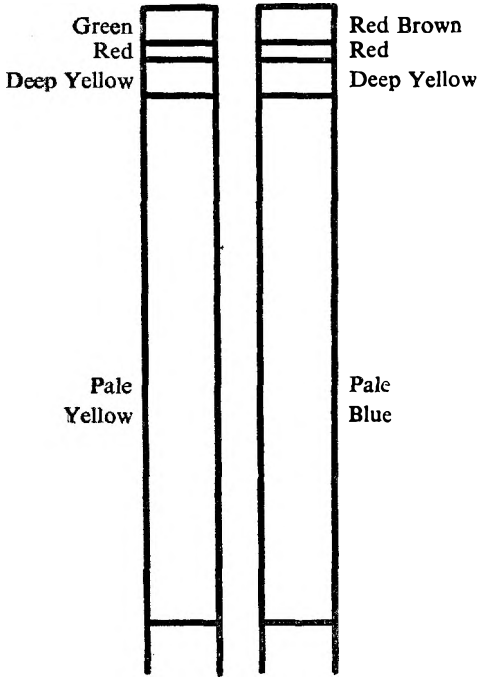


Fig. 7. Aloin triacetate in chloroform, on neutral alumina. Left, in daylight; right, in ultra violet light.

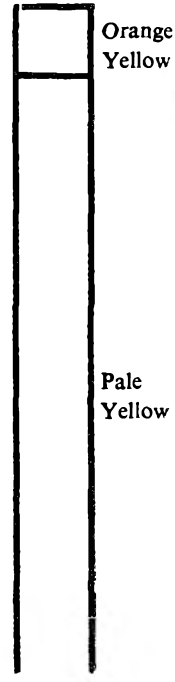


Fig. 8. 1-Chloro-9-anthrone in benzene, on neutral alumina (daylight).

chromatographed on neutral alumina. Almost the whole column was orange (red in ultra-violet light), but there was a narrow yellow band at the bottom. A satisfactory eluant was not found.

1-Chloro-9-anthrone. This substance was prepared by the method of Barnett and Matthews[9]. 1 g. of the crude product was dissolved in benzene and chromatographed on a column of neutral alumina (25 cm. × 12 mm.) with the result shown in Figure 8. No difference was observed in ultra-violet light. On addition of more benzene, the pale yellow zone filtered through. The filtrate was concentrated and allowed to evaporate. Yellow needles were obtained weighing 0·9 g., m.pt. 117°C. (lit. m.pt. 118°C.).

4-Chloro-9-anthrone. This was prepared by the method given by Barnett and Matthews[9]. 1 g. of the crude product was dissolved in benzene, and chromatographed on a column of neutral alumina as above. The whole column was yellow (orange in ultra-violet light with a narrow violet zone at the bottom). Elution was readily effected by addition of more benzene. The violet fluorescent zone was collected separately,

but on evaporation yielded little or nothing. The filtrate from the yellow zone on evaporation yielded yellow needles, m.pt. 119°C. (lit. m.pt. 118°C.), yield 0·83 g. The mixed melting-point with 1-chloro-9-anthrone was 108° to 112°C.

3-Chloro-9-anthrone. 3-chloro-anthrone dissolved completely in warm caustic soda solution, giving a yellow solution, which on addition of sodium hydrosulphite ($Na_2S_2O_4$) did not become red. It was thus free from anthraquinones. M.pt., 154° to 156°C. (lit. m.pt. 156°C.).

1 g. of the crude product was dissolved in benzene, and chromatographed on a column of neutral alumina, 40 cm. × 2 cm. The chromatogram was yellow, the upper third being slightly darker than the remainder. When viewed under ultra-violet light there was no significant change. The column was extruded and divided into three approximately equal parts, which were eluted with warm alcohol.

Bottom zone. Evaporation of the eluate gave 0·3 g. yellow residue melting between 146° and 200°C. It gave a red colour on warming with warm sodium hydroxide and sodium hydrosulphite.

Middle zone. 0·51 g. of residue, melting between 143° and 210°C. It also gave a red colour with warm sodium hydroxide solution and sodium hydrosulphite.

Top zone. 0·15 g. residue, which again gave the red colour as above, and melted between 150° and 195°C.

The three residues which had been recovered from the column were mixed, washed twice with boiling alcohol, and the insoluble residue filtered off and dried. This product was insoluble in warm caustic soda solution; on addition of sodium hydrosulphite to this, a red colour was obtained.

M.pt., 195° to 212°C.; mixed m.pt. with 2-chloroanthraquinone, 195° to 209°C. Found: C, 70·94; H, 3·48; $C_{14}H_7O_2Cl$ requires C, 69·28; H, 2·91 per cent.

The above evidence therefore points to the anthrone having been oxidised on the column of alumina, with formation of the anthraquinone.

Oxidation of Anthrone in presence of alkali. Anthrone ($C_{14}H_{10}O$) m.pt. 153° to 156°C., which was free from anthraquinone, was dissolved in warm dilute caustic soda solution, and allowed to stand in an open beaker for 3 days. A small proportion of the solution was then warmed with sodium hydrosulphite—the solution became red. The remainder of the solution was acidified with hydrochloric acid, filtered, and the precipitate washed and dried. The crude product melted between 190° and 250°C. It was washed with boiling alcohol, and the residue dried. M.pt., 235° to 247°C.; mixed m.pt. with anthraquinone, 240° to 255°C. On warming with caustic soda solution (in which it was insoluble) and adding sodium hydrosulphite, a red colour was produced.

Discussion

Alumina was first used as the adsorbent in the chromatographic examination of senna extracts. Separation into coloured zones took

place readily but elution of the adsorbed substances was very difficult. Continuous extraction with an organic solvent such as chloroform or ether was necessary to elute the zones at the top of the column which contained the hydroxyanthraquinones. The difficulty experienced in the elution of the hydroxyanthraquinones is probably due to their combination with the alumina, forming lakes (as suggested by Cropper[3]). It was found that a column prepared from a chloroform extract gave reactions for free anthraquinones only, while one prepared from an alcoholic extract gave reactions for both free and combined anthraquinones. This confirms that the glycosidal substances are insoluble in chloroform. In the examination of alcoholic extracts some of the lower zones gave a positive reaction with Molisch's Test, but gave no reaction for anthraquinones. This may have been due to the hydrolysis of some of the glycosides, and the free sugars, being less strongly adsorbed than the anthraquinones, were carried lower down the column.

The search for a more suitable adsorbent was not entirely successful. The fineness of powder was a disadvantage in many cases, resulting in extremely slow percolation of the liquid. Heavy magnesium carbonate gave most promise, one advantage being that elution can be effected by dissolving the magnesium carbonate in dilute mineral acid and extracting with chloroform in a separating funnel. One column was prepared using a mixture of magnesium carbonate 1 part and potato starch 3 parts. Through this was passed the chloroform eluate from the red (top) zone of an alumina column prepared from a chloroform extract. Development with methyl alcohol produced separation into three zones, the two top ones giving reactions for hydroxyanthraquinones, thus showing that at least partial separation of these constituents had been effected. The use of neutral alumina did allow of separation into a larger number of zones than previously, but the same difficulty was experienced in the elution of these zones.

Acetylation of the hydroxy-groups in the anthraquinones was then carried out, in order to prevent them reacting with the alumina. Although separation into six zones was achieved, elution was again very difficult.

The chromatography of single compounds, similar in nature to the active constituents of senna, was now considered. Aloin, which is an anthranol glycoside very similar in nature to those found in senna, was successfully chromatographed on neutral alumina, but on a very small scale (5 ml. of a 1 per cent. solution in methyl alcohol). Elution was very slow, and several days would have been required for elution of quantities similar to those of the senna extracts. The use of magnesium carbonate caused decomposition of the aloin, probably due to hydrolysis on the alkaline adsorbent, and possibly subsequent oxidation. Aloin triacetate was chromatographed successfully on both neutral alumina and on magnesium carbonate, thus proving that the "blocking" of the hydroxyl groups did prevent their reaction with the adsorbent.

1:8-Dihydroxyanthranol presented the same difficulty of elution from columns of neutral alumina and magnesium carbonate. Acetylation of this substance reduced the firmness with which it was adsorbed on

magnesium carbonate and thus rendered it suitable for chromatographing on that substance.

Three chloroanthrones were chromatographed on neutral alumina. 1-chloro-9-anthrone, and 4-chloro-9-anthrone were both successfully purified by chromatographing on neutral alumina, using benzene as the solvent and the eluant. 3-Chloro-9-anthrone when similarly treated, was partially oxidised to 2-chloroanthraquinone. Anthrone ($C_{14}H_{10}O$) was allowed to stand in dilute caustic soda solution for three days. At the end of that time it had been partially oxidised to anthraquinone.

These results, together with other evidence to be published elsewhere. suggest that certain anthrones are readily oxidised in presence of alkali. The alkalinity of alumina is evidently sufficient to bring about this change in some cases. Cahn and Simonsen[10] have shown that aloe-emodin-anthranol is converted to aloe-emodin by aerial oxidation in alkaline solution.

Conclusions

1. Alumina is too strong an adsorbent for use with hydroxyanthraquinone derivatives, probably due to the formation of lakes.

2. Heavy magnesium carbonate gives more promising results. The fact that it dissolves readily in dilute acids, thus liberating the adsorbed substances, which may be extracted by an immiscible solvent, is very useful. Its alkalinity is a disadvantage, and may lead to decomposition of the adsorbate.

3. Acetylation of the hydroxyanthraquinone derivatives before chromatography reduces the extent to which they are adsorbed, and so makes elution easier.

4. Atmospheric oxidation of anthrones to anthraquinones may take place in presence of alkali, and may occur on a chromatographic column.

The author is greatly indebted to Dr. Neil Campbell, F.R.S.E., for advice and encouragement while carrying out the above work.

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DISCUSSION

The paper was read by Dr. G. H. Macmorran.

The Chairman said that it would be interesting to know whether the author had anything to say about the pharmacological activity of the various adsorbates.

DR. J. W. FAIRBAIRN (London) said that he could confirm the author's findings, that alumina was far too strong, and the best adsorbent he had tried was magnesium carbonate. Figure 1 showed a column which was red at the top, but in the paper itself there was no mention of anthraquinones being contained in that column. That might be due to a misprint because later it was stated that chloroform did extract free anthraquinones, and therefore they should appear on the column. In Figure 4, the pale pink and deep pink bands gave no reaction with the anthraquinones. Was it possible to elute the anthraquinones, and should one resort to treatment with acid and extraction with chloroform? Although ethyl alcohol extracted a certain amount of glycosides, it was a very poor solvent for the purpose; one could not hope to get all the glycosides of senna out with ethyl alcohol.

DR. G. H. MACMORRAN, replying, said that he had done no work on the pharmacological activity of any of the zones on the chromatographic columns. The top zone of Figure 1 gave no reaction for combined anthraquinones, and that was to be expected, but he thought that it did give a reaction for free anthraquinones. With regard to Figures 4 and 5, he did not think that there were any reactions given for free anthraquinones. In the paper there was a table showing that the first three zones gave reactions with Bornträger's test, indicating the presence of free anthraquinones, and zones 2, 3, 4 and 5 gave a positive reaction with Molisch's test, so that only zones 2 and 3 gave reactions for both the anthraquinones and the carbohydrates. It was suggested that some of the glycosides had been hydrolysed on the column and the sugars carried further down the column, and therefore in zones 4 and 5 there was a positive reaction with Molisch's test but not for the anthraquinones.

THE PARTITION CHROMATOGRAPHY OF ALKALOIDS

PART III.—THE ALKALOIDS OF *PUNICA GRANATUM*

BY J. CHILTON AND M. W. PARTRIDGE

From the University, Nottingham.

Received June 21, 1950.

THE systematic study of anthelmintics appears to have been hitherto totally incommensurate with the importance of helminthiasis, which in various forms affects an estimated 800 million people[1]. In particular, traditional remedies have been subjected to little critical examination. The difficulties encountered in the *in vitro* culture of many parasitic worms[2] and in testing anthelmintics are amongst the important reasons for this. Recently progress has been made in the development of *in vitro* tests, one of which[3] employs the liver fluke and would appear to be of value in estimating the comparative activities of anthelmintics, particularly against trematode and cestode parasites.

Preparations of the root of *Punica Granatum* have long been in use as tænifuges and the so-called "pelletierine tannate" of the Pharmacopœia is an example of such a preparation which is intended to contain the active constituents of this drug. A thorough search of the literature has revealed only the work of von Schroeder[4] in 1884, who showed that pelletierine was toxic to tapeworms; this alkaloid has, as a consequence, been considered to be the active component of the mixture of alkaloids present in *Punica Granatum*. It therefore appeared of interest to investigate the separation of the alkaloids of pomegranate, their relative proportions in the crude drug and in commercial samples of "pelletierine tannate" and to obtain more information on their relative activities as anthelmintics.

Four alkaloids of *Punica Granatum* have been isolated and characterised, namely, pelletierine, methyl*iso*pelletierine, *pseudo*pelletierine and *iso*pelletierine[5]. Because of the inaccessibility of experimental material and since a partial fractionation of pomegranate alkaloids by fractional liberation of the bases from their sulphates has already been effected[6,7,8], it was considered that an extension of the methods described in previous parts of this series of communications would provide a simple means of separating these alkaloids.

EXPERIMENTAL

Fractionation of "Pelletierine Tannate."—The conditions applicable to the partition chromatographic separation of pomegranate alkaloids were investigated, using two commercial samples of "pelletierine tannate," one purchased in England, the other in France.

The " pelletierine tannate " was assayed; the total bases were liberated with sodium hydroxide, collected in chloroform, recovered as their sulphates in an excess of standard acid and, after evaporating the chloroform, their titre was determined by back titration of the excess of acid, using bromocresol green as indicator.

Because it was found in *ad hoc* experiments that some of the basic material co-distilled with chloroform during evaporation of a chloroform solution of the alkaloids, and that aqueous sodium hydroxide caused their resinification, a special procedure was adopted for the preparation of the alkaloids for chromatographic separation. The " pelletierine tannate " was dissolved in N sodium hydroxide and the mixed bases were immediately extracted with chloroform; from the chloroform solution, the alkaloids were collected in a small excess of N sulphuric acid and the aqueous solution was concentrated under reduced pressure to a small volume. This solution was saturated with sodium phosphate, and 4N sodium hydroxide, equivalent to the N sulphuric acid used in extracting the alkaloids, was added. The resulting solution was absorbed on twice its weight of kieselguhr, and this material was packed on top of a previously prepared chromatographic column.

The partition chromatographic columns were similar to those described in Parts I and II[9,10] and consisted of kieselguhr on which was distributed phosphate buffer. The technique for the elution and recovery of the alkaloids was the same as that previously described. Throughout these experiments, we have found the method of packing chromatographic columns described by Martin[11] to be extremely useful. By systematic experiments, using quantities of mixed bases equivalent to about 30 to 40 mg. of *pseudo*pelletierine, a study was made of the optimum conditions for fractionation on a column containing 5 ml. of 0·5 M phosphate buffer distributed on 10 g. of kieselguhr. The homogeneity of each alkaloid fraction was checked by repeating the process on a column containing a buffer of lower *p*H value and a higher proportion of kieselguhr and buffer to alkaloid.

Although kieselguhr is usually regarded as having a low adsorptive capacity for alkaloids, it was found that even after development of the column with a chloroform solution of ammonia, some bases were retained on the column. After extrusion of the column, the alkaloids could still not be desorbed by shaking with N sodium hydroxide and chloroform. Accordingly the adsorbed alkaloids were recovered as their sulphates. It was found that the major portion of the alkaloids which were adsorbed by kieselguhr could be submitted to chromatographic separation on a column consisting of phosphate buffer distributed on " Pyrex " glass in No. 60 powder; 7 g. of the glass supported 1 ml. of buffer.

For the identification of the alkaloids, and for their isolation in quantities sufficient for comparison of their anthelmintic activity, the small-scale experiments which afforded the maximum enrichment of the different fractions were repeated on a larger scale. In order to obtain sharp separations in these experiments, it was found necessary to employ

a column built up of two units as described by Claesson[12]. By this means, quantities of mixed alkaloids equivalent to up to 5 g. of *pseudo*-pelletierine were fractionated.

The procedures described in Parts I and II were adopted for the identification of the alkaloids as their picrates and reineckates in the eluate fractions corresponding to peaks in the graphs.

Fractionation of the alkaloids of Punica Granatum.—The materials available consisted of four samples (20 to 90 g.) of museum specimens of pomegranate root bark, the bark (190 g.) and wood (300 g.) of fresh young roots from Poona, bark (180 g.) of old roots from Poona and fresh whole roots (770 g.) from Hong Kong.

The crude drug, dried at below 60°C. and in moderately coarse powder, was mixed with 30 per cent. of its weight of calcium hydroxide, moistened with water, and kept for 4 hours. After packing in a percolator, the drug was macerated for 24 hours with alcohol (70 per cent.) and percolated with alcohol (70 per cent.). The percolate was acidified with dilute sulphuric acid, precipitated calcium sulphate was removed, the filtrate was concentrated to a small volume, filtered to remove resin and washed with chloroform. Alkaloids liberated by sodium hydroxide were collected in chloroform as rapidly as possible. The total titre of the alkaloids in the chloroform, and hence also the proportion of alkaloids in the drug, were calculated from the titre of an aliquot portion. An equivalent of N sulphuric acid was shaken with the chloroform in order to recover the mixed alkaloids as an aqueous solution of their sulphates. This solution was treated in the manner described earlier for the preparation of the alkaloids derived from " pelletierine tannate " for chromatographic separation. Chromatographic separation and identification of the alkaloids were effected in the same way as outlined in the description of the fractionation of " pelletierine tannate."

Results

In the course of experiments designed to yield information on the conditions necessary to achieve separation of the alkaloids, the effect of changes in the *p*H value of the phosphate buffer, of the quantity of mixed alkaloids placed on the chromatographic column, the dimensions of the column and the rate of flow of eluting solvent were studied. The results obtained revealed no new feature additional to those recorded in Part I, and are therefore not described in detail here.

Alkaloids of " Pelletierine Tannate."—Figure 1 shows the separation of the alkaloids from " pelletierine tannate " under the empirically determined optimum conditions at *p*H 6·8, using ether for the elution of alkaloids corresponding to peaks 1A and 1B and chloroform for alkaloids corresponding to peak 1C. From an examination of Figure 2, it can be seen that no further fractionation was achieved when the alkaloids corresponding to Figure 1, peaks 1A and 1B, were refractionated at *p*H 6·5 (curve 2A, 2B), and those corresponding to Figure 1,

peak 1C, were refractionated at *p*H 5·9 (curve 2C). The course of the fractionation of alkaloids equivalent to 5 g. of *pseudo*pelletierine on the Claesson multiple column is shown in Figure 3 (curve 3B, 3C) and in curve 3B′, 3C′, the fractionation of alkaloids equivalent to 0·6 g. of *pseudo*pelletierine from the second sample of "pelletierine tannate" is shown.

Figure 4 refers to the fractionation of the alkaloids from the English sample of "pelletierine tannate" which are adsorbed by kieselguhr, but

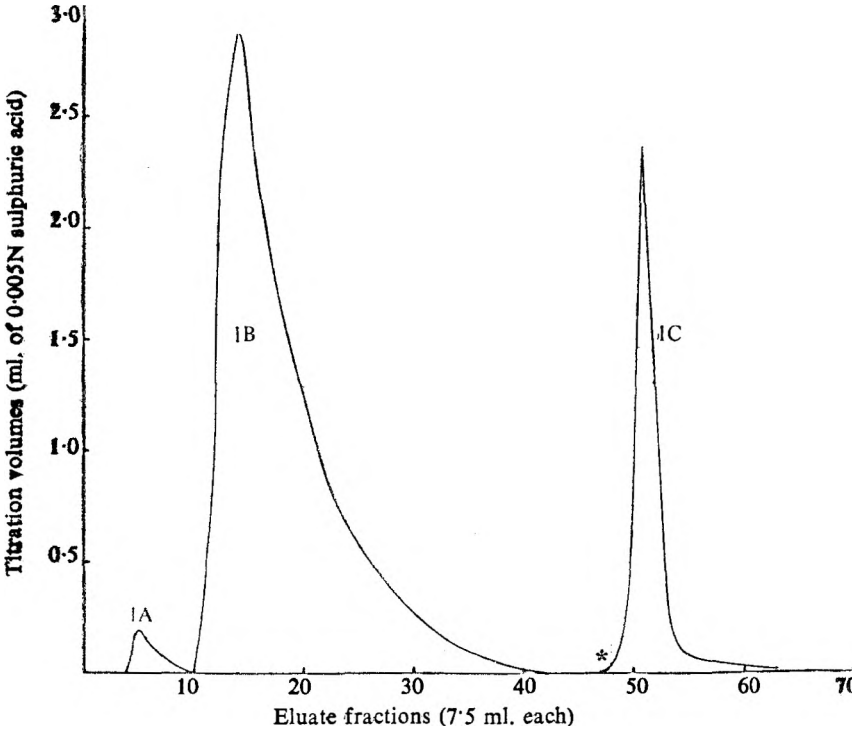

FIG. 1. Separation of alkaloids from "pelletierine tannate" equivalent to 30 mg. of *pseudo*pelletierine. Column, 10 g. of kieselguhr with 5 ml. of 0·5M phosphate buffer, *p*H 6·8.

* Chloroform used as eluant.

may be submitted to partition chromatography on phosphate buffer—glass powder columns. From curve 4D, 4E, it is seen that only a partial separation of two bases can be achieved. The phosphate buffer—glass powder partition chromatogram of the kieselguhr-adsorbed alkaloids from "pelletierine tannate" of French origin is summarised in Figure 5. This material contained one major basic component (curve 5D). Curve 5E refers to authentic pelletierine isolated from the crude drug and passed through a partition chromatographic column under identical conditions; comparison of curves 5D and 5E indicates that the two bases corresponding to the major peaks are not identical.

The characters of derivatives prepared from eluate fractions corresponding to peaks on the curves representing the different partition chromatograms are given below:—*Peaks 1B, 2B, 3B:* Picrate, needles from water, m.pt. 252°C. (with decomposition); Hess[13] records the same m.pt. for *pseudo*pelletierine picrate.

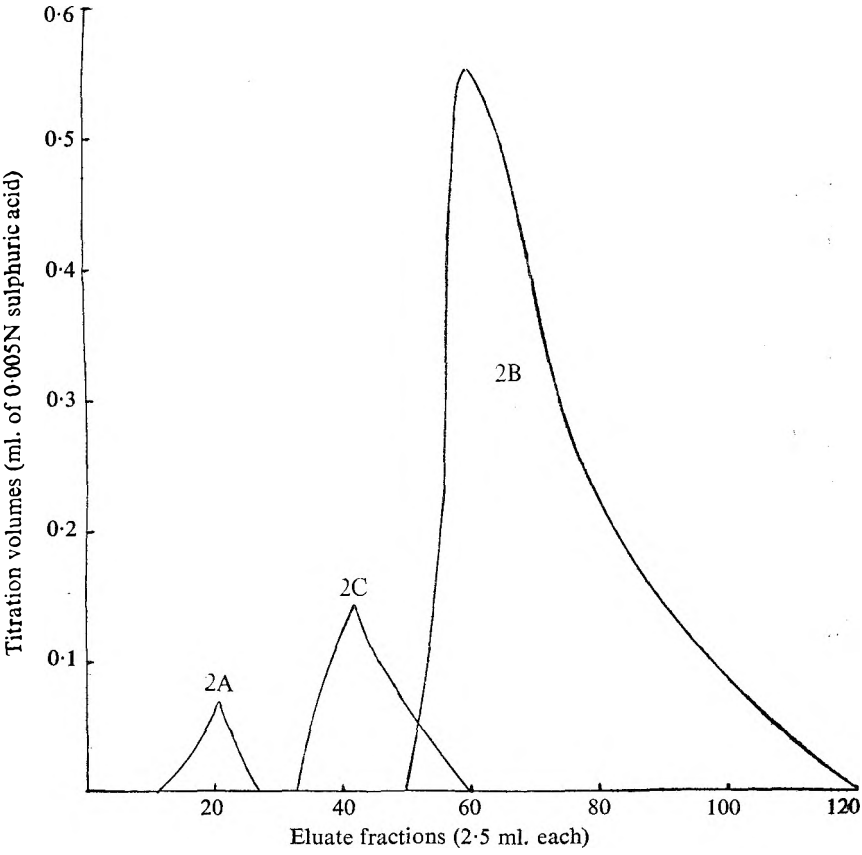

FIG. 2. 2A, 2B—Refractionation of alkaloids corresponding to 1A, 1B at *p*H 6·5; eluant, ether. 2C—Refractionation of alkaloid corresponding to 1C at *p*H 5·9; eluant, chloroform. Columns, 10 g. of kieselguhr with 5 ml. of 0·5M phosphate buffer.

Peaks 1C, 2C, 3C: Picrate, rosettes from absolute alcohol, m.pt. 154°C.; methyl*iso*pelletierine isolate by Hess[13] had identical m.pt.

Peaks 4D, 5D: Picrate, insoluble amorphous powder, m.pt. 208°C. (with decomposition), which decomposed when recrystallisation was attempted; Reineckate, leaflets from aqueous acetone, m.pt. 240°C. (with decomposition); found SCN, 49·6, 49·2 per cent.; *Z*, 150, 154.

Peak 4E: Picrate, after two recrystallisations from water separated as rosettes, m.pt. 150°C., depressed to 128° to 130°C. by methyl*iso*pelletie-

vine picrate. Hess[14] describes pelletierine picrate as having m.pt. 150° to 151°C.

A further fraction of basic material which was not eluted from the phosphate buffer—glass powder columns by chloroform failed to yield any solid derivative, even after extensive further fractionations.

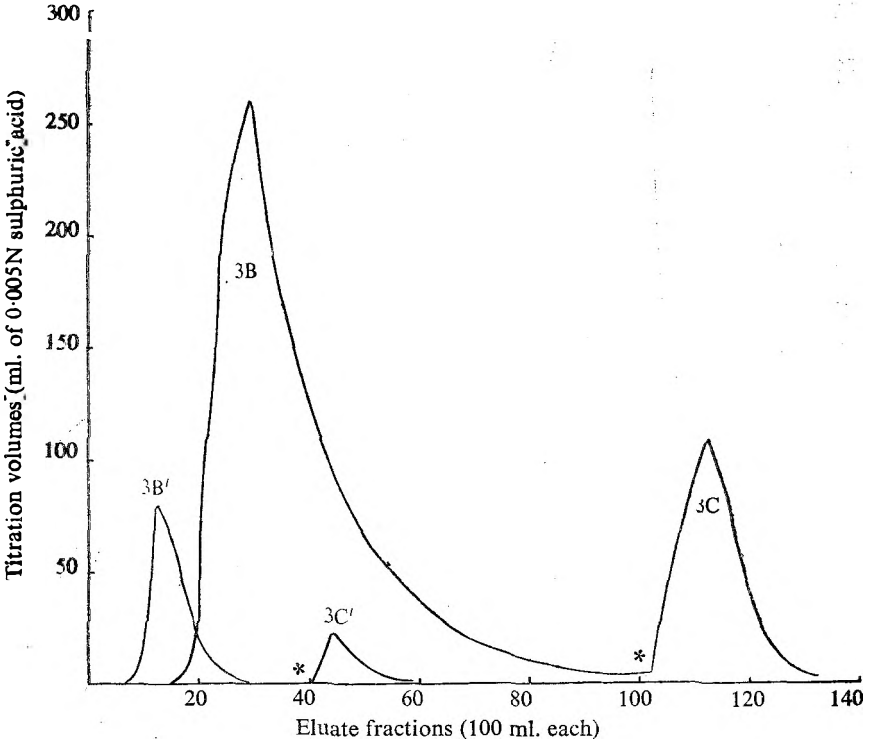

FIG. 3. 3B, 3C—Separation of alkaloids from "pelletierine tannate" equivalent to 5 g. of *pseudo*pelletierine. Column, 650 g. of kieselguhr with 325 ml. of 0·5M. phosphate buffer, *p*H 6·8. 3B′, 3C′—Separation of alkaloids from "pelletierine tannate" equivalent to 0·6 g. of *pseudo*pelletierine. Column, 200 g. of kieselguhr with 100 ml. of 0·5M phosphate buffer, *p*H 7·0.

* Chloroform used as eluant.

TABLE I

ALKALOIDS OF "PELLETIERINE TANNATE"

Sample	Peak 2A* bases per cent.	Peaks 3B, 3B′, ψ-pelletierine per cent.	Peaks 3C, 3C′ methyl*iso*-pelletierine per cent.	Peak 4E, pelletierine per cent.	Unidentified bases* per cent.	Total bases† per cent.
English "pelletierine tannate" ...	0·85	63	14	4·1	18	9·2
French "pelletierine tannate" ...	0·70	64	16	nil	19	10·3

* Calculated as ψ-pelletierine. † Calculated as ψ-pelletierine in original "pelletierine tannate."

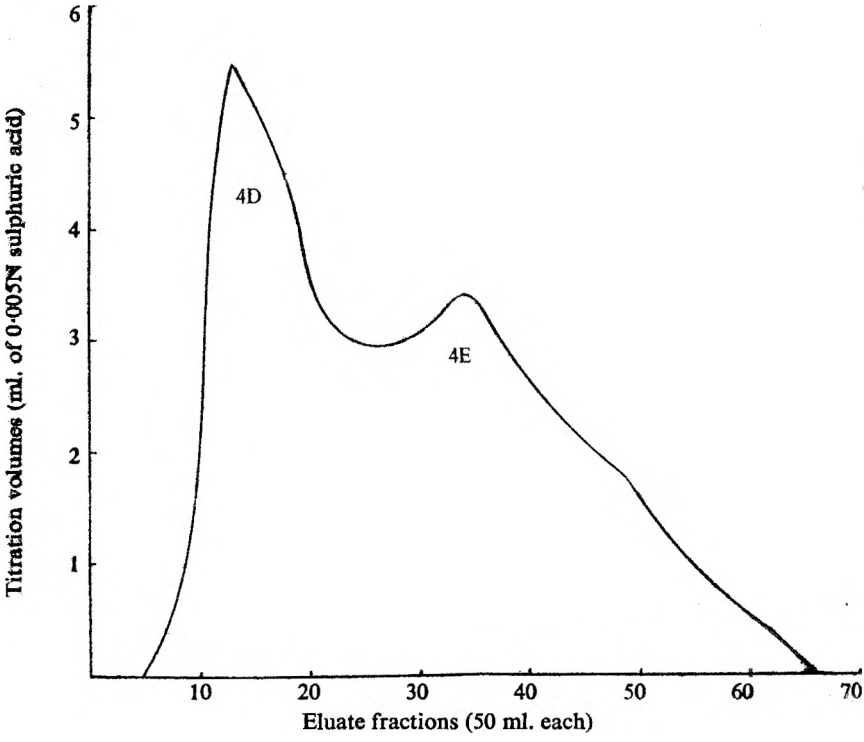

FIG. 4. Fractionation of English "pelletierine tannate" alkaloids equivalent to 250 mg. of *pseudo*pelletierine. Column, 560 g. of glass powder with 80 ml. of 0·5M phosphate buffer, *p*H 7·4; eluant, chloroform.

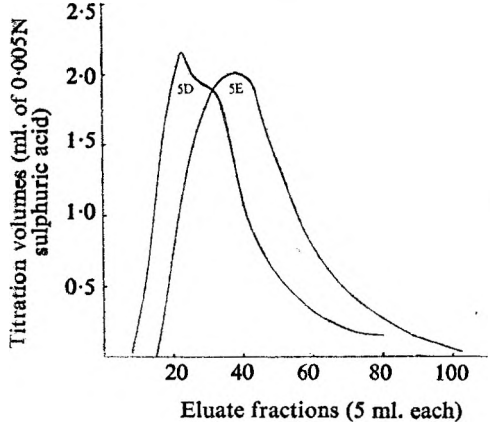

FIG. 5. 5D—Fractionation of French "pelletierine tannate" alkaloids equivalent to 75 mg. of *pseudo*pelletierine. 5E—Partition chromatogram of pelletierine (75 mg.). Columns, 140 g. of glass powder with 20 ml. of 0·5M phosphate buffer, *p*H 7·3; eluant, chloroform.

The quantitative data obtained from the two samples of "pelletierine tannate" are summarised in Table I.

Alkaloids of Punica Granatum.—A typical curve obtained with a phosphate buffer—kieselguhr column showing the course of fractionation of the alkaloids extracted from the fresh bark of the roots of *Punica Granatum* is given in Figure 6.

Alkaloids adsorbed by the kieselguhr afforded a single peak (Figure 5, curve 5E) after recovery and passage through a phosphate buffer-

glass powder column. The characters of the alkaloids in the eluate fractions are summarised below:—

*Peak 6B: pseudo*pelletierine picrate, needles from water, m.pt. 252°C. (with decomposition); *Reineckate*, plates from aqueous acetone, m.pt. 216°C. (with decomposition); found SCN, 49·3 per cent.; $C_9H_{15}ON$, $H[Cr(SCN)_4(NH_3)_2]$ requires SCN, 49·2 per cent.

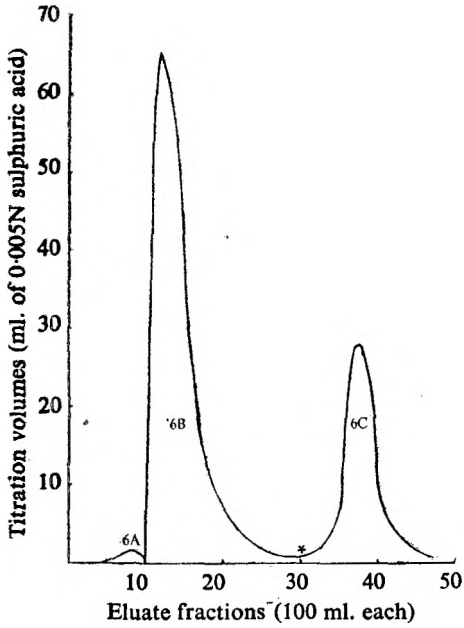

FIG. 6. Separation of alkaloids from root bark of *Punica Granatum* equivalent to 600 mg. of *pseudo*pelletierine. Column, 160 g. of kieselguhr with 80 ml. of 0·5M phosphate buffer, *p*H 7·0. Chloroform used as eluant.

Peak 6C: methyl*iso*pelletierine picrate, rosettes from absolute alcohol, m.pt. 154°C.; *Reineckate*, leaflets from aqueous acetone, m.pt. 228°C. with decomposition); found SCN, 49·0 per cent. $C_9H_{17}ON$, $H[Cr(SCN)_4(NH_3)_2]$ requires SCN, 48·9 per cent.

Peak 5E: pelletierine picrate, rosettes from water, m.pt., 150°C.; *Reineckate*, clusters of needles from aqueous acetone, m.pt., 254°C. with decomposition); found SCN, 50·6 per cent. $C_8H_{15}ON$, $H[Cr(SCN)_4(NH_3)_2]$ requires SCN 50·5 per cent.; sulphate $[\alpha]_D^{18°C.}$ −10·5° (c=11·8 in water).

The quantitative results obtained with the samples of crude drug are summarised in Table II.

Biological activities of the alkaloids of Punica Granatum.—The activities of pelletierine, *pseudo*pelletierine and methyl*iso*pelletierine against

TABLE II

ALKALOIDS OF *Punica Granatum*

Sample	Total Alkaloids* per cent.	Peak 6A* per cent.	ψPelletierine per cent.	Methyl*iso*pelletierine per cent.	Pelletierine per cent.	Recovery of total alkaloids per cent.
1. Old bark	0·10	trace	58	24	17	71
2. ,, ...	0·10	trace	27	41	32	96
3. ,, ...	0·23	1·6	25	36	38	91
4. ,, ...	0·19	0·4	38	33	28	90
5. Fresh bark of young roots ...	0·38	nil	32	36	32	82
6. Fresh wood of young roots ...	0·115	nil	21	32	47	76
7. Fresh bark of older roots ...	0·44	1·4	54	20	25	84
8. Fresh whole roots	0·39	0·5	22	35	42	95

* Calculated as ψ-pelletierine.

the liver fluke were determined by Drs. M. R. A. Chance and T. E. Mansour*, to whom we are greatly indebted for permission to quote their results (Table III); a fuller account of this work will be reported elsewhere.

TABLE III

ACTIVITIES OF ALKALOIDS OF *Punica Granatum* AGAINST LIVER FLUKE

Alkaloid	Maximum dilution causing	
	cessation of movement	visible interference with movement
Pelletierine sulphate	1 : 8000	1 : 64,000
Methyl*iso*pelletierine sulphate	1 : 4000	1 : 16,000
*Pseudo*pelletierine sulphate	1 : 2000	1 : 4000

DISCUSSION OF RESULTS

The results described in this communication provide evidence additional to that reported in Parts I and II of the effectiveness of partition chromatography as a means of fractionating mixtures of alkaloids. No new factors governing the enrichment of the fractions were observed. The high adsorptive capacity of kieselguhr was contrary to expectation since this material is frequently used in the clarification of liquids during the isolation of alkaloids[15]. Adsorption during partition chromatography is usually undesirable since it tends to cause spreading of the bands of material undergoing partition and thereby hinders separation[16].

Powdered glass provides a satisfactory means of overcoming this difficulty. Its capacity as a carrier of the stationary aqueous phase is about one-third of that of kieselguhr as a volume-weight ratio but as a volume-volume ratio, which is of greater practical importance in partition chromatography, the two carriers have about equal capacity. Powdered glass has the additional advantage that it is easily prepared in a standard and reproducible form, whereas kieselguhr is an extremely variable material. Experiments on the applicability of different grades of kieselguhr in partition chromatography will be described in a forthcoming communication.

The proportion of total alkaloids in pomegranate root and root bark varies widely; in agreement with the findings of Goodson[7] and of Ewers[17], we find that fresh samples generally contain a higher concentration of total alkaloids than older samples. Although the bark of pomegranate root has been traditionally used as the source of the alkaloids, the sample of wood examined by us was found to contain a useful proportion of alkaloids. From the restricted number of samples examined, there appears to be no regularity in the relative proportions of pelletierine, *pseudo*-pelletierine and methyl*iso*pelletierine; the highest proportion of pelletierine relative to the other alkaloids was found in the fresh sample of wood of the root.

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For the characterisation of pelletierine, *pseudo*pelletierine and methyl-*iso*pelletierine, the properties of the picrates were in good agreement with those recorded in the literature. Unfortunately authentic specimens of these alkaloids were not available for the determination of mixed melting-points. The characters of the Reineckates provided further confirmation of the identity of these alkaloids, particularly of pelletierine and methyl-*iso*pelletierine which afford picrates of similar melting-point. Hess[13] reported the isolation of *iso*pelletierine in 0·0015 per cent. yield from pomegranate root bark. We failed to find this alkaloid; attempts to characterise the alkaloid corresponding to peak A in Figure 6 as *iso*-pelletierine were unsuccessful.

The optical activity of pelletierine has been the subject of controversy. Hess and Eichel[8] found no optically active base in pomegranate root bark, whereas the results of Tanret[18] and Goodson[7] showed that the bark contains *l*-pelletierine. Hess and Eichel[19] resolved racemic pelletierine and for the *l*-sulphate record $[\alpha]_D^{18°C.}$ - 5·33°; whereas Tanret[18] reported - 30·3° as the specific rotation of the sulphate of pelletierine isolated from the bark. We now find that the sulphate of pelletierine extracted from the root and isolated by partition chromatography has $[\alpha]_D^{18°C.}$ - 10·5° (c. = 11·8 in water).

The two samples of " pelletierine tannate " examined by us bore little resemblance in composition to the mixed alkaloids extracted from the crude drug; one contained only a minor proportion and the other was apparently devoid of pelletierine. The unidentified basic material, which bore some resemblance to pelletierine in its behaviour on a chromatographic column and in its equivalent weight, may possibly have been a polymer of pelletierine formed during preparation of the " pelletierine tannate." It is unlikely that this material has any connection with the suggested bicyclic tautomeride of pelletierine[20]. The failure of " pelletierine tannate " accurately to represent the total alkaloids of the crude drug has previously been reported by Tanret[6] and Goodson[7], who ascribe this feature to the preferential precipitation of *pseudo*pelletierine and methyl*iso*pelletierine tannates during manufacture. In the process for the large-scale manufacture of " pelletierine tannate " given by Schwyzer[21], such preferential precipitation would be impossible. However, in Schwyzer's process, loss of pelletierine due to formation of a polymeric resin during maceration of the drug with 2·5N sodium hydroxide would doubtless take place, and some loss of volatile alkaloid would occur during evaporation of the ether-chloroform solution of the bases.

The biological results recorded in Table III may be regarded as an indication of the relative activities of pelletierine, *pseudo*pelletierine and of methyl*iso*pelletierine as anthelmintics (see Chance and Mansour[3]). Tanret's[6] and Goodson's[7] observations and those reported here indicate that " pelletierine tannate " is a relatively inefficient form of presentation of the anthelmintic alkaloids present in *Punica Granatum*. The assay process of the Pharmacopœia, which is designed to determine total bases in " pelletierine tannate " affords no evidence of the proportion of active

alkaloids in this material. It is apparent that pelletierine or one of its salts, isolated from the crude drug, would provide the most satisfactory anthelmintic preparation derived from pomegranate. For its isolation, fractional distillation or exploitation of the adsorptive capacity of kieselguhr under conditions similar to those described here would appear to be suitable. A preparation of the sulphates of the total alkaloids of pomegranate by the method described in the experimental section would be more likely to contain the active alkaloids than the " pelletierine tannate " at present available. We observed that aqueous solutions of these alkaloidal sulphates undergo no decomposition on storage for 6 to 7 months. A general study of the pharmaceutical aspects of this subject was not possible, owing to the great difficulty of obtaining even small quantities of the crude drug.

We are greatly indebted to Professor J. E. Driver, Mr. F. Fish, Dr. J. W. Fairbairn and Mr. S. G. Naravane for their assistance in procuring samples of the crude drug.

SUMMARY

1. The application of partition chromatography to the separation of the alkaloids of *Punica Granatum* and of " pelletierine tannate " has been studied.

2. Different samples of *Punica Granatum* contain widely different proportions of pelletierine, *pseudo*pelletierine and methyl*iso*pelletierine. Commercial samples of " pelletierine tannate " may contain little or no pelletierine, which exhibits the highest activity of the pomegranate alkaloids against the liver fluke.

3. Powdered glass has been found suitable as a carrier for the stationary phase of partition chromatographic columns.

This communication is abstracted from a thesis submitted by one of us (J. C.) in partial fulfilment of the requirements for the degree of Master of Pharmacy in the University of Nottingham.

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The paper was read in abstract by Mr. J. Chilton.

There was no discussion.

A COMPARATIVE STUDY OF AGARS FROM VARIOUS GEOGRAPHICAL SOURCES

By J. L. Forsdike

From the Laboratories of Boots Pure Drug Company, Nottingham

Received June 8, 1950

Although small amounts of agar were produced in other countries, prior to the outbreak of war in 1939 almost the whole of the world's supply of this material came from Japan. During the period of the war, however, production of agar was developed in many countries of the British Empire, including New Zealand, South Africa, Australia, Canada and India, as well as England; also in some foreign countries, the United States of America, Denmark, China, Russia, Spain and Italy among others. Agars of New Zealand, South African, Australian, British and Danish origin are commercially available in this country at the present time, and it was, therefore, thought that it would be of some value to make a comparison of these varieties of agar with Japanese strip agar, normally in use before the war and now again on the market. The points to which attention has been particularly directed are:—(1) The character and strength of the gel produced, (2) The melting- and setting-points of the gel, (3) Ash values and the microscopical characters of the ash.

The agars have also been examined for their reaction to the British Pharmacopœia tests for identity and purity.

Sources and Materials

New Zealand Agar. Agar is prepared in New Zealand from *Pterocladia lucida* and *P. capillacea*[1,2]. The production has reached 100 tons per annum[3]. Four commercial samples have been examined (Nos. 5, 6, 7 and 18). The agar occurs as a coarse greyish-white powder.

Australian Agar. This is derived from *Gracilaria confervoides*[4]. Its manufacture has been described by Wood[5]. Of the two specimens of this variety examined, No. 11 consisted of coarse brown flakes and No. 12 of a greyish-brown powder.

South African Agar. The chief sources of agar prepared in South Africa are *Gelidium cartilagineum, Gracilaria confervoides* and *Suhria vittata*[6]. Five commercial samples have been examined (Nos. 8, 9, 10, 17 and 19). Each consisted of a coarse, light-brown powder.

British Agar. This is prepared from *Gigartina stellata* and *Chrondrus crispus*. The sources, production and properties of British agar have been described, in considerable detail, by Newton *et al.*[7] in a recently published monograph. The manufacture of British agar has also been described and illustrated in an anonymous communication[8]. The two specimens examined (Nos. 13 and 14) were more finely powdered than the other varieties and yellowish in colour.

Danish Agar. Danish agar has been presented in two forms, one consisting of thin strips, very much crinkled and twisted and almost white in colour, and the other of a fine white powder. Two samples of strip (Nos. 15 and 20) and one of powder (No. 16) have been examined. The botanical source of this agar is not known.

Japanese Agar. 24 different batches of Japanese strip agar (Kobe No. 1) (Nos. 1 to 4 and 21 to 40) have been tested to obtain quantitative data for comparison with those of the other varieties. All of these have been obtained during the last three years with the exception of No. 2, which was imported in 1937. A report of the British Intelligence Objectives Sub-Committee, issued in 1946[9], describes the conditions under which agar is manufactured in Japan at the present time. The report names 34 different species of seaweed which are used for the purpose: of these *Gelidium amansii* and *G. pacificum* are the most important. A detailed description of the collection of the seaweed and of the manufacture, testing and grading of the agar is given and also figures for the quantities produced and exported.

Properties of the Gel

Gel Strength. In the work on British agar[7], the gel strength was measured by a modification of the method suggested by Campbell[10], which consists of determining the force required to turn a vane through a predetermined angle when immersed in the jelly. Chakraborty[11] immersed one pan of a balance in the jelly at a known level and determined the weight required to pull the pan out. Another method for determining the gel strength of agar, suggested by Lockwood and Hayes[12], depends on measuring the change in height of a column of jelly when removed from its containing cylinder. The apparatus is called a "ridgelimeter."

In the present work, the gel strength was measured by the Bloom gelometer. This instrument is regularly in use for the determination of the jelly strength of gelatin, and forms the subject of a British Standard Specification[13]. It has also been described by the National Association of Glue Manufacturers of the United States of America[14]. In adapting this method for use on agar, the principal modifications necessary are in the method of preparation and strength of the jelly employed. It was found that 1 per cent. was normally a suitable strength, and the method of procedure was as follows: Heat 500 ml. of water to boiling point, add 5 g. of agar, in moderately coarse powder, and boil gently for 5 minutes, with constant stirring. Cool somewhat, adjust the total weight of the solution to 500 g. by the addition of water and mix thoroughly. Allow to cool to about 60°C. and pour into the standard bottles. Three bottles should be filled with each solution under test. Leave the bottles until the contents have cooled to room temperature, then stopper and place them in the chill bath at a temperature of 10° ± 0·1°C. for not less than 16 and not more than 18 hours. The jelly strength is then determined in triplicate, exactly as described in the standard method and the average value taken.

The gel strengths of the specimens under examination, determined by the above method, are recorded in Table I. The results on sample No. 2 confirm the observation of Chakraborty[11] that Japanese agar loses its setting power, to some extent, after long storage. The melting- and setting-temperatures of this sample also seem to have been affected by age.

TABLE I

	Country of Origin	Gel Strength 1 per cent. g.	Setting Temperature 2 per cent. °C.	Melting Temperature 2 per cent. °C.
Average of 23 samples ...	Japanese	260 to 310	33 to 34	89 to 93
Sample 2	,, 1937	177	35	82
,, 5	New Zealand	625	35·5	92
,, 6	,,	618	36	90
,, 7	,,	610	36	91
,, 18	,,	620	35	92
,, 11	Australian	56	34	76
,, 12	,,	85	34·5	77
,, 8	South African	280	36	86
,, 9	,,	306	36·5	86
,, 10	,,	243	36·5	88
,, 17	,,	287	36·5	88
,, 19	,,	250	36	87
,, 13	British	55	40	56
,, 14	,,	100	40	57
,, 15	Danish	135	44	64
,, 16	,,	135	44	65
,, 20	,,	130	43	64

Some determinations were also made, at strengths other than 1 per cent., with the object of finding what concentration of the various agars would be required to produce a jelly of strength equal to one made with a given amount of Japanese agar. The results are stated in Table II.

TABLE II

CONCENTRATION OF AGAR REQUIRED TO PRODUCE A GEL EQUAL IN STRENGTH TO THAT PRODUCED BY 1 PER CENT. OF JAPANESE AGAR

New Zealand	0·7 per cent.
Australian	2·0 ,,
South African	1·0 ,,
British	1·5 ,,
Danish	1·5 ,,

Melting- and Setting-Temperatures. These were determined on 2 per cent. gels, since this is the concentration most usually used in bacteriological work. The solution was prepared by boiling the agar in water for 5 minutes, as described above, and adjusting to the required weight. From this solution 10 ml. was poured into a test tube, a thermometer inserted and the whole allowed to cool slowly, the temperature at which the gel solidified being taken. It was found that this temperature was quite sharply defined. The tube was kept in a thermostat, at 15°C., overnight and the next day was placed in a water-bath and gradually heated and the temperature at which the gel melted was recorded. The

results will be found in Table I. Melting- and setting-temperatures of agar have also been recorded by Chakraborty[11] and by Newton *et al*[7].

Sensory Characters. The visible characteristics of the 1 per cent. gels, prepared as described above, were as follows. *Japanese.* Slightly brownish, somewhat opalescent, a trace of insoluble matter present. *New Zealand.* Colourless, somewhat opalescent, a slight trace of insoluble matter present. *Australian.* Distinctly brownish, very opalescent, some insoluble matter. *South African.* Brownish, opalescent, a trace of insoluble matter. *British.* Very pale yellow, clear, no insoluble matter. *Danish.* Colourless, slightly opalescent, a slight trace of insoluble matter.

Ash and Microscopical Characters of the Ash

Ash and Loss. Table III shows the total and acid-insoluble ash contents of the various specimens, determined by the methods of the British Pharmacopœia. The losses sustained, by most of the samples, on drying to constant weight at 100°C. are also shown in this table. The official limits and the average values for 24 samples of Japanese strip agar are included for comparison. These results show that, other considerations apart, British and Danish agars are ruled out, so far as the official standards are concerned, by their high ash values.

TABLE III

	Country of Origin	Total ash per cent.	Acid-insoluble ash per cent.	Loss at 100°C. per cent.
	B.P. limits	5·0	1·0	18·0
Average of 24 samples ...	Japanese	2·3 to 3·6	0·02 to 0·30	12·0 to 20·0
Sample 5	New Zealand	1·20	0·20	12·2
" 6	"	1·07	0·18	—
" 7	"	0·92	0·06	11·9
" 18	"	1·05	0·15	16·1
" 11	Australian	5·90	0·56	14·8
" 12	"	3·24	0·02	16·1
" 8	South African	2·80	0·20	14·6
" 9	"	2·70	0·18	8·2
" 10	"	2·30	0·10	—
" 17	"	3·00	0·10	8·1
" 19	"	2·50	0·13	15·9
" 13	British	35·1	0·40	8·4
" 14	"	37·2	0·31	10·4
" 15	Danish	18·3	0·80	13·2
" 16	"	16·4	0·09	16·8
" 20	"	16·5	0·13	—

Microscopical Characters of the Ash. The part of the ash insoluble in hydrochloric acid was examined microscopically and a search made, in particular, for diatoms and sponge spicules. All samples of a common national origin showed similar microscopical characters, which are summarised below. The diatoms of Japanese agar are well known. King[15] has listed and illustrated characteristic species.

New Zealand. A few small diatoms, mainly species of *Melosira*, and sponge spicules. In addition, large masses of silica and much amorphous débris are present (Fig. 1).

Australian. Mainly amorphous debris, a small amount of silica and a few sponge spicules and diatoms, also *Melosira* sp. (Fig. 2).

South African. Diatoms, whole and broken, of a species of *Coscinodiscus* with a hexagonal pattern on the surface are fairly numerous, also present are a few silico-flagellates, fragments of silica and amorphous débris. Sponge spicules are absent (Fig. 3).

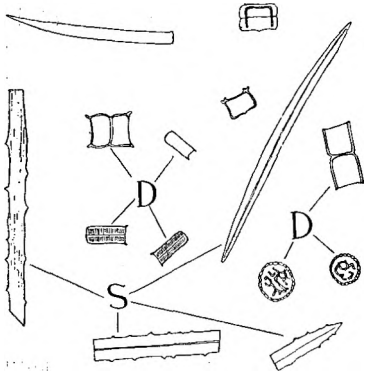

FIG. 1. Diatoms and sponge spicules from New Zealand agar. D, Diatoms, *Melosira sp.* S, spicules. × 330.

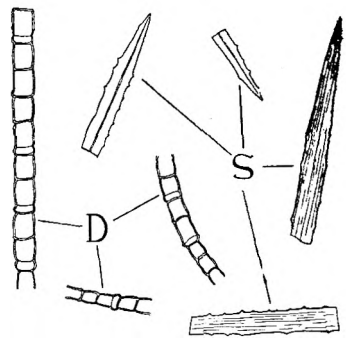

FIG. 2. Diatoms and sponge spicules from Australian agar. D, Diatoms, *Melosira sp.* S, spicules. × 330.

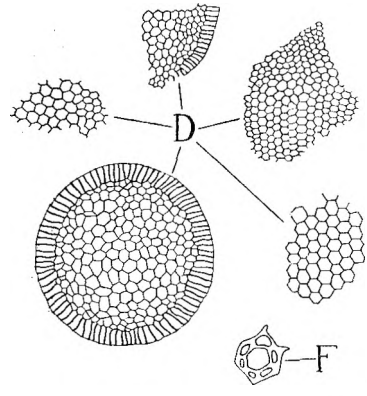

FIG. 3. Diatoms from South African agar. D, Diatoms, *Coscinodiscus sp.* F, A silico-flagellate. × 330.

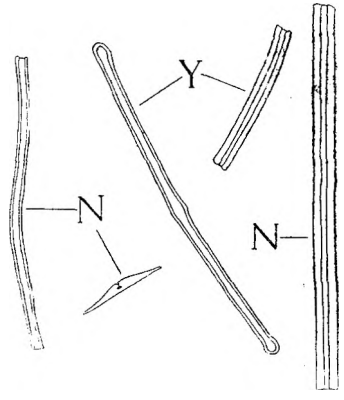

FIG. 4. Diatoms from Danish agar. N. *Nitzschia sp.* Y, *Synedra sp.* × 330.

British. Consists almost entirely of finely-divided amorphous matter, fragments of silica are very rare and diatoms and sponge spicules absent.

Danish. Mainly silica; some amorphous debris and a few diatoms, including species of *Nitzschia* and *Synedra,* no sponge spicules (Fig. 4).

PHARMACOPŒIAL TESTS FOR IDENTIFICATION

Ruthenium Red. All varieties are stained pink by solution of ruthenium red.

Potassium Hydroxide. All varieties give a yellow colour on warming with a 5 per cent. solution of potassium hydroxide but, with British agar, this colour is paler and longer in developing than with the other types.

Hydrolysis Test. All varieties of agar, after hydrolysis with hydrochloric acid, will reduce Fehling's solution. The barium chloride test for sulphate, however, when carried out in accordance with the instructions given in the British Pharmacopœia, gives no reaction with New Zealand, Australian and South African or even with Japanese agar. British and Danish agars do give a precipitate in this test.

Iodine Test. Powdered Japanese, New Zealand, South African and Australian agars give a deep crimson colour with a dilute solution of iodine. British and Danish agars give only a brown colour. It was found that 0·5 ml. of 0·05 N iodine with 0·1 g. of powder gave a more distinctive colour than 1 ml. of 0·05 N iodine recommended by the B.P.

Tannic Acid Test for Gelatin. This test will give satisfactory results with all varieties of agar, but when carrying out the test it is essential that the temperature of the mixed agar and tannic acid solutions be between 80° and 90°C. Within this range, pure agar gives no precipitate; the presence of 10 per cent. of gelatin will give rise to a distinct opalescence, while 20 per cent. produces a milky white turbidity. If, however, the temperature falls much below 80°C., an exactly similar turbidity is obtained from agar alone, if the agar be of Japanese, New Zealand, South African or Australian origin. British and Danish agars give no precipitate with tannic acid solution even in the cold.

Picric Acid Test for Gelatin. The United States Pharmacopœia gives a test for gelatin using picric acid. A 1 per cent. solution of the agar is prepared by boiling with water; this solution is cooled to about 50°C. and to 5 ml. is added an equal volume of a 1 per cent. solution of picric acid. None of the varieties of agar gave any precipitate when tested in this way. However, 10 per cent. of gelatin in the agar will give a marked turbidity and 5 per cent. is just detectable. This test is superior to the British Pharmacopœia test using tannic acid, mentioned above, because it is more sensitive and, even more, because there is no risk of getting false positives; no turbidity being produced by agar alone even on cooling to normal room temperature.

Conclusions

New Zealand, South African and Australian agars are of the same type as Japanese agar. New Zealand agar is generally superior to Japanese: South African is about equal to Japanese: Australian is much inferior in gel strength and colour, and contains more ash and insoluble matter. So-called British and Danish agars, on the other hand, apart from their inferiority in gel strength and in melting- and setting-temperatures. are of quite different character from the Japanese material and

are to be regarded as substitutes for, rather than as varieties of, agar. It would be preferable that these materials should be sold under some name other than "agar."

SUMMARY

1. New Zealand, South African, Australian, British and Danish agars have been examined. These agars are compared with Japanese agar in respect of the strength of the gels which they produce and also the melting- and setting-points of the gels.

2. The ashes, acid-insoluble ashes and losses on drying of the 5 varieties of agar are recorded and the microscopical characters of the ashes have been described and illustrated.

3. The agars have been tested by the British Pharmacopœia tests for identification of agar and also by the United States Pharmacopœia test for gelatin in agar.

I am indebted to the Directors of Boots Pure Drug Company for permission to publish results obtained in their laboratory.

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DISCUSSION

THE CHAIRMAN said that the results of this work were disappointing as far as British agar was concerned. Fortunately agars from Commonwealth sources were as good as the Japanese, but it was obvious that something giving 35 per cent. of ash and not giving the reactions of agar ought not to be called agar.

DR. T. E. WALLIS (London) said that one of the most important points arising from the paper was the quantity of ash in the different varieties. He thought that the ash in the Japanese, New Zealand and South African agars probably represented the ash in the seaweeds from which they were made, but with the British agar he understood that a considerable proportion of calcium salts was added in the course of preparation in order to make the material obtained from the algae gel when it was used. Perhaps this was also the case with the Danish variety. The picric acid test was a far better test for the presence of gelatin than

that now in the B.P. Some of the differences between the different agars depended upon the particular composition of the mucilaginous matter which was obtained from them. That obtained from the Japanese seaweeds was said to contain the sulphuric ester of galacturonic acid, and that was the cause of the positive reaction in the sulphate test. He could not explain why the author had failed to get a positive result in the test for sulphate after hydrolysis. Perhaps the process of preparation of Japanese agars had been altered recently. Did the author regard the diatoms as really good differential diagnostic characters for the different kinds of agar?

DR. J. M. ROWSON (London) said that he could confirm the author's findings with regard to the failure of the gelatin test. Students repeatedly reported to him the presence of gelatin in genuine agar because they did not get the temperature right. The tannic acid test required careful control to give a satisfactory indication, and the picric acid test was better. Like Dr. Wallis, he had been surprised to find that the author did not get a reaction with the sulphate test on Japanese agar. Also, he wondered whether the author had made any chemical investigation of the ash in British agar. Had the author plotted the log concentration graph for the concentrations to give equivalent gels as that would be interesting from the physico-chemical point of view? He also wondered whether the author had investigated the viscosities of dilute agar solutions and compared them with the gel strengths. This might form a further standard for agar, and, since U-tube viscometers were in the B.P. appendix, it might be more convenient to establish a viscosity strength than a gel strength, if the two were comparable.

DR. J. W. FAIRBAIRN (London) asked if there was any correlation between the botanical source of the agars and the gel strength. The Japanese and South African agars were similar in strength and both contained *Gelidium* species. The New Zealand agar, which was outstanding, was characterised by *Pterocladia*, of which there was a little in the Japanese agar. It was possible that the most important factor for any country wanting to cultivate seaweed was to select those species which yield a gel of very high strength. In evaluating gel strength the author had tried two methods. The results in each case were quite different, and even the order of gel strength varied a little. It seemed very important to get a method which would give the same results every time. He was sorry that it did not seem possible, from the paper, to distinguish with certainty the various commercial agars. Could the author guarantee, if he were given a powdered agar, to identify its geographical source?

MR. T. D. WHITTET (London) said that he had investigated a number of chemical tests to distinguish between these agars and had discovered a number of useful reactions, but, as yet, the work was uncompleted. He had also examined Norwegian agar (which seemed very similar to the British and Danish), a Californian sample (which was almost identical with Japanese) and Indian agar (which was more like South African).

He was able to get a positive reaction for sulphates with all the agars, and a very dense one indeed with the British agar. In the tannic acid test he had had the same results as the author except with Australian agar, which gave a slight precipitate even with the hot solution. This became very dense on cooling, and there was still a slight turbidity even on boiling the solution. He had included sodium alginate in his tests, and the only difference between this and British agar was the precipitate with calcium salts which occurred only with the former.

MR. G. SYKES (Nottingham) agreed that British and Danish agars probably did not justify the name of "agar" in the pharmacopœial sense. He wondered whether the author had considered the clarity of the agar solution in different solvents. British agar gave a clear solution in water but not in the presence of peptone. It was, therefore, no good for certain culture media. Instead of the Bloom gelometer for testing gel strength, had the author used the finger pressure test specified for gelatin in a British Standards Specification? As regards relative gel strengths he had found a 3 per cent. gel of British agar to have a weaker strength than a 2 per cent. one, and that the strength of gels in different solvents varied considerably before and after sterilisation.

DR. K. R. CAPPER (London) said that agar was sometimes regarded as being relatively inert in bacteriological media, but that was by no means true. The nature of the ash could have a great deal to do with the effect of agar on bacterial growth. With *B. subtilis*, for instance, the whole character and appearance of the growth on New Zealand agar was different from when Japanese agar was used. Certain ions, particularly cations, could have an effect on the degree of inhibition produced by some antibiotics, and by adding certain metallic salts to agar it was possible to obtain quite different results.

MR. A. R. G. CHAMINGS (Horsham) referred to the efforts and expense incurred during the war in the production of British agars, which, it appeared, unfortunately did not meet the B.P. requirements.

MR. H. DEANE (Sudbury) pointed out that large quantities of agar are used in the food industry.

PROFESSOR H. BERRY (London) said that he would like to know the nature of the water-soluble extractive. Before the war good quality Japanese agar had given little trouble, but since the war many difficulties had been due to variations in the quality of agar. It would be interesting to have some information on the quality of Japanese agar now on the market. One particular difficulty was with Brewer's medium, which should be perfectly clear. With post-war samples a gelatinous precipitate formed and was very troublesome. It would be interesting if the author could make arrangements, now that he had these specimens typed, to have bacteriological tests done on the various specimens. Was it possible to rely on samples of New Zealand agar as being of a constant type?

MR. R. L. STEPHENS (London) referred to the use of agar in emulsion of liquid paraffin with phenolphthalein B.P.C. It was a matter of considerable embarrassment to make this preparation with New Zealand agar and find that it was almost solid. The B.P.C. gave no instructions for adjusting the formula of that emulsion in the way in which the B.P. directed that the proportions of emulsifying agents might be changed according to the method of preparation. Had the author considered this aspect of agar?

DR. NORMAN EVERS (Ware) said that some agars had an effect on bacterial growth because they contained copper.

DR. W. P. KENNEDY (London) mentioned that material coming from India had been found to be heavily contaminated with copper, and it was discovered that this was due to the method of preparation, in which large copper pans were used. There was enough copper in some Indian agars to inhibit bacterial growth appreciably.

MR. G. R. A. SHORT (London) said that the objection to the use of British agar in confectionery would be its peculiar flavour, due probably to its high content of potassium chloride.

THE CHAIRMAN, referring to Professor Berry's suggestion for an investigation of the water-soluble matter of these agars, said it was to be noted that the unsatisfactory agars came from *Chondrus crispus*. This gave two extracts, one separable by hot water and the other by cold. They both contained sulphonated carbohydrates. The cold water extract had an inhibiting effect on the gelling of the hot water extract, but if one separated it one got a very viscous preparation derived from the hot water extract only. That might perhaps give a line to be followed in the investigation of the differences between the water-extracted matter of these different agars.

MR. J. L. FORSDIKE, in his reply, said that he had not investigated the ash of British agar, but it was stated by the manufacturers that it was principally potassium chloride, which was added to produce the gel. The preparation of agar in Japan had been described in the report of the British Intelligence Objectives Sub-Committee, 1946, No. JAP/PR/814. All the samples examined contained the same diatoms. They had tried the sulphate test on a hundred or more samples of Japanese agar and had never found it to work satisfactorily. They had not made concentration graphs, and they had not determined the viscosity. The reason for choosing the Bloom gelometer was that it was used for determining the gel strength of gelatin and was the subject of a British Standard Specification. It was, therefore, a well-established and quite invariable instrument. As to botanical source and gel strength, there was little doubt that the type of seaweed used to produce the agar was the principal factor in determining the gel strength, though no doubt the method of manufacture had something to do with it. In Japan 34 different species of seaweed were used to make agar, and that would cover most of the species in use in other parts of the world. He did

not think that the cultivation of seaweed for the production of agar had ever been attempted, and it would be rather impracticable. The usual practice was simply to collect the seaweeds which happened to grow in the locality where agar was to be produced. He did not quite understand Dr. Fairbairn's remarks about the gel strengths obtained by the two different methods; all the gel strengths were determined by the same method, although with gels of different strengths.

With regard to the differentiation of the various types, in those samples which he had examined he had found the same kind of diatoms and spicules present in the ash of every sample of a particular geographical variety, and therefore he thought it would be possible to identify Australian, New Zealand or South African agar by microscopical examination of the acid-insoluble ash. He had not examined either Californian or Indian agars. Only two samples of Australian agar had been examined and neither of them gave any precipitate in the gelatin test. He could not say whether the presence of peptone or any other substance would affect the gel as he had not tried making gels containing other substances and he had not examined the effect of sterilisation on gel strengths. He would try to determine what the water-soluble extractive was and also investigate the presence of copper. Whether the quality of Japanese agar was exactly the same as that obtained before the war, he did not know, but he was unaware of any obvious differences. He would make an attempt to have some bacteriological tests done on the samples of agar as had been suggested. If New Zealand agar was used in a formula intended for the Japanese variety, one would get very much stronger gelling.

VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

PART II.—THE EVALUATION OF SENNA POD AND ITS PREPARATIONS

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INTRODUCTION

THE quality of senna pod is usually assessed by its appearance, its water-soluble extractive and, to a certain extent, by its geographical source, the Alexandrian pod (*Cassia acutifolia* Delile) being more highly esteemed than the Tinnevelly (*C. angustifolia* Vahl). In a previous paper[1] it was shown that the glycosides (sennosides A and B) rather than the free anthraquinones are the active constituents and hence an estimation of the glycosidal content of a sample of pod should be a reliable indication of its biological activity. Further experiments have confirmed the close relationship between glycosidal content and biological activity and the results of these comparative experiments are recorded in this paper. The analyses of a large number of commercial samples of senna pod have shown that the above mentioned criteria of appearance and water-soluble extractive are no guide to the glycosidal content, though there is justification for considering the Alexandrian pod to be better than the Tinnevelly (see Part III).

The method used for estimating the glycosidal content was based on that of Kussmaul and Becker[2] and a detailed account of the modified chemical assay process which has been developed is given in this paper.

THE CHEMICAL ASSAY OF SENNA POD

Extraction of the glycosides. As the chief object has been the assay of the crude drug and its preparations, it has been essential to devise a reliable means of extracting the glycosides completely for assay purposes; the rest of the process is similar to that of Kussmaul and Becker except for alteration in details. For the sake of completeness a detailed account of the whole assay process is included. As a preliminary, the crude drug in fine powder was used to determine the true glycosidal content by hydrolysing a suspension directly; this was obviously necessary as a basis for comparative work. The use of a suspension of the powdered drug, however, is inconvenient for regular assay purposes and is inapplicable when whole or coarsely powdered pod is being dealt with; a suitable extraction process, therefore, is an essential stage.

Though the glycosides are sparingly soluble in water they dissolve readily in the presence of alkali, due to the formation of soluble salts:

it was therefore decided to use water as a solvent and to adjust the *p*H suitably. Hot water will penetrate the tissues of the pod more rapidly than cold water and thereby facilitate solution, but because of the somewhat thermolabile nature of the glycosides prolonged exposure to high temperature must be avoided.

Table I summarises the results obtained by assaying 2 per cent. infusions prepared by infusing 10 g. of pod in coarse powder under various conditions. It is seen that the active principles are incompletely soluble in water alone, but dissolve entirely and without loss when the infusion is maintained at a temperature approaching 100°C. for 10 to 15 minutes followed by the addition of alkali to *p*H 6 to 7. Immersion in a boiling water bath for less than 10 minutes may be sufficient for complete extraction, whereas immersion for longer than 20 minutes results in gradual decomposition of the active principles.

TABLE I

EFFICIENCY OF VARIOUS INFUSION TECHNIQUES FOR THE EXTRACTION OF GLYCOSIDES FOR THE ASSAY OF SENNA POD

Method of preparation of infusion			Proportion of active principles present
Vehicle	Procedure	Length of time of infusion	
			Per cent.
Cold water	Shaken occasionally ...	24 hours	84
Boiling water ...	Immersed in boiling water bath ; cooled and adjusted to volume.	15 minutes 20	75·0 75·0
Boiling water ...	Immersed in boiling water bath ; adjusted to *p*H 6 to 7 ; cooled and adjusted to volume.	5 minutes 10 15 20 30	83·4 100 100 100 85·6

Preliminary experiments showed that, in order to reduce sampling errors to a minimum, it is necessary to use a larger amount of whole pod and pod in coarse powder than when pod in fine powder is to be assayed.

The standard procedures for preparing infusions of senna pod which have been developed for chemical assay are as follows.

(a) *Whole pod.* 10 g. is cut into strips 2 to 3 mm. wide and transferred to a 500-ml. graduated flask. About 450 ml. of boiling water is added, the flask is immersed in a boiling water-bath for 10 minutes and frequently agitated. After removal from the bath, sufficient N sodium hydroxide is added to adjust to *p*H 6 to 7. The infusion is immediately cooled and made up to 500 ml.

(b) *Pod in coarse powder.* 10 g. is infused in about 450 ml. of boiling water for 10 minutes, the *p*H is adjusted to 6 to 7 as in (a), and the volume made up to 500 ml.

(c) *Pod in fine powder.* 1 g. is infused in about 90 ml. of boiling water for 10 minutes, the *p*H is adjusted to 6 to 7 as in (a) and the volume made up to 100 ml.

In every instance 10 ml. of the filtered infusion is taken for assay.

The Assay Process

(i) *Removal of the free anthraquinones.* N hydrochloric acid is added to adjust the 10 ml. of infusion to about *p*H 3, which is then shaken with 60 ml. and then 40 ml. quantities of ether until the ether extract is colourless. The combined ether fractions are washed with small quantities of acidified water and the washings added to the original aqueous solution. This solution contains only glycosides and no free anthraquinones.

(ii) *Hydrolysis of the glycosides.* The aqueous solution is heated with half its volume of 10N sulphuric acid in a boiling water bath for 15 minutes. The aglycone separates as a brown flocculent precipitate on cooling.

(iii) *Extraction and purification of the aglycones.* The liquid is shaken with 80 ml. of ether and allowed to settle; the aqueous portion is separated and the yellow ether solution decanted from the brown residue which forms as a layer between the ether and aqueous layers. The brown layer is dissolved in a small quantity of 30 per cent. sodium hydroxide solution and to this solution is added the separated aqueous portion (which contains excess of acid). The extraction is continued with 40 ml. quantities of ether in a similar manner, until the ether fraction is colourless, indicating complete extraction of the aglycone.

In order to separate those aglycones derived from the sennosides A and B, i.e., those containing carboxyl-groupings on the anthracene nucleus, the combined ether portions are extracted repeatedly with small volumes of N sodium bicarbonate until an extract is colourless. To the combined bicarbonate solutions ether is added, the solution is acidified with 50 per cent. sulphuric acid and, when effervescence has ceased, the mixture is shaken to transfer the aglycones to the ethereal layer. The aqueous layer is run off and the yellow ethereal solution filtered into a graduated flask. Any brown residue is dissolved in sodium hydroxide solution as before, re-acidified and the extraction repeated with 20 ml. quantities of ether. The solution is finally adjusted to volume with ether.

(iv) *Colorimetric estimation.* A suitable volume of the aglycone solution in ether is extracted by shaking with small portions of N caustic soda solution. To the combined alkali solutions 0·2 ml. of 3 per cent. hydrogen peroxide solution per 10 ml. of alkaline liquid is added and the mixture heated in a boiling water-bath for 4 to 5 minutes cooled, made up to a suitable volume with N caustic soda, and the intensity of the purple-red colour determined colorimetrically. For this purpose, three types of instruments have been used, viz., the EEL colorimeter (a simple instrument with a single photocell connected to a sensitive galvanometer); the *Spekker Absorptiometer* (with two balanced photocells and a null-point reading on the galvanometer) and the *Unicam Diffraction Grating Spectrophotometer* (with a single photocell connected to a sensitive galvanometer). In every instance calibration curves were made using pure sennosides A and B, and from these a final curve to represent a

mixture of equal parts of the sennosides was constructed; the peak of the wavelength of the incident light used was 520 mμ.

Limits of Accuracy of the Assay Process

The limits of accuracy of the assay process were estimated by carrying out a number of replicate assays on infusions prepared by the standard procedures from the drug (a) in fine powder (1 g. of drug in 100 ml.), and (b) in coarse powder (10 g. of drug in 500 ml.), and (c) on infusions prepared from the whole pod (10 g. in 500 ml.). The results are recorded in Table II and indicate that with (a) fine powder, (b) coarse powder and (c) whole pod, the coefficients of variation are 1·3 per cent., 5·71 per cent. and 6·14 per cent. respectively, which mean that for a probability of $P = 0{\cdot}99$, the results of assays for the fine powder would be within 4 per cent. of the true value, whereas those for the coarse powder and whole pod would be within 17 to 18 per cent. of the true value. The results from duplicate assays of an infusion carried out on the same day, were frequently identical and never deviated from each other by more than 5 per cent. It is likely that the limits of error in the assay of whole pod and coarse powder could be decreased by using a sample larger than 10 g.

These figures show that, apart from sampling errors, the chemical assay process described is accurate to within ± 4 per cent.

TABLE II

Limits of accuracy of the chemical assay process when applied to (a) pod in fine powder, (b) pod in coarse powder, and (c) whole pod

	Fine powder	Coarse powder	Whole pod
	Glycosides/g of drug	Glycosides/g. of drug	Glycosides/g. of drug
	32·5 mg.	30·5 mg.	30·8 mg.
	32·5	33·5	33·8
	31·8	29·5	31·2
	32·2	31·8	28·8
	31·2	31·1	31·1
	31·5	28·5	28·4
Mean	32·0 mg./g.	30·8 mg./g.	30·7 mg./g.
Coefficient of variation	1·30 per cent.	5·71 per cent.	6·14 per cent.

Note.—The coarse powder and whole pod represented the same sample, whereas the fine powder was made from a different sample.

Correlation Between Biological Activity and Glycosidal Content

In Table III are set out the results of comparative assays of biological activity and glycosidal content of various samples of pod and simple dilutions. The glycosidal content was determined by the chemical assay process already described and the biological activity by Lou's[3] process. These results reveal a remarkably high degree of correlation and indicate that the chemical assay is a reliable method for the evaluation of the pod, but do not necessarily prove that the activity of the crude drug is entirely

due to the sennosides. However, for different samples of pod, the relative activities can be reliably assessed by means of the chemical assay.

TABLE III

BIOLOGICAL ACTIVITY AND GLYCOSIDAL CONTENT OF SENNA POD

Sample	Biological activity		Glycosidal content as sennosides A+B determined chemically	Ratio of Biological activity to Glycosidal content
	$P_S = 100$	As sensosides A+B		
		Per cent.	Per cent.	
Standard Pod, P_S ...	100	3·200	3·20	1·00
Powdered pod	(i) 148 } Mean = 135 (ii) 122 }	4·320	4·60	0·94
Powdered pod, mixed with inert material.	(i) 15·1 } Mean = 15·0 (ii) 14·6 }	0·480	0·475	1·01
Powdered pod, mixed with inert material.	(i) 16·6 (ii) 20·3 } Mean = 16·7 (iii) 13·2 }	0·534	0·523	1·02
Powdered pod	(i) 86 } Mean = 91 (ii) 95 }	2·910	2·830	1·03

Note.—The biological assay results have been expressed in relation to the Standard Pod, $P_S = 100$; as this sample contains 3·2 per cent. of sennosides A and B, the biological assay results have been expressed in terms of sennosides A+B in order to compare with the chemical assay results.

THE EFFECT OF INTERFERING SUBSTANCES ON THE CHEMICAL ASSAY PROCESS

It was noticed that when certain non-official preparations of senna pod were assayed biologically and chemically, the ratios between biological activity and glycosidal content, as shown in Table IV, were much higher than those given from the comparative assays of pod described above. It was inferred that ingredients were present which interfered with the extraction of the anthracene derivatives in the colorimetric assay process. That liquorice interferes with the Borntträger reaction has already been noted[4], and until this point is clarified it is recommended that the chemical assay process be used only to evaluate samples of the crude drug and its simple preparations, and the bio-assay for those preparations of senna pod, the exact composition of which is not known.

TABLE IV

BIOLOGICAL ACTIVITY AND GLYCOSIDAL CONTENT OF NON-OFFICIAL PREPARATIONS OF SENNA POD

Description	Biological activity		Glycosidal content as sennosides A+B determined chemically	Ratio of Biological activity to Glycosidal content
	$P_S = 100$	As sennosides A+B		
		Per cent.	Per cent.	
A liquid preparation...	(i) 9·9 } Mean = 12·1/ml. (ii) 14·3 }	0·387 $^{W}/_{v}$	0·300 $^{W}/_{v}$	1·29
A liquid preparation...	(i) 17·4 } Mean = 15·5/ml. (ii) 13·6 }	0·500 $^{W}/_{v}$	0·340 $^{W}/_{v}$	1·48
A pastille	(i) 10·5 } Mean = 10·8/g. (ii) 11·1 }	0·346 $^{W}/_{w}$	0·248 $^{W}/_{w}$	1·40

Note.—See Table III.

Summary

1. A technique is described for preparing an infusion of senna pod (whole and in coarse and fine powder) containing all the active glycosides.

2. Details are given of a method, based on that of Kussmaul and Becker[2], for estimating the glycosides colorimetrically; the method is accurate to within ± 4 per cent.

3. A high degree of correlation has been shown to exist between the biological activity and glycosidal content, indicating that the chemical assay is a reliable method for evaluation of senna pod.

4. It has been recommended that since other ingredients, such as liquorice, may interfere with the chemical assay process, the bio-assay be used for estimating the activity of senna pod preparations, the exact composition of which is not known.

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VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

Part III. Galenical Preparations of Senna Pod

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In Part I of this series of papers[1] reference was made to a preliminary test on an extract of senna pod which was found to contain only about 5 per cent. of its theoretical activity. Biological and chemical tests have since been carried out on a number of galenical preparations of senna pod and the results have confirmed that only a small proportion of the activity of the pod is present in these concentrated preparations. The ultimate aim of the work described in this paper has been to devise a method of preparing an extract representing the full activity of the pod from which it was made, and as a result, it has been possible to set out the principles underlying efficient extraction and concentration processes.

Evaluation of Samples of Senna Pod

To assess the efficiency of an extraction and concentration process it is necessary to know the glycosidal content and water-soluble extractive of the pod. Table I summarises the results obtained from the examination of a number of samples of Alexandrian (*Cassia acutifolia*, Delile) and Tinnevelly (*C. angustifolia*, Vahl) pod of manufacturing and hand-picked grades. The glycosidal content was determined by the assay process described in Part II[2] and the water-soluble extractive by the B.P. method. Moisture content determination was carried out by drying at 100°C. in an oven. These figures show that (a) appearance is not a reliable method of evaluating the pod, as the best quality hand-picked Alexandrian pod sometimes has a lower glycosidal content than the dark and much broken manufacturing grade; (b) the average glycosidal content and range for Alexandrian pod was 2·37 to **3·22** to 4·34 per cent., and for Tinnevelly 1·22 to **1·96** to 2·78 per cent.; (c) the average water-soluble extractive and range for Alexandrian pod was 25·8 to **28·3** to 31·2 per cent. and for Tinnevelly 20·7 to **23·0** to 25·5 per cent.; the Alexandrian pod contains more activity and water-soluble extractive than the Tinnevelly, but within each species no correlation was apparent between glycosidal content and water-soluble extractive. It is interesting to note that not all samples of Alexandrian pod and none of the samples of Tinnevelly pod examined complied with the B.P. requirement of containing not less than 28 per cent. of water-soluble extractive. The B.P.C., on the other hand, states that senna fruit has a water-soluble extractive of 18 to 30 per cent.; the extractives of all samples exceeded the lower

limit, and in three samples of Alexandrian pod the upper limit was exceeded. Since no indication is available for ascertaining the source of pod used in commercial preparations, 2·59 per cent. (average of 3·22 and 1·96 per cent.) has been taken as the "average glycosidal content of senna pod," and 25·65 per cent. (average of 28·3 and 23·0 per cent.) as the "average water-soluble extractive."

TABLE I

THE POTENCY OF COMMERCIAL SAMPLES OF SENNA POD

Sample	Source	Grade	Year purchased	Glycosidal content as sennosides A and B per cent.	Water-soluble extractive, per cent.	Moisture content, per cent.
1	Alexandrian	Hand-picked	1946	3·20	—	—
2	"	"	1949	2·37	27·1	10·0
3	"	"	1950	3·05	26·7	10·5
4	"	Manufacturing	1949	4·34	30·1	8·5
5	"	"	1949	3·32	31·2	8·3
6	"	"	1949	2·83	26·8	10·2
7	"	"	1950	3·07	28·9	8·4
8	"	"	1948	2·78	30·2	10·4
9	"	"	1950	2·91	27·2	9·9
10	"	"	1950	3·78	28·6	10·2
11	"	"	1949	3·28	26·9	8·7
12	"	"	1946	2·98	29·6	10·0
13	"	"	1949	3·21	28·5	9·0
14	"	"	1949	3·89	25·8	—
15	"	"	1950	3·30	28·9	—
16	Tinnevelly	Hand-picked	1950	2·78	23·3	9·8
17	"	"	1950	2·64	23·7	9·8
18	"	Manufacturing	1950	1·63	20·7	8·8
19	"	"	1949	1·29	21·3	11·5
20	"	"	1949	2·23	25·4	9·6
21	"	"	1950	1·35	23·3	10·3
22	"	"	1949	2·55	25·5	11·0
23	"	"	1950	1·22	20·8	10·5

THE POTENCY OF COMMERCIAL SAMPLES OF LIQUID EXTRACT OF SENNA B.P.

Liquid extract of senna B.P. (which is prepared by a triple maceration process with cold water) is a 1 : 1 extract, *i.e.*, 1 ml. should represent the activity of 1 g. of pods. Samples of this galenical prepared by a number of manufacturers were purchased on the open market and were assayed for glycosidal content and for total solids. The values are recorded in Table II, as the potencies found experimentally and as a proportion of the glycosidal content of the "average" pod in order to indicate the efficiency of the complete process of manufacture and storage. Samples A to F were assayed within a month of purchase, G and H six months after purchase. It is seen that less than 5 per cent. of the original activity exists in the majority of these extracts; even if one of the poorest grades of Tinnevelly pod (possessing, *e.g.*, 1·22 per cent. glycosides) had been used, the proportion of glycosides in the best sample assayed would be only 17 per cent. of that in the pod. The total solids content of the extracts is very low when compared with the average water-soluble extractives of the pod, even taking into account that priteinous matter is precipitated and removed during preparation. These figures suggest that the pharmacopœial process of triple maceration does not allow complete

extraction; this fact could be responsible, partly or completely, for the remarkably low glycosidal contents of the commercial extracts.

TABLE II

POTENCY OF COMMERCIAL SAMPLES OF LIQUID EXTRACT OF SENNA, B.P.

Manufacturer	Glycosidal content	Proportion of theoretical glycosidal content	Total solids
	per cent. w/v	per cent.	per cent. w/v
A	0·210	8·1	14·3
B	0·170	6·6	14·6
C	0·087	3·3	11·1
D	0·080	3·1	15·4
E	0·068	2·6	11·4
F	0·057	2·2	15·8
G	0·030	1·2	10·7
H	0·027	1·0	24·0

Note—Liquid extract of senna, B.P. should contain in 1 ml. the activity of 1 g. of pod. If the average glycosidal content of the pod is assumed to be 2·59 per cent., liquid extract of senna should contain 2·59 per cent. w/v of glycosides. This figure has been used in calculating the values in column 3.

THE POTENCY OF NON-OFFICIAL PREPARATIONS OF SENNA POD

For reasons already stated (see Part II[2]), non-official preparations of senna pod were assayed biologically. Table III records the results of the bio-assays of several preparations. The reputed activity has been calculated on the assumption that a pod of average potency had been used in the preparation; by comparing the theoretical values with those found experimentally, the proportion of glycosides surviving the manufacturing process has been computed. The results indicate that the proportion of the theoretical glycosidal content is higher than that in pharmacopœial preparations; this may be due to (a) the use of a better quality pod than the "average", (b) more care being taken to standardise and stabilise

TABLE III

BIOLOGICAL ASSAYS OF NON-OFFICIAL PREPARATIONS OF SENNA POD

	Description	Reputed activity	Biological activity	Proportion of theoretical glycosidal content
		Per cent. glycosides	Per cent. glycosides	Per cent.
1	A liquid	1·295 w/v	0·387 w/v	30·7
2	A liquid	1·295 w/v	0·500 w/v	38·6
3	A pastille	0·856 w/w	0·346 w/w	40·5
4	Granules	0·500 w/w	0·534 w/w	107·0

Note—The biological activity has been expressed in relation to the Standard Pod, Ps, which contains 3·20 per cent. of sennosides A + B.

the product, and (c) the presence of other ingredients which may have a synergistic effect on the purgative activity of the glycosides.

The Potency of Commercial Samples of Concentrated Infusion of Senna B.P.

Concentrated infusion of senna, B.P. is prepared by a reserved percolation process using alcohol (20 per cent.) and should be eight times as active as fresh infusion of senna, B.P.C. Samples of this galenical prepared by the same manufacturers as those listed in Table II, were analysed. The results, recorded in Table IV, show once more that there was little activity present. It was therefore necessary to establish the glycosidal content of the fresh infusion in order to be able to compute the theoretical potency of the concentrated infusion. Results of experiments recorded later show that the B.P.C. fresh infusion contains approximately 1·75 mg. of glycosides per ml.; the concentrated infusion should therefore contain 14 mg. per ml.

TABLE IV

Potency of commercial samples of concentrated infusion of senna B.P.

Manufacturer	Glycosidal content	Proportion of theoretical glycosidal content	Total solids
	Per cent. w/v	Per cent.	Per cent. w/v
A	0·076	5·58	7·1
B	0·026	1·85	9·4
C	0·060	4·29	11·1
D	0·073	5·21	15·9
E	0·012	0·86	11·9
F	0·054	3·86	14·5
G	0·035	2·50	9·3
H	0·210	15·00	16·4

Note—Concentrated infusion of senna, B.P. should have eight times the activity of fresh infusion of senna, B.P.C. The latter preparation has been found to contain approximately 1·75 mg. of glycosides per ml.; concentrated infusion of senna should therefore contain approximately 1·4 per cent. w/v of glycosides. This figure has been used in calculating the values in column 3.

Infusions of Senna Pod

(a) *Simple Infusions.* The B.P.C. 1949, p. 797, states that a simple infusion may be made by soaking 4 to 12 pods in about 5 fl. oz. of warm water for about 12 hours. As the mean weight of a pod is given as 0·16 g. the maximum recommended concentration is approximately 12 in 1,000. On packets of pods bought from pharmacies, in some instances the directions recommend that pods be soaked in cold water and in others in hot water, for periods varying from 3 to 4 hours to overnight. Infusions were prepared by adding 1,000 g. of (i) boiling water, (ii) water at 50°C. and (iii) cold water, to 12 g. samples of best quality hand-picked Alexandrian pod (containing 3·05 per cent. of glycosides) and assayed after varying periods of time. The results are presented in Table V. It

has previously been shown (Part II)[2] that not more than approximately 4/5 of the active glycosides in the pod can be dissolved by water alone. It is seen, therefore, that a 1·2 per cent. infusion made with boiling water extracts in 15 minutes the maximum possible activity; water at 50°C., and cold water, even after 24 hours, extract sub-maximal amounts. If it is assumed that a simple infusion extracts approximately 3/4 of the activity of the pod, then 5 fl. oz. of an infusion made from 4 to 12 pods would therefore contain about 15 to 45 mg. of glycosides.

(b) *Fresh Infusion of Senna B.P.C.* The fresh infusion was prepared using 50 g. of the sample of pod used in (a) and 500 g. of boiling water. The preparation was assayed after the prescribed infusion time and after further periods. A similar infusion made with 25 g. of pod in 500 g. of water was also prepared and assayed. The results (Table V) show that in the B.P.C. infusion, 53 per cent. of the activity of the pod has been extracted after 15 minutes and that no more active principles are extracted even after 1 hour's contact. With the 5 per cent. infusion, 65 per cent. of the pod's activity has been extracted. The construction of the B.P.C. fresh infusion was approximately 1·75 mg. of glycosides per ml. The official dose range of 15 to 60 ml. would therefore offer 21·5 to 105 mg. of glycosides; this is considerably in excess of that administered in a simple infusion of from 4 to 12 pods and also of the B.P. dosage of senna pod (*viz.* 0·6 to 2 g., representing approximately 18 to 60 mg. of glycosides).

TABLE V

POTENCY OF INFUSIONS OF SENNA POD AND EFFICIENCY OF EXTRACTION PROCESS

Vehicle	Method of preparation: Proportion of pod Per cent.	Method of preparation: Length of time of infusion Hours	Concentration of infusion Glycosides mg./ml.	Proportion of active principles extracted Per cent.
Boiling water ...	1·2	¼	0·298	81·5
		24	0·307	84·0
Water at 50°C. ...	1·2	4	0·258	71·0
		24	0·258	71·0
Cold water ...	1·2	24	0·263	72·0
Boiling water ...	5·0	¼	0·990	65·0
Boiling water ...	10·0	¼	1·69	53·0
		½	1·69	53·0
		1	1·69	53·0

The results, so far, indicate that, whereas fresh infusions of senna contain approximately 1/2 to 3/4 of the activity of the pod, commercial samples of concentrated preparations such as liquid extract of senna and concentrated infusion of senna contain, at the most, about 1/10 of the activity. The fresh infusion therefore represents the most active of the official galenicals. The low glycosidal content of the concentrated preparations suggests that in addition to incomplete extraction, losses may occur during evaporation and possibly during subsequent storage. Thus, in order to locate the stages at which losses in activity take place, it is

necessary to investigate the efficiency of the extraction processes of the B.P. (triple maceration, reserved, and also general percolation), followed by studies of the effect of heat on the glycosidal content of aqueous macerates and percolates of senna pod.

The Efficiency of the Pharmacopœial Processes for the Extraction of Senna Pod

(a) *Triple Maceration.* The B.P. triple maceration process for liquid extract of senna was carried out using pod containing 3·07 per cent. of glycosides and 28·9 per cent. of water-soluble extractive. Each macerate was assayed for glycosides and total solids. The results are submitted in Table VI and show that only about 2/3 of the active principles and 3/4 of the water-soluble extractive have been extracted by the process. These figures help to explain, in some measure the low glycosidal content of commercial preparations of liquid extract of senna; nevertheless the discrepancy is still great and suggests that activity is also lost during the subsequent concentration stage and possibly during storage.

TABLE VI

Efficiency of triple maceration extraction process for preparation of liquid extract of senna, B.P.

Macerate	Volume	Proportion of glycosides extracted	Proportion of total solids extracted
	ml.	per cent.	per cent.
1st	400	34	37
2nd	390	17	22
3rd	430	13	18
Pressings ...	130		
Totals ...	1350 ml.	64	77

Weight of pod used = 200 g.

(b) *Reserved Percolation.* Concentrated infusion of senna, B.P. was prepared, using a sample of pod in coarse powder containing 3·08 per cent. of glycosides and 35·3 per cent. of water-soluble extractive. Each of the percolates and the finished preparation was assayed for glycosides and total solids. (The second percolate was concentrated by heating under reduced pressure at a temperature which never exceeded 40°C.) The results are submitted in Table VII. Since approximately half of the activity of the concentrated infusion is derived from the reserved percolate (which in the above preparation had a potency of 0·76 per cent. w/v of glycosides), even if all the activity in the second percolate were destroyed by overheating during concentration, the finished product should not contain less than about 3/4 of this value. The freshly prepared concentrated infusion contained 1·02 per cent. w/v of glycosides, indicating that there had been no loss during concentration of the second percolate. The proportion of the theoretical glycosidal content (see note

to Table IV) was therefore 73 per cent. However, the concentrated infusion contained only 6 instead of 8 times the activity of the fresh infusion. This discrepancy exists because the pharmacopœial directions for the preparation of the concentrated infusion permit the extraction of only about 43 per cent. of glycosides, whereas in the preparation of the fresh infusion approximately 53 per cent. is extracted (Table V). The remarkably low glycosidal content of commercial samples suggests that deterioration takes place subsequent to manufacture during storage.

TABLE VII

EFFICIENCY OF RESERVED PERCOLATION PROCESS FOR PREPARATION OF CONCENTRATED INFUSION OF SENNA B.P.

Percolate fraction	Proportion of glycosides extracted	Proportion of total solids extracted
	per cent.	per cent.
0 to 140 ml.	21·7	35
140 to 340 ml.	21·0	31
Totals	42·7	66

Weight of pod used = 160 g.

(c) *General Percolation Process.* Senna pod in coarse powder containing 3·08 per cent. of glycosides and 35·3 per cent. of water-soluble extractive was percolated with chloroform water as in the B.P. general process. The percolate was collected in fractions which were assayed for glycosides and total solids. The results, recorded in Table VIII, show that with a percolate : drug ratio of 15, all the soluble extractive but only 3/4 of the active principles are extracted, and that practically the whole of this quantity is contained in the first six volumes of percolate.

PROPORTION OF GLYCOSIDES TO WATER-SOLUBLE EXTRACTIVE (OR TOTAL SOLIDS)

The proportion of glycosides to water-soluble extractive or total solids is useful and important, as it offers a ready means of ascertaining the efficiency of the extraction and concentration processes. For the sample of pod used above the value is 87 mg./g., which means that if the active principles were extracted completely with water and the solution evaporated to dryness without loss, the glycosidal content of the product would be 87 mg./g.

EFFECT OF HEAT ON THE GLYCOSIDAL CONTENT OF AQUEOUS PERCOLATES OF SENNA POD

The usual method of concentration is evaporation by heat, and accordingly time-temperature effects on aqueous percolates of senna pod were studied. Portions of a percolate (the reaction of which is usually *p*H 5·1 to 5·2) were heated at different temperatures for various periods of time and the loss in glycosidal content was determined. The results.

recorded in Table IX, show that the deterioration is less at lower temperatures and for shorter periods of time as might be expected. By using reduced pressure, evaporation at temperatures between 50° and 60°C.

TABLE VIII

POTENCY OF SUCCESSIVE FRACTIONS OBTAINED BY PERCOLATION OF SENNA POD IN COARSE POWDER WITH WATER

Percolate fraction in ml.	Proportion of glycosides extracted	Proportion of total solids extracted
	per cent.	per cent.
0 to 300	63	85
300 to 600	10	12
600 to 900	1	2
900 to 1500	1	1
Totals	75	100

Weight of pod used = 100 g.

is fairly convenient on a manufacturing scale, but even at this temperature there was a loss of $\frac{1}{3}$ to 1/5 of the initial activity during an 8-hour period of heating. The effect of increasing alkalinity was studied by adjusting portions of a percolate to *p*H values between 5·1 and 8·4, heating at 60°C. for various periods and determining the glycosidal content.

TABLE IX

EFFECT OF HEAT ON GLYCOSIDAL CONTENT OF SENNA PERCOLATE (*p*H 5.1)

Temperature	Period of heating	Proportion of initial glycosidal concentration, per cent.
100°C.	0 minutes	100·0
	5	91·0
	10	84·5
	15	70·4
	30	56·3
	60	53·4
	120	28·2
80°C.	0 hours	100·0
	1	86·0
	2	76·6
	3½	42·9
	6½	23·0
70°C.	0 hours	100·0
	1	92·4
	4	81·0
	6	57·6
	8	51·5
60°C.	0 hours	100·0
	4	82·6
	7½	64·0
	16	60·5
	24	44·2
	48	18·1
50°C.	0 hours	100·0
	2	88·8
	8	81·0
	15	76·0
	24	70·4
	48	67·5
	96	35·2

As the results submitted in Tables IX and X and shown graphically in Figures 1 and 2 indicate, at values more alkaline than *p*H 7·1, there was a striking and rapid loss of activity in comparatively short periods of time.

TABLE X

EFFECT OF HEAT AT 60°C. ON THE GLYCOSIDAL CONTENT OF SENNA PERCOLATE AT DIFFERENT *p*H VALUES

*p*H	Period of heating	Proportion of initial glycosidal concentration, per cent.
6·00	0 hours	100·0
	2	75·0
	4	63·0
	7½	49·0
	16	29·7
	24	23·0
7·10	0 hours	100·0
	2	76·0
	6	30·2
	7½	20·0
8·38	0 hours	100·0
	1	47·0
	2	26·5
	4	12·5

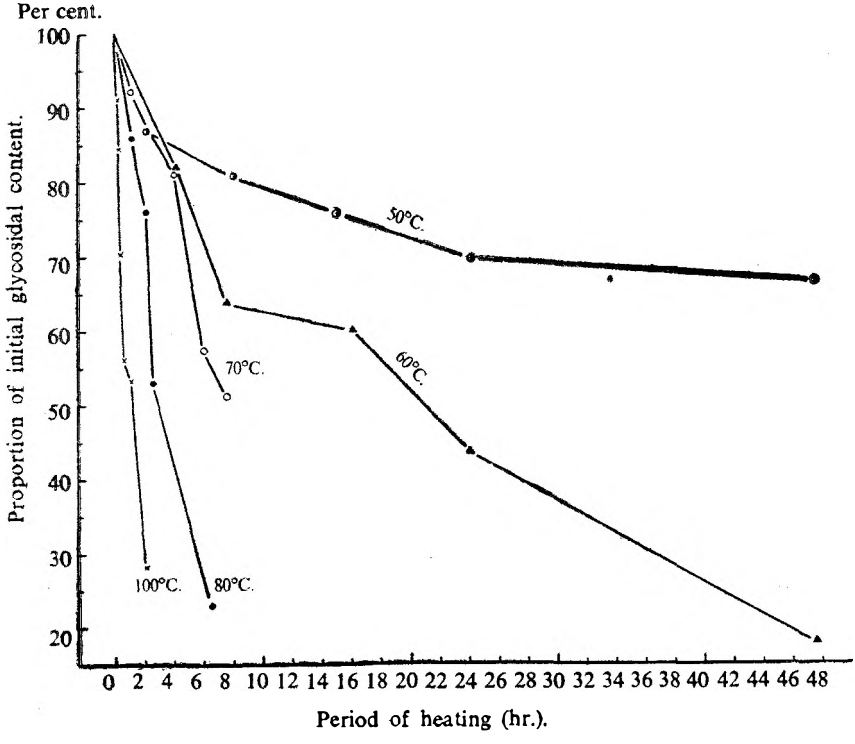

FIG. 1. Effect of heat on glycosidal content of senna pod percolate (*p*H 5·1) (Table 9).

Preparation of Potent Extracts by Concentration of Aqueous Percolates

(a) *A Soft Extract.* A senna pod percolate containing 95·5 mg. of glycosides per g. of total solids was evaporated to a soft extract under reduced pressure at a temperature not exceeding 40°C. The evaporation took 10 hours. The product was assayed and found to contain 56 mg. of glycosides per g. of extract and 93·3 mg. of glycosides per g. of total solids, indicating that 98 per cent. of the activity of the percolate had survived the concentration process.

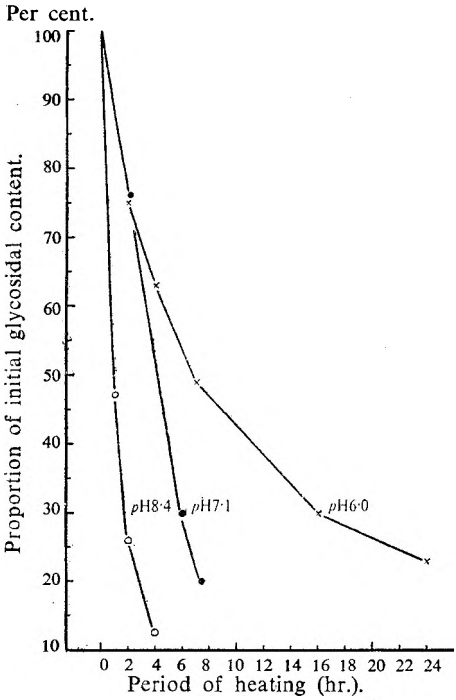

Fig. 2. Effect of heat at 60°C. on senna pod percolate at different *p*H values (Table 10).

(b) *A Liquid Extract.* A percolate containing 83 mg. of glycosides per g. of total solids was heated at 80°C. for 3 minutes, cooled rapidly, set aside for 48 hours to allow proteinous matter to settle, and the liquid decanted. The percolate, when reassayed, showed no loss in activity. It was then concentrated by heating under reduced pressure at a temperature not exceeding 45°C. to a thick liquid (the specific gravity of which was 1·21), which contained 33·4 mg. of glycosides per g. of extract, and 74 mg. of glycosides per g. of total solids, indicating that 89 per cent. of the activity of the percolate had survived the concentration process.

To this extract 25 per cent. of alcohol (90 per cent.) was added (as in the preparation of liquid extract of senna B.P.). The finished preparation was assayed and found to contain 34·8 per cent. of total solids and 2·5 per cent. w/v of glycosides.

Extraction of Senna Pod with Organic Solvents and their Aqueous Dilutions

According to Stoll *et al.*[3] the active principles, sennosides A and B, exist in the crude drug as water-soluble salts and as free glycosides which are almost insoluble in cold water. This explains why it is practically impossible to extract the glycosides completely with water, unless hot water with traces of alkali are used (as in the standard assay process, Part II[2]). Stoll also states that sennoside A is only sparingly soluble in ethyl alcohol, and slightly soluble in methyl alcohol and acetone; sennoside B is more readily soluble in these solvents. However, if the solvents

are diluted to contain about 30 per cent. of water, they then readily dissolve both of the sennosides and their salts. These facts have been confirmed by shaking known amounts of pod in coarse powder with the above-mentioned pure solvents and their aqueous dilutions and assaying the filtered solutions; isopropyl alcohol and its dilutions were also used. The results clearly demonstrated that the solvent action for the glycosides is at a peak when 30 to 40 per cent. of water is present.

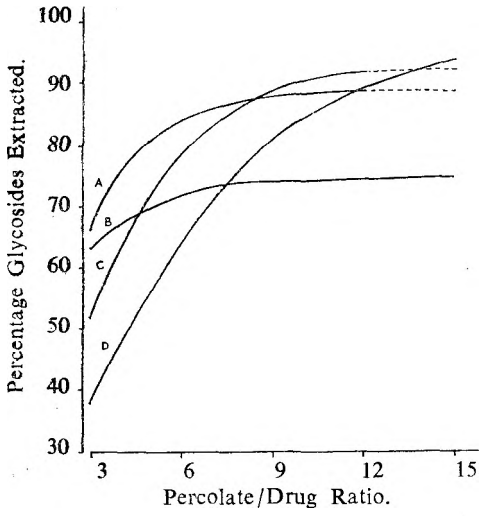

FIG. 3. Extraction of glycosides of senna pod, in coarse powder, by percolation with aqueous dilutions of organic solvents. A, 70 per cent. methyl alcohol; B, 70 per cent. acetone; C, water; D, 70 per cent. ethyl alcohol.

In order to investigate the efficiency of these solvents for the extraction of senna pod, the drug in coarse powder was percolated with 70 per cent. dilutions with water of acetone and ethyl, methyl, and isopropyl alcohols. The proportions of glycosides and total solids extracted in successive fractions of percolates were determined and the results submitted in Table II and expressed graphically in Figures 3 and 4. The results for percolation with water (see Table VIII) are also included for comparison. Percolation with 20 per cent. ethyl alcohol gave results

TABLE XI

POTENCY OF SUCCESSIVE FRACTIONS OBTAINED BY PERCOLATION OF SENNA POD IN COARSE POWDER WITH AQUEOUS DILUTIONS OF CERTAIN ORGANIC SOLVENTS

Weight of pod used = 100 g.

Percolate fraction	70 per cent. v/v acetone		70 per cent. v/v ethyl alcohol		70 per cent. v/v methyl alcohol		20 per cent. v/v ethyl alcohol	
	Proportion of glycosides extracted	Amount of total solids extracted	Proportion of glycosides extracted	Amount of total solids extracted	Proportion of glycosides extracted	Amount of total solids extracted	Proportion of glycosides extracted	Amount of total solids extracted
ml.	per cent.	g.	per cent.	g.	per cent.	g.	per cent.	g.
0 — 300	52·0	16·5	38·7	16·2	61·3	20·8	59·4	27·1
300 — 600	27·1	5·6	27·6	5·1	23·8	4·6	9·3	3·8
600 — 900	10·0	2·0	16·4	2·0	4·0	1·3	1·6	0·9
900 —1200	3·3	1·0	7·9	1·1	1·0	0·6	0·2	0·5
1200 —1500	—	—	4·8	0·7	—	—	—	—
1500 —1800	—	—	2·7	0·5	—	—	—	—
Totals ...	92·4	25·1	98·1	25·6	90·1	27·3	70·5	32·3

almost identical with those for water. (Percolation with 70 per cent. *iso*propyl alcohol failed to extract the active principles to the same extent as the other solvents and the results have therefore not been included.)

The diagrams clearly show that the drug can be exhausted almost completely by 70 per cent. dilutions of acetone, ethyl alcohol and methyl alcohol at a drug : percolate ratio of 1 : 15. Even at a ratio of 1 : 9, 80 to 90 per cent. of the glycosides have been extracted; moreover these solvents extract less solid matter than does water. These combined effects increase the proportion of glycosides to total solids in the percolate to values considerably in excess of that given by aqueous percolates and yield solutions from which extracts of much higher potency can be made. The proportion of glycosides to total solids in percolates using 70 per cent. dilutions of acetone, ethyl alcohol and methyl alcohol, ethyl alcohol (20 per cent.), and water, were as follows: 113·5, 117, 101, 67 and 68 mg./g. respectively.

Preparation of Potent Extracts by Concentration of Percolates Made with 70 per cent. Dilutions of Certain Organic Solvents

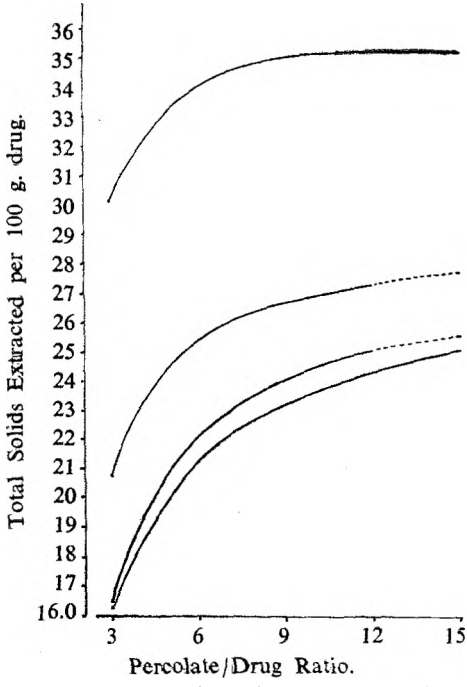

Fig. 4. Extraction of soluble extractive of senna pod, in coarse powder, by percolation with aqueous dilutions of organic solvents. Topmost curve, water; 2nd, 70 per cent. methyl alcohol; 3rd, 70 per cent. acetone; lowest, 70 per cent. ethyl alcohol.

The percolates previously made with 70 per cent. dilutions of acetone, ethyl alcohol and methyl alcohol were evaporated under reduced pressure at as low temperatures as possible. The soft extracts so prepared were assayed, and the proportion of glycosides to total solids calculated and compared with those from the corresponding percolates. Only when the temperature of evaporation did not exceed 40°C. did the values indicate that no loss of activity had occurred. For example, the 70 per cent. methyl alcohol percolate contained 101 mg. of glycosides per g. total solids; the soft extract prepared from it contained 61 mg. of glycosides per g. extract and 100 mg. of glycosides per g. total solids. This indicated that there had been a complete survival of the glycosides and the product represented an extract containing all the activity of the pod.

Discussion

Two considerations must be borne in mind when selecting a suitable method for the extraction of a drug, namely, (a) the proportion of active principles extracted and (b) the volume of solvent required. Since the active principles of senna are thermolabile, as has been shown by this investigation, it is essential that the solvent used should extract most of the activity in a reasonably small volume, in order to minimise losses during evaporation. A review of the results recorded in Tables V, VI and VIII shows that percolation is the most efficient method of extraction: with hot infusion (Table V) a drug: solvent ratio of 1 : 10 extracts only approximately 56 per cent. of the available glycosides; with triple maceration (Table VI) a ratio of 1 : 7 extracts 64 per cent., whereas with percolation (Table VIII) a ratio of 1 : 6 extracts 73 per cent. Furthermore, percolation is the most convenient process for dealing with large quantities of crude drug. The large volume of water needed for the extraction of the active principles is an outstanding feature and renders the concentrating of the extractive more difficult and costly.

In addition to losses attributed to incomplete extraction, evaporation of aqueous extractives at a temperature not exceeding 60°C. could be responsible for a further 20 to 30 per cent. loss in activity (Table IX). Studies on the effect of heat on percolates have shown that the glycosides are more sensitive to heat (especially in the alkaline *p*H range) than has been generally believed, and that losses in activity during concentration of extractives will be less the lower the temperature and shorter the time of evaporation. However, by adhering rigidly to the pharmacopœial instructions for extraction and concentration, the potency of liquid extract of senna is not likely to be more than about 50 per cent. of that of the pod; extraction by the more efficient general percolation process would increase this proportion to about 60 per cent. This appears to be the maximum which could be present in the concentrated products of commerce which have been made using water as solvent. Analyses of such extracts (Table II) indicate that less than 10 per cent. of the glycosidal content of the drug is present. These facts suggest that there may be considerable loss on storage, and it is proposed to investigate this matter in greater detail later. It is interesting to record that fresh infusion of senna B.P.C. is the most active and reliable of the official galenicals.

The amount of active principles present in extracts could be increased if evaporation were conducted at temperatures lower than 60°C. and could approach a maximum of approximately 75 per cent. of that of the pod by concentrating at a temperature which does not exceed 40°C., but such a low temperature may not be practicable on a manufacturing scale.

The use of dilutions of organic solvents is attractive because they can dissolve a higher proportion of glycosides in smaller volumes; furthermore, since the solutions can be concentrated at lower temperatures than can aqueous solutions, there will be smaller losses of activity. How-

ever, before organic solvents can be recommended for the preparation of pharmaceutical extracts, the question of their possible toxicity, and whether they extract undesirable materials, must first be considered. If the last traces of the solvent can be removed completely from the extract, and provided that the cost of the process is economic, then solvents which are more efficient than water have obvious advantages.

Summary

1. The glycosidal content (as sennosides A + B) of a number of commercial samples of senna pod has been determined by chemical assay, and it has been shown that the usual criteria, appearance and water-soluble extractive, are not sufficiently reliable as methods of evaluation.

2. Alexandrian pod (*Cassia acutifolia,* Delile) contains a higher proportion of glycosides and water-soluble extractive than Tinnevelly (*C. angustifolia,* Vahl), the averages and ranges being 2·37 to **3·22** to 4·34 per cent. and 1·22 to **1·96** to 2·78 per cent. of glycosides, and 25·8 to **28·3** to 31·2 per cent. and 20·7 to **23·0** to 25.5 per cent. of water-soluble extractives, respectively.

3. The glycosidal content of a number of commercial samples of liquid extract of senna B.P. has been determined; the extracts were found to contain not more than 1/10 of the activity of the pod. Samples of concentrated infusion of senna B.P. contained not more than 1/6 of the theoretical glycosidal content.

4. The low values, in both instances, have been traced to incomplete extraction, loss on concentration and to deterioration during storage.

5. Certain non-official preparations of senna pod were assayed biologically and their glycosidal content calculated. The proportion of the theoretical activity was higher than that in official preparations.

6. Approximately 3/4 of the active principles of the pod are extracted in a simple (1·2 per cent.) infusion. Fresh infusion of senna B.P.C. (which has a drug: menstruum ratio 1 : 10) extracts only about 1/2 of the activity.

7. The B.P. extraction processes have been investigated; the general percolation process was found to be the most efficient, about 3/4 of the activity being extracted in a drug; percolate ratio of 1 : 6.

8. The effect of heat on the glycosidal content of aqueous percolates of senna pod has been studied. Between 50° and 60°C. there was a loss of 1/3 to 1/5 of the initial activity during 8 hours heating. No loss in activity took place during concentration of a percolate at a temperature not exceeding 40°C. during 10 hours' heating.

9. The active principles are almost completely soluble in 70 per cent. acetone, 70 per cent. ethyl alcohol, and 70 per cent. methyl alcohol; less solid matter is extracted by these solvents than with water. Extracts prepared by percolation with these solvents and evaporation of the percolate below 40°C. contained all the activity of the pod. The advisability of using these solvents for the preparation of an extract of senna is discussed.

We would like to thank the Directors of Westminster Laboratories Ltd. for certain facilities generously provided during the course of this investigation, and for permission for one of us (I.M.) to publish the results. We would also like to thank Mr. T. C. Lou and Mr. M. R. I. Saleh for carrying out the bio-assays.

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3. Stoll, Becker and Kussmaul, *Helv. chim. Acta*, 1949, **32**, 1892.

DISCUSSION

Part II was presented by Dr. I. Michaels and Part III by Dr. Fairbairn, and the two papers were discussed together.

PROFESSOR H. BRINDLE (Manchester) asked whether the authors had considered the possibility of spray drying their aqueous extract of senna. He thought that they could have produced a 100 per cent. extract. Blood complement, which decomposed almost completely at 40°C., had been dried almost completely without loss and, therefore, there should be no loss whatever in drying extract of senna. It would be quite possible to extract senna pods with water, spray dry to a powder and compress into a tablet.

MR. T. D. WHITTET (London) asked whether the authors had tried propylene glycol as a solvent. It was a suitable solvent for a number of galenical preparations and he had found that several substances which were virtually insoluble in water were quite soluble in solutions of propylene glycol. As it toxicity was even lower than that of glycerin, it might be a suitable solvent for this type of preparation.

DR. D. C. GARRATT (Nottingham) asked if the accuracy of the chemical assays in question (± 4 per cent.) was based on a correlation with the biological activities, because he would submit that that was not permissible in view of the small number of biological assays. If it were based on the reproducibility of the chemical assays it was a reproducibility and not an accuracy which was involved.

MR. C. J. EASTLAND (London) said that he was pleased to find that the authors confirmed the results of a similar investigation by two colleagues of his, Dr. Collier and W. Bellis, which was reported to the Brighton Conference. Like the present authors, they had found a fair degree of agreement between the chemical and biological assays; later work, however, suggested that the chemical assay was likely to give rather high results when applied to really old extracts or preparations which had been artificially aged by storage at high temperature. Perhaps the modified Kussmaul and Becker's method overcame this difficulty. If not, it would indicate that the breakdown on hydrolysis of the glycosides during prolonged storage periods was different from the breakdown caused by subjecting the extracts to high temperatures.

MR. J. L. FORSDIKE (Nottingham) asked whether the authors had considered the use of dilute alcohols in the preliminary extraction of the drug for the assay. When senna pods were extracted with 45 per cent. alcohol and the extracts assayed, one would get a considerably higher result than if one extracted the same pods with water. He had extracted two samples of senna pod exactly as in Part II of the paper and also by using 70 per cent. and 45 per cent. alcohol, the filtrate in each case being assayed as described by the authors. The 70 per cent. alcohol gave a 5 to 10 per cent. higher red colour intensity than the aqueous alkali extraction and 45 per cent. alcohol gave as much as 20 per cent. more colour, which indicated a higher combined anthraquinone content in the drug and seemed to suggest that the whole of the anthraquinone glycosides present was not extracted by the method described in the paper.

Three different methods of extraction had been described, one for the whole pod, one for the coarsely-powdered pod and one for the fine powder. In the first two the volume was made up to 10 g. in 500 ml., but for the fine powder it was 1 g. in 100 ml. Was there any reason for this difference, or any objection to powdering the whole or partially-crushed material finely before use? The authors had heated the alkaline solution for 4 to 5 minutes. He thought it necessary to control that time of heating very accurately to 4 minutes, as after this time the colour decreased. It was also essential to examine the colour immediately, and this was not specified in the paper, because the red colour faded within a period of an hour or so. In Part III of the paper the authors had examined the potency of commercial samples of liquid extract of senna B.P. by chemical assay and found only 1 to 8 per cent. of the theoretical sennoside content (Table II) but in non-official preparations of senna pod, assayed biologically (Table III), they had found 30 to 40 per cent. and in one case over 100 per cent. Were the B.P. extracts also assayed biologically, as the results might prove to be of some significance. Tables VI, VII and VIII (Part III) showed that the authors had found only a proportion of the expected amount of glycosides to be extracted by the three methods. Had they determined whether the missing glycosides were left in the marc or whether they had been destroyed in the process of extraction?

MR. H. DEANE (London) said that Dr. Fairbairn had referred to certain proprietary preparations as being highly active, but was he sure that they did not contain other purgatives than senna, as that was quite a likely possibility? He thought that they should investigate the deposit which forms when these preparations are stored. The authors had not given any details about the colour standard. He presumed that the pure glycosides were not commercially available and that it was necessary for the analyst to prepare them and to make his own standards with the colorimeter which he was using.

MR. G. R. A. SHORT (London) commented on the absence of any correlation between the appearance of the pods and their activity. Pods

used in manufacturing often gave a highly active product in spite of their poor condition. He wondered whether the degree of ripeness affected the activity of the pods. He suggested that a creeping film evaporator would be very suitable for the evaporation of the senna extracts. The period of heating would only be a few seconds, and, even when the concentrated liquid was subsequently evaporated to dryness, it would not be necessary to heat for 10 hours as suggested by the authors. There was no need to fear the toxic effects of the solvents used, as manufacturers made sure that they were entirely removed from the final product.

DR. G. H. MACMORRAN (Edinburgh) asked why the authors had adjusted the infusion to *p*H 3. Straub and Gebhardt had reported that one of the glycosides was hydrolysed by acids during extraction. This being so, the aglycone would be extracted by the organic solvent and the final figure in the chemical assay would be smaller than the correct figure. He thought that the similarity between the chemical and biological assays was very strange as biological methods were generally considered to have a wide variability. It might be that a low figure was being shown in each, due, in the case of the chemical assay, to the hydrolysis of one of the glycosides.

DR. G. E. FOSTER (Dartford) asked which filter had been used with the Spekker absorptionmeter. DR. MICHAELS replied that it was the 604.

DR. F. J. DYER (London) asked whether the authors had tried the use of 60 per cent. alcohol plus glycerin, as was done in the extraction of the digitalis glycosides by one or two of the older commercial methods.

DR. I. MICHAELS, in reply, said that they had no spray drier and they had read that spray drying resulted in the extracts being somewhat hygroscopic. Propylene glycol was not used as its boiling-point was rather high and the glycosides were thermolabile. They agreed with Dr. Garratt that they should have referred to limits of reproducibility rather than limits of accuracy. The assays had been done a number of times on the powders and on the whole pod, on different days, as the process was rather prolonged at first, and they statistically analysed the results. One person was able to do two assays in a working day. The results were often identical and never varied by more than about 5 per cent. Mr. Eastland had pointed out that an old extract frequently gave high chemical results. The extracts which they had bought must have been several months old, and they were stored in the laboratory for six months before being assayed. These samples gave very low results. The effect of ripeness on the activity of samples of pods had not been investigated, but they would bear this in mind. The reason for the period of 10 hours being required for the evaporation of one extract was that they were careful to keep the temperature of the water-bath below 50°C. Although this was a long time, there was small likelihood of much decomposition. They had thought of using the creeping film evaporator, but had then given up investigating aqueous extracts

as they were not satisfied that they had extracted all the active principles, and their goal was to produce an extract which represented 100 per cent. of the activity of the product. The creeping film evaporator would have been useful for producing a 75 per cent. active preparation.

DR. J. W. FAIRBAIRN said that he had drawn the attention of Dr. Collier to the fact that the chemical assay process estimated only glycosides, and therefore he could not understand why with an old preparation, which had a low biological activity, a high result was obtained with the chemical assay. Mr. Eastland's suggestion that there might be different types of breakdown on hydrolysis was worth thinking about. With regard to Mr. Forsdike's remarks, it was important to remember that there were other glycosides and other anthraquinone compounds in senna which were not assayed by the Kussmaul and Becker process. Their results, however, had shown that, while the sennosides might not account for the total activity, they very nearly did so. They had been very surprised by the close agreement between the biological and chemical assay results. The biological process was accurate only within 15 to 20 per cent. He expected that future results would differ more widely. The exact composition of the proprietary preparations tested had not been known. There might have been some synergistic effect of other ingredients, and they had had to use the biological rather than the chemical assay. The pure glycosides had been obtained from Professor A. Stoll. If one had to make them, the instructions were given in the literature for doing so, but it might entail three months' work. In his opinion, the easily hydrolysable glycoside of Straub and Gebhardt did not exist.

THE PREPARATION OF LIQUID HUMAN PLASMA BY THE KAOLIN PROCESS

By G. R. Milne and G. M. Todd

From the Glasgow and West of Scotland Blood Transfusion Service

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The British Pharmaceutical Codex 1949 includes monographs on human blood and blood products. It is also proposed to include standards for these in the next Pharmacopœia or in an Addendum to the present Pharmacopœia (Report of the British Pharmacopœia Commission to the Pharmacopœia Committee of the General Medical Council, May, 1950)[1].

The Codex Monograph on liquid human plasma states that it is the liquid portion of whole blood. The plasma is removed from the red cells by aseptic methods and mixed aseptically with plasma from other samples of whole blood. Pooled human plasma so obtained is normally opaque due to the presence of fat and, on storage, clots and particles of fibrin may also develop. The cloudy appearance of the liquid makes it difficult to distinguish between sterile and infected plasma.

Two methods of treatment are available. Firstly, the plasma may be dried from the frozen state as soon as possible after collection. The alternative is to sterilise the pooled plasma by filtration through sterilising asbestos pads and this yields a clear fluid which, should it become contaminated, is self-indicating. The filtration of plasma through an asbestos filtering medium is made difficult by the activation of the plasma prothrombin by contact with the asbestos (Bushby and Whitby[2]). Clotting occurs in the filtered fluid and later, on the surface of the pads and filtration comes to an end.

Several methods are available for removing fibrinogen and prothrombin from plasma. McFarlane[3] has developed an ether-freeze process for the removal of fibrinogen and the unstable lipoid-globulin complex from plasma. This method, for technical reasons, has not yet been fully developed for large scale use. Fibrinogen may be precipitated by the addition of acids or by heating at 55°C. Both these methods need careful control during the process, and even then, precipitation is not always complete and the resulting fluid is not easily cleared by filtration. In addition, the fluid does not store well. Clegg and Dible[4] have suggested the addition of excess of calcium to the citrated plasma to remove fibrinogen. Our experience using this method has not been uniformly successful and the calcium content of the plasma is increased much above the physiological level. Maizels[5] has described a method using kaolin for the adsorption of fibrinogen and prothrombin and suggests that the product is less likely to produce toxic reactions than material untreated with kaolin.

A modification of the process suggested by Maizels has been used in our laboratories and is briefly as follows:— 400 g. of kaolin B.P. is weighed into a wide-mouthed bottle of 5 l. capacity. To this 400 ml. of

distilled water is added and well mixed. The addition of the water is necessary to ensure efficient sterilisation of the kaolin in the autoclave. The flask is closed with a two-holed rubber stopper fitted with suitable glass tubing and is then sterilised at 121°C. for one hour. After pressure in the autoclave has been released, 15 inches of vacuum is applied in order to dry the kaolin as much as possible. Plasma (approximately 4 l.) is withdrawn under aseptic conditions from 16 bottles of blood into each of the kaolin bottles. The pooled plasma and kaolin are well mixed by repeated shaking during 15 minutes and then stored frozen at −10°C. for not less than 5 days.

When required for processing, the plasma is allowed to thaw at 4°C. When completely thawed out, it is gently mixed and allowed to stand at 4°C. for 24 hours to allow the kaolin to settle. The supernatant is first clarified by passage through asbestos pads of clarifying quality and then sterilised by passage through sterilising asbestos pads in a frame filter. The sterile plasma is filled aseptically, in 400 ml. quantities, into sterile transfusion bottles. The processed plasma is incubated at 20°C. in a dark cupboard for 21 days. Before issue each bottle is carefully inspected for the presence of foreign bodies and bacterial or fungal contamination.

Plasma prepared in this way is clear and, being practically free from fibrinogen and prothrombin, will not form fibrin clots on storage for periods of about 4 to 6 months.

Standards for Liquid Plasma. The B.P.C. gives standards for liquid human plasma which include appearance, preparation, protein content and sterility. It is with the protein content of kaolin-processed plasma that this paper is mainly concerned. The standard laid down for protein is not less than 4·5 g. per cent. This is also the standard required by the Therapeutic Substances Act, 1925; Amendment Regulations, 1948. The object of the experimental work described below is to determine whether, by using the kaolin method, this standard of protein content can be attained.

Experimental

The protein content of pooled plasma was determined before and after treatment with kaolin and after clarification and sterilisation by filtration. All protein estimations were made by a modification of the micro-Kjeldahl method.

Method. 4 ml. of plasma was diluted to 100 ml. with water and carefully mixed. 5 ml. of this dilution (= 0·2 ml. of plasma) was pipetted into a micro-Kjeldahl flask. To this was added 0·2 ml. of a 7·5 per cent. solution of sodium molybdate and 0·2 ml. of 2/3 N sulphuric acid. The mixture was centrifuged in the flask for 15 minutes at 2,000 r.p.m. The supernatant liquid was decanted off and the flask allowed to drain on filter paper. Approximately 0·2 g. of potassium sulphate, 0·2 g. of copper sulphate, 1 ml. of concentrated sulphuric acid and a few glass beads were added to the flask which was then heated on a hot-plate until charring ceased and the liquid had a clear green colour. The flask was then fitted

to the micro-Kjeldahl distillation apparatus with the condenser delivery tube immersed in 15 ml. of N/70 sulphuric acid. 10 ml. of water and 5 ml. of 40 per cent. sodium hydroxide solution were then added and distillation by steam was carried out until the volume in the receiver had been doubled. The unused sulphuric acid was titrated with N/70 sodium hydroxide and the protein content of the sample calculated on the basis: 1 ml. of N/70 sulphuric acid = 0·2 g. of N and total protein per 100 ml. = ml. of N/70 sulphuric acid × 0·625.

Blank estimations on the reagents were carried out and any necessary adjustments were made.

Results. The results of estimations on 4 pools of plasma are shown in Table I. The figures are the mean of two estimations at each stage.

TABLE I

	Protein (g. per cent.) in Pool No.				Average (30 pools)
	1	2	3	4	
Raw plasma before treatment with kaolin ...	5·40	5·25	5·40	5·30	5·34
After treatment with kaolin	3·75	3·99	3·94	3·63	3·80
After filtration through clarifying pads ...	3·78	3·94	3·78	3·75	3·85
After filtration through sterilising pads ...	3·19	3·56	3·38	3·56	3·45

It will be seen from Table I that 1·54 g. per cent. of protein is lost by adsorption on the kaolin. Estimations of the protein on the kaolin sludge were carried out. Results corresponded to 1·40 g. per 100 ml. of plasma (i.e., 91 per cent. of the total adsorbed protein). The results shown in Table I have been confirmed by many estimations made during the routine processing of plasma.

Discussion

The use of kaolin-processed liquid plasma has a number of advantages. Firstly, the end product is liquid and does not require reconstitution with sterile water before use. Secondly, it is self indicating for sterility, and in addition, it is stable when stored for reasonable periods at room temperature.

The main disadvantages are in the lowered protein content, and in the fact that during transport over long distances there may be some precipitation due to breakdown of the lipoid-globulin complex. It may be inferred from the B.P.C. Monograph that the kaolin process is an "approved method." The results shown above, however, would indicate that plasma prepared by the kaolin process does not satisfy the official requirements for protein content. The official minimal protein content (4·5 per cent.) has, presumably, been decided upon using dried raw plasma as the standard.

The loss of 1·54 g. per cent. of protein on the kaolin is more than can be accounted for in terms of fibrinogen of which the average plasma content is stated to be 0·3 g. per cent. Whilst prothrombin and fibrinogen are selectively adsorbed by the kaolin there is evidence that other proteins are adsorbed at the same time. This fact has been pointed out by Maizels[5]. It is interesting to note that passage through the clarifying pads does not remove protein, but passage through the sterilising pads results in a further loss of 0·35 g. per cent. This may be accounted for by the much higher asbestos content of the sterilising pads. If the kaolin process is to be considered as a suitable method of treatment for plasma, then the official standard for protein in the finished product may have to be adjusted.

The kaolin process is a useful and safe method for preparing human plasma for use and it has been suggested by Maizels that pyrogens and other toxic substances may also be removed by the kaolin. The work of Chute and Vaughan[6] would appear to support this theory.

Summary

1. The kaolin process for the preparation of human liquid plasma is described.

2. Results are given for the estimation of the protein content of the fluid at the various stages of the process. A loss of protein to the extent of 1·54 g. per cent. by adsorption on the kaolin is shown. A further loss of 0·35 g. per cent. is sustained by passage through sterilising asbestos pads.

3. It is suggested that, if the kaolin process is to be considered an "approved method," then the minimal standard for protein content of the fluid should be revised.

Our thanks are due to Dr. John Wallace, Regional Director of the Glasgow and West of Scotland Blood Transfusion Service for helpful advice and criticism.

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DISCUSSION

The paper was read in abstract by Mr. G. M. Todd.

Mr. G. Sykes (Nottingham) said that he was surprised at the enormous loss of protein on passing the plasma through the sterilising pads. He calculated that 14 g. of protein had been lost. That might be accounted for by the use of a large number of pads, but he imagined that not more than 1 or 2 14-cm. pads would be used to filter 4 litres of solution and it was difficult to believe that such an enormous amount could be

adsorbed on such a comparatively small pad. He wondered whether those figures could be accounted for by the fact that the whole 4-litre volume would not be filtered in the work reported, but only a comparatively small fraction. It was important from the manufacturing point of view because they were continually faced with the problem of the adsorption of all sorts of drugs, from protein to simple salts. In the case of the clarifying pads, there was comparatively no loss.

MR. F. H. OLIVER (Sunderland) asked whether the authors had any information on the ratio of globulins to albumins, because plasma was used largely for restoring the volume of the blood, and, since the albumins were more useful for the capillaries than the globulins, the proportion of globulins would be very important.

MR. G. R. MILNE (Glasgow) said that the B.P.C. monograph on whole blood gave details about the collection of blood into anti-coagulant solutions, and mentioned specifically three types of anti-coagulant. The first was 3 per cent. solution of trisodium citrate using 100 ml. to 440 ml. of blood. The second was 2 per cent. solution of sodium acid citrate with 2·5 per cent. of glucose, 120 ml. to 420 ml. of blood, and the third was a solution of 1·6 g. of trisodium citrate, 0·56 g. of citric acid and 1·5 g. of glucose, in 75 ml. which is used with 500 ml. of blood. In Glasgow, they used 100 ml. of the second solution with 440 ml. of blood. If one took the B.P.C. recommendation of 120 ml. of solution to 420 ml. of blood and assumed (1) that the volume of plasma to the volume of red cells was in the ratio of 55:45, which was normal, and (2) that the average protein content of human plasma was 6·75 per cent., then the amount of protein in the diluted plasma would be only 4·44, which was below the B.P.C. limit of 4·5, which was also given in the Therapeutic Substances Regulations. The other two methods were satisfactory, and gave figures of 4·77 and 5·4 respectively. The quality of kaolin needed investigation, as its adsorptive properties varied, although it satisfied the requirements of the British Pharmacopœia. With regard to the adsorption on the asbestos pad, the filtration of 4 litres of plasma had been described, but in point of fact they were filtering as many as 8 such quantities through a frame filter which would contain anything from 8 to 12 asbestos pads. There was no doubt that the ratio of the area of asbestos pads to the amount of fluid chemical being filtered was very important. They had satisfactorily prepared solutions of thrombin on a small scale, but when they tried larger quantities they lost the thrombin in the process, by adsorption on the large area of the filter pad. They had, therefore, reverted to the use of smaller pads which took more time, but the ratio of filter pad area to the volume being filtered was extremely important. The asbestos content of the pads was also an important factor and their quality was now being improved.

MR. G. M. TODD, also replying, said that the size of the pads used was 400 sq. cm. They had not yet done any work on the globulin-albumin ratio, but it was on their programme.

THE USE OF SULPHATED WHOLE BLOOD IN THE ASSAY OF HEPARIN

By S. S. Adams

With an Interpretation of the Data by K. L. Smith

From Boots Pure Drug Co., Ltd., Nottingham

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Numerous *in vitro* methods have been described for the assay of heparin. The more popular ones have been reviewed by Jorpes[1], and it is not proposed to consider them in detail here. They include both physicochemical and biological methods, but only the latter, which determine the inhibitory effects of heparin on the clotting of fresh blood or stored blood systems, in comparison with a standard preparation, give results which are related to the activity of the anticoagulant in the animal organism[1,2].

On theoretical grounds, assays using fresh whole blood are to be preferred to those using stored blood or plasma systems as in them the heparin activity is more closely related to that which occurs *in vivo*. The practical disadvantages of the fresh blood methods are considerable[3], and stored blood systems, since they are easier to manipulate, are therefore favoured. These systems consist of blood, or more usually plasma, which is preserved by oxalate or citrate, and in which clotting is induced by the addition of calcium, calcium and thrombokinase, or thrombin. Possible sources of error are, however, introduced, due to the effect of the salts on the blood proteins, and the addition of unsuitable amounts of clotting promoters.

When blood is collected into an equal volume of half-saturated sodium sulphate solution it will remain fluid, but simple dilution with water will promote coagulation[4]. If aqueous heparin solutions are used to dilute this "salted" blood, and thus induce coagulation, they cause prolongation of the clotting times over the water controls. The application of this system to the assay of heparin was considered; its use for this purpose has not hitherto been recorded. It was hoped many of the objections raised against other artificial systems would be avoided since aqueous dilution would produce a reconstituted though diluted whole blood, in which the clotting components would be in normal physiological proportions. Unfortunately the recorded coagulation times were too long for a rapid assay, and the addition of an unsuitable excess of thrombokinase extract was necessary to reduce them to a favourable range. Data presented later will show that this addition did not lead to erroneous results.

The design of heparin assays can be divided into two classes as described by Jaques and Charles[3].

(a) The clotting time method, in which clotting times are determined

for one or a given number of heparin solutions and which entails continuous examination.

(b) The titration method, in which the clotting mixture and a series of heparin dilutions are examined once only, after a certain fixed incubation time varying from 2 to 24 hours.

In the design of this assay, the clotting time method was chosen, so that data amenable to standard methods of analysis could be obtained and the lengthy incubation periods which many titration methods require could be avoided.

Reagents Used

Sulphated Whole Blood. Ox blood is collected from a freshly slaughtered beast into a 250-ml. wide neck glass stoppered bottle, containing 50 ml. of a 7 per cent. w/v solution of exsiccated sodium sulphate B.P., and stored below 4°C. until required.

Thrombokinase Extract. 1·5 g. of acetone-dried ox brain is extracted with 60 ml. of distilled water for 10 to 15 minutes at 50°C., centrifuged for 2 minutes at 1,500 r.p.m., and the suspension filtered through a Whatman No. 1 filter paper. This extract will retain its activity for several days when kept in the refrigerator, but rapidly undergoes bacterial decomposition at room temperature. The addition of tricresol (0·3 per cent.) added as a bacteriostatic does not affect the accuracy of the method.

Acetone-Dried Ox Brain. A fresh ox brain, freed from vascular and connective tissue, is cut into pea-size pieces and placed in acetone for preliminary dehydration. 30 g. of this material is pounded in a mortar with successive 75 ml. volumes of acetone until dehydration is complete. This is evident when a dry whitish buff powder remains, after Buchner funnel filtration. This preparation is finally dried at 37°C. for 2 hours to remove all traces of acetone.

Procedure

The clotting times produced by 3 dilutions of standard heparin and 3 equivalent dilutions of the unknown heparin are determined simultaneously, and this is repeated 4 times for a complete assay. It has been found convenient to make the highest concentration of heparin 2 units/ml., and to use an 80 per cent. interval between successive levels. The method of determining the clotting times is as follows.

1 ml. of a heparin dilution in distilled water is pipetted into a 6″ × ½″ soda glass test tube followed by 0·1 to 0·2 ml. of thrombokinase extract. 1 ml. of sulphated ox blood is then added, and the whole mixed by gentle inversion to prevent the formation of air bubbles. The time from this addition to the formation of a firm clot which remains in the bottom of the tube when it is completely inverted, is recorded.

The change in the fluidity of the blood observed by gentle tilting indicates the onset of coagulation, and by shortening the interval between examinations the operator can determine the end-point to within 15 seconds and with practice avoid the breaking of the clot by premature

inversion. If a tube is inverted before complete coagulation, the formation of the clot is hindered, and the whole run of 6 tubes is abandoned. This, however, seldom happens except in the initial training of an operator, as the end-point is extremely good. The amount of thrombokinase extract to be added may be varied slightly according to conditions, but is generally 0·2 ml. if correctly prepared. The most accurate assays are obtained when the longest clotting times range from 9 to 12 minutes, and longer times than these are not recommended. This can be arranged either by altering the range of heparin concentrations or the concentration or volume of the thrombokinase extract. It is perhaps easier to keep the heparin concentrations fixed and to dilute the thrombokinase extract until favourable clotting times are obtained with the 2 units/ml. standard solution.

Table I shows the clotting times in minutes recorded during a normal assay. The interpretation of such data, using the linear relationship which exists between the log. coagulation time and the log. concentration

TABLE I

CLOTTING TIMES IN MINUTES

Runs	Standard Heparin			Unknown Heparin—Assumed Strength		
	1·28 μ/ml.	1·6 μ/ml.	2 μ/ml.	1·28 μ/ml.	1·6 μ/ml.	2 μ/ml.
1	3¼	5¼	10¼	2¾	4⅜	9⅜
2	2½	4	6¼	2⅜	3½	5¼
3	3½	5½	10¼	3¼	5	8⅜
4	3¼	5	8½	3	4½	7½

of heparin gives potency estimates with a high order of accuracy, as is shown later in this paper. More rapid assays with a slight loss of accuracy may be carried out using 2 doses only of standard and single doses of the unknown. The potency estimates for such assays may be made graphically or arithmetically.

Sulphated ox blood is a more stable system than many citrated or oxalated bloods or plasmas. It is stable for a period of at least 3 to 4 weeks if stored below 4°C., and small granular clots which do sometimes form do not affect the assay and can be filtered off through muslin.

FACTORS INFLUENCING THE ACCURACY OF THE ASSAY

Tube Diameter. The choice of a test tube of a definite size, in which to conduct the assay, is of great importance, since tubes of varying diameters give clotting times which differ appreciably. Tubes of ½-inch diameter give excellent results, smaller ones than this causing errors in the end-point determinations, due to surface tension effects.

The Clotting End-Point. The clotting end-point is a very definite one, easily determined, and of rapid onset, such that it can be assessed to within 15 seconds of its formation. This enables different operators to obtain readily reproducible results after a very brief training.

Temperature at which the Assay is Conducted. Nearly all types of heparin assay are conducted at a constant temperature, usually 37°C. With sulphated blood however this is not necessary, and all operations are performed at room temperature. Experiments in which the assay was conducted at 37°C. showed no increase in accuracy. Furthermore it is not essential to keep the system in a refrigerator immediately prior to use, as is the case with some clotting time methods involving oxalated or citrated plasmas[5,6].

The Effect of Thrombokinase on the Accuracy of the Assay. Jalling, Jorpes and Linden deprecate the use of thrombokinase in heparin assays, because of the possibility of it removing some of the heparin, especially if the barium salt is used[2]. They quote results obtained by MacIntosh[7], in substantiation of this, in which he obtained lower values with recalcified plasma, and thrombokinase, than with fresh whole blood.

In order to determine if similar results were to be expected using the sulphated blood assay, four samples of sodium heparin were tested, and compared with results obtained using fresh whole blood. Table II shows that there is no significant difference between the two sets of figures.

TABLE II

Heparin Sample	Sulphated Blood Assay		Fresh Blood Assay
	Potency μ/mg.	Limits P = ·95	Potency μ/mg.
Crude Heparin	25	23·7—26·3	25·3
H 217—226	59·6	57·1—62·4	56·3
HP 50	100·7	92·7–108·6	101·2
HP 50 Hydrolysed	50	46·3—53·5	51·7

The Influence of Electrolytes on Clotting Times. The presence of electrolytes in heparin solutions was found to delay considerably the clotting times of sulphated blood, and this is in accord with the findings of other workers using other coagulation systems[3,5]. For this reason all solutions and extracts are made in distilled water. The maximal concentration of sodium chloride in the heparin solutions which causes no added anticoagulant action varies from 0·5 to 0·15 per cent. w/v. This factor has however no effect in the assay of injection of heparin B.P., as the chloride concentrations in the final dilutions are much less than those which show an apparent anticoagulant action.

Interpretation of the Data and Accuracy of the Method

The use of sulphated whole blood with added thrombokinase for the assay of heparin yields data in a form amenable to standard methods of examination, since, over the practical range, the log. of the time for coagulation is linear to log. concentration; this is a relationship which we have used also for the treatment of heparin assays by the method of MacIntosh[7] following a suggestion made to us by Mr. E. C. Fieller. The data are so readily obtained that the various hypotheses needed to calculate potency and its fiducial limits may be confirmed separately for each assay by conducting four runs in each of which both the standard and the unknown are represented at three concentrations. If the concentrations are in geometric progression the arithmetical procedures described by Bliss and Marks[8], and the British Standards Institution[9] may be applied. In practice we use parts of each method and have retained the symbols used respectively by them to denote the equivalent numerical values. The example Table I represents the data obtained in one of two assays carried out on a sample of heparin assumed to obtain 5,000 units per ml. and will be used to illustrate the analysis.

Arithmetical Procedures. The logarithms of the coagulation time are recorded to the nearest second decimal place, and these together with the appropriate summations are shown in Table III.

TABLE III

Run	1·28 units S_1	1·6 units S_2	2·0 units S_3	1·28 units U_1	1·6 units U_2	2·0 units U_3	Sum
1	0·51	0·72	1·01	0·44	0·68	0·99	4·35
2	0·40	0·60	0·80	0·44	0·54	0·72	3·50
3	0·54	0·74	1·01	0·51	0·70	0·94	4·44
4	0·51	0·70	0·93	0·48	0·65	0·88	4·15
Sum	1·96	2·76	3·75	1·87	2·57	3·53	16·44

An analysis of variance is made by the recognised method in which errors between runs and concentrations are removed (Table IV).

In practice we do not isolate the variance due to the separate effects but calculate the Residual Sum of Squares as Total Sum of Squares + Correction Term − Sum of Squares for Runs − Sum of Squares for Concentrations.

The further essential numerical values are then obtained using factorial coefficients after the method described by Bliss and Marks[8] with the exception that we prefer to examine the linearity of separate log. concentration response lines. The extraction of these values are shown in Table V.

Since the values A_1, A_2, A_3, in turn do not exceed $s^2 \times 4{\cdot}54$ the linearity of the respective lines and their parallelism is accepted. It may happen that one or more of the criteria will exceed $s^2 \times 4{\cdot}54$ but in this

TABLE IV

ANALYSIS OF VARIANCE ON DATA IN TABLE I

Source	Sum of squares	Correction term	Reduced sum of squares	df	Variance
Total	$0{\cdot}51^2 + \text{———} + 0{\cdot}88^2$	$16{\cdot}44^2/24$	0·8722	23	
Between runs ...	$\left[4{\cdot}35^2 + \text{———} + 4{\cdot}15^2\right]/6$	$16{\cdot}44^2/24$	0·0900	3	0·0300
Between doses ...	$\left[1{\cdot}96^2 + \text{———} + 3{\cdot}53^2\right]/4$	$16{\cdot}44^2/24$	0·7597	5	0·1519
Residual ...			0·0225	15	$0{\cdot}0015 = s^2$

Reduced sum of squares = Sum of squares − Correction term.

TABLE V

Treatment effect	Coefficients for x S_1	S_2	S_3	U_1	U_2	U_3	Nx^2	SN(xYp)	$\frac{S^2N \times Yp}{Nx^2}$
Samples	−1	−1	−1	+1	+1	+1	24	−0·5 = T	
Slope	−1	0	+1	−1	0	+1	16 = P	3·45 = Q	
Linearity of curve for standard	+1	−2	+1				24	0·19	$0{\cdot}0015 = A_1$
Linearity of curve for unknown				+1	−2	+1	24	0·26	$0{\cdot}0029 = A_2$
Parallelism of lines	+1	0	−1	−1	0	+1	16	−0·13	$0{\cdot}0011 = A_3$
Yp	1·96	2·76	3·75	1·87	2·57	3·53			$0{\cdot}0018 = A = \frac{A_1 + A_2 + A_3}{3}$

case we still proceed with the analysis providing the value A does not exceed $s^2 \times 3{\cdot}29$. The values 4·54 and 3·29 being the respective 5 per cent. variance ratios for $n_1 = 1$ and 3 when $n_2 = 15$ (Fisher and Yates[10]) which are the conditions that apply in the given example.

Calculation of Potency and its Fiducial Limits. In general the log. activity ratio of the unknown to the standard may be calculated from tests with three concentrations of standard and three of unknown

as $M = 1{\cdot}33\ TI/Q$

where I is the log. of the ratio of successive concentrations.

Its fiducial limits at a prescribed probability level may be obtained by using the appropriate Student value t and calculating

$$C = Q^2/[Q^2 - t^2 s^2 P]$$

and applying this in the expression

$$CM \pm \sqrt{(C-1)\,(2{\cdot}667\ I^2 + CM^2)}$$

which is the appropriate modification of that given by Fieller[11].

In designs similar to that discussed, and working at P = 0·95 these expressions reduce to

$$M = 0{\cdot}1292\ T/Q$$

$$C = Q^2/Q^2 - 72{\cdot}659\ s^2$$

$$CM \pm \sqrt{(C-1)\,(0{\cdot}02507 + CM^2)}$$

and when applied to the specific example yield the following estimates.

$$M = -0{\cdot}01872 = \bar{1}{\cdot}98128 = \text{log. } 0{\cdot}9579$$

$$C = 1{\cdot}00924$$

$$CM \pm \sqrt{(C-1)\,(0{\cdot}0257 + CM^2)} = -0{\cdot}00356 \text{ and } -0{\cdot}03421$$

$$= \bar{1}{\cdot}99644 \text{ and } \bar{1}{\cdot}96578 = \text{log. } 0{\cdot}9917 \text{ and log. } 0{\cdot}9243$$

Thus we estimate the potency of the unknown to be 95·79 per cent. of that assumed with fiducial limits P = 0·95 of 99·17 per cent. and 92·43 per cent.

In a second assay conducted on this sample the following values were obtained.

T = −0·16	Q = 2·94	s^2	= 0·0003
A1 = 0·0028		$s^2 \times 4{\cdot}54$	= 0·0013
A2 = 0·0021	A = 0·0017	$s^2 \times 3{\cdot}29$	= 0.0010
A3 = 0·0002			

Since in this instance both A_1 and A_2 exceed $s^2 \times 4{\cdot}54$ and A exceeds $s^2 \times 3{\cdot}29$, it would not be permissible to use s^2 in the analysis of this assay but we may proceed using A in place of s^2 taking due note that A has been established with 3 df.

The same formulae are used to calculate M and its fiducial limits at P = 0·95 except that the value of C is now taken as

$$C = Q^2/[Q^2 - t^2 AP]$$

which in examples similar to that described reduces to

$$C = Q^2/[Q^2 - 162A].$$

Applying the formulæ to the above example we obtain the following estimates

$$M = -0{\cdot}00703 = \bar{1}\ 99297 = \text{log. } 0{\cdot}9840$$

$$C = 1{\cdot}03291$$

$$CM \pm \sqrt{(C-1)\,(0{\cdot}02507 + CM^2)} = +0{\cdot}02146 \text{ and } \bar{1}{\cdot}96402$$

$$= \text{log. } 1{\cdot}051 \text{ and log. } 0{\cdot}9204$$

Concordancy and Combination of Results. It is our practice to weight each estimate inversely as its variance and to use this to establish the concordancy of the estimates and to calculate the mean potency.

In general terms the variance is calculated as

$$s_m^2 = \frac{s^2}{b^2}\left(\frac{1}{N_{St}} + \frac{1}{N_u} + \frac{M^2}{PI^2}\right) = \frac{1}{w}$$

where $b = Q/PI$, N_{st} and N_u = the number of responses to standard and unknown s^2 being replaced by A on the appropriate occasions.

If X represents the estimated per cent. potency the concordancy is checked by comparing the value

$$Sw(X - \bar{X})^2 \text{ calculated as } SwX^2 - S^2wX/Sw$$

with the appropriate value of χ^2 with n corresponding to the number of estimate less 1.

The mean log. estimated percentage potency is then calculated as

$$\bar{X} = SwX/Sw$$

and its limits of error as $\bar{X} \pm \sqrt{6^2/Sw}$. Where t is the appropriate Student value corresponding to the summed degrees of freedom with which s^2 (or A) have been established. In a design similar to that described the weight may be calculated as

$$w = \frac{6b^2}{s^2} \Big/ [1 + 40M^2]$$

In applying this treatment to the examples given we have used the value (X – 2) in place of X to reduce the number of digits to be handled and have extracted the values shown in Table VI.

TABLE VI

Test	Estimated potency	X	w	w(X – 2)	w(X – 2)²
	per cent.				
1	95·79	1·9813	19136	–357·8432	6·6917
2	98·4	1·9930	12628	–88·3960	·6188
			31764	–446·2392	7·3105
			Less correction term $S^2w(X-2)/Sw$		6·2690
				$Sw(X-\bar{X})^2$	1·0415

The value $Sw(X - X)^2$ of 1·0415 for two estimates indicates satisfactory agreement between them at P = 0·95. We therefore calculate the mean log. estimated potency as $2 - \frac{446{\cdot}2392}{31764} = 2 - 0{\cdot}0140 = 1{\cdot}9860 = \log.$ 96·83 per cent. and using in this instance the value t = 2·1 the limits of error of this estimate at P = ·95 as 1·9860 ± 0·0118 = 1·9978 and 1·9742 = log. 99·49 per cent. and 94·23 per cent.

Characteristics of the log. concentration response lines. During 115 tests of the linearity of the l.c.r.l. for standard and for the unknown and their parallelism were acceptable without question 83 times. In 15 of the remaining tests one or more of the criteria were in doubt, but since A did not exceed $s^2 \times 3{\cdot}29$ the linearity and parallelism of the lines were still accepted, but in the other 17 tests A had to be used in place of s^2 for the subsequent calculations. In all, the linearity of the l.c.r.l. for standard was questioned on 8 occasions, that of the unknown on 19 occasions, and the parallelism on 14 occasions.

Theoretical Accuracy of the Assay. In the 83 tests acceptable without question the value of b^2/s^2 ranged from 92·5 to 9586·0 and had a mean value of 2,764·0. (In the 17 tests in which A was used in place of s^2 the mean value for b^2/A was 755·3.)

Thus on the average when M is zero the weight of the test described should be 16,584, which at P=0·95 would indicate an error of the order ±3·5 per cent.

The accuracy of the method in practice. The data shown in Table VII illustrate the accuracy of the method in practice. The results are those obtained during the examination of dilutions of the standard treated as samples of unknown strength. The error of these estimates are within that expected from the internal evidence of the tests.

TABLE VII

Standard prepared to be	Potency found	P=0·95 limits
76·9 per cent.	80·16 per cent.	75·89—84·8
91·6 ,,	89·12 ,,	82·4 —97·12
110 ,,	108·2 ,,	103 —114
110 ,,	109·2 ,,	104·5 —114·5

The application of the method to a rough assay. In tests carried out at intermediate stages in the production of heparin B.P. the linearity of the line relating log. coagulation time to log. concentration is accepted and assays are conducted using two levels of standard (2 U/ml., 1·28 U/ml.) and one only of the sample under test. This is prepared so that it would be equivalent to the high concentration of standard if the potency assumption has been made correctly. On these occasions four samples are examined at one time and the assay is completed by carrying out 6 runs. The estimation of potency could be made graphically, but it is perhaps easier to calculate it arithmetically.

If S_2, S_1 and U_2 represent the sums of the log. coagulation times for high and low concentratilons of standard and for the unknown respectively.

Then $$Q = S_2 - S_1 \text{ and } T = U_2 - S_2$$
$$\text{and } M = TI/Q$$

where I = log. ratio of the Standard concentrations.

On theoretical grounds the accuracy of such an estimate when $b^2/s^2 = 2700$ would be of the order ± 5 per cent at P=0·95. There is some objection to calculating the fiducial limits from such an assay since occasions do occur when the linearity or parallelism of the respective lines are in question. When fiducial limits are desired it is preferable to carry out the full assay which has been described.

Summary

1. A new method of heparin assay using " sulphated " ox blood, and thrombokinase is described.

2. The method has the following advantages:—

(a) The method is rapid, accurate, and agrees well with whole blood methods.

(b) Equipment and technique are simple.

(c) The blood system, if stored below 4°C. is stable for at least 3 weeks.

(d) Reproducibility of results between different operations is good.

(e) The assay is conducted at room temperature.

(f) The data may be interpreted by standard methods of analysis and a treatment is described.

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DISCUSSION

The paper was presented in abstract by Mr. S. S. Adams.

THE CHAIRMAN asked if any members had compared the existing methods with that proposed in the paper.

MR. R. MAXWELL SAVAGE (Barnet) reported some observations on clotting times. They occasionally used a method for determining clotting times which was almost identical with that described in the paper, and he could confirm that it worked very well. It gave a sharp end-point. They did not do it at room temperature, because it was so easy to control the temperature with the water-bath. The method had the drawback that it could not be used for systems which were clotting very rapidly. There was an alternative method which consisted of putting very small drops of plasma and of the solution of heparin side by side on a microscope slide and mixing them with a capillary stirrer. The time was observed with a stop-watch and clotting times as low as 15 seconds were recorded with quite small errors. His next point concerned the preparation of a clotting system. Dried plasma, if stored in a screw-capped bottle in a small desiccator in a refrigerator, retained its clotting properties for periods of a year or more. Thrombin was a stable reagent, so that, by the use of dried plasma and thrombin solution, with a little attention to storage, a clotting system could be set up very easily without previous notice and in a few minutes. Previous reports had suggested 25°C., i.e., the temperature of the body at the normal site of clotting,

to be a more suitable temperature than 37°C. At about that temperature many clotting processes changed very little and control of temperature might therefore not be necassary. In the author's method, the standard obviated the need for controlling the temperature, as both the standard and test solution were equally affected, but in the method he had described there were difficulties in operating without a thermostat. The clotting process was not completely indifferent to temperature, and if one did the determination of the standard and of the test solution at too great an interval of time, the disturbing effects of, say, a shaft of sunlight on a neighbouring bench might put up the temperature sufficiently to upset the experiment.

DR. G. E. FOSTER (Dartford) asked if the author had tried to make heparin possessing a retarded action, and whether he had tried hirudin which was used a great deal in physiological work.

MR. S. S. ADAMS, in reply, said he was aware that the system which they had used was not very suitable for the more rapid clotting times, but they were not very interested in that, because with clotting times which ranged from 3 to 10 minutes the operator had plenty of time in which to put down the clotting times accurately. When the clotting times varied only between about 30 and 90 seconds, it was difficult to record the times for 3 or 4 test tubes accurately. He had not tried a method using dried plasma, but he had tried several methods, and their modifications, using thrombin. The major difficulty was that both the plasma and thrombin had to be kept at 0°C. immediately prior to use, because otherwise the clotting times differed quite appreciably from minute to minute. He had done some work with retarded heparins, and this method would give a result for absolute potency. He was referring to preparations in which the heparin was in a gelatin base. The measurement of retarded effect *in vivo* was made by injecting a standard solution of normal heparin into rabbits and determining the clotting times of the blood at half-hourly or hourly intervals, and then injecting the retarded heparin into another batch of rabbits and determining the clotting times for those. It was not a quantitative method. They had no experience of hirudin.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Carvone in Essential Oils, Determination of. C. Schooltens. (*Pharm. Weekbl.*, 1950, **85**, 738.) Considerable variations are found in the results of determinations of carvone by different methods. Using a mixture of 65 per cent. of pure carvone with 35 per cent. of limonene, assays were performed by 7 different methods with, generally, low results. The U.S.P. process gave results ranging from 56·7 to 58·5 per cent.; that of the British Pharmacopœia from 60·3 to 63·9 per cent.; the sulphite method of the National Formulary VII gave 64·5 to 65·9 per cent. The latter method is thus to be preferred. Gravimetric determination with 2:4-dinitrophenylhydrazine is fairly satisfactory; results being from 63·7 to 66·0 per cent.

G. M.

Cocaine and Ethocaine, Chromatographic Separation of. R. Fischer and E. Buchegger. (*Pharm. Zentralh.*, 1950, **89**, 185.) The mixed bases (about 20 mg.), dissolved in carbon tetrachloride, are passed through a column of 5 g. of alumina (9 mm. diameter). Cocaine is then eluted by 20 ml. of carbon tetrachloride containing 4·5 per cent. of acetone, and subsequent treatment with 20 ml. of chloroform removes the ethocaine.

G. M.

Isopropyl Alcohol, Identification of. H. Auterhoff. (*Pharm. Zentralh.*, 1950, **89**, 293.) A number of colour reactions given by isopropyl alcohol with phenols are described. These are in general not very characteristic. More satisfactory is that with *p*-dimethylaminobenzaldehyde, as follows. A solution, containing about 20 per cent. of the alcohol, is treated with a little charcoal to remove higher alcohols and other impurities, and the mixture is filtered. A few ml. of the filtrate is layered on a 1 per cent. solution of *p*-dimethylaminobenzaldehyde in sulphuric acid. Isopropyl alcohol gives a bright red-violet ring in a few minutes, gradually becoming brown. Higher alcohols give an immediate brown colour. Brown or reddish brown rings are given by *n*-propyl alcohol, *n*-butylalcohol, *iso*butyl alcohol and *iso*amyl alcohol.

G. M.

Mineral Oil, in Fatty Oils, Detection of. H. Patzsch. (*Pharm. Zentralh.* 1950, **89**, 302.) The presence of unsaponifiable matter in an oil is not conclusive evidence of the presence of mineral oil. The unsaponifiable matter should be refluxed for 6 hours with its own volume of acetic anhydride. If it dissolves completely, the presence of lower wax alcohols is probable, but separation on cooling suggests sterols or high-melting fatty alcohols (e.g., myricyl alcohol). Undissolved material indicates mineral oil, or solid paraffins, which solidify as an upper layer on cooling. The latter may be distinguished by their melting-point, density and insolubility in a mixture of equal parts of alcohol and ether.

G. M.

Sugars, Reducing, Quantitative Paper Chromatography of. J. Montreuil. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1639.) A series of unidimensional chromatograms are produced on a sheet of paper, the initial spots of 5 to 50 μg. of the sugars being spaced 4 cm. apart. After drying,

a single reference band is cut off and developed in order to give the exact localisation of the different spots. From the other part of the paper, a square, cut out large enough to include a spot, is extracted with water at 40°C. for 12 hours. To 5 ml. of the solution obtained 1 ml. of a 0·2 per cent. solution of potassium ferricyanide and 1 ml. of a reagent containing 0·3 per cent. of potassium cyanide and 1·6 per cent. of sodium carbonate are added. The mixture is placed on the water-bath for exactly 8 minutes, cooled quickly, and treated with 0·5 ml. of 2 per cent. oxalic acid solution and 2 ml. of a solution containing 5 g. of ferric sulphate and 75 ml. of phosphoric acid (d=1·7) in 500 ml. The volume is then made up to 25 or 50 ml. After standing for 30 minutes in the dark, the colour is determined. A similar blank test is made on a piece of paper of the same dimensions.

G. M.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Digitalis Glycosides, Paper Chromatography of. A. B. Svendsen and K. B. Jensen. (*Pharm. Acta Helvet.*, 1950, **25**, 241.) Mixtures of chloroform, methanol and water were found to be suitable for the paper chromatography of digitalis glucosides. The chloroform was first freed from alcohol by washing and drying, and the solvent mixtures were prepared by prolonged shaking in a thermostat, being then allowed to settle. In order to detect the position of the spots, the paper, after drying, was sprayed with a 25 per cent. solution of trichloracetic acid in chloroform, then heated for 2 minutes at 100°C. A positive result is shown by a fluorescence varying in intensity and colour with different glucosides. R_F values observed for 10 of these substances are given in the table below, the solvent mixtures (I, II and III) being prepared from 10 parts of chloroform, 5 of water, and respectively 2, 4 or 8 of methyl alcohol.

	Solvent Mixture		
	I	II	III
Purpurea glucoside A	0·07	0·10	0·15
Digitoxin	0·88	0·91	0·90
Digitoxigenin	0·92	0·93	0·92
Purpurea glucoside B	0·02	0·03	0·05
Gitoxin	0·76	0·81	0·82
Gitoxigenin	0·82	0·84	0·84
Digilanid A	0·37	0·46	0·49
Desacetyldigilanid A	0·07	0·10	0·15
Digilanid B	0·14	0·18	0·25
Desacetyldigilanid B	0·02	0·03	0·05
Digilanid C	0·08	0·12	0·15
Desacetyldigilanid C	0·01	0·01	0·02
Digoxin	0·68	0·75	0·76
Digoxigenin	0·58	0·64	0·61

The process was applied to *Digitalis purpurea* leaves prepared in different ways. The drug, stabilised by alcohol vapour, contained mainly primary glycoside, with small amounts of secondary glycoside and genin. Leaves dried by moderate heat showed only small amounts of primary glycoside, but secondary glycoside and genin in larger amounts. The amount of genuine glycoside appeared somewhat greater in leaves dried at 80°C. than in those treated at 55° to 60°C.

G. M.

Digitoxoside and Gitoxoside, Colour Reactions of. P. Bellet. (*Ann. pharm. franc.*, 1950, **8**, 471.) Suspend about 0·5 mg. of commercial

digitoxoside (" digitaline ") in 5 ml. of phosphoric acid and allow to stand for 5 minutes; an intense yellow colour is produced, and the presence of gitoxoside is indicated by an intense yellow-green fluorescence in filtered ultra-violet light. Less than 1 per cent. of gitoxoside can be detected by this method, compared with 10 per cent., using the Keller-Kiliani reaction. Although the yellow colour in daylight is due to the sugar moiety, the fluorescence in filtered ultra-violet light is due to the aglucone, the actual fluorescent substance being dianhydrogitoxigenin, having conjugated double bands in positions 14-15, 16-17 and 20-22. It may be postulated that *resonance at carbon atoms 14, 16 and 20 leads to the formation of mesomeric* states responsible for the fluorescence. In anhydrodigitoxigenin, the double bonds are too far apart for appreciable resonance to occur. Oleandrin and honghelin also give a fluorescence with phosphoric acid in ultra-violet light.

G. B.

Starch, Hydrolysis of, by Hydrochloric Acid. A. Leman and P. Didry. (*C.R. Acad. Sci. Paris*, 1950, **231**, 443.) The first stage of the breakdown of starch into non-reducing intermediate products proceeds the more rapidly the more concentrated the acid, but the optimum acidity for complete and rapid conversion into glucose is approximately normal. The actual volume of acid used is unimportant, provided that it is at least 20 ml. per 0·01 mol. of starch. For a given quantity of hydrochloric acid, hydrolysis proceeds much further when it is more concentrated, provided that the concentration does not exceed normal. Using N hydrochloric acid, hydrolysis is practically complete on the water-bath in 50 minutes; 0·5 N acid requires 2½ hours; and 0·4 N 4 hours. G. M.

TOXICOLOGY

Anthisan Poisoning, Acute. A. A. Miller and E. Pedley. (*Brit. med. J., 1950, 1, 1115.*) *The authors describe a fatal case of* poisoning *in* a child of 16 months who ate several tablets containing 0·1 g. of anthisan; the actual number taken was unknown, but probably was not more than 6. No effects appeared after about 2 hours, when the child began to moan and became unconscious; muscular twitchings occurred and there was profuse catarrhal discharge and foaming at the mouth and nose. Death occurred about 3 hours after eating the tablets. At necropsy a greenish-coloured watery fluid poured from the mouth and nose on moving the body. The main signs were those of acute passive congestion; the meninges, spleen. kidneys and liver were all congested. The stomach contained about 6 oz. of watery undigested food showing a marked greenish tinge. A solution of the base in dilute hydrochloric acid gives a yellowish-green precipitate with Mayer's reagent and a trace of the dry base gives a bright cherry red colour with concentrated sulphuric acid. These reactions were obtained from the stomach contents submitted to the Stas-Otto extraction process. S. L. W.

Barium, Toxicological Determination of. I. L. Castagnou and S. Larcebau. (*Bull. Trav. Soc. Pharm. Bordeaux*, 1950, **88**, 23.) After the administration of a fatal dose (0·11 g./kg. of body weight) to a cat, 76·8 per cent. was recovered from the organs, by far the greater part being in the intestines. With other cats, which received larger doses causing considerable vomiting, *the recovery was less than 1 per cent. Since in all* these cases the difference between the total barium recovered from all organs, and that from the stomach and intestines, was of the order of 1

to 2 mg., it may be stated that only a few mg., absorbed and fixed in these organs, is fatal. If similar results had been obtained in a toxicological examination, it would not have been possible to affirm that death was due to barium poisoning. Rabbits are much less affected by barium, and in this case appreciable amounts were recovered from all organs. The method adopted for the determination of barium (ashing and fusion with sodium and potassium carbonates) does not appear to be altogether satisfactory, and this question should be investigated. G. M.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Chorionic Gonadotropin, Effect of Colloids on Action of. K. Pedersen-Bjergaard and M. Tønnesen. (*Dansk Tidsskr. Farm.*, 1950, **24,** 271.) In view of the reported action of polyvinylpyrrolidone in delaying the absorption of extracts of the posterior lobe of the pituitary gland, the effect of a number of colloids on the action of gonadotropin was examined, using immature female rats. With hormone from the serum of pregnant mares, the action was not altered by colloids. With subcutaneous injection of chorionic gonadotropin, the effect was increased 4 times by the addition of colloids. The minimum percentage concentration of the colloids which, when used in solution as solvent for 0·3 I.U. of chorionic gonadotropin, caused a positive Allen-Doisy reaction in 50 per cent. of the immature rats used, was as follows: polyvinylpyrrolidone, 25; gelatin, 15; acacia, 15; soluble starch, 10; agar, 1; carboxymethylcellulose, 0·75; tragacanth, 0·125; Irish moss, 0·0625. A similar increase of action was also observed, with polyvinylpyrrolidone, when the chorionic gonadotropin was administered to female Rhesus monkeys. In man, the excretion of the hormone was decreased by 50 per cent. by the addition of this colloid. G. M.

Ribonucleotides, Chromatography of. J. Montreuil and P. Boulanger. (*C. R. Acad. Sci. Paris*, 1950, **231,** 247.) Chromatography has already been applied to the separation of the bases produced by the hydrolysis of nucleic acid. In order to avoid the difficulties resulting from de-amination during acid hydrolysis, the authors have applied the method to the nucleotides. The solution, obtained by hydrolysis with 2 per cent. sodium hydroxide for 24 hours at ordinary temperature, is applied directly to the paper. Alternatively, separation may be facilitated by passing the solution through a cation exchange column, which holds back the adenylic and cytidylic acids, which may be eluted by dilute ammonia. Solvents used and R_F values obtained are as follows:—

	Phosphoric acid	Xanthylic acid	Uridylic acid	Guanylic acid	Cytidylic acid	Adenylic acid
Phenol 40 ; isopropyl alcohol 5 ; formic acid 5 ; water 50 ...	0·17	0·23	0·33	0·44	0·50	0·64
Phenol 50 ; N sulphuric acid 50	—	0·19	0·24	0·28	0·37	0·50
Phenol 45 ; formic acid 5 ; water 50	0·31	0·36	0·44	0·52	0·60	0·70
Isopropyl alcohol 60 ; glycol monochlorhydrin 30 ...	—	—	0·30	0·14	0·24	0·26
N hydrochloric acid 10 ...	0·70	—	0·62	0·36	0·43	0·43

The papers are dried at 80° C. and sprayed with Hanes and Isherwood *reagent* (ammonium molybdate in hydrochloric-perchloric acids). The colour appears on exposure to sunlight; it may be increased by treatment with hydrogen sulphide. The nucleotide may be determined by cutting out the spot, digesting with sulphuric and nitric acids, and determining the phosphate colorimetrically. The chromatography may be made two-dimensional by using phenolic and alcoholic solvents. G. M.

Vitamin A, Synthesis of Ether-Oxides of. R. Golse and J. Gavarret. (*Bull. Trav. Soc. Pharm. Bordeaux*, 1950, **88**, 57.) β-Ionone is condensed in presence of zinc with propargyl bromide, giving a β-acetylenic alcohol. This compound is condensed with methoxy-4-butanone-2, giving (trimethyl-2′:6′:6′-cyclohexene-1′-yl)-9-dimethyl-3:7-methoxy-1-nonene-8-yne-4-diol-3:7. This compound is converted into vitamin A methyl ether by partial reduction with colloidal palladium, treatment with phosphorus bromide, and removal of hydrogen bromide by potassium hydroxide. By this synthesis, only two stages of condensation are required, and, unlike the similar method of Milas and others, there is no question of any change in the position of the double bond. G. M.

Vitamin B_{12} and Desoxyribosides as Growth Factors for Lactic Acid Bacteria. E. Kitay, W. S. McNutt and E. E. Snell. (*J. Bact.*, 1950, **59**, 727.) Eighteen strains of lactic acid bacteria, representative of 5 different species of *Lactobacillus* and one species of *Leuconostoc*, were found not to grow in a medium complete with respect to known amino-acids and synthetic vitamins, and supplemented by tomato juice and an enzymatic digest of casein. All grew when thymidine was added to the medium. The thymidine could be replaced in most cases by hypoxanthine desoxyriboside, guanine desoxyriboside, adenine desoxyriboside, cytosine desoxyriboside, or by high levels of desoxyribonucleic acid. Vitamin B_{12} could replace thymidine or other desoxyribosides for many, but not all, of the organisms. Ascorbic acid, thioglycollic acid, cysteine or glutathione could also replace thymidine. Vitamin B_{12} and vitamin B_{12b} added aseptically were equally active growth-promoters, but if autoclaved in the medium, the activity of vitamin B_{12b} was only about one-seventh that of vitamin B_{12}. H. T. B.

BIOCHEMICAL ANALYSIS

Creatine, Creatinine and Related Compounds in Urine; Determination by Paper Chromatography. S. R. Ames and H. A. Risley. (*Proc. Soc. exp. Biol. N.Y.*, 1948, **69**, 267.) A 0·025-ml. aliquot of urine is placed on the paper strip and developed for 15 to 20 hours with water-saturated solutions of butyl alcohol, phenol, or lutidine-collidine (equal parts). The strip is air-dried, heated at 100°C. for one hour to convert creatinine to creatine and glycocyamine to glycocyamidine, cooled to room temperature and sprayed with a mixture of 1 part of 10 per cent. sodium hydroxide and 5 parts of saturated trinitrophenol solution. The presence of creatinine or related compounds is shown by the appearance of orange spots. Water-saturated solutions of butyl alcohol and lutidine-collidine separate creatine and creatinine better than water-saturated phenol, although the latter gives an excellent resolution between glycocyamine and the others. The smallest amount of creatine or creatinine which could be detected was 11μg. It is possible to test for amino-acids and creatinine and related compounds on the same strip by spraying the reagent on to the paper previously treated with ninhydrin. In an alternative

procedure for the determination of creatine the strip is not heated, but is air-dried and sprayed with a solution of diacetyl in alcoholic sodium carbonate, when the presence of creatine is indicated by a pink colour. The smallest amount of creatine detectable by this method is 33μg.; creatinine does not react and glycocyamine and glycocyamidine give no colour in quantities up to 200g., but several amino-acids interfere. G. R. K.

Prothrombin Levels in Blood, A New Improved Method for Determination of. A. Goldfeder, D. Bloom and M. Weiner. (*Science*, 1950, **111**, 365.) A simple method of determination of the prothrombin time, using only 5 cmm. of whole blood, is as follows. Calibrated 5 cmm. capillary tubes are filled with a 2 per cent. solution of potassium oxalate and dried in an oven. A prepared capillary tube is filled with blood and, with the aid of a small rubber bulb, the blood is quickly expelled into 15 cmm. thromboplastin solution on a glass slide, and gently mixed. The mixture is drawn gently in and out of the capillary tube, the tube being raised at each filling until a fibrin strand forms. A stop-watch is used to record the time required after the addition of the blood to the thromboplastin solution, for the formation of a fibrin strand. In preparing the thromboplastin solution, whole blood, instead of plasma, is used, the blood being taken into tubes with dry oxalate. In precise work, it is necessary to take into account viriations due to changes in room temperature and, in subjects with low hæmocrit values, the variable plasma volume per ml. of whole blood. G. B.

Testosterone, Colorimetric Assay of. A. T. Nielsen. (*Acta Endocrinol.*, 1948, **1**, 362.) The procedure is based upon the blue colour formed when testosterone is heated with sulphuric acid and subsequently mixed with alcoholic sulphuric acid. Two modifications are described, as follows :—(a) a test-tube (180 × 20 mm.) containing dry testosterone is placed in an ice-bath and 1ml. of sulphuric acid added with stirring. The tube is heated in boiling water for 5 minutes, replaced in the ice-bath, 4ml. of 25 per cent. alcoholic sulphuric acid (1 vol. sulphuric acid diluted with 3 vol. methyl alcohol) added with stirring, and the mixture placed in a thermostat at 29°C. for 30 minutes. After transferring to a photometer cell (1cm.) the extinction is read at 600mμ. The blank consists of reagents without testosterone. (b) The testosterone is dissolved in 0·2 ml. of methyl alcohol prior to the addition of sulphuric acid. The mixture is heated in boiling water for 30 seconds and the test continued as described under (a). At room temperature maximum colour is obtained within 15 to 25 minutes after admixture of the alcoholic sulphuric acid reagent. Colour intensity is plotted against the time (in minutes) elapsing after the addition of alcoholic sulphuric acid. The colour is stable for at least an hour. The colour intensity obtained with testosterone dissolved in methyl alcohol is definitely higher than that given by the dry substance but a more accurate and reproducible action is obtained with the latter, but method (b) is necessary for the determination of testosterone isolated from oily solutions. The accuracy of the method is of the order of ± 5 per cent. 3 substances are known to give a colour reaction very similar to testosterone; Δ^4-androstene-3β,17-dione, deoxycorticosterone and dehydroandrosterone, but the first of these may be effectively removed by means of nicotinic acid hydrazide and in the case of the last 2 the colour intensity may be decreased by extending the heating time. The authors outline the practical application of the method to the analysis of pharmaceutical preparations. S. L. W.

Tyrothricin, *in vitro* Assay of. R. J. Reedy and S. W. Wolfson. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 1.) A routine procedure is reported

in which the normal serial dilution method, a modification of that of Dubos, is used. The organism used, *Streptococcus fæcalis* (M-19), has been found more sensitive to the action of tyrothricin than streptococcus H-69D-5, and the possibility of interference by other ingredients of emulsions and ointment-type products is thus reduced. Details of the method are given for alcohol and propylene glycol solutions and for extraction of tyrothricin from oily and ointment preparations. It was noted that the organism occasionally dissociated, with the development of gramicidin-resistant variants, when transferred daily in the assay broth. This may be overcome by dilution of the nutrients or sub-culturing until the desired sensitivity is regained. A supplementary method for use as a check is also given since the tyrothricin present in many commercial preparations cannot be completely recovered by the routine procedure. It was found necessary to extract the tyrothricin before assay to avoid interference by the bases. G. R. K.

PHARMACY

DISPENSING

Adrenaline, Preservation of Solutions of. M. P. Girard and G. Kerny. (*Ann. pharm. franc.*, 1950, **8**, 463.) Solutions containing 0·025 per cent. of adrenaline in distilled water with the addition of sodium bisulphite, filled into ampoules under nitrogen or carbon dioxide and sterilised by tyndallisation, retain at least 90 per cent. of their adrenaline content for five years. The solution prepared with hydrochloric acid and sodium bisulphite keeps equally well, but the solutions are not well preserved by hydrochloric acid alone. Solutions stored in ampoules of certain kinds of yellow glass deteriorate rapidly; apparently the decomposition is accelerated by the iron which the solution absorbs from the glass. There is good agreement between the colorimetric assay and the biological assay, when bisulphite alone is the preservative, but in the presence of hydrochloric acid and bisulphite, the chemical assay gives the higher result. Colour of the adrenaline solutions cannot be used as a criterion of their state of preservation because some colourless solutions have been found to have lost 60 per cent. of their titre. G. B.

Morphine Solutions, Decomposition of, on sterilisation. C. G. van Arkel and J. H. van Waert. (*Pharm. Weekbl.*, 1950, **85**, 319.) The absorption spectrum of morphine hydrochloride has a maximum at 282·5 mμ and a minimum at 260 mμ. Pseudomorphine, the oxidation product of morphine, shows a higher absorption, so that an increase in the absorption minimum is the best indication of decomposition of a solution of morphine hydrochloride. It was found that a 1 per cent. solution of morphine hydrochloride heated for 1 hour at 100°C., showed 1 per cent. of decomposition, but that this could be completely prevented by the presence of 0·05 per cent. of sodium bisulphite. The decomposition is somewhat greater in more dilute solutions. Keeping of the sterilised solution in brown ampoules for 3 months resulted in a slight discoloration in the absence of sodium bisulphite, but the actual decomposition was too small to permit of spectrographic determination. G. M.

Sterilisation in Free Steam. K. Steiger. (*Pharm. Acta Helvet.*, 1950, **25**, 107.) Tests were made to determine if reliable sterilisation could be attained in 30 minutes at 100°C. in presence of bactericidal substances. The

test organism was a suspension of highly resistant soil spores, which was added in moderate quantity to clear injection solutions. The results showed that sterilisation was attained, without any addition, at *p*H 3·2 or lower. The addition of 0·01 per cent. of sodium fluoride or of 0·1 per cent. of chlorbutol was of little advantage, but 5 per cent. of alcohol resulted in complete sterilisation up to a *p*H of 6·1. Esters of *p*-hydroxybenzoic acid, also in presence of 5 per cent. of alcohol, produced no gain in efficacy, but the combination of 0·1 per cent. of chlorbutol with 5 per cent. of alcohol increased the range of effective action up to *p*H 9. Equally effective (without alcohol) were 0·01 per cent. of potassium oxyquinoline sulphate or 0·0001 per cent. of phenylmercuric borate. With this last addition, 5 minutes' heating in flowing steam was sufficient to produce complete sterility under the conditions of the experiments. G. M.

NOTES AND FORMULÆ

Aurothioglucose (Solganal). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950,**142,** 816.) Aurothioglucose $C_6H_{11}AuO_5S$, is a yellow to yellowish-green, odourless, tasteless powder, soluble in water and insoluble in acetone, alcohol, chloroform and ether; aqueous solutions decompose on standing. When dried *in vacuo* over phosphorus pentoxide for 24 hours the loss in weight is not greater than 1 per cent.; specific rotation $[\alpha]_D^{25°C}$, +65° to + 73°. It contains 49·4 to 51·0 of gold and 8·0 to 8·4 per cent. of sulphur; it is assayed for gold by boiling with nitric acid, filtering and igniting the residue; the filtrate is used for the assay of sulphur by boiling with hydrochloric acid, diluting with water and precipitating the sulphate with barium chloride. Aurothioglucose is administered by the intramuscular injection of a suspension in oil in the treatment of active rheumatoid arthritis and non-disseminated lupus erythematosus. G. R. K.

Diglycocoll Hydroiodide-Iodine (Bursoline). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950, **142,** 990.) Diglycocoll hydroiodide-iodine, $2(HO_2C.CH_2.NH_2—HI—NH_2.CH_2.CO_2H) + I_2$, is a dark lumpy powder with a strong odour of iodine, freely soluble in water, almost insoluble in chloroform and only very slightly soluble in alcohol, although the iodine component is soluble in alcohol; a 0·1 per cent. solution in water has *p*H about 3·0. It contains 6·82 to 7·02 per cent. of nitrogen (determined by the Kjeldahl method), 30·5 to 32·0 per cent. of titratable iodine (determined by adding sulphuric acid and potassium iodide and titrating with sodium thiosulphate) and 30·5 to 32·0 per cent. of iodine present as iodide (determined by boiling with potassium iodate and sulphuric acid to remove free iodine, cooling, adding potassium iodide and titrating with sodium thiosulphate). Diglycocoll hydroiodide-iodine is used for the disinfection of drinking water. G. R. K.

Dimethyltubocurarine Iodide (Metubine Iodide). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950, **142,** 1142.) Dimethyltubocurarine iodide is the dimethyl ether of *d*-tubocurarine iodide and occurs as a white to pale yellow, odourless, crystalline powder, slightly soluble in water, dilute hydrochloric acid and dilute sodium hydroxide, very slightly soluble in alcohol and almost insoluble in benzene, chloroform and ether; when heated to 257°C. it decomposes with evolution of gas. The extinction coefficent $E\,\frac{1\ \text{per cent.}}{1\ \text{cm.}}$ at 2800 Å is 74±1·5. It gives a pink precipitate with ammonium reineckate and a yellow precipitate with trinitrophenol; it is distinguished from tubo-

curarine chloride by treating with Folin-Ciocalteu reagent, diluting with water, adding sodium carbonate and heating in a water-bath; the final solution is colourless or very faintly blue. Dimethyltubocurarine iodide loses not more than 7 per cent. of its weight when dried *in vacuo* at 75°C. for 8 hours. It contains 2·80 to 3·10 per cent. of nitrogen (by the Kjeldahl method) and 98 to 102 per cent. of dimethyltubocurarine iodide, determined by measuring the optical density of a 0·005 per cent. solution at 2800 Å and dividing by 7·4; the specific rotation $[\alpha]_D^{25°C.}$ of a 0·25 per cent. solution in water is +150° to +160°. The potency is determined by observing the head-drop response following intravenous injection in rabbits. Dimethyltubocurarine iodide has an action similar to that of tubocurarine chloride but is more potent, having a shorter onset and more prolonged action. G. R. K.

Hydroxyamphetamine Hydrobromide (Paredrine Hydrobromide). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950, **142**, 816.) Hydroxyamphetamine hydrobromide is 1-(*p*-hydroxyphenyl)-2-aminopropane hydrobromide, $HO.C_6H_4.CH_2CH(NH_2).CH_3,HBr$. and occurs as a white crystalline powder with a faint odour, m.pt. 189° to 192°C., very soluble in water and alcohol, and almost insoluble in benzene and ether; a 2 per cent. aqueous solution has *p*H 4·5 to 5·5. The free base, obtained by saturating an aqueous solution with potassium carbonate, extracting with ether and evaporating, is a white to faintly yellow crystalline solid, m.pt. 127° to 129°C., soluble in acids and alkalis. Hydroxyamphetamine hydrobromide is distinguished from other amines by the emerald-blue colour it gives with ammonium molybdate and sulphuric acid, and the purple colour with ferric chloride. When diazotised and extracted with chloroform, the chloroform is coloured amber. It loses not more than 0·5 per cent. of its weight when dried at 110°C. for 3 hours; ash, not more than 0·1 per cent. A 0·0015 per cent. solution shows ultraviolet absorption maxima at 2550 Å ($E^{1\ per\ cent.}_{1\ cm.}$ = 370±5) and 2780 Å, with a minimum at 2440 Å; the ratio of the observed optical densities at 2250 Å and 2780 Å is 4·3 to 5·4. The content of hydrogen bromide is 34·0 to 35·2 per cent. and the content of hydroxyamphetamine hydrobromide, 97·5 to 101·0 per cent.; the latter is determined by liberating the base by the method described above, dissolving in sulphuric acid and titrating the excess of acid. Hydroxyamphetamine hydrobromide is used locally as a 1 per cent. solution to reduce swelling of the nasal mucosa. G. R. K.

Mephenesin (Oranixon). (*New and Nonofficial Remedies; J. Amer. med. Ass.*, 1950, **143**, 655.) Mephenesin is 3-*o*-toloxypropane-1:2-diol, $CH_3.C_6H_4.O.CH_2.CHOH.CH_2OH$, and occurs as an odourless, crystalline white powder, m.pt. 67° to 72°C. It is freely soluble in alcohol, chloroform and ether, and sparingly soluble in benzene and water; a saturated aqueous solution has *p*H 6·0. The extinction coefficient $E^{1\ per\ cent.}_{1\ cm.}$ at 2700 Å is 81±3. When mephenesin is dissolved in sulphuric acid and treated with formaldehyde an intense red colour develops. The loss on drying *in vacuo* over phosphorus pentoxide for 24 hours is not more than 0·1 per cent. The content of mephenesin is 98 to 102 per cent., determined by measuring the light absorption of a 0·006 per cent. solution at 2700 Å. Mephenesin resembles curare in action and is used for the production of muscular relaxation in light surgical anaesthesia. It may also be tried in the treatment of spasticity and tremor in Parkinson's disease, of muscle spasm in hemiplegia, tetanus and certain other spastic conditions, and of athetoid or

choreiform movement. It antagonises the action of strychnine and potentiates that of barbiturates. It has a low toxicity and is given in a dose of 1 g. 3 to 5 times a day, usually as tablets or elixir. G. R. K.

PHARMACOGNOSY

***Colchicum Autumnale*, New Compounds from.** F. Santavy. (*Pharm. Acta Helvet.*, 1950, **25,** 248.) By chromatographic separation of an extract of *Colchicum autumnale*, the following substances were separated: substance I, substance F, substance G, substance J, colchicine, substance D, substance B and a crystalline mixture of substances C and E_1, a phytosterol mixture, saccharose and 2-hydroxy-6-methoxybenzoic acid. The quantities of substances F and G obtained were comparable with that of colchicine. Substance G was partially converted into colchicine by chromatography on alumina. G. M.

Coriander Fruit, Dutch. F. H. L. van Os. (*Pharm. Weekbl.*, 1950, **85,** 732.) A number of strains of coriander, differing in the size of fruit, are generally recognised. An examination was made of coriander grown in Holland with a view to determining whether different varieties, characterised by large and small fruits, were present. This did not appear to be the case, the different sizes of fruit in the harvest being apparently derived from umbels flowering at different periods. In the determination of the essential oil, prolonged distillation results in fatty acids distilling over. A correction should be made for this by determining the acid value of the distilled oil. It is important that the material should not be ground too finely before distillation, as this results in a loss of essential oil. G. M.

Stramonium, Assay and Comparative Method of Drying. M. Rubin and L. E. Harris. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39,** 477.) Freshly collected stramonium leaves were dried (1) in air, (2) at 40°C. and (3) by freeze-drying. The fresh leaves contained 89 per cent. of water, and after drying in air they contained about 9·45 per cent. At 40° C. the water content was 8 per cent. and 5·45 per cent. after freeze-drying. These figures were determined by the U.S.P. toluene method. Assays were then carried out on each sample by (a) U.S.P. XIII method of acid titration of the isolated bases (b) the hydrolytic method of Reimers (*Quart. J. Pharm. Pharmacol.*, 1948, **21,** 470), and (c) a chromatographic method. For the last method 10 g. of the sample was macerated with 10 ml. of ammonia solution (27 per cent. NH_3 w/w) and 30 ml. of ether for 1 hour. This extract was adjusted to 100 ml. It was then run through an alumina column and the adsorbed alkaloids were eluted with alcohol. After elution the alkaloidal solution was evaporated to dryness on a water bath, heated for 15 minutes and the residue was dissolved in 20 ml. of ether and 15 ml. of 0·02N sulphuric acid was added. The ether was then driven off and the excess of acid titrated against 0·02N sodium hydroxide with methyl red as the indicator. Results obtained by the U.S.P. method were slightly lower than those obtained by the other methods, which were almost identical. The hydrolytic method was the least time-consuming. A. D. O.

PHARMACOLOGY AND THERAPEUTICS

Aureomycin in the Treatment of Poliomyelitis. E. Appelbaum and R. Saigh. (*J. Amer. med. Ass.*, 1950, **143,** 538.) 38 patients with non-paralytic poliomyelitis were treated with aureomycin during the early phase

of the disease, while 66 patients served as controls. Aureomycin was given in a dosage of 1 to 1·5 g. daily to children up to 4 years of age, 4 g. to those of 5 to 16 years of age, and 6 g. to patients above that age. The treatment was continued for 7 days. The drug was well tolerated except for transient nausea or vomiting in some cases. The clinical results were about the same in the treated and the control patients and the authors conclude that in this study the early use of aureomycin did not appear to exert any favourable effect on the clinical course of the disease. S. L. W.

Benzodioxan, Action of, in Man. F. T. G. Prunty and H. J. C. Swan. (*Lancet*, 1950, **258**, 759.) The experiments described were planned to contrast the effect of benzodioxan (933F, 2-(1-piperidylmethyl)-1 : 4-benzodioxan) on hypertension produced by adrenaline in normal subjects with that produced by noradrenaline and to observe the resultant circulatory changes. The benzodioxan was given intravenously in a dose of 0·25 mg./kg. of body weight to 4 patients undergoing adrenaline infusion and 3 undergoing noradrenaline infusion. Following the injections there was an initial fall in blood pressure due to peripheral vasodilatation succeeded after a few seconds by a rise to a level above that observed before injection. Both the fall and the rise were accompanied by tachycardia, which may have been responsible for an increase in cardiac output, resulting in the rise in blood pressure. In no patient was there a sustained fall in blood pressure as reported by other workers, and there is therefore some doubt about the reliability of the drug in differentiating phæochromocytoma with hypertension from hypertension due to other causes. G. R. K.

Chloramphenicol in Typhoid Fever. R. A. Good and R. D. MacKenzie. (*Lancet*, 1950, **258**, 611.) A clinical trial in 13 cases of typhoid fever, alternate cases being given chloramphenicol, showed that this antibiotic has a specific clinical effect on the disease. The most striking effect was its control over the patients' temperature which fell to normal in 72 hours. At the same time it relieved the persistent headache and reduced the toxæmia, the patients felt better, appetites returned and abdominal discomfort was lessened. Half the treated cases developed relapse symptoms and in 2 these were sufficiently severe to justify further treatment. All responded satisfactorily to a second course of treatment, which may indicate that longer courses of treatment, larger doses, or a routine second course should be given to all primary cases. The relapses in the treated cases occurred later than in the untreated controls. The condition of the 3 control cases which relapsed became so severe that chloramphenical was given, to which they responded satisfactorily. The course of treatment with chloramphenicol lasted 8 days, 4 g. being given in the first hour, followed by 0·25 g. two-hourly until temperature was normal, and then 4-hourly for the rest of the course. No toxic effects due to the drug were noted, and there was no evidence that *S. typhi* developed an increased resistance to the drug. From the bacteriological point of view the drug did not prove efficient as in 3 of the treated cases fæcal excretion of *S. typhi* which stopped temporarily during treatment started again either before or at the end of the course. S. L. W.

Chloramphenicol in Typhoid Fever. A. L. K. Rankin and A. S. Grimble. (*Lancet*, 1950, **258**, 615.) Chloramphenicol is effective in the treatment of typhoid if given in adequate dosage over a minimum period of 18 to 20 days, an initial dose of 4 g. being followed by 3 g. daily until the patient is apyrexial, then 1·5 g. daily for a week, and 1 g. daily for the final

week. The incidence of relapses is about the same with or without the drug. Thus, in this series of 17 cases there were 8 relapses, 4 among the 9 cases treated with chloramphenicol and 4 among the 8 cases not treated with the drug. In cases treated with chloramphenicol for 8 or 9 days *S. typhi* was isolated from the fæces and/or urine both during and after treatment, but in cases treated for 18 days *S. typhi* was not isolated from the fæces and/or urine after completion of the course. The average duration of fever in cases treated with chloramphenicol was 3 days whereas in controls it was 14½ days; there was very noticeable, and in some cases dramatic, improvement in the patients' condition within 48 to 72 hours. No toxic or side-effects were observed in any of the patients treated with chloramphenicol. S. L. W.

Chloramphenicol in Typhoid Fever. A. H. El Ramli. (*Lancet*, 1950, **258**, 618.) In a series of 200 cases treated with chloramphenicol the average duration of fever after commencement of treatment was 3·5 days, the relapse rate in patients followed up for at least three weeks was 27·5 per cent., and the mortality was 6·5 per cent. Relapses were fewest on 12-hourly doses. The response to the drug was little affected by the severity of the condition or duration of symptoms before treatment. Toxic effects observed, due to chloramphenicol, were anorexia and gastric upset, stomatitis and glossitis, and mental apathy. The best scheme of dosage appears to be 50 mg./kg. of body weight over the first 2 hours, and then 25 mg./kg. every 12 hours until the temperature becomes normal, and half that dose for 14 days in convalescence. Rest in bed and symptomatic treatment should continue, as before chloramphenicol therapy, for at least another week. Chloramphenicol provides the most efficient treatment for typhoid fever so far devised. S. L. W.

Digitalis, Differences in Standardisation of. E. Keeser. (*Arch. Pharm., Berl.*, 1950, **283**, 166.) Considerable differences were found between biological assays of digitalis preparations in different laboratories, although the results in both cases were compared with the international standards. In order to obtain uniform results, it is essential to specify details of the method of assay, and also of the preparation of the solution. It is well known that the rate of administration of the preparation is of importance, especially with slow-acting glucosides such as digitoxin. Exact definition of the end-point is essential. In the case of specialities produced by commercial firms, it is desirable that they should publish the essential features of the method of preparation and declare any additions. G. M.

Dinaphthalene Methane Silver Disulphonate, Trichomonicidal Action of. G. R. Sluming. (*Brit. med. J.*, 1950, **1**, 1116.) A 1 per cent. aqueous solution of this substance, known under the proprietary name of viacutan, was successfully employed in the treatment of 20 resistant cases of trichomonal vaginitis. The treatment consisted of 6 weekly paintings of the vagina with the solution without any preliminary cleaning, followed by insufflation of acetarsol compound powder in non-pregnant cases and acetarsol compound pessaries daily during the course of treatment. In none of the cases was a relapse reported within 6 months. Viacutan has *p*H 4·5 to 5 and contains a wetting agent to assist spreading and a water-soluble yellow dye to indicate the areas treated. It has the property of attaching itself to and penetrating animal tissue. It inhibits the growth of both Gram-positive and Gram-negative organisms in 24-hour broth cultures in a dilution of 1 in 16,000, and its activity is enhanced by the presence of blood, pus or serum.

In addition to its use in trichomonal vaginitis it may also be employed for pre-operative sterilisation of the skin in obstetrical and gynæcological operations.

S. L. W.

β-Naphthyldi-2-chloroethylamine (R48) in Leukæmia, Hodgkin's Disease and Allied Diseases. W. B. Matthews. (*Lancet,* 1950, **258,** 896.) The author reports on clinical trials with this substance on 17 patients. Five cases of Hodgkin's disease all showed some improvement, but the results were on the whole disappointing and were certainly no better than those to be expected with nitrogen mustard. Two cases of reticulosarcoma and 2 of acute leukæmia did not respond. Good remissions were obtained in 2 out of 3 cases of chronic myeloid leukæmia, and in 2 out of 4 cases of chronic lymphatic leukæmia. One case of polycythemia vera was still in remission a year after treatment. Gastric disturbances with R48 were less common and less severe than with nitrogen mustard, and the only other toxic effect was cystitis which occurred in one patient. The drug was given by mouth in tablets each containing 100 mg., which were chewed before being swallowed and taken after meals; the taste was inoffensive. The daily dose never exceeded 600 mg., and usually 300 to 400 mg. was given in divided doses. The duration of each course of treatment was determined by the response of the leucocyte count and varied within wide limits. In patients with a non-leukæmic blood picture treatment proceeded until the leucocyte count fell to about 3,000 per c.mm.; in chronic leukæmia the aim was to reduce the leucocyte count to about 20,000 per c.mm. On roughly comparable doses the average duration of a course in Hodgkin's disease was 48 days; in chronic myeloid leukæmia 45 days; and in chronic lymphatic leukæmia 20 days. The author concludes that the action of R48 is essentially similar to that of nitrogen mustard but slower and more easily controlled.

S. L. W.

Œstrogens, Artificial, Activity of, Determined by Experiments on Rats. G. L. M. Harmer and W. A. Broom. (*Lancet,* 1950, **258,** 850.) Using stilbœstrol as the reference substance, and giving it an arbitrary value of 100 for its potency by mouth and subcutaneously, the following relative values were obtained for other œstrogens in large scale tests with ovariectomised rats (the subcutaneous value is given in parentheses): hexœstrol 10 (66), dienœstrol 68 (28), potassium hexœstrol sulphate 7·3 (0·98), 7-methyl-bis-dehydrodoisynolic acid 500 (46), œstrone 2 (29), premarin 5 (8), ethinyl-œstradiol 18 (233) and divinyl stilbœstrol 103 (45). Premarin consisted of the naturally occurring œstrogens in their water-soluble form, expressed as sodium œstrone sulphate. The results agree with clinical findings for some of the substances but with others such as ethinylœstradiol and 7-methylbisdehydro-doisynolic acid there were large discrepancies. Such discrepancies are serious because they may lead to a clinically useful synthetic œstrogen being discarded because of unfavourable animal tests. It is also clear that clinical assay is still necessary before the value of a new œstrogen can be established and that there is an urgent need for an animal test which will give the same results as clinical tests.

G. R. K.

Procaine Penicillin. Choice of Preparation. R. W. Fairbrother and K. S. Daber. (*Brit. med. J.,* 1950, **1,** 1098.) Two different types of preparation, with a particle size of 20μ or less, were subjected to investigation, the main object of which was to determine the blood levels produced by a standard intramuscular injection of 1 ml. The preparations were:—Type A: procaine penicillin (300,000 units) in arachis oil with 2 per cent. w/v

aluminium monostearate, plus crystalline potassium penicillin G (100,000 units) in 1 ml. Type B: a stable aqueous suspension of procaine penicillin in a finely divided state and containing 400,000 units of procaine penicillin in 1 ml. With Type A preparation effective levels of penicillin were maintained for the 24-hour period in all of 28 individuals (15 out of 21 gave assayable levels after 48 hours) but some difficulty was experienced in administration owing to its viscosity. Type B was injected without difficulty and gave satisfactory levels for a 24-hour period in all of 40 cases. In the authors' view this is the most convenient preparation for general use, though Type A should be very useful when it is desirable to give the injection at longer intervals than 24 hours. S. L. W.

Procaine Penicillin in Oil, Histological Reactions to. P. Story. (*Brit. med. J.*, 1950, **1**, 1467.) This paper gives an account of the histological changes occurring in the deltoid muscles and adjacent lymph nodes of a patient who had received intramuscular injections of procaine penicillin in arachis oil. The preparation used contained 300,000 units of penicillin G in combination with 120 mg. of procaine in 1 ml. of oil. Examinations at necropsy showed that underneath the needle marks seen on the skin of each deltoid region was an area in the muscle about 6 x 3 cm. which was greasy to the touch and showed some free oil in its deepest part. No scarring of muscle was observed macroscopically but some minute cystic areas and one small hæmorrhage were observed. Histological examination of tissues from both deltoid muscles showed that the muscle was œdematous and the perimysium contained a large number of oily globules. This oily material was associated with a marked cellular reaction in the connective tissue and some degeneration of adjoining muscle bundles. The cellular reaction was composed mainly of small round cells and large mononuclear phagocytes with smaller numbers of eosinophils and a few multinucleate giant cells. In some places however eosinophils were particularly numerous and in areas immediately surrounding the oil globules large mononuclear and multinucleate phagocytes were predominant. Fat-bearing phagocytes were plentiful around the oil and in adjacent lymph nodes. S. L. W.

Progesterone and Anhydrohydroxyprogesterone. A Comparative Study of Oral Administration. W. Bickers. (*J. Lab. clin. Med.*, 1950, **35**, 265.) Patients who had had a bilateral oophorectomy or who had amenorrhœa secondary to functional ovarian failure received 4,000 I.U. of mixed natural œstrogens daily for 20 days. A progestational response was produced by subsequent oral treatment with progesterone or anhydrohydroxyprogesterone (120 mg. daily for 5 days). Secretory response in the endometrium was less with anhydrohydroxyprogesterone than with progesterone. The invagination of the glandular epithelium and migration of nuclei was similar for the two drugs but secretion in the gland lumen, œdema in the stroma and tendency towards decidua-like cell formation in the stroma occurred only with progesterone treatment. G. B.

Salicyl Derivatives, Renal Excretion of during Aspirin Therapy, Influence of Urinary *p*H on. W. S. Hoffman and C. Noble. (*J. Lab. clin. Med.*, 1950, **35**, 237.) When aspirin is administered, the renal clearance of free salicylate, calculated on the total plasma salicylate, is generally below 2 per cent. of the creatinine clearance. The true value, calculated on an estimate of the unbound plasma salicylate is 4 to 5 times higher. This represents about 20 per cent. of the total salicylate, the remainder appearing in the urine in conjugated forms. When sufficient sodium bicarbonate is adminis-

tered to render the urine alkaline the free salicylate clearance is increased 3 to 13 times, in proportion to the urinary *p*H. It is suggested that the greater part of the free salicylate in the glomerular filtrate is reabsorbed by the tubules if the urine is acid, but not if it is alkaline. Conjugated forms of salicylate occur only in traces in the plasma, and must be either produced in the renal tubules, or excreted by them. A modification of a method for estimating urinary free salicylate, salicylurate and salicyl glycuronides, based on the differential ease of extraction with ethylene dichloride and carbon tetrachloride, and on the ease of hydrolysis with boiling acid, is described.

G.B.

BACTERIOLOGY AND CLINICAL TESTS

Ethylene Oxide, for the Sterilisation of Hospital Equipment. S. Kaye. (*J. Lab. clin. Med.*, 1950, **35**, 823.) Bedding, books and papers known to be contaminated with *Streptococcus hœmolyticus, Bacillus globigii* and BCG organism were rendered completely sterile by treatment with ethylene oxide. A suitable method for the sterilisation of hospital blankets, etc., is as follows. The materials are placed in an autoclave which is then closed and evacuated. Carboxide (ethylene oxide, 10 per cent., in carbon dioxide) is introduced from a cylinder and allowed to remain in the autoclave overnight, after which it is removed by vacuum pump, and passing air through the material for about one hour. Woollen and other delicate fabrics are not harmed by this treatment. G. B.

Menstruum for Drying Organisms and Viruses. J. W. Hornibrook. (*J. Lab. clin. Med.*, 1950, **35**, 788.) A suitable suspending medium for the freeze-drying of organisms and viruses which are required to retain their viability may be prepared as follows. Dissolve 1·35 g. of potassium citrate (monohydrate), 2·45 g. of sodium citrate (dihydrate), 0·61 g. of potassium phosphate, 0·6 g. of magnesium chloride (hexahydrate), 1·0 g. of potassium carbonate (sesquihydrate) and 57·5 g. of lactose in 500 ml. of water, mix with a solution of 1·33 g. of anhydrous calcium chloride in 500 ml. of water, adjust to *p*H 7·0 by addition of lactic acid, and filter through a Berkfeld filter. This solution approximates in composition to milk dialysate. For cholera organisms, this medium is preferable to Naylor's solution, milk, broth or lactose solution, and the medium may also be used for drying yellow fever vaccine. The inclusion of cysteine does not improve the medium, but it is possible that some of the salts are superfluous. G. B.

Toxin Production in Aerated Cultures of *Corynebacterium Diphtheriæ*. F. F. Howatt and G. B. Reed. (*Canad. J. Res., Sect. E.* 1950, **28**, 23.) When *C. diphtheriæ* is grown in aerated cultures, toxin production occurs earlier than in pellicle cultures. The yield of toxin is increased, provided that a further quantity of fermentable carbohydrate is added to the aerated cultures. The following method of aerated culture was adopted. 200 ml. of Wadsworth and Wheelers' infusion free proteose peptone medium was used in a 1000-ml. flask. 5 hours after inoculation the flask was placed on a mechanical shaker to give aeration at the rate of 2 to 3 volumes of air per minute. A further amount of a solution of sodium lactate, maltose and glucose, equivalent to half the amount in the original medium was added after 24 to 36 hours' incubation. The toxin concentration reached a maximum in 60 hours, compared with 120 hours for non-aerated (pellicle) cultures.

G. B.

BOOK REVIEWS

ANATOMY OF THE DICOTYLEDONS, VOLUMES I AND II, by C. R. Metcalfe and L. Chalk. Pp. lxiv + 1.500 + Plates. Clarendon Press, Oxford. 1950. £6 6s.

For over half a century the standard work of reference on the anatomy of the Dicotyledons has been Solereder's *Systematic Anatomy of the Dicotyledons* published in 1899, the English translation by Boodle and Fritsch from the original German appearing in 1908. Drs. C. R. Metcalfe and L. Chalk have produced, in the *Anatomy of the Dicotyledons,* a work which may well become the standard work on this subject for the second half of the present century. This volume aims at providing a summary of our present knowledge of the anatomy of the vegetative organs of the Dicotyledons, it has been written mainly so as to emphasise the taxonomic and phylogenetic value of histology, thus perpetuating but also extending Solereder's chief aim. A large mass of new material has become available for study during the past forty years which has enabled generalisations to be based on a surer foundation and has provided the means of filling many of the gaps in Solereder's work; studies of the secondary wood have also made extensive progress in recent years and much information of value to wood anatomists has been added. This, as the successor to Solereder's classic work, is therefore in effect a new book providing a comprehensive survey of the anatomical characters of leaf, axis and wood of dicotyledonous plants in relation to taxonomy. Some attention has also been given by the authors to ecological and developmental anatomy and to the economic uses of plant materials, thus providing a mine of useful information now made accessible to all who have to deal with plant products of economic importance and with their microscopical structure.

The choice of anatomical characters which may be used to indicate taxonomic affinity is fraught with difficulties, for a similar anatomical response may arise in the members of unrelated groups which are found in the same ecological environment, whilst, on the other hand, some diagnostic characters of no ecological significance have arisen independently in unrelated families. Bearing in mind these factors, the authors have drawn up a list of those characters whose taxonomic value has become well established. These include the hairs, stomata, epidermal cells, veins, petiole, cell contents, cork, endodermis, "pericyclic" sclerenchyma, medullary rays, abnormal bundles, and characters of the wood. It is suggested that the following more descriptive terms be used for the four main types of dicotyledonous stomata:—type A—the "ranunculaceous" or anomocytic (irregular-celled) type: type B—the "cruciferous" or anisocytic (unequal-celled) type: type C—the "rubiaceous" or paracytic (parallel-celled) type: type D —the "caryophyllaceous" or diacytic (cross-celled) type. Since the work is concerned only with the vegetative organs, the anatomical characters of flowers, fruits and seeds are omitted. The anatomy of fruits and seeds has, however, been adequately summarised by Netolitzky.

In order to show the taxonomic significance of anatomical characters the main work is arranged systematically as a description of each family, using a modification of the system of classification described by Bentham and Hooker

in their *Genera Plantarum*. The monograph on each family indicates the range of anatomical characters observed in the different genera and the descriptions have been arranged under the general plan of Summary (general, wood); Leaf; Axis (stem, wood, root, anomalous structure); Taxonomic notes; Economic uses; Genera described; Literature. The text is freely illustrated, some of the diagrams are from the original Solereder but the greater number are new figures drawn according to a definite plan and employing throughout a uniform system of shading to indicate the different tissues. The work is based upon the examination of a very wide range of materials and of prepared slides fom all parts of the world and no large groups of known dicotyledonous plants are omitted from this monumental compilation. The authors and their assistants are to be complimented upon the broad concept upon which the work is based and upon the authoritative anatomical evidence presented with such lucidity. No attempt has been made to over-stress the taxonomic notes for each family, although many valid suggestions are made. The genus *Krameria* is raised to the status of a separate family Krameriaceæ distinct from either the Polygalaceæ or the Leguminosæ-Cæsalpiniaceæ. The phylogenetic implications based upon a study of anatomy are fully discussed and these support the view that angiosperms may be polyphyletic in their evolution from the Cretaceous period, some phyla only being derived from a Magnolian or allied ancestor.

Although the authors have made use of anatomical characters to provide evidence of relationship between families or genera rather than to distinguish between species or groups of less than specific rank, they have also produced much evidence for the use of microscopical methods in the identification of individual species. To pharmacognosists or public analysts who are faced with the problems of identifying unknown samples, this work will provide much help. An examination of such material will allow it to be assigned to its correct family, by reference to thirty-six pages of tables which contain lists of families in which certain diagnostic features occur; these include all the diagnostic characters employed in the microscopical characterisation of leaf, stem, wood or root as well as the aids to diagnosis provided by plant habit and geographical distribution. From these tables one must turn to the description of families for further identification of genus or species and since these sections are not primarily designed for this purpose the work is more difficult. We have here no complete or artificial key for the identification of leaf, stem, wood or root of every dicotyledonous plant, we have however a most comprehensive description of the anatomical characters of the Dicotyledons. Detailed pharmacognostical studies have produced much anatomical information which is recorded in this treatise, although much of the work of the last six years is only included by references in the list of the literature.

The two volumes, extending to 1,500 pages, are well produced, the diagrams and plates are clear and distinct whilst the text appears to be almost completely free from typographical errors and the work is fully indexed. A most useful character of the book is a bibliography of 2,535 references covering the period 1908 to present date; no references given by Solereder in the English translation of 1908 are quoted and thus the two publications provide a complete bibliography of the anatomy of the Dicotyledons. To pharmacognosists and to all others concerned with plant anatomy this publication is indispensable.

J. M. ROWSON.

LETTERS TO THE EDITOR

Stability of Solutions of Adrenaline Tartrate

SIR,—The stability of solutions of adrenaline in hydrochloric acid, in tartaric acid, and in ascorbic acid was investigated in previous studies[1,2,3,4,5]. Based chiefly on the results of short-term heating experiments, the optimal conditions of preparation and storage were suggested. The opportunity for investigating the stability of solutions of adrenaline in varying amounts of tartaric acid and 0·1 per cent. sodium metabisulphite after long-term storage has now arisen, and results clearly confirm the conditions previously stated and now incorporated in the British Pharmacopœia, 1948. Nearly all the samples were prepared and sterilised in 1944, and since that time have been retained in ampoules in the dark at room temperature. They were all colourless. For activity, they were compared against a fresh standard adrenaline solution (1 in 1000 of base) by the method of Burn, Hutcheon and Parker[6] which utilises the simultaneous recording of contractions of the normal nictitating membrane and the rise of blood pressure of a spinal cat. In many cases relaxation of the non-pregnant uterus of the cat was also measured, this being a very sensitive test for adrenaline activity. The results shown in the accompanying Table indicate that the optimal *p*H value is about 3·6, similar to that found by taking a solution of adrenaline acid tartrate containing sodium metabisulphite (0·1 per cent.). Solutions of the latter mixture stored for 6 years in vaccine bottles after sterilisation (115°C. for 30 minutes) showed little loss of activity. From the point of view of both long-term and short-term experiments, the Pharmacopœial conditions of preparation and storage for injection of adrenaline tartrate are adequate. Loss of activity will occur if the containers are not full or nearly full when sterilized.

THE EFFECT OF STORAGE FOR 6 YEARS ON THE PHYSIOLOGICAL ACTIVITY OF TREATED SOLUTIONS OF ADRENALINE IN TARTARIC ACID CONTAINED IN FULL 2 ML. AMPOULES. (EXPRESSED AS PERCENTAGE ACTIVITY COMPARED WITH FRESH STANDARD ADRENALINE SOLUTION.)

Original *p*H of solution	Treatment before storage			
	Untreated	115°C.		
		30 minutes	3 hr.	6 hr.
3·0	91	87	81	81
3·6	95	91	91	87
4·2	91	87	81	74

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