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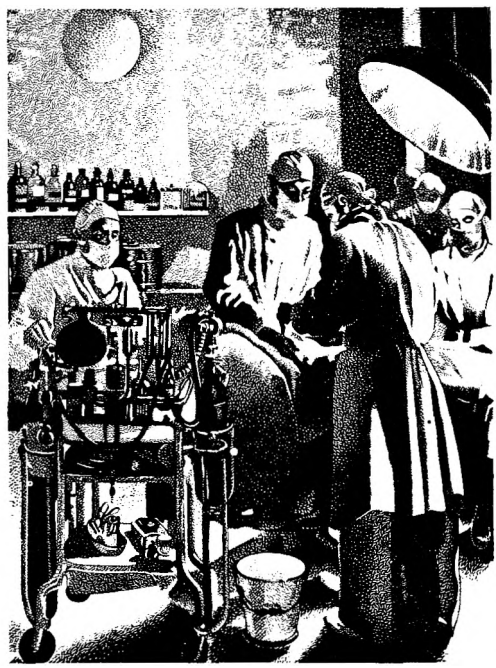

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REVIEW ARTICLE

THE INTERNATIONAL STANDARD FOR VITAMIN D

By KATHARINE H. COWARD, D.Sc.

Head of the Nutrition Department, School of Pharmacy, University of London

THE International Standards for the determination of the different vitamins are part of the indispensable equipment of the laboratories in which this work is carried out. Though the standard materials themselves are so well known, the amount of labour which has been devoted to their creation and adoption and the infinite care which has been taken to ensure their suitability, accessibility and integrity are not so well known or appreciated. The adoption of a crystalline preparation of vitamin D_3 to serve as the International Standard for Vitamin D affords a suitable opportunity for outlining the nature of this work, the importance of which to scientific research, medical practice, the problems of nutrition, and the international exchange of ideas, results and materials is now well recognised.

In this country the Therapeutic Substances Act (1925) was passed to deal with the administrative control of those substances of therapeutic value, the potency of which could not be adequately tested by chemical and physical means. Such substances are of many different kinds and, since their potency could only be determined by biological tests carried out in strict comparison with a standard preparation, the necessity for these was fully recognised; and, since the same materials and medicaments are used in many lands, it was obvious that the standards for their measurement should have an International standing and application. International Standards for the assay of antitoxins, insulin, pituitary (posterior lobe) powder and digitalis had been established before the similar work for vitamins was begun. Very few firms, however, were equipped to carry out the necessary biological determinations on their products of therapeutic application. Accordingly, to meet the demand for such tests, the Pharmaceutical Society of Great Britain established its Pharmacological Laboratory in January, 1926. Within a very few months requests for the determination of vitamin D in cod-liver oils were made by firms who realised that the vitamin D content of these oils varied within wide limits and who knew also that it was possible to extract the vitamin D of the oil without detection of the loss, and the Pharmaceutical Society after consulting the Accessory Food Factors Committee of the Medical Research Council set up a Vitamin-testing Department within the Pharmacological Department. The writer of this review was appointed to take charge of it and in January, 1927, the available laboratories had been put into a condition suitable for the satisfactory performance of the tests. Actually, the task of devising a reasonable uniform diet for albino rats—on which they would grow, reproduce and lactate and, moreover, would yield a progeny having minimum reserves of vitamins A and D, so that they could be used at an early age for the comparative tests—proved

to be of paramount importance and has so remained throughout the years. The account of the work in this field is described elsewhere[1]. Suffice to say here that the diet found to be satisfactory by 1928 has, with only minor modifications such as the exigencies of the times demanded, proved to be satisfactory up to the present time.

Up to the time of setting up this laboratory, tests had been really only qualitative, and the relative vitamin potencies of foods indicated by the signs +, ++, +++, or ++++, according to the amounts, roughly, of the different foods required for bringing a certain amount of reaction which was known to be extremely variable and which could only be controlled by comparative tests in relation to Standard preparations; and it was not until 1931 that the extremely important step of establishing International Standards for the assay of vitamins A, B_1, C and D was taken by the Permanent Standards Commission of the Health Organisation of the League of Nations[2].

By 1926 certain criteria for measuring the response of an animal to vitamin D were already known and considered to be adaptable for use as the basis for quantitative assay; of these, the ash content of the bone (generally expressed as the percentage ash of the dry, fat-extracted bone) had been used in various laboratories for comparing different cod-liver oils; and the " line test " had been used for the same purpose and, particularly in Steenbock's laboratory, for following the antirachitic activation of foods, food-constituents and cholesterol by irradiation with ultra-violet rays. In his laboratory the amount of healing, indicated by the width of the line of calcification, was assessed in five grades, 0, +, ++, +++, ++++, the last indicating complete healing. It was decided to adopt the " line test " in our own laboratory as it was quicker and less laborious than the " ash content of the bone " method. While we were raising our colony of rats to the right condition for these tests, we used litters drawn partly from our own colony and partly from those of other laboratories. The inconvenience of this was offset by the value of the information it gave on how greatly rats obtained from different colonies and subjected to the same treatment may differ in their response to the same dose of vitamin D. Ample evidence was also obtained of the difference in response of different litters from the same colony. All this afforded proof—if any were needed—of the necessity to provide for the comparative tests some stable preparation of vitamin D which could be used as a standard of reference and tested simultaneously with every vitamin D determination.

About this time Rosenheim and Webster[3] were irradiating ergosterol with ultra-violet light and getting intensely active antirachitic material as shown by tests on rats very similar to the line test, the width of the line of healing in their experiments being measured by X-ray examination of the bones. They had determined that a certain degree of activity could be produced in a solution of ergosterol by irradiating it for a certain time, that further irradiation for some time did not increase or decrease the activity but that finally the activity did fall to zero. They were therefore able to undertake to make a solution of activated ergosterol, and by

using the same conditions of irradiation, strength of solution, etc., they could reasonably expect to make a similar solution of equal potency at any future time. Such a preparation would be very nearly ideal for a standard of reference, the only point left to be determined being the stability of the preparation. This could only be done in the course of time or by expedited experiments in which a solution was subjected to more stringent conditions of temperature, etc., than it was likely to encounter during its normal use. Such experiments were carried out and no loss of activity found[4]. In February, 1927, a particular sample of irradiated ergosterol in olive oil, containing the equivalent of 0·0001 mg. of the original ergosterol in 1·0 mg. of oil, was prepared by Mr. T. A. Webster of the Rosenheim and Webster team at the National Institute for Medical Research, London and adopted by the Pharmaceutical Society as its standard of reference. The unit of activity adopted was that contained in 1 mg. of this oily solution. This was the first standard of reference for any vitamin to be adopted in any country[5].

The first way in which the standard was used was generally to give to three rats of a litter daily doses of, say, 0·00001, 0·00002, and 0·00005 mg. of original sterol (i.e., 0·1, 0·2, 0·5 units) respectively, and to three other rats of the same litter doses of the oil under test in the same proportions. Comparisons were then made between the responses of rats on corresponding doses, and an estimate of potency made by the consideration of all the possible comparisons without the litter. Several litters were used in exactly the same way and an average of the several estimates calculated.

Later, in 1931, Dyer[6] worked out a method of making the estimation more quantitative. He selected a series of cut and stained bones of rats which had been used in "line tests" which showed graded stages in healing, the widths of the lines chosen being as nearly proportional to the figures 0 – 6 as could be judged by the naked eye, 0 representing no healing and 6, complete healing. Thus it became possible to assign a numerical value to the healing of each rat and then total and average the healing of any number of rats all of which had received the same dose. He then constructed a curve of response to graded doses of vitamin D. 15 litters of 7 rats each were prepared in the usual way. In each litter different rats received the following daily doses of vitamin D:— no dose, 0·0625, 0·125, 0·250, 0·5, 1·0, 2·0 units. The responses of the 15 rats receiving each dose were averaged and plotted against the dose given. The curve of response was used in future tests to compare the average response of a group of rats receiving one dose of, say, cod-liver oil with that of another group receiving one dose of the standard, by finding the abscissa of the curve corresponding with the healing of each group and calculating the potency from the respective abscissae. Further work, however, showed that the curve of response to vitamin D was logarithmic in shape, whether the ash content of the bone or the line of healing was the criterion used. The slope varied from time to time and, accordingly the method of testing 2 doses of Standard against 2 doses of test substance was adopted, the average slope of the 2 curves thus obtained in each

assay being used for the calculation of potency and of error of that assay.

Meanwhile, the need of a standard of reference for vitamin D was being recognised in other laboratories and in other countries. It was obviously desirable that workers in different laboratories should use the same standard and unit of activity. A second larger batch of irradiated ergosterol in olive oil had been prepared in 1928 at the National Institute for Medical Research under conditions similar to those of the first batch, part being kept at -4° to 0°C. and part at room temperature. A further large batch was prepared early in 1929 and extensive biological comparisons made between it and the second and first batches. It proved to have the same activity as the first batch and also the same as the part of the second batch which had been kept at the very low temperature. The part of the second batch which had been kept at room temperature, however, was very much less active than the third batch and a direct comparison between the two parts of the second batch confirmed the loss of activity of the part kept at room temperature. The Medical Research Council[7] therefore made available a standard of reference of vitamin D and recommended its adoption. It also recommended that the unit of activity should be defined as the antirachitic potency of a quantity of this preparation corresponding to 0·0001 mg. of the ergosterol used in its production. Thus the unit, as far as biological tests could determine, had the same value as the one already used for some years in the Pharmaceutical Society's laboratory. It was of convenient size for laboratory use on test animals; that is to say, the doses required for tests were neither a large multiple nor a small fraction of the unit proposed. The solution for distribution was made up to contain 1,000 units/g. It was ready for issue the following September, and a memorandum giving details for the use of the standard was sent out with the first issue to each laboratory proposing to use it. Thereafter, fresh supplies were sent out every 6 months. Still another large preparation of irradiated ergosterol was made in 1931 and by biological tests it was shown to be indistinguishable in antirachitic activity from the previous one.

The need for standards of reference for other vitamins also had become urgent, not only in Great Britain but also in other countries. In 1931 a conference on Vitamin Standards[2] was held in London at the invitation of the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations and standards of reference were adopted for International use for vitamins A, B_1, C and D.

The 1931 Conference recommended that the Standard solution of irradiated ergosterol at that time issued from the National Institute for Medical Research, London, should be adopted as International Vitamin D Standard for the next two years. If, within that period, it should become necessary, owing to exhaustion of the supply, to replace that solution by a fresh standard, the equivalence should be determined by experts of different countries. It also recommended for adoption as international

unit of vitamin D, the activity of 1 mg. of the International Standard solution of irradiated ergosterol.

It was hoped at that time that a crystalline substance more stable than the solution of irradiated ergosterol was expected to be would soon become available. Bourdillon in this country, Windaus in Germany and Reerink and van Wijk in Holland had each prepared a crystallised substance which they thought was pure vitamin D. The three substances, however, differed in certain physical properties and each worker realised that his product was not pure. Callow purified Bourdillon's preparation by treating it with 3-5-dinitrobenzoyl chloride in pyridine and crystallising the product from acetone. Close examination revealed the fact that the product consisted of two kinds of crystals, one yellow and the other orange. Callow separated them into two groups by means of a pin and obtained 50 mg. of the yellow and 40 mg. of the orange form. He hydrolysed each and found one to be inactive biologically and the other active. He gave the name pyrocalciferol to the inactive form and retained the original name of the mixture, calciferol, for the active substance, which had about twice the antirachitic activity of the original. Meanwhile Windaus showed that his original product (which he now called D_1), really consisted of a molecular compound of lumisterol and a substance which he called D_2. This he sent to Hampstead for comparison with Bourdillon's calciferol and it proved to have the same potency as that substance and the same physical properties. Windaus and Bourdillon concluded that they had at last arrived at the same substance. It has since been called calciferol or vitamin D_2. It is now made on a small manufacturing scale and has been available for some years to replace the standard preparation of irradiated ergosterol, as recommended by the Second International Conference on Vitamin Standardisation. This has, however, never been necessary except on one occasion during the War when the dilution of the Standard was made with an oil which rapidly inactivated it. Reports from various workers that the standard did not appear to have its usual activity resulted in an immediate comparison between that issue and the remains of previous issues in several laboratories. It showed beyond question that that issue was not " up to Standard " and a solution of pure calciferol was made up with a carefully chosen oil and compared by several workers with a fresh dilution of the standard made with the fresh oil. The two solutions proved to be of equal potency and the activity of the original solution has never since been doubted.

The Second International Conference on Vitamin Standardisation[8] held by the Permanent Commission on Biological Standardisation of the League of Nations in London, 1934, recommended that the Standard Solution of irradiated ergosterol prepared at the National Institute for Medical Research, London, and issued as International Vitamin D Standard, should be retained. It also recommended that when the present International solution was exhausted, or if it should become unsatisfactory for any reason, it should be replaced by an equivalent solution of pure crystalline vitamin D in olive oil of such strength that 1 mg.

contained 0·025 μg. of crystalline vitamin D, a definition for which was added. The unit recommended for adoption by the 1931 Conference was to remain unaltered: namely, the vitamin D activity of 1 mg. of the International Standard solution of irradiated ergosterol which had been found equal to that of 0·025 μg. of crystalline vitamin D.

By this time it was becoming more and more evident that irradiated ergosterol had very little antirachitic activity for chicks; hence a determination of the vitamin D potency of a cod-liver oil by comparison with the standard by means of tests on chicks would give a very much larger value for the oil than a similar test carried out on rats. Therefore, the Conference stated that the assay of materials for vitamin D content should be carried out by comparative tests on rats, and their value in International units should be derived from the results of such tests. If other species were employed for these tests, the values could not be expressed in International units.

With regard to the method of determination, the 1931 Conference considered it permissible to use various biological methods of estimation, either prophylactic or therapeutic, e.g., the "line-test," X-ray examination or determination of the bone ash, provided that not less than (and preferably more than) 20 rats were used, one half receiving doses of the Standard and the other receiving doses of the substance under test. The Conference did not consider it desirable to draft detailed procedures for the determinations. Indeed, Dr. R. Gautier[9], now Assistant Director-General to the World Health Organisation and in 1945 Secretary to the Permanent Commission on Biological Standardisation of the Health Organisation of the League of Nations, wrote at that time (with regard to all biological assays), "When the value of an international unit has thus been given an agreed definition, it has been recognised that the nature and details of the biological method used in making the comparative measurements in units should be left, so far as possible, to the free choice and judgment of the expert conducting the test. Each worker is likely to make the most accurate assays when using a method with which experience and opportunity have made him familiar. Freedom of choice in this matter also affords an incentive to researches aiming at the improvement of existing methods and the discovery of better ones, whereas the adoption of a single method by agreement and the standardisation of its details must tend to stereotype knowledge and hinder progress in this field. There are certain conditions, however, by which the choice of a method of biological measurement must be guided. It should, of course, be capable of yielding results of the greatest practicable precision; but the fact is apt to be overlooked that the most accurately reproducible results are of doubtful value, and may even be misleading, unless it is certain that the method is measuring the therapeutically important constituent of the product under test. In a case where the assay can be assumed to be dealing with a single and uniform active principle, the position is simple. Any biological

test will then be acceptable if it will give an accurate comparative measure of the quantity or proportion of that principle; and provided it so measures the right thing, and measures it accurately enough, the activity on which the measurement depends need have no more relation to the therapeutic action than would the property forming the basis of a chemical determination of the active principle, if such were applicable. The position becomes at once more complicated when several active principles are in question, which may be present in varying proportions, and concerning the relative therapeutic importance of which there is no sufficient ground for decision or differentiation. . . ."

The Second Conference on the Standardisation of Vitamins held in 1934 recommended that "the experience gained in the use of cod-liver oils standardised against the International Standard by means of tests on rats might be devoted to an attempt to elucidate the questions involved in the anomalous action on certain species of different sources of vitamin D." It was Waddell[10] who first threw light on this subject. Reverting to the antirachitic substance generated in cholesterol on irradiation, he found it to be at least as active as the vitamin D of cod-liver oil in the prevention of rickets in chicks, the respective doses being determined by tests on rats, and he concluded that the pro-vitamin D of cholesterol was not ergosterol. Work by Windaus, Lettre and Schenck, Grab, Brockmann, Brockmann and Busse led to the isolation of a substance from the products of the irradiation of 7-dehydrocholesterol and its identification with the vitamin D of tunny-liver oil and halibut-liver oil. This substance was named vitamin D_3 and it was reported to have the same antirachitic potency (40,000 I.U./mg.) for rats as calciferol. A collaborative test to compare the antirachitic potencies of calciferol (vitamin D_2) and vitamin D_3 was organised by the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute.[11]. Workers in 9 different laboratories in Great Britain made the comparison by means of tests on rats, and the relative potencies of the two preparations, as indicated by the different workers, were all so nearly equal to unity that statistical analysis of the result was considered unnecessary. Vitamin D_3 was declared to contain 40,000,000 International units of Vitamin D per gram. Moreover, a comparison of these two preparations on the healing of rickets in children carried out by Morris and Stephenson in Glasgow, on cases of osteomalacia and late rickets by Wilson in India and on a case of parathyroid tetany by Himsworth and Maizels in London failed to show any difference in antirachitic potency and, indeed, offered a certain amount of positive evidence that the two substances were equally potent for human beings. Various other workers had shown that the vitamin D of cod-liver oil (whatever might be its nature) and calciferol were equally potent for human beings. Thus it was possible to adopt vitamin D_3 as a standard of reference for determining the potency of oils intended for rats, chicks or human beings. The possibility of its adoption was to have been included in the Agenda of the Third International Conference

on Vitamin Standardisation planned for September, 1939, but not held at that time on account of the outbreak of war.

Meanwhile the British Standards Institution had adopted a particular sample of vitamin D_3 as a Provisional Standard Preparation for assaying the vitamin D potency of cod-liver oils intended for chick feeding. It was dissolved in pure vitamin D-free olive oil; 1 mg. of the solution contained 0·000025 mg. of the crystalline vitamin, and this amount was adopted as the B.S.I. unit of antirachitic activity. Since rats appear to use vitamin D_2 and vitamin D_3 equally well, 1 unit of the B.S.I. Standard was equivalent to 1 International unit as far as rats were concerned. It was obvious, however, that a cod-liver oil intended for chick feeding must be assayed by tests on chicks, for a cod-liver oil containing added calciferol would have a high value according to tests on rats, but would be less potent when given to chicks. The British Standards Institution drew up a plan[12] for determining the vitamin D content of cod-liver oils by using chicks as test animals and the ash content of the bone as criterion. An example worked out for potency and approximate and fiducial errors was added. The result of such a test, however, could only be expressed in B.S.I. units since the oil was compared with the B.S.I. Standard.

The work carried out on this B.S.I. Standard in many laboratories in Britain, Canada and the U.S.A. proved of the greatest value and paved the way towards the adoption of an International Standard of vitamin D_3. As soon as the World Health Organisation was formed, the International Standards for vitamin A and vitamin D came under review. The story of the vitamin A standard will be told later. Work for this vitamin D standard began in 1946. The Accessory Food Factors Committee of the Medical Research Council and Lister Institute organised, through its vitamin D sub-committee, a large collaborative experiment to investigate the possibility of adopting vitamin D_3 as the International Standard of reference for vitamin D. Several manufacturers were making crystalline vitamin D_3, and samples of this substance were generously contributed by them. The total weight contributed was about 29 g. The physical constants of each sample were determined. The samples were pooled, and the physical constants of the pooled sample were determined. The solution for issue was prepared at the National Institute for Medical Research, London, and was of the same strength as that issued by the B.S.I., viz., 0·000025 mg. in 1 mg. of oil. It was decided at a meeting of the Vitamin D sub-committee to compare the following preparations:—

1. The present International Standard for vitamin D (irradiated ergosterol in olive oil).
2. The new preparation of pooled samples of vitamin D_3.
3. The British Standards Institution Standard for vitamin D_3.
4. A preparation of the purest sample of calciferol obtainable.

The Committee invited laboratories in as many countries as possible to take part in the comparative tests. Eventually workers in Great

Britain, Denmark, Holland, Norway, Sweden, New Zealand, Canada and the U.S.A. did so. It was impossible to find experts able to carry out the comparisons in France, Spain, Portugal, India or South Africa. A most complete and exhaustive series of comparative tests was carried out and investigators in 33 laboratories sent in reports, some concerning tests with chicks, some with rats, some with both. Some even sent in duplicate tests with rats, one to compare results obtained with albino and piebald rats respectively.

The British participants and Dr. J. O. Irwin met to arrange the design of the experiment, and a scheme put forward by N. T. Gridgeman was adopted. It entailed randomisation of litters between doses (x, 2x, 4x) and randomisation of rats within each litter between the four samples. Thus all rats of any one litter had the same size of dose, but of different preparations. Altogether there were to be at least 10 litters on each dose, i.e., at least 30 litters in all, comprising at least 120 rats. Details of procedure (diet, criterion, duration of test, etc.) were left to individual workers. The work in the U.S.A. and Canada was organised by the U.S.P. Committee, who accepted the design planned by the British workers and issued certain instructions to their participants to accord with their own experience. In addition, they issued 2 bottles of cod-liver oil, 1 to be assayed on the assumption that it contained 115 U.S.P. units (International units)/g. and the other as 96 U.S.P. units (International units)/g. Actually these were one and the same oil, but it seemed a good opportunity for investigating the possible influence of "level" of testing on the result obtained.

In all, 54 assays were completed, 29 using rats and 25 using chicks. Dr. J. O. Irwin and his colleagues calculated the results of each test and its fiducial limits of accuracy and the ratios of the potencies (with their fiducial limits) of the various preparations. Their summary[13] is as follows:—

	Result	Fiducial Limits (P = ·95)
(a) New Standard/Old Standard	0·981	0·925 to 1·009
(b) B.S.I. Standard/Old Standard	0·916	0·902 to 0·983
(c) Calciferol/Old Standard	0·933	0·896 to 0·972
(d) U.S.P. Ref. C.L.O./Old Standard...	0·869	0·839 to 1·004
(e) New Standard/B.S.I. Standard	1·071	1·036 to 1·098
(f) U.S.P. Ref. C.L.O./B.S.I. Standard	0·949	0·843 to 0·958

Irwin says, "Thus the B.S.I. Standard and the calciferol are less potent than the Old Standard and the New Standard may be slightly so. The new standard is somewhat more potent than the B.S.I. Standard, while the calciferol and B.S.I. Standard do not differ significantly. The U.S.P. Reference Oil is less potent than the B.S.I. Standard. This could not be concluded from the rat assays only, and so may be due to the presence of a little vitamin D_2 in the oil. The results are remarkably uniform. For (a), (b), (c), the fiducial range is less than 10 per cent., for (e) it is about 6 per cent., for (d) and (f) it is rather greater, but still of a satisfactory order, for a biological test."

A Sub-Committee on Fat-soluble Vitamins of the Expert Committee on Biological Standardisation of the World Health Organisation held a meeting in London in 1949[13], and recommended that the preparation of crystalline vitamin D_3, as described below, at present held at the National Institute for Medical Research, London, should be adopted as the International Standard for vitamin D. This new standard should replace the existing solution of irradiated ergosterol and the latter should be retained as a reference preparation only and not as an International Standard. The Committee also recommended that the International Unit of vitamin D should be the vitamin D activity of 0·025 μg. of the International Standard preparation of crystalline vitamin D_3. The Standard should be issued as a solution containing 1000 I.U. per gram. The properties of the recrystallised vitamin D_3 freshly withdrawn from a sealed ampoule were determined as:—

m.pt. 87° to 89°C. (corr.)

$[\alpha]_D^{20°C.} = +110°$ (ethanol)

$E\ {}^{1\ \text{per cent.}}_{1\ \text{cm.}}$ 265 mμ = 490 (ethanol) corresponding to a molecular extinction coefficient of 18,800.

The recommendations of the sub-committee were adopted by the General Assembly of the World Health Organisation held in Rome in June, 1949.

Step by step with the growing use of international standards of reference for assaying the biological potency of therapeutic substances has been the adaptation of existing methods and development of further methods and refinements of methods for estimating the accuracy of assays. The first edition of Fisher's "Statistical Methods for Research Workers" was published in 1925. It has now reached its tenth edition; several other writers have published somewhat similar books and a few mathematicians, such as J. O. Irwin, E. C. Fieller, D. J. Finney and C. I. Bliss, have applied themselves to original investigations in this subject. Biologists have followed the trail these pioneers have blazed and now they know just how much as well as how little faith they may place in their results. The one is quite as important as the other.

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BRITISH PHARMACEUTICAL CONFERENCE BLACKPOOL, 1949

RESEARCH PAPERS

PENICILLIN FORMULATIONS: THE EFFICACY OF OILY INJECTIONS

By J. C. Floyd

From the Pharmaceutical Research and Service Laboratories, Imperial Chemical Industries, Ltd., Blackley, Manchester, 9

Received July 1, 1949

The rapid excretion of penicillin when administered parenterally in aqueous solution has led to the development of several non-aqueous formulations designed to prolong the therapeutic blood level after injection. The object of "slow-release" is two-fold; firstly to eliminate the inconvenience and pain to the patient of frequent injections and secondly to avoid wastage of penicillin. Its adoption is based on the now generally accepted principle that a continuous therapeutic level is more effective than intermittent "peaks." The three main principles so far employed in such preparations are: (a) the suspending of penicillin in vegetable oil, usually in presence of a thickening or dispersing agent, (b) the utilisation in these suspensions of penicillin salts which are only sparingly soluble in water, and (c) the control of particle size of the suspended penicillin. The first slow-release formulation was devised by Romansky[1], who suspended a water-soluble salt of penicillin in arachis oil containing 4·8 per cent. of beeswax. Numerous clinical reports have shown that this preparation achieves therapeutic levels (>0·03 u./ml.) for periods up to 24 hours after injection. Later, the preparation of sparingly soluble salts of penicillin containing either heavy-metal or organic bases was followed by their trial in slow-release formulations. Of these, the most effective so far has been procaine penicillin. Clinical reports on preparations containing procaine penicillin suspended in untreated arachis oil were made by Herrell, Nichols and Heilmann[2], and by Hobby Brown and Patelski[3], both claiming therapeutic levels for periods greater than 24 hours. At about the same time, attention was drawn to the significance of the particle size of the suspended penicillin by Romansky and Dowling[4], who reported that in oil/wax suspensions of soluble salts the best results were obtained when the particle size of the latter exceeded 50μ. This view was supported by Sullivan[5], who used large particle size procaine penicillin in untreated arachis oil. Shortly after the introduction of procaine penicillin Buckwalter and Dickison[6] developed a new technique of suspending penicillin by using an aluminium stearate/arachis oil gel instead of oil/wax or untreated oil. Clinical reports on the new suspensions were made by Thomas and his co-

workers[7], who obtained therapeutic levels for as long as 96 hours but who simultaneously showed that small particle size penicillin was more effective than large in stearate/oil suspensions. Beyond relatively simple theories such as "water-proofing" of penicillin, no satisfactory explanation of the mechanism of slow-release has been advanced. A survey of earlier work also shows that not all the variants of the slow release formulations have a common basis for comparison. This is largely due to the fact that clinical and laboratory evaluation has been by a variety of techniques. The object of the work described here was to compare as accurately as possible the efficacy of a series of preparations embodying the most important of the possible variants, viz. (a) soluble and sparingly soluble penicillin (potassium and procaine salts), (b) gelling and suspending agents (beeswax and aluminium stearate), and (c) particle size of penicillin. To permit comparison of a large number of variants with sufficient accuracy it was decided to adopt a laboratory animal test for evaluation, and to use statistical methods for the efficient design of the experiments and analysis of the results.

Experimental

This consisted essentially of two groups of tests described here as Experiments 1 and 2. In both experiments the orders of preparation of samples, dosing and inoculation were randomised to ensure that any extraneous non-random variation would not vitiate the comparison between the main factors of the experiments. The following standard techniques were used:—

(1) *Biological Evaluation.* Three groups, each of 10 mice, were injected subcutaneously with the test preparation, each mouse receiving 30,000 units in 0·1 ml. The mice were then infected intraperitoneally with a 24-hour culture of *Streptococcus pyogenes* Krüger strain. All three groups were infected simultaneously, but the test preparation was injected in advance at different time intervals for the three groups. The usual plan was to inject the first group 5 days before infection, the second 3 days, and the third 2 days. The number of deaths occurring up to 72 hours after infection was recorded for each group. A similar test has been used by Miller[8]. In general the number of deaths increased with the time interval between dosage and infection and the criterion used for comparing the effectiveness of the different formulations studied here was the estimated time between dosage and subsequent infection required to produce a 50 per cent. mortality in the mice. The greater this time the more effective the preparation.

(2) *Determination of Penicillin Potency of Test Preparation.* The penicillin salts were assayed both microbiologically (*Staphylococcus aureus*) and iodimetrically[9]. Suspensions were assayed iodimetrically only and the results interpreted by means of a factor obtained in the assay of the penicillin salt.

(3) *Control and Measurement of Particle Size of Penicillin.* Three ranges of particle size were selected for testing and are described here

arbitrarily as "coarse," "medium," and "fine." Reduction to appropriate particle size was effected by dry grinding, followed, where necessary, by grinding in oil (with subsequent removal of oil before incorporation in the arachis oil base). Representative particle size analyses are expressed graphically in Figure 1, the measurements being made using a technique based on that of Fairs[10]. The graph expresses the relationship between the ranges and shows that the distribution in each range does not exhibit any unusual feature.

(4) *Preparation of Base.* (i) *Stearate/Arachis Oil*: Four methods were used, differing essentially in the temperatures at which the oil and stearate

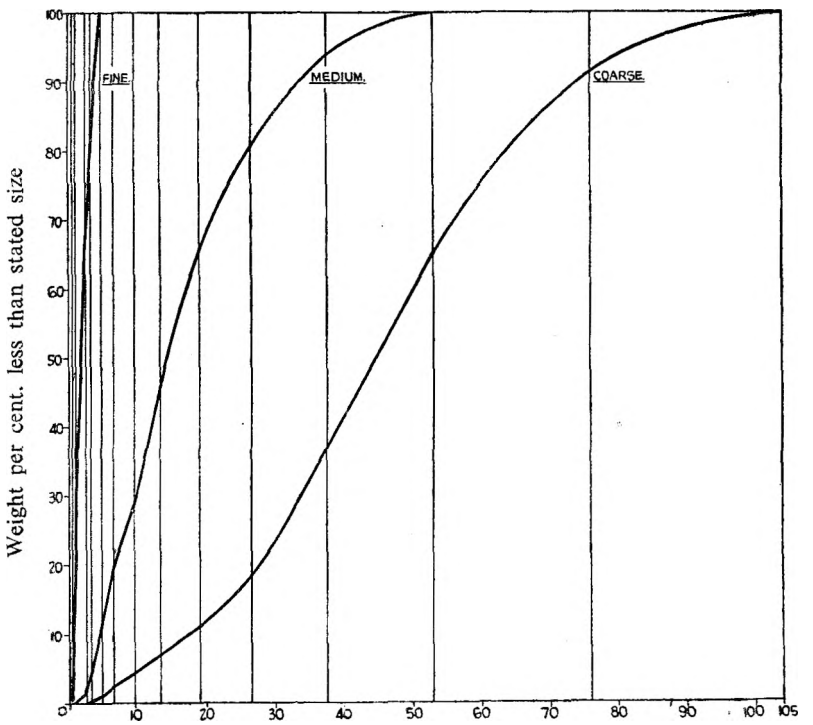

FIG. 1. Particle size distribution of typical preparations.

were gelled. (a) *High temperature*: Powdered aluminium stearate was dispersed in arachis oil, and the mixture heated slowly and with constant stirring to within the range 117° to 122°C. At this point the mixture became extremely viscous. The temperature was then raised to 150°C. and maintained at this level for 1 hour to effect sterilisation. The final product was rather less viscous than at 117°C. (b) *Medium temperature:* Aluminium stearate and arachis oil were separately sterilised by maintaining at 140°C. for 4 hours. The stearate was then dispersed in the oil, using aseptic technique, and the mixture raised slowly, and with stirring, to within the range 125° to 130°C. At this temperature the mixture

gelled and developed maximum viscosity, which was retained on cooling. (c) *Low temperature:* As for method (b), but the gelling temperature was raised to within the range 117° to 122°C. This gave a gel of slightly higher viscosity than (b). (d) *Unheated:* Aluminium stearate and arachis oil were separately sterilised by maintaining at 140°C. for 4 hours and then mixed by ball-milling for 24 hours. This yielded a fine dispersion of stearate in oil approximately equal in viscosity to the original oil.

(ii) *Beeswax/Arachis Oil:* The beeswax and arachis oil were mixed in the cold and the temperature raised slowly, with stirring, to 150°C., at which temperature it was maintained for 1 hour. The solution was allowed to cool and was agitated gently during cooling from 60°C. to room temperature to ensure dispersion of beeswax in a finely divided state.

(5) *Incorporation of Penicillin in Beeswax or Stearate Base:* The penicillin was mixed with the prepared sterile base in a mortar and the mixture tranferred to a standard vessel containing a fixed charge of steel balls. The vessel was sealed and rotated for 1 hour, the speed being adjusted so as to ensure efficient mixing with minimum alteration of particle size.

Experiment No. 1: Using the standard techniques already described, the effect of varying the following factors was investigated: (a) penicillin salt (procaine and potassium), (b) nature of suspending agent (beeswax and aluminium mono-, di-, and tristearates), (c) concentration of suspending agent (1 per cent. and 2 per cent. for both beeswax and stearate), (d) particle size of penicillin (medium and fine). The samples of aluminium stearate used are described here arbitrarily as mono-, di-, and tri-stearates since analysis showed that the base/acid ratio was approximately of this order. Typical analyses are given in the Appendix. All stearate/oil gels were prepared by the high temperature method, variations in the method of gelling being studied in Experiment 2. It was not considered desirable at this stage to examine the four factors in all combinations, and in the first part of the experiment (1a) one half of the combinations representing a half replicate of the full factorial design[11] was

TABLE I

LOGARITHMS OF ESTIMATED TIMES BETWEEN DOSAGE AND SUBSEQUENT INFECTION TO GIVE 50 PER CENT. MORTALITY

Penicillin Salt	Particle Size	Monostearate		Distearate		Tristearate		Beeswax	
Procaine ...	Medium ...	0·36	per cent. (1)	0·85	per cent. (2)	0·55	per cent. (2)	−0·14	per cent. (1)
	Fine ...	0·68	(2)	0·44	(1)	0·99	(1)	0·57	(2)
Potassium ...	Medium ...	0·14	(2)	−0·48	(1)	All mice died	(1)	All mice died	(2)
	Fine ...	0·01	(1)	0·14	(2)	0·16	(2)	All mice died	(1)

The figures between the brackets refer to the concentration of stearate or beeswax.

carried out. The results are given in Table I expressed as logarithms of the time required between dosage and subsequent infection to produce 50 per cent. mortality. In deriving these results it was found that the mortality measured as a probit was linear with respect to the logarithm of the time between dosage and infection. The well-known method of probits[12] was then used to calculate the 50 per cent. point. The slope of the probit log-time line was calculated for each sample. Each slope had a fairly large standard error and the assumption could be made that all the slopes were equal. The combined slop was then calculated and used to obtain an estimate of the 50 per cent. point for all preparations.

The reliability of the results differed from sample to sample but the variation was not large and an average figure of 0·14 for the standard deviation could be used without risk of serious error. It is clear from this table that potassium is much inferior to procaine, and beeswax inferior to aluminium stearate but the 6 remaining samples of procaine penicillin in stearate gels do not give sufficient information on the comparison between the particle size of penicillin and the three types of stearate. A further 6 samples were therefore prepared (Experiment Ib), with the 2 different particle sizes of procaine penicillin, using the three stearates at concentrations of 1 per cent. and 2 per cent. but with these concentrations reversed. Table II shows the results of this experiment together with the relevant portion from Table I. The two halves of the experiment then consisted of samples prepared using all combinations of the variables, particle size, concentration, and type of stearate.

TABLE II

LOGARITHMS OF ESTIMATED TIMES BETWEEN DOSAGE AND SUBSEQUENT INFECTION TO GIVE 50 PER CENT. MORTALITY

	Particle Size	Monostearate		Distearate		Tristearate	
			per cent.		per cent.		per cent.
Experiment Ia	Medium ...	0·36	(1)	0·85	(2)	0·55	(2)
	Fine	0·68	(2)	0·44	(1)	0·99	(1)
Experiment Ib	Medium ...	0·32	(2)	0·32	(1)	0·34	(1)
	Fine	0·28	(1)	0·11	(2)	0·18	(2)

The figures between the brackets refer to the concentration of stearate.

It should be noted that the average result for Experiment Ib is appreciably lower than that for Experiment Ia. This is probably due to the different batch of mice or the different suspension of infecting organism, or both, the experiments being carried out at different times. The design is such, however, that all the important comparisons are made within experiments and the comparison between the experiments has been made to coincide with the higher and less important interactions between the factors. The analysis of variance is given in Table II.

The standard deviation of each result calculated from the detailed observation on the mice is approximately the same for both experiments, the average value being 0·130, which corresponds to a variance of 0·0169.

From Table III it is seen that the efficacy of the preparations is influenced by the amount of the stearate used. The magnitude of this

effect is shown by a comparison of the averages for all the 1 per cent. and all the 2 per cent. preparations given in Table II: mean for 2 per cent. stearate, 0·549; mean for 1 per cent. stearate, 0·355. The 2 per cent. preparations are clearly more effective.

Table III also shows that efficacy is influenced by the particular combination of type of stearate and particle size of the procaine penicillin

TABLE III

ANALYSIS OF VARIANCE

Source of Variation	Sum of Squares	D/F	Variance
Type of stearate	0·0258	2	0·0129
Amount of stearate	0·1121	1	0·1121
Particle size	0·0003	1	0·0003
Interactions :—			
Stearates with particle size	0·1347	2	0·0674
Stearates with amount	0·0230	2	0·0115
Between experiments	0·4454	1	0·4454
Remainder	0·0440	2	0·0220
Total	0·7853	11	—
Error	—	Large	0·0169

used. This is seen more clearly in Table IV, in which the results of Experiments 1a and 1b have been averaged.

Table IV shows that fine particles are better with mono- and tri-stearate, but worse with distearate, and that medium particles are best with distearate. This type of interaction was unexpected and requires verification. Should this interaction be fortuitous, it is unlikely to affect the further conclusion that fine particles are not significantly different from medium ones. Table IV also shows an apparent trend to higher results with increasing stearic acid content of the aluminium stearate.

TABLE IV

(*a*) INTERACTION OF TYPE OF STEARATE WITH PARTICLE SIZE

Particle Size	Type of Stearate			Means
	Mono	Di	Tri	
Medium	0·340	0·584	0·445	0·456
Fine	0·478	0·275	0·588	0·447
Mean	0·410	0·430	0·516	

Experiment No. 2: This was designed to assess the effect of varying (a) the gelling method (high, medium and low temperature and unheated), (b) the type of aluminium stearate (aluminium monostearate and an aluminium stearate of no specified composition, referred to as "Technical"), (c) concentration of stearate (2 per cent. and 3 per cent.), and (d) particle size of procaine penicillin (medium and coarse). The statistical design used to examine these factors was a half replicate of the factorial design. This involved 16 samples, and the results expressed as logarithms of the estimated time between dosage and subsequent infection to give 50 per cent. mortality in mice are given in Table V.

TABLE V

LOGARITHMS OF ESTIMATED TIMES BETWEEN DOSAGE AND SUBSEQUENT INFECTION TO GIVE 50 PER CENT. MORTALITY

Particle Size	Type of Stearate	High Temperature		Medium Temperature		Low Temperature		Unheated	
			per cent.		per cent.		per cent.		per cent.
Coarse ...	Mono- ...	0·40	(3)	0·25	(2)	0·39	(3)	−0·23	(2)
	Technical ...	0·49	(2)	0·38	(3)	0·40	(2)	−0·35	(3)
Medium ...	Mono- ...	0·50	(2)	0·43	(3)	0·60	(2)	0·06	(3)
	Technical ...	0·56	(3)	0·44	(2)	0·44	(3)	−0·03	(2)

The analysis of variance of these results is given in Table VI.

The only significant effects are particle size of penicillin and temperature of gelling. The residual variance, which is a mixture of interactions, is no greater than the error variance due to the inherent variability of the mice. Had the reverse been the case, it would have been necessary to carry out the second half replicate to complete the factorial design

TABLE VI

ANALYSIS OF VARIANCE

Source of Variation	Sum of Squares	D/F	Variance
Concentration of stearate	0·0038	1	0·0038
Particle size	0·0848	1	0·0848
Type of stearate	0·0002	1	0·0002
Temperature of gelling	1·1367	3	0·3789
Residual	0·0460	9	0·0051
Total	1·2715	15	—
Error	—	Large	0·0177

involving all combinations of the factors. The mean results are as follows:—

Particle Size: Coarse = 0·217; Medium = 0·362.

Temperature of gelling: High = 0·489; Medium = 0·378; Low = 0·485; Unheated = − 0·167.

Medium particle size is thus better than coarse, and unheated gels inferior to heated. There is no clear distinction between the high, medium and low temperatures of gelling. Similarly, there is no detectable difference between the two types of stearate and between 2 per cent. and 3 per cent. concentrations of stearate.

DISCUSSION

The reliability of the mouse protection test has already been discussed by Miller[8], whose conclusions are confirmed by comparison of his results and those reported here with the clinical data of Thomas[7]. Assuming that the test gives a good indication of the prolongation of blood levels achievable in man, it would thus appear that the most efficient of the oily injections so far examined consists of procaine penicillin (fine particle size) dispersed in oil gelled by aluminium stearate. Whether the three factors of penicillin salt, particle size, and gelling agent are closely inter-

related is not yet established, but the superiority of procaine penicillin over the potassium salt clearly extends to all formulations and all particle sizes. As far as penicillin salt is concerned, this might lead to a theory of delayed absorption due to reduced water-solubility. This explanation cannot be accepted, however, until a series of water insoluble salts have been studied. It has already been shown[7] that aluminium penicillin is less effective than the procaine salt and this difference cannot be explained on the grounds of solubility alone. The influence of particle size presents an interesting problem. The first reports[4,5] favoured large particles but the position was reversed when stearate gels were introduced, suggesting that the action of the latter is connected with the surface area of penicillin. The fact that gels of different viscosity do not differ significantly in biological behaviour indicates that aluminium stearate does not merely act as a thickening agent, although it is equally clear that it loses its effect if gelling does not take place. The chemical composition of the stearate is apparently not a significant factor and therefore samples may be selected on purely pharmaceutical grounds without risk of sacrificing activity. The optimum proportion of stearate seems to be approximately 2 per cent. of the oil base. Preparations containing more than this have no increased activity and are more difficult to manipulate.

In the light of the foregoing, it is suggested that the mechanism of aluminium stearate probably involves the formation of a protective film around the penicillin particles. Such a film might be created by interaction at the penicillin surface to produce "procaine stearate," the molecules of which could be orientated with the carbon chain in the oil phase. This would retain a film of oil over the penicillin surface, the former being held more tenaciously than would be a film of untreated oil. This theory would explain the effect of penicillin particle size since the area of adsorbed film would increase with reduction of the particle size. The theory is also compatible with the other observed effects of concentration of stearate and disappearance of activity in non-gelled material. In the former case the optimum concentration required to establish the protective film is apparently of the order of 2 per cent., and nothing is gained by raising this figure. For the latter, it is suggested that the interaction with procaine penicillin can only take place when the stearate is in the gelled state.

Summary

(a) A comparison has been made of the efficiency of various oily injections of penicillin using a mouse-protection test.

(b) It is concluded that the most satisfactory preparation consists of procaine penicillin of small particle size dispersed in a stearate/arachis oil gel.

(c) A theory is suggested for the mechanism by which aluminium stearate induces "slow-release."

I am indebted to Dr. O. L. Davies for the design of the tests and the analysis of results, to Dr. A. R. Martin for supervision of the biological work, and to Mr. A. G. Fishburn for help in the preparation of the paper.

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APPENDIX

ANALYSIS OF ALUMINIUM STEARATES

Test	Monostearate	Distearate	Tristearate	Technical
	per cent.	per cent.	per cent.	per cent.
Loss at 100°C. for 3 hours ...	1·5	0·66	0·96	0·9
Ash	16·20	8·75	6·20	0·05
Water-insoluble ash ...	15·90	8·2	5·3	9·69
Aluminium (as Al_2O_3) ...	16·2	8·8	6·4	10·10

DISCUSSION

The CHAIRMAN said that the paper was an interesting illustration of the use of the mouse protection test as an alternative to the rabbit method of studying the persistence of penicillin in the blood. It was also a very good example of the value of physical chemistry in pharmacy.

DR. K. BULLOCK (Manchester) observed that procaine penicillin had been chosen from a number of salts of penicillin and organic bases. Could the author give the references to the other organic bases or indicate the types of other organic bases tried?

PROFESSOR H. BRINDLE (Manchester) said he was interested in slow release vehicles for penicillin and in the use of the mouse protection test as a criterion. He had always used the rabbit and, in common with other workers, had found some difficulty in getting a sample of the blood which was sufficiently sterile to give satisfactory results.

In the mouse protection test the solution or suspension was injected subcutaneously into the mouse. Everything depended on the relationship between the oily depot and the surrounding tissue and this might give very different results from those which one would get by intramuscular injection in human beings. He had not consulted the references cited by the author but thought that Thomas and Miller had worked with aqueous solutions, which were very different from oily suspensions. Did the author think it quite fair to relate his results on mice to what might happen when suspensions were administered intramuscularly in human beings?

DR. R. E. STUCKEY (London) said that various papers had been published recently giving varying opinions on the efficacy of penicillin suspensions. Had the author any experience with the aqueous procaine penicillin suspension tested by his method?

DR. F. HARTLEY (London) mentioned that the author suggested that the viscosity of the solution made very little difference to the duration of action of the material. Did the extent of gelling vary with the age of the suspension? If so, the ease of manipulation would also vary.

MR. J. C. FLOYD, in reply, said that a number of salts were mentioned in the literature, and they had used a number of organic bases, including *p*-chlorophenyl biguanide. In reply to Professor Brindle, he thought that the question was really concerned with whether the test gave results comparable with those obtained in man. He thought this question was answered by Thomas's findings which showed excellent correlation between the results in mice and in human beings.

They had examined the aqueous procaine penicillin suspension by the method described, but the results were not very good; the effect was so rapid that the test became too insensitive.

He was not quite clear what Dr. Hartley meant by the extent of gelling. If one gelled under optimum conditions that gel on standing would eventually break. He thought that all aluminium stearate gels eventually broke on standing, and presumably to incorporate penicillin into a broken gel would be a much more simple matter than to incorporate it into an extremely viscous one. Whether there was any biological difference between a gel before and after breaking he could not say.

THE EFFECT OF THE SULPHONE GROUP IN HYPNOTICS

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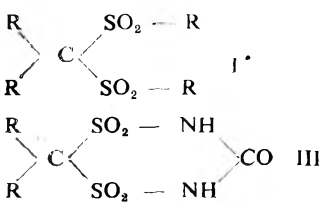
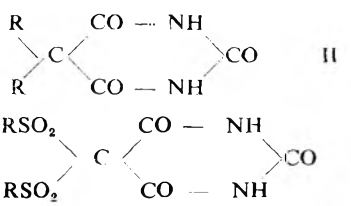
Sulphones of the type I possess remarkable narcotic properties and bear some structural resemblance to the substituted malonic acid fragment of barbituric acid derivatives II, another group of excellent hypnotics. Such resemblances has encouraged some workers to attempt the preparation of " Veronalsulphone " (III), but such attempts failed[1,2,3].

Another type of compound that would reflect both the sulphonals and the barbitones, is a barbituric acid substituted at position 5 with alkyl sulphone groups (IV).



I

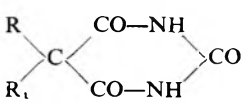


II

III

IV

By preparing such compounds the effect of introducing the sulphone groups into the barbituric acid nucleus can be tested; and as typical compounds 5-ethylsulphonylbarbituric acid (V), 5-ethyl-5-ethylsulphonylbarbituric acid (VI) and 5 : 5-diethylsulphonylbarbituric acid (VII) were prepared and their hypnotic potency compared with that of diethylbarbituric acid (VIII).



V : R = H, $R_1 = C_2H_5SO_2$

VI : R = C_2H_5, $R_1 = C_2H_5SO_2$

VII : R and $R_1 = C_2H_5SO_2$

VIII : R and $R_1 = C_2H_5$

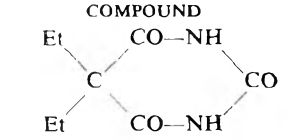
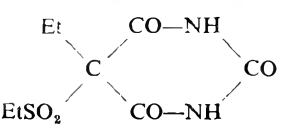
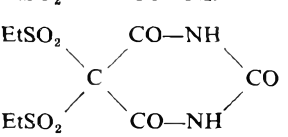
The syntheses were achieved by methods parallel to those used for substituted barbituric acids, viz.: the condensation of ethylsulphone malonic esters with urea, the substituted malonic esters being prepared by the interaction of ethylsulphonyl chloride and ethyl sodio-malonate in etheral medium. In the case of ethyl-ethylsulphonylbarbituric acid, the compound was prepared also by the interaction of sodium sulphinate and 5-ethyl-5-bromobarbituric acid[4] in order to confirm the configuration of the condensation product. This latter process also gave better yields. The physical and chemical characters of the new products are tabulated in the experimental part.

We are indebted to Professor M. Sherif of the Pharmacology Department of the Faculty for the biological investigation of the new barbiturates. The relative activities of the compounds are tabulated as follows taking the activity of veronal as a reference.

Evidently, therefore, the introduction of sulphone groups into the molecule of barbitone has decreased its activity. The toxicity of the new products is greater than that of barbitone, thus the possibility of their administration may be restricted and unsafe.

Experimental

Sodium ethanesulphonate was prepared by a modification of the method of Hemillian[5]. A clear solution of anhydrous sodium sulphite (35 g.) in water (250 ml.) and ethyl iodide (30 g.) were heated on a water-bath under a reflux condenser until all the iodide disappeared (7 hours).

COMPOUND	SO_2 GROUPS	ACTIVITY
	0	100
	1	80
	2	10

Copper sulphate solution (100 ml. of 20 per cent.) was added with stirring to the warm reaction mixture and the precipitate so formed removed by filtration. The filtrate was neutralised with sodium hydroxide solution and concentrated on a water-bath till a scum formed on the surface, then allowed to crystallise, and filtered again. The filtrate was evaporated to dryness, the residue extracted with boiling alcohol (80 per cent.) and filtered whilst hot. On cooling the alcoholic filtrate sodium ethanesulphonate crystallised in thin wide plates. Yield, 78 per cent.

Ethylsulphonyl chloride was prepared by a modification of the method of Gerhardt[6]. Sodium ethanesulphonate (13 g.), dried at 120°C. under reduced pressure for 2 hours, was finely powdered and mixed with phosphorus pentachloride (20 g.) and then heated on a water-bath for 10 minutes. The cooled mixture was decomposed by pouring into ice-cold water and the acid chloride produced extracted with ether. After drying the extract and removal of the solvent, the acid chloride was distilled at 75° to 77°C./25 mm.Hg. Yield, 68 per cent.

Ethylsulphonylmalonic Ester (I). Diethylmalonate (30 g.) was added gradually with stirring to sodium wire (2·3 g.) covered with ether (150 ml.), the whole being heated on a water-bath under a reflux condenser. Slow addition of ethanesulphonyl chloride (10 g.) dissolved in ether (50 ml.) commenced after all the sodium had disappeared (1½ hours), stirring and refluxing being continued during the addition and for a further 7 hours until the mixture became neutral to litmus. Water was then added, the mixture acidified, the ethereal layer separated and the aqueous layer repeatedly washed with ether. The mixed ethereal solutions were dried over calcium chloride, the ether removed, and the residue distilled *in vacuo*.

Ethyl-ethylsulphonylmalonic ester (II) was prepared by the same procedure as I using ethylsulphonylmalonic ester (30 g.) in place of malonic ester and ethyl iodide (20 g.) in place of ethylsulphonyl chloride.

Diethylsulphonyl-malonic ester (III) was prepared by the same procedure as I using ethylsulphonylmalonic ester (30 g.) in place of the malonic ester.

All the esters occurred in the form of pale yellow oils soluble in ether and organic solvents, insoluble in water and showing a sp. gr. greater than 1. Table I gives their constants.

TABLE I

Ester	Boiling point	Sp. Gr. at 20°C.	Sulphur found	Content required	Yield
					per cent.
I	145 to 147°C./5 mm. Hg	1·36	12·68	12·7	48
II	160 to 163°C./5 mm. Hg	1·42	11·40	11·43	42
III	172 to 174°C./5 mm. Hg	1·48	18·41	18·6	29

Condensation with Urea.

5-Ethylsulphonylbarbituric Acid (IV). Sodium (5·1 g.) was dissolved in absolute methyl alcohol (120 ml.). To the solution of 1 ml. of ethyl acetate was added followed, after 10 minutes at 60°C., by urea (12·7 g.). When the urea had dissolved, ethylsulphonylmalonic ester (15 g.) was added and the apparatus transferred to an oil-bath. A condenser for distillation and a sealed stirrer were fitted. The temperature of the reaction mixture was gradually raised to 130°C., in the course of 3½ hours, then the viscous mass was treated with ice and ice-cold water; when the bulk of the melt had dissolved, the alkaline solution was treated with 20 ml. of benzene, then rendered distinctly alkaline to congo red with hydrochloric acid, concentrated and allowed to crystallise. The crude barbituric acid was recrystallised from an acetone-benzene mixture.

5 : 5-Diethylsulphonyl-barbituric acid (V). The above procedure was

applied using diethylsulphonemalonic ester (21 g.) in place of the monoethylsulphonemalonic ester. The product was recrystallised from 95 per cent. alcohol.

5-Ethyl-5-ethylsulphonylbarbituric acid (VI). (a) The same procedure was applied using ethyl-ethylsulphonylmalonic ester; the crude product was crystallised from 85 per cent. alcohol. (b) 5-Bromo-5-ethylbarbituric acid[7] (2·7 g.) and sodium ethanesulphinate[8] (1·2 g.) were dissolved in absolute methyl alcohol (20 ml.) and the mixture refluxed for 8 hours. The precipitated sodium chloride was filtered and the filtrate concentrated and allowed to crystallise. The product was identical with that above as confirmed by a mixed melting-point.

All the barbituric acid derivatives obtained were crystalline solids soluble in ether, alcohol and dilute alkalis; some of their constants are shown in Table II.

TABLE II

Acid	Melting point	Yield per cent.	Analyses			
			Nitrogen		Sulphur	
			Found	Required	Found	Required
IV	145 to 146°C.	27	12·74	12·73	14·46	14·55
V	156 to 157°C.	41	8·96	8·97	20·28	20·26
VI	163 to 164°C.	44	11·31	11·29	12·87	12·90

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DISCUSSION

The paper was read by Dr. Said.

The CHAIRMAN said that the Conference would welcome this contribution on synthetic work.

DR. HARTLEY (London) asked, firstly, if the introduction of the ethylsulphone group modified in any way the dissociation of the barbiturate portion of the molecule; that is, was the sodium salt formed readily hydrolysed or comparatively neutral? Secondly, if the introduction of

the ethyl-sulphone groups modified the way in which the molecule could be broken down by acid hydrolysis?

MR. D. E. SEYMOUR (Welwyn) asked what methods were used to make compound III. The combination of sulphones with the barbiturate would be a most difficult problem, and the compound probably would not have any value in this particular field. It was a sulphonamide, and might have quite different properties. Had Dr. Said examined his compounds for biological activity other than hypnotic value?

DR. K. BULLOCK (Manchester) asked if Dr. Said could tell them a little about the method of testing hypnotics. In relating constitution to physiological action, the method of testing was important. He asked whether the decreasing activity with increasing number of sulphone groups could be related to such properties as decreasing solubility, or change of *p*H.

DR. R. E. STUCKEY (London) said he could not quite follow Mr. Seymour's remark about compound III being essentially a sulphonamide, and would like that explained. It was interesting that the compound, with free hydrogen in position 5, showed no activity; with such a compound, the molecule was more acidic, and had a considerably lower K_A value.

DR. F. SAID, in reply, said that with one ethylsulphone group the compounds were more soluble in liquids than in water, and with two ethylsulphone groups the difference was even more marked. For testing the compounds dogs had been used, the relative activities being determined from the amounts needed to produce sleep. One group of dogs was given barbitone and another the compound to be tested, but he could not give details of the method as the test was done in the Pharmacological Department. Other biological activities had not been investigated. He agreed that it was desirable to investigate the germicidal activity, but doubted whether compound III could be described as a sulphonamide.

WOOL ALCOHOLS

PART I—OBSERVATIONS ON CHANGES IN PHYSICAL AND CHEMICAL PROPERTIES IN WOOL ALCOHOLS AS A RESULT OF OXIDATION

BY G. S. MUIRHEAD, K. H. OBERWEGER, D. E. SEYMOUR AND D. SIMMONITE

(From the Research Department, Herts Pharmaceuticals Ltd.)

Received July 1, 1949

IN view of the increase in the use of wool alcohols* as an emulsifying agent in the preparation of pharmaceutical creams and ointments, and in particular, the inclusion of monographs on wool alcohols and ointments derived therefrom in the British Pharmacopoeia, it was considered that some results of investigations carried out in these laboratories on various aspects of the chemical and physical behaviour of wool alcohols would be of value to pharmaceutical workers.

A review of the literature indicates that very little information exists concerning the properties of wool alcohols and their behaviour when stored under different conditions. The results presented in this paper refer specifically to one particular grade of wool alcohols available in this country,† although experience gained by us suggests that wool alcohols produced in these and other associated laboratories differ in certain properties. It has been known for some considerable time that certain characteristic changes occur when wool alcohols are stored in air.[1,2] For example, exposure of samples of the material to the atmosphere gives rise to a change in surface characteristics and suggestions have been made in the literature that this change is due to oxidation. Gillam,[3] for example, reports that wool alcohols when exposed to atmospheric conditions, for considerable periods of time, show a marked increase in fatty acids, as denoted by rise in acid value, and it has been noted by us that wool alcohols, when subjected to irradiation with ultraviolet light showed marked changes in physical characteristics and rise in acid value. It has also been observed that these changes are slowed down by the addition of small quantities of antioxidants (e.g., pyrogallol, α-tocopherol). It has been our experience over several years that samples of wool alcohols when stored in bulk prior to use in a technical process, showed marked rises in acid value and such samples, on examination according to the B.P. monograph, possessed acid values as high as 30, and some samples contained negligible digitonin-precipitable material. It was also noted that such wool alcohols were unsuitable for use in the manufacture of water-in-oil emulsions.

Although we have shown that breakdown of water-in-oil emulsions is due to several factors other than the nature of the emulsifying agent itself (e.g. nature of hydrocarbon used, conditions employed in

* The term wool alcohols throughout this paper refers to the total unsaponifiable fraction of lanoline and is not necessarily synonymous with Wool Alcohols B.P.

† The wool alcohols used throughout this investigation were obtained from Messrs. Croda Ltd., and are marketed under the trade name "Hartolan."

emulsification), it has been apparent that the initial nature of the wool alcohols or changes which they might have undergone during storage in the emulsion, may adversely affect the stability of the product. For example, a series of comparable emulsions prepared from wool alcohols of different origin showed marked differences in stability and those which tended to separate more readily showed a marked rise in acid value of the isolated oil phase, such rise being retarded by the inclusion of antioxidants.

In view of these observations it was considered that a quantitative study should be made of some of the physical and chemical properties of wool alcohols when treated as follows :—irradiation by means of ultra-violet light, exposure of the solid material to air at normal or elevated temperatures, and treatment of molten material with oxygen. This paper records some of the results obtained and the methods used. The changes in physical and chemical constants were measured by periodic determination of some or all* of the following :—

1. Surface activity ; 2. Appearance ; 3. Acid value ; 4. Saponification value ; 5. Acetyl value ; 6. Digitonin-precipitable fraction.

Experimental

For convenience, the work will be described under the following sub-headings :—1. Treatment of molten wool alcohols with gaseous oxygen ; 2. Exposure of wool alcohols to atmospheric oxygen ; 3. Irradiation of wool alcohols with ultra-violet light ; 4. Examination of wool alcohol emulsions after storage.

1. *Treatment of molten wool alcohols with gaseous oxygen.* The following method and equipment were devised with a view to producing a uniform rate of oxidation and causing appreciable changes in the material within reasonable periods of time. The apparatus is illustrated in Figure 1.

Oxygen enters at A and passes through the aperture B of the hollow glass stirrer G and partially through the stirrer guide C. It enters the molten wool alcohols through the holes D, E & F, in the stirrer and also through the base of the stirrer guide at H. Gas leaves the vessel at the side tube I and its flow rate was measured by a suitable meter. The lower portion of the apparatus was surrounded by a removable steam jacket so that test samples could be withdrawn via J. The stirrer speed was approximately 300 revolutions per minute. Samples for analysis were withdrawn at the intervals quoted and the results obtained are tabulated in Table I. A control experiment was carried out substituting carbon dioxide for oxygen. In this way it was shown that negligible changes of the wool alcohols occurred in the absence of oxygen even on heating for the time quoted. As a further precaution the effluent gases were trapped in a wash bottle cooled with solid carbon dioxide so that any acidic volatile matter produced could be estimated by titration

* The changes which wool alcohols undergo on treatment with gaseous oxygen have been chosen as typical of those which occur in the other three methods and for convenience the full physical and chemical constants were determined only in Experiment 1.

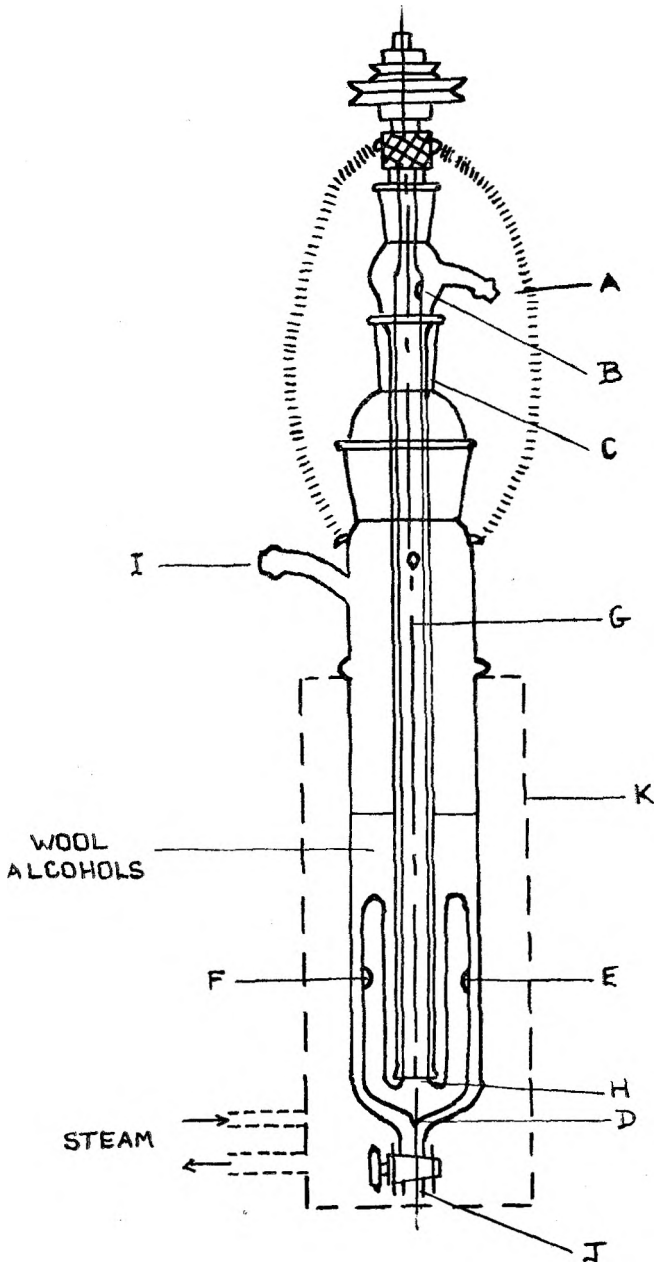


FIG. 1. Apparatus for the treatment of molten wool alcohols with gaseous oxygen.

in alcoholic solution. A small acidic fraction was obtained in this way which constituted approximately 1 per cent. of the total acidity and this was ignored in the acid values quoted in Table I.

2. *Exposure of wool alcohols to atmospheric oxygen.* The wool

TABLE I

CHANGES IN CHEMICAL AND PHYSICAL PROPERTIES OF WOOL ALCOHOLS DURING OXIDATION

Time of oxygenation in hours	Acid value	Ester* value	Acetyl value	Cholesterol content (digitonide method) per cent.	Interfacial Tension Dynes/cm. Age of interface	
					60 secs.	120 secs.
0	2·5	4·7	134	30·40	8·5	7·4
5	4·0	23·4	132	25·49	10·6	9·6
10	5·3	24·1	122	23·85	—	9·2
20	8·1	32·5	—	20·43	8·1	7·2
30	12·0	38·6	110	15·14	7·6	6·5
40	16·0	61·2	—	8·32	6·1	4·7
50	25·4	62·0	—	4·99	4·3	3·9
60	31·1	78·6	86	0·81	4·5	3·7
70	34·0	96·0	—	—	5·2	4·5
80	35·3	96·7	55	—	4·3	4·0
90	38·4	96·6	—	—	5·1	4·4

* Difference between saponification value and acid value.

alcohols were obtained in suitably thin layers by pouring the molten material into large photographic developing dishes to a depth of 1/16 inch. The samples were then exposed to the atmosphere at the normal temperature and in an incubator at 37°C. Samples were withdrawn periodically and their acid values determined. The results are shown graphically in Figure 2.

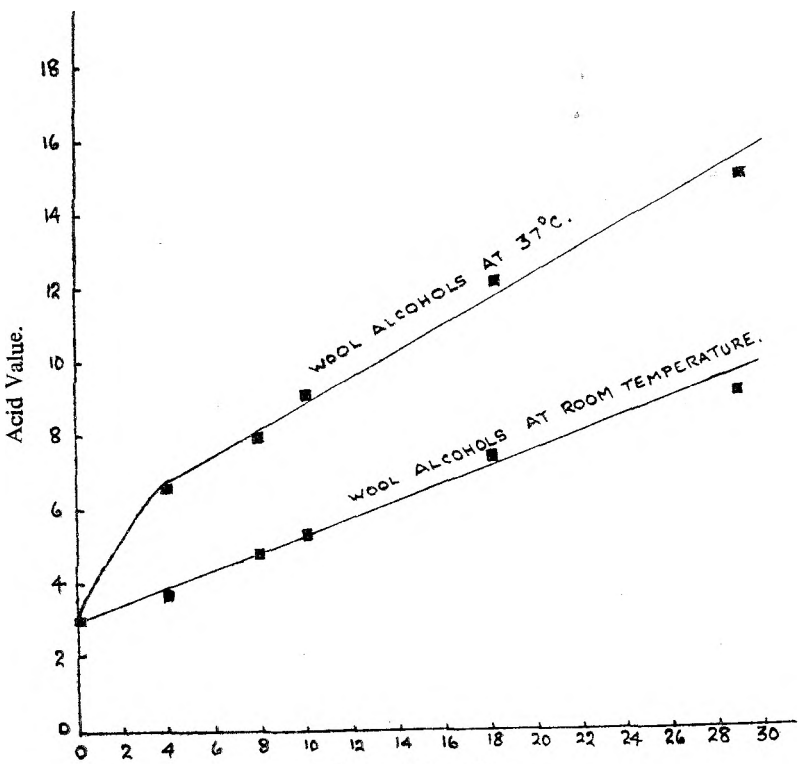


FIG. 2.

3. *Irradiation of wool alcohols with ultra-violet light.* The material was prepared in thin layers as in the previous experiment and exposed to the radiation of an Hanovia ultra-violet lamp, the quartz tube being approximately 8 inches from the surface of the wool alcohols. Samples were withdrawn at intervals and their acid values determined. The experiment was repeated using wool alcohols containing antioxidants. The results are shown graphically in Figure 3.

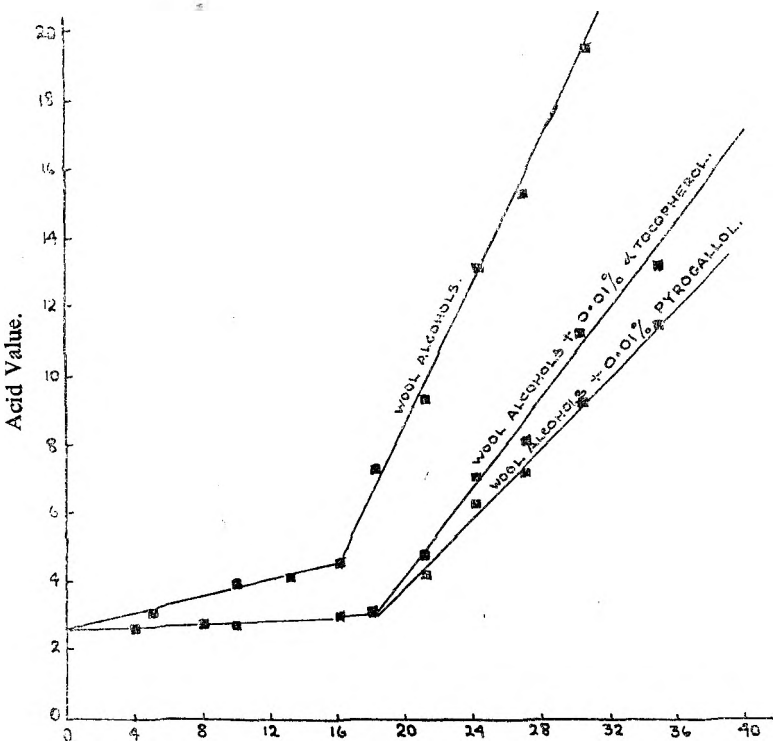


FIG. 3.

4. *Examination of wool alcohol emulsions after storage.* Two emulsions were prepared according to the following formula, one containing 0·007 per cent. w/w of propyl gallate.

Wool Alcohols	20
Paraffin wax	100
White oil (viscosity 74 Redwood secs.) ...	200
Water	680

The samples were packed in lacquered tinplate containers leaving a considerable air space above the emulsion and stored at room temperature and at 37°C. Samples were observed at various intervals of time, the appearance being noted and the acid value of the total oil phase determined by the following procedure :—A sample was withdrawn and twice its weight of warm water added. It was then heated on a steam bath until the emulsion had separated into distinct clear layers. The

oily layer was then separated and a suitable weight used for the determination of acid value as described later. It was assumed that only wool alcohols were responsible for any change in acid value of the oily phase and the value was calculated with reference to the known wool alcohol content. During the experiment some of the emulsions partially separated but the acid value determinations were nevertheless continued. The results are illustrated in Figure 4.

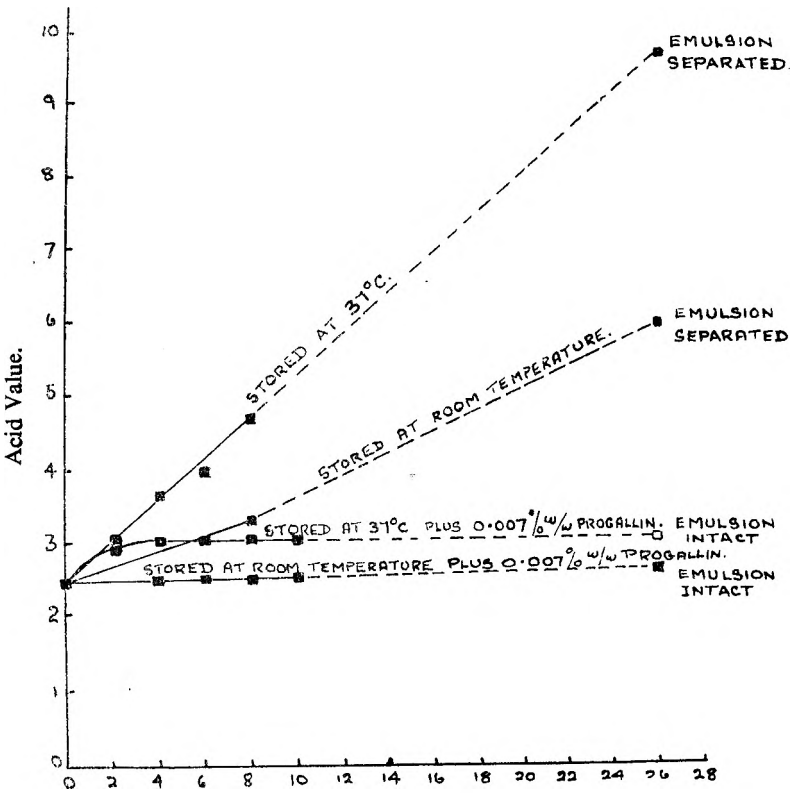


FIG. 4.

METHODS USED FOR THE DETERMINATION OF PHYSICAL AND CHEMICAL CONSTANTS

Acid value, cholesterol (digitonide method), and saponification value were determined by the method described in the British Pharmacopoeia 1948.

Acetyl value. The method described in the British Pharmacopoeia 1948, page 762, was found inconvenient for ordinary samples of wool alcohols and could not be applied at all to highly oxidised samples since the high viscosity of the acetylated material rendered its washing and recovery from the separating funnel extremely difficult. The following modification was therefore devised and proved satisfactory in use. After acetylation according to the B.P. method, the mixed acetates were

dissolved in chloroform, filtered and the extract washed with brine until neutral. After finally washing with water and drying over anhydrous sodium sulphate the solvent was removed. The determination was then completed in the normal manner.

Determination of surface activity by measurement of interfacial tension. Interfacial tension measurements were undertaken in an attempt to assess quantitatively any changes of emulsifying power which wool alcohols may have undergone as a result of oxidation. Heinrich,[4] who well realised the usefulness of this tool for the study of cosmetic emulsions, made use of the pendant drop method for the measurement of interfacial tension. In addition to measurements on total wool alcohols, experiments with fractions were carried out.

The results are set out in Table II.

TABLE II

INTERFACIAL TENSION VALUES OF SOME WOOL ALCOHOL FRACTIONS

Fraction	Interfacial tension in dynes/cm. Age of interface 120 secs.
Total wool alcohols (1 per cent. w/v in white oil*)	5·0
Cholesterol (1 per cent. w/v in white oil)	5·6
iso-Cholesterol (1 per cent. w/v in white oil) (Lanosterol-agnosterol fraction)	16·4
Cetyl alcohol (1 per cent. w/v in white oil)	13·8
Blank using water and white oil	52·5
Total wool alcohols (1 per cent. w/v in benzene)	7·4
Optically inactive alcohols (1 per cent. w/v in benzene)	12·4
Blank using water and benzene	32·2

* Viscosity 74 seconds Redwood

The possibilities of this method were extensively investigated by Andreas *et al.*[5] whose techniques and calculations have been freely adopted in our work. Certain mathematical functions necessary for the application of their technique have recently been published in an improved form by Fordham[6] whose tables have been used throughout this study.

The method consists of developing a suitably shaped drop of one phase beneath the surface of the other. From measurements of the drop aided by suitable magnification the interfacial tension of the particular system can be calculated. The apparatus is illustrated in Figure 5 and is substantially that used by Andreas *et al.*[5]. Certain features of difference will be described to supplement the diagram.

1. The lighting was obtained from a high power microscope lamp using 6 volt 8 amp. bulbs and fitted with adjustable lenses and an iris diaphragm.

2. The drops were expelled from a glass jet of suitable size by means of a 10 ml. hypodermic syringe fitted with a micrometer adjustment. A similar device had been used by Smith.[7]

3. The constant temperature cell was mounted together with the drop-forming mechanism in place of the stage of a microscope, the barrel being horizontal, so that multi-directional adjustment could be easily obtained. The camera was a Kodak ¼ plate, fitted with Compur shutter and ground glass focussing back. In practice it was found that suitable magnification to cover the different sizes of drops could be obtained by the use of 1 inch or 2/3 inch microscope objectives.

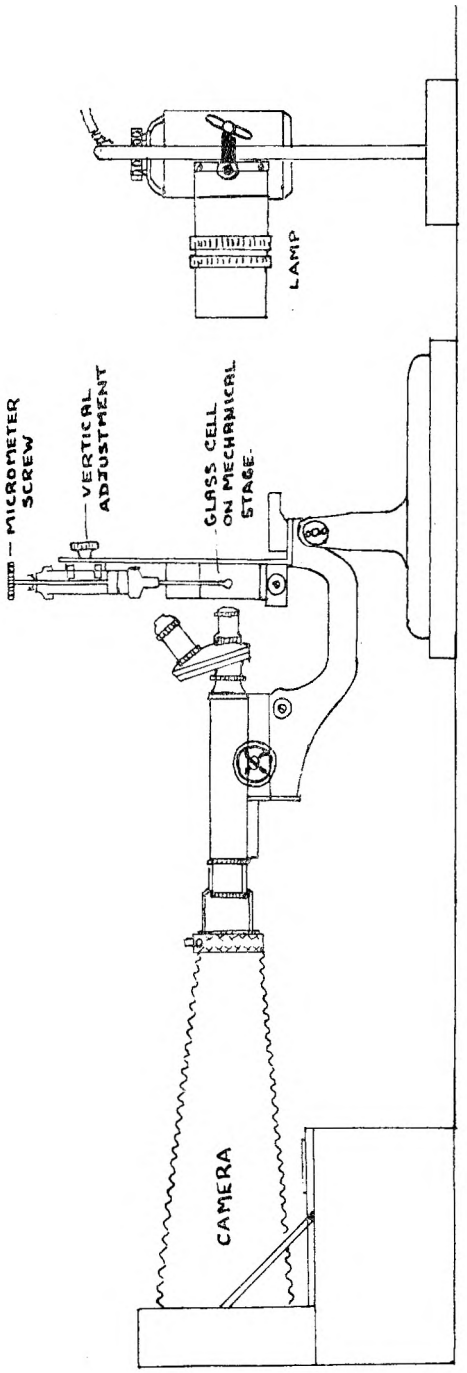
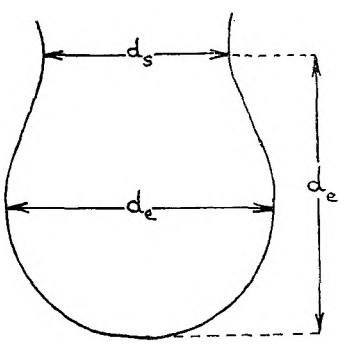


FIG. 5. Apparatus for the measurement of interfacial tension.

Briefly the experimental procedure was as follows :—The sample of wool alcohols under test was accurately weighed out dissolved in benzene* and adjusted to volume to give a 1 per cent. w/v solution. The solution was placed in the glass cell of the apparatus and drops of water contained in the syringe were developed on the end of a suitable jet as illustrated. The rate at which successive drops were formed was kept as uniform as possible and when the drop had the correct shape (possessed a suitable waist) it was photographed. The drop was photographed at intervals of 60 and 120 seconds after formation, since a finite time is necessary for the establishment of the interface and the attainment of a mimimum value of interfacial tension. The plate was placed in a photographic enlarger and projected on to squared paper. The dimensions needed for the calculation were then measured directly and the interfacial tension calculated using the tables published by Fordham[6] in conjunction with the following formula quoted by Andreas *et al.*[5] :—

$$\gamma = \frac{(\rho^1 - \rho^2) \times g d_e^2}{H} \qquad S = \frac{d_s}{d_e}$$



where γ=interfacial tension in dynes per cm. between the two phases under consideration $\rho^1 - \rho^2$ = difference in densities of the two phases at the temperature of the experiment. g = acceleration due to gravity. H = function of S, which can be obtained from the HS functions for pendant drops given by Andreas *et al.*[5] and Fordham.[6] For a derivation of this relationship the original paper by Andreas *et al.* should be consulted.

Discussion

The composition of wool alcohols has been the subject of study by a number of workers and although a full analysis has not been completely worked out for any one specimen of the material, it is fairly clear that it consists essentially of a mixture of optically inactive aliphatic alcohols, the most prevalent of which is ceryl alcohol,[8] sterols, principally cholesterol[9], and triterpenes[10] (essentially a mixture of two triterpene alcohols lanosterol and agnosterol, originally termed '*iso*cholesterol' by Schulze[11]).

Gillam[3] in his paper has suggested that wool alcohols on oxidation produce a mixture of fatty acids, esters and alcohols, and infers that sterols are primarily concerned in the degradative changes involved. We are of the opinion that evidence obtained by Gillam,[3] coupled with that obtained by ourselves in this study, is insufficiently complete to elucidate the mechanism of the degradation of wool alcohols resulting from

* Benzene was used in this series of experiments since oxidised samples of wool alcohols become increasingly insoluble in liquid paraffin which was used in the preparation of emulsions.

oxidation. Our results show, however, that the material is readily prone to oxidation. These findings appear to be at variance with statements by Lower[2] that wool alcohols are extremely resistant to oxidation and do not decompose or go rancid. A progressive rise in acid value, coupled with the fall in acetyl value and rising ester value, suggests that wool alcohols as a result of oxidative degradation become partially converted into carboxylic acids which can then undergo esterification either with unchanged fractions of the wool alcohols or their oxidation products which have retained a functional hydroxyl group. Such a mechanism has already been envisaged by Gillam.[3] A steady fall in the precipitable digitonides suggests that the sterols are involved in one or other of these degradations. For example, the increase in ester value and the steady fall in yield of the precipitable digitonides are linearly related, and Figures 6, 7 and 8 indicate the interdependence of some of the changes

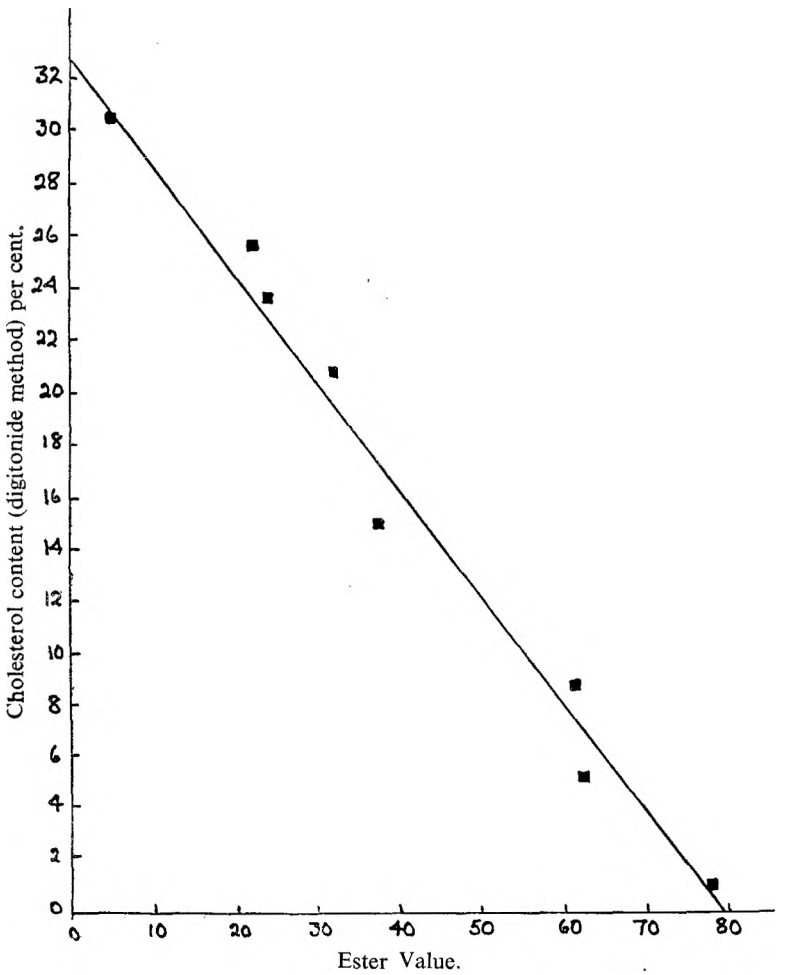


Fig. 6.

we have observed. It is interesting to note that the oxidised wool alcohols appear to show changes in physical properties which are suggestive of polymerisation, thus the material becomes increasingly vitreous and viscous, its melting point rises and its solubility in certain hydrocarbons decreases.

It is not proposed to elaborate however on possible mechanisms of degradation with the limited data which is available at this stage of the work. Further investigations on this subject will appear in subsequent communications.

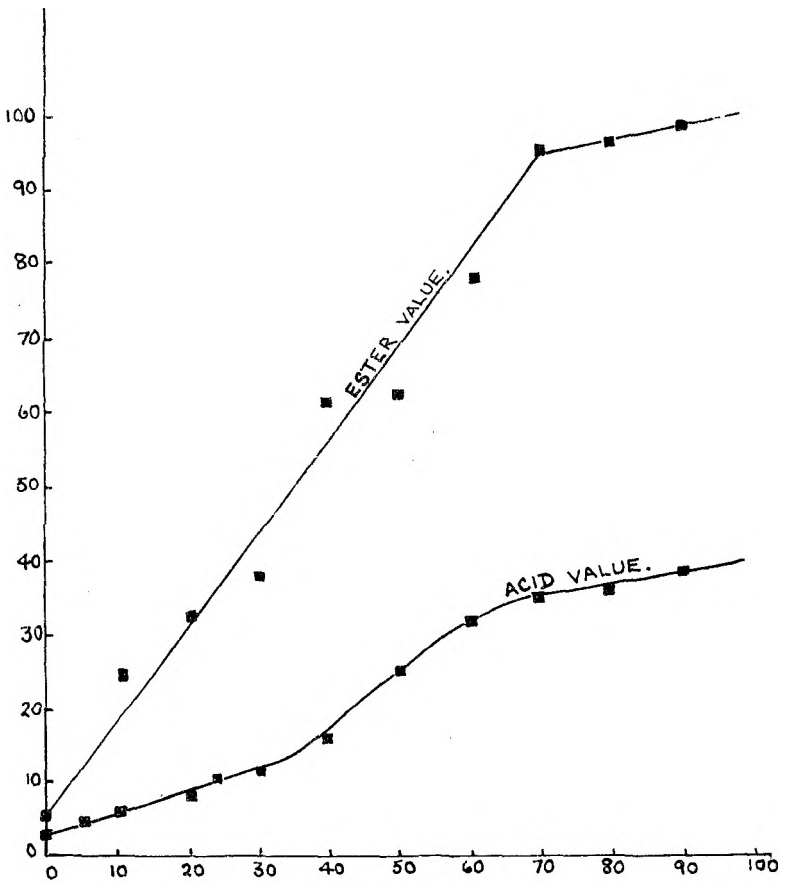


FIG. 7.

Interfacial tension measurements when carried out in a solvent in which samples at all stages of oxidation are completely soluble, show an initial fall with the attainment of a steady value when other chemical changes have become negligible. This suggests that wool alcohols do not suffer a deterioration of surface activity on oxidation. Their diminished efficiency as an emulgent may be attributable to loss of solubility in the hydrocarbon phase of the emulsion. The observations

concerning antioxidants are of interest and may be of practical value in the preservation of wool alcohols and emulsions prepared therefrom.

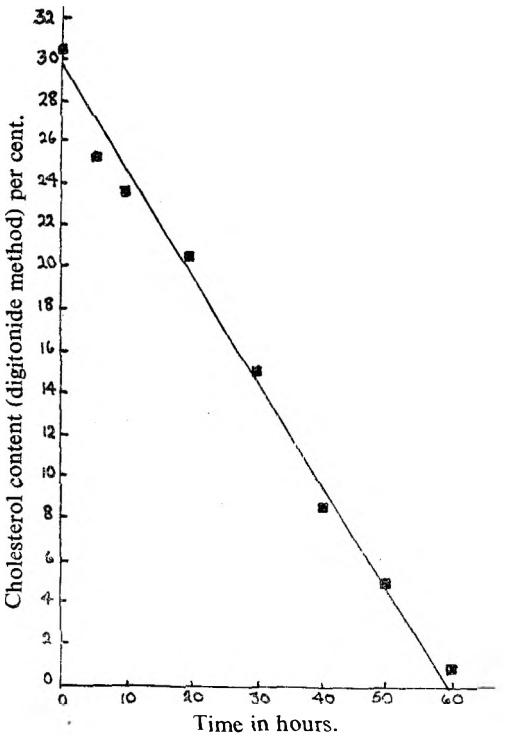


FIG. 8.

SUMMARY

1. Wool alcohols have been subjected to oxidation under varying conditions and progressive changes in physical and chemical properties have been observed. Such changes include—rise in acid and saponification value, decrease in acetyl value and cholesterol content.

2. The interfacial tension-reducing power of total wool alcohols and some isolated fractions have been measured using the pendant drop method. This value does not show any appreciable change as oxidation proceeds.

3. Wool alcohol emulsions suffer breakdown on storage. This is accompanied by rise in acid value of the oil phase. Both effects can be retarded by the inclusion of antioxidants in the formula.

4. Some tentative mechanisms based on the results obtained have been put forward to explain the changes which wool alcohols undergo on oxidation.

Much of the practical work was carried out by Miss E. Graydon, P. Hills and G. Sumpter. The authors wish to thank A. G. Wright for his work in connection with the design and construction of the two types of apparatus illustrated and the Directors of Herts Pharmaceuticals Limited for permission to publish this work.

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DISCUSSION

The Paper was read by Mr. D. E. Seymour.

The CHAIRMAN said that the increasing use of wool alcohols in pharmacy made it important to study what happened when the material deteriorated, as it undoubtedly did. One wondered whether the British Pharmacopœia Commission should not consider the use of antioxidants in some of the rather unstable preparations in question.

PROFESSOR SPRING (Glasgow) asked whether the authors had any evidence to show whether the oxidation was solely confined to the cholesterol fraction. He assumed from the evidence put forward that the cholesterol fraction was involved. He would like to know the views of the authors on the increase in the acetyl value.

DR. K. BULLOCK (Manchester) said he was very interested in Fig. 3, where apparently a clear light phase was shown. Had the authors any theory as to what went on in that light phase?

MR. A. W. BULL (Nottingham) said that the authors had shown that oxidation was a factor to be seriously considered in the use of wool alcohols in pharmaceutical preparations, and had indicated that the use of antioxidants would reduce deterioration. Some indication was given in the paper of the rate of deterioration with and without antioxidants under rather drastic conditions—i.e., with a relatively large surface area compared with total bulk. Could the authors give some information on the rate of deterioration of wool alcohols in lump form, both with and without the antioxidants?

Woodard had referred to the deterioration of penicillin ointment and to the fact that the variations in stability appeared to be connected with differences in the appearance of the wool alcohols and had drawn attention to the oxidised film. He thought that the Pharmacopœia Commission, in considering the formula for penicillin ointment, should pay special attention to the points brought out in the present paper and consider whether wool alcohols was a good ingredient in penicillin ointment. They themselves found that penicillin ointment was far less stable than penicillin eye ointment, for example.

MR. R. W. GILLHAM (Leeds) asked whether the authors could suggest a practical method of storing wool alcohols in bulk to prevent surface oxidation. The best method seemed to be to keep the stock down to the minimum and to order freshly as required. Had the authors carried out any work on the stability of penicillin ointment? Two years ago, at the Torquay Conference, a member mentioned that he had made some ointment of penicillin and found it too weak, had added some more penicillin and found that it was still weak, then added more and found it still below strength, and finally he had thrown it away. The suggestion was made that it had something to do with the condition of the wool alcohols used.

MR. J. H. OAKLEY (London) said that this work might partly explain why samples of hydrous ointment made on a larger scale were frequently

unsatisfactory. Was it possible that the storage of large quantities of the wool alcohols, coupled with the greater heat treatment necessary with larger quantities, would make the product less satisfactory? Had the authors any information on the larger-scale handling of wool alcohols?

MR. C. J. EASTLAND (London) asked whether the authors had studied the effect of traces of copper on wool alcohols, and whether they had been able to correlate the copper content with the formation of these oxidation products. Some samples of wool alcohols contained considerable amounts of copper; moreover, the copper content appeared to vary in different parts of any given batch, possibly due to variations in the duration of contact with the copper vessel used.

MR. R. L. STEPHENS (Brighton) asked whether the authors had tried using chelating agents such as ethylene diamine tetracetate. He had seen samples of ointment of resorcin kept for 3 or 4 months which had not become discoloured, while similar samples without this agent had gone a darker colour.

PROFESSOR H. BRINDLE (Manchester) referred to the authors' statement that the higher gallate esters give better results than the propyl esters. Had the authors gone lower in the series? In his experience ethyl gallate was more efficient than propyl gallate. Ethyl gallate had been used by the Ministry of Food during the war as an antioxidant for food fats.

DR. W. MITCHELL (London) suggested that there was a possible danger in introducing antioxidants of a phenolic nature. It was common in cosmetic work to find that the inclusion of traces of such antioxidants caused the development of objectionable colours.

DR. H. DAVIS (London) said that if one dispensed hydrous ointment from the same batch in different types of container the sample in a wide shallow container did not separate as rapidly as the one in a narrow deep container, although this method increased the surface exposed to oxidation. The addition of an antioxidant and a direction that it was best dispensed in a wide flat container would be useful in the Pharmacopœial monograph for this preparation.

MR. D. E. SEYMOUR, in reply, said he would leave the question of the clear light phase to one of his collaborators who did the photographic work, and go straight to the question put by Professor Spring. The authors were not clear about the fate of the cholesterol in this oxidation, but their theory at the moment was as follows. It would appear that the triterpene alcohols, which were polycyclic in nature, produced an acid which condensed with cholesterol, thus reducing the acetyl value. There should be a decrease in acetyl value and not an increase. Some early experiments carried out in their laboratories on the chemical oxidation of the triterpene alcohols showed that it was possible to oxidise completely the triterpenes by chromic acid and be left with cholesterol.

They had had a great deal of experience in the bulk handling of wool

alcohols, and the point about not having too much in stock at a time was very important. It should be stored in the dark and in sealed containers. It was very apparent if one looked at wool alcohols that there was an oxidised surface film, and material which they had had in stock for some time had failed to make an emulsion at all. On examining it afterwards they found that it had an acid value as high as 30; it was totally insoluble in the hydrocarbon phase. It was also important to keep it in large pieces and before use to scrape off the oxidised surface film containing the polymer.

They had recently commenced an investigation of penicillin ointment and were obtaining some surprising results, but they were not consistent.

Mr. Oakley's question about working on a large scale with wool alcohols was important. They had even considered working in an inert atmosphere, for example, under a blanket of carbon dioxide in the homogeniser, but had not yet adopted that procedure because of the technical problems which obviously arose. The possible effect of packing the material when the emulsion might be still warm had occurred to them.

Traces of metals might act as oxidation catalysts, and they were at the moment examining wool alcohols from different sources, to see what metals were present and what the effects might be on oxidation. The materials with which they had worked had been made in all-glass plant, and they did not get these problems so much with that type of material. They had not had any experience with chelating agents.

They had used the higher gallate esters because they were not soluble in water and had even considered cholesterol gallate. The lower esters were soluble in water and might cause darkening by contact with the iron of the plant. They understood that there was not much difference in efficacy between the lower esters and the higher ones, and ethyl gallate was unsuitable in a hydrous preparation because of its solubility in water.

Shallow containers were ideal for wool alcohol emulsions, though there was one great snag about them, namely, the very large surface area exposed to oxidation, which they now believed to be the main cause of breakdown.

MR. K. H. OBERWEGER, dealing with Dr. Bullock's point about the light phase, said they had put it down to an induction phase, which was not uncommon with natural materials of the type in question, possibly due to the presence of some natural antioxidant which would eventually be swamped by oxidation, and thus lose its activity.

THE OCCURRENCE OF METHYL COMPOUNDS IN GALENICALS

By H. E. Brookes and H. K. Johnson

From the Analytical Laboratory, Boots Pure Drug Co., Ltd., Nottingham

Received July 9, 1949

The presence of traces of methyl alcohol in the galenicals prepared from certain vegetable drugs has been previously recorded, and the fact is of considerable interest since the Board of Customs and Excise prohibits its presence in rebateable preparations. Richardson[1] showed evidence of such a possibility and found amounts varying from 0·01 to 0·10 per cent (0·045 to 0·48 per cent. based on alcohol content) in simple preparations of orange peel, gentian root, lemon peel, buchu and compound preparations of orange, gentian and rhubarb. In his opinion, the derivation of the colour in the reaction obtained in the British Pharmacopœia's modification of the Denigés test[2] was not the presence of essential oils or methyl esters, but of methyl alcohol itself, since adequate means were employed for removal of the former[3]. It was suggested that the methyl alcohol was derived from the decomposition of pectin present in orange and lemon peels. No observations on the technique of distillation were given.

Our interest in this matter was renewed recently, because of a complaint by the Customs and Excise Authorities with reference to a rebate claim on a production batch of concentrated compound infusion of gentian. They accepted that certain drugs on distillation may produce small amounts of methyl alcohol or substances giving similar reactions, but maintained that the proportion found, 0·21 per cent., was abnormally high and that the preparation must, therefore, be considered to contain industrial methylated spirit. Since methylated spirit was not used in the manufacture of the concentrated compound infusion of gentian, this and subsequent batches, made with pure alcohol, were tested for methyl alcohol and compared with samples of material marketed by other pharmaceutical houses. The test used was the B.P. test for the presence of methyl alcohol in alcohol which, as stated in Appendix XII G may give a positive response to the presence of methyl compounds as well as to methyl alcohol in the preparation. The former are converted to methyl alcohol in the test and the figures for methyl alcohol in this paper include that derived from such compounds. The results are recorded in Table I.

Methyl alcohol determinations on a number of batches of other galenicals prepared with pure rectified alcohol carried out as routine control, are recorded in Table II.

These results substantiated our contention that methyl compounds can be present in galenicals prepared according to the British Pharmacopœia and justified our intention to reinvestigate the original observations of Richardson, to show whether amounts of methyl compounds which the Customs and Excise Authorities had stated to be inadmissible were, in fact, liable to be present and if possible to determine the source or reasons for their production.

TABLE I

METHYL ALCOHOL IN CONCENTRATED COMPOUND INFUSION OF GENTIAN

Sample No.	Alcohol content per cent.	Methyl alcohol in galenical per cent.	Methyl alcohol per cent. of alcohol content
1	21·9	0·088	0·41
2	20·8	0·046	0·22
3	22·6	0·041	0·18
4	22·0	0·044	0·20
5	22·2	0·089	0·40
6	21·8	0·12	0·55
7	21·8	0·12	0·55
8	21·6	0·10	0·46
9	21·6	0·05	0·22
10	18·2	0·05	0·28
11	18·4	0·05	0·28
12	19·5	0·05	0·26
13	21·3	0·05	0·23
14	22·4	0·025	0·11
15	21·3	0·05	0·24
16	20·2	nil	nil
17	21·4	0·10	0·47

Samples numbered 1 to 5, 8 and 9 are taken from normal manufacturing batches. Those numbered 6 and 7 are from manufacturing batches made with previously disintegrated orange and lemon peels. Numbers 10 to 13 were made on a laboratory scale. Numbers 14 to 17 were materials purchased in the normal way from other drug houses.

TABLE II

METHYL ALCOHOL IN NORMAL BATCHES OF GALENICALS
(Carried out as routine control)

Preparation	Alcohol content per cent.	Methyl alcohol in galenical per cent.	Methyl alcohol per cent. of alcohol content
Ammoniated tincture of valerian	50·1	0·10	0·20
Compound tincture of gentian	41·2	0·008	0·019
Concentrated compound tincture of gentian B.P. 1932, 5th addendum	35·2	0·008	0·023
Camphorated tincture of opium	58·0	0·0025	0·004
Tincture of belladonna	68·4	0·0055	0·008
Tincture of ipecacuanha	23·6	nil	nil
Tincture of orange	73·6	0·007	0·009
Tincture of squill	55·2	nil	nil
Tincture of calumba	58·4	nil	nil
Tincture of lemon (for syrup of lemon)	34·4	0·11	0·32
Tincture of hyoscyamus	66·4	0·007	0·011
Strong tincture of ginger	82·8	0·008	0·01
Tincture of aloes	39·6	0·008	0·02
Tincture of opium	42·8	0·008	0·019
Compound tincture of rhubarb	50·0	0·005	0·01
Tincture of serpentary	59·0	0·006	0·01
Tincture of myrrh	84·8	0·02	0·024
Tincture of strophanthus	67·6	0·001	0·001
Liquid extract of senega	39·6	0·44	1·1
Liquid extract of cascara	22·4	nil	nil
Liquid extract of liquorice	17·8	nil	nil
Liquid extract of ergot	48·4	0·005	0·010
Liquid extract of sarsaparilla	13·2	0·004	0·030
Concentrated infusion of orange peel	21·2	0·12	0·57
" " " "	22·0	0·12	0·55
" " " "	22·0	0·08	0·36
Concentrated infusion of valerian	23·0	0·033	0·14
Elixir of senna	12·8	0·08	0·62
Concentrated compound decoction of sarsaparilla	21·0	0·0018	0·009

EXPERIMENTAL

The alcohol content was obtained (a) by the British Pharmacopœial method or (b) by direct distillation; where ammonia was present, the distillate was neutralised to solid phenolphthalein and redistilled.

The method of the British Pharmacopœia for detection of methyl

alcohol was adapted to quantitative use by comparison against controls as follows:

An amount of the distillate obtained by method (a) or (b) calculated by preliminary experiment to contain the equivalent of between 0·0001 ml. and 0·001 ml. of methyl alcohol was taken, sufficient alcohol added to produce finally a 10 per cent. solution, and diluted to 5 ml. with water. The pharmacopœial test for methyl alcohol was carried out on this solution and controls containing 0·0001, 0·00015, 0·0002, etc., ml. of methyl alcohol in 5 ml. of 10 per cent. methyl alcohol-free ethyl alcohol, and the colour produced compared.

All alcohol used in the experiment and on production tests was checked for methyl alcohol content, and all results recorded were corrected for the trace of methyl alcohol present in the ethyl alcohol used. The results given in Tables I and II were obtained on distillates obtained by the British Pharmacopœial method.

In order to account for the high methyl alcohol content of liquid extract of senega recorded in Table II, a batch was examined at each stage of manufacture. The alcohol was determined by the method of the British Pharmacopœia (a) and by direct distillation (b), the tests being carried out on the distillates. The methyl alcohol content of the menstruum was not detectable by the method of the British Pharmacopœia. The results are given in Table III.

TABLE III

METHYL ALCOHOL FOUND DURING THE MANUFACTURE OF LIQUID EXTRACT OF SENEGA

	Methyl alcohol by method (*a*) per cent.	Methyl alcohol by method (*b*) per cent.
Reserve portion of the percolate	0·28	0·01
Soft extract from the remainder of the percolate...	0·74	0·02
Liquid extract of senega	1·40	0·01

The above results indicate that the methyl alcohol obtained was produced during the assay. To confirm this, water, ethyl alcohol and an excess of sulphuric acid were added to the residue from the distillation of the liquid extract by method (b), and a normal distillation performed. The methyl alcohol content of the distillate was 0·52 per cent. calculated to the liquid extract. Since this was insufficient to account for the high methyl alcohol content of the final product, this process was repeated, starting with a distillation from the liquid extract by method (b). When the acid, alcohol and water had been added to the residue, it was distilled very slowly. The distillate gave 0·88 per cent, methyl alcohol calculated to the liquid extract, a quantity greater than the previous figure, but less than that obtained for the galenical. A further addition of water and alcohol to the residue was again slowly distilled. The methyl alcohol content of the distillate was 0·48 per cent. calculated to the liquid extract. The sum of the percentages of methyl alcohol obtained from the residue is 1·36, an amount comparable with that given for the finished product in Table III. This shows that the length of time taken in the distillation

by the method of the British Pharmacopœia affects the amount of methyl alcohol formed, and, further, that its source is undoubtedly the senega.

A production batch of concentrated infusion of senega B.P. gave methyl alcohol when the method of the British Pharmacopœia for alcohol content by acid distillation was used, to the extent of 0·36 per cent., whereas by direct distillation the amount was only 0·04 per cent. This latter figure was higher than expected and suggested that some alkaline hydrolysis occurred during preparation.

Owing to our Tinctures Department finding the recovered alcohol from the preparation of this galenical to be contaminated with methyl alcohol, the process was examined in detail, using the same drugs. The schematic diagram indicates the method of production, points of control testing and amounts of methyl alcohol found at each stage of production.

The method of preparation used in our Tinctures Department differs from that described in the British Pharmacopœia by the use of ammonia in the percolation, the modification aiding preparation and giving a better final product. That this small deviation from the British Pharmacopœia directions does not affect the findings was shown by the results of testing a sample prepared strictly by the British Pharmacopœia procedure, in which closely similar figures for the final methyl alcohol content were obtained.

In conclusion we determined the methyl alcohol in the distillate from a mixture of powdered senega, pure ethyl alcohol and water:

	per cent. methyl alcohol calc. to senega
1. Direct distillation, adding no acid or alkali	0·10
2. Distilled in presence of acid	1·20
3. Distilled in presence of ammonia, neutralised and redistilled	0·80

Hence it is demonstrated that for senega root and its galenical preparations, a small amount of preformed methyl alcohol may be present, but alkaline or acid distillation produces considerable amounts probably due to hydrolysis, acid giving the greater. The presence of methyl alcohol was confirmed by the Riche and Bardy[4] test.

The presence of methyl salicylate in senega to an extent of 0·25 per cent has been recorded[5,6]. Distillation of small amounts of methyl salicylate with dilute alcohol and water showed that ammonia will hydrolyse this ester completely in dilute alcohol, but dilute acid has no effect.

Richardson[1] suggested that the methyl alcohol might be derived from the hydrolysis of pectin and the gelatinisation of senega preparations has been attributed to the occurrence of pectinous substances in the drug[6,7]. By distillation of pectin far in excess of the proportions likely to be present in the galenicals tested, some methyl alcohol was obtained, the

amount from acid distillation being comparable with that from alkaline distillation. Pectin should not be present in senega preparations to any extent, since alkaline distillation of senega produces much less methyl alcohol than acid. This suggests other substances to be the main source of the methyl alcohol.

In our opinion, the production of methyl alcohol by hydrolysis during distillation may not necessarily always account for its presence in galenicals. In support of this, we can quote some experiments on concentrated infusion of orange which was examined at all stages of manufacture, showing relatively high proportions of methyl alcohol on each test using the method of the British Pharmacopœia for alcohol determination (which does not require acid distillation and eliminates esters by light petroleum extraction of the distillate) but also showing similar results by a direct neutral distillation. These results suggest that in preparations of orange, the methyl alcohol may occur in the orange peel.

Conclusions

Based on our work with senega and the results given by routine examinations of other galenicals, it may be inferred that methyl compounds may occur in many galenicals in more than traces and that some of these may be hydrolysed to methyl alcohol. Since acid or alkaline media increase this hydrolysis, it would be desirable for the test for methyl alcohol to be made on a distillate from neutral solution. As considerable frothing impedes distillation of many galenicals, especially senega, unless acid is present, a correct alcohol content should be determined by the method of the British Pharmacopœia, and the methyl alcohol test carried out separately, distilling from neutral solution.

Summary

1. The presence of methyl alcohol in galenicals in more than traces is recorded and previous observations confirmed.

2. The source of the methyl alcohol in senega preparations has been closely investigated.

3. The method of the British Pharmacopœia for alcohol content has been shown to be the main contributory cause of the production of methyl alcohol by acid hydrolysis of the senega extractives.

4. In these and other galenicals some preformed methyl alcohol is present.

W wish to express our thanks to Mr. D. A. Hughes for his co-operation in the preparation of samples for examination, and to the Directors of Boots Pure Drug Co., Ltd., for permission to publish this work.

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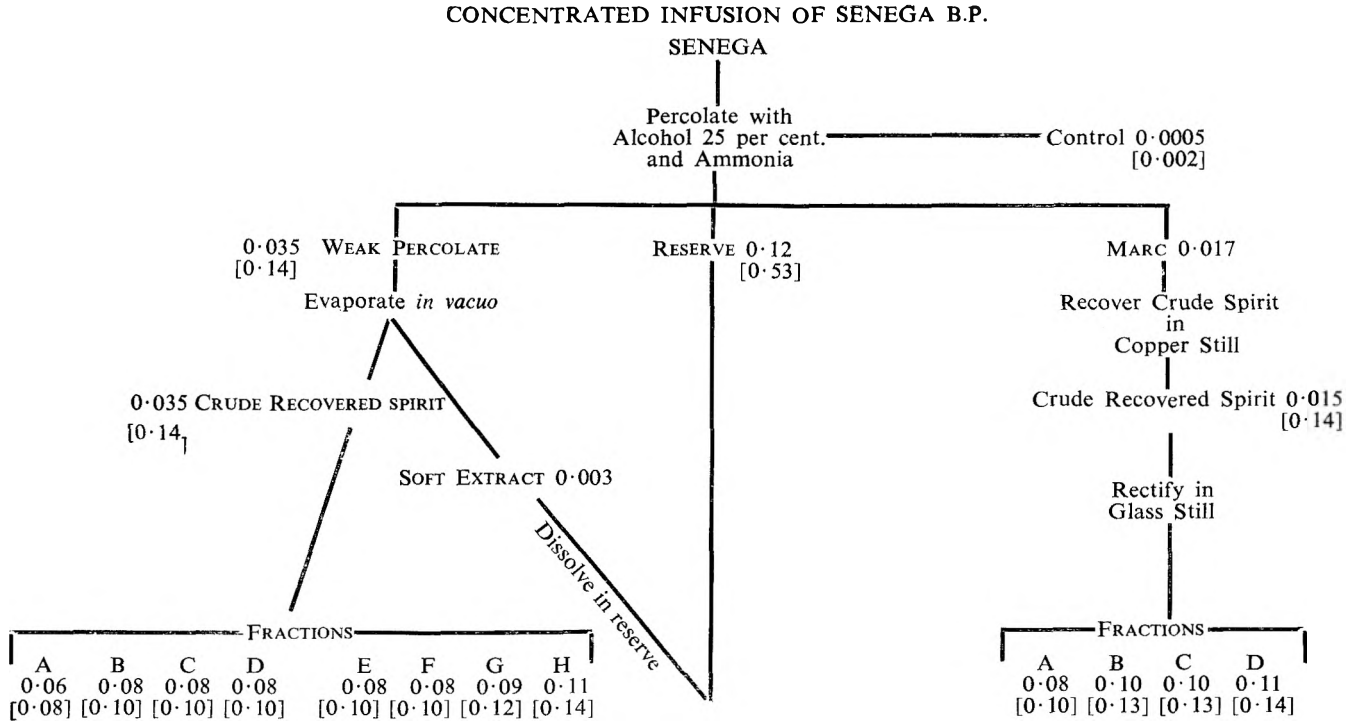

Figures are percentages of Methyl Alcohol determined by Method [b].

Bracketed figures are percentages of Methyl Alcohol calculated to the Alcohol content.

DISCUSSION

The paper was read by Mr H. E. Brookes.

The CHAIRMAN said he thought most people who dealt with galenicals had been aware that traces of methyl alcohol were frequently found when no methyl alcohol had been used in their preparation, particularly in the case of senega. A most interesting observation in this paper was the fact that the acid distillation used in the B.P. assay increased the amount of methyl alcohol in the case of senega.

DR. G. E. FOSTER (Dartford) said that he had also met this problem of methyl alcohol in galenicals. The Customs and Excise Authorities would not allow a rebate in certain cases, although only pure ethyl alcohol had been used. If anyone implied that methylated spirit had been used, it was up to them to prove it, and he did not think they would be able to prove it against the manufacturing records that could be produced.

DR. W. MITCHELL (London) said that these results would be welcomed, not only by manufacturers, but by the Government Chemist's Department. The Customs and Excise Department were not so accommodating as Dr. Foster had suggested, and the line they always took was that no manufacturing records could cover the possibility of accidental mixing of a small amount of industrial spirit with S.V.R. The Customs and Excise Authorities had complained to his Company that a sample of concentrated infusion of senega contained an excessive amount of methyl alcohol. After much correspondence the Authorities had accepted the Company's figure of 0·1 per cent., but had said that the maximum they could allow was 0·03 per cent. and anything in excess of that must have been added, accidentally or deliberately. They had admitted that they did not suspect the Company's bona fides, but were tied by the literature. The Authorities had further said that if evidence could be provided to support the claim that infusion of senega could contain 0·1 per cent. or more they would consider it. In his laboratory they had recently tested many batches of senega preparations, and so far had not found any containing as much as 0·1 per cent. of methyl alcohol. He was interested that the present authors had found these higher figures, and he asked how much methyl alcohol they had found in their alcohol. Had they used the chromotropic acid test as a confirmatory test? He had found it satisfactory in confirming the B.P. test.

MR. BROOKES, in reply, said that they also had obtained very little redress from the Customs and Excise Authorities. He was pleased to have the figure of the maximum amount of methyl alcohol allowed as 0·03 per cent. By the usual Schiffs reagent method it was difficult to determine the content of methyl alcohol in alcohol, but they had managed to get some very good Schiffs reagent and found it to be 0·0005 per cent. of methyl alcohol. He had not used the chromotropic acid test.

PARA-AMINOSALICYLIC ACID—PART III

SOME FURTHER STUDIES ON THE *IN VITRO* TUBERCULOSTATIC BEHAVIOUR OF *PARA*-AMINOSALICYLIC ACID AND RELATED COMPOUNDS

BY D. J. DRAIN, C. L. GOODACRE AND D. E. SEYMOUR

From the Research Department, Herts Pharmaceuticals, Ltd.

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IN our previous report[1], some preliminary results showed that simple substitution in the *p*-aminosalicylic acid molecule did not produce any compound possessing an *in vitro* tuberculostatic activity markedly higher when using *Mycobacterium tuberculosis* H37RV strain. This paper is concerned with the *in vitro* study of a wider range of 1-2-4-tri-substituted aromatic compounds, the majority of which have not been previously described; their chemistry will be reported upon elsewhere. The culture medium used throughout the work and the technique for the determination of activity was similar to that described in our earlier paper[1]. It may be of interest to note that we have confirmed the observations made by Youmans *et al.*[2] that the presence of tween 80 (polyoxyethylene sorbitan mono-oleate) in the medium markedly influences the tuberculostatic behaviour of *p*-aminosalicylic acid and other compounds; this

TABLE I

THE INHIBITORY CONCENTRATION OF *p*-AMINOSALICYLIC ACID AND RELATED COMPOUNDS. INOCULUM OF 0·001 MG/ML. OF THE H37RV STRAIN

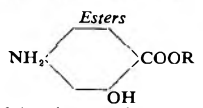
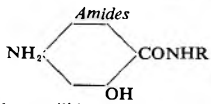
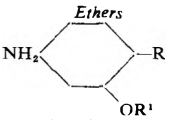
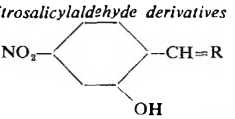
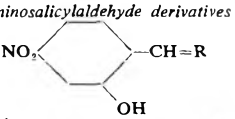
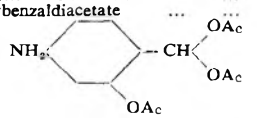
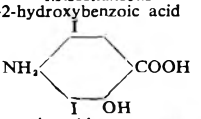
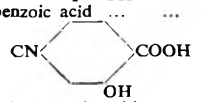
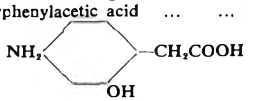
	Standards	*Inhibitory concentration mg. 100 ml. (after 14 days at 37° C.)*
GROUP A		
1	*p*-Aminosalicylic acid	0·0487—0·0243
2	Streptomycin	0·0121—0·006
GROUP B	*Esters* 	
3	β-Diethylaminoethyl-4-amino-2-hydroxybenzoate (R = -$CH_2.CH_2.N(C_2H_5)_2$	0·1 — 0·01 (0·195— 0·0975)
4	β-Hydroxyethyl-4-amino-2-hydroxybenzoate (R = -CH_2-CH_2-OH)	100—10
GROUP C	*Amides* 	
5	4-Amino-2-hydroxybenzanilide (R = -C_6H_5)	10—1
6	4-Amino-2-hydroxybenz-*p*-toluidide (R = -$C_6H_4.CH_3$)	10—1
7	4-Amino-2-hydroxyhippuric acid (R = -CH_2COOH)	100—10
8	4-Amino-2-hydroxybenzoyl alanine (R = -CH(CH_3)-COOH)—	10—1
9	4-Amino-2-hydroxybenzoyl-DL-aspartic acid (R = -CH(CH_2COOH)-COOH)	100—10

TABLE I—*continued*

	Standards	Inhibition concentration mg. per 100 ml. (after 14 days at 37°C.)
GROUP C—cont.		
10	4-Amino-2-hydroxybenzoic acid hydrazide (R=-NH_2)	0·1—0·01 (0·0975—0·0487)
GROUP D	*Ethers* 	
11	* 4-Amino-2-methoxybenzoic acid· (R=-COOH.R′,-CH_3)	> 100
12	4-Amino-2-methoxytoluene hydrochloride (R=-CH_3.R′,-CH_3)	100—10
13	4-Amino-2-ethoxytoluene hydrochloride... (R=-CH_3,R′,-C_2H_5)	100—10
14	4-Amino-2-*n*-propoxytoluene hydrochloride (R=CH_3,R′,-CH_2-CH_2-CH_3)	100—10
15	4-Amino-2-*n*-butoxytoluene hydrochloride (R-CH_3,R′,-CH_2 $(CH_2)_2$ CH_3)	100—10
GROUP E	*4-Nitrosalicylaldehyde derivatives* 	
16	4-Nitrosalicylaldehyde (R=O)	1—0·1
17	4-Nitrosalicylaldoxime (R=N.OH)	10—1
18	4-Nitrosalicylaldehyde semicarbazone (R=N.NHCONH_2)	10—1
19	4-Nitrosalicylaldehyde thiosemicarbazone (R=N.NHCSNH_2)	1—0·1
GROUP F	*4-Aminosalicylaldehyde derivatives* 	
20	4-Aminosalicylaldoxime (R,N.OH)	10—1
21	4-Aminosalicylaldehyde thiosemicarbazone R=N.NHCS.NH_2)...	0·01 —0·001 (0·003 —0·0015)
22	4-Amino-2-acetoxybenzaldiacetate ... ... 	10—1
GROUP G	*Miscellaneous*	
23	3-5-diiodo-4-amino-2-hydroxybenzoic acid ... ... 	0·1—0·01 (0·0975—0·0487)
24	4-cyano-2-hydroxybenzoic acid ... ... 	100—10
25	4-Amino-2-hydroxyphenylacetic acid ... ... 	> 100

* Youmans, Raleigh, and Youmans[7] reported that this compound does not inhibit growth of 0·01mg/ml of *M. tuberculosis* H37RV at a concentration of 10 mg/100 ml.

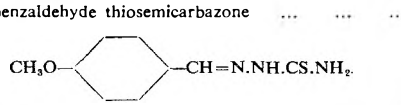
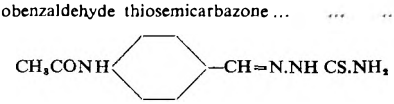
substance was, however, retained in our medium as its presence facilitates the turbidimetric standardisation of inoculum. The less soluble compounds were dissolved in propylene glycol, which was found to have no tuberculostatic effect when used in concentrations of 1 per cent. and below.

Table I shows the inhibitory concentrations of the compounds examined when using a standard inoculum of 0·001 mg./ml. (dry bacterial substance) of M. *tuberculosis* H37RV strain. The standards used were *p*-aminosalicyclic acid and streptomycin and compounds exhibiting activities of an order similar to the two standards were examined in closer dilutions.

The above results coupled with those obtained by others[3,4,5,6,7,8] suggest that there is no apparent relationship between chemical structure and *in vitro* tuberculostatic activity in this group of compounds, and from data obtained it would appear difficult to predict the effect of a simple variation in structure on *in vitro* activity. One can conclude that there is a high degree of specificity of the *p*-aminosalicylic acid molecule for tuberculostatic activity, and with the possible exception of the esters, alteration in molecular structure gives rise to markedly diminished activity. The anomalous results relating to the activity of the esters[1,4,5,6,7] may be due to their low solubility and tendency to hydrolyse under conditions of test. The effect of nuclear substitution with the exception of halogens has received no attention, and we propose to investigate this type of compound and report more fully.

We are of the opinion, however, that some of the compounds referred to in our previous paper, together with compounds No. 3,4,7 and 10, are worthy of a preliminary *in vivo* examination in view of the possibility that they may possess certain advantages over *p*-aminosalicylic acid by being less rapidly absorbed and excreted. The aldehyde derivatives (Groups E and F) have special interest for animal work in view of the reports by Domagk *et al.*[9] that certain benzaldehyde thiosemicarbazone derivatives have promising properties in the treatment of some forms of tuberculosis. Compound No. 21 (the thiosemicarbazone of *p*-aminosalicylaldehyde) is considered to possess sufficiently high *in vitro* activity to justify a trial in animals. For comparison purposes we report in Table II the *in vitro* activities of two of the compounds studied by

TABLE II

		Inhibition concentration mg./100 ml.
26	*p*-methoxybenzaldehyde thiosemicarbazone ... ... 	0·0487—0·0243
27	*p*-acetylaminobenzaldehyde thiosemicarbazone ... ... 	0·0975—0·0487

Domagk, namely, *p*-methoxybenzaldehyde thiosemicarbazone and *p*-acetylaminobenzaldehyde thiosemicarbazone.

The observation that the action of *p*-aminosalicylic acid is antagonised by *p*-aminobenzoic acid[1,7] has led us to study the possible effects of other members of the vitamin B group; at this stage it is possible to report that such antagonism is not displayed by pteroylglutamic acid. The significance of this cannot be realised until further results are obtained. It is interesting to note that the activity of sulphathiazole against *Staphylococcus aureus*[10] is similarly antagonised by *p*-aminobenzoic acid and not by folic acid.

Summary

1. A series of tri-substituted aromatic compounds have been synthesised and their *in vitro* activities against the tubercle bacillus determined. The results of the study indicate no apparent relationship between structure and activity.

2. The thiosemicarbazone of *p*-aminosalicylaldehyde has been synthesised and its activity compared with the thiosemicarbazones of *p*-acetylaminobenzaldehyde and *p*-methoxybenzaldehyde.

The authors wish to thank Mr. D. Suddaby, B.Sc., A.R.J.C., and Mr. B. W. Mitchell, B.A. (Cantab.), A.R.I.C., for the preparation of some of the compounds and the Directors of Herts Pharmaceuticals Limited for permission to publish these results.

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PARA-AMINOSALICYLIC ACID—PART IV

ATTEMPTS TO INDUCE RESISTANCE TO *PARA*-AMINOSALICYLIC ACID, IN STRAINS OF *MYCOBACTERIUM TUBERCULOSIS*

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Received, June 23, 1949

THE unfortunate limitation of streptomycin in the treatment of tuberculosis, due to the development of drug resistance, suggested to us the necessity for an investigation as to whether similar phenomena might occur with *p*-aminosalicylic acid.

The investigation was concerned with attempts to induce drug-resistance *in vitro*, and with a study of strains of *M. tuberculosis* isolated from patients before and during *p*-aminosalicylic acid treatment. The H37RV strain was cultivated in Dubos medium containing decreasing amounts of the acid. After a suitable incubation period, the bacilli in the tubes containing the highest concentration of *p*-aminosalicylic acid which was still allowing growth were freed from it by washing, and used to inoculate a similar series of tubes. No increased resistance was demonstrated by this method, in fact, no growth at all occurred at the dilutions used after two or more passages. These results are confirmed by Hurni[1] who reported on a similar study while this work was in progress. In view of the unsatisfactory results obtained with the above method, the following procedure was adopted in an attempt to induce drug resistance. A large inoculum (0.5 mg./ml. of dry bacterial substance) of *M. tuberculosis* H37RV was introduced into Dubos medium containing *p*-aminosalicylic acid in a concentration of of 100 mg./ml. After 14 days' incubation, a similar concentration of *p*-aminosalicylic acid in Dubos medium was inoculated from this culture. Inhibition concentration tests were made at monthly intervals, and after 10 months (20 passages) the organisms showed a similar *p*-aminosalicylic acid sensitivity to that at the beginning of the experiment. A duplicate experiment was carried out using a medium containing no tween 80 with similar results.

These results suggest that under the above conditions the H37RV strain does not become resistant to the tuberculostatic action of *p*-aminosalicylic acid. In an attempt to obtain further and possibly more significant data we obtained cultures of *M. tuberculosis* isolated before and during treatment from patients suffering from pulmonary tuberculosis who received 20 g./day for 6/day week of sodium *p*-aminosalicylate given orally in divided doses. The strains, after cultivation in Dubos medium, were subjected to sensitivity tests by the method described in our previous paper[4] using a standard inoculum (0·001 mg./ml. of dry bacterial substance). The results of these experiments are shown in Table I and it will be seen that in only one instance (case 9) was there any indication of development of resistance to *p*-aminosalicylic acid. It is of interest to note, however, that there does exist a slight difference in sensitivity between different strains. These results together with those of

Lehmann[2], Hurni[1], and Seivers[3] suggest that "drug-fastness" is not a significant problem in the treatment of tuberculosis with this drug.

Whilst this paper was in course of preparation, Graessle and Pietrowski[5] reported that repeated exposure of *M. tuberculosis* H37RV to *p*-aminosalicylic acid for 120 days failed to produce an increase in the resistance of the strain.

TABLE I

Case No.	Before treatment	*p*-Aminosalicylic Acid treatment		
		2 months	3 months	4 months
1	0·0243—0·0121			0·0975—0·0487
2	0·0487—0·0243	0·0243—0·0121		
3	0·0243—0·0121			0·0487—0·0243
4	0·0243—0·0121			0·0487—0·0243
5	0·0243—0·0121			0·0487—0·0243
6	0·0243—0·0121			0·0243—0·0121
7	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
8	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
9	0·0243—0·0121			3·125 —1·56
10	0·0243—0·0121	0·0243—0·0121		0·0121—0·006
11	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
12	0·0487—0·0243	0·0243—0·0121		0·0487—0·0243
13	0·39 —0·195	0·39 —0·195		0·0975—0·0487
14	0·0487—0·0243	0·0487—0·0243		0·0487—0·0243
15	0·0243—0·0121	0·0243—0·0121		0·0243—0·0121
16	0·0243—0·0121			0·0243—0·0121
17	0·0487—0·0243		0·0487—0·0243	
18	0·0487—0·0243		0·0487—0·0243	
19	0·0243—0·0121	0·0243—0·0121		
20	0·0243—0·0121	0·0243—0·0121		
21	0·0487—0·0243			0·0487—0·0243
22	0·0243—0·0121			0·0243—0·0121
23	0·0243—0·0121	0·0243—0·0121		
24	0·0243—0·0121	0·0243—0·0121		
25	0·0487—0·0243		0·0487—0·0243	

The authors are indebted to Dr. R. Shoulman of the Highlands Hospital, Winchmore Hill, and Dr. J. Alston of the Archway Hospital, N.19, for the supply of the cultures. They also wish to thank the Directors of Herts Pharmaceuticals Ltd., for permission to publish.

SUMMARY

1. After repeated exposure of *M. tuberculosis* H37RV to *p*-aminosalicylic acid for 10 months, no increase in resistance developed.

2. Out of a total of 25 strains of *M. tuberculosis* isolated from patients receiving *p*-aminosalicylic acid, only one developed any increase in resistance after four months' treatment.

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

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Para-Aminosalicylic acid has now become of such general interest that it is hardly necessary to quote in detail the many references in the literature to its chemistry, pharmacology and clinical value. Most of the studies on the pharmacology of this substance have been carried out in Sweden[1,2,3,4,5] and America [6,7,8,9,10] and little has so far been published in this country. The object of the present series of experiments was to confirm, if possible, certain aspects of the published work and to extend the study of the pharmacology of *p*-amino-salicylic acid in other directions.

The acute and chronic toxicity of the drug in the strain of mice used in this laboratory was first determined, together with the pathological effects of prolonged administration. Blood levels and rate of excretion in mice were studied and following the work of Beyer[11,12] the possible effect of 4-carboxy-phenylmethanesulphonanilide (Caronamide) in retarding excretion was investigated. Erdei and Snell[13], Nagley and Logg[14] and other clinical workers have commented on the fall in temperature produced in tuberculous patients treated with *p*-aminosalicylic acid and it seemed desirable to study the possible antipyretic effect of the drug. O'Connor[15], bearing in mind the known action of salicylates, referred to the possibility of causing hypoprothrombinæmia by the use of *p*-aminosalicylic acid, and it was felt that experimental evidence on this point, also, would be useful.

The chemotherapeutic action on *Mycobacterium tuberculosis* in mice was investigated, using a technique very similar to that described by Youmans and Mc Carter[16]. A report by Woody and Avery[17] that potassium iodide potentiated the action of streptomycin in guinea-pigs infected with tubercle suggested that this work should be repeated using *p*-amino-salicylic acid as the tuberculostatic drug.

Experimental Methods

Toxicity. White Swiss mice of both sexes weighing 20 to 25 g. were used. In acute experiments, the drug was administered intravenously, subcutaneously, intraperitoneally and orally as an aqueous solution of the sodium salt, and observations of mortality were continued for 7 days after injection. Oral doses were given by stomach tube under light ether anaesthesia. In chronic experiments, the drug was given dissolved in the drinking water and the amount consumed daily per cage of 10 animals was measured. The solution was provided in drinking bulbs with constricted outlets, so that the animals were able to obtain liquid by licking the ends of the tubes but no loss of the solution by spilling occurred.

Blood levels. Blood samples were obtained at intervals after administration, by decapitation of the mice. Estimations were carried out by the method described below.

Antipyretic effect. Rabbits of a mixed stock and both sexes, weighing 2 to 3 kg., were used. Pyrexia was induced by intravenous injections of a solution of pyrogen prepared from *Pseudomonas æruginosa* by the method of Welch *et al.*[18]. Rectal temperatures were determined with clinical thermometers. *p*-Aminosalicylic acid and known antipyretics were administered intravenously and orally.

Prothrombin Times. Prothrombin times on rabbits were determined by Quick's method[19]. Blood samples were removed from the ear vein by syringe containing 0·1 ml. of 0·10M sodium oxalate per ml. of blood.

Chemotherapy. Mice were used of the same strain as those used for the toxicity experiments. They were infected with *M. tuberculosis* H37 Rv. These organisms were cultured in a modified Dubos medium containing NaH_2PO_4 1·0 g.; Na_2HPO_4, $12H_2O$ 6·25 g.; Sodium citrate 1·5 g.; $MgSO_4,7H_2O$ 0·6 g.; Casein hydrolysate 2·0 g.; Tween 80 0·5 g .; tap water 1000 ml. Cultures for mouse inoculation were grown in the above medium for 10 days at 37°C. Most of the clear supernatant was poured off and the deposit harvested. A 5 ml. sample of the thick suspension was assayed for moist weight of organism per ml. by centrifuging, resuspending in a small amount of alcohol (50 per cent.) and centrifuging again in a tared tube. The supernatant liquid was poured off and all excess of moisture was removed with a small cotton wool swab. This method appeared to give reasonably reproducible results when several assays were made on one suspension. The assayed suspension was standardised to twice the concentration required finally, using the modified Dubos medium.

Fresh egg yolks were separated and shaken with an equal volume of sterile saline and strained through several layers of muslin. Material for inoculation was prepared by mixing equal parts of egg yolk mixture and double strength culture suspension. 0·25 ml. of this suspension containing 0·3 mg. moist weight of organisms (occasionally 0·1 or 1·0 mg.) was injected intravenously into each mouse.

The survival time of untreated mice infected with 0·3 mg. of organisms was usually between 15 and 30 days and survival time over a period of 28 days was used as the main criterion of the protective action of drugs. Post-mortem examinations were carried out on the heart, lung, liver, spleen, kidney and gut for macroscopic lesions and in certain cases microscopical examinations of fixed sections were made. We have not found the close correlation between post-mortem appearance and dose of drug reported by Raleigh and Youmans.[20]

The drug under examination was administered either subcutaneously, twice a day, into the interscapular region or orally in the drinking water, as described for the toxicity experiments. In the latter case, measurements were made of the daily consumption per cage of 5 mice. Dosage was commenced within an hour or two of infection, with the exception of the curative experiments, where the drug was given 14 days after infection.

Estimation of *p*-Aminosalicylic Acid

After trials of the numerous published methods, we find the following modification of the method described by Klyne and Newhouse[21] the most useful for our purpose.

(i) *Determination in Blood.*

Reagents: Solution of *p*-toluenesulphonic acid 20 per cent. in 0·2N hydrochloric acid.

Hydrochloric acid, 10 per cent. v/v.

0·75M disodium hydrogen citrate prepared by mixing 39·4 g. of citric acid with 16·8 g. of sodium hydroxide and diluting to 250 ml.

Solution of *p*-dimethylaminobenzaldehyde 2 per cent. in alcohol (95 per cent.).

1·0 ml. of oxalated blood is added to 13 ml. of distilled water with shaking. After 3 minutes, 6 ml. of *p*-toluenesulphonic acid reagent is added slowly with shaking. The mixture is filtered through a Whatman No. 4 paper and refiltered if necessary to give a clear solution. To 5 ml. of the filtrate, add 1·0 ml. of the citrate buffer, 0·4 ml. of 10 per cent. hydrochloric acid and 1·0 ml. of *p*-dimethylaminobenzaldehyde reagent. Make up to 10 ml. and read on the Spekker absorptiometer after 15 minutes, using 0 B1 filters and a 1 cm. cell. A blank is similarly prepared from normal blood.

(ii) *Determination in Urine.*

Reagents:—Trichloracetic acid, 10 per cent. w/v.

Solution of sodium hydroxide 1 per cent.

Hydrochloric acid, 10 per cent. v/v.

Ehrlich's reagent (1 g. of *p*-dimethylaminobenzaldehyde in 3·3 ml. of concentrated sulphuric acid diluted to 50 ml. with distilled water).

(*a*) *Procedure for free* p-*aminosalicylic acid.*

The urine is diluted to 50 volumes with distilled water and 1 ml. of the diluted urine added to 9·0 ml. of the following mixture:—Trichloracetic acid 10 per cent. w/v 32 ml.; solution of sodium hydroxide 1 per cent. 30 ml.; Ehrlich's reagent 10 ml.; distilled water 18 ml. The colour is read on the Spekker absorptiometer using 0B1 filters and 1 cm. cells.

(*b*) *Procedure for total* p-*aminosalicylic acid.*

To 1·0 ml. of urine 2 ml. of hydrochloric acid 10 per cent. v/v is added and the whole diluted to 10 ml. with distilled water. This diluted urine is heated on a water-bath at 100°C. for 1 hour, when all the acid free, and conjugated, is decarboxylated to *m*-aminophenol. The solution is then diluted to 25 ml. and the *m*-aminophenol estimated by the method described for free acid, substituting the 1 : 25 acid solution for the 1 : 50 aqueous dilution. Standard curves are used to convert the extinction coefficients into *p*-aminosalicylic acid concentrations in all estimations.

RESULTS

Toxicity.

(*a*) *Acute Experiments.* The sodium salt was administered but the doses in Table I are expressed as the acid. Mortality figures give the number of mice dead 7 days after administration.

TABLE I

ACUTE TOXICITY OF *p*-AMINOSALICYLIC ACID IN MICE

Dose in mg./g.	6·0	5·0	4·0	3·5	3·25	3·0	2·5	2·0
Mortality intraperitoneally	5/5		1/5	0/5		0/5		
Mortality intravenously ...			2/2	3/3	5/5	5/5	2/5	0/5
Mortality subcutaneously	5/5	5/5	2/5			0/5		
Mortality orally		5/5	2/5			1/5		

From these figures, the approximate LD50 for the various routes appear to be as follows:—Intraperitoneal 4·5 mg./g.; Intravenous 2·5 mg./g.; Subcutaneous 4·0 mg./g.; Oral 4·0 mg./g.

(*b*) *Chronic Experiments.*

TABLE II

CHRONIC TOXICITY OF *p*-AMINOSALICYLIC ACID IN MICE

Concentration in Drinking water	Average Consumption in mg./mouse/day	Limits of Average Consumption mg./mouse/day	Mortality after 12 weeks
per cent.			
1·0	53·3	22·0—70·0	7/10
0·75	40·6	27·0—50·0	5/9
0·5	24·6	13·4—40·0	0/10

From these figures, assuming the average weight of a mouse to be 20 g., it would appear that the approximate LD50 for daily administration over a period of 3 months is slightly less than 2·0 mg./g. The maximum tolerated dose under the same conditions would be rather more than 1·25 mg./g.

Subcutaneously, 1·25 mg. and 2·5 mg./mouse given twice daily for 8 weeks caused no deaths in groups of 5 mice.

Comparable figures are not readily available in the literature, the majority of workers being content to quote the concentration of drug in the diet. McClosky *et al.*[10] found the maximum tolerated intravenous dose to rats and rabbits to be more than 2·0 mg./g. and 0·5 mg./g. respectively. In guinea-pigs, single oral doses of 3 mg./g. produced 30 per cent. mortality. The same authors found that daily oral doses of 0·5 mg./g. to guinea-pigs produced a mortality of 70 per cent. after 32 doses. It would seem, therefore, that mice are rather more resistant than guinea-pigs to prolonged oral administration of *p*-aminosalicylic acid. Other workers, [2,7,8] have reported toxicity figures based on diet concentrations but, in the absence of records of consumption, it is not possible to compare them with those obtained in the present experiments. Levaditi *et al.*[22] appear to be the only other workers who have administered

oral doses to mice over a long period (75 days) and their figures of 2·5 mg./g. for a toxic dose and 1·0 to 1·5 mg./g. for a tolerated dose agree well with those given above.

A histological examination of the tissues of the mice used in the chronic series of experiments was made either at the time of death or, if the animals survived the test period, at the end of the test. Many of the kidney sections showed a cloudy swelling with some congestion of the tubules and the appearance of a deposit in the lumen. The glomeruli appeared normal. Many of the liver sections showed a loss of cell outline, with granulation of the cytoplasm and signs of nuclear degeneration. The hearts, lungs and small intestines appeared normal in nearly all cases.

Blood levels. Following oral doses of 0·5 mg./g to mice, the blood level rose rapidly to about 30 mg./100 ml. in 1 hour and fell quickly to zero after about 4 to 5 hours. This is in agreement with earlier reports using rabbits and guinea-pigs[2,10]. Caronamide given simultaneously with *p*-aminosalicylic acid in oral doses of 0·125 to 0·5 mg./g. had no effect on the peak blood level but appeared to delay somewhat the fall in blood levels. (Fig. 1.)

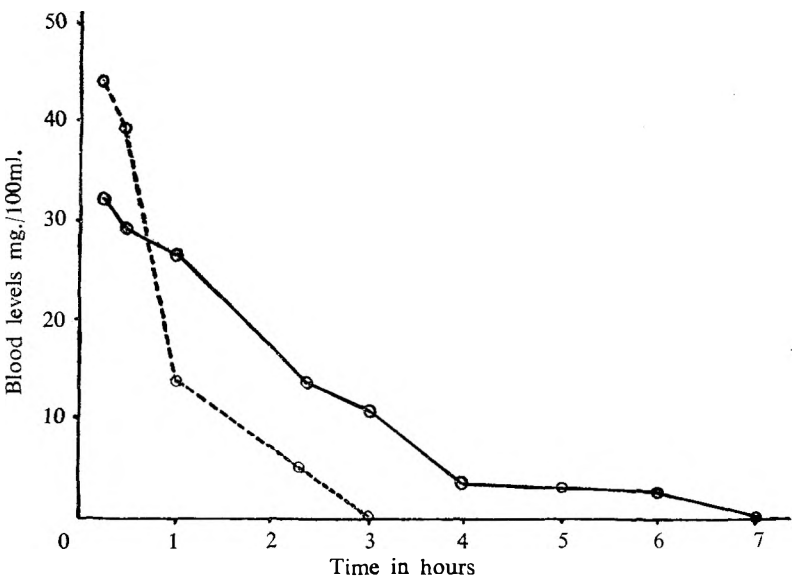

FIG. 1.—Effect of Caronamide on *p*-Aminosalicylic Acid blood levels in mice

O———O { *p*-Aminosalicylic acid 0·5 mg./g. orally
Caronamide 0·5 mg./g. orally

O- - - - -O *p*-Aminosalicylic acid 0·5 mg./g. orally

This result was not obtained regularly and, in any case, did not appear to be of the same magnitude as that obtained with penicillin (Fig. 2). However, it seemed worthy of trial in human volunteers and Figure 3. shows the negligible effect exerted by caronamide on the blood levels of *p*-aminosalicylic acid in two normal men.

Apart from the blood concentration produced in mice by one large

dose, it appeared desirable to examine the blood levels occurring under the conditions of the therapeutic test. Accordingly, determinations were made on mice which had been receiving 0·125 per cent. of the acid in their drinking water (=0·25 mg./g./day) for 4 days. The value, 1·2 mg./100 ml., was almost too low to measure with any degree of accuracy. Nevertheless, this level, as will be seen, is adequate to protect mice for a considerable time against tuberculosis infection, and this fact would seem to suggest that high blood levels may not be essential in clinical treatment, or that the acid is converted *in vivo* into a more active compound.

Antipyretic effect. *p*-Aminosalicylic acid and aspirin were administered orally to rabbits, simultaneously with an intravenous injection of bacterial pyrogen. Rectal temperatures were taken hourly for 5 hours thereafter,

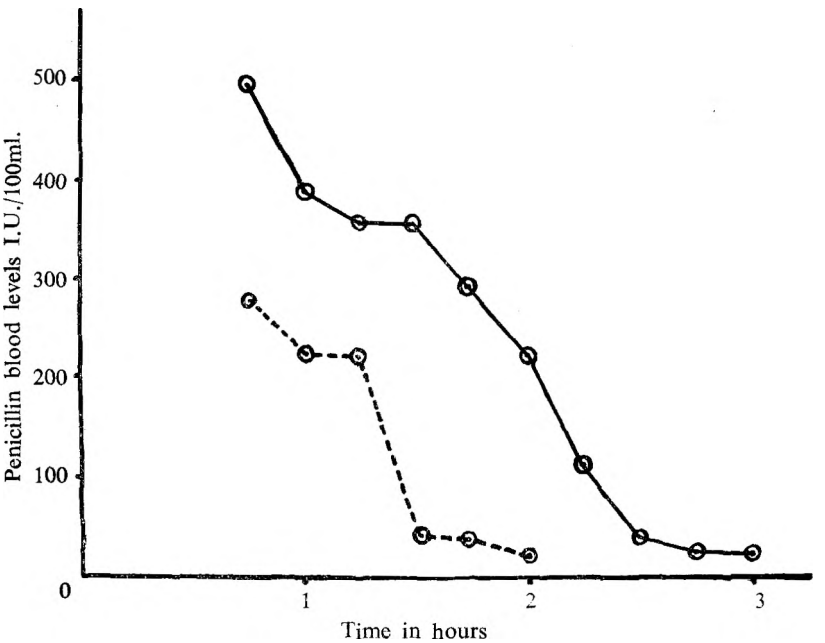

FIG. 2.—Effect of Caronamide on Penicillin blood levels in mice

O———O { Penicillin 25 I.U./g. subcutaneously
Caronamide 0·5 mg./g. orally
O- - - - - -O Penicillin 25 I.U./g. subcutaneously

and the average maximum rise of temperature calculated. No significant difference was noted between the control group and that receiving the acid, whereas a significant fall in temperature occurred in those animals treated with aspirin. It seems clear that *p*-aminosalicylic acid, under these conditions, has no antipyretic action.

Effect on Prothrombin Times. Two experiments on rabbits were carried out. In experiment 1, normal prothrombin times were determined twice at an interval of 1 week, followed by daily oral doses of 0·5 g. After 3 days' treatment no significant change of prothrombin time had

TABLE III

ANTIPYRETIC EFFECT OF *p*-AMINOSALICYLIC ACID

Number of Rabbits	Dosage	Average maximum rise in temperature °F.
12	Standard Pyrogen 1 : 250 Dilution 5 ml./rabbit intravenously	1·91
11	Standard Pyrogen as above + 1·0 g./kg. of acid orally	1·7
12	Standard Pyrogen as above + 200 mg./kg. of aspirin orally	1·27

occurred. In experiment 2, only one normal level was determined, followed by daily intravenous doses of 0·5 g. After 5 days, a statistically significant increase in prothrombin time was observed, but this fell again to a non-significant level after 12 days. Table IV gives the results of these experiments and it would appear that, on the whole, *p*-aminosalicylic acid, in the dose used, has very little effect on the prothrombin times of rabbits.

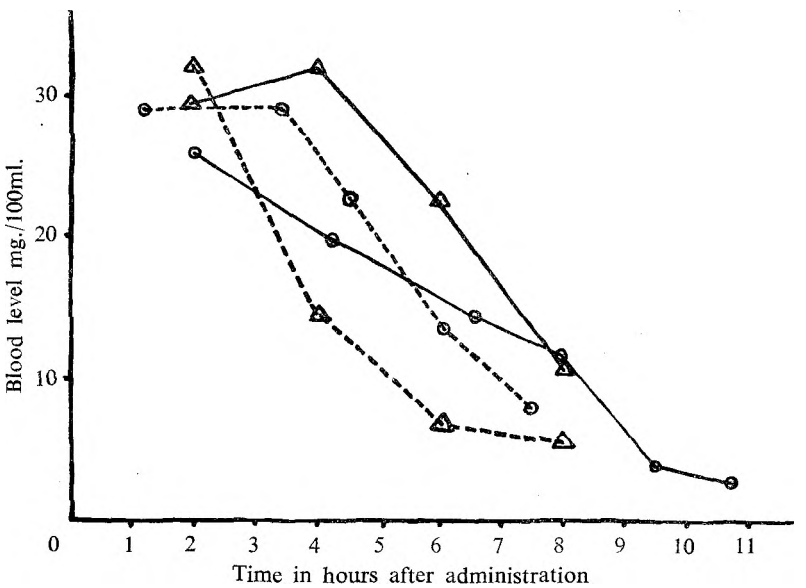

FIG. 3.—Effect of Caronamide on *p*-Aminosalicylic Acid blood levels in human subjects

(All doses administered orally)

Δ————Δ Subject A 20 g. of *p*-aminosalicylic acid + 3 g. of caronamide; 4 hours later 3 g. of caronamide.

Δ------Δ Subject A 20 g. of *p*-aminosalicylic acid

O————O Subject B 20 g. of *p*-aminosalicylic acid + 3 g. of caronamide; 4 hours later 3 g. of caronamide.

O------O Subject B 20 g. of *p*-aminosalicylic acid

TABLE IV

EFFECT OF *p*-AMINOSALICYLIC ACID ON THE PROTHROMBIN TIMES OF RABBITS

Experiment	Number of Rabbits	Daily Dose	Average Prothrombin times in seconds			
			Normal	After 3 days treatment	After 5 days treatment	After 12 days treatment
1	4	0·5 g. of acid per rabbit orally	10·5 ; 9·25	9·95		
2	5	0·5 g. of acid per rabbit intravenously	8·1		10·5	9·5

Therapeutic action. The first experiments were directed towards determining the sensitivity of the test to graded doses of drug and infective organisms. Table IV gives the average survival rates of fairly large numbers of mice under varying conditions, treated with graded oral doses.

TABLE V

EFFECT OF *p*-AMINOSALICYLIC ACID ON SURVIVAL RATES OF MICE INFECTED WITH *M. tuberculosis*

Concentration in drinking water	Acid consumed mg./mouse/day	Infecting dose of organism H37 RV (intravenously)		
		1·0 mg.	0·3 mg.	0·1 mg.
per cent.		per cent.	per cent.	per cent.
0·25	10·0 approx.	68 (15)	90 (20)	90 (20)
0·125	5·0 approx.	30 (20)	83 (35)	100 (20)
0·0625	2·5 approx.	20 (15)	55 (40)	87 (15)
nil	nil	0 (25)	18 (65)	56 (25)

Figures in brackets indicate the number of mice used.

It will be seen that the test offers a reasonable degree of discrimination, particularly in the group receiving 0·3 mg. of organism. Streptomycin was used as a standard of comparison and Figure IV shows the survival rates of mice infected with 0·3 mg. H37 RV and treated with twice-daily subcutaneous injections of streptomycin or oral doses of *p*-aminosalicylic acid.

Taking the area of each curve as a measure of the comparative action of the respective doses of the two drugs, we found that streptomycin administered subcutaneously was between 3 and 6 times more active than *p*-aminosalicylic acid orally.

TABLE VI

COMPARATIVE THERAPEUTIC ACTION OF *p*-AMINOSALICYLIC ACID AND STREPTOMYCIN SUBCUTANEOUSLY

Daily dose	Mortality after 8 weeks	Average survival times	Average grade of lung lesions
2·5 mg. of acid	0/5	56 days (max.)	3·75
750μ g. of streptomycin	0/5	56 days (max.)	2·6
375μ g. of streptomycin	2/5	44 days	4·0
Nil	5/5	18·2 days	4·0

When both drugs were given subcutaneously, the results were as shown in Table VI. Giving some weight to the observations on the lung lesions, it would appear that streptomycin is about 5 times more active than *p*-aminosalicylic acid when both drugs are given subcutaneously.

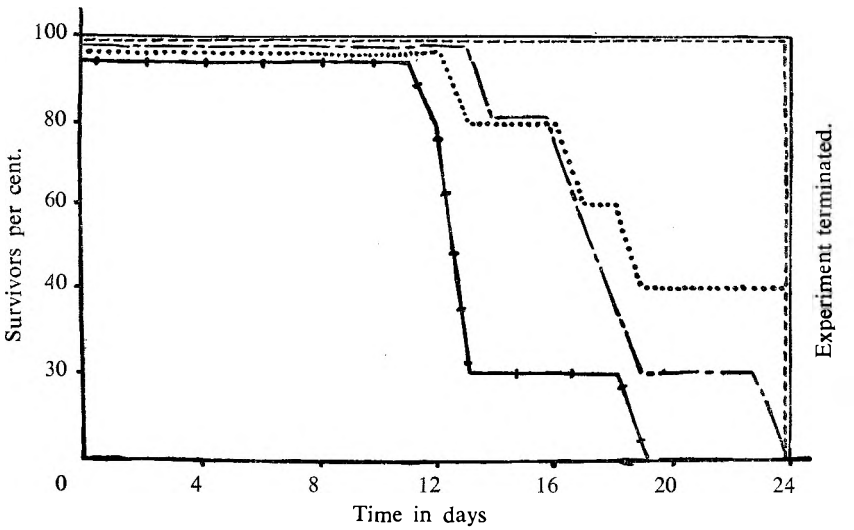

FIG. 4.—Comparison of *p*-Amineosalicylic acid (orally) with Streptomycin (subcutaneously) against experimental tuberculosis in mice

———————— 2 × 375 μg. of streptomycin daily.
– – – – – – – – – – 0·125 per cent. *p*-aminosalicylic acid (*c.* 5·0 mg./day)
— — — — — — — 2 × 187·5 μg. of streptomycin daily
..... 0·0625 per cent. *p*-aminosalicylic acid (*c.* 2·5 mg./day)
—+—+—+— Controls

Curative Action. It was realised that the above type of experiment, although quite useful for screening purposes, did not reproduce the conditions occurring clinically, where an established infection has to be treated. Some experiments were, therefore, carried out using mice in which the infection had been allowed to develop, as judged by the histological examination of control animals, before treatment with *p*-aminosalicylic acid was commenced. Figure V gives the result of such an experiment, from which it will be seen that the acid has some curative effect, although, naturally, it is not so effective as when given from the commencement of the infection. Further experiments on these lines are being carried out.

Development of resistance. Evidence that resistance does not develop either experimentally or clinically has already been reported [4,14,23,24,25]. Our own experiments, so far, confirm these reports and it is unnecessary to report them in detail. Briefly, the experiments were of two types, one in which the infective organism (H37 RV) had been grown in medium containing the acid and had then been used to infect mice which were subsequently treated with *p*-aminosalicylic acid, and the second in which the infective organism was obtained from mice which had been treated with *p*-aminosalicylic acid for a considerable period.

In the first case, the organism had been grown in a medium containing 1·0 mg./ml. of *p*-aminosalicylic acid and mice infected with this strain were found to be quite as responsive to the therapeutic action of the acid as mice infected with a similar strain grown in a normal medium.

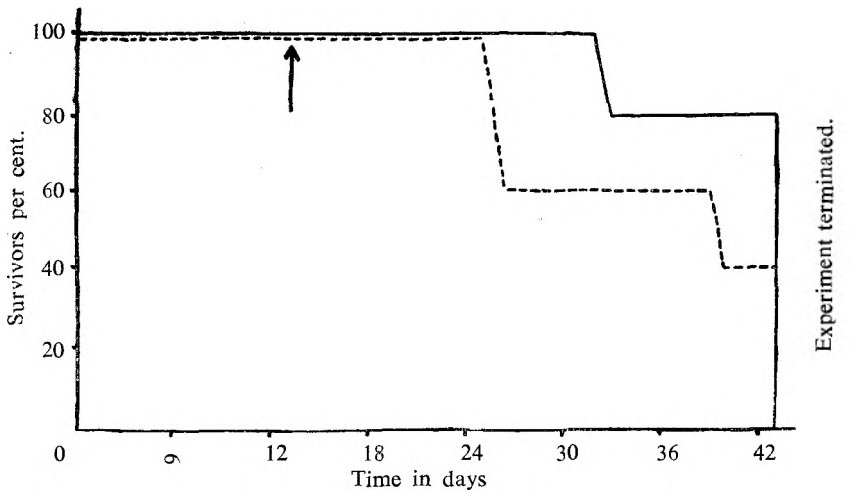

FIG. 5.—Effect of *p*-aminosalicylic acid against an established tubercular infection in mice.

———— 0·125 per cent. *p*-aminosalicylic acid (*c.* 5·0 mg./day)
– – – – – – Controls
↑ Dosage of *p*-aminosalicylic acid started

In the second case, the strain of organism was isolated from mice which had received 10 mg./day orally for 42 days and, here again, mice infected with this strain showed no sign of resistance. These results would appear to support previous work that resistance does not occur to any large extent, but, in view of the suggestion[26] that it is not easy to demonstrate resistance to streptomycin experimentally in mice, it was thought advisable to repeat the second type of experiment using more passages through *p*-aminosalicylic acid-treated animals and with streptomycin controls. These experiments are still in progress and will be reported at a later date.

TABLE VII

EFFECT OF POTASSIUM IODIDE ON THE TUBERCULOSTATIC ACTION OF *p*-AMINOSALICYLIC ACID

Concentration in drinking water	Mortality	Average survival times
0·0625 per cent. of acid	7/10	31·3 days
0·0625 per cent. of acid + 0·04 per cent. of potassium iodide ...	7/10	39·3 days
0.04 per cent. of potassium iodide	8/10	19.0 days
Nil	5/5	18·2 days

Potentiation by Potassium Iodide. Woody and Avery[17] have reported that potassium iodide has a marked potentiating effect on the action of streptomycin in tuberculous guinea-pigs. It was decided to investigate the effect of potassium iodide on the therapeutic action of *p*-aminosalicylic acid, and Table VII gives the results. Statistically, the difference in survival time is not significant. The effect of potassium iodide is not so marked as reported by Woody and Avery for streptomycin, but the above experiment is being repeated using increased concentrations.

Discussion

The experiments in which *p*-aminosalicylic acid was given in the drinking water are of a more quantitative character than those previously described, inasmuch as the daily drug consumption has been measured. This has enabled us to determine a rather more accurate maximum oral tolerated dose over a period of 3 months and to make a comparison of the tuberculostatic action of streptomycin and *p*-aminosalicylic acid. Parenterally or orally, *p*-aminosalicylic acid appears to be much less active than streptomycin parenterally.

Caronamide has been shown to have little or no action on *p*-aminosalicylic acid blood levels and it seems likely therefore that the renal mechanism for the excretion of the acid is different from that obtaining in the case of penicillin. This antibiotic has been shown to be excreted largely *via* the tubules and the experimental result suggests that *p*-aminosalicylic acid may be excreted mainly by the glomeruli.

p-Aminosalicylic acid has been shown to have no effect on the prothrombin times of rabbits, and it would appear therefore that it does not form any derivative of salicylic acid capable of affecting the prothrombin times and that prolonged use is unlikely to lead to hæmorrhagic states.

The contrast between the lack of antipyretic effect shown by *p*-aminosalicylic acid experimentally and the reports of such an effect clinically may be due to the difference in sensitivity of rabbits and man to antipyretic drugs. The dose of acetylsalicylic acid necessary to exert a marked antipyretic effect in rabbits is considerably larger, weight for weight, than for a similar effect in man, and other workers[27] have reported similarly large doses of other antipyretics as being required by rabbits. Co Tui and Schrift's report[28] of the relative insensitivity of rabbits to pyrogen compared with man is probably another demonstration of this disparity. Brownlee[29] has very recently shown that *p*-aminosalicylic acid has a peripheral vaso-dilating action in the human subject, and suggests that the heat loss so produced may explain the drug's antipyretic effect. In a fur bearing animal, such as the rabbit, the vascular effect would be unlikely to produce such a marked heat loss and this may be an alternative explanation of the different action of the drug in rabbits and man. It is proposed to investigate further the antipyretic action of *p*-aminosalicylic acid using partially depilated rabbits.

Resistance to *p*-aminosalicylic acid does not seem to occur, nor does

potentiation by potassium iodide, but further work on both these points is desirable.

SUMMARY

1. Acute and chronic toxic doses of *p*-aminosalicylic acid to mice have been determined by various routes of administration.

2. Prolonged oral and subcutaneous administration to mice produces some pathological effects on the liver and kidney.

3. A method is described for the estimation in blood and urine, and blood levels curves are given in mice and man.

4. Caronamide has no effect on the blood levels in man.

5. It appears to have no hypoprothrombinæmic or antipyretic effect in rabbits.

6. It exerts a protective effect in mice infected with *M. tuberculosis* H37 Rv, but it is not so effective an antitubercular drug as streptomycin.

My grateful thanks are due to my colleagues Mr. A. S. Beach, Mr. J. H. Marvin, Mr. T. R. Middleton and Mr. C. R. B. Williamson for their assistance in this work, and I am indebted to the Directors of British Chemicals and Biologicals Ltd., for permission to publish this paper.

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THE SEPARATION AND IDENTIFICATION OF ERGOT ALKALOIDS BY PAPER PARTITION CHROMATOGRAPHY

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During the long history of ergot much time has been devoted to the study of the alkaloids, to which the drug owes its therapeutic and toxic properties. Many workers have contributed to our knowledge of this subject and, in particular, the problem of assaying ergot has been a major topic, engaging the attention of laboratories all over the world. A good summary of analytical work up to 1937 has been given by Barger[1] and a later account has more recently been published by the American Pharmaceutical Association[2]. In spite of the effort already expended on this project, however, available methods still lack specificity; biological assays measure the total potency due to the alkaloids in the preparation under examination, while colorimetric and other chemical methods estimate the total alkaloids, the water-insoluble or the water-soluble alkaloids. The results may be stated in terms of alkaloid calculated as ergotoxine, ergotamine or ergometrine but no method so far available will allow the actual amounts of these alkaloids in a specimen of ergot to be determined.

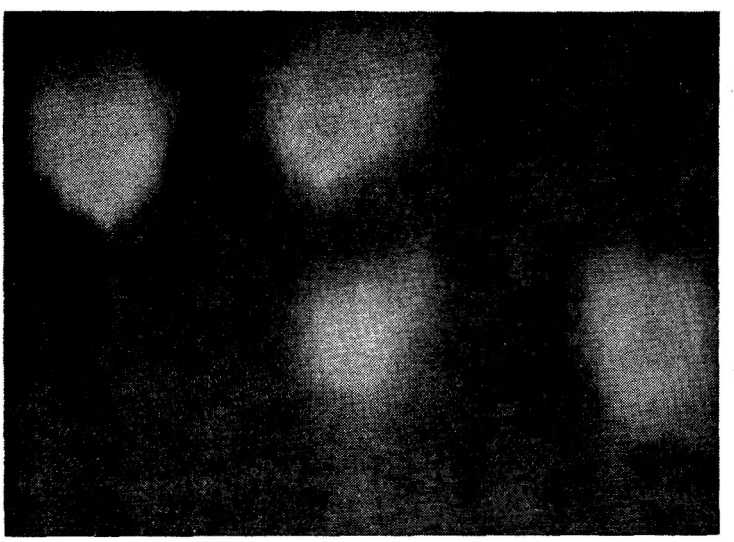
It was the purpose of the work, described in the present communication, to apply the technique of paper partition chromatography[3] to this problem for, in view of the remarkable success of this new technique in other fields, there was good reason to believe that results of interest would emerge.

Separation of Alkaloids

The separation of amino-acids on paper chromatograms using a water saturated solvent, first described by Consden, Gordon and Martin[3], is well known. These workers used strips of filter paper, on which were placed spots of the solutions under test, the upper end of the paper being immersed in a horizontal trough containing the water-saturated solvent. The strips were hung in an airtight chamber in an atmosphere saturated with water and solvent. The solvent from the trough gradually passed down the paper causing separation of the amino-acids which were subsequently located on the paper by use of the ninhydrin reagent. A suitable chamber was provided by using a stoneware drain-pipe standing vertically and closed by a lead tray at the bottom and a sheet of plate glass at the top. Water saturated with solvent was placed at the bottom of the chamber in order to maintain the required atmosphere. Full details are given in the original paper, to which the reader is referred.

Using this technique and employing *n*-butyl alcohol-acetic acid-water mixture as solvent we investigated the behaviour of ergot alkaloids on Whatman No. 1 paper. The alkaloids gave little or no colour with the ninhydrin reagent but their positions on the paper were readily detected by their fluorescence in ultra-violet light. It became immediately evident

that the water-insoluble alkaloids, of the ergotoxine and ergotamine groups, passed down the paper with the solvent front. However, separation of the water-insoluble from the water-soluble alkaloids occurred and, what is more important, ergometrine and ergometrinine passed down the paper at different rates and consequently could be separated and identified. The solvent employed was prepared by shaking together *n*-butyl alcohol (4 vols.), glacial acetic acid (1 vol.) and water (5 vols.). After standing the upper layer of *n*-butyl alcohol was placed in the trough and used as the moving phase on the chromatogram, while the lower aqueous acetic acid layer was placed in a dish at the bottom of the drain-pipe. Satisfactory chromatograms resulted when the alkaloids were employed as tartrates, lactates or maleates, but when present as sulphates little movement on the paper took place. A chromatogram was prepared by placing 0·05 ml. of solution containing 5 to 10 μg. of alkaloid on the paper. After allowing the solvent to run down the paper for 12 to 18 hours the chromatogram was air dried and examined using a suitable source of filtered ultra-violet light. With samples of pure ergometrine and ergometrinine, in solution as maleates, the following R_F values were obtained: —Ergometrine, 0·59; ergometrinine, 0·68.



Left: Ergometrine. *Right*: Ergometrinine.
Centre: Mixture of Ergometrine and Ergometrinine.

Fig. 1. Section of chromatogram of ergot alkaloids in ultra-violet light, showing the relative positions of ergometrine and ergometrinine.

Figure 1 shows a typical chromatogram, photographed while exposed to ultra-violet light, and illustrates the application of the method for purposes of identification.

An obvious extension of this work is the development of a method for estimating ergometrine, the most important ergot alkaloid. In this we

have only been partially successful. The fluorescent spots corresponding to the respective alkaloids may be marked on the chromatogram with pencil and subsequently measured in area or cut out and extracted to remove the alkaloids. These methods did not yield satisfactory results, however, for the quantitative removal of the alkaloids from the paper proved surprisingly difficult and the area of the spots could not be used for quantitative work. Our most successful results were obtained by preparing a series of standard spots of ergometrine on a paper chromatogram at the same time as the sample under test was examined, and matching the fluorescence of the respective spots under ultra-violet light. As with most fluorescent methods difficulties caused by quenching of the fluorescence at high concentrations were encountered and it was necessary to use solutions containing less than 0·001 per cent. of ergometrine before the fluorescence approached a linear relationship to concentration. Matching was performed visually and the precision was not high, the experimental error being of the order of ±20 per cent. As an alternative procedure chromatograms were prepared using serial dilutions of the standard and test solutions until dilutions were reached at which the fluorescence of the alkaloidal spots disappeared. This technique failed owing to the difficulty of determining the end-point, for even at extreme dilutions the fluorescence persisted.

The Ergometrine Content of Ergot

The first attempt to determine chemically the water-soluble alkaloids in ergot was made by Hampshire and Page[4] and their method has formed the basis of the B.P. 1948 assay process, in which the water-soluble alkaloidal content is expressed in terms of ergometrine and estimated from the difference between estimations of the total and water-insoluble alkaloids. Numerous other researches on the same topic have been published but the only processes of note are those developed by Grove[5] and by Powell *et al.*[6] by which the ergometrine is extracted and determined directly by colorimetric assay. These latter processes have been further developed in a collaborative study described by Smith[7] and have also been included in a report on the assay of ergot issued by the American National Formulary Committee[8].

We have estimated the ergometrine content of ergot, using our chromatographic technique and, for this purpose, the following process was used. 5 g. of ergot, ground to No. 60 powder, was defatted by extraction with light petroleum and air dried at room temperature. The resulting powder was thoroughly mixed with 0·3 g. of sodium bicarbonate and water was added, drop by drop, with stirring until there was obtained a well damped mass, which was then placed in a percolator (made from a piece of glass tubing 1 inch in diameter) and extracted with peroxide-free ether containing 5 per cent. of alcohol. Extraction of the alkaloids was slow and was best performed by drawing off 10 ml. of percolate at hourly intervals until about 70 ml. had been collected, after which the marc and solvent were allowed to remain in contact overnight before further percolate was withdrawn. Collection of the percolate was then continued, as described above, until another 100 ml. had been withdrawn, when the

process was stopped and the marc allowed to remain in contact with the solvent overnight. The extraction was completed in the morning by drawing off portions of percolate at half-hourly intervals until the total volume of extract amounted to 200 to 250 ml. The percolate was collected in an amber glass bottle and the whole process carried out in a dark room. After transferring the ethereal extract to a separating funnel the alkaloids were removed by shaking with 6 quantities, each of 10 ml., of 5 per cent. lactic acid; the acid extracts being collected in a graduated cylinder and the volume adjusted to 100 ml. with distilled water. Portions of this extract were then suitably diluted with 1 per cent. lactic acid until 0·05 ml. placed on a No. 1 Whatman paper strip and developed with *n*-butyl alcohol-acetic acid-water mixture, as described in the first section, gave a fluorescent spot approximately equal in intensity to that obtained with an ergometrine standard containing 0·2 to 0·5 μg. of ergometrine in 0·05 ml. By running a series of standards on the same paper the ergometrine content of the ergot was estimated.

Table I summarises the results obtained on samples of ergot, which were also assayed by the process of the B.P. 1948 and by that of the American National Formulary Committee[5].

It will be seen that the N.F. and chromatographic methods, in most cases, gave results for the ergometrine contents which were in reasonable agreement, but that the B.P. process afforded figures for the water-soluble alkaloids far in excess of the ergometrine present. Accordingly some of the final tartaric acid extract containing the total alkaloids, obtained by the B.P. process, was submitted to chromatographic analysis. Besides alkaloids of the ergotoxine group ergometrine and ergometrinine were detected. In addition a slower moving band was present above the ergometrine and this we were able to identify as being due to lysergic and *iso*-lysergic acids. It thus became clear that the use of boiling ether for extraction resulted in partial hydrolysis of the alkaloids with the production of lysergic acid, which was removed together with the water-soluble alkaloids and was estimated as ergometrine. The B.P. process therefore, did not yield reliable figures for the ergometrine content of the drug.

TABLE I

Sample of Ergot	B.P. 1948 Process		National Formulary Committee Process		Ergometrine determined chromatographically
	Total alkaloids expressed as ergotoxine	Water-soluble alkaloids expressed as ergometrine	Total alkaloids expressed as ergotoxine	Water-soluble alkaloids expressed as ergometrine	
	per cent.	per cent.	per cent.	per cent.	per cent.
1	0·22	0·043	0·215	0·023	0·019
2	0·10	0·0125	0·12	0·0086	0·006
3	0·19	0·038	0·195	0·025	0·026
4	0·16	0·040	0·195	0·020	0·016
5	0·20	0·045	0·21	0·022	0·024

Some preliminary experiments with liquid extract of ergot B.P. 1914 and liquid extract of ergot B.P. 1932 showed that the presence of ergometrine in these preparations could readily be confirmed by suitably

diluting with 1 per cent. lactic acid and preparing a chromatogram. Rough assays could be carried out as the colouring matter of the extracts remained almost stationary at the top of the chromatograms. Old extracts contained both ergometrinine and lysergic acid.

Ergometrine Preparations

At the British Pharmaceutical Conference 1948 Foster and Stewart[9] gave an account of the stability of ergometrine preparations. During the discussion on the paper Eastland[10] questioned the conclusion of the authors that the drop in biological potency of ergometrine maleate injection on storage was due to conversion of ergometrine into ergometrinine, and stated that this was at least partially due to hydrolysis of ergometrine to lysergic acid. Eastland supported his views by experimental data showing that while colorimetric assays of ergometrine injection, filled into ampoules under nitrogen and incubated for some months at 45°C., showed little loss of alkaloid by direct assay, a lower figure for the alkaloidal content was obtained if the injection were rendered alkaline and the alkaloid extracted before being estimated. It was suggested that the difference was due to lysergic acid.

During the past year we have carried out direct and indirect assays on ampoules of injection of ergometrine maleate B.P. 1948 which had been stored at room temperature for periods up to 10 years. For indirect assays the alkaloid was extracted with ether after making alkaline and saturating the solution with sodium chloride as described by the N.F. Committee[8]. Preliminary extraction experiments using solutions of pure ergometrine and ergometrinine maleates, of the same strength as used for the injection, showed that 90 to 95 per cent. recovery of ergometrine and 95 to 100 per cent. recovery of ergometrinine resulted. When applied to ergometrine maleate injection the process gave a recovery of 90 to 95 per cent. of the total alkaloidal content when freshly prepared ampoules were employed, but with 5-year-old ampoules the recovery was only 75 to 80 per cent. Allowing for a 10 per cent. loss during the extraction it would appear that some 15 per cent. of alkaloid in the older ampoules remains to be accounted for. The results of Eastland were therefore confirmed.

In order to study the composition of the injection more closely

TABLE II

Sample of ergometrine maleate injection B.P. 1948	Time of storage at room temperature	Components identified on chromatogram
Freshly made and unsterilised ...	—	Ergometrine
Freshly made and sterilised at 10 lb. pressure of steam for 30 minutes	—	Ergometrine Ergometrinine Traces of lysergic and *iso*-lysergic acids
Sterilised and stored at room temperature	5 years	Ergometrine Ergometrinine Lysergic and *iso*-lysergic acids

samples were examined by paper partition chromatography using *n*-butyl alcohol-acetic acid-water mixture as solvent. The results are summarised in Table II.

The general results indicated that on sterilisation of the injection some conversion, estimated to be approximately 20 per cent., of ergometrine to ergometrinine occurs. Very little further conversion appears to take place on storage at room temperature which, however, results in slow hydrolysis of the alkaloids with the formation of lysergic and *iso*-lysergic acids. The presence of the lysergic acids is better shown by using a basic solvent prepared by shaking a mixture of *n*-butyl alcohol (4 vols.), water (5 vols.) and pyridine A.R. (1 vol.), allowing to separate and using the *n*-butyl alcohol layer as the moving phase on the chromatogram, while the aqueous layer is used for saturating the atmosphere of the chamber. Under these conditions and using No. 1 Whatman paper the R_F values were as follows:—Lysergic acid, 0·2; *iso*-lysergic acid, 0·4.

The presence of lysergic acid was further confirmed by extracting the alkaloid from some old injection, the final alkaloidal extract being made with 1 per cent. lactic acid. On preparing a chromatogram with this extract ergometrine and ergometrinine were identified but the lysergic acids, present in the chromatogram of the original injection, had disappeared.

It was of interest to examine chromatographically tablets of ergometrine maleate which had been stored at room temperature and, for this purpose, the tablets were extracted with, or dissolved in, 1 per cent. lactic acid. Very little, if any, formation of ergometrinine or lysergic acid was detected in tablets which had been stored for periods up to 5 years.

Identification of Water-Insoluble Alkaloids

The water-insoluble alkaloids of ergot are distinguished from ergometrine by the presence in their molecular structures of certain amino-acids. Jacobs and Craig[11] found among the products of alkaline hydrolysis of ergotinine the lactam of a dipeptide, derived from L-phenylalanine and D-proline, while Smith and Timmis[12] showed that ergosine gave a similar lactam of the dipeptide of L-leucine and D-proline. Stoll, Hofmann and Becker[13] showed that ergocornine, ergocristine and ergokryptine, isolated from the ergotoxine group of alkaloids, also contained amino-acids of the L-series in addition to D-proline.

TABLE III

ALKALOIDS CHARACTERISED BY STRUCTURES DERIVED FROM LYSERGIC OR *iso*-LYSERGIC ACID, AMMONIA, A KETO ACID, D-PROLINE AND ONE OTHER AMINO-ACID

Additional amino acid	Ergotamine group (pyruvic acid group)	Ergotoxine group (Dimethyl-pyruvic acid group)
L-phenylalanine	Ergotamine Ergotaminine $C_{33}H_{35}O_5N_5$	Ergocristine Ergocristinine $C_{35}H_{39}O_5N_5$
L-leucine	Ergosine Ergosinine $C_{30}H_{37}O_5N_5$	Ergokryptine Ergokryptinine $C_{32}H_{41}O_5N_5$
L-valine	—	Ergocornine Ergocorninine $C_{31}H_{39}O_5N_5$

The relationships of the known water-insoluble alkaloids are summarised in Table III embodying data by Stoll *et al*[13].

The exact mode of linkage of the amino-acids in these alkaloids is not known but Stoll and Hofmann[14] have reported that thermal degradation of ergotamine yielded pyruvyl-phenylalanyl-proline, and Stoll[15] has further reported finding dimethylpyruvyl-valyl-proline, together with lysergic acid amide, in the products from the alkaline hydrolysis of ergocornine.

It is thus clear that the identification of the amino-acids obtained on acid hydrolysis would be of great assistance in the identification of pure alkaloids and, in the absence of major quantities of extraneous amino-acids, might be of help in the examination of cruder preparations. The occurrence of valine in ergocornine and ergocorninine is a specific test for this inter-convertible pair of alkaloids. The presence of phenylalanine would indicate ergotamine or ergocristine and their isomers, further tests such as the identification of the keto-acid and the determination of physical properties being necessary for complete identification of the alkaloids. The finding of leucine would be equally significant for the detection of ergosine and ergokryptine and their isomers.

We have identified the amino-acids in the acid hydrolysates of the alkaloids by the method of paper partition chromatography in the following manner. 10 mg. of the alkaloidal preparation together with 1 to 2 ml. of concentrated hydrochloric acid was heated in a sealed tube at 100°C. for 16 hours. After cooling, the contents of the tube were transferred to an open dish and evaporated to dryness on a steam bath. The dark residue was extracted with 0·2 ml. of distilled water and, without separating the insoluble matter 0·01 ml. of the suspension was placed on the paper. The chromatogram was prepared on Whatman No. 4 paper using *n*-butyl alcohol-acetic acid-water mixture. Although the positions of the amino-acids on the well-dried paper were revealed by their bluish-white fluorescence in filtered ultra-violet light, a more specific test in the presence of fluorescent alkaloids was afforded by the ninhydrin reaction, carried out by spraying the paper with 0·1 per cent. ninhydrin in equal parts of *n*-butyl alcohol and chloroform, drying and then developing by heating in an oven at 100°C. The amino-acids were identified by their R_F values and their ninhydrin colour reactions, leucine and valine yielding reddish purple spots while those due to phenylalanine and proline were grey-blue and yellow respectively. Under the above conditions hydro-

TABLE IV

Alkaloid	Amino-acids, identified chromatographically
Ergotamine	Proline, phenylalanine
Ergotaminine...	Proline, phenylalanine
Ergocristine	Proline, phenylalanine and faint trace of valine
Ergocristinine (ergotinine)	Proline, phenylalanine and faint trace of valine
Ergocornine (ergotoxine)	Proline, valine and trace of phenylalanine

lysis of ergine, the amide of lysergic acid, gave a continuous streak showing a variety of colours, but no major spots reacting with ninhydrin. A similar streak often appeared on chromatograms prepared with hydrolysates of water-insoluble alkaloids but was fainter and caused no difficulty in the identification of the amino-acids.

Using the above technique the results shown in Table IV were obtained with samples of " pure " alkaloids.

From these preliminary experiments it is obvious that not only is paper chromatography a valuable tool for identifying but also for assessing the purity of ergot alkaloids. For example, it is clear that in the cases of the ergocristine and ergocornine examined each alkaloid was contaminated with traces of the other. When assessing the degree of contamination it is necessary to remember that phenylalanine, under the conditions employed, gives less colour than leucine or valine and one of the quantitative applications of paper chromatography should be applied.

Other minor ninhydrin spots have been noted in the chromatograms, but at present no assignment of these to any known constituent of the hydrolysate can be made. Alkaline hydrolysates have not shown the presence of amino-acids but, in general, give two elongated spots reacting with ninhydrin. The substances in these spots have yet to be identified.

Discussion

It was stated early in this paper that available methods of ergot assay lacked specificity and it is felt that the application of paper partition chromatography, now described, has done something to remedy this deficiency.

The need for a specific identification test for ergometrine has long been felt by workers in this field and it is a matter of great importance to the chemical manufacturer who wishes to purchase ergot for the manufacture of ergometrine. By paper chromatography it is a simple matter, even with a small sample of drug, to state whether ergometrine is present and approximately in what quantity. The application to liquid extracts of ergot has also been described.

Of all ergot preparations perhaps injection of ergometrine maleate is the most important. As a result of the present work a much clearer picture of the changes which occur during the manufacture and storage of the injection has been obtained and, in view of the very small quantity of injection required for a test, the chromatographic technique has opened the field for much fuller investigation. It has been seen that conversion of ergometrine to ergometrinine occurs on heat sterilisation of the injection and it may be that sterilisation at room temperature by candle filtration might afford a better preparation. The *p*H of the injection may influence the alkaloidal equilibrium and we have experiments in progress to explore this aspect of the problem. Hydrolysis of the alkaloid in the injection has been confirmed and this factor must be added to the previously accepted causes of deterioration[9]. It is considered unlikely that the hydrolysis of ergometrine in solution can be avoided.

The close relationship of the ergot alkaloids to the polypeptides has been emphasised by the use made of chromatograms, prepared from the

hydrolysates of the alkaloids. In spite of the amount of work so far done on the ergotoxine group of alkaloids it seems doubtful whether complete separation of the individual alkaloids has yet been achieved and, in this connection, it is certain that the chromatograms of the amino-acids will be of great value in testing highly purified specimens for traces of other alkaloids. A long-standing controversy as to whether ergotamine and ergotoxine ever occur together in the same ergot[16] has not been resolved and chromatography may well have something to contribute in this field. Preliminary results with chromatograms, prepared from hydrolysates of single sclerotium of *Claviceps purpurea* have indicated that the amino-acids found are a good indication of the alkaloids present in the drug, about which there is still much to be learnt.

SUMMARY

1. A study has been made of the application of paper partition chromatography in the ergot field.

2. A technique for the separation and identification of ergometrine and ergometrinine, when present in mixtures of total ergot alkaloids, has been described.

3. The method has been extended so that approximately quantitative results may be obtained and, in this way, the ergometrine contents of samples of ergot have been estimated.

4. The changes which take place in injection of ergometrine maleate B.P. 1948, during manufacture and storage have been studied chromatographically. It was found that during heat sterilisation some conversion of ergometrine to ergometrinine occurs. On storage some hydrolysis giving rise to lysergic and *iso*-lysergic acids takes place.

5. By preparing chromatograms from the hydrolysates of water-insoluble ergot alkaloids, identification tests for individual alkaloids have been developed.

We wish to thank Mr. H. M. Hood, B.Sc., for the photograph of a chromatogram, Mr. R. L. Grant, M.Sc., for a sample of ergometrinine and Dr. S. Smith for specimens of lysergic and *iso*-lysergric acids. We are also indebted to the Directors of The Wellcome Foundation for permission to publish this paper.

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DISCUSSION

The paper was read by Dr. G. E. Foster.

The CHAIRMAN said that he would particularly like to congratulate the authors on the ingenious method of hydrolysing the alkaloids and separating the elements chromatographically. At last year's Conference he had suggested that the colour test was not really an indication of the amount of deterioration. The work reported in this paper confirmed that and gave valuable new methods of determining the amount of deterioration. He hoped some agreement would be reached on the names for ergot alkaloids.

DR. F. HARTLEY (London) said that preliminary experiments carried out by his colleagues with a similar object to that of Dr. Foster had shown interesting results but they had not been able to pursue them to the extent that Dr. Foster had done. In examining different solvents for the separation of ergot alkaloids on a chromatogram, they had found that *iso*butyric acid gave R_F 0·90. *n*-Butanol-acetic acid gave an R_F value of 0·55 which agreed well with the figure 0·59 given by Dr. Foster.

In addition to the amino acids obtained by the hydrolysis of ergometrine, 2-amino-propanol was produced. This was readily distinguishable from the amino acids by the ninhydrin reaction and in their hands, using *n*-butanol-acetic acid it had an R_F value of 0·33. This observation might assist in studying the deterioration of ergometrine injection and perhaps Dr. Foster could determine the 2-amino-propanol in his five years old sample.

PROFESSOR BRINDLE (Manchester) said that they had been trying chromatography for the estimation of ergot alkaloids and had found difficulty in extracting the alkaloids from the chromatogram. They had tried to extract the ergotoxine and ergometrine (quantitatively) by prolonged extraction in a continuous extractor with ether, but on examination of the chromatograms in ultra-violet light, a fluorescence persisted. They had had the same difficulty in extracting the alkaloids from silica gel. They had achieved more success using kieselguhr and a citrate-phosphate buffer at *p*H5. The ergotoxine had been recovered quantitatively, but the ergometrine was not so easy. In a deteriorated solution of ergotoxine they had found a difference between the colorimetric assay, done directly on the solution, and that performed on the extracted alkaloids. They were satisfied it was due to lysergic acid, which did not affect the colour test if the alkaloids were extracted first. He had noticed that deterioration varied according to *p*H. With a solution at *p*H3 there was good agreement between the colorimetric and biological assays, with the biological result a little below the colorimetric. If deterioration occurred at *p*H5 or over there was a big difference between the two assays, with the biological result about half that of the colorimetric assay. Had Dr. Foster any observations to make on the considerable difference in the type of deterioration according to *p*H?

DR. W. MITCHELL (London) asked if the colours of the ultra-violet fluorescence were distinctive for ergometrine and ergometrinine. Dr. Foster had said that the accuracy of the method of comparing the intensity of

fluorescence was about ± 20 per cent. That was not a very high accuracy. Was it also necessary to make allowance for the size of the spot? If the standard spot was smaller than the test spot, presumably it would have some effect on the intensity. Was there any possibility of alteration of the ergometrine due to the somewhat prolonged exposure to air? This method for the testing of ergot would be useful, but the limiting factor was the time involved since it appeared to require at least five working days.

DR. R. E. STUCKEY (London) said that in his laboratory they had had some experience in removing amino-acids from chromatograms for their quantitative estimation. Was Dr. Foster sure that the residual fluorescence was due to traces of ergometrine remaining in the paper and not to peptisation of the paper fibres which was liable to occur? He would like to suggest that the chromatogram be run not as a spot but as a band with ten or more spots. This would increase the amount of ergometrine making a chemical or spectrophotometric estimation possible and the amount of ergometrine left in the paper might then be low in comparison with that extracted. Was any information available on that point?

DR. G. E. FOSTER (Dartford) said he was very interested to learn about Dr. Hartley's experience with other solvents. In view of Dr. Stuckey's suggestion they might have to look into the problem of extraction more closely, but so far they had found it impossible to remove the fluorescence completely. As to the difference of *p*H, they had prepared an ergometrine maleate injection, and adjusted the *p*H to various values from 3 to 6 by the addition of maleic acid. The chromatograms were obtained after sterilisation, all the spots being put on one paper. As the *p*H decreased, the amount of ergometrine gradually increased until at *p*H3 or 3·5 there had been very little ergometrinine there at all, and the amount of lysergic acid seemed to decrease at the same time.

A recent sample of ergometrine maleate injection from the U.S. had been found to contain very little ergometrinine, but the acidity had been much higher than that of the B.P. injection.

The colour of the fluorescence was the same for ergometrine and ergometrinine. The 20 per cent. error of visual comparison of the fluorescence was within the limits usual in this kind of work. The size of the spots did not seem to be important. Four of the five days required for the method were taken up with percolation. The ratio of total alkaloids to ergometrine was more or less constant in Spanish or Portuguese ergot, and by doing a rough chromatograph extraction it was possible to obtain a quick qualitative result overnight.

Conversion of the alkaloids on the paper did not appear to occur. They had no information on peptisation of the paper fibres. Neither had they done any tests using a band technique.

PROFESSOR H. BRINDLE (Manchester), in answer to Dr. R. E. Stuckey said they had tried increasing the amount of the alkaloids used in paper chromatography. They could not get a quantitative recovery from the band. As they increased the band, the amount of paper was increased, and it was adsorption on the paper which caused the difficulty.

THE DETERMINATION OF BENZENE HEXACHLORIDE (HEXACHLOROCYCLOHEXANE) IN PHARMACEUTICAL PREPARATIONS

By W. H. C. Shaw

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In addition to its use as an industrial insecticide benzene hexachloride (Gammexane) has proved of value as an external parasiticide in human and veterinary medicine. For this purpose, the biologically active γ-isomer (Lorexane) has been isolated and formulated to yield a variety of pharmaceutical products. The object of the work described here was to provide a simple method for the determination of benzene hexachloride in the presence of the excipients likely to be found in such formulations. Earlier analytical studies of benzene hexachloride have been concerned chiefly with mixed isomers and have described the determination of either (a) γ-isomer in presence of others, or (b) total benzene hexachlorides. Of these, (a) usually involve biological or physicochemical methods, while (b) nearly all depend on the alkaline dehydrochlorination to trichlorobenzene originally reported by Van der Linden[1].

$$C_6H_6Cl_6 + 3OH' = C_6H_3Cl_3 + 3Cl' + 3H_2O$$

Several workers have shown that this reaction is quantitative for all isomers when benzene hexachloride is refluxed with alcoholic potassium hydroxide, and that the determination may be completed by either acid or silver nitrate titration of the resultant solution. Goldenson and Sass[2] have studied the effect of replacing potassium hydroxide by other alkalis, and Howard[3] has drawn attention to the possible use of monoethanolamine, which has the advantages of being easily freed from chloride impurity and of not reacting with vegetable oils.

Since the quality of the active agent is capable of independent analytical control, it was considered sufficient that the method should determine total benzene hexachloride, i.e., there was no need for it to be specific for γ-isomer. The main criterion was that it should be effective in the presence of relatively large amounts of excipients and diluents, since the biological potency of the γ-isomer is such that it is rarely used at concentrations above 1 per cent. In view of the simplicity of the reaction, it was decided to investigate more fully alkaline dehydrochlorination as a basis for a general method.

Methods of Dehydrochlorination:

(1) *By Alcoholic Potassium Hydroxide:* The limits of time, temperature and concentration to ensure a quantitative reaction were investigated as follows. A known weight of pure γ-isomer (about 0·4 g.) was reacted with an excess of alcoholic potassium hydroxide at controlled time and temperature, and the ionisable chlorine produced was titrated with silver nitrate. The results obtained are recorded in Table I, and

show that : (a) Using 1 per cent, potassium hydroxide the reaction is quantitative in 15 minutes at room temperature, provided that sufficient alcohol is present to dissolve the benzene hexachloride. (b) No further reaction involving the trichlorobenzenes takes place after at least 1 hour's refluxing with 4 per cent. potassium hydroxide in alcohol (95 per cent.).

TABLE I

	Reaction time	Alcohol strength	Potassium hydroxide solution	Recovery (percentage of theory)
	minutes	per cent. v/v	per cent. w/v	
Room temperature 20° C. approx.	1	95	4	66
	2			89
	5			100·0
	15			100·2
	15		1	99·4
Refluxing	15	Nil (aqueous)	1	16
	15	20	1	51
	15	70	1	100·1
	15	95	1	100·1
	60	95	4	100·0

(2) *By Monoethanolamine:* Howard[3] has already shown that monoethanolamine produces quantitative dehydrochlorination when heated either with solid benzene hexachloride or its oily solutions. To compare this method with (1) the action of an excess of monoethanolamine when used both alone and in alcoholic solution was investigated under similar conditions of time, temperature, and concentration. The results are recorded in Table II and show that:—

(a) *For the alcoholic solution:* (i) Even a large excess of monoethanolamine produced practically no reaction after 1 hour at room temperature. (ii) To obtain a quantitative reaction it was necessary to boil for 2 hours with 8 per cent. of monoethanolamine in alcohol (95 per cent.).

(b) *For monoethanolamine alone:* (i) The reaction was not complete in 18 hours at room temperature. (ii) The reaction was quantitative after 5 minutes heating on the water-bath, with frequent shaking.

The action of monoethanolamine in alcoholic solution is thus much

TABLE II

	Reaction time	Monoethanolamine alone (ml.)	Monoethanolamine (ml. in 25 ml. of alcohol)	Recovery (percentage of theory)
Room temperature	1 hour	—	1	Trace
	18 hours	—	1	16
	18 hours	2	—	91
Refluxing	15 minutes	—	1	24
	60 ,,	—	1	75
	120 ,,	—	1	94
	60 ,,	—	2	97·5
	120 ,,	—	2	100·0
	5 minutes	2	—	100·0
	15 ,,	2	—	99·7

slower than that of potassium hydroxide. This confirms the findings of Howard, who recommended heating with undiluted monoethanolamine for 1 hour at 100°C. as a general procedure for dehydrochlorination.

Application to Pharmaceutical Preparations:

The relative merits of the potash and monoethanolomine methods were assessed for the following types of pharmaceutical preparations, selected as being the most commonly encountered. (a) Solutions in mineral oil (including ointments with paraffin base); (b) solutions in vegetable oil (including sulphonated oils); (c) alcoholic preparations; (d) dusting powders; (e) emulsions and creams. In testing the suitability of each method the following processes, described here as General Methods, were first applied and subsequently modified, where necessary, to suit special requirements.

General Method A (Potassium Hydroxide): To an appropriate weight of sample is added 25 ml. of alcohol, with sufficient potassium hydroxide to react with the benzene hexachloride and with any saponifiable matter present. The mixture is refluxed for 1 hour, cooled, and acidified with dilute nitric acid. Fatty acids, trichlorobenzene, and unsaponifiable matter are extracted with ether and the ionisable chlorine in the aqueous layer determined by any convenient method. A control titration is made to compensate for the presence of ionisable chlorine in sample and reagents. For this purpose, dilute nitric acid is first added in sufficient quantity to ensure that the solution remains acid during and after the addition of potassium hydroxide. It has already been established that benzene hexachloride itself is unaffected by nitric acid[4].

General Method B (Monoethanolamine): To an appropriate weight of sample is added an excess of monoethanolamine (1 ml. per g. of benzene hexachloride) with 0·1 ml. extra per 1 ml. of oil, and the mixture heated for 1 hour on the water-bath, with frequent shaking. After cooling, the mixture is acidified with dilute nitric acid, diluted with water, and extracted with ether. The ionisable chlorine in the aqueous layer is then determined as before. A blank titration is also carried out as described in Method A. Experimental results are summarised in Table III.

Discussion

The potassium hydroxide method is, in general, the more attractive since the reagent is readily available and the reaction proceeds rapidly in alcohol even at room temperature. It is particularly useful when the analysis involves a break-down of emulsions where monoethanolamine is markedly inefficient. The presence of a trace of chloride impurity in potassium hydroxide of A.R. quality is not a serious disadvantage, firstly because it is small in relation to the total ionisable chlorine produced in the assay and, secondly, because it is compensated by a control titration. This method is thus the more suitable for preparations of types (a), (c), (d) and (e). The only circumstances in which the monoethanolamine method is to be preferred are when it is desired to avoid

TABLE III

Preparation	Prepared Strength per cent.w/w	Modifications to Method A	Strength Found per cent.w/w	Modifications to Method B	Strength Found per cent.w/w
1. Solution in liquid paraffin	1·970	Nil	1·97	Nil	1·96
2. Ointment containing liquid, hard and soft paraffins and wool fat.	1·964	Nil	1·96	Not suitable, due to formation of emulsions on extraction.	—
3. Solution in arachis oil	1·027	Nil	1·01	Nil	1·02
4. Solution in sulphonated castor oil ...	5·058	Reflux with nitric acid for control titration	5·09	As for Method A	5·06
5. Solution in aqueous alcohol	0·1013	Add 50 per cent. aqueous solution of potassium hydroxide to give a concentration of 1 per cent. and allow to stand for 15 minutes. No heating necessary.	0·101	Add momoethanolamine to give a concentration of 8 per cent. Reflux 2 hours. Cool, acidify, and complete by General Method.	0·101
6. Self emulsifying concentrate containing castor oil and polyglyceryl ricinoleate in alcohol.	0·2014	Reflux with dilute nitric acid for control titration.	0·202	As for No. 5	0·200
7. Dusting powder containing talc and starch.	0·102	Extract by shaking with a known volume of cold alcohol (95 per cent.). Filter or centrifuge. To an aliquot add sufficient 50 per cent. aqueous potassium hydroxide to give a concentration of 1 per cent. Allow to stand 15 minutes, acidify, and complete.	0·105	Less suitable than potassium hydroxide due to long procedure.	—
8. Ointment with oil in water emulsion base containing sulphonated castor oil, diethylene glycol distearate, and emulsifying wax.	0·111	Reflux with dilute nitric acid for control titration.	0·107	10 per cent. of added momoethanolamine at 100° C. for 2 hours failed to give quantitative recovery.	—
9. Ointment with oil in water emulsion base containing castor oil, cetyl alcohol, diethylene glycol distearate, polyglyceryl ricinoleate and non-ionic emulsifying agent.	0·100	After saponification extract oils, etc. with petroleum ether in presence of a high concentration of alcohol.	0·103	Comments as for No. 8	—
10. Fluid oil in water emulsion containing kerosene and sulphonated castor oil	0·211	Reflux with dilute nitric acid for control titration.	0·214	Comments as for No. 8	—

saponification of oils and fats, where the comparatively large quantities of potash required for saponification may introduce an undesirably large amount of chloride. Otherwise, monoethanolamine has the disadvantage of always requiring heat and agitation unless used in high concentration in the presence of alcohol, which eliminates the necessity for shaking. It also becomes less efficient as a dehydrochlorinating agent in the presence of solvents. It is thus recommended, as far as pharmaceutical preparations are concerned, only for the determination of benzene hexachloride in simple solutions in vegetable oils, particularly when the concentration is very small.

SUMMARY

Methods are suggested for the determination of benzene hexachloride in certain pharmaceutical preparations. These are based on alkaline dehydrochlorination using monoethanolamine or alcoholic potassium hydroxide.

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DISCUSSION

DR. C. GARRATT (Nottingham) suggested that the author had obtained a surprising degree of accuracy in the analysis of the difficult mixtures mentioned in Table III. He asked whether the author could give some idea of the degree of variation obtained in successive determinations, and whether the results recorded were typical, or picked from a series which showed the method to advantage.

MR. SEYMOUR (Welwyn) asked whether the author had considered the use of bases other than ethanolamine.

MR. W. H. C. SHAW, replying, said that a certain amount of variation in the analytical results was to be expected. This really depended on the strength of the preparation which was being assayed. With the stronger preparations, the results came in general within $\pm$ 0·5 per cent. of the actual amount of benzene hexachloride used, but for the less concentrated preparations such as a 0·1 per cent. cream or emulsion the accuracy probably fell to about $\pm$ 2 per cent. As to bases other than monoethanolamine, pyridine had been tried by Howard in the work which led him to recommend monoethanolamine.

AN EXAMINATION OF PYROGEN FROM VARIOUS SOURCES

By D. W. Wylie and J. P. Todd

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Whether bacteria in general produce the same pyretic substance or each bacterial species a specific pyrogen is not yet known. During our investigation of pyrogen we have discovered several variations, such as different types of fever curves following injection of modifications of the same culture and variations in stability of pyrogen, from different bacterial sources, when heated at 120°C., which suggest that different pyrogens exist.

Fever Curves

A study of published fever curves produced as the result of injection of pyrogen into rabbits[1,2,3,4,5,6] shows that these can be placed in one or other of three main classes:—1. Curves in which the body temperature started to rise shortly after the injection, reached a peak and then returned to normal again. 2. Curves in which the body temperature started to rise shortly after the injection as in (1), fell slightly, but instead of returning to normal, rose to a second peak, which was then followed by a return to normal. 3. Curves in which there seemed to be a delay before the body temperature rose to its peak, this again being followed by a return to normal. In some of the fevers of this latter type the temperature fell distinctly during the initial period of no reaction, in a few cases this fall being so great that the animals collapsed and sometimes died[1,6].

None of the workers in this field appear to have observed any significant difference between the single- and double-peak curves which appeared in graphical form in their publications. This may have been due to the same culture, under what appeared to be exactly the same conditions, stimulating both the single- and double-peak types of curves. It has been shown here that this failure to observe significant differences in the shapes of the curves was probably caused by taking the temperature of the rabbits at hourly intervals only.

In the course of this work, the three types of fevers already discussed were frequently obtained. As the work proceeded, it became apparent that the differences in the curves were significant and were not caused by biological variation or by the method of temperature determination used, but by actual differences in the constitution of the solutions. In experiments in which a fall in body temperature occurred after injection of solutions, the cause was found to be associated not only with the delayed fever (type 3), as suggested in the literature, but to be due to a depressant substance produced by some organisms. After removal of this depressant substance, which can be driven off by gentle heat, the solutions stimulated the single- and double-peak types of fevers.

Typical examples of the three types of fevers are shown in Figure 1. In the single-peak curve (type 1) in which the rise in temperature is immediate, the peak temperature is reached in 70 to 120 minutes, the

average time being 86 minutes; in the double-peak curve (type 2) the first peak is identical with curve type 1, but this falls slightly, to be followed by a second rise, reaching its peak in 3 to 4 hours after the

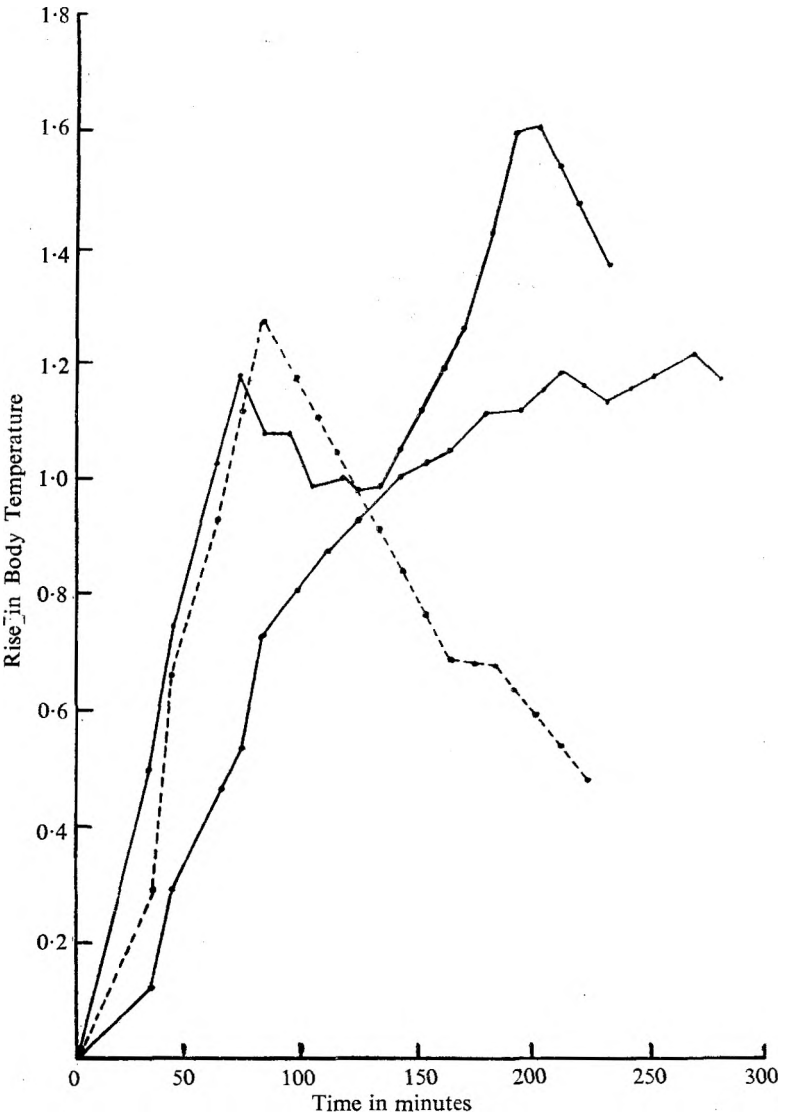

FIG. 1.—Typical examples of three types of fevers stimulated by pyrogenic cultures.

injection; in the delayed-peak fever curve (type 3), the time to reach the peak temperature is similar to that of the second peak in the double-peak curve. From a study of the time to reach the peak, it would seem that the double peak curve (type 2) is a combination of the two single-peak curves.

Figure 2 demonstrates how completely the shape of the curve is masked

by determining the rectal temperatures at hourly intervals only, the technique used by most of the investigators, and it is probably due to this that no significant conclusions were drawn from the shapes of the curves, as, when using this method, slight changes in the time to reach the peak could change what appeared to be a single-peak curve in one test to a

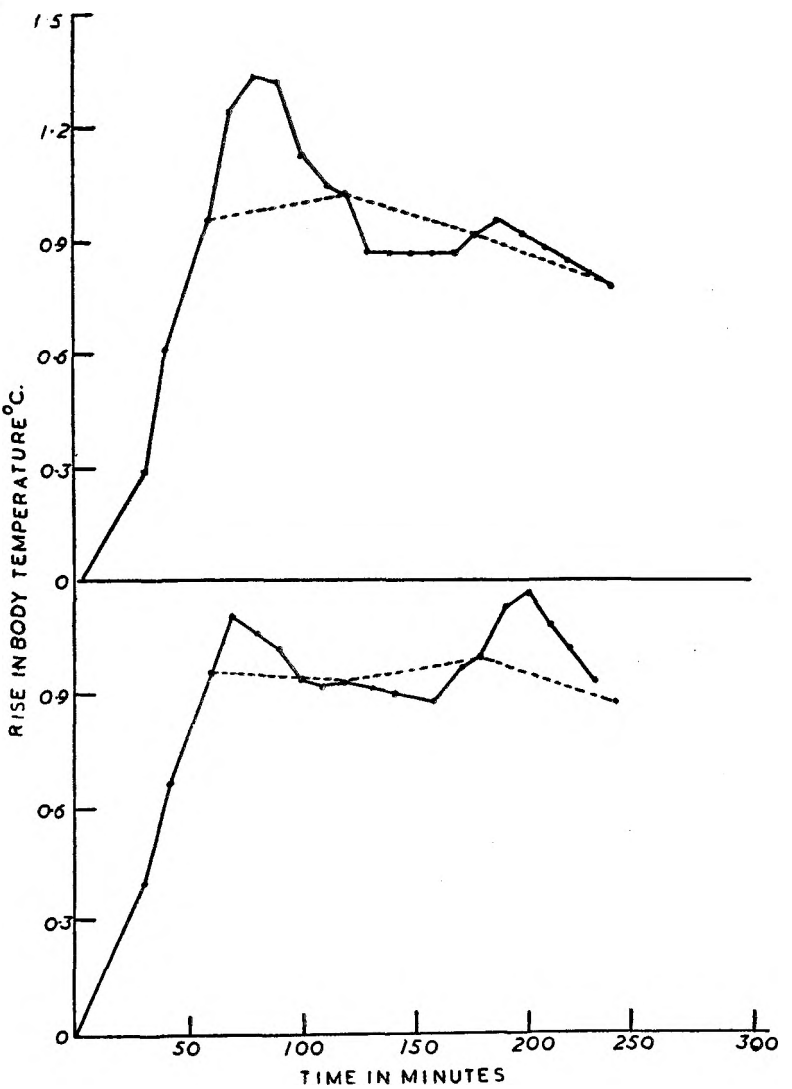

FIG. 2.—Showing effect of method of temperature determination on the shape of the fever curve.
———— Thermocouple method.
— — — — — Thermometer method.

double-peak curve in a repeat of that test. Four explanations can be advanced for the differences in the shapes of the curves.

1. That pyrogen in a whole culture containing the organism and its metabolic products (such a whole culture has been found to stimulate the double-peak reaction) is both dissolved in the medium and contained within the bacterial cell. Here the first rapid rise in temperature may be produced by the pyrogen dissolved in the medium, and the second fever by the slow liberation of pyrogen from the bacterial cells as they are broken down in the blood-stream. The cell-free filtrate therefore should cause the immediate reaction only, and a suspension of the cells the delayed reaction.

2. That each of the three types of fevers is stimulated by a different substance.

3. That the immediate response fever is caused by one type of pyrogen, the delayed response by another type, and the double-peak response by a mixture of the two substances.

4. That the actual dosage of pyrogen is the controlling factor and that by varying the dose, the shape of the curve can be altered.

These explanations have been investigated in the experiments described below.

EXPERIMENTAL

A culture of *Proteus vulgaris* was grown for 4 weeks at 37°C. in gelatin hydrolysate synthetic medium and the following experiments carried out:

1. Samples of the whole culture were sterilised by autoclaving at 115°C. for 30 minutes and tested on two different groups of 5 rabbits at a dose level of 0·002 ml./kg. of body-weight. Both tests showed the double-peak type of fever, one of which is shown in Figure 3.

2. Samples of the viable culture were filtered free of bacterial cells by passage through Berkefeld filter candles and the filtrate sterilised by autoclaving at 115°C. for 30 minutes. This filtrate was clear to the eye and no cells could be detected on microscopical examination. At a dose of 0·002 ml./kg. of body-weight, similar to the dose of the whole culture, this filtrate stimulated only the single peak fever of the immediate response type, as is shown in Figure 3.

3. Samples of the autoclaved whole culture were centrifuged at 4,000 r.p.m. for 45 minutes, and the supernatant liquid decanted and retained. The cells were then washed 3 times with pyrogen-free saline, separated by centrifuging each time, and the washings discarded. The washed cells were suspended in pyrogen-free saline and the cell-count adjusted to approximately the same as that of the original culture, as determined by Brown's Opacity Tubes. Both the supernatant and the suspension of washed cells were tested. The suspension of cells stimulated the single-peak fever of the delayed type and the supernatant liquid the double-peak fever (Figure 3).

From the results of this experiment it is seen that the same culture can produce all three types of fever depending on the state of the sample when injected, that the fraction stimulating the immediate response, the cell-free filtrate of the viable culture, can be separated from that causing the delayed response (the washed cells) and that the combination of the

two fractions, i.e., the whole culture, causes the double-peak type of fever. This then tends to discount the theory that there are three separate substances and, also, the theory that the dosage of pyrogen is the important controlling factor; further proof of this latter point is that removal of the cells has little effect on the first peak but removes the second peak.

The fact that the supernatant liquid from the centrifuged sample caused

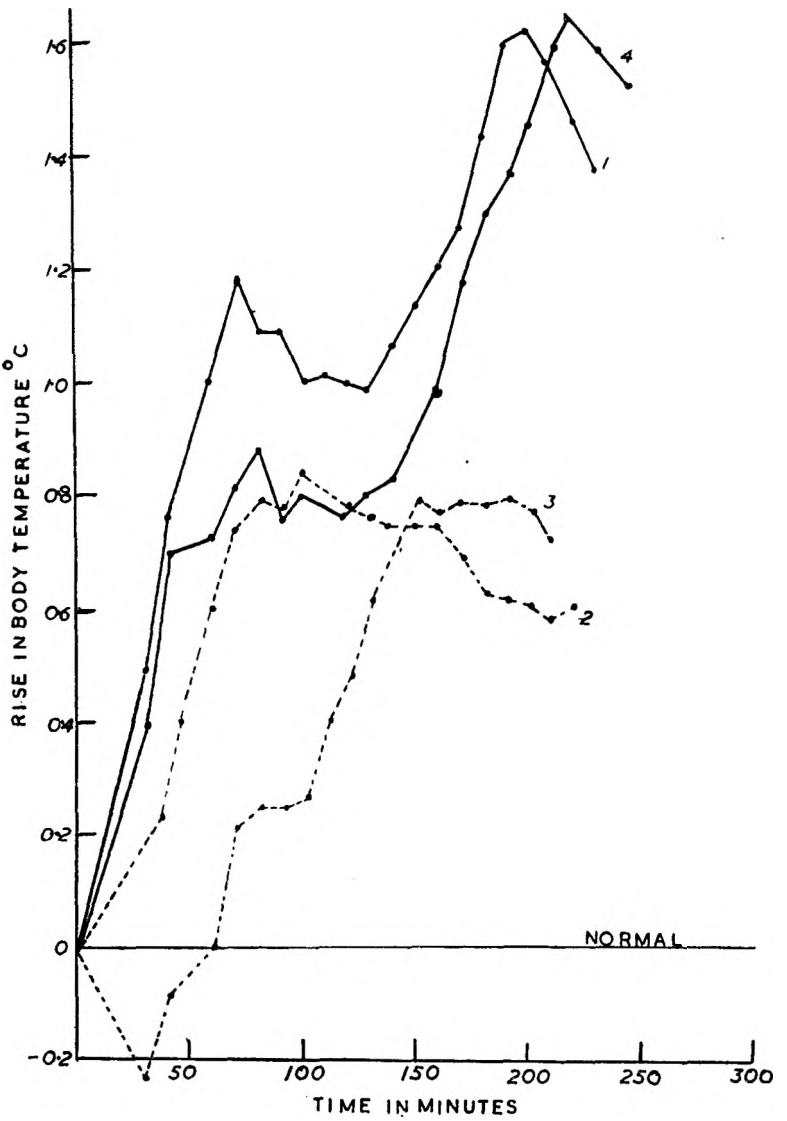

FIG. 3.—Showing different fever curves stimulated by modifications of the same culture of *Proteus vulgaris*.
Curve 1.—Whole culture. Curve 2.—Cell-free filtrate.
Curve 3.—Washed bacterial cells.
Curve 4.—Centrifuged supernatant of sterlised culture.

the double-peak type of reaction was unexpected, if this supernatant liquid were really cell-free, as it appeared to be when examined, the hypothesis that the double-peak fever is attributable to the same pyretic substance being present in the medium and in the bacterial cells is not tenable. Since the other results of the experiment supported the hypothesis it was possible that the centrifuged supernatant liquid still contained sufficient cells to cause the second peak. The above experiments were therefore repeated with additional refinements, mainly to find how many cells, if any, were necessary to stimulate the second peak, or

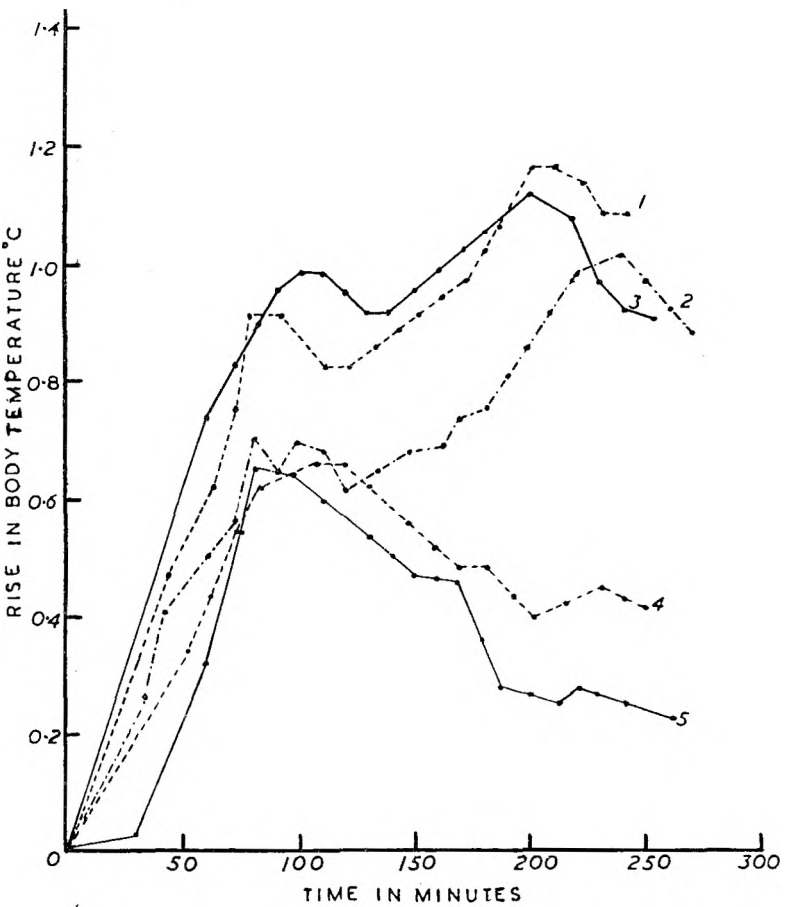

FIG. 4.—Elimination of second peak by reducing the dose.
Curve 1.—Dose in ml./kg. of body-weight.
Curve 1.—0·02 Curve 2.—0·02 Curve 3.—0·004
Curve 4.—0·002. Curve 5.—0·0002.

whether it was possible to stimulate the double-peak type of fever by injection of a completely cell-free solution.

Escherichia coli was grown on a gelatin hydrolysate medium at 37°C. for 5 weeks and samples of this culture tested as follows:—

1. Samples of the whole culture were sterilised by autoclaving at

115°C. for 30 minutes; these were tested on groups of five rabbits in doses of 0·02, 0·004, 0·002 and 0·0002 ml./kg of body weight. The curves for these experiments are shown in Figure 4. The larger doses of 0·02 and 0·004 ml./kg. of body-weight stimulated the double-peak fever but those of 0·002 and 0·0002 ml./kg. of body-weight only the single-peak.

2. Samples of the viable culture were filtered free of cells by filtration through a Berkefeld filter candle, and the filtrate sterilised by autoclaving at 115°C. for 30 minutes. A dose of 0·02 ml./kg. of body-weight was tested on groups of 5 rabbits on 3 separate occasions, only the single-peak fevers of the immediate response type were stimulated. The curve for

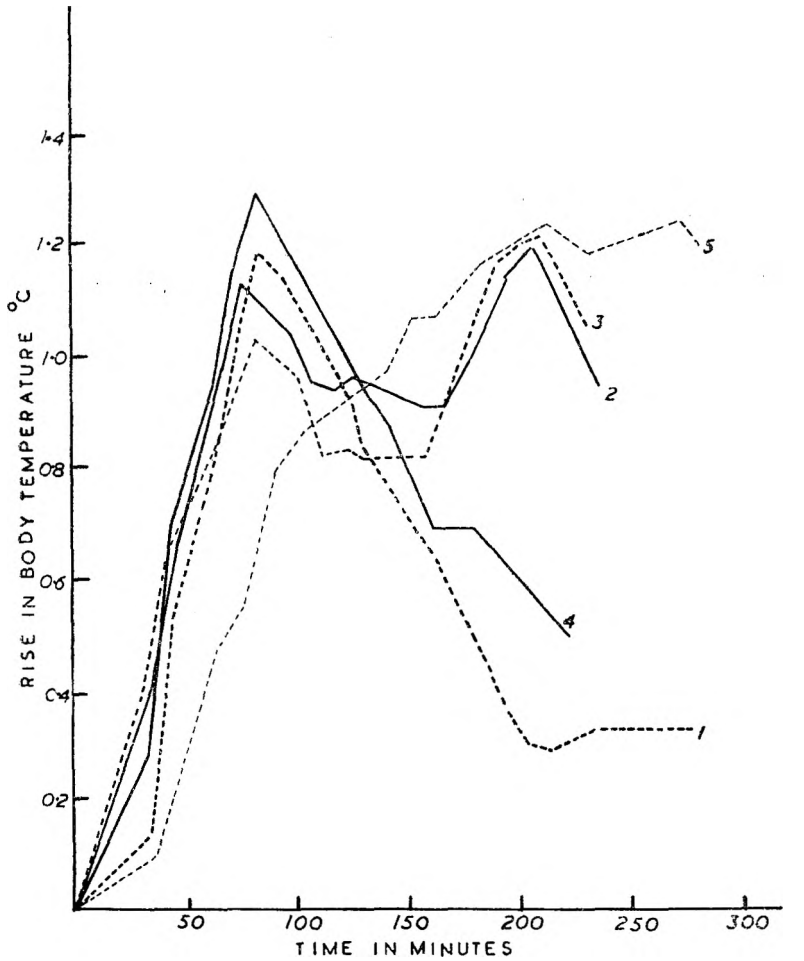

FIG. 5.—Showing different fevers stimulated by modifications of the same culture of *Escherichia coli*.

Curve 1.—Filtrate of viable culture.
Curve 2.—Filtrate of autoclaved culture.
Curve 3.—Centrifuged supernatant of autoclaved culture.
Curve 4.—Centrifuged supernatant of viable culture.
Curve 5.—Washed bacterial cells.

one test is shown in Figure 5 (curve 1). However, in the previous set of experiments on *P. vulgaris*, the supposedly cell-free supernatant which unexpectedly gave rise to the double-peak reaction was prepared by sterilising the culture before the cells were removd by centrifugation and, to bring the filtration experiments into line with this, samples of the whole culture of *E. coli* were first sterilised by autoclaving and then filtered free of bacterial cells. A dose of 0·02 ml./kg. of body-weight of this filtrate was tested on groups of 5 rabbits on 2 separate occasions and the double-peak type of fever was obtained (Figure 5, curve 2). A dose of 0·002 ml./kg. of body-weight of the whole culture would contain more cells than a dose of 0·02 ml./kg. of body-weight of the filtered culture, but such a dose of the whole culture does not stimulate the double-peak fever and the filtrate does; it would appear, therefore, that, the few cells possibly present in the filtrate would not cause the double-peak. Therefore of the 4 theories advanced, the most probable one is that the solutions stimulating the double-peak of reaction contain two pyretic substances, one causing the immediate rise in body temperature and the other a delayed rise, a mixture of the two causing the double rise. By the fact that the second peak is either eliminated or reduced by removal of the cells, it would seem that most of the substance stimulating the second peak is within the cell, whereas that stimulating the first peak is mainly dissolved in the medium.

3. The tests on the centrifuged supernatant liquid were also carried out using the supernatant liquid of both the viable and the sterilised whole culture. In all cases the centrifuging was at 3,750 r.p.m. for 3½ hours in an attempt to produce complete deposition of the cells, and no cells could be detected on microscopical examination. In four tests, each at a dose of 0·02 ml./kg. of body-weight, the supernatant liquid of the previously sterilised whole culture caused the double-peak fever, one of which is shown in Figure 5 (curve 3). The supernatant of the viable culture at the same dose stimulated only the single-peak fever (Fig. 5, curve 4). These results therefore support those obtained for the filtrate. The cells obtained by centrifugation of the sterilised whole culture were washed 3 times with pyrogen-free water and finally diluted with the required amount of saline solution to bring the cell count back to approximately that of the original culture, and tested at a dose of 0·02 ml./kg. of body-weight. The fever developed was of the delayed type as was found in the previous experiment (Figure 5, curve 5).

A third series of experiments were carried out, again on *P. vulgaris*, using a seven-day culture:

1. Three doses of the whole culture, 0·02, 0·002 and 0·0002 ml./kg. of body-weight, were tested. The two higher doses stimulated the double-peak reaction and the dose of 0·0002 ml./kg. of body-weight the single-peak reaction, showing again that the second peak can be diluted out more readily than the first.

2. Filtrates from both the viable and the sterilised cultures were tested, that of the autoclaved culture stimulated a definite double-peak response and that of the viable culture the single-peak reaction.

Summarising these results we have, that the second peak of a double-peak fever is more readily diluted out than the first, that the cell-free filtrate or centrifuged supernatant stimulates the single-peak type of fever if the cells are removed before autoclaving, and the double-peak type if the whole culture is autoclaved before removal of the cells, and that the washed cells of the centrifuged culture cause the production of the delayed fever. From the results therefore it appears that there are two pyretic substances, one causing the immediate fever and the other the delayed fever, and a mixture of both the double-peak fever. However, it also appears that the substance causing the second peak might be formed by the autoclaving of the cells. If the culture is autoclaved while the cells are present this substance is found in the medium, whereas, if the cells are first removed before autoclaving, the substance is usually absent. To clarify this point, a further set of experiments were carried out on a seven-day culture of *Proteus vulgaris*. The whole culture was divided into three parts, the first was sterilised by autoclaving at 115°C. for 30 minutes, the second by heating at 60°C. for 1 hour and the third by addition of 0·3 per cent. of chlorocresol. At a dose of 0·02 ml./kg. of body-weight all three solutions caused the double-peak type of reaction. The actual quantity of chlorocresol injected into the rabbits was 0·1 mg. and would have little effect. Thus the substance stimulating the double-peak fever is not formed by autoclaving the cells. Another possible explanation is that in the viable culture the substance causing the first peak is mainly dissolved in the medium and that stimulating the second peak mainly within the cell, and if there is any of this latter substance in the medium it is not usually present in sufficient quantity to stimulate the second peak. Autoclaving however increases the quantity of this substance in the medium sufficiently that the cell-free solution can stimulate the double-peak type of fever. This explanation is further supported by the fact that *Serratia marcescens (Bacillus prodigiosus)* stimulated the double-peak fever whether the culture was sterilised before or after removal of the cells, showing that the second-peak substance is not an artifact produced by autoclaving the cells. It may be assumed that in this case the second substance passes more readily from the cell into the medium than it does in either *Escherischia coli* or *P. vulgaris*.

The organisms examined which have stimulated the double-peak fevers are *P. vulgaris, P. morganii, Ps. fluorescens, Ps. æruginosa, S. marcescens, S. keilensis, E. coli, Eberthella typhosa, B. mycoides* and *Staphylococcus aureus*. On the other hand it cannot be claimed that other organisms tested in the course of this work which did not stimulate the double-peak type of reaction do not produce the necessary substance, as it may not have been present in the solutions tested in sufficient quantity to stimulate the second peak.

Production of Depressant Substance by some Organisms and the Effect this has on the Fever Curve

Reactions, following injection of cultures of *P. vulgaris* and *Ps. fluorescens*, were obtained from time to time which differed from the

usual type of fever reaction. In these cases the body temperature commenced to rise sharply in the first hour, as it would in the usual fever reaction, but this was followed by a rapid fall in body temperature to well below normal. In one of the tests the average fall for five rabbits was 1·34°C. below the initial temperature (Figure 6), one of the rabbits actually showing a fall of 3·4°C. within 1½ hours of the injection. In the more severely affected rabbits of the groups, the other symptoms were signs of general collapse, the hind quarters were paralysed and there was no control over urination, the animals could not stand and the head

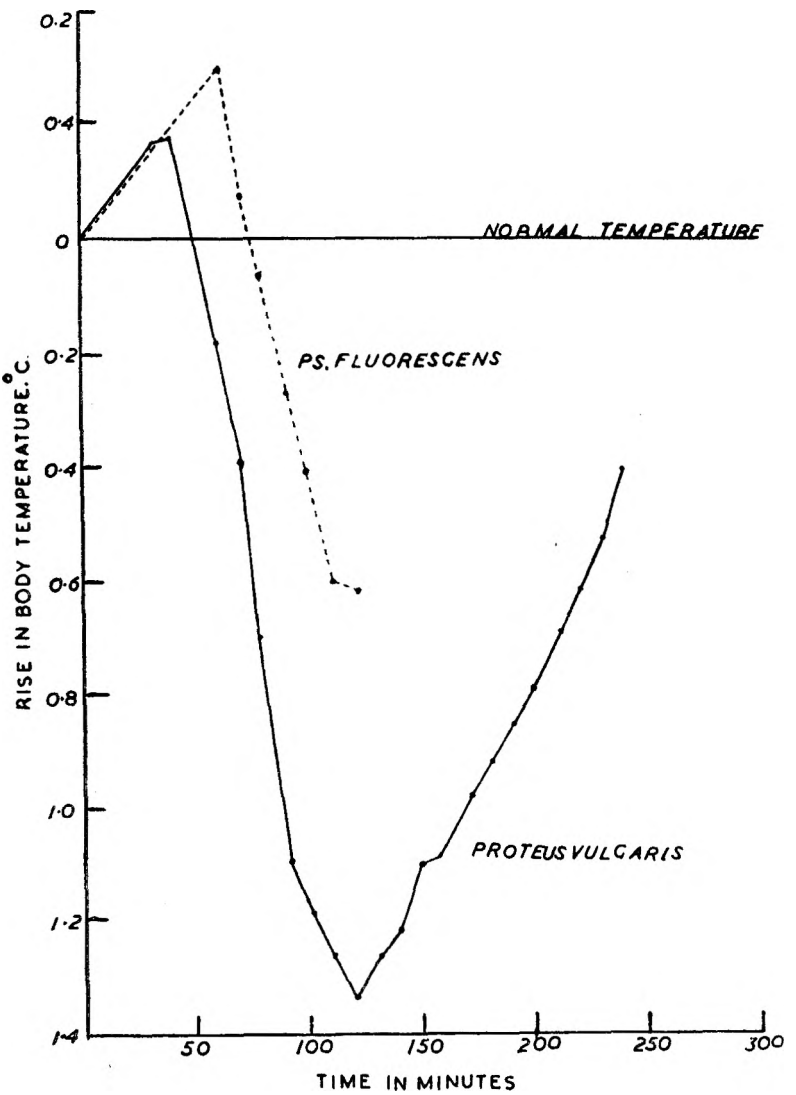

FIG. 6.—Fall in body temperature caused by a depressant substance present in cultures of *Ps. fluorescens* and *Proteus vulgaris*.

could only be moved slightly. These symptoms appeared in $1\frac{1}{4}$ to $1\frac{3}{4}$ hours after the injection. Two hours after the injection the animals usually began to recover from this state, the temperature rose slowly and the animal was soon able to stand and move about. The symptom which was apparent in all of the animals injected, whether severely affected or not, was the loss of appetite, the animal refusing food for a number of days after the experiment.

The depressant effect was not repeated when further samples of the same cultures were retested, and it was also observed that when the reaction occurred, the first rabbit in each group to be injected was usually the most severely affected. As the solution to be injected is heated to approximately body temperature before injection, it was realised that if the depressant were very volatile it might be slowly driven off and, therefore, the solution injected into the first rabbit might contain a higher concentration of the depressant substance than that injected into the following rabbits. On this assumption experiments were carried out on a seven-day culture of *P. vulgaris*. Samples of the whole culture, in sealed ampoules, were sterilised by autoclaving at 115°C. for 30 minutes, and tested in doses of 0·02 and 0·002 ml./kg. of body-weight. In these tests the solution was diluted, placed in an open beaker and heated to 30° to 40°C. for about 10 minutes before injection. This solution produced the usual fever reaction (Figure 7), no depressant action or loss of appetite being observed. Similar samples of this culture were tested in doses of 0·04, 0·02 and 0·01 ml./kg. of body-weight, this time taking care during the preparation of the solution to prevent loss of any volatile substance, and the solution was not heated before injection. Here the depressant action was apparent at a dose of 0·04 ml./kg. of body-weight, there being a distinct fall in body temperature and development of paralysis (Figure 7). The rabbits receiving the other two doses, although not showing the fall in body temperature nor the paralysis, lost appetite for food for a few days. Thus the substance causing this fall in body temperature and loss of appetite is volatile, and since it is present in the cultures autoclaved in sealed ampoules, it appears to be relatively stable towards heat.

Modifying effect of depressant on the fever.—Figure 8 shows how the height of the fever stimulated by a culture may be modified by this depressant substance. When the solution was heated before injection it caused the usual type of fever, the modifying effect of the depressant is seen in the fevers stimulated by the solution injected without previous heating, the temperature rose sharply in the first hour but instead of continuing to the usual peak in $1\frac{1}{2}$ hours, it either fell sharply, or tended to remain at the point reached. Such a reaction, in which the depressant effect is not very obvious, might lead to the wrong conclusions concerning the pyrogenic activity of the solution. The depressant was inferred to be present in these solutions, for, although the temperature of the animals did not fall below normal, they refused food for a number of days following the experiment. It is clear that when testing cultures of bacteria it is advisable to heat the solutions before injection and the result of any

experiment which is followed by the refusal of the animals to eat should be examined for signs of the modifying effect of the depressant, and the experiment repeated if necessary.

This depressant might also account for the distinct fall in body temperature following the injection of the washed cells of *P. vulgaris*, as is seen in Figure 3. In this delayed fever the temperature fell before beginning to rise, and this reaction seems to be similar to the reactions referred to by Seibert[3] and Hort and Penfold[6].

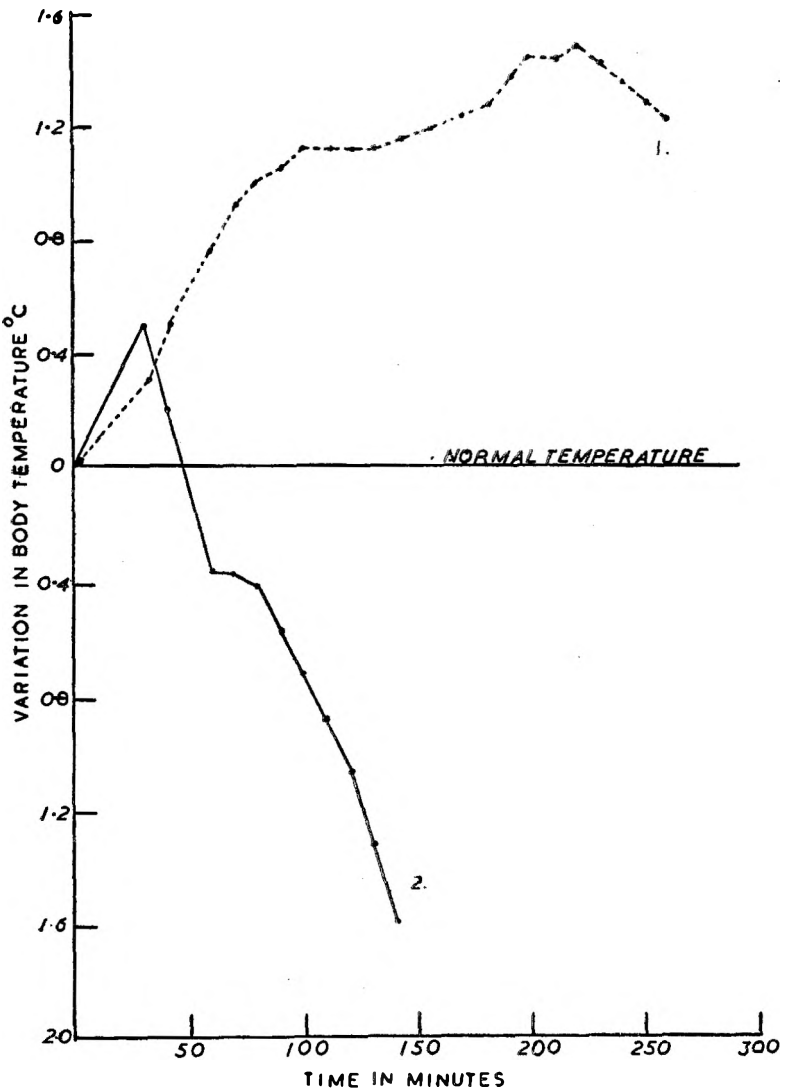

FIG. 7.—Showing effect of heating solution before injection.
Curve 1.—Solution heated before injection.
Curve 2.—Solution not heated before injection.

Variations in stability to heat.—The stability of pyrogen to heat has been investigated by Seibert[3] and Banks[7], both concluded that it was a thermostable substance. In a previous paper, however, we showed that a cell-free filtrate of a 48-hour culture of *P. vulgaris* showed an initial rapid loss in activity when heated at 120°C., but that the solution was still pyrogenic after 4 hours at 120°C.

A further 48-hour and a 31-day culture of *P. vulgaris* and also cultures of *Ps. æruginosa, B. subtilis* and *M. tetragenes* of 3, 2 and 1 weeks incubation at 37°C. respectively, have since been examined for stability

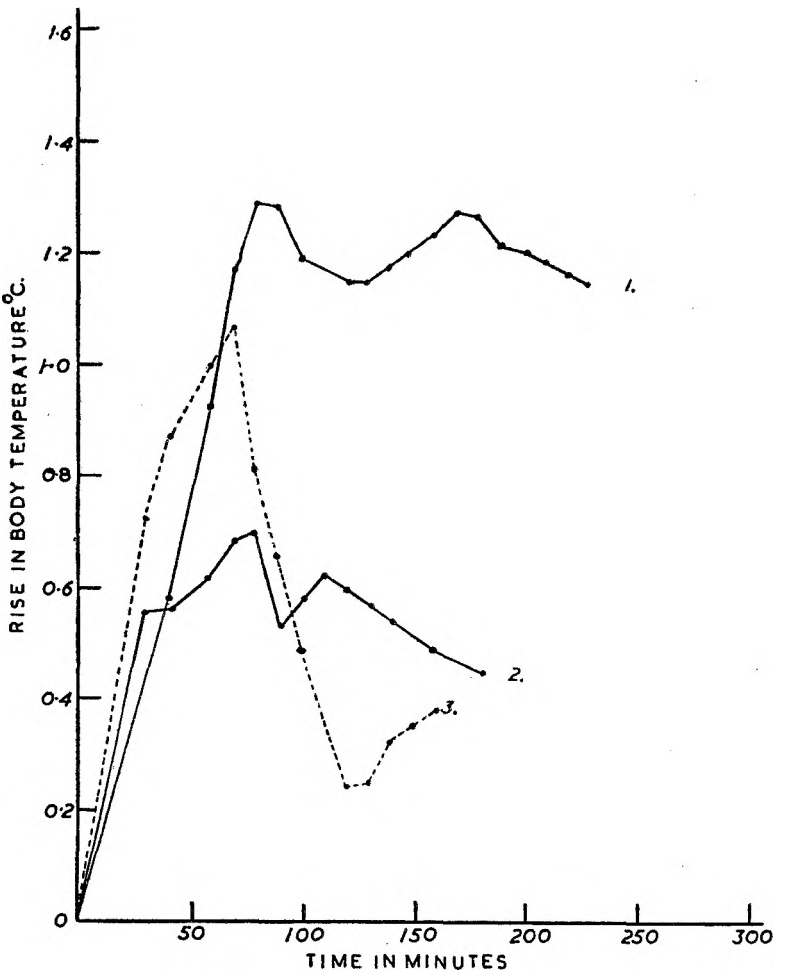

FIG. 8.—Modifying effect of depressant on the fever stimulated by a whole culture of *Proteus vulgaris*.

Curve 1.—Dose—0·002 ml./kg. of body weight, no depressant effect.
Curve 2.—Dose—0·02 ml./kg. of body weight, depressant effect.
Curve 3.—Dose—0·005 ml./kg. of body weight, depressant effect.

to heat at 120°C. As the method of heating at 120°C. by autoclaving, as described in the previous paper, is not easily controlled or reproduced, the cultures were filtered free of bacterial cells, sealed in thick-glass ampoules and immersed in an oil-bath maintained at 120 ± 1°C. The reaction of each solution was previously adjusted to *p*H7, it was not considered necessary to add buffering agents to the solution, as it was the buffered medium in which the organisms had been grown. Samples of each of the cultures were heated at 120°C. for 30 minutes, 1 hour, 1½ hours and 2 hours. The strengths of the unheated cultures were calculated by extrapolation of the log. concentration against time curve, which is a straight line. Table I shows the results for this set of experiments.

It would seem, from the results, that the rate of loss inactivity of cultures of *P. vulgaris, B. subtilis* and *Ps. æruginosa* is somewhat similar, in that approximately 50 per cent. is destroyed after 30 minutes and 95 per cent. after 2 hours. The activity of the *M. tetragenes* culture, on the other hand, was much more difficult to reduce, only 20 per cent. of the original activity being destroyed after 2 hours at 120°C.

TABLE I

SHOWING THE RATE OF DESTRUCTION OF THE PYROGEN FROM *PS. ÆRUGINOSA, B. SUBTILIS AND M. TETRAGENES*

Heat Treatment		*Pseudomonas æruginosa*		*Bacillus subtilis*		*Micrococcus tetragenes*	
Temp. °C.	Duration hours	Units/ml.	Percentage destroyed	Units/ml.	Percentage destroyed	Units/ml.	Percentage destroyed
Extrapolation		214	0	19	0	9·1	0
120	½	75	65	9	52·6	8·75	3·8
120	1	51	76	4·4	76·8	8·0	12·6
120	1½	26·9	87	2·4	87·4	7·5	17·6
120	2	10·6	95	0·9	95	7·3	19·8

		Proteus vulgaris						
		48-hour culture		48-hour culture		31-day culture		Average percentage destroyed
60	1	46	0	87·5	0	825	0	0
120	½	31	33	42·5	38	362	56	42
120	1	6·75	85	21·9	76	400	52	71
120	2	2·0	96	6·2	93	41	95	94
120	4	0·75	98	1·9	98	14	98	98

Linear relationship between the log. of concentration and time.—By determining the percentage of the original concentration left at each stage, and plotting the logarithm of this against time, a linear relationship was found to exist for all four cultures, which satisfied all points within the experimental error of the quantitative test, i.e. about 15 per cent. Wylie and Todd[8]. In the case of *Proteus vulgaris*, a batch was heated at 120°C. for 4 hours, but this point deviated in all three experiments from the linear relationship of the other points.

Figure 9 shows that the same straight line, the " calculated line of best fit " in this case, can be drawn to represent the rate of destruction of the pyrogen produced by *P. vulgaris, Ps. æruginosa* and *B. subtilis* within the

experimental error of the quantitative test. The results for *M. tetragenes* although still linear are quite different.

Summarising these results, we find that the pyrogen present in the cell-free filtrate of cultures of *Ps. æruginosa, P. vulgaris* and *B. subtilis* show very similar rates of destruction. *M. tetragenes*, on the other hand, seems to produce a pyretic substance of totally different stability. How far these results would agree with those obtained by the use of pure pyrogen is unknown, and continuation of the work on these lines, for purposes of comparing the stability of pyrogen produced by different organisms, would be of little value unless carried out on the purified substances. The results obtained here fulfil the desired requirements, in that they give an indication of how much pyrogen may be lost during any heating process, and also of the degree of severity of treatment permissible in the isolation of pyrogen.

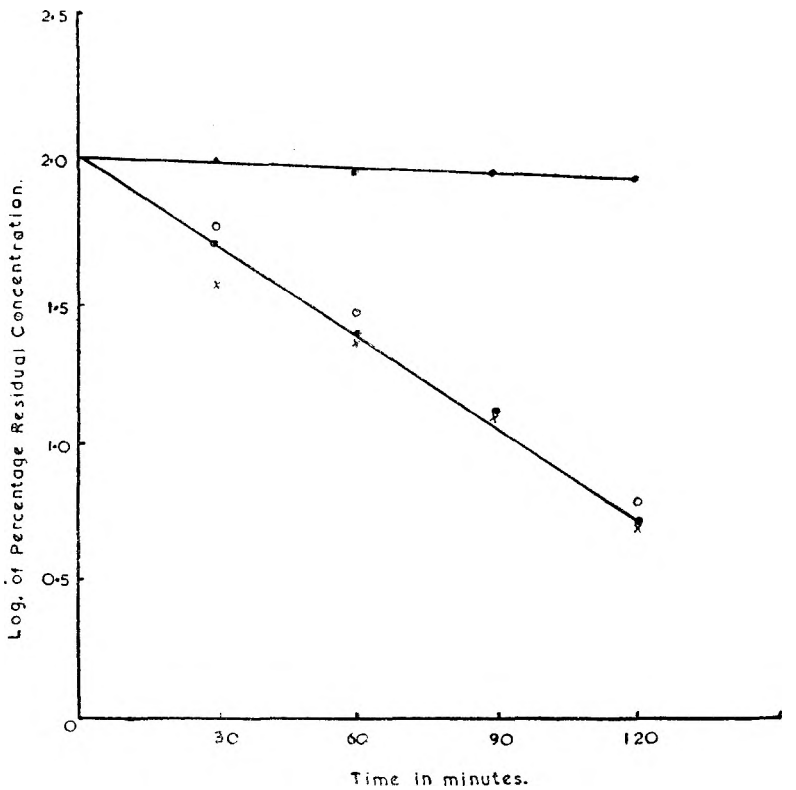

FIG. 9.—Showing linear relationship between logarithm of residual concentration and duration of heating at 120°C.

Upper graph — *Micrococcus tetragenes.*
Lower graph — o *Proteus vulgaris.*
● *Bacillus subtilis.*
× *Pseudomonas æruginosa.*

Summary

1. Three distinct types of fever curves can be stimulated by injection of modifications of the same bacterial culture.

2. Injection of a sterilised whole culture causes a double-peak fever; the cell-free filtrate or supernatant liquid of a culture stimulates a single-peak fever, rising to a peak in an average time of 86 minutes if the cells are removed before autoclaving, and a double-peak reaction if the culture is autoclaved before removal of the cells; the fever caused by injection of a suspension of washed bacterial cells is a single-peak fever of the delayed reaction type, reaching its peak in 3 to 4 hours.

3. The results show that certain bacteria probably produce two pyretic substances, one of which, in the viable culture, is dissolved in the medium and stimulates the single-peak fever of the immediate reaction type, and the other is contained mainly in the bacterial cell and stimulates the single-peak fever of the delayed reaction type, a mixture of both causing the double-peak fever.

4. *Proteus vulgaris* and *Ps. fluorescens* can produce a volatile depressant substance, which either lowers the body temperature to below normal, or prevents the pyrogen from causing a rise in body temperature. This depressant causes general paralysis if injected in sufficiently large doses, in smaller quantities the most obvious effect is the loss of appetite. It has been shown that any pyrogen test followed by the rabbits refusing to eat for a few days should be examined for other effects of the depressant substance, and should be repeated, taking care to heat the solution to between 30° and 40°C. for about 10 minutes before injection.

5. An investigation of the stability of pyrogen to heat at 120°C. showed that *Ps. æruginosa, B. subtilis* and *P. vulgaris* produce pyretic substances of similar stability, whereas *M. tetragenes* produces a much more thermostable substance.

6. The results from the investigation of the stability showed that the pyrogens of *P. vulgaris, Ps. æruginosa* and *B. subtilis* are sufficiently labile to require care when using heat during isolation, as approximately 95 per cent. is destroyed after 2 hours at 120°C. Finally it must be emphasised that further investigation into these points would be of little value unless carried out on the pure pyretic substances isolated from various bacterial sources.

This work was carried out during the tenure by one of us (D.W.W.) of a Carnegie Scholarship and a Wellcome Research Fellowship to the trustees of which we wish to express our thanks.

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DISCUSSION

The paper was read by Mr. Wylie.

The CHAIRMAN said that the subject of pyrogens appeared to be more complicated than had been thought originally; he assumed the authors were intending to continue their work, and he would suggest to them that they should try chromatography as a method of separating pyrogens.

PROFESSOR H. BRINDLE (Manchester) said that the presence of the depressant seemed important, and it should be taken note of in testing for pyrogens. Had the authors distilled their cultures in order to make certain that this depressant substance was volatile?

In Figure 4 the authors used dilutions in order to show that the second factor could be diluted out. This introduced difficulties as regards quantitative work. If the Figure was correctly printed it would appear that there was a considerable difference between the action of two exactly similar doses of 0·02 ml., and it also seemed that 0·004 ml., only of 1/5 of this dose would produce a greater response than the whole dose.

DR. G. E. FOSTER (Dartford) asked whether the authors had been able to confirm the destruction of pyrogens in solution on keeping as had been reported at the Torquay Conference. His Company had been asked by an outside organisation to do some pyrogen tests for them, and one of the substances had made the rabbits' temperature fall very markedly. They had subsequently found out that the material submitted to them was a salicylate, which was an antipyretic. Had the authors any experience of testing for pyrogens in antipyretics?

MR. E. H. REID (Dagenham) asked whether the culture of *B. prodigiosus* had been treated in the same way as that of *P. vulgaris*, i.e., by autoclaving at 115°C. for 30 minutes, by heating at 60°C. for an hour or by the addition of 0·3 per cent. of chlorocresol. If not, the possibility that the double peak was due to the sterilisation procedure could not be excluded.

MR. G. E. SHAW (Runcorn) said that in work on scour in calves and on infantile gastro-enteritis they had found that by extracting a culture of organisms by boiling they could obtain a toxic substance which did not come out into the culture medium.

This substance, which was only present in extremely small amounts, had been found to contain about 15 per cent. of carbohydrate. When concentrated it produced a temperature reaction in calves with a characteristic gastro-intestinal lesion. It also killed rabbits, guinea-pigs, and mice. It would also act as a rather feeble antigen, but one could prepare an antiserum against it. The organism was grown on a medium containing ammonium sulphate, protein acid hydrolysate and glucose, and in deep culture the medium was aerated. After about 20 hours the count was about 3,000 million organisms per ml. Could Mr. Wylie give the total count of the culture he had used, and also the viable count? Was the pyrogen reaction simply a form of antigen-antibody reaction, or was it the effect of a pyretic chemical? He thought that different pyrogens might be of a similar type, all producing an antigen

tissue reaction. If they shook up pyrogen with an adsorbent such as zeolite, did they remove the constituent responsible for the rapid effect, or that causing the more slow reaction?

MR. J. A. MYERS (Bradford) asked if the authors had compared their results with the temperature curves of patients after transfusions. Quite frequently a rise of temperature was ascribed to the presence of pyrogens, but it might be due to the patient's clinical condition. Such comparisons might roughly indicate whether a patient's rise of temperature was due to something within the solution, such as bacteria, or due to some clinical interference related to the patient's condition.

MR. G. R. MILNE (Glasgow) said that the presence of a depressant substance was of special interest in connection with blood transfusion. In a well organised transfusion unit large quantities of transfusion fluids were produced with no trouble from pyrogenic reactions, but with blood and blood products, no matter how careful one was, there were always spells when reactions occurred. Unfortunately when they occurred careful records were not usually made, and it was therefore difficult to find an explanation for them. Many samples of blood had been returned to his depot to be tested for bacterial contamination and all the reports were negative. He was interested in the report of the depressant substance, because the condition of the patient might be such that the ratio of the amount of depressant substance to that of the pyrogen would turn the balance.

MR. D. W. WYLIE, in reply, said that they were not directly concerned with the depressant substance and had merely investigated it to find how it was produced, and how it could be eliminated before they tested the pyrogenic culture. The substance could be distilled off. The double peak response curve upset the quantitative test to some extent, but they had found the test still accurate provided one regarded only the first peak. The experiments in Figure 4 were not necessarily carried out at doses which were in the quantitative range; therefore one might get one-fifth of a dose stimulating response similar to that of the full dose.

In this paper and in the previous one, they had given results of the stability of pyrogen at 120°C. They had tested various solutions for pyrogen but not, so far, antipyretic solutions. Their culture of *B. prodigiosus* had been sterilised by autoclaving at 115°C. for 30 minutes. In this case the intracellular substance passed into the medium.

They had done the total counts and viable counts asked for by Mr. Shaw and he would send them on. They did not seem to be very important. They did not think the pyrogenic response was an antibody antigen reaction. They had not compared their fever counts with patient's curves, but the rise of temperature in rabbits had been similar.

PROFESSOR J. P. TODD, the joint author of the paper, said he would like to add that the depressant substances were being investigated, and Mr. Wylie's successor had started on the work.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

***m*- and *p*-Cresol in their Mixtures, Colorimetric Analysis of.** S. A. Savitt, A. M. Goldberg, and D. Othmer. (*Anal., Chem.*, 1949, **21**, 516.) The method used was based on the production of coloured nitrosophenols. The cresols were dissolved in a potassium hydroxide-glacial acetic acid buffer solution, and sulphuric acid followed by sodium nitrite solution was added. After standing, alcoholic ammonium hydroxide was added, the solution allowed to stand overnight, and the colour read photoelectrically using a violet light filter (492 mμ). Standard curves of the colour transmissions of the nitroso-solutions are given together with the calibration curves, for known concentrations of *m*- and *p*-cresol and their mixtures. The rate of absorption of water by the cresols in the atmosphere was determined and a graph is given for *m*- and *p*-cresols; it was concluded that the small amount of water absorbed over the initial 15 minutes of exposure would have no effect on the precision and accuracy of the analytical procedure. Numerous unknown samples were analysed and gave results which were reproducible to within 1 per cent. If phenol or *o*-cresol were present, it was found to be necessary to remove it by an efficient distillation and rectification prior to analysis for the binary mixture itself. R. E. S.

Iron, Volumetric Determination of. W. D. Cooke, F. Hazel and W. M. McNabb. (*Anal. chem.*, 1949, **21**, 643.) Solutions of ferric salts, acidified with either sulphuric or hydrochloric acid, were reduced by treating with liquid zinc amalgam. After separation of the amalgam, the residual ferric ion was reduced by the addition of a few drops of chromous chloride solution, the chromous ion reactions being followed by a low potential redox indicator, phenosafranine (oxidation-reduction potential, −0·28 volt). Complete reduction of the ferric ion was indicated by the colour change of the indicator from pink (oxidised form) to colourless, while the reverse colour change indicated complete oxidation of the excess chromous ion by atmospheric oxygen. No evidence of oxidation of ferrous iron by air was observed under the conditions of the experiment, thus eliminating the necessity of maintaining an inert atmosphere during liquid zinc amalgam reductions. In the actual titration of a ferric salt liquid amalgam was added followed by sulphuric or hydrochloric acids. After 1 minute carbon tetrachloride was added, the amalgam was removed (the reactions being carried out in a separating funnel), 2 drops of phenosafranine indicator were added followed by chromous chloride (usually 4 to 5 drops) until the pink colour of the indicator disappears and a clear green tint was visible; at this stage the solution was swirled until the pink colour reappeared. Phosphoric acid and diphenylamine sulphonate were then added and the titration was completed with standard potassium dichromate solution. An indicator correction was found to be necessary. R. E. S.

Lævulose, Determination of. D. T. Englis and J. E. Miles. (*Anal. chem.* 1949, **21**, 583.) The fact that lævulose was found to produce consider-

able colour, while dextrose produced practically no colour, when treated with the Folin-Denis phosphotungstic-phosphomolybdate reagent, was used as a basis for the colorimetric determination of lævulose in the presence of dextrose. A 20 per cent. solution of trisodium phosphate was added finally in place of the usual sodium carbonate solution, and a series of trial determinations indicated that 10 minutes was the optimum time for heating in a water-bath at 100°C. Graphs are given for the extinction values (determined photoelectrically) of the blue colours produced by solutions of lævulose and dextrose of known concentrations when treated under the conditions recommended. In order to determine the amount of lævulose in a mixture a procedure is outlined which necessitates the determination of total reducing sugars by another method. Using the known values, the percentage of lævulose can then be determined since the blue colour produced by the dextrose in the Folin-Denis reaction is relatively low in intensity. R. E. S.

Potassium, Colorimetric Determination of, by Folin-Ciocalteu Phenol Reagent. M. A. M. Abul-Fadl. (*Biochem. J.*, 1949, **44**, 282.) The method depends on the fact that alkaline solutions of cobalt salts, in the presence of a trace of an amino-acid (glycine or alanine), reduce the phosphomolybdic-phosphotungstic acid phenol reagent to a blue colour, the intensity of which is directly proportional to the amount of cobalt present, and hence, if potassium has been precipitated as cobaltinitrite, to the amount of potassium in the original solution. Details are given for the precipitation of the potassium from the serum as cobaltinitrite and for the washing of the precipitate. The precipitate is dissolved in water, and glycine, sodium carbonate and Folin's solutions added when, after standing at 37°C. for 10-15 minutes, the blue colour is read photoelectrically. The reduction of the phosphotungstic-phosphomolybdic acids by cobalt salts could not be effected in the absence of amino acids; the intensity of the blue colour was dependent on the amino-acid concentration, the optimum colour being obtained with 0·2 to 0·5 M-glycine. Amino-acids of higher molecular weight than alanine gave a blue colour with the phenol reagent in the absence of cobalt, the intensity of the colour depending on the molecular weight. The method can conveniently be carried out on 0·2 ml. serum instead of 0·5 ml. required for previous methods. R. E. S.

Riboflavine in Foodstuffs and Biological Material, Fluorimetric Estimation of. E. Kodicek and Y. L. Wang. (*Biochem. J.*, 1949, **44**, 340.) The material under examination is first extracted on a water-bath with 0·1N hydrochloric acid, the proteins being precipitated by the use of meta-phosphoric acid and/or digestion with takadiastase (for starchy materials.) The extract is washed with chloroform in acid solution and oxidised with saturated potassium permanganate solution followed by treatment with hydrogen peroxide to decolorise the remaining permanganate. At this stage the extract is adjusted to *p*H 6, methyl alcohol is added to clarify if necessary, and fluoriphotometric readings are taken using a blue filter for the incident light and a yellow filter between the solution and the photocell. A sensitive photocell-galvanometer circuit is used and in the blank riboflavine is reduced to a non-fluorescent compound with a neutralised solution of sodium dithionite. Several samples were analysed in 8 to 12 replicates and the coefficient of variation ranged from 2·2 to 4·6 per cent. The accuracy of the method decreases with decreasing concentration of riboflavine in the

material examined. Details of procedure are given together with a standard curve for pure riboflavine. R. E. S.

ORGANIC CHEMISTRY

Aloin. Characters and Reactions of. C. L. Harders. (*Pharm. Weekbl.* 1949, **84,** 250, 273.) The solubility of aloin from Curaçao aloes was determined for a number of solvents at 20°C., with the following results:—

Solvent	Solubility
Chloroform	1:33333
Carbon tetrachloride	1:20000
Ether	1: 3333
Ether (saturated with water)	1: 1818
Ethyl acetate	1: 56·4
Amyl alcohol	1: 523·6
Dichlorhydrin	1: 34·8
Dioxan	1: 13·3
Water	1: 155·4
Methyl alcohol	1: 13·2
Ethyl alcohol	1: 46

Impure (commercial) aloin has a higher solubility in water. The molecular weight of aloin, determined cryoscopically in ethyl urethane, was found to be 413. By hydrolysis in the absence of oxygen, no anthranols are formed, as under these conditions they give condensation products. When oxygen is present hydrolysis gives an emodin. A preliminary oxidation is also necessary in order to remove a pentose from the molecule. The fresh sap of the plant contains no emodin. Results suggesting the presence of an easily split glucoside of an anthraquinone derivative are due to the presence of mucilage, derived from the leaf, and to the action of oxygen on aloin. A new compound which was isolated from the sap is the cause of the intense colour. This compound crystallises in needles (m.pt. 179°C.), is readily soluble in benzene, and has a molecular weight of 230·5. For the determination of aloin in aloes four methods are suggested: chlorination (Leger); determination of pentose (Goldner); determination by persulphate (Seel) and determination with ferric chloride. Details of the last method are as follows: 10 mg. of aloes is dissolved in 0·5 ml. of water and treated with a solution of 0·3 g. of ferric chloride and 38 g. of calcium chloride ($2H_2O$) in 20 ml. of 0·5N hydrochloric acid. The mixture is boiled under a reflux for 45 minutes, diluted with an equal volume of water, and filtered through glass wool. The filtrate is shaken out successively with 25, 25, and 15 ml. of ether. The residue on the glass wool is dissolved in 4N sodium hydroxide and poured into a slight excess of N hydrochloric acid. The suspension obtained is also shaken out with ether. The combined ethereal solutions are washed successively with 5 ml. of water, 2·5 ml. of phosphate buffer (*p*H8) and 5 ml. of water The solution is then shaken with 20 per cent. ammonia until no more emodin is extracted, the volume of the ammoniacal solution being finally made up to 250 ml. The emodin is determined colorimetrically, using as standard a solution prepared in a similar manner from pure aloin. Good samples of Curaçao aloes contain nearly 40 per cent. of aloin. G. M

Myristicin, Synthesis of. V. M. Trikojus and D. E. White. (*J. chem. Soc.*, 1949, 436.) The constitution of myristicin as 1-methoxy-2:3-methylenedioxy-5-allylbenzene has been confirmed by synthesis (preliminary report *Nature*, 1939, **144,** 1016). Allylation of pyrogallol 1-methyl ether gave

two monoallyl ethers, the lower boiling ether being characterised as pyrogallol 1-methyl 2-allyl ether. Pyrolysis of this ether gave 2:3 dihydroxy-1-methoxy-5-allylbenzene which on treatment with methylene iodide and potassium carbinate gave myristicin, identical with the natural product obtained from oil of nutmeg. This identity was established by comparison of samples of tetrabromomyristicin, *iso*myristicin, and tetrabromo*iso*myristicin prepared from natural and synthetic myristicin. Pyrolysis of the higher boiling monoallyl ether (pyrogallol 1-methyl-3-allyl ether) and methylenation of the product produced myristicin in smaller yield. R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Vitamin A, Refining of Oils containing. By J. H. Burgoyne and F. A. Burden. (*Nature*, 1949, **163**, 722.) It has been thought that alkali-refining of fish-liver oils containing vitamin A decreases their potency because of the absorption of vitamin A by soaps formed *in situ*. Experiments with a number of oils seem to indicate that just the opposite is the case. The neutralised oil, far from losing anything of its original potency, extracts to a large extent vitamin A from the soap formed during the neutralisation. In oils with high potency and high acid value this increase can be quite substantial, as illustrated by the following example.

	Weight (g.)	Vitamin A Potency (I.U./g.)	Total content of Vitamin A. (I.U.)
Original oil	24·50	120,000	2,940,000
Neutralised oil	18·75	140,000	2,625,000
Oil in soapstock	5·75 (by difference)	44,000	253,000

The loss of vitamin A during the alkali-refining is due to some neutral oil being carried away with the soap-stock, while the potency of the neutralised oil has increased. As the latest refining methods allow of a loss closely approaching the theoretical Wesson loss, it seems beyond doubt that alkali-refining provides the most economical and simple method of purifying oils containing vitamin A. S. L. W.

BIOCHEMICAL ANALYSIS

***p*-Aminosalicylic Acid in Body Fluids, Determination of.** J. P. Newhouse and W. Klyne. (*Biochem. J.*, 1949, **44**, VII.) The method employs diazotisation and coupling at room temperature instead of at 0°C. Oxalated blood, plasma or cerebro-spinal fluid (0·2 ml.) is measured into a test-tube containing 6·7 ml. of water, the contents of the tube are shaken, allowed to stand for 3 minutes and trichloracetic acid (0·6 ml. of a 25 per cent. w/v solution) is added; after shaking, the mixture is allowed to stand for 15 minutes and is then filtered through a small Whatman paper (No. 40 or 42) into a graduated centrifuge tube. To the clear filtrate (5·0 ml.) concentrated hydrochloric acid (1·5 ml.) and a 1 per cent. solution of sodium mitrate (0·2 ml.) are added, the tube is shaken for 30 to 40 seconds and 1·0 ml. of ammonium sulphamate reagent (consisting of 2·0 g. ammonium suphamate in 50 ml. of glacial acetic acid and 50 ml. of water) is added. The resulting solution is shaken for 10 seconds and 1·0 ml. of naphthylethylenediamine

dihydrochloride solution (0·2 per cent.) is added. *p*-Aminosalicylic acid gies a purple colour attaining its maximum intensity after standing for 15 minutes at room temperature and stable for at least 3 hours. The colour intensity is measured with a photoelectric photometer, using a yellow-green filter (e.g. Ilford No. 605) and is compared with that obtained from a standard representing 10 mg. of *p*-aminosalicylic acid per 100 ml. of blood. In the preparation of the standard, 5·0 ml. of a stock solution (containing 183·9 mg. of sodium *p*-aminosalicylate dihydrate/l.) is diluted to 100 ml., 2·0 ml. of this solution, 2·6 ml. of water, and 0·4 ml. of trichloracetic acid solution are mixed and treated as the 5·0 ml. of blood filtrate. In setting the instrument to zero a blank consisting of 0·4 ml. of trichloracetic acid solution and 4·6 ml. of water is used and is treated as the 5·0 ml. of blood filtrate; a blank determination on blood is not usually necessary. The colour developed is directly proportional to the *p*-aminosalicylic acid concentration in the range 0 to 10 mg./100 ml. of blood, but this linear relationship does not hold strictly at higher concentrations. When known quantities of *p*-aminosalicylic acid (1 to 20 mg./100 ml.) were added to normal blood or to cerebro-spinal fluid the following recoveries were obtained: blood 77 to 94 per cent., mean 85 per cent.; cerebro-spinal fluid 90 to 100 per cent., mean 94 per cent. Duplicates agreed within about 0·3 mg./100 ml. Streptomycin did not interfere in the determination R. E. S

Heparin, A New Method for the Assay of. H. Kjems and H. Wagner. (*Acta Pharmacol. Toxicol.*, 1948, **4**, 155.) A method for the assay of heparin is described based on the fact that, under suitable conditions, the clotting times in mixtures of varying amounts of heparin and constant amounts of oxalated ox plasma and thrombin are gradually prolonged between 20 and 60 seconds, so that a smooth curve is obtained for clotting time plotted against amount of heparin. 0·07 ml. of heparin standard solution and 0·13 ml. of 0·9 per cent. sodium chloride solution are pipetted into a test tube, 1 ml. of oxalated ox plasma is added and the contents are mixed by swinging the test tube before the addition of 0·1 ml. of thrombin solution. The test tube is at once placed in a water thermostat at 37°C., and during the first 5 sec. the test tube is vigorously moved in order to produce rapid temperature equilibrium; it is then swung to and fro with a quiet pendulum movement, being raised slightly over the horizontal position each time it is moved to the left. The moment of clotting is noted when the mixture does not run but slides down the wall of the test tube. Sodium chloride solution (0·9 per cent.) must be added to the thrombin solution until a clotting time of 30 to 35 sec. is attained. The oxalated plasma is made by centrifuging fresh ox blood with 10 per cent. of a 5 per cent. potassium oxalate solution, stored at 0°C. for at least 24 hours, and again centrifuged immediately before use. A standard curve of clotting times using known amounts of heparin is prepared and the strength of an unknown solution of heparin can thus be determined. A number of variables in this assay have been studied including the preparation of the plasma, the volume of the heparin solution and the influence of *p*H. The presence of a hitherto unknown factor taking part in the coagulation of the blood is shown. R. E. S.

Œstrogens, A New Method for the Separation of Androgens from, and for the Partition of Œstriol from, the Œstrone-Œstradiol Fraction. N. B. Friedgood, J. B. Garst and A. J. Haagen-Smit. (*J. biol. Chem.*, 1948, **174**, 523.) The application of ultra-violet absorption spectrophoto-

metry to the quantitative determination of the urinary œstrogens was investigated with a view to developing an objective physical method for their accurate determination. The causes of inaccuracies of the methods in current use were investigated, including the following aspects of the problem: the spectrophotometric identification and quantitative micro-determination of crystalline œstrogens; the detection by spectrophotometric assay of gross errors in current methods for extraction and partition of œstrogens; studies on the ultraviolet absorption of substances used for the extraction and partition procedures; the separation of the phenolic œstrogens from the androgen steroid fraction; the separation of urinary œstrogens from the other urinary phenolic substances by steam distillation; and the micro-Girard separation of œstrone from œstradiol. A new method was developed for the extraction, partition and quantitative determination of crystalline œstrone, œstradiol and œstriol. The œstrogens were separated from the androgens by partition between N potassium hydroxide and a mixture of 1 volume of solvent ether and 18 volumes of carbon tetrachloride. Benzene and disodium hydrogen phosphate were used for the separation of the œstrogens into strongly and weakly phenolic fractions because 0·3M sodium carbonate carries one-third of the œstradiol over into the œstriol fraction. A specially designed apparatus made it possible to reduce to a minimum the number of transfers of extracts and residues. The sensitive ultra-violet spectrophotometric method which was used for the assay of crystalline œstrone, œstradiol and œstriol gave consistent results and crystalline œstrogens were recovered quantitatively. L. H. P.

Phenazone in Biological Materials, Estimation of. B. B. Brodie, J. Axelrod, R. Soberman, and B. B. Levy. (*J. biol. Chem.*, 1949, **179**, 25.) Two methods for the determination of this compound in biological materials are described. One procedure involves the extraction of phenazone from an alkaline solution of the biological material (organ tissues and fæces are prepared for analysis by emulsification in acid) with chloroform, and the evaporation of the solvent. The residue is dissolved on dilute sulphuric acid, sodium nitdite is added and the resulting 4-nitrosophenazone is determined by spectrophotometric measurement of the light absorption at 250 mμ. The other procedure, suitable for plasma, involves the estimation of the phenazone directly in the plasma filtrate after removal of the protein with zinc hydroxide. A table is given, showing satisfactory recovery experiments in both procedures when known amounts of phenazone were added to tissues and to plasma. Reproducible analyses of plasma and urine stored in the refrigerator for several days showed that the compound is stable in biological fluids. Distribution experiments between plasma and ethylene dichloride indicated that the substance estimated was actually phenazone. Many drugs found in plasma did not interfere. Sulphadiazine interferes since it absorbs light at 350 mμ, the absorption being increased by the addition of nitrous acid.

R. E. S.

Urobilinogen, Ehrlich's Aldehyde Test for. T. M. Wilson and L. S. P. Davidson. (*Brit. med. J.*, 1949, **1**, 884.) Owing to the considerable variation in the reagents used, and to the fact that there are numerous substances other than urobilinogen which can give colour reactions with *p*-dimethylaminobenzaldehyde the aldehyde test lacks specificity. With the various reagents in current use the final concentration of hydrochloric acid varies between 0·35 and 2·5 per cent. (excluding Watson's reagent in

which the hydrochloric acid is immediately neutralised by sodium acetate) and the final concentration of *p*-dimethylaminobenzaldehyde varies between 0·06 and 0·6 per cent. Ehrlich's rosindole reagent, which is still used by some workers, has the composition: *p*-dimethylaminobenzaldehyde 4 g., absolute alcohol 380 ml., and concentrated hydrochloric acid 80 ml. Apart from urobilinogen the substances most liable to give rise to a reddish-pink colour when urine is tested with Ehrlich's aldehyde reagent are indole, the precursors of two indole pigments—indirubin and urorosein—and porphobilinogen. The reaction is more sensitive if urines are tested while fresh and warm, and precautions detailed should be rigidly observed. Further work is required to determine the optimal quantities of *p*-dimethylaminobenzaldehyde and hydrochloric acid to be used and the relative amounts of reagent and urine to be employed in the test. S. L. W.

CHEMOTHERAPY

***p*-Hydroxybenzenesulphonamides.** K. A. Jensen and S. Å. K. Christensen. (*Acta chem. scand.*, 1949, **87**, 207.) The following compounds were prepared by diazotising the corresponding *p*-amino compounds and boiling the diazonium chlorides so obtained with water:—2-(*p*-hydroxybenzenesulphonamido)-thiazole, m.pt. 221 to 222°C., 2-(*p*-hydroxybenzenesulphonamido)-benzthiazole, m.pt. 292°C., 2-(*p*-hydroxybenzenesulphonamido)-5-methylthiazole, m.pt. 231°C., 2-(*p*-hydroxybenzenesulphonamido)-5-methyl-1:3:4-thiadiazole, m.pt. 217 to 218°C., 6-methoxy-8-(*p*-hydroxybenzenesulphonamido)-quinoline hydrochloride, m.pt. 268°C. N-(3:4-dimethylbenzoyl)-*p*-hydroxybenzenesulphonamide, m.pt. 187°C., 6-(*p*-hydroxybenzenesulphonamido)-coumarin, m.pt. 230 to 231°C., *p*-hydroxybenzenesulphony guanidine, m.pt. 160 to 162°C., *p*-hydroxyphenyl-2-aminothiazolyl(5)-sulphone, m.pt. 260°C., *p*-hydroxyphenyl-2-aminothiazoly(5)-sulphone hydrochloride m.pt. 247°C. These compounds in a concentration of 1 in 5000 had no bacteriostatic action on *Diplococcus pneumoniæ* (type 1), *Eberthella typhosa*, *Staphylococcus aureus* and *Escherichia coli*. G. R. K.

Salicylic Acid, Some Substituted Amides of. K. A. Jensen and S. C. Linholt. (*Acta chem. scand.*, 1949, **87**, 205.) Six amides were prepared by treating the appropriate amino compound in pyridine solution with acetylsalicyloyl chloride and removing the acetyl group by hydrolysis. The amino compounds used were 2-aminothiazole, 2-aminopyridine,2-amino-5-methyl-1:3:4-thiadiazole, sulphanilamide, sulphathiazole and *pp'*-diaminodiphenylsulphone. When tested against *Diplococcus pneumoniæ* (type 1), *Eberthella typhosa*, *Staphylococcus aureus* and *Escherichia coli*, they were without bacterial effect in a concentration of 1 in 5,000. G. R. K.

PHARMACOLOGY AND THERAPEUTICS

***iso*-Amidone, a New Analgesic Drug Analogous to.** P. Ofner, R. H. Thorp and E. Walton. (*Nature*, 1949, **163**, 479). 6-Piperidino-4:4-diphenyl-5-methyl-3-hexanone (I) (the piperidyl analogue of *iso*midone) has been synthesised by a route involving the preparation of a nitrile $Ph_2C(CN).CHMe.CH_2NC_5H_{10}$ (II) as an intermediate. The constitution of the nitrile (II) was confirmed by exhaustive methylation and identification of the product, while work is proceeding with a view to isolating other isomers

of (I) which may have been formed in the synthesis. The piperidyl analogue of *iso*amidone prepared showed the smallest degree of undesirable side-actions of any of the active analgesic drugs yet studied. It showed the same effects in acute animal toxicity experiments as amidone or *iso*amidone, death in all cases resulting from acute cardiac failure; all these compounds differed from morphine since they were more toxic upon rapid intravenous injection. In man "piperidyl *iso*amidone" (I) in doses of 12·5 mg. produced no appreciable side-actions. After a 25mg. dose some of the volunteers described a sensation of warmth, were flushed and slightly dizzy, although the symptoms were less pronounced than those observed with other analgesic drugs. There was no significant effect upon the cardio-vascular system. The drug produced analgesia similar in duration to that of amidone, and showed sufficient promise to warrant extensive examination and clinical trials, particularly in obstetrics. Recent work has produced a nitrile, isomeric with (II), which yielded 6-piperidino-4:4-diphenyl-3-heptanone; preliminary results on this compound using young rats indicated that this ketone was approximately twice as active as morphine analgesically, yet only equal to it as a respiratory depressant. R. E. S.

Curare and Erythroidine Alkaloids, a Biological Method for Determination of. E. P. Pick and G. V. Richards. (*Proc. Soc. exp. Biol., N.Y.*, 1949, **67,** 329.) Groups of 5 to 10 white mice, weighing 18 to 20 g. each, are injected subcutaneously with 0·5 mg. of morphine sulphate. Restlessness and the typical tail reflex occur generally within 5 to 10 minutes and persist for 2 hours or more. Curare and erythrina preparations, dissolved in 0·85 per cent. sodium chloride solution, are injected intraperitoneally in a volume not exceeding 0·5 ml. into animals exhibiting the typical morphine reaction. A positive effect appears within 5 to 10 minutes and is characterised by disappearance of the excitement phenomena and relaxation of the tails. This period lasts usually for 10 to 25 minutes and is followed by a gradual reappearance of restlessness and tail phenomena. The median effective dose and standard error for crystalline *d*-tubocurarine chloride is 2·8± 0·2 μg., for *Strychnos* curare 24·0 ± 2·0 μg., for dihydro-β-erythroidine bromide 44·0 ± 3·0 ug., and for intocostrin 20·0 ± 2·0 milliunits. Much higher doses of other drugs, including quaternary alkaloids, are necessary to antagonise the effect of small amounts of morphine in mice. Myanesin, in doses of 8 mg. per mouse, despite its curare-like action on the striated muscles, is not able to depress the tail reflex in morphinised mice. S. L. W.

Curarising and Anti-curarising Action, Prolongation of. H. J. Chase, B. K. Bhattacharya and J. L. Schmidt. (*J. Pharmacol.*, 1949, **95,** 95.) Measurable curare action has been demonstrated in rabbits by the "head-drop" assay method to be present 48 hours after the subcutaneous injection of a suspension containing 30 mg. (200 units)/ml. of *d*-tubocurarine chloride in a peanut oil and 4·8 per cent. beeswax vehicle. Suspension of neostigmine in the vehicle failed significantly to prolong the action of neostigmine. The duration of the anti-curare effect of neostigmine intravenously and subcutaneously, when measured in rabbits, corresponds closely with the duration of clinical effects of this drug seen in the treatment of myasthenia gravis. The duration of the anti-curare action of a single intravenous injection of di-*iso*propylfluorophosphonate solution containing 1 mg./ml. was also determined in rabbits. The maximum antagonism to curare occurred following a dose of 2 mg./kg. and the duration of this action for 12

days or more simulates what is known of the duration of di-*iso*propylfluorophosphonate effect on serum cholinesterase. The prolonged effect of large subcutaneous doses of neostigmine and large intravenous doses of di-*iso*propylfluorophosphonate resulted in an increased sensitivity to curare.
S L. W.

Digitalis, Assay of, with Lupin Seedlings. W. Hauser and K. Rosenberger. (*Scientia pharm.*, 1949, **17**, 11.) It has been proposed to assay digitalis by measuring the retarding effect of its extract on the growth of seedlings of *Lupinus albus*. The authors have shown that this effect is due to a saponin fraction which can be isolated by means of the cholesterol compound, and that the active glycosides have no influence on the growth of the seedlings. The method is more sensitive to saponins than the hæmolytic index, and would appear to be useful, since it is known that the absorption of the heart-active glycosides is favoured by the presence of the saponins of digitalis leaf.
G. M.

Flaxedil, Curare-like Actions of. W. W. Mushin, R. Wien, D. F. G. Mason and G. T. Langston. (*Lancet*, 1949, **256**, 726.) Flaxedil, tri-(diethylaminoethoxy)-benzene triethyliodide, is a curarising agent with an action similar to that of curare, inhibiting the transmission of nervous impulses by acetylcholine and blocking transmission across the myoneural junction of voluntary muscle. It is a stable, water-soluble compound, and aqueous solutions are miscible with those of thiopentone sodium. Assays by the rabbit head-drop method indicate that it is about one-third as potent as *d*-tubocurarine. The dose needed to arrest respiration is about 1·7 times the curarising dose, and the ratio for *d*-tubocurarine is 1·5. The neuromuscular blocking effects are easily reversible by neostigmine or physostigmine. It has no eserine-like properties and it stimulates isolated rabbit gut only in comparatively high concentrations. It causes no vasodepressor effect even with many times the paralysing dose, provided there is adequate oxygenation. It has but little paralysing action on ganglia. Given in doses of about 1 mg./kg. intravenously in conscious men it caused complete paralysis of the flexor muscles of the forearm and of the abdominal muscles within 4 minutes, the paralysis passing off within about 25 minutes. At this dosage there was no demonstrable decrease in pulmonary ventilation, there was no pronounced alteration of blood pressure, sweating was not marked, and there was no cyanosis. When used during light cyclopropane and ether anæsthesia in 45 adult patients, to paralyse the abdominal wall during major abdominal operations, it was usually necessary to give up to 100 to 120 mg., and 2 doses were necessary, the first wearing off after about half an hour.
S. L. W.

Insulin, Subcutaneous Implantation of, in Diabetes Mellitus. L. Vargas. (*Lancet*, 1949, **256**, 598.) The material for making the pellets was obtained from the complex of protamine zinc insulin precipitated by addition of the buffer solution. This emulsion was filtered, and the residue from the filter paper dried *in vacuo*, powdered, and mixed with an equal amount of cholesterol after both substances had been passed through ether. This contained 8·6 to 17 I.U./mg. The pellets, prepared aseptically by compression, weighed 3 to 14 mg. and measured 1·5 mm. across and 2 to 10 mm. long. On the basis of rabbit experiments the daily absorption was calculated to be about 1 per cent. of the amount implanted. In the 7 cases presented it

was shown that the pellets implanted subcutaneously are absorbed as slowly as fat-soluble hormones. The dose used in the implant varied from 20·0 to 92·7 I.U./kg. of bodyweight, except in one case in which 450 units were given. The calculation of a daily absorption of 1 per cent. was confirmed in these cases, which assures a maximum duration of activity of the implant of about 100 days. Massive absorption has never taken place and there is therefore no danger of hypoglycæmic shock. Protamine zinc insulin alone is absorbed as slowly as the cholesterol complex, so that even if the pellets were to break there would be no risk. This method of administering insulin not only abolishes injections but may achieve better results since the continuous action of the implant imitates the effect of an artificial endocrine pancreas. S. L. W.

Iron Therapy of Anæmia, Intravenous. J. A. Nissim and J. M. Robson. (*Lancet*, 1949, **256**, 326.) Saccharated iron oxide was prepared from the following analytical grade reagents: ferric chloride hexahydrate, anhydrous sodium carbonate, sodium hydroxide and sucrose. It was found that there was a linear relationship between toxicity and the effect of heating, as measured by the physical characteristics of the substance produced, thus making it possible to select the best samples for intravenous injection. The best sample was found to have LD50 300 mg. or 15 ml./kg. of a 2 per cent. solution, as compared with the 180 mg. or 9 ml./kg. of the original sample reported on. This new preparation has been repeatedly given intravenously to several patients in doses of 300 mg. of elemental iron without toxic reaction, and it is possible that even larger doses might be tolerated. Compounds of iron with glucose or fructose instead of sucrose were found to form much more easily and require less heat for their preparation, but they were also more toxic, having an LD50 of about 150 mg. or 7·5 ml./kg. in mice. Samples prepared according to the method described by Stack and Wilkinson invariably precipitated on autoclaving, probably due to the formation of ferric glucosate. True saccharated iron oxide is much more resistant to heat and shows hardly any breakdown when autoclaved.

S. L. W

Iron Therapy, Intravenous. G. Hemmeler. (*Acta med. scand.*, 1949, **132**, 364.) Iron given by intravenous injection has a much greater erythropoietic action than when given by mouth. Moreover iron may be administered in this manner with advantage to patients who are intolerant of iron by mouth or in whom there is faulty absorption of iron. Ferrous or ferric salts are not well tolerated by injection owing to the fact that the iron is present in the ionised form. If, on the other hand, the medicament is given in the form of a complex salt in which the iron is only slowly ionised in the system after injection secondary effects are less liable to occur. A non-ionised preparation which meets this requirement, namely, the sodium salt of ferri-di-($\alpha\gamma$-dioxy-$\beta\beta$-dimethyl) butyrate, has been successfully used by the author in the treatment of iron-deficiency anæmia. The therapeutic dose of this preparation is well below the dose which is likely to give rise to toxic effects, and a dose of 40 mg. daily is well tolerated and cures even severe cases of iron-deficiency anæmia within 3 or 4 weeks. Intravenous iron therapy should not be used except in patients suffering from iron deficiency, since animal experiments show that iron in excess of that required for the synthesis of hæmoglobin is not eliminated but is stored in the liver and spleen. S. L. W.

N-Methyloxyacanthine Iodide, Curariform Activity of. D. F. Marsh, D. A. Herring and C. K. Sleeth (*J. Pharmacol.*, 1949, **95**, 100.) Oxyacanthine, isolated from *Berberis vulgaris*, has many structural components found in *d*-tubocurarine. A comparison of the pharmacological activity of its quaternary derivative, N-methyloxyacanthine iodide, in rats, rabbits, dogs and man, with that of *d*-tubocurarine chloride shows that it is one-half to three fourths as active as paralysing agent. It differs however in that it possesses a weak atropine-like action. In trained dogs, paralysing doses of *d*-tubocurarine chloride produce copious salivation, but paralysing doses of N-methyloxyacanthine iodide produce no salivation. A similar effect occurs in man; during experiments with N-methyloxyacanthine, subjects experienced a dryness of the mouth which persisted for several hours after the experiment had terminated. S. L. W.

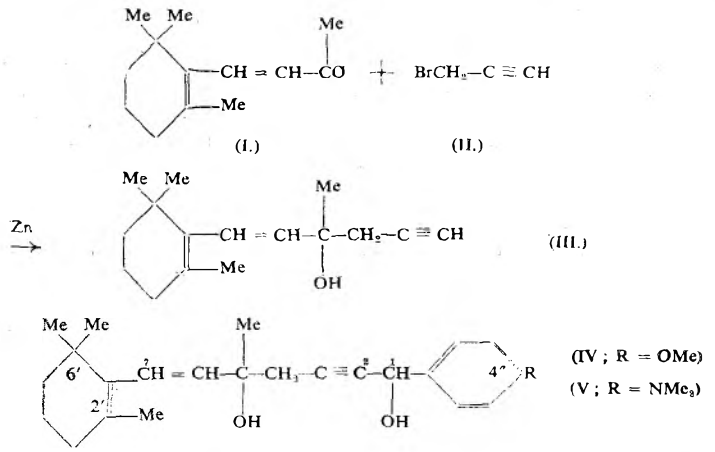
Myanesin in the Treatment of Tetanus. M. H. A. Davison, A. B. Ward and E. A. Pask. (*Brit. med. J.*, 1949, **1**, 616.) Hæmoglobinuria following the use of myanesin in tetanus has been reported in about 20 cases. Two cases are described. The first patient was treated mainly by sedatives and *d*-tubocurarine chloride and received 58 ml. of myanesin intravenously over a period of 60 hours. He recovered and had no hæmoglobinuria. The second, a much more severe case, was treated solely with myanesin, which efficiently controlled the spasms until hæmoglobinuria developed, after 265 ml. had been given, in 26½ hours. The myanesin was then discontinued; sedatives were given but were ineffective and the patient died 2 hours later. During these two hours hæmoglobinuria almost disappeared, and the kidneys were normal to the maked eye and on section. This supports the view that hæmoglobinuria may be temporary and might perhaps be disregarded. These cases indicate not only that myanesin acts efficiently in controlling the spasms of tetanus, without paralysing the muscles of respiration, but that its use in massive doses will bear further investigation. Tolerance did not develop, which was in marked contrast to the reaction of the first case to sedative drugs. S. L. W.

Thiourea in the Treatment of Thyrotoxicosis. G. T. Kent, R. A. Shipley and K. D. Rundell. (*Amer. J. med. Sci.*, 1949, **217**, 627.) A series of cases of thyrotoxicosis were treated with daily doses of 0·1 to 0·3 g. of propylthiouracil or with thiourea in a similar dosage. The latter usually received also 15 minims daily of Lugol's solution of iodine. The clinical efficacy of the two substances was found to be of similar magnitude but thiourea was inferior to propylthiouracil in that it gave a higher incidence of side reactions, chiefly fever, which occurred in 16 per cent. of cases. The relapse rate in 27 patients, treated for from 3 months to 2 years before the drug was withheld, was 77 per cent. H. T. B.

LETTER TO THE EDITOR

The Synthesis of 4-hydroxy-6(2′:6′:6′-trimethylcyclohex-1′-enyl)-4-methyl-hex-5-en-1-yne

SIR,—By condensation of β-ionone (I) with propargyl bromide (II) employing a Reformatsky type of reaction, 4-*hydroxy*-6(2′:6′:6′-*trimethylcyclohex*-1′-*enyl*)-4-*methyl-hex*-5-*en*-1-*yne* (III) has been obtained in good yield. The



product formed a yellow oil (Found : C, 83·2; H, 10·4; $C_{16}H_{24}O$ requires C, 82·7; H, 10·4 per cent.) which showed selective light absorption at λ_{max} = 232mμ, $E^{1\ per\ cent.}_{1\ cm.}$ = 255, and gave a violet-blue colour with the Carr-Price reagent. On catalytic microhydrogenation it absorbed 2·7 molar equivalents of hydrogen, the 1′:2′-*cyclo*hexenyl double bond evidently resisting hydrogenation because of its tertiary character. Zerewitinoff determinations revealed the presence of two active hydrogen atoms.

The structure assigned to (III) has been confirmed by reaction of its Grignard reagent with *p*-methoxybenzaldehyde when 1:5-*dihydroxy*-7(2′:6′:6′-*trimethylcyclohex*-1′-*enyl*)-1(4″-*methoxyphenyl*)-5-*methyl-hept*-6-*en*-2-*yne* (IV) Found : C, 77·8; H, 9·7; OMe, 7·6. $C_{24}H_{32}O_3$ requires C, 78·2; H, 8·8; OMe, 8·4 per cent. was obtained. Reaction with *p*-dimethylaminobenzaldehyde gave the corresponding 4″-*dimethylamino*-analogue (V) (Found : C, 78·4; H, 9·4; N, 3·7; $C_{25}H_{35}O_2N$ requires C, 78·7; H, 9·2; N, 3·7 per cent.), characterised by selective light absorption at λ_{max} = 260 mμ, $E^{1\ per\ cent.}_{1\ cm.}$ = 470.

Experiments on the conversion of (III) into vitamin A alcohol are in progress.

The authors thank Dr. R. E. Stuckey and Mr. P. S. Stross for the absorption data, and the Directors of The British Drug Houses, Ltd., for permission to publish these results.

Research Department,
The British Drug Houses, Ltd.,
London, N.1.
10th October, 1949.

B. N. FEITELSON
V. PETROW
O. STEPHENSON.

NEW REMEDIES

The asterisk (*) after the name of an article indicates that the information given is derived from the makers' publications. Further information regarding these articles may be obtained by application to the Editor.

Diparcol* is a proprietary brand of diethazine hydrochloride or β-diethylaminoethyl-N-phenthiazine hydrochloride, a pinkish-white crystalline powder, almost odourless and having a bitter, numbing taste. M.pt. 175°C.; soluble 1 in 10 in water at 25°C., in ethyl alcohol and in chloroform, but insoluble in ether. It is rapidly absorbed from the gastro-intestinal tract and is especially indicated in Parkinson's syndrome. It acts mainly upon hypertonia, treatment leading to disappearance of rigidity; tremor is reduced, and cramps and paræsthesia relieved. Treatment is started with from 2 to 5 0·05 g. tablets every 4 to 5 days until the most effective dosage is reached. Full improvement usually appears after 6 to 10 weeks of treatment. Untoward reactions include mental confusion, sometimes with nausea and vomiting, somnolence and occasionally exacerbation of symptoms. Diparcol is issued in sugar-coated tablets containing 0·05 and 0·25 g. in bottles of 100 and 500, and in 5 ml. ampoules. S. L. W.

Disecron* is a compound solution containing 2·5 mg. of œstradiol monobenzoate and 12·5 mg. of progesterone in 1 ml. of ethyl oleate, for intramuscular injection. It is indicated in the treatment of certain forms of secondary anæmia, the most satisfactory result being obtained in cases of less than 2 years' duration. It it most likely to be successful in secondary amenorrhœa of the emotional and environmental type. One injection is given intramuscularly on each of 2 consecutive days, succeeding 2-day courses being repeated at 28-day intervals. It is issued in boxes of 2, 10 and 25 ampoules each containing 1 ml., and in vials of 10 ml. S. L. W.

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Neo-Octon* is a proprietary brand of *iso*amylaminomethylheptene hydrochloride, a synthetic antispasmodic with low toxicity and freedom from side-effects. It is indicated in conditions in which it is desired to relieve spasm of the smooth musculature, e.g., spastic conditions of the gastro-intestinal tract, ureter and bladder, dysmenorrhœa and biliary colic. Orally, 1 or 2 tablets (0·05 to 0·1 g.), or 10 to 20 drops of 5 per cent. solution, are given twice or three times daily; parenterally, 0·5 to 1 ml. (0·025 to 0·05 g.) is given twice or three times daily subcutaneously, intramuscularly, or if necessary, intravenously. Neo-Octon is issued in tubes of 10 or bottles of 100 and 500 tablets; in bottle of 10 ml. and 100 ml. of a 5 per cent. solution; and in boxes of 6 and 30 ampoules. S. L. W.

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