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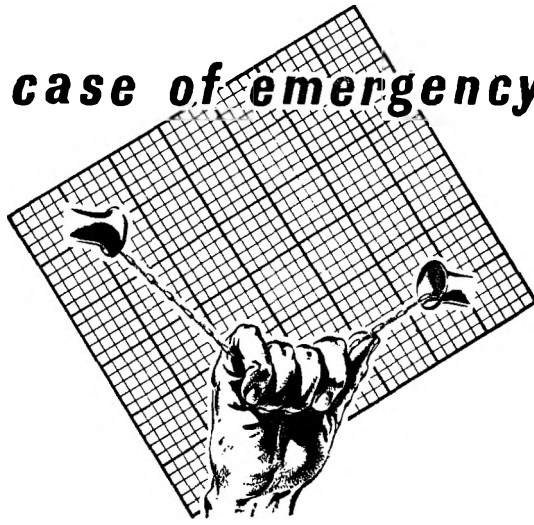

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REVIEW ARTICLE

ALKALOID FORMATION IN PLANTS*

By W. O. James, M.A., F.R.S.
Department of Botany, Oxford

The Accumulation of Alkaloids in Plants

In speaking of the relations between alkaloids and the plants that form them, it is convenient to begin with the distribution of the alkaloids within the plants' tissues. This is something that can be described with a good degree of accuracy and certainty, virtues which are not shared by all branches of our inquiry. The pioneer work was done by Errera and his school[1–6], working at Brussels over the 20 years from 1886 to 1906; and they did the job so thoroughly that little that is really new has been added since. Their aim was to show where alkaloids accumulate in plants, not limiting themselves to a single kind of alkaloid nor a single species of plant. They therefore needed a reagent of low specificity, reacting generally with alkaloids; and which should also have the additional property of readily entering cells and reacting with the alkaloids *in situ*. After many trials, they decided that the best for their purpose was Bouchardat's reagent, i.e., iodine (1 per cent.) in potassium iodide solution (1 per cent.). To show that their precipitations were alkaloidal and to prevent any confusion with the quite different glycogen and protein reactions, they showed that the reactions were absent from the same tissues previously treated with ethanolic tartaric acid solution (5 per cent.) to remove the alkaloids. More specific reagents, such as methylal-sulphuric acid for morphine, were used as appropriate for confirmation. They examined a very wide range of alkaloid-bearing plants, covering alkaloids of such diverse types as nicotine, the tropane series, morphine and its allies, colchicine, the glycosidal alkaloid solanine, and many groups such as those of the legumes, orchids and Amaryllidaceæ, which at that time were little known and imperfectly characterised.

One of the plants most searchingly examined in this way was *Atropa belladonna*, and it has since been examined by other workers including those in my own laboratory[8,9]. No serious discrepancies have come to light. Moreover, the same general results apply to all the numerous plants examined by the Brussels school, and Errera was able to sum up by drawing attention to 4 main tendencies[10].

Alkaloids tend to accumulate in:—

(1) *Very active tissues*, such as meristems of stem and root apices, including the apices of lateral roots while still embedded in the parent pericycle. Young potato sprouts are particularly rich in solanine and should not be eaten. Particularly interesting is the accumulation in wound cambia arising below the cut surfaces.

* Based on two University of London lectures delivered at The School of Pharmacy on May 6 and 7, 1953.

Molle, of the Brussels school, showed these accumulations in the regenerating cells below the actually damaged ones of *Clivea miniata* leaves.

(2) *Epidermal and hypodermal tissues*, often including the hairs of the epidermis. It has been suggested that the relatively volatile nicotine may be lost in this way from the surface of *Nicotiana* plants.

(3) *Vascular sheaths*, pericycle and endodermis, but not the conducting tissues themselves[1].

(4) *Latex vessels* (where present) as in all parts of the opium poppy plant, but especially in the capsules[2].

At the other end of the scale, dead cells within the living plant rarely contain much alkaloid. *Cinchona* barks may hold as much as 10 per cent. of total alkaloids; but they occur strictly within the living parenchyma, not in the dead cork and fibres[11]. An exception is provided by *Datura* seeds. After fertilisation the perisperm contains alkaloids and, during the development of the seed, the perisperm cells are exhausted and dry to a thin membrane of crushed cells. These dead cells do contain much of their original alkaloid content, which is readily leached out with water[12]. *Berberis* alkaloids accumulate in the wood of old stems, partly in the walls of dead cells, but still mostly in living cells adjacent to the vascular bundles[13,14].

Within the living cell itself the alkaloids are almost always accumulated in the vacuole as water-soluble salts of the usual vegetable acids, or sometimes as salts of special acids, such as cinchotannic acid in *Cinchona*. The salts remain in solution and are not precipitated even in *Cinchona*. The limitation of the alkaloids to the cell contents can be made more visible by plasmolysing the cells before applying the alkaloid reagent. Wildeman's demonstration[5] with the epidermal cells of orchid petals seems to have been amongst the earliest. Further confirmation comes from the simple observation that, if tissue sections are cut so thin that the individual cells are opened, the alkaloids are lost from them. Any treatment, such as etherisation, that destroys the semipermeability of the protoplast allows the vacuole contents to escape, and the alkaloids are lost with them. If, instead of immersing a root tip directly in Bouchardat's reagent, it is first treated with a drop of ether and the ether afterwards removed, the alkaloid streams out from the root tips and forms a cloud of precipitate in the surrounding iodine[8].

Chaze[15] made careful observations on radicles of germinating *Nicotiana* seeds. He found the meristematic cells to contain aleurone, i.e., storage protein, grains which broke down as germination proceeded. As the proteins disappeared, they were replaced by minute fluid droplets which took up neutral red, i.e., behaved as vacuoles. These slowly increased and ran together, finally forming the large central vacuole. From the earliest stages of liquefaction, the vacuoles gave alkaloid reactions with iodine. The vacuole is the only part of the cell in which alkaloid accumulation can normally be demonstrated. With the exceptions already mentioned, the impregnation of cell walls by alkaloids, found in some crude

drugs, is a *post mortem* happening permitted by the breakdown of the protoplasmic membranes.

The life history of alkaloid accumulation in cells can also be followed by histochemical methods up to a point. That is, major changes of concentration can be followed, but the methods cannot be made quantitative within a cell, so minor changes are doubtful. Many alkaloid-bearing cells, such as those of *Cinchona* leaf primordia, are devoid of alkaloids in their very early stages[16]; then, during vacuolation, alkaloids accumulate rapidly to a maximum. This may be maintained throughout the further life of the cell, as in the bark parenchyma of *Cinchona*, or there may be a slight falling away. This is characteristic of peripheral pith cells. Cells towards the centre of the pith usually lose their alkaloids entirely as they reach maturity. It is still uncertain whether the loss is due to translocation towards the peripheral tissues of the stem, as the early investigators supposed, or to actual breakdown *in situ*.

Similar ontogenetic sequences occur in plant organs as a whole, and can be established by quantitative macroscopic analysis from a fairly early stage. Leaves are the most convenient and most frequently investigated parts. The time curve reflects that of individual cells. In the basal leaves of belladonna plants investigated through their growing season from April to June, the absolute amount of total alkaloids in the leaf at first increased; but from a fairly early stage began to decline, either on account of transport out of the leaf, or on account of decomposition. It is worth noting that the total alkaloid as a percentage of dry weight decreased from the earliest analyses obtained, emphasising the relative slowness of alkaloid synthesis[17,18].

The accumulation of alkaloids during the life history of the plant as a whole shows several interesting variations. The seeds of alkaloid-forming plants may themselves have no, little, or much, alkaloid in their tissues. *Nicotiana* and opium poppy seeds contain no nicotine nor morphine respectively. *Atropa*, *Datura* and *Erythroxylon coca* seeds have only scanty alkaloids, usually located in peripheral tissues. *Strychnos* and some lupins have larger quantities located in the storage tissues. There is a characteristic behaviour during germination for each of these three classes[18].

As representative of the first, barley will serve. There is no hordenine in the grain. Quantities equivalent to about 0·5 per cent. dry weight appear in the radicle during the first few days of germination[19]. They increase during the first 7 to 10 days and disappear rather rapidly between 14 and 18 days[20]. No hordenine appears again during the life-history of the plant. It is notable that it is lost while the seedling is in vigorous juvenile growth, not when it has become senescent. It is formed during a period of temporary excess of mobilised reserves; and one might suppose that, at this time, more of its amino-acid precursor is formed than can be accommodated in the protein pattern. The germination of *Nicotiana* seedlings shows a similar start; but the alkaloid goes on increasing during the life of the plant.

The second type of behaviour is typified by *Atropa* and *Datura*[21,22]. The small amount of alkaloid in the seed itself disappears. The first

appearance of alkaloids in the germling is again in the radicle and at a very early stage. I have found alkaloids in *Datura* radicles when they were only 2 mm. long. Nevertheless, these alkaloids seemed to be formed *de novo* and not to be merely translocated from the seed's original supply; because the peripheral tissues containing the preformed alkaloids can be removed without detriment to the new formations in the radicle. Again it is noticeable, as before, that the very youngest stages of the radicle, before it is 2 mm. long, are alkaloid-free[8].

The third and more complex story is illustrated by the seeds and seedlings of *Strychnos*, which have alkaloids both in the endosperms and in the embryos[23]. During germination, the alkaloids in the endosperm are broken down under the influence of the embryo; those of detached endosperms kept under the same conditions remain unaltered[24]. This is the way in which the reserve starch behaves in cereal endosperms. Alkaloids in the embryo are also broken down at first and later begin to accumulate again. Sucrose behaves like this in the embryos of barley. These alkaloids, and the more fully investigated alkaloids of lupin seedlings[25], behave like metabolically labile reserves; but their significance in this way should not be exaggerated, because they account at best for so small a proportion of the nitrogen and other materials of the seed.

The behaviour of the alkaloids during the later development of the plant is well illustrated by that of nicotine in tobacco[26]. It is interesting to note how closely nicotine accumulation runs with that of protein in the period of rapid growth, though of course on a smaller scale. Later, nicotine tends to be lost more than protein, and both show secondary increases when the growth of lateral shoots is encouraged. Nevertheless, too much significance must not be read into these parallels, because the accumulation of inorganic salts shows just the same behaviour. They are all consequences of the plant's general growth rather than closely related to one another.

It may be as well to emphasise at this point that everything so far said has applied to the accumulation of alkaloids rather than to their formation. We cannot, for example, assume that the cells in which alkaloids can be shown to accumulate are necessarily the cells in which they were synthesised; but this rather trite observation has not always had the attention it demands.

Solanaceous Grafts

The Solanaceæ are conspicuous for the ease with which they will form interspecific grafts, and these have played a considerable part in recent alkaloid studies; in fact, ever since Strasburger (1885) grafted thornapple cuttings on to potato stocks and had the tubers examined for alkaloids[27]. The chemists who did the analyses for him reported traces of atropine in the tubers.

The alkaloid-forming species in the family are very numerous and from this standpoint they fall into three classes; those that form

(*a*) *solanine and its allies*, i.e., the species of *Solanum* and *Lycopersicum*, including potatoes and tomatoes;

(*b*) *the tropane alkaloids*, particularly the species of *Atropa*, *Datura* and *Duboisia*;

(*c*) *nicotine and its allies*, mainly *Nicotiana*.

Strasburger's original experiment used a scion of type (*b*) grafted on to a stock of type (*a*). All the possible combinations of *a*/*b*, *b*/*c* and *a*/*c*, and their reciprocals, have now been performed with a great variety of species and the resulting grafts analysed in their various parts for tropane and nicotine alkaloids. The solanine-formers have simply been regarded as "alkaloid-free", so far as these other two types of alkaloid are concerned. The general result has been that the alkaloid, characteristic of the stock species, has been found in both stock and scion in about the usual concentrations; and the alkaloid characteristic of the scion species has been virtually absent from both the stock and the scion itself[18]. To this there seem to be some exceptions, and Mothes and Romeike have recently claimed that, when belladonna is grafted on tomato, the results differ with the variety of tomato used[28]. With some tomato strains no tropane alkaloids are demonstrable in the belladonna scions; but with others detectable quantities are formed. If this is confirmed it will be an important result.

Recently, it has become possible to perform an elegant variation on the grafting technique. In all the above an alkaloid "unnatural" for one or the other species is concerned. Evans and Partridge published a method for the separate estimation of hyoscine and hyoscyamine in mixtures[29]. The method depends upon a simple partition chromato-

TABLE I

ALKALOID ANALYSES OF *Atropa belladonna* AND *Datura innoxia*
Normal plants

Species	Sample	Total alkaloids	Hyoscine	Hyoscyamine	Ratio
		mg./g. dry weight			
Atropa belladonna ..	leaves	2·25	0·19	2·20	0·09
Datura innoxia	leaves	0·93	0·79	0·15	4·6
	stems	3·25	2·52	0·54	4·6
	tap roots	0·82	0·81	0·05	17·4
	fine roots	2·00	1·69	0·34	5·0
	leaves	1·80	1·65	0·14	11·4
	tap roots	1·75	1·65	0·10	16·9

graphy and can readily be adapted to the natural mixtures occurring in plants of the type (*b*) above. James and Thewlis[30] have examined the two species *Atropa belladonna* and *Datura innoxia* (frequently spoken of as *D. metel*) in this way. It turns out that the ratio of the amounts of hyoscine/hyoscyamine are very different in the two plants and surprisingly constant for each species. Table I shows the original series of results obtained, and these have since been confirmed. The mean value of the ratio for a considerable number of belladonna analyses was 0·12. The leaves, stems and fibrous roots of the *Datura* species show an excess of hyoscine, and much less hyoscyamine, with the result that the ratio rises about 50-fold. In tap roots it rises even higher, due to the almost

total disappearance of hyoscyamine, and this is the only considerable variation of the ratio noted within either species.

Using these two species it is possible to create a graft of the type *b/b* in which no alkaloids foreign to either component are introduced. The results are a confirmation of the older ones. The results in Table II show that, whichever way round the graft is made, the ratio characteristic of the stock appears in the scion as well as in the stock itself. The constancy of the ratio in belladonna is very striking; the somewhat greater variation of the ratio in *Datura* results from the error in estimating the very small amounts to which the hyoscyamine is reduced. Evans and Partridge, in a recent letter to *Nature*[31], say that they have themselves obtained similar results and have succeeded in analysing the hyoscyamine fraction further, revealing the presence of small quantities of meteloidine, in *Datura innoxia*.

TABLE II

ALKALOIDS IN RECIPROCAL GRAFTS OF *Atropa belladonna* AND *Datura innoxia*

Graft	Sample	Total alkaloids	Hyoscine	Hyoscyamine	Ratio
		mg./g. dry weight			
A. belladonna on *D. innoxia*	scion	1·07	0·91	0·08	11·4
	stock	1·20	0·90	0·12	7·6
D. innoxia on *A. belladonna*	scion	1·75	0·16	1·60	0·10
	scion	1·81	0·15	1·60	0·09
	stock	1·10	0·13	1·03	0·12

The distribution of the alkaloid in the scions receiving them has received some attention. The concentration in the leaves may frequently reach a level commensurate with the normal concentration in the leaves of the stock species. It is possible to grow the grafts to maturity and obtain a crop of fruit from the scions. When tomatoes are grown upon belladonna or thornapple stocks, great interest attaches to the question whether the fruit will contain alkaloids also, though remaining—as they do—to all appearances normal. There has been considerable diversity of opinion about the answer. Continental workers have frequently reported the identification of mydriatic alkaloids in such fruits[32,33,34]. Our own experience has been consistently negative, as in the results of Table III. Mothes[38] has reported that, of 3 varieties of tomato raised

TABLE III

ALKALOIDS IN TOMATO ON BELLADONNA GRAFT

		mg./g. dry weight
Tomato scion	leaves	8·05
	fruit 1–2 cm.	0·00
	2–3 cm.	0·00
	>3 cm.	0·00
	ripe	0·00
	pedicels	1·00
	stem	1·90
Graft union	—	2·25
Belladonna stock	stem	0·90
	roots	2·85

on *Datura* stocks, one had no alkaloid in the fruit, one had a little, and the third relatively large quantities. Miss Steenstra, in Amsterdam, similarly reported that some varieties of tomato accumulate alkaloids while others do not. Mrs. Wilson, recently at Oxford and now at Reading, arranged exchanges of seed with Miss Steenstra, and has now examined 10 different varieties of tomato grown as scions on belladonna stocks, and has been unable to demonstrate tropane alkaloids in any of them. She has also compared analytical methods with Miss Steenstra. It has become the practice in some southern states in America to grow tomatoes commercially upon *Datura* stocks. This is done because tomato roots suffer heavy infections to which *Datura* is immune. Although traces of alkaloids can be detected in the fruits by large-scale reductions of material, no harmful results seem to have afflicted the population.

Translocation of Alkaloids

The alkaloids exist in the plant exclusively as water-soluble salts. They are therefore likely to be moved from one part of it to another. There are two main directions of translocation: (*a*) towards the leaves and (*b*) away from them; whether the movement is physically upwards or downwards is relatively unimportant. Movement of solutes into the leaves mainly depends on the movement of the transpiration stream, the water of which is evaporated from the internal leaf surfaces, leaving the solutes stranded. Movement away from the leaves is mainly by way of the phlœm towards points of consumption of metabolites, such as the stem, root and flowering apices.

Movement of alkaloids in the transpiration stream passing from roots to leaves is an established fact. It is rendered probable by the graft results already discussed, and confirmation comes from the analysis of the sap seen to extrude from the stumps of decapitated tobacco plants. Dawson has shown this to contain 0·2 mg. of nicotine per ml.[36]

Movement in the phlœm presents a prettier problem. There is general agreement that alkaloids cannot be demonstrated within sieve tubes by histochemical methods, although neighbouring cells, such as phlœm parenchyma and bundle sheaths, may contain considerable quantities. This may only mean that movement keeps the concentrations in the sieve tubes too low for detection. There are, on the other hand, numerous results which are consistent with the idea of a phlœm transport. It was shown earlier, for example, that mature belladonna leaves lose alkaloids while attached to the plant; whereas detached leaves of a comparable age do not lose alkaloids until a general breakdown sets in.

The usual device of stem-ringing is not available for examining translocation among the Solanaceæ, because they have internal phlœm; and with the aim of getting round this difficulty Mrs. Wilson has performed a number of variations of the grafting method.

Her first device was the rooting of alkaloid-free scions, as illustrated in Figure 1. Belladonna was side-grafted on to *Physalis* or tomato and grown on to a convenient size. Then a downward-pointing flap

was cut from one side of the young belladonna stem and surrounded by damp moss in a split pot. Rooting took place after about a fortnight and the graft was grown on for 2 or 3 months. Without the roots it would have remained alkaloid-free in all its parts. Analysis of a belladonna/tomato graft with roots on the scion revealed 1·84 mg. of alkaloid in the scion roots themselves, 0·35 mg. in the scion shoot, a doubtful trace in the stem below the scion roots, and 0·51 mg. in the tomato stump and roots. It can only be supposed that the alkaloids found in the tomato stock had travelled down the belladonna stem between the two root systems. One would not expect to find appreciable quantities of alkaloids in the stem itself, since they do not accumulate in the basal parts of the stems of normal belladonna plants.

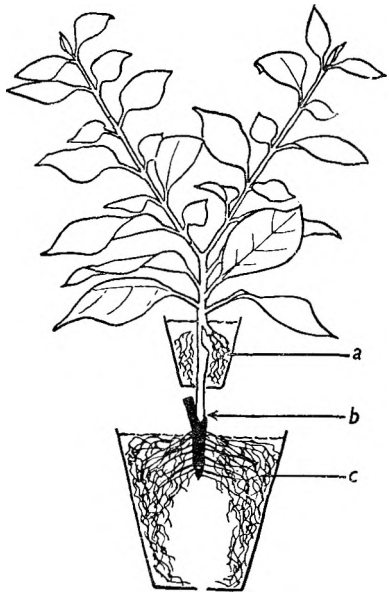


FIG. 1. Diagram of an *Atropa belladonna*/tomato "flap-rooted" graft.

a. Scion roots.
b. Side graft union.
c. Tomato stock.

After Wilson[37].

Approach grafts also gave corresponding results. A belladonna and tomato plant grown together in a single pot were side-grafted together and grown on without further manipulation. At harvest the tomato roots were found to contain 0·94 mg., or nearly a third, of the belladonna alkaloids in the graft. Similarly, grafting a rooted belladonna plant to an alkaloid-free belladonna scion carried by a tomato stock caused the accumulation of belladonna alkaloids in the stem and roots of the tomato stock. As in the first manipulation, the alkaloid must have travelled down through a considerable length of belladonna stem—initially free from alkaloids—to reach the tomato roots.

An interesting variation of this technique was obtained by grafting belladonna and potato together. Here the potato plant formed young tubers as well as roots below the graft union. At the time of harvest the new potatoes were to all appearances normal; but a pair taken for analysis contained 0·74 mg. of belladonna alkaloids. The occurrence of tropane alkaloids in potatoes raised on *Datura* stocks has been observed by Mothes[38,39].

Bridge-grafts provided another method. In these a downwardly-directed flap, an inch or more long, was raised from the side of a normal belladonna stem. A converse upwardly-directed flap was cut in an alkaloid-free belladonna scion, and the two grafted together. After 17½ weeks' growth, belladonna alkaloids were recovered from the tomato

stock carrying the scion to the extent of 23·71 mg. These experiments have been frequently repeated with consistent results, and seem to make it clear that belladonna alkaloids do travel along belladonna, and other, stems in a direction opposite to that of the transpiration stream and, therefore, probably in the phlœm. It was shown that they travel into fine roots and young tubers, i.e., into tissues which are rapidly growing and metabolising. The one unexpected exception seems to be the young fruits, and it is still uncertain whether the alkaloids that are normally abundant in belladonna berries are conveyed there from the roots or are synthesised *in situ*.

The Site of Alkaloid Synthesis

All parts of a plant are involved in the earlier stages of alkaloid synthesis, but, if attention is limited to the final and most characteristic steps, it is possible that the site is more limited. The results of the grafting experiments described above are usually taken to indicate that synthesis is predominantly in the root. Confirmation comes from the observation of alkaloid synthesis in tissue cultures of detached tobacco[40] and *Datura* roots[41], and probably from the early appearance of alkaloids in root meristems of barley, tobacco and opium poppy when all other parts, including the seed itself, are alkaloid-free, and from the rapid appearance of alkaloids in alkaloid-free scions, or even cut leaves of scions, when they are induced to root.

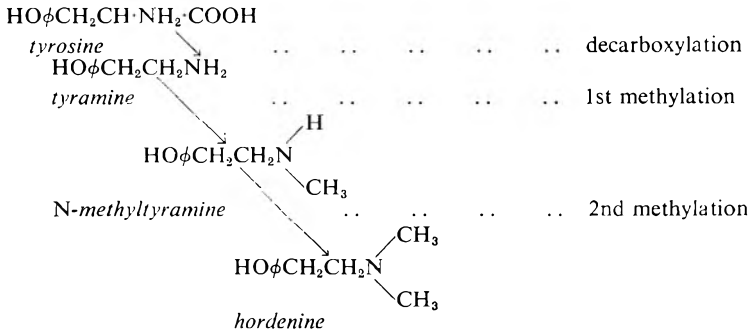
A number of exceptions has been listed. Detached *Cinchona succirubra* leaves are reported to increase their quinine when kept in the dark; *Nicotiana glauca* forms anabasine either as stock or scion[43]; and *Nicotiana glutinosa* scions form nornicotine, apparently by demethylating nicotine received from the roots[43,44]. In reciprocal grafts of *Datura tatula* and *Datura ferox*, the former continues to yield its normal hyoscyamine when a scion. *Datura ferox*, as a scion, contains its normal meteloidine. Both contain hyoscine, and it has been suggested that hyoscyamine and meteloidine are either being formed *de novo* or by reduction of hyoscine in their respective shoots[45]. It is difficult to assess the significance of these results at present, because the mixture of alkaloids in the stocks has not yet been analysed, and because the ratio of hyoscine/hyoscyamine, or hyoscine/meteloidine, is very different in the scion from the value given for the normal top.

Attempts to form nicotine and tropane alkaloids in cut leaves and shoots have led to confused results, and the most usual and vigorous site of synthesis certainly seems to be the young root, probably in vacuolating cells. The evidence from grafts that alkaloids are not normally formed in shoots is not quite conclusive on account, for example, of the possibility of a suppressing influence of a foreign stock (cf. also Mothes[38]).

Mechanism of Alkaloid Synthesis

It is a convention, credible at present, that alkaloid synthesis may be taken as starting with an appropriate amino-acid, such as ornithine leading to tropane alkaloids, or proline to the nicotine series. Numerous

attempts have been made to obtain evidence for the occurrence of such series in plant tissues, so far with very little success, the main obstacle being, apparently, the extreme sluggishness of the reactions which it is wished to investigate. As a type, the relatively simple formation of the alkaloidal amine hordenine may be briefly discussed. Its isolation and characterisation were accomplished by Léger[19] in 1906, and the following mechanism of formation from tyrosine was suggested by Raoul[46] in 1937.



Formation of hordenine is restricted to young barley roots during the first 3 weeks. It increases more or less steadily to a maximum during the first fortnight, and then more rapidly disappears. Evidence of the reaction-path comes from identification of the substances concerned and from feeding experiments. It has been shown that free tyrosine is present in 3-day-old barley seedlings, though it may be present in the seed only combined in its proteins[47]. Tyramine and *N*-methyltyramine have been identified by separation on paper chromatograms. They are present only in small amounts and could be recognised from about the 10th to the 14th day[20]. *N*-methyltyramine has been isolated in larger quantities by V. S. Butt at Oxford. After separation on a cellulose column it was obtained as its hydrochloride giving an undepressed melting point at 148° C. 15 mg. was obtained from the germination of 1 kg. of Spratt-Archer barley. *N*-methyltyramine has also been isolated by Kirkwood and Marion[45] from a strain of barley that does not produce hordenine.

Two major difficulties are encountered in feeding experiments; hordenine is formed only at a time when the endosperm is providing abundant amino-acids, and the surface of barley seedlings is normally infested with micro-organisms that may affect external nutrient additions. This is particularly important in the initial decarboxylation, since some bacteria appear to be particularly active in decarboxylating amino-acids.

To get round the first difficulty, Kirkwood and Marion[49] fed labelled tyramine to barley seedlings and subsequently identified similarly labelled *N*-methyltyramine and hordenine chromatographically. The intensity of the activity was higher in the *N*-methyltyramine than in the hordenine. Labelled tyrosine (*dl*-tyrosine-2-C^{14}) gave similar results[50], and the evidence was taken to support the existence of the series of reactions

proposed. Since no labelled tyramine was found, it was presumed that its existence was transient. No evidence of the absence of contaminating micro-organisms was provided.

To overcome both difficulties a series of experiments has been made in the Oxford laboratories by Mrs. S. V. Barber, using excised barley embryos in sterile culture. The embryos were deprived of their endosperms, which were replaced as a source of nutrients by White's medium, without the glycine usually included. They were then germinated, suspended in the medium by vigorous æration in tubes containing 15 embryos each. About 1 tube in 10 became infected after several days and was discarded. After 9 days the embryos had roots about 1 inch long and, grown in this way, were entirely devoid of hordenine or its suggested precursors. When an endosperm extract was added to the White's medium, hordenine was formed. This method, therefore, provided a suitable means of testing possible intermediates. Addition of tyramine and *N*-methyltyramine did not result in any formation of hordenine detectable on chromatograms, even though the bases could be shown to enter the embryonic tissues by the staining due to their partial oxidation to melanins.

The failure to form hordenine might be due to lack of methyl donors. The experiments were, therefore, continued with additions of formate, betaïne, choline or methionine as well as the base. Betaïne proved toxic at the concentration used (15 mg./l.). Formate + tyramine produced no synthesis of hordenine, but choline and methionine when added with tyramine both caused the formation of small amounts of *N*-methyltyramine and hordenine. Methionine with added N-methyltyramine also gave hordenine. The efficiency of methionine as a methyl donor in plant tissues has also recently been emphasised by its action in *Dicentra* seedlings during the synthesis of protopine[56]. There is therefore good evidence that hordenine may be formed in barley by two successive methylations of tyramine, but the evidence that the tyramine comes from tyrosine is much less conclusive. Prolonged attempts to identify a tyrosine decarboxylase in barley gave consistently negative results. A vigorous glutamic decarboxylase was, however, isolated, the produce of whose action was γ-amino-butyric acid[51]. It is, therefore, conceivable that hordenine may originate from glutamic acid rather than from tyrosine, and that addition of the phenolic group may come after the decarboxylation instead of before. Whichever is the order of events, the origin of the phenol is at present equally uncertain.

Consequences of Alkaloid Formation

As mentioned earlier, the alkaloids in the reserve tissues of seedlings may disappear and their carbon and nitrogen probably be returned to metabolic circulation. The amount of material concerned in these changes is, however, very small, usually less than 1 or 2 per cent. of the nitrogen, and probably much less of the carbon. When alkaloids accumulate in any considerable quantity it is usually in tissues such as the bark of *Cinchona* or the old stems of *Berberis*, from which they are

not lost. The alkaloids cannot be regarded as having much significance as metabolic reserves.

Attempts to show that they exercise a protective function have also met with little success. While it may be that alkaloids are toxic to many predators if taken in sufficient dosages, it appears to be very rarely that this happens in practice. For example, aphids parasitise *Nicotiana* to the great detriment of the plants; they are not poisoned by the nicotine in the plant saps, but can be conveniently destroyed with nicotine sprays. Alkaloid toxicity is specific and often too limited to protect against species dangerous to the plant. The alkaloids of *Atropa belladonna* are highly toxic to the human species, which is not an eater of belladonna, but are innocuous in quite large doses to farm animals, rabbits, birds, aphids, caterpillars and flea-beetles. The last two are common and dangerous pests of belladonna crops. Plant parasites, such as mistletoe on *Duboisia* and dodder on *Conium* and *Delphinium*, are not controlled by the alkaloids of the host, though the alkaloid may pass in considerable quantity into the parasite. Fungal and bacterial attacks are not averted either. Any protective results of alkaloid formation must be slight and erratic. So far no convincing evidence has been produced that alkaloids serve any significant rôle in the plants that produce them, and alkaloid-free scions grown on stocks that form no alkaloids show no abnormalities that might be attributed to their absence.

The Causes of Alkaloid Production

Alkaloid-forming plants, probably about 10 per cent. of the known flora, may be regarded as those in which an additional metabolic reaction-chain has been evolved. The taxonomic distribution of related groups of alkaloids in related species is consistent with this suggestion. Qualitative changes in the alkaloids formed by a particular species, or even changes in their relative quantities, are very difficult to bring about physiologically. The primary control lies with the gene complex. It is known that single genes may directly control the existence or operation of individual enzymes. In crosses of *Nicotiana tabacum* with *N. glauca* the principal alkaloids of the F_1 generation were anabasine and nornicotine, characteristic of *N. glauca*. The formation of nornicotine could readily be explained on the assumption that *N. glauca* possesses a gene controlling the demethylation of nicotine to nornicotine, which is absent from *N. tabacum*, in which the main alkaloid is nicotine itself[44,54].

Any new enzyme formed owing to the mutation of a gene might bring a whole chain of new reactions into being. It is not necessary to suppose that each additional step would require a further mutation. Adaptive enzymes may arise as a response to the presence of a new compound capable of acting as substrate. Such enzymes, stable only in the presence of their substrates, are already known to exist in the higher plants. Pre-existing enzymes of low specificity might react with the new products and modify them further, and some reactions may even be spontaneous, requiring no catalysis. By such processes, discussed in rather more detail elsewhere[55], it is possible to suppose that the great wealth of alkaloids

(not to mention pigments, tannins, glycosides and other plant products) have come into being. Whether they will have any significance for the existence of the plant that forms them is entirely secondary. The alkaloids are evolutionary try-outs. They may be called waste, more or less in the sense that experimental models and chance by-products are waste. They are created by a blind designer, who scores far more failures than successes, but the failures are just as necessary and as inevitable a part of the evolutionary process as the triumphs.

For permission to cite unpublished data in the foregoing, I am greatly indebted to Mrs. S. V. Barber, Mrs. P. M. Wilson and Dr. V. S. Butt.

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BRITISH PHARMACEUTICAL CONFERENCE LONDON, 1953

SCIENCE PAPERS AND DISCUSSIONS

(*continued from page 793*)

THE PREPARATION OF DRY EXTRACTS OF CASCARA

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A DRY extract of cascara sagrada has been official in the British Pharmacopœia since 1885. The first method of extraction was to macerate the bark in No. 40 powder for 48 hours with proof spirit (57 per cent. w/v of ethanol) and then to pack the moist powder into a percolator and percolate with water to a stated volume. The resulting percolate was evaporated on a water bath until a suitable consistency was obtained.

In 1898 the directions were altered to percolation to exhaustion with distilled water and evaporating to dryness on a water bath. This method was retained virtually unchanged in the 1914 Pharmacopœia. The 1932 B.P. directed that the liquid obtained by percolating the bark to exhaustion must be evaporated to dryness under reduced pressure.

In 1948 the official directions were amended to allow the percolate to be evaporated to a viscous liquid at atmospheric pressure, before completing the evaporation to dryness under reduced pressure at a temperature not exceeding 100° C. The same method is included in the 1953 Pharmacopœia.

It is generally considered that the reason for the use of reduced pressure in the preparation of this extract is to render the final product in a suitable form for the preparation of granules and not for reasons of conserving activity—hence the alteration of the method of preparation in 1948.

The method official in the United States Pharmacopœia XIV is to macerate cascara in coarse powder with boiling water for three hours followed by percolation to dryness with boiling water. The resulting percolate is evaporated to exhaustion, reduced to a fine powder and is mixed with sufficient starch to give a specified weight. This method was allowed as an alternative to the official 1932 B.P. method by the Sixth Addendum (1943) to permit the use of extracts obtained from America during the war.

Greco and Dumez[1] have published a modification of this method using a pressure cooker for the preparation of liquid extract of cascara and we have investigated their method and some modifications of it for both dry and liquid extract of cascara.

Until 1948 the only criteria for the quality of cascara extracts were their

physical properties, such as colour, taste and smell. The Pharmacopœia of that year introduced a minimum requirement of 80 per cent. of water-soluble extractive matter and this test is retained in the 1953 edition.

Fairbairn *et al.*[2,3,4,5] have now devised chemical and biological assays for several of the anthraquinone drugs and these have now been applied to cascara and its extracts[6].

As a result of assays of senna extracts Fairbairn and Michaels[7] found that the glycosides of senna are damaged by prolonged heat and Fairbairn[8] suggests that the same may be true of cascara.

We, therefore, decided to compare the activity of extracts prepared by the methods of the 1932 B.P., the 1953 B.P., Greco and Dumez's modification of the U.S.P. XIV method, and a simplification of this method devised by one of us (W. B.).

Experimental

4 extracts were prepared from the same batch of bark:—

A. By the 1932 B.P. method.

Percolation to exhaustion with distilled water and evaporation to dryness under reduced pressure. Sample A.

B. By the 1953 B.P. method.

Percolation to exhaustion with distilled water, evaporation to a syrupy extract at atmospheric pressure, followed by final evaporation to dryness under reduced pressure. Sample B.

C. The method of Greco and Dumez.

The drug in very coarse powder is placed in a suitable vessel in an autoclave and 4 times its weight of boiling water is poured over it. The mixture is allowed to macerate for 15 minutes and is then heated for 10 minutes at 15 lbs. pressure. The material is then packed into a percolator and boiling water is passed through it until it is exhausted. The percolate is evaporated to dryness at atmospheric pressure. Sample C.

D. Bruce's modification of the method of Greco and Dumez.

The drug in very coarse powder is placed in a suitable vessel in an autoclave and 4 times its weight of boiling water is poured over it. It is then immediately heated for 10 minutes at 15 lbs. pressure. The material is drained and transferred to a tincture press and the marc is pressed as strongly as possible. The expressed liquid is added to that previously drained from the drug and the mixed liquids are evaporated to dryness under atmospheric pressure. Sample D.

These extracts and the bark from which they were made were assayed chemically and, in some instances, biologically and the results are shown in Table I.

Each extract was prepared from 500 g. of bark and the yield of extracts are recorded in Table I; it is thus possible to calculate the amount of glycosides originally in the bark which has been retained in the final extracts. These proportions are given in the last column of the table.

TABLE I

CHEMICAL AND BIOLOGICAL ASSAYS OF DRY CASCARA EXTRACTS, EACH PREPARED FROM 500 G. OF CASCARA BARK

SAMPLE	YIELD OF EXTRACT	CHEMICAL ASSAY mg. of glycoside (as aloe emodin) per g.	BIOLOGICAL ASSAY mg. per g. as sennosides A and B	B/C* RATIO	AMOUNTS OF GLYCOSIDES PRESENT IN EXTRACTS	PERCENTAGE OF GLYCOSIDES EXTRACTED
Cascara bark ..	—	7·30, 7·42 } 7·36	—	—	3·68 g.	—
A B.P. 1932 method. 10 l. of percolate	100 g.	7·37	4·86	0·65	0·74 g.	20·04
B B.P. 1948 and 1953 method	122 g.	10·8, 10·8, 10·8, 10·8 } 10·8	6·60	0·611	1·32 g.	35·82
C Method of Greco and Dumez. 9 l. of percolate	143 g.	18·4, 19·0 } 18·7	9·96	0·53	2·67 g.	72·69
D Bruce's method ..	107 g.	10·8, 10·8 } 10·8	—	—	1·15 g.	31·40

* For explanation of B/C ratio *see* Fairbairn and Mahran (this Journal, p. 827).

DISCUSSION

From the results it appears that percolation with boiling water is a more efficient method of extracting cascara than with cold water. The use of autoclaving as suggested by Greco and Dumez, also appears to increase efficiency of extraction.

The method of Greco and Dumez extracted 72·69 per cent. of the glycosides present in the bark compared with 20·04 per cent. by the 1932 B.P. method and 35·82 per cent. by the present official method. Bruce's method extracted 31·4 per cent. and is, therefore, more efficient than the 1932 B.P. method. The Greco and Dumez method shows some saving of time compared with the official method. Bruce's method is very convenient and results in a very great saving of time compared with the other methods and our results show that it gives as good an extract as the official method. This extract was only assayed chemically and gave the same value as the official method with a slightly smaller yield.

The use of reduced pressure in evaporating the extracts to dryness does not result in a more potent product, and, in fact, an extract prepared by evaporation entirely under reduced pressure was slightly less potent than one where reduced pressure was only used in the final stages. This may be due to the greater time necessary to remove all the water when using reduced pressure.

Another possible reason for the greater potency of extracts prepared by the autoclaving method may be that this treatment inactivates enzymes which may cause decomposition of the glycosides. This may also be the reason for the lower potency of extracts prepared by evaporating the percolate entirely under reduced pressure. The lower temperature might

conserve the activity of such enzymes. This explanation would not, however, account for the greater yield of the autoclaving method.

Summary

1. The efficiency of 4 methods of preparing dry extract of cascara is compared.
2. The use of boiling water and autoclaving gives more efficient extraction than cold water.
3. The use of reduced pressure for evaporating the percolate does not give an extract with increased potency and may cause reduction in potency.
4. A method using autoclaving followed by pressing of the marc is as efficient as the official method and results in a great saving of time.

We wish to thank Dr. J. W. Fairbairn and Mr. G. E. D. Mahran for carrying out the chemical and biological assays on these extracts.

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VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

PART VII. THE EVALUATION OF CASCARA SAGRADA AND ITS PREPARATIONS

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INTRODUCTION

CASCARA bark is the dried bark of *Rhamnus purshianus*, D.C. collected at least one year before being used. It has been used as a tonic laxative in medicine since about 1804, and is now a well-established drug for the treatment of constipation. However, there is still no generally accepted method of measuring the activity apart from determining the water-soluble extractive, which is obviously a rather crude method of assessing activity. Numerous methods of chemical assay have been published based on the determination either by gravimetric or colorimetric means of the amount of anthraquinone compounds present, but no-one has been able to correlate their results with the biological activity. Fairbairn[1] has already shown the importance of the form in which the anthraquinones occur and has demonstrated that for cascara, the free anthraquinones contribute little to the purgative activity. He has also shown that for senna and rhubarb, the main activity is associated with the glycosidal or "combined" anthracene derivatives. We therefore decided to estimate the amount of anthracene glycosides in various samples of cascara bark and its preparations and see if the results were correlated with the biological activity.

DETERMINATION OF THE CONTENT OF ANTHRACENE GLYCOSIDES

As already stated, numerous methods of chemical assay of cascara and the closely related drug frangula (*Rhamnus frangula* L.) have been published. Most of them are based on the red colour produced by the well-known Bornträger reaction or one of its modifications; the intensity of the colour is matched against standard solutions of anthraquinones in alkali, or measured photoelectrically. It is obvious, however, that a colorimetric method will only be satisfactory if the anthraquinones can be extracted in a fairly pure form for measuring, but few of the published methods have taken into account the fact that interfering substances are present in cascara and readily pass into the final solution being measured. Furthermore, the absorption curves of the final red colour are seldom given so that it is not possible to know whether the standard used or the wave-length are suitable. In view of our experience with the chemical assay of senna[2] and rhubarb[3], we decided to apply similar methods of colorimetric assay to cascara. This necessitated carefully testing each stage of the process, e.g., removal of free compounds, hydrolysis, etc., as

they applied to cascara. As a result, we found difficulties peculiar to cascara and they had to be overcome before a satisfactory method was evolved.

Difficulties Peculiar to the Chemical Assay of Cascara. Cascara contains a much larger proportion of free anthraquinones than does senna, so that the removal of these requires a comparatively large amount of organic solvent. If ether is used for this purpose as in the senna assay[2], a certain amount of glycoside is also removed and cannot be recovered conveniently. Consequently we used chloroform instead, as had been used with rhubarb.[3] A more serious difficulty was the presence of some pigments which were carried with the liberated aglycones into the final alkaline solution and imparted to it a dirty brown tint. This interfering substance has already been reported by Fairbairn[4] and several other workers have attempted to destroy it. Thus Brandt[5] proposed a 5 per cent. solution of totally effloresced ammonium carbonate. Björling and Ehrlén[6] working on frangula used sodium metabisulphite and sodium bicarbonate and Fairbairn and Lou[3] found that the use of potassium metabisulphite was sufficient for the removal of interfering substances in the assay of rhubarb. Gibson and Schwarting[7] used a chromatographic method of assay and claimed that they could obtain the anthraquinones in a pure form and separate from a yellow pigment which was retained on the column.

We decided to test these methods for eliminating the interfering substance by preparing the absorption curves of the final coloured solutions and comparing them with those of pure aloe-emodin and of pure emodin in alkaline solution. Glycosides of aloe-emodin[8] and of emodin[9] have already been shown to occur in cascara. In view of the fact that Gibson and Schwarting publish absorption curves of their purified fractions and claim they are identical with those of the pure anthraquinones, we firstly tried out their chromatographic method. A disadvantage common to most methods of absorption chromatography is the difficulty of obtaining consistency in successive batches of absorbent; this difficulty is increased when, as in Gibson and Schwarting's work, a commercial product like "Celite" was used. However, we managed partly to overcome this difficulty by obtaining from America supplies of celite and the special magnesia used. Another serious objection to the method was the fact that it took the authors over a week to develop the column; we found that even after 10 days' elution, no differentiation of the anthraquinones had occurred. It is obvious that even if we had been able to differentiate and elute all the anthraquinones, the method would still be inconvenient in practice. We did make further attempts at using absorption chromatography with sucrose, magnesium carbonate, magnesium oxide, alumina and charcoal as absorbents, but were unsuccessful and we concluded that while absorption chromatography is extremely useful for qualitative work, it was not so useful for quantitative work.

We made further attempts at eliminating the interfering substances by the use of various organic solvents to extract the aglycones after hydrolysis and by treatment of these solutions with metabisulphites and bicarbonates

as advocated by Brandt[5], Björling and Ehrlén[6] and others, but in no instance were the interfering substances completely removed (*see* Figure 1 (b)).

During the course of the work, it was noticed that a sample of bark about 6 years old had little interfering substance in the final solution (*see*

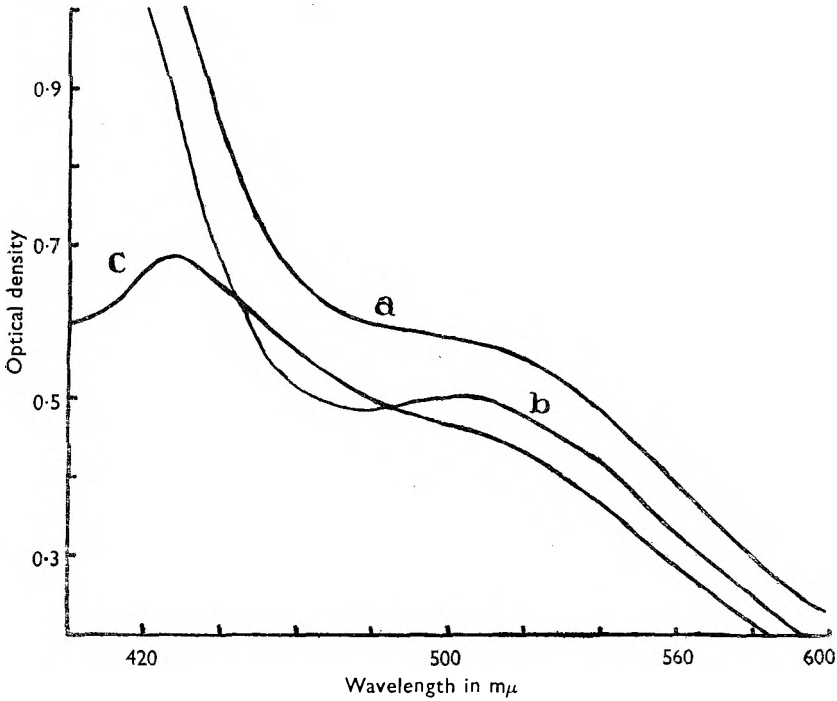

FIG. 1. Absorption curves, based on readings made with a Uvispek of the red alkaline solutions prepared from (a) 1 year old bark C_3 without special treatment; (b) C_3 after metabisulphite and bicarbonate purification stage; (c) 6 year old bark C_1.

Figure 1 (c)). A comparison of the curves 1 (b) and 1 (c) shows that the metabisulphite treatment has removed some colour interfering at about 480 mμ and that prolonged storage has removed substances interfering at about 420 mμ. A combination therefore of these two methods of treatment might well remove the bulk of the interfering substances. It is generally assumed that during storage, a griping or an emetic principle is gradually destroyed; furthermore some workers claim that this prolonged natural process can be substituted by treating the bark with hydrogen peroxide. Though there was no reason to connect the interfering substances with this griping principle, we decided to try whether treatment with peroxide at various stages in our chemical assay process would destroy some of the interfering substances. This quite empirical idea turned out to be successful in practice, though another equally empirically determined stage was necessary before the final solution was pure enough for colorimetric assay. We found that if a fairly large proportion of hydrogen peroxide was added during the acid hydrolysis of the glycosides, and the final

alkaline solution of the liberated aglycones was also oxidised with hydrogen peroxide as in the assay of the other anthraquinone drugs, the solution was practically free from interfering substance. If, however, the final alkaline solution was acidified, extracted with ether and the ether solution re-extracted with sodium hydroxide solution this new alkaline solution is now quite pure as is indicated by its absorption curve which is very close to that of a mixture of aloe-emodin and emodin (Figure 2).

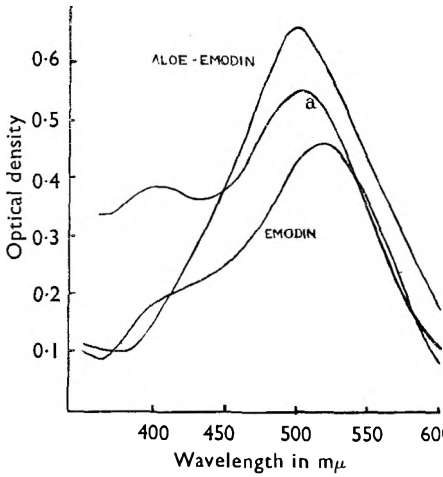

FIG. 2. Absorption curves, based on readings made with a Uvispek, of the red alkaline solutions prepared from (a) cascara by the method advocated in this paper, from pure aloe-emodin; (21·4 mg./l.; $E_{1\text{ cm.}}^{1\text{ per cent.}} = 308$) and pure emodin, (16·8 mg./l.; $E_{1\text{ cm.}}^{1\text{ per cent.}} = 274$).

Further experiments showed that the conditions for dissolving the glycosides and hydrolysing them and for the alkaline oxidation of the aglycones as used for senna and rhubarb were equally applicable to cascara. We also passed a known amount of pure aloe-emodin through all the stages of the assay process and found there was no loss involved.

The peak of the absorption curve of the final alkaline solution is 500 mμ; that of emodin is 520 mμ and that of aloe-emodin is 500 mμ. This suggests that aloe-emodin is present in the final alkaline solution in greater proportion than emodin, and it was therefore decided to use pure aloe-emodin as a standard and measure the intensity of the colour at 500 mμ. Since the molecular weights and extinction coefficients of both compounds are similar; there will be little error in using this standard and, as will be shown later, the results of the chemical assays, calculated in terms of aloe-emodin, are closely correlated with those of the bioassays. Details of the chemical assay process appear at the end of this paper.

The fact that the interfering substances partly disappear with age may be of use in determining the approximate age of a sample of cascara bark.

THE BIOLOGICAL ASSAY

The biological assay was based on that of Lou and Fairbairn[10] for cascara. In contrast to their earlier method, however, we were able to use the crude drug as standard providing a fairly potent sample was used, it was in fine powder and that 1 ml. of suspension was given to each mouse.

As a standard, we used a good sample of powdered cascara bark (C_S) in the same way as powdered senna pod (P_1) and powdered rhubarb (R_S) had been used with the other anthraquinone drugs. However, in order to relate the activities of our cascara standard with that of other drugs and

particularly with that of a pure compound, we assayed C_S against P_1 (Table 1) and against pure sennosides A + B (two assays). It was found that

1 g. of Cascara $C_S \equiv$ 0.185 g. of senna pod P_1
1 g. of $P_1 \equiv$ 58 mg. of sennosides A + B*
1 g. of $C_S \equiv$ 10·7 mg. of sennosides A + B

Accuracy of the Bioassay Process. Table I shows the results of 10 replicate assays of C_S against P_1 as standard and Table II the results of 11 replicate assays of another sample of powdered cascara C_4 against C_S as standard. In Table I the potency of P_1 is taken to be 100, and the mean of the values for the potencies of C_S is found to be 18·5, with a standard deviation of 1·27, i.e., 6·9 per cent. of the mean. In Table II the potency of C_S is taken to be 100 and the mean of the values for the potency of C^4 is found to be 133 with a standard deviation of 16·4, i.e., 12·4 per cent. of the mean. The limits of error for a single determination (P = 0·99) based on these figures would be ± 17·8 per cent. of the mean and ± 31·9 per cent. of the mean; these limits compare well with those given by Lou for senna[12] and Lou and Fairbairn[13] for rhubarb. As we always carry out the bioassay in duplicate, the error of the mean based on these two determinations should very rarely exceed ± 23 per cent.

TABLE I

RESULTS OF REPLICATE BIOASSAYS OF CASCARA BARK C_S AGAINST SENNA POD P_1 AS STANDARD

Bioassay	Potency of P_1	Potency of C_S
1	100	17·5
2	100	18·2
3	100	17·8
4	100	19·4
5	100	20·2
6	100	15·7
7	100	18·8
8	100	19·0
9	100	18·6
10	100	19·5
Mean	100	18·5

Standard deviation = 1·3 (6·9 per cent. of the mean)

CORRELATION OF CHEMICAL AND BIOLOGICAL ASSAYS

7 different samples of cascara bark, purchased within the last 8 years, were assayed chemically and biologically and the results recorded in Table III show that there is a close correlation between the two sets of figures. The standard sample C_S was exhausted with chloroform to remove the free compounds; there was no loss in activity, thus confirming the general statement made by Fairbairn[1], that the free compounds contribute very little to the activity of the anthracene purgatives. When a similar

TABLE II

RESULTS OF REPLICATE BIOASSAYS OF CASCARA BARK C_4 AGAINST CASCARA BARK C_S AS STANDARD

Bioassay	Potency of C_S	Potency of C_4
1	100	123·1
2	100	126·0
3	100	135·0
4	100	162·2
5	100	135·8
6	100	135·5
7	100	113·7
8	100	124·8
9	100	106·8
10	100	149·1
11	100	150·0
Mean	100	132·9

Standard deviation = 16·4 (12·4 per cent. of the mean)

* This figure is in close agreement with an earlier estimation where Fairbairn and Saleh[11] found that 1 g. of $P_1 \equiv$ 64 mg. of Sennosides A + B.

series of assays were carried out on various batches of dry extract o cascara B.P. a curious fact came to light. Though the chemical and biological assay results were correlated, the biological assay results were all about one half of the value to be expected from the chemical assays. Thus if a dose of crude drug containing say 10 mg. of glycoside had a biological activity of 100 units, a dose of dry extract containing 10 mg. of glycoside had only an activity of 50 units. This reduction in activity was also found to hold for samples of liquid extract, elixir and tablets.

TABLE III

CHEMICAL AND BIOLOGICAL ASSAYS OF COMMERCIAL SAMPLES OF CASCARA BARK

Sample	Water soluble extractive	Chemical assay, mg. of glycosides per g. (as aloe-emodin)	Biological assay		B/C ratio
			mg. of P_1 per g.	mg. Sennosides A + B per g.	
C_8—received 1951 ..	23·77	11·7	269 (mean of 3 assays)	16·14	1·38
C_4—received 1946 ..	24·92	11·9	242 (mean of 6 assays)	14·52	1·22
C_6—received 1948 ..	24·12	11·8	(i) 225, (ii) 259 } 245	14·7	1·25
C_7—received 1947 ..	*23·34*	*12·0*	*252*	*15·1*	*1·26*
C_8—received 1945 ..	23·00	8·6	170	10·2	1·18
C_{10}—received 1953 ..		13·25	265	15·9	1·20
Cs—received 1952 ..	24·49	9·5	185 (mean of 10 assays)	11·1	1·168
Cs—exhausted with chloroform to remove the free compounds		9·95	184 (mean of 4 assays)	11·04	1·11
C_1—received 1945 ..		*9·0*			
C_2—received 1945 ..		8·0			
C_5—received 1952 ..		8·62			
C_9—received 1952 ..		5·75			

We have attempted to express this fact by calculating the ratio of biological activity to glycosidal content for each sample examined; this ratio is called the B/C ratio and is obtained as follows:

$$B/C = \frac{\text{Biological activity of 1 g. expressed as mg. of sennosides A + B}}{\text{Chemical assay for glycosides per g. expressed as mg. of aloe-emodin.}}$$

We are attempting at present to find out why the B/C ratio for the crude drug is twice that for the galenical preparations.

The results, however, show that the chemical assay provides a good criterion of the biological activity provided samples of crude drug are being compared with each other, or samples of galenicals are being compared. They also give some indication of the low efficiency of the present methods of preparing galenicals of cascara sagrada. The mean glycosidal content of the 11 samples of bark in Table III is 10·0 mg./g. On the assumption that the water-soluble extractive of B.P. samples of bark is 25 per cent., dry extract of cascara should contain 40 mg. of glycosides

per g. The mean for the 7 commercial samples in Table IV is only 11·1 mg./g. thus indicating that only 28 per cent. of the original glycosidal content has been retained. Similar calculations for the commercial samples of liquid galenicals mentioned in Table IV show an average yield of only 29 per cent. In the same way calculations based on the figures given by Gibson and Schwarting[14] show a similar low efficiency of about

TABLE IV

CHEMICAL AND BIOLOGICAL ASSAYS OF COMMERCIAL SAMPLES OF GALENICAL PREPARATIONS OF CASCARA

Sample	Water soluble extractive	Chemical assay, mg. of glycosides per g. or ml. (as aloe-emodin)	Biological assay		B/C ratio
			mg. of P_1 per g. or ml. of extract	mg. of sennosides A + B per g. or ml. of extract	
Dry extract—E_1 ..		17·8	200 (mean of 7 assays)	12·0	0·67
Dry extract—E_2 ..		9·0	105, 126 } 115	6·9	0·76
Dry extract—E_6 ..	87·0	12·25	135 (mean of 3 assays)	8·1	0·66
Dry extract E_9 ..	80·4	11·0	140, 90 } 115	6·9	0·63
Dry extract—E_3 ..	84	11·75			
Dry extract—E_4 ..	86	5·0			
Dry extract—E_5 ..	81	10·8			
Tablet (5 gr.)—T_1 ..		4·9 (mg. per tablet)	51 (mg. P_1 per tablet)	3·0 (mg. per tablet)	0·61
Tablet (5 gr.)—T_2 ..		3·0 (mg. per tablet)	34 (mg. P_1 per tablet)	2·04 (mg. per tablet)	0·68
Tablet (5 gr.)—T_3 ..		4·04 (mg. per tablet)	42 (mg. P_1 per tablet)	2·52 (mg. per tablet)	0·62
Liquid extract—LE_1		3·3	31, 40 } 35	2·1	0·64
Liquid extract—LE_2		2·9	35, 35 } 35	2·1	0·7
Liquid extract—LE_8		3·9	44	2·64	0·67
Elixir—LX_1 ..		2·14	21	1·26	0·58
Elixir—LX_2		2·02	14	0·84	0·42
Elixir LX_3		3·26	19	1·14	0·35

20 to 30 per cent. Though the method of chemical assay used by these authors did not then take into account the presence of an interfering substance, their figures are probably a sufficiently good guide for comparative purposes. Since the B/C ratio for all these preparations is only half of that for the crude drug it appears that they represent only about 15 per cent. of the original biological activity. Work on improving the present methods of preparing galencials is being carried out.

THE CHEMICAL ASSAY PROCESS

About 1·0 g. of the powdered bark, accurately weighed is transferred to a graduated 100-ml. flask by means of about 80 ml. of hot water and

the flask placed in a boiling water bath for 15 minutes. The flask is cooled to room temperature, the *p*H of the solution adjusted to 6 and the contents shaken vigorously; it is then made up to volume and filtered or centrifuged. Extracts and liquid preparations of cascara are suitably diluted, adjusted to *p*H 6 and made up to a definite volume.

(i) *Removal of the free Anthraquinones.* 10 ml. of the above filtrate or solution is brought to *p*H 3 by addition of N hydrochloric acid and shaken vigorously with successive quantities, each of 30 ml., of chloroform till the chloroform is colourless after shaking. The combined chloroform extracts are washed with 2 × 5 ml. of water and the washings added to the aqueous fraction. The chloroform layer which contains all the free compounds is now discarded.

(ii) *Hydrolysis of the glycosides.* To the aqueous fraction, which now measures about 20 ml., is added 12 ml. of 6 per cent. hydrogen peroxide and 16 ml. of 10 N sulphuric acid, thus making the final concentration of acid about 3·3N. The mixture is heated for 15 minutes in a boiling water bath and then cooled under the tap, when the aglycones may appear as a brown precipitate.

(iii) *Extraction and Purification of the Aglycones.* The previously cooled liquid is shaken vigorously in a separating funnel with 80 ml. of ether, and allowed to stand. The lower aqueous portion is separated and the yellow ethereal layer decanted from the brown residue which forms as a layer between the ether and aqueous layers. The brown layer is dissolved in a small quantity of sodium hydroxide solution and this solution is added to the separated aqueous portion (which contains an excess of acid). The extraction is continued in a similar manner with successive portions of ether, until the ether fraction is colourless. The combined ether fractions are shaken with 3 quantities, each of 20 ml., of 10 per cent. sodium metabisulphite solution and with 3 or 4 quantities, each of 20 ml., of 1 per cent. sodium bicarbonate solution, the aqueous layers being discarded.

Colorimetric Estimation. The purified ether solution is extracted with 10 to 20 ml. of N sodium hydroxide, in small portions, till the alkaline extract is colourless. To the combined alkaline solution is added 0·2 ml. of 3 per cent. hydrogen peroxide per 10 ml. and heated for 4 minutes in a boiling water bath. The solution is rapidly cooled, excess of sulphuric acid is added and the liquid extracted with ether. The ether solution is re-extracted with N sodium hydroxide; the alkaline solution is warmed to drive off dissolved ether and made up to suitable volume. The intensity of the red colour is determined photoelectrically at 500 mμ, within half an hour, and the amount of aglycone present, calculated as aloe-emodin, is read off from calibration curves previously prepared from pure aloe-emodin.

Summary

1. A satisfactory colorimetric method for the determination of the amount of anthracene glycosides in samples of cascara sagrada and its preparations is described.

2. The main difficulty encountered was the presence of pigments

which passed into the final coloured solution being measured; it was found possible to destroy these pigments by oxidation in acid medium and by treatment, at one stage in the assay process, with sodium metabisulphite and sodium bicarbonate.

3. The method of biological assay described by Lou and Fairbairn[13] has been used extensively and we have found that the standard error of the mean based on two assays ($P = 0{\cdot}99$) was never more than ± 23 per cent.

4. 7 samples of crude drug were assayed chemically and biologically by the above methods and the results showed very good correlation; similarly with 12 commercial samples of dry extract, liquid extract and elixir.

5. One curious fact, however, came to light; galenical preparations containing glycosides in amounts similar to those in the crude drug were only half as active. This problem is being investigated further.

6. The results show that commercial samples of official cascara preparations contain only about 15 per cent. of the original activity of the crude drug; we are attempting to devise more efficient methods of preparation.

We wish to thank Professor H. W. Youngken, Jnr., University of Washington, Seattle, for supplying us with the celite and the special magnesia used in the chromatographic work; Mr. J. H. Davey for assistance with the biological assays; Dr. A. L. Glenn, Department of Chemistry, School of Pharmacy, for advice in using the Uvispek spectrophotometer in his department in order to construct the curves in Figures 1 and 2; and the University of London for a grant made to one of us (J. W. F.) to cover the cost of the Unicam spectrophotometer used mainly in this work. Some of this work has been carried out by one of us (G. E. D. H. M.) as part of the requirements for the Ph.D. degree of the University of London.

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DISCUSSION

The papers on cascara were taken together. The first was presented by MR. T. D. WHITTET and the second by MR. G. E. D. H. MAHRAN.

MR. T. C. DENSTON (London) referred to the variation in activity of different batches of liquid extract of cascara and asked the authors of

the first paper whether they had investigated the effects of time and temperature of autoclaving on their preparations. He wondered whether the method was applicable on a commercial scale.

MR. H. DEANE (Long Melford) said it would be interesting if Mr. Whittet could compare an alcoholic extract with the B.P. extract. Some years ago it was found that treatment of the powdered bark with hydrogen peroxide before extracting with spirit gave an extract which was free from griping properties.

DR. W. MITCHELL (London) said that as both Mr. Deane and himself had pointed out in a paper to the 1948 Conference, the application of a vacuum for drying had little or no effect on the temperature in the final stages of drying, but heating over 100° C. resulted in the extract failing to comply with the B.P. limit test for water-soluble extractive. Did the authors' products comply with that test? The suggestion that concentration took longer under vacuum was surprising. With properly designed equipment the drying process was shortened. That was very desirable in large-scale manufacture.

Turning to the results in Table 1, it was puzzling that the yield of extract by the B.P. 1932 method was lower than that obtained from the same bark by the B.P. 1948 process. It seemed possible that the initial cold water extraction was, for some reason, more efficient in the authors' B.P. 1948 preparation. That might also account for the higher recovery of glycosides as shown by the chemical and biological assays. That seemed more likely than the authors' suggestion that vacuum evaporation had caused greater destruction of glycosides, and in any case vacuum evaporation would not account for the lower yield.

The results did not prove that the Greco and Dumez autoclaving method was more efficient than simple extraction with boiling water. The Bruce method would appear to be quite impracticable for large-scale working, and the fact that it gave a yield similar to the B.P. 1932 method suggested that it was not autoclaving but the subsequent boiling water percolation that made the Greco and Dumez method show better results. He was under the impression that the belief that a griping or emetic principle was present in cascara bark and disappeared on storage was now more or less abandoned. The fact that the ratio between the results of biological and chemical assays was lower in galenicals (presumably made by cold water extraction) than in cascara bark could suggest two things: (*a*) that cold water not only extracted less total anthraquinone glycosides, but also relatively still less of the most biologically active compounds or (*b*) that in the concentration of the original water extracts to give the galenicals, the glycosides were partially inactivated. Had the authors any information on the relative average amounts of free anthraquinones in the bark and in galenicals?

MR. A. W. BULL (Nottingham) asked Mr. Whittet within what order he would expect his results to be reproducible. Did the authors still consider that the variation in the method of concentration would account for the surprising variation in yields shown in Table 1? Many

problems would be involved in operating methods (C) and (D) on a large scale.

MR. BOARDMAN (Manchester) said that apparently the present B.P. method of extraction was not so bad after all. Hot water would improve the extractive yield, but might make it necesssary to reduce the figure for water-soluble extractive to 75 instead of 80 per cent. From experiments which he had carried out he had been unable to convince himself that there was any difference in the extractive from new or old bark.

MR. J. H. OAKLEY (London) pointed out that for the bark, the biological assay result exceeded that of the chemical assay; whereas for the galenical preparation the converse was the case. Both the liquid and the dry extract were prepared by extraction of the bark by cold water as compared with the elixir which was extracted by boiling water in the presence of magnesia. There appeared to be no difference according to the paper, yet he felt convinced that hot extraction was more effective than cold. On a large scale the methods of Whittet and Bruce would be uneconomical. The higher activity of the bark as determined biologically might be due to the digestive system of the animals being better able to extract the active principle. Had Mr. Mahran any information on whether an acid or alkaline extraction medium was more effective than plain distilled water? Was the figure of 3·68 for glycosides present in the bark, obtained by Mr. Whittet, derived by extraction with water and assaying, or by administration of the bark to animals?

MR. R. L. STEPHENS (Brighton) asked Dr. Fairbairn and Mr. Mahran where the loss of activity had occurred in the preparation of the dried extracts. If any biological activity were left in the apparently exhausted bark, that would answer the question, and show that an animal could extract active material which was not extractable by water.

MR. T. D. WHITTET asked whether Dr. Fairbairn and Mr. Mahran had noticed similar anomalous results in B/C ratios with other anthraquinone drugs. The work he had done suggested that high B/C ratios did not depend on efficiency of extraction, since the ratio was inversely proportional to the efficiency of the extraction. In some of Dr. Fairbairn's work with senna he believed that he had found that alcoholic extraction was more efficient than aqueous extraction. He agreed that the difference between the 1932 and 1948 figures was puzzling, and it was being investigated further. One of the difficulties was the criterion for the exhaustion of cascara. Both taste and a test for glycosides had been used but neither was quite satisfactory. He did not think that failure to comply with the B.P. limit test for water-soluble extractive was of great importance; he regarded the test as a temporary expedient for standardising the extract before chemical or biological tests were available. All the extracts were prepared from the same bark, which was assayed by the methods of Fairbairn and Mahran as described in their paper. He agreed that his methods were not applicable on a large scale. Various times and temperatures for autoclaving had been tried, but in his view they were not critical. It might be that the use of boiling water was the critical factor. He considered that the criterion

of a good extract was its activity, and it might be that some preparation of the crude bark would have to be formulated instead of using elaborate extraction methods.

MR. G. E. D. H. MAHRAN, in reply, said there was a difference in the chemical assay of a fresh and an old bark. The fresh bark showed a much larger amount of colouring or interfering substances than the old bark, as confirmed by the final shapes of absorption curves of each. This was being investigated as a method of testing the age of bark, and work was also being carried out to show to what extent the presence of colouring matter may be correlated with the griping effect. The chemical assay could be used to determine the amount of free anthraquinone compounds in bark and galenicals. The amount varied in different samples, but removal by extraction with ether or chloroform did not result in any loss of activity. This supported the theory of Fairbairn that anthracene derivatives are highly active as anthranol glycosides, less active as free anthranols, and much less active as free anthraquinones. The biological activity ran parallel with the content of combined anthraquinone compounds. In senna, biological activity ran parallel with glycosidal content and in rhubarb, with the content of combined rhein-like compounds. It was found that the active principles dissolved without any loss when the infusion was maintained at a temperature approaching 100° C. for 15 minutes followed by the addition of alkali to *p*H 6. He considered that no activity could still be retained in the marc left after preparing a 70 per cent. alcoholic extract of cascara.

With regard to the loss of activity in making galenicals, when carrying out the chemical and biological assays on the bark, the B/C ratio was 1·2; when the extracts were assayed the B/C ratio was found to be only half this value, i.e. 0·6. This was noticed only in cascara. It appeared that there might be a factor lost during the preparation of the galenicals. The presence of an enzyme which might hydrolyse or destroy the glycosides was considered, but it was found that the method of preparation was the cause. The bark was extracted with cold and hot water, and extracts were always found to have a B/C of 0·6. Bark was also extracted with absolute methanol and ethanol but the B/C ratio for the extract remained low. It appeared that there was a factor which was not extracted by water or alcohol alone, and it seemed that the selection of solvents accounted for the phenomenon. By extracting the bark with 70 per cent. ethanol and evaporating the liquid to dryness under vacuum at a temperature not exceeding 40° C., a dry extract was obtained which had a B/C ratio the same as that of the crude drug, i.e. 1·2, and which possessed corresponding activity.

THE ESTIMATION OF TROPANE ALKALOIDS IN VEGETABLE DRUGS BY PAPER PARTITION CHROMATOGRAPHY, WITH SPECIAL REFERENCE TO THE ALKALOIDS OF *DATURA SANGUINEA*

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Although the estimation of tropane alkaloids in plant products has been investigated extensively, no generally accepted method of determination of the individual alkaloids is yet available, and the problem continues to be the subject of research. Assay processes for the official solanaceous drugs estimate the total alkaloidal contents, expressed as hyoscyamine, while, following the early chromatographic work of Roberts and James[1] and of Trautner and Roberts[2], Evans and Partridge[3,4] devised a technique for separation and estimation of hyoscine and hyoscyamine using kieselguhr columns, which have been used similarly by Schill and Ågren[5].

These methods suffer from three main disadvantages. Firstly, there is no provision for establishing with certainty that complete separation of the alkaloids has been achieved since they are estimated titrimetrically. Secondly, if either of the alkaloids is present in small amount only, its estimation by adsorption on a column, followed by elution with an appropriate solvent and titration, is subject to a relatively large error. For example, the titration equivalent to 10 g. of drug containing 0·01 per cent. of hyoscine is only 0·165 ml. of 0·02N acid. Thirdly, when a mixture of tropane alkaloids is separated on a column in a routine analytical laboratory account cannot be taken of the possible presence of alkaloids other than hyoscine and hyoscyamine, as such work presents considerable experimental difficulties and is time consuming. In this connection Hills and Rodwell[6], who investigated the alkaloids of *Duboisia myoporoides* using the column technique, have stated that alkaloids other than hyoscine can account for 30 to 90 per cent. of the total alkaloidal content and that, even in samples yielding maximal amounts of hyoscine, it may not be possible to account for more than half of the total alkaloid.

In view of the above considerations, it was felt that a more specific assay process might be developed by paper partition chromatography using buffered papers such as employed by Brindle, Carless and Woodhead[7] for the separation of hyoscine and hyoscyamine. By this method it is possible to identify all non-volatile alkaloids which differ in R_F value from hyoscyamine and hyoscine, providing that an adequate range of reference alkaloids is available.

Accordingly, we have applied ourselves to this problem and have devised an assay process based upon paper partition chromatography. Our results are described in the present paper, which gives details of the method and its application to the assay of certain solanaceous drugs.

Preliminary Experiments

In our preliminary investigations we followed closely the work of Brindle *et al*[7]. Suitable volumes of a solution of alkaloids in chloroform were applied to filter paper buffered to *p*H 7·4 and the bases separated chromatographically by running with water-saturated *n*-butanol. After development of the chromatograms the alkaloidal spots were located by treatment with Dragendorff's reagent. Some estimate of the relative amounts of alkaloids could be obtained by comparison of the intensities of the spots, but it was considered that removal of the alkaloids from the paper and subsequent assay would afford greater precision. For this purpose, reference chromatograms carrying standard spots of hyoscine, hyoscyamine and other appropriate alkaloids were developed simultaneously with the chromatograms carrying the test material. Only the reference chromatograms were treated with Dragendorff's reagent and the spots, so obtained, employed to mark the alkaloid carrying areas on the test chromatograms. The untreated papers were then cut into strips carrying the individual alkaloids, which were then removed by elution with ethanol. Suitable volumes of eluate were freed from interfering buffer salts and the alkaloidal contents determined by the Vitali-Morin reaction, which unfortunately is not given by all tropane alkaloids.

In experiments, using a standard solution containing hyoscine and hyoscyamine, we were unable to recover completely the alkaloids by the above chromatographic process, in spite of the fact that the paper strips after elution of the alkaloids gave no reaction with Dragendorff's reagent. Similar losses have been reported in separations of amino-acids[8], thiohydantoins[9], and ergot alkaloids[7]. We observed, however, that in the case of these alkaloids any such losses occurred substantially in proportion to the amount of alkaloid present. Typical results are summarised in Table I.

TABLE I

Assay	Alkaloid	Weight placed on chromatogram	Weight recovered by assay
1	Atropine	0·10 mg.	0·062 mg.
	Hyoscine	0·10 mg.	0·0665 mg.
2	Atropine	0·10 mg.	0·076 mg.
	Hyoscine	0·10 mg.	0·073 mg.

It therefore became possible to obtain satisfactory results by determination of the mixed alkaloids by classical methods, followed by chromatographic separation, elution and colorimetric estimation of the individual alkaloids. The chromatographic results may be used to calculate the relative proportions of the individual alkaloids and these figures, in conjunction with the total alkaloidal content, give the percentages of hyoscine and hyoscyamine present. This method of assay has been successfully applied to solanaceous plants containing hyoscine and hyoscyamine together with very small amounts of other alkaloids.

Determination of Total Alkaloids

Numerous methods have been described for the determination of total tropane alkaloids in plant materials. In our work the following process, based upon Allport's[10] procedure, has been used.

Transfer 10 g. of drug in No. 60 powder to a 150-ml. beaker. Add 20 ml. of ethanol (95 per cent.), mix well, allow to stand for 10 minutes and treat with 3 ml. of dilute ammonia solution. Mix well and, after 10 minutes, add with stirring 50 ml. of chloroform and set aside for a further 15 minutes. Transfer the mixture to a percolator plugged with cotton wool, collecting the percolate in a graduated 350-ml. separating funnel. Gently compress the contents of the percolator with a glass plunger and continue percolation with chloroform until complete extraction of the alkaloid is achieved (about 200 to 250 ml. required). Extract the percolate with 4 quantities (30 ml.) of 6 per cent. acetic acid in 5 per cent. ethanol, and extract the mixed acid layers with three quantities (25, 20 and 15 ml.) of chloroform, washing each chloroform extract with the same 20 ml. of 6 per cent. acetic acid. Reject the chloroform extracts and add the washings to the main acid solution. Render distinctly alkaline with dilute ammonia solution and extract 4 times with chloroform (25 ml.), washing each extract with the same 20 ml. of water. Transfer the mixed extracts to a wide-necked flask and remove the solvent by evaporation. Add 3 ml. of absolute ethanol, again evaporate to dryness and dry the residue at 100° C. for 30 minutes to remove volatile bases. Dissolve the residue in 10 ml. of 0·1N sulphuric acid, warming the flask to assist solution, and titrate the excess of acid with 0·02N sodium hydroxide, using methyl red as indicator.

Each millilitre of 0·02N sulphuric acid is equivalent to 0·005787 g. of hyoscyamine or 0·006067 g. of hyoscine, the actual factor used for calculating the total alkaloidal content being selected according to which alkaloid predominates, as shown by chromatographic examination.

Chromatography

Reagents

Sörensen's Phosphate Buffer Solution, p*H* 7·4

Sodium Phosphate, anhydrous, Na_2HPO_4	— 0·76 g.
Potassium Dihydrogen Phosphate, Anhydrous, KH_2PO_4	— 0·18 g.
Water	to 100 ml.

Buffered Papers (p*H* 7·4)

Cut sheets of Whatman's filter paper No. 1 into strips 22½ in. long and of suitable widths. Immerse the strips in phosphate buffer solution *p*H 7·4 for about 5 minutes. Remove surplus buffer by draining and dry the strips in air for not less than 5 hours.

Dragendorff's Reagent[11]

(a) *Stock Solution.* Mix 0·85 g. of bismuth subnitrate with a mixture of 40 ml. of water and 10 ml. of glacial acetic acid. Add 50 ml. of 50 per cent. potassium iodide solution and shake the liquid until all solid matter has dissolved. Store the solution in an amber glass bottle.

(b) *Reagent.* Add 10 ml. of stock solution to 20 ml. of glacial acetic acid and adjust the volume to 100 ml. with water. The reagent should be freshly-prepared before use.

Standard 1 : 1 *Atropine-Hyoscine Solution in Chloroform*

Dissolve 0·723 g. of hyoscine hydrobromide in 15 ml. of water, render alkaline with 2 ml. of dilute ammonia solution and extract 4 times with chloroform (25, 25, 20 and 20 ml.). Wash each chloroform extract with the same 12 ml. of water and filter through a dry filter into a graduated flask containing 0·500 g. of atropine. Adjust the volume to 100 ml. with chloroform and mix well. To prepare a 0·2 per cent. solution dilute the above 1 per cent. solution in the ratio of 1 to 5 with chloroform.

Separation of Alkaloids

Add 3 ml. of dilute ammonia solution to the titrated solution, obtained during the estimation of the total alkaloids, and transfer to a separating funnel, rinsing the flask successively with two quantities of water (10 ml. and 5 ml.) and one quantity of chloroform (25 ml.) and adding the washings to the bulk. Shake, allow the mixture to separate and remove the chloroform layer and wash it with 10 ml. of water. Continue the extraction with 3 quantities, each of 20 ml., of chloroform, washing each extract with the same 10 ml. of water. Remove the solvent from the mixed extracts by evaporation, using a flask with a ground joint to enable it to be well closed with a glass stopper. Add 3 ml. of ethanol to the residue and evaporate to dryness on a water bath.

Dissolve the residue in an accurately measured volume of chloroform (a volume of 6x to 10x ml. is suitable, where x = per cent. of total alkaloids in sample of drug and using at least 1 ml. of chloroform). By means of a micrometer syringe transfer appropriate volumes of the solution to the starting lines of buffered paper strips, according to the scheme shown in Table II. When more than 0·03 ml. of solution is required, apply no more than 0·03 ml. at a time and allow each portion to evaporate before the next application. Develop the chromatograms by irrigating with water saturated *n*-butanol in an atmosphere saturated with water and *n*-butanol, according to the technique of Consden, Gordon and Martin[12].

TABLE II

VOLUME OF STANDARD 1 : 1 ATROPINE-HYOSCINE SOLUTION IN CHLOROFORM APPLIED TO PAPER-STRIP

Buffered Paper-Strip	1 per cent. solution	0·2 per cent. solution	Volume of Alkaloidal Extract applied to Paper-Strip
1.	0·02 ml.	—	0·005, 0·010, 0·015, 0·020 ml.
2.	0·02 ml.	—	0·025, 0·030, 0·040 ml.
3.	—	0·04 ml.	0·020, 0·030, 0·040 ml.
4.	—	0·04 ml.	0.060, 0·080, 0·100 ml.
5.	—	—	0.020, 0.020 ml.
6.	—	—	0·040, 0·040 ml.
7.	—	—	0·060, 0·060 ml.
8.	—	—	0·090, 0·090 ml.

Allow the chromatograms to develop overnight and remove the paper strips from tanks and allow them to dry in the air at room temperature for not less than $2\frac{1}{2}$ hours. Cut strips 5, 6, 7 and 8 length-wise into equal portions, and immerse one half of each, together with strips 1, 2, 3 and 4 in Dragendorff's reagent. Allow to drain and dry in the air.

Approximate Assay

An approximate but useful guide to the hyoscyamine and hyoscine contents of a sample, which will also serve to determine the dilution required for the subsequent colorimetric estimation, may be obtained by matching the "unknown" spots on strips 1 to 4 with the corresponding "standard" spots. If it is found that one alkaloid predominates and the other is present to the amount of no more than 5 per cent. of the total, it will be unnecessary to proceed beyond this stage, owing to the difficulty of estimating very small amounts of alkaloid by the Vitali-Morin reaction. Alkaloids present in minor amounts are estimated semi-quantitatively by visual inspection of the chromatograms.

In addition to the above evaluation of hyoscyamine and hyoscine strips 4 and 8 should be carefully examined for the presence of other alkaloids. In this connection it should be noted that hydrolysis products (tropine and oscine) from the alkaloids, afford relatively weak pink or purple spots, in contrast to the characteristic orange spots given by the parent alkaloids[13]. Similar pink or purple spots are given by norhyoscyamine, pseudo-hyoscyamine, noratropine and pseudo-atropine.

Any spots differing in R_F value from those of hyoscyamine and hyoscine may be identified and estimated semi-quantitatively by running suitable amounts of chloroform extract containing the "unknown" alkaloid or alkaloids alongside small volumes of standard solutions of each suspected alkaloid.

Determination of Hyoscyamine and Hyoscine

Place the reagent-treated portions of strips 5, 6, 7 and 8, against their respective untreated portions and cut out the alkaloid bearing areas of the latter, along lines across the strips midway between neighbouring spots. Remove the alkaloid from each section by elution overnight with ethanol, collecting the eluate in a 5 cm. diameter glass dish (a large desiccator arranged as a chromatographic chamber forms a suitable apparatus for this work). At the end of each run, dry the paper sections and test them for absence of alkaloid with Dragendorff's reagent.

Adjust each eluate to a suitable volume with ethanol and transfer an amount, equivalent to 0·06 to 0·12 mg. of alkaloid, to a small glass dish and evaporate to dryness. Extract the residue with small volumes of chloroform and filter each extract through a No. 1 Whatman paper into a second glass dish. Evaporate the filtrate to dryness, moisten the residue with 1 ml. of 6 per cent. acetic acid and again evaporate to dryness. Complete the colorimetric determination by the Vitali-Morin reaction, as described by Allport and Wilson[14], but using 0·5 per cent. methanolic potassium hydroxide solution, as recommended by Ashley[15], for development of the colour. Compare the standard test solutions by measuring their light absorption in 1 cm. cells at 550 mμ.

Assay of Solanaceous Drugs

The chromatographic assay has been applied to the examination of *Datura sanguinea*[16], a species of *Datura* which has been little studied. Our

results on specimens of the plant grown from seed at Dartford and also imported from S. America are summarised in Table III. While our chemical work has been in progress, *Dr. A. C. C. Newman and Dr.* J. M. Rowson have been carrying out a botanical study of the species, of which they hope to publish in due course a full description.

TABLE III

ASSAY RESULTS FOR SAMPLES OF *Datura sanguinea*

Sample		Alkaloidal content, with reference to sample as received			
		Total Alkaloids per cent.	Hyoscine per cent.	Hyoscyamine and Atropine per cent.	Other Alkaloids per cent.
1952 Harvest: First year Seedlings	Dry Leaf	0·511	0·49	0·02	
	" Stalk	0·311	0·28	0·03	
	" Root	0·542	0·21	0·29	Unidentified Alkaloid "A" 0·04
1952 Harvest: Plants from cuttings Taken in 1951	Dry Leaf	0·409	0·41	Not more than 0·004	
	" Stalk	0·296	0·27	0·02	
	" Root	0·762	0·18	0·42	Unidentified Alkaloid "A" 0·16
1952 Harvest: Tops from 1951 Plants Wintered under Glass	Dry Leaf	0·342	0·34	nil	
	" Stalk	0·302	0·29	0·014	
Specimens received from S. America (Ecuador)	Dry Leaf	0·267	0·27	*nil*	*Valeroidine* not more than 0·004
	" Flowers	0·618	0·55	0·04	Valeroidine 0·03; unidentified Alkaloid "A" not more than 0·004
	" Bark	0·257	0·19	0·02	Valeroidine 0·04; unidentified Alkaloid "B" 0·01; unidentified Alkaloid "A" not more than 0·007
	" Seeds	0·172	0·17	nil	Valeroidine not more than 0·003

NOTE:

REPRESENTATIVE R_F VALUES (Water-saturated *n*-butanol; downward runs)

Hyoscine	0·84
Valeroidine	0·70
Hyoscyamine and Atropine	0·61
*Unidentified Alkaloid "A"	0·49
*Unidentified Alkaloid "B"	0·33

* *Not* noratropine, norhyoscyamine, pseudo-Hyoscyamine, Meteloidine, Nicotine, Oscine, Tropine, or Valeroidine

Samples of leaves of some *Duboisia* species have also been examined for alkaloids and these results are included in Table IV.

TABLE IV

ASSAY RESULTS FOR SAMPLES OF LEAVES OF *Duboisia spp.*

Sample	Total Alkaloids	Hyoscine per cent.	Hyoscyamine and Atropine per cent.	Other Alkaloids per cent.
Duboisia myoporoides	1·60	1·4	0·1	0·1 Valeroidine
Duboisia leichhardtii No. 1	2·10	0·2	1·9	—
Duboisia leichhardtii No. 2	4·07	0·6	3·5	—

DISCUSSION

It will be noted that the proposed method of assay depends upon the proportions of the individual alkaloids, as estimated colorimetrically after chromatographic separation, being the same as in the total alkaloids extracted from the drug. This will be so if the losses, during chromatography, occur in proportion to the amounts of alkaloids applied to the paper. We are satisfied that, in our experiments, this condition was

substantially fulfilled, for close agreement was obtained between the approximate assay figures, estimated by matching of the spots located with Dragendorff's reagent, and the subsequent figures afforded by colorimetric assay using the Vitali-Morin reaction.

Volatile alkaloids in the drug may give rise to error, and care must be taken that these bases are removed from the alkaloids, obtained in the estimation of total alkaloids, before titration. For the plant products used in the present work, heating the alkaloids for 30 minutes at 100° C. was adequate but longer heating may be necessary for other materials. When the sample available is small it may not be possible to titrate the total alkaloids and in such cases the colorimetric assay of Allport[10] may be used. Any fat-containing samples, such as seeds, should be defatted with light petroleum before carrying out the determination of total alkaloids.

Some workers, when using paper chromatography, equilibrate the paper strips with the solvent saturated atmosphere prior to commencement of the runs. In the present work this was found unnecessary. Other factors having little effect upon the development of the chromatograms were (1) minor variations in the composition of the phosphate buffer solution, (2) slight variations in the filter paper used; both Whatman's No. 1 and No. 2 papers gave satisfactory results, and (3) the period of immersion of the papers in the buffer solution. Citrate buffer of *p*H 7·4 was unsuitable for quantitative work owing to large losses of alkaloid. Both downward and upward runs are suitable, but the descending technique was mainly employed in view of the greater distance travelled by the solvent front and alkaloidal spots in a given time. Both A.R. and commercial grades of *n*-butanol have been successfully used and no special precautions were taken to maintain the tanks at constant temperature during the course of runs. Glass tanks have been used throughout the work; in some preliminary experiments using a large ebonite tank the results were unsatisfactory.

The presence of buffer salts as well as traces of moisture is liable to cause unduly rapid fading of the colour in the Vitali-Morin reaction. For this reason it is important that the ethanolic eluates should be freed from buffer salts by extraction with chloroform, as described in the assay. The colour reaction must also be carried out in thoroughly clean and dry apparatus.

Examination of *Datura sanguinea* gave interesting results. The aerial parts contain sufficient hyoscine to make the plant a possible commercial source of the alkaloid. Besides hyoscyamine a number of minor alkaloids, of which valeroidine was identified, are present. It should be noted, however, that under the experimental conditions given above, hyoscine and tigloidine have almost identical R_F values and consequently it was not feasible to take into account the possible occurrence of the latter. Work is in progress with a view to separating tigloidine and hyoscine on two-dimensional paper chromatograms using a suitable pair of solvents. In preliminary experiments a mixture of chloroform, carbon tetrachloride and light petroleum showed promise as a secondary eluent. There is also

no separation of atropine and hyoscyamine and these alkaloids are determined together.

Hyoscyamine and hyoscine predominate respectively in the root and aerial parts of *Datura sanguinea*. Our results with *Duboisia* species confirm known data regarding the alkaloids present.

There has been a revival of interest during recent years in alkaloid biogenesis and much research has been done with solanaceous plants. It is hoped that the present work may find some application in this important field.

Summary

1. A study has been made of the application of paper partition chromatography to the separation and estimation of tropane alkaloids.
2. The separation of the alkaloids on buffered paper and the subsequent elution of the individual alkaloids is described.
3. The estimation of the recovered alkaloids has been carried out by the Vitali-Morin reaction.
4. The chromatographic results taken in conjunction with the total alkaloidal content of a plant material has been used to determine the hyoscine and hyoscyamine contents of the sample.
5. The proposed assay has been applied to samples of *Datura sanguinea* and of *Duboisia* species.

We wish to thank Dr. A. C. C. Newman for supplying the specimens of vegetable drugs used in this investigation. We also wish to thank the Directors of The Wellcome Foundation for permission to publish our results.

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DISCUSSION

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

Dr. W. Mitchell (London) said he was particularly interested to learn that valeroidine had been detected in *Datura sanguinea*. That alkaloid along with tigloidine, poroidine and *iso*poroidine, was first isolated from *Duboisia myoporoides* by Martin in about 1935, and he (Dr. Mitchell) subsequently established their structures. The hydrobromides

of tigloidine, poroidine and *iso*poroidine could readily be extracted from reasonably concentrated aqueous solutions of the total hydrobromides by means of chloroform. Valeroidine was not extracted because its hydrobromide was even more soluble in water. By applying that technique could not the authors make a preliminary separation of the total alkaloids and apply the material so extracted to a separate chromatogram? That should certainly eliminate any doubt as to tigloidine being obscured by the hyoscine spot. Was it possible that either of the unknown alkaloids "A" and "B" referred to in the paper was the base "D" that he isolated from *Duboisia leichhardtii* and believed probably to be *iso*valeryltropene, or could they be either poroidine (*iso*valerylnortropene) or *iso*poroidine (α-methylbutyrylnortropene) from *D. myoporoides*?

DR. W. C. EVANS (Nottingham) said he desired to correct the impression given by the authors that the method of assaying the drugs was an absorption process. It was not. It had a purely partition effect. Judging by the inability of the authors to obtain complete recovery of their alkaloids, it would seem that absorption on cellulose was a far greater problem than absorption on kieselguhr. How did the authors identify their valeroidine? Was it by the preparation of crystalline derivatives or by the measurement of the R_F value? That value was liable to be misleading, since a number of alkaloids gave similar R_F values. In that connection he asked whether the authors could give figures for the other alkaloids which were listed at the bottom of Table III as not being identical with the positions of the unidentified alkaloids A and B.

MR. H. B. WOODHEAD (Manchester) said that the recoveries in Table I were said to be typical results, but he wondered whether there was any reason for the recoveries in assay 1 being different from those in assay 2. At what stage of the process was alkaloid being lost?

DR. J. B. STENLAKE (Glasgow) said that Shute in *Nature* had shown that when alkaloids and acids are chromatographed on paper with water as solvent, ion exchange occurred between the ions of the alkaloid or acid and certain ions which were present either in or on the surface of the paper; losses might be explicable on that basis. There was no doubt that under the *p*H conditions which the authors were using any such ion exchange would be minimised, but if such a process occurred, Shute had also shown that there was a definite tailing of spots, and he asked the authors whether they had observed any tailing which might be indicative of ion exchange. It might be possible at least to repress ion exchange of that type, by saturating the paper in a salt solution such as potassium chloride.

MR. J. E. CARLESS (Manchester) said that he would have thought it would have been better to show a wider range of alkaloidal loads than the 0·1 mg. of alkaloid in each case as shown in Table I.

MR. R. E. A. DREY, in reply, said that after the experimental work described had been completed, the effect on their method of the solubility in chloroform of tigloidine hydrobromide was investigated. It was concluded that if tigloidine and also poroidine and *iso*poroidine were to

be determined quantitatively in solanaceous plant materials it would be necessary to adjust the method of extraction, described under "determination of total alkaloids," so that no chloroform liquor or washings would be rejected. It would also be necessary for a second chromatographic separation to be carried out, when tigloidine hydrobromide, and presumably the hydrobromides of poroidine and *iso*poroidine, would be found to have moved with the solvent front, leaving the hydrobromides of the other tropane alkaloids on or close to the starting line. They were unable to identify the alkaloids "A" and "B" with poroidine, *iso*-poroidine or "base D," as no samples of the latter alkaloids were available for comparison. They did not attempt to identify valeroidine beyond running a "marker" spot of pure valeroidine alongside the alkaloidal extract obtained from the vegetable material, in view of the small quantity of alkaloid available. Noratropine, norhyoscyamine and pseudo-hyoscyamine gave relatively weak pink spots of R_F slightly less than that of hyoscyamine. Meteloidine gave very weak ill-defined spots. Tropine and hyoscine gave weak pink spots at a relatively short distance from the starting point, whilst nicotine (only found in *D. myoporoides*) had an R_F value identical with that of hyoscine. No explanation for the losses of alkaloid on the paper (Table I) could be offered apart from the more obvious explanation of losses by retention of alkaloidal matter on the paper and decomposition of alkaloid in the course of runs. It was particularly difficult to attribute such losses to any specific cause, as they might occur both during chromatographic separation and in the course of subsequent elution. Little trouble was experienced due to "tailing."

STUDIES IN THE PHARMACOLOGY OF 4-BENZAMIDOSALICYLIC ACID AND ITS SALTS

By E. M. Bavin and Barbara James

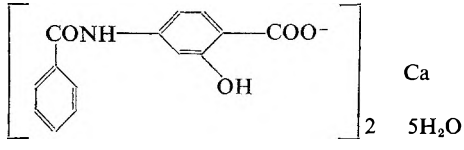
From Smith and Nephew Research Ltd., Hunsdon Laboratories, Ware, Herts

Received July 13, 1953

Recent reports[1,2] have shown that *p*-aminosalicylic acid delays to a remarkable extent the development of streptomycin resistance by *Mycobacterium tuberculosis*, and investigations are in progress to determine whether it has the same effect on the development of isoniazid resistance. The combined use of streptomycin and *p*-aminosalicylic acid is now regarded as essential when the former drug is given in the chemotherapeutic treatment of tuberculosis and most workers feel that this is the main use for *p*-aminosalicylic acid, in spite of its own definite tuberculostatic effect. The nauseous taste of *p*-aminosalicylic acid, however, still remains a difficulty, especially since the drug has to be given in large doses daily over a long period, and a number of patients find it almost impossible to continue the treatment in spite of its unquestioned value. Many attempts have been made to overcome this difficulty, mainly by the use of different pharmaceutical presentations of the drug such as cachets, tablets and granules but these forms are necessarily somewhat more costly.

Recently we have been able to study a derivative of *p*-aminosalicylic acid which, because of its almost complete lack of taste, may be one answer to the difficulty referred to above.

This derivative is the calcium salt of 4-benzamidosalicylic acid with the formula (I).



I

Its laboratory number is H.P. 170.

The free acid was first reported by Rosdahl[3] and independently by Seymour and co-workers[4,5], the latter group showing it to possess *in vitro* tuberculostatic activity inferior to *p*-aminosalicylic acid[5]. A chance observation, recently, however, showed that the calcium salt was very insoluble and, presumably because of this insolubility, was almost tasteless. It occurred to us that if this calcium compound was decomposed in the body to liberate free *p*-aminosalicylic acid, it might provide a more tolerable method of administering this latter drug. Theoretically, the compound could liberate 47·6 per cent. by weight of *p*-aminosalicylic acid. It was decided therefore, to make a study of some aspects of the pharmacology of calcium 4-benzamidosalicylate with particular reference

to its absorption, plasma levels and excretion. The toxicity and tuberculostatic effects of the compound were also investigated.

Experimental Methods

Toxicity. Acute and chronic toxicity tests were carried out on white Swiss male mice between 20 and 30 g. in weight, the drug being given by gastric tube in aqueous gum acacia suspension. Chronic toxicity tests were also carried out on albino male rats, to which the drug was administered mixed with the diet (Diet 41).

Plasma, Urine and Tissue levels. (*a*) *p*-Aminosalicylic acid was determined in plasma and urine by the method of Newhouse and Klyne[6].

(*b*) A method was evolved for the determination of 4-benzamidosalicylic acid in biological tissues based on the separation from *p*-aminosalicylic acid by chloroform extraction at an acid *p*H, hydrolysis by prolonged boiling with sulphuric acid, followed by diazotisation and coupling with a standard *p*-aminosalicylic acid solution as described by Pesez[7].

The details of the method are as follows:—

Reagents

Hydrochloric acid —5 N solution
Sulphuric acid —10 per cent. v/v solution
Sodium hydroxide —2 N solution
Sodium *p*-aminosalicylate—0.15 per cent. w/v solution (*freshly prepared*)
Sodium nitrite —1 per cent. w/v solution
Sodium carbonate —saturated solution
Chloroform.

Standard solution. 10 μg./ml. aqueous solution of sodium 4-benzamidosalicylate.

Method. Place 1 ml. of oxalated plasma in a small separating funnel, add 9 ml. of distilled water and 1 ml. of 5 N hydrochloric acid. Mix and extract 3 times with 5 ml. quantities of chloroform. The funnel is shaken for 1 minute with each extraction. If the separation is incomplete, the bottom layer is centrifuged and the chloroform layer removed with a pipette. The chloroform extract is evaporated to dryness in a boiling tube (6″ × 1″) on a boiling water bath. 3 ml. of 10 per cent. sulphuric acid are added to the dried residue, which is then heated on a boiling water bath for 4 hours. The boiling tubes are fitted with air condensers consisting of 9″ of glass tubing passing through rubber bungs of a suitable size. Colour is extracted from some makes of rubber bung during hydrolysis and these must be avoided.

The tubes are removed from the water bath. 2 ml. of 5 N sodium hydroxide is added and the tubes are placed in a freezing mixture between 0° C. and −5° C. In quick succession, 0·5 ml. of freshly prepared 0·15 per cent. sodium *p*-aminosalicylate and 0·5 ml. of 1 per cent. sodium nitrite solution are added and the tubes are shaken. The tubes are removed from the freezing mixture after exactly 5 minutes and 3 ml. of saturated sodium carbonate solution is added. The tubes are shaken and allowed to stand

for 15 minutes at room temperature. 1 ml. of water is added to each tube and the colour read on a Unicam spectrophotometer or similar instrument at wavelength 435λ.

The colour is stable for 1 hour after the addition of the carbonate solution. The amount of 4-benzamidosalicylic acid present is calculated from the colour produced by a standard solution containing 10 μg./ml. sodium 4-benzamido-salicylate, which is treated as plasma in the above method. Two blank determinations are carried out. One is a reagent blank, in which plasma is replaced by water, the optical density of which is subtracted from the optical density of the standard solution. The second blank is a plasma blank, taken either before dosage or from a nontreated control, the optical density of which is subtracted from the optical density of the unknown sample.

Concentration of 4-benzamido salicylic acid (μg./ml. plasma) =

$$\frac{\text{Optical density of unknown—Optical density of plasma blank}}{\text{Optical density of standard—Optical density of reagent blank}} \times \frac{257}{279} \times 10$$

Recovery of known amounts of the soluble sodium salt of 4-benzamido-salicylic acid added to plasma are shown in Table I and will be seen to be reasonably satisfactory. Recoveries from urine are also good provided the sample is diluted to contain not more than 40 μg./ml.

TABLE I

RECOVERY OF ADDED 4-BENZAMIDOSALICYLIC ACID FROM PLASMA AND URINE

Plasma		Urine	
Content	Found	Content	Found
7 mcg./ml.	9·6 μg./ml.	5 μg./ml.	5·45 μg./ml.
17 "	20·2 "	10 "	9·6 "
21 "	23·2 "	20 "	15·0 "
35 "	36·2 "	40 "	41·8 "
40 "	38·8 "	80 "	63·0 "
		120 "	101·0 "

(*c*) Tissue levels of *p*-aminosalicylic acid and 4-benzamidosalicylic acid were estimated by homogenising the weighed whole tissues in 100 ml. of Krebs-Phosphate-Ringer solution[8] in a high-speed homogeniser and carrying out the estimations in an aliquot of the homogenate. For the investigation of the action of tissue homogenates on the decomposition of 4-benzamidosalicylic acid, the homogenates were prepared as already described and the soluble sodium salt of 4-benzamidosalicylic acid was used as substrate. The mixtures of homogenates and substrate were incubated at 37° C. Controls were employed to exclude any effect by the Ringer solution or any non-enzymatic effect of the tissue.

Prothrombin times were estimated by Quick's method[9].

Clotting times were estimated by Lee and White's method[10].

Chemotherapeutic activity was measured by the mouse corneal technique[11] using the H.37 Rv strain of organism.

RESULTS

1. *Toxicity.* Single doses of calcium 4-benzamidosalicylate up to 10 g/kg., administered orally to mice produced no mortality. This low

toxicity may be partly due to the insolubility of the drug and the resulting deficient absorption, particularly with high doses. A group of 10 mice was given 5 g./kg. per day orally for 1 month without any effect on their rate of growth. Experiments on chronic toxicity were carried out in rats, using daily doses of 0·5, 1·25 and 5·0 g./kg. over a period of 18 weeks. No animals died and on the lowest 2 doses the rate of growth of the animals was identical with that of the control group. On the highest dose, however, there was an early check in the rate of growth which lasted for about 3 weeks and the loss was not recovered during the period of the experiment. (Figure 1).

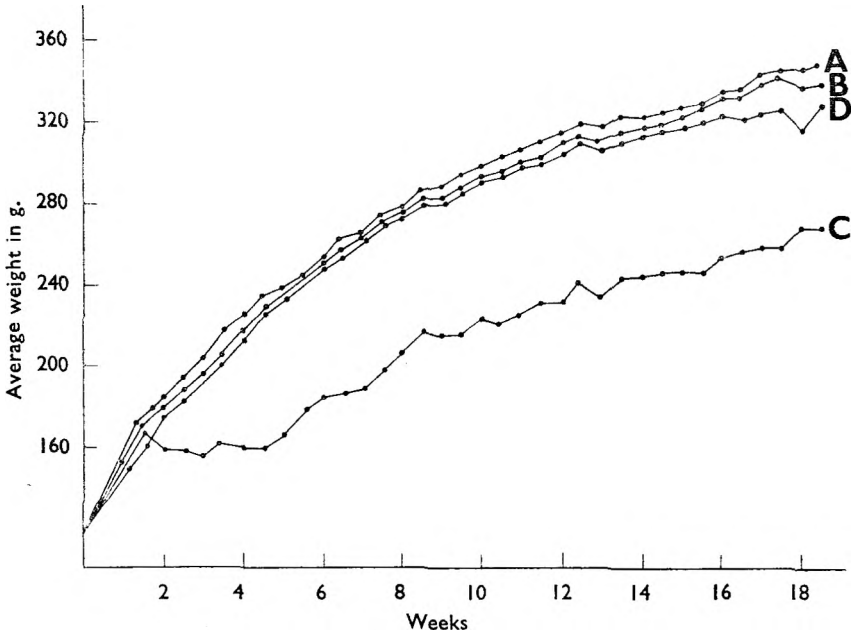


FIG. 1. The effect of calcium 4-benzamidosalicylate on the growth of rats (10 animals/group).

A. 0·5 g./kg./day. C. 5·0 g./kg./day.
B. 1·25 g./kg./day. D. Control group.

Since we had previously found that *p*-aminosalicylic acid produces thyroid hyperplasia[12] in rats, we examined microscopically the thyroids of the rats used on the highest dose in the above chronic toxicity experiments. The glands showed loss of colloid material and proliferation of the cubical epithelium. Little change was observed in the thyroids from rats receiving the lower doses.

The mean weight for the thyroid glands (both lobes) from 6 rats treated with daily doses of 1·25 g./kg. for 9 weeks was 6·66 ± 0·62 mg./100 g. The mean weight of the glands from the control animals was 7·03 ± 0·46 mg./100 g. The difference in weight between the two groups of thyroids was 0·37 mg./100 g. and this was not statistically significant. These results showed that calcium 4-benzamidosalicylate resembled *p*-aminosalicylic

acid in its effect on the structure of the rat thyroid and in not producing any marked hypertrophy of the gland. This action may be due to the effect of the substance itself or to its decomposition to *p*-aminosalicylic acid. Histological examination of heart, lung, liver, kidney, spleen, pituitary and adrenal glands showed no effect. There was no effect on the red and white blood cell counts, clotting and prothrombin times.

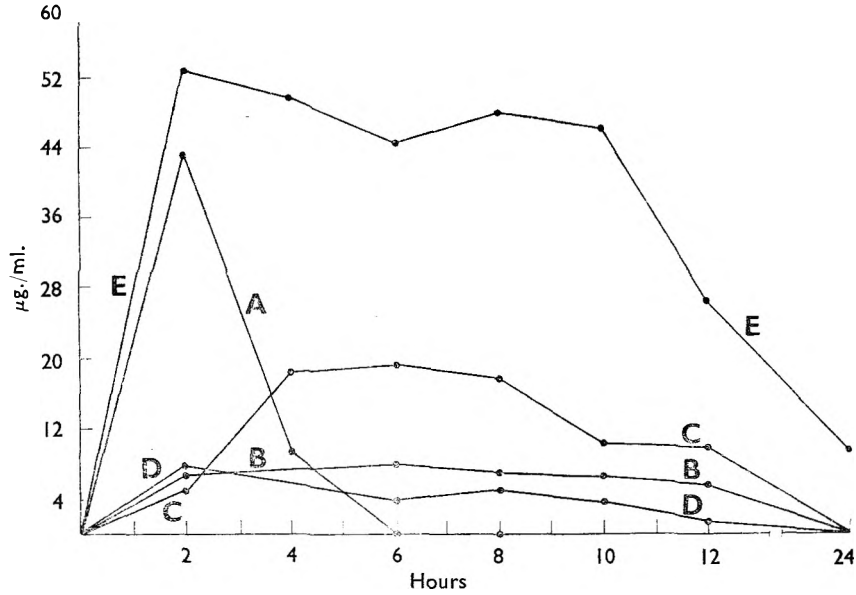


FIG. 2. Plasma levels of *p*-aminosalicylic acid and 4-benzamidosalicylic acid in rabbits after oral dosage.

A. *p*-Aminosalicylic acid level after 0·213 g./kg. of sodium *p*-aminosalicylate.
B. *p*-Aminosalicylic acid level after 0·39 g./kg. of calcium 4-benzamidosalicylate.
C. 4-Benzamidosalicylic acid level after 0·39 g./kg. of calcium 4-benzamidosalicylate.
D. *p*-Aminosalicylic acid level after 1 g./kg. of calcium 4-benzamidosalicylate.
E. 4-Benzamidosalicylic acid level after 1 g./kg. of calcium 4-benzamidosalicylate.

II. *Plasma Levels.* Figure 2 gives the mean plasma levels of *p*-aminosalicylic acid and 4-benzamidosalicylic acid obtained in 3 groups of 3 rabbits each. The first group received the normal clinical dose of sodium *p*-aminosalicylate orally (i.e., 15 g./70 kg. = 0·213 g./kg.); the second group received the stoichiometric equivalent of calcium 4-benzamidosalicylate (i.e. 0·392 g./kg.); and the third group received 1·0 g./kg. of calcium 4-benzamidosalicylate. As will be seen, the first group rapidly attained a fairly high peak plasma level of *p*-aminosalicylic acid, but this fell to zero within 6 hours. In contrast the second group showed that much lower but much more prolonged plasma level of *p*-aminosalicylic acid, accompanied by a higher and prolonged plasma level of 4-benzamidosalicylic acid. The third group showed higher values for 4-benzamidosalicylic acid with little difference in the *p*-aminosalicylic acid levels.

The result of this experiment showed that calcium 4-benzamidosalicylate

was absorbed from the gastro-intestinal tract, presumably in the form of free 4-benzamidosalicylic acid or its soluble sodium salt, and then broken down in the body to yield *p*-aminosalicylic acid at a steady rate over a period of about 24 hours.

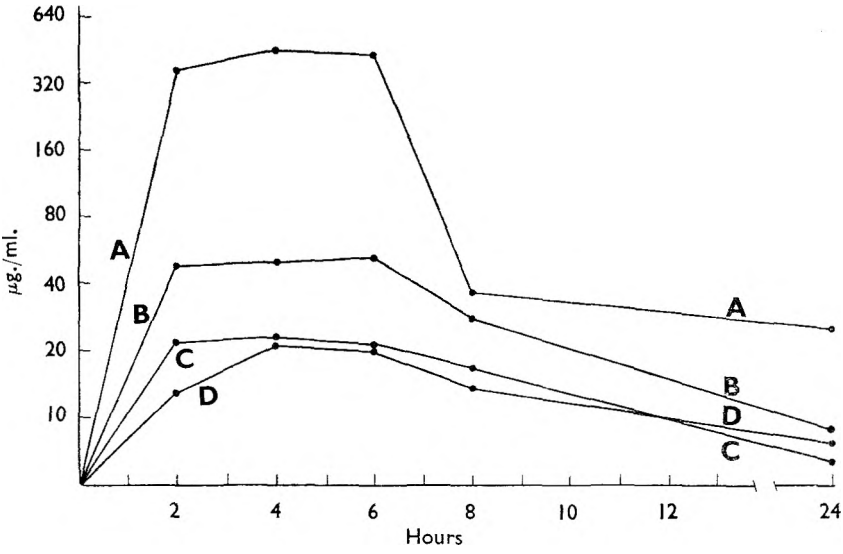


FIG. 3. Plasma and urine levels in man after an oral dose of 15 g. calcium 4-benzamidosalicylate in milk.

A. *p*-Aminosalicylic acid level in urine.
B. 4-Benzamidosalicylic acid level in urine.
C. *p*-Aminosalicylic acid level in plasma.
D. 4-Benzamidosalicylic acid level in plasma.

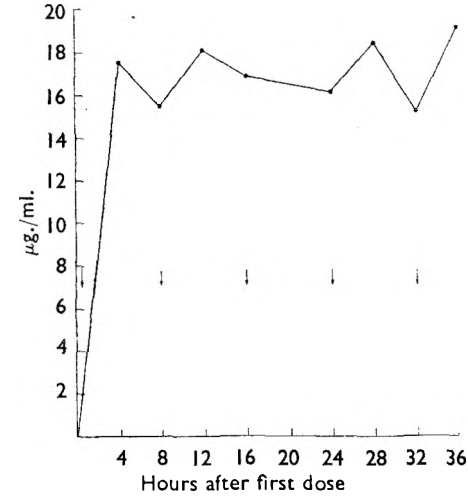


FIG. 4. Plasma level of *p*-aminosalicylic acid in man after divided doses of calcium 4-benzamidosalicylate. Arrows denote doses of 5 g.

In Figures 3 and 4 are shown plasma levels obtained in human volunteers. Figure 3 gives the results obtained with a single dose of 15 g. of calcium 4-benzamidosalicylate and Figure 4 shows the effect of repeated doses of 5 g. of the compound at intervals of 8 hours. Both figures show approximately the same maximum level of *p*-aminosalicylic acid of about 20 μg./ml. but the effect of the repeated doses is clearly to maintain the plasma content of *p*-aminosalicylic acid at an approximately steady level for the period of administration of the compound. Figure 3 also shows that a very high

concentration of *p*-aminosalicylic acid and free 4-benzamidosalicylic acid is attained in the urine. These urinary concentrations are about 25 times and twice the blood plasma levels of the respective constituents.

Plasma levels were also investigated in three groups of mice which had received 2 per cent. and 1 per cent. of calcium 4-benzamidosalicylate in their diet for a period of 1 week. Comparison was made with a similar group of mice receiving 1 per cent. of sodium *p*-aminosalicylate for a similar period. It was felt that the information from this experiment would be of value in itself and would also be of use in assessing the result of chemotherapeutic tests on the compound. The results are given in Table II obtained 2 hours, 4 hours and 6 hours after giving the medicated diet. This Table shows that, even when given at twice the dose, calcium 4-benzamidosalicylate produces somewhat smaller plasma levels than does *p*-aminosalicylic acid, although this difference appears to become less a few hours after administration.

TABLE II

p-AMINOSALICYLIC ACID LEVELS IN PLASMA OF MICE AFTER RECEIVING CALCIUM 4-BENZAMIDOSALICYLATE IN THE DIET FOR ONE WEEK

Group	*p*-Aminosalicylic acid µg./ml.		
	2 hours	4 hours	6 hours
2 per cent.	30	4·1	14·1
1 per cent.	0	0	4·7
1 per cent. sodium *p*-aminosalicylate ..	55·5	12·5	17·4

III. *Urinary excretion.* Preliminary experiments on rats and rabbits showed that after a single dose of 2 g. of calcium 4-benzamidosalicylate a marked and prolonged urinary excretion occurred of both *p*-aminosalicylic acid and 4-benzamidosalicylic acid. The result of a typical experiment on a rabbit is given in Table III.

TABLE III

4-BENZAMIDOSALICYLIC ACID AND *p*-AMINOSALICYLIC ACID LEVELS IN URINE OF RABBITS AFTER A DOSE OF 2 g. OF CALCIUM 4-BENZAMIDOSALICYLATE

	Time	*p*-amino-salicylic acid µg./ml.	4-benzamido-salicylic acid µg./ml.
Rabbit 1	0 to 3 hours	276	12·8
" "	3 to 24 "	394	24·4
Rabbit 2	0 to 24 "	254	18·1

Similar results were obtained in human volunteers, and are illustrated in Figure 3. From this it appears that the urinary excretion of *p*-aminosalicylic acid and 4-benzamidosalicylic acid following one dose of calcium 4-benzamidosalicylate is not fully complete in 24 hours. This is in marked contrast to the very rapid excretion which occurs when sodium *p*-aminosalicylate is given orally. The unusually high concentration of *p*-aminosalicylic acid found in the urine was of particular interest in relation to the possible clinical use of calcium 4-benzamidosalicylate in genito-urinary tuberculosis. This is discussed at greater length below.

This same finding of high urinary levels of *p*-aminosalicylic acid also suggested that the kidney might play an important part in the breakdown of 4-benzamidosalicylic acid and this possibility was investigated as part of a general study of the action of tissue homogenates on the compound.

IV. *Tissue Levels.* A group of 3 rats was treated with daily oral doses of 1·25 g./kg. of calcium 4-benzamidosalicylate for a period of 1 week and the animals were then killed 16 hours after the last dose. The levels of *p*-aminosalicylic acid and 4-benzamidosalicylic acid in the liver, lung, spleen and kidney were found to be very variable between 0 and 150 μg./g., with a tendency for the *p*-aminosalicylic acid content of the lung to be lower than that of the other tissues.

V. *Tissue Homogenates.* The effect of homogenates of various tissues on sodium 4-benzamidosalicylate after incubation for 24 hours is shown in Table IV. It will be seen that all the tissues produced hydrolysis of the

TABLE IV

DECOMPOSITION OF SODIUM 4-BENZAMIDOSALICYLATE BY TISSUES

Tissue	*p*-Aminosalicylic acid (μg./ml. of homogenate)				
	Normal tissue and 100 μg./ml. of sodium 4-benzamido-salicylate	Boiled tissue and 100 μg./ml. of sodium 4-benzamido-salicylate	Normal tissue and 50 μg./ml. of sodium *p*-amino-salicylate	Krebs Ringer and 100 μg./ml. of sodium 4-benzamido-salicylate	Krebs Ringer and 50 μg./ml. of sodium *p*-amino-salicylate
Liver	31·0	0	30·7	0	30·4
Lung	27·0	0	34·8	0	36·3
Kidney	57·5	0	37·0	—	—
Blood	15·2	—	23·0	—	—

compound, but the kidney appeared to be the most active in this respect. This result lends support to the suggestion made above as to the origin of the urinary *p*-aminosalicylic acid. The lack of effect by boiled tissue suggests that the decomposition is brought about enzymatically, but whether the enzyme responsible for the hydrolysis is some form of peptidase or an enzyme of less specific action is not at present known. The experiments of incubating tissue homogenates with *p*-aminosalicylic acid showed that some loss of *p*-aminosalicylic acid occurred, possibly due to decarboxylation, during 24 hours' incubation. Theoretically, the figures in the first column of the Table should be corrected for this loss.

VI. *Chemotherapeutic effect.* The result of a typical chemotherapeutic test is given in Table V. It will be seen that calcium 4-benzamidosalicylate is less active than *p*-aminosalicylic acid, weight for weight, and from an approximate calculation, it appears that it may have 50 per cent. of the activity of *p*-aminosalicylic acid. This agrees quite closely with the percentage of *p*-aminosalicylic acid theoretically obtainable from calcium 4-benzamidosalicylate and suggests that the tuberculostatic effect is due to the liberation of *p*-aminosalicylic acid and not due to an activity *per se* of the compound. This suggestion is supported by the plasma levels of *p*-aminosalicylic acid and 4-benzamidosalicylic acid in rabbits and mice shown in Figure 2 and Table II. If the molecule of 4-benzamidosalicylic

acid is itself tuberculostatic, the high plasma levels of this acid attained in these experiments would have led to an expectation of a high tuberculostatic activity, when tested *in vivo* in other groups of mice. The low plasma level of *p*-aminosalicylic acid obtained simultaneously with the higher levels of 4-benzamidosalicylic acid further reinforces the argument

TABLE V

CHEMOTHERAPEUTIC EFFECT OF CALCIUM 4-BENZAMIDOSALICYLATE COMPARED WITH *p*-AMINOSALICYLIC ACID IN MICE

Group	Number of animals	Compound	Percentage level in diet	Percentage without lesions
1	10	Calcium 4-benzamidosalicylate	0·5	50
2	10	" "	1·0	60
3	8	" "	2·0	87·5
4	6	*p*-Aminosalicylic acid	0·25	50
5	12	" "	0·5	66·7
6	9	" "	1·0	88·9
7	10	Untreated	—	10·0

that the lower activity of calcium 4-benzamidosalicylate, as compared with *p*-aminosalicylic acid, is due solely to the liberation of small concentrations of *p*-aminosalicylic acid *in situ*.

DISCUSSION

The present investigation has shown that the calcium salt of 4-benzamidosalicylic acid is decomposed, at least partially, in the animal body to form *p*-aminosalicylic acid and benzoic acid. The mechanisms of this reaction is obscure at the moment, but it is apparently enzymatic in character and brought about by most of the tissues of the body, predominantly by the kidney. It is hoped to investigate this reaction more closely by the use of specific enzymes.

The liberation of *p*-aminosalicylic acid occurs slowly and steadily and it is possible to maintain comparatively steady, rather low, plasma levels of *p*-aminosalicylic acid over a protracted period, particularly when divided doses are administered. It is perhaps worth recording that a recurrent maximum plasma level of *p*-aminosalicylic acid of 20 μg./ml. was reached under various conditions of dosage. This effect may be merely fortuitous or may indicate the existence of some kind of chemical equilibrium. Further work may throw more light on this point.

The fact that high urine levels of *p*-aminosalicylic acid occur after treatment with calcium 4-benzamidosalicylate, combined with the probability that the tuberculostatic action of the compound is due solely to *p*-aminosalicylic acid liberated, suggested that calcium 4-benzamidosalicylate might be of value in genito-urinary tuberculosis. Clinical trials of the substance in this condition have been inaugurated through the kindness of Mr. J. G. Gow and are still in progress. Two recent reports[13,14], however, suggest that calcium 4-benzamidosalicylate is of considerable value as a substitute for *p*-aminosalicylic acid in the treatment of genito-urinary tuberculosis. The advantages reported are lack of toxicity and taste and prolonged *p*-aminosalicylic acid blood levels.

It is also reported that it appears to delay the development of streptomycin resistance equally as well as *p*-aminosalicylic acid. With these facts

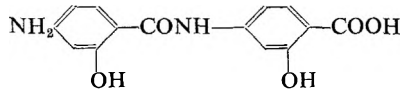
in mind, the clinical trials of calcium 4-benzamidosalicylate in pulmonary tuberculosis were commenced and the results will be reported in due course. Interim observations suggest that the drug is well received by the patient because of the absence of nausea following its use.

During our work on this compound, we considered the possibility that the *in situ* liberation of 0·36 g. of benzoic acid per g. of drug might have toxic effects or alternatively the detoxication of this amount of benzoic acid by glycine conjugation might disturb the amino-acid balance. However, it was clear from the animal experiments and later, from the clinical trials that the overall toxicity of calcium 4-benzamidosalicylate is very low. Experiments to investigate more fully the fate of the benzoic acid part of the molecule are planned.

The fate of the calcium ion has, also, not yet been fully investigated. Preliminary work has shown that urinary calcium is not greatly increased after administration to volunteers and presumably most of the calcium is excreted *via* the fæces.

It seems that calcium 4-benzamidosalicylate resembles *p*-aminosalicylic acid in causing a loss of colloid material from the rat thyroid gland and the possibility must, therefore, be considered of occasional cases of myxœdema occurring during clinical use. These cases, however, have been mild and relatively rare during treatment with *p*-aminosalicylic acid, and it seems likely that same will be the case with calcium 4-benzamidosalicylate.

The facts that the molecule appears to be, itself, tuberculostatically inactive and that only half of the molecule is converted into *p*-aminosalicylic acid suggested that a modification of the structure of the compound might be advantageous. Consequently, consideration was given to the use of 4-(4-amino-2-hydroxy-benzamido)-salicylic acid (*II*).



II

This will be seen to be a compound of two molecules of *p*-aminosalicylic acid, instead of one molecule of *p*-aminosalicylic acid and one molecule of benzoic acid as occurs in calcium 4-benzamidosalicylate, and has already been shown to have an *in vitro* activity equal to that of *p*-aminosalicylic acid[15]. If decomposition occurs in the body on the same lines as with calcium 4-benzamidosalicylic acid, this substance should yield approximately twice as much *p*-aminosalicylic acid, and, presumably, should therefore, have a correspondingly increased therapeutic activity. Examination of the calcium salt of this acid showed that it resembled the calcium salt of 4-benzamidosalicylic acid, in insolubility and tastelessness and an investigation of this substance (H.P 354) is now in progress.

Summary

1. The calcium salt of 4-benzamidosalicylic acid (H.P. 170) is a very insoluble, almost tasteless compound, which decomposes in the body to

liberate *p*-aminosalicylic acid. Plasma levels of *p*-aminosalicylic acid are low but prolonged, and urine levels are much higher and equally prolonged.

2. It has a very low toxicity to mice and rats. It produces loss of colloid material from the rat thyroid and thus resembles *p*-aminosalicylic acid.

3. It has a tuberculostatic activity of approximately 50 per cent. of that of *p*-aminosalicylic acid. This activity is probably entirely due to liberated *p*-aminosalicylic acid.

4. It is decomposed to *p*-aminosalicylic acid by homogenates of several tissues. The action is probably enzymatic.

5. It has been found to be of value as a substitute for *p*-aminosalicylic acid in the treatment of genito-urinary tuberculosis. It appears to delay the emergence of streptomycin resistance.

6. A method is described for the determination of 4-benzamidosalicylic acid in biological fluids.

We are indebted to Mr. D. E. Seymour and Mr. J. G. Gow for much helpful discussion during the course of this work, and our thanks are also due to Mr. B. W. Mitchell for valuable help and advice with the analytical methods and Dr. M. Seiler for the chemotherapeutic experiments. We would also like to record our indebtedness to Mr. P. Wagner and (the late) Mr. J. M. Sells for much careful laboratory work.

Our thanks are also due to the Directors of Smith and Nephew Research, Ltd. for permission to publish this work.

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DISCUSSION

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

DR. G. E. FOSTER (Dartford) said it was not clear what constituted the mechanism of the reaction in the colorimetric assay.

MR. T. H. ELLIOTT (Singapore) asked why the authors had not considered the use of *p*-phenylene-ethylenediamine which could be used in acid solution, was relatively stable, and was likely to give more satisfactory recoveries.

MR. E. M. BAVIN, in reply, pointed out that the mechanism of the reaction was explained in Pesez's paper (reference 7). If the hydrolysis broke down the calcium benzamidosalicylate to *p*-aminosalicylic acid, the latter was merely a coupling agent. There was nothing peculiar about *p*-aminosalicylic acid as a final coupling agent, and one could use any other phenol. *m*-Aminophenol could have been estimated by the use of *p*-phenylene-ethylenediamine or by other methods, but the method used was chosen because of previous experience of it. He agreed that the recoveries shown in Table I were not good, but it had been found that the method used gave quite satisfactory results from the clinical point of view.

21-ACETOXY-12α : 17α -DIHYDROXYPREGN-4-ENE-3 : 20-DIONE AND ITS ANTICORTISONE PROPERTIES

BY (MRS.) W. J. ADAMS, B. G. CROSS, A. DAVID, F. HARTLEY, D. PATEL, V. PETROW and (MRS.) I. A. STUART-WEBB

From The Research Laboratories, The British Drug Houses, Ltd., London, N.1

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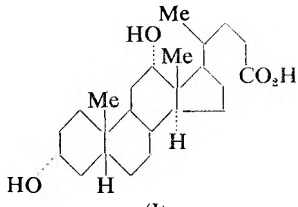
SOME fifty years ago Still[1] and Wishart[2] noted that jaundice exercised a profound and beneficial effect upon the syndrome of rheumatoid arthritis. Initial attempts by Hench[3] to apply this finding to the clinical treatment of rheumatism by inducing artificial hyperbilirubinæmia by intravenous injection of bile salts and bilirubin, however, were not entirely successful, only slight transitory benefits, not comparable with those produced by naturally-acquired jaundice, being observed. More encouraging results were subsequently reported by MacCallum and Bradley[4] and by Gordon, Stewart and MacCallum[5] who induced jaundice with icterogenic serum in volunteer rheumatoid arthritic patients. Their results led them to conclude that the remissions induced in rheumatoid arthritis by an attack of jaundice were striking and showed that the disease was capable of undergoing considerable regression.

Parallel with these investigations Hench, in America, was continuing studies initiated in 1929 on the beneficial effects of pregnancy and jaundice upon rheumatoid arthritis. By 1949 he was able to state[6] that "the inherent reversibility of rheumatoid arthritis is activated more effectively by the intercurrence of jaundice or pregnancy than by any other condition or agent thus far known." He further conjectured that the beneficial effects thus encountered were due to a hypothetical "antirheumatic Substance X" which was not a disintegration product from a damaged liver, but a compound specific in nature and function and normal to the human organism. As temporary remissions of arthritis were known to follow procedures capable of stimulating the adrenal cortices, Hench turned his attention to the adrenal hormones, finally associating "antirheumatic Substance X" with cortisone[6]. The dramatic results, thereby achieved have inevitably given a powerful stimulus to the furtherance of studies on the relation between structure and action in the steroid group.

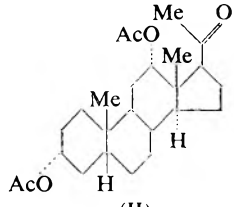
Early in 1951 a programme of work was initiated in these laboratories on the preparation of certain analogues of cortisone for study as anti-phlogistic agents. We had been greatly impressed by the clinical evidence linking remissions in rheumatoid arthritis with intercurrent jaundice. We had also accepted in large measure the explanation of this effect on the basis of Selye's "General Adaptation Syndrome" whereby jaundice is regarded as a stressor agent which imitates the physiological defence mechanism of the body by calling for increased production of antiphlogistic steroids[7]. At the same time we thought the results were capable of a different interpretation and one worthy of consideration.

Disturbance of the excretory mechanism of the liver leads to jaundice

through reabsorption into the blood stream of the bile pigments, bile salts, cholesterol and other constituents normally voided through the bile ducts into the intestine. Reabsorption of the bile salts would clearly lead to a raised level of desoxycholic acid (I) (and cholic acid) in the



(I)



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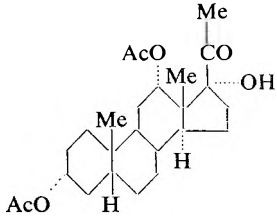

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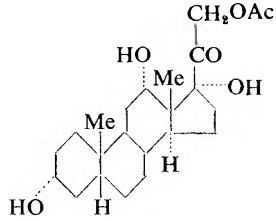

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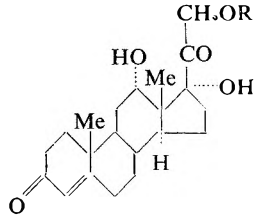
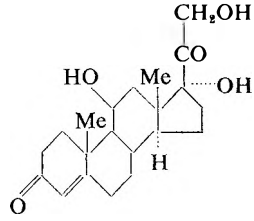


(V; R = H)
(Va; R = Ac)



(VI)

blood stream[8] and hence to a raised level of this steroid in the blood perfusing the adrenals. These glands, in turn, were known[9] to convert compounds such as cholesterol into hydrocortisone (VI) and could therefore also possess the capacity to transform desoxycholic acid (I), or a precursor thereof, into 12α:17α:21-trihydroxypregn-4-ene-3:20-dione (V) which bears a close structural resemblance to the antiphlogistic hormone hydrocortisone (VI). If this were indeed the case, it follows that (V) might correspond to the "antirheumatic Substance X" postulated by Hench as responsible for the remissions produced by jaundice in arthritic patients.

It was against this background that we began the tedious and difficult transformations required to convert (I) into (Va). Desoxycholic acid was first converted into 3α:12α-diacetoxypregnan-20-one (II). The 17α-hydroxyl group was then introduced to give 3α:12α-diacetoxy-17α-hydroxypregnan-20-one (III). The constitution assigned to this compound was confirmed by (i) dehydration by the Darzen method when 3α:12α-diacetoxypregn-16-en-20-one was obtained, identical with an

authentic sample prepared by the method of Djerassi[10] and (ii) chromic acid oxidation to a product, $C_{23}H_{34}O_5$, which, from its melting point and optical rotation appeared to be 3α:12α-diacetoxy-testan-17-one[11]. Hydrolysis of (III), followed by introduction of a 21-acetoxyl group, furnished 21-acetoxy-3α:12α:17α-trihydroxypregnan-20-one (IV). Partial oxidation of this compound gave a product which was undoubtedly the desired 21-acetoxy-12α:17α-dihydroxypregnane-3:20-dione. Its alternative formulation as the corresponding 12-keto-3α-hydroxy-derivative was rendered unlikely by the observation that the material failed to react with ethyl chloroformate, a reagent specific for C_3-hydroxyl groups[12]. (IV), in contrast, passed smoothly into the 3-cathyl-derivative, converted into 12α:21-diacetoxy-3-cathyl-17α-hydroxypregnan-20-one by acetylation. Bromination of the foregoing 3-keto compound, followed by dehydrobromination, furnished 21-acetoxy-12α:17α-dihydroxypregn-4-ene-3:20-dione (Va). The overall yield of (Va) from (I), however, was very low. This fact was largely accounted for by the use of more than 20 stages in its preparation. Thus an 80 per cent. yield at each stage, even if it could invariably be reached, would only give an overall yield of *ca.* 1 per cent. In addition, the marked interference exercised by the 12α-hydroxyl group upon the introduction of the 17α-hydroxyl group with consequent drop in yield, combined to make the preparation of (Va) a formidable undertaking. It may be of interest to record, in this connection, that nearly 100 kg. of desoxycholic acid were used in devising the process and preparing sufficient material to allow a preliminary biological evaluation of the product.

21-Acetoxy-12α:17α-dihydroxypregn-4-ene-3:20-dione (Va), obtained in this way, separated from acetone-hexane in white needles, m.pt. 195° to 197° C. Found: C, 68·3; H, 8·0. $C_{23}H_{32}O_6$ requires C, 68·3; H, 7·9 per cent., $[\alpha]_D^{24^\circ C.}$ + 146° (c, 0·422 in chloroform). The presence of the 3-keto-Δ^4 system in this compound followed from its ultra-violet absorption spectrum which showed a maximum at 240 mμ, ϵ_{2380} 16,600 (in *iso*-propanol). The 12α-hydroxyl group was characterised by acetylation. The α-ketol side chain was revealed by use of the 3:3′-dianisole bis-4:4′:(35-diphenyl) tetrazolium chloride reagent[13] with which a purple coloration was obtained on paper chromatograms. Infra-red absorption spectra measurements, kindly determined by Dr. A. E. Kellie (Courtauld Institute of Biochemistry) provided independent confirmation for the presence of a free hydroxyl group, a 3-keto-Δ^4 system and of an acetylated α-ketol side chain in the compound.

Examination of (Va) for cortisone-like activity proved disappointing. Thus the compound was unable to produce involution of the thymus in infantile rats at a dose level effective for cortisone. In addition, it failed to raise the liver glycogen level in the adrenalectomised rat[14]. Examination for androgenic[15], progestational and œstrogenic activity showed the material to be inactive at the dose levels employed. Its effect on mineral metabolism, kindly determined by Dr. J. F. Tait and Mrs. S. A. Simpson (Middlesex Hospital Medical School) employing a bioassay based on the depression of the urinary $^{24}Na/^{42}K$ ratio in adrenalectomised rats[16],

revealed that it possessed not more than 2 per cent. of the activity of desoxycorticosterone employed as a standard.

In spite of these negative results, which clearly did not support the hypothesis that (V) must correspond to Hench's "antirheumatic Substance X," we nevertheless retained a conviction that the steroid was sufficiently closely related in structure to the adrenal cortical hormones to show some form of biological activity. A purely empirical search for such action, however, was contra-indicated by the relative inaccessibility of the material. We therefore turned our attention to some theoretical concepts relating changes in structure of active compounds to the effect of such changes upon biological activity, hoping thereby to obtain some indications for further work.

Last year we[17] presented a discussion to the British Pharmaceutical Conference on the mechanism of biological action and put forward therein the novel concept of specific (σ) and non-specific (ν) pharmacodynamical groups. In accordance with these views the molecule of a biologically active compound was pictured as being partly embedded in the receptor system, the σ-groups lying within the system and the ν-groups resting upon its surface. Any changes in the σ-groups were held to alter the delicate spatial relationship between the biologically active compound and the receptor centres thus making juxtaposition impossible with consequent loss of activity. Limited structural changes in the surface ν-groups were nevertheless regarded as permissible for quantitative retention of activity. It was pointed out, however, that such changes could also lead to structures lacking the activity of the biologically active compound but able to take its place in the receptor system thereby blocking the approach of the active molecule. Inhibition would consequently occur.

Applying these concepts to the cortisone structure, it appeared that the 3-keto, 20-keto, 17-hydroxyl, 21-hydroxyl and the Δ^4-unsaturated linkage could be regarded as σ-pharmacodynamical groups as any changes therein lead to loss of antiphlogistic activity[18]. The 11-substituent, however, seemed to fall within the ν-category as both the 11-keto and 11 β-hydroxy compounds show similar biological action[18]. Modification of this pharmacodynamical centre, as in (V) could therefore have the effect of altering quantitatively the antiphlogistic action of the compound or else could lead to a steroid with inhibitory properties. As the former alternative was ruled out by the experimental evidence outlined on p. 863, above we turned our attention to the second possibility, namely that (Va) may have the properties of an anticortisone. In so far as the preliminary results indicate, this has indeed proved to be the case.

We have already referred to the action of cortisone in restoring the liver glycogen of the adrenalectomised rat[14]. We now find that simultaneous administration of (Va) with cortisone acetate leads to a markedly smaller increase in the level of the liver glycogen compared to controls receiving cortisone acetate alone. As (Va), *per se*, did not appear to influence glyconeogenesis (p. 863 above) these results indicate that (Va) inhibited the action of the cortisone acetate, i.e., it functioned as an *anticortisone* in this particular assay.

It cannot, of course, be inferred from these observations that (Va) must necessarily inhibit all the biological functions of cortisone in the animal organism. The full extent of its antihormonal action has still to be determined. The results obtained do, nevertheless, indicate the importance of further study of the properties of (Va) in relation to the pathological states adversely affected by the antiphlogistic steroids. For example, cortisone is known to be able to suppress the inflammatory reaction irrespective of the nature of the causative agent, thereby enabling pathogenic organisms to disseminate rapidly[19]. Again, under certain conditions it appears to prevent the rapid production of antibodies[20,21]. It also exercises an undesirable effect upon the growth of certain neoplasms in that it induces the production of metastases[22,23,24]. There are, consequently, many potential uses for inhibitors of cortisone action.

In conclusion, it seems opportune to speculate on the possible existence of natural steroids with such inhibitory functions. The liver is known to synthesise cholesterol from acetate and thence convert it into cholic acid, desoxycholic acid, etc. by oxidative and reductive procedures[25]. In addition, it is now clear that the liver also contains enzyme systems able to convert simple steroids into cortisone[26,27,28]. Thus nearly all the mechanisms required to transform (I), or a cholesterol-like precursor thereof, into (V) are proven to operate in the liver. Legitimate grounds consequently exist for the assertion that (V) may possibly be formed in the body and thus play a part in the dichotomy of the inflammatory response.

Experimental

Examination of 21-*Acetoxy*-12α:17α-*dihydroxypregn*-4-*ene*-3:20-*dione* (Va) *for Cortisone-like Activity.*—(i) Dr. S. W. F. Underhill and Miss B. M. Manly, B.Sc., of these laboratories, have kindly examined (Va) for its hypotrophic effect on the thymus of nestling rats[29]. They found that the compound was inactive at a total dose level of 3 mg./22 g. rat.

(ii) The glyconeogenetic activity of (Va) was determined in the following way[30]: Young male albino rats of B.D.H. stock whose ages ranged from 50 to 150 days were weighed and adrenalectomised under ether anæsthesia. A high protein diet was provided *ad libitum* to the fourth postoperative morning and 1 per cent. sodium chloride drinking water to the fifth post-operative morning. In some of the preliminary studies food was given twice daily at regular intervals for a limited period, but in spite of training prior to adrenalectomy this feeding routine was found to be unsatisfactory and was therefore discontinued. After removal of the drinking water the compound under test was injected subcutaneously in aqueous suspension, the dose being given in 4 portions at 2-hourly intervals. The volume of the suspension used was adjusted such that each animal received 0·5 ml./150 g. body weight, at each injection. Hepatectomy under hexobarbitone anæsthesia (0·2 ml. to 0·4 ml. of a 10 per cent. aqueous solution intraperitoneally) was performed 2 hours after the last injection. Immediately on removal each liver was washed in iced saline solution, dried with filter paper, weighed and digested in 15 ml.

of 30 per cent. potassium hydroxide in a boiling water bath for approx. 60 minutes. The glycogen was precipitated by the addition of 16·5 ml. of 95 per cent. ethanol, heating to boiling on the water bath and cooling. The precipitated glycogen was separated by centrifuging (2000 r.p.m.) for 10 minutes. It was dissolved in 5 ml. of distilled water, transferred to a 100-ml. round-bottomed flask and hydrolysed by heating on a boiling water bath for 3 hours with 10 ml. of N sulphuric acid. The solution, on cooling, was neutralised with N sodium hydroxide using 3 drops of phenol-red as indicator. The volume of each hydrolysate was made up to 50 ml. and each portion strained through cotton wool. Aliquots of 5 ml. each were mixed with an equal volume of Schaffer Hartmann reagent[31] in small conical flasks and placed in a vigorously boiling water bath for 15 minutes. This was followed by rapidly cooling in iced water to 35° to 40° C., adding 2 ml. of 5N sulphuric acid and placing for 2 minutes in a bath maintained at 35° to 37° C. The mixture was then titrated with 0·005N sodium thiosulphate solution using starch mucilage as indicator and the results calculated as described by Somogyi[31]. Table I shows the values obtained, the liver glycogen content being expressed as a percentage of fresh liver weight.

TABLE I

Rat	Number of animals	Compound	Total dose mg./150 g. of body weight	Mean percentage glycogen (fresh liver weight)
Intact (Control)	28	—	—	0·37
Adrenalectomised (Control)	5	—	—	0·024
Adrenalectomised	3	(Va)	4	0·036
	3	(Va)	8	0·037
	3	(Va)	16	0·034

Examination of Table I shows that the results obtained with the control groups accord well with earlier values given by Pabst, *et al.*[30], who report liver glycogen values of 0·4 per cent. and 0·016 per cent. for intact and adrenalectomised rats, respectively. (Va) is seen to exert no significant effect. Cortisone acetate, in contrast, is found to restore the liver glycogen content, typical results obtained being given in Table II.

TABLE II

Number of rats	Dose of cortisone acetate mg.	Dose of Va in mg./150 g. of body weight	Mean percentage glycogen (fresh liver weight)
7	0·25	—	0·087
3	0·25	4·0	0·04
2	0·25	12·0	0·0
7	0·5	—	0·103
3	0·5	8·0	0·08
3	0·5	12·0	0·053
7	1·0	—	0·301
2	1·0	12·0	0·093
4	1·0	16·0	0·17

Examination of 21-*Acetoxy*-12α:17α-*dihydroxypregn*-4-*ene*-3:20-*dione* (Va) *for Androgenic, Progestational and Œstrogenic Activity.*—Dr. S. W. F. Underhill and Mr. W. S. Parr have kindly examined (Va) for androgenic[32], progestational[33] and œstrogenic[34] activity using the methods indicated but found no evidence for activity at doses of 50 mg., 200 mg., and 3 mg., respectively.

Examination of 21-*Acetoxy*-12α:17α-*dihydroxypregn*-4-*ene*-3:20-*dione* (Va) *for Inhibition of the Glyconeogenetic Activity of Cortisone in the Adrenalectomised Rat.*—Rats were adrenalectomised and the liver glycogen content determined as described above. Our aim to use not less than 3 animals at each dose level was not fully realised as the numbers were reduced to 2 in two instances owing to post-operative deaths. The results obtained are recorded in Table II.

Examination of Table II shows the effect of cortisone acetate in restoring liver glycogen. A linear relationship was found between log dose and liver glycogen per cent. of fresh liver weight (cf. Pabst *et al.*[30]). Comparison of groups of rats in which one group received (Va) additionally to the cortisone acetate shows that (Va) inhibits the glyconeogenetic effect produced by the cortisone acetate.

Summary and Conclusions

1. 21-Acetoxy-12α:17α-dihydroxypregn-4-ene-3:20-dione (Va), a structural analogue of hydrocortisone (acetate), has been prepared from desoxycholic acid.

2. A preliminary evaluation of its biological properties has been made.

3. It has been found that this compound (Va) inhibits the glyconeogenetic activity of cortisone acetate in the adrenalectomised rat.

4. The possible implications of this result are discussed.

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DISCUSSION

The paper was presented by DR. F. HARTLEY.

DR. G. E. FOSTER (Dartford) said that there was a theory that adrenocorticotrophic hormone stimulated the adrenals to produce cortisone, and he wondered whether the substance described in the paper would inhibit adrenocorticotrophic hormone as well as cortisone activity.

DR. F. HARTLEY, in reply, said that the action of adrenocorticotrophic hormone was not merely to liberate cortisone, and it would be important to consider to what extent their substance might reverse the action of other adrenal cortex secretions. The latter were capable of influencing liver glycogen, carbohydrate metabolism and androgenic and shock-inhibiting characteristics, so that while at first sight it might seem attractive, in his view it would lead to difficulties in interpreting the results if such a blunderbuss method of stimulating adrenal activity as adrenocorticotrophic hormone were used in an attempt to antagonise the carbohydrate metabolism factor. It was emphasised in the paper that the results could not be interpreted at present as meaning that the compound would antagonise all the actions of cortisone. That that cautionary remark was justified was shown by the fact that the help of Dr. Long and his colleagues at the Medical Research Council had been sought to ascertain the behaviour of the substance in the guinea-pig tuberculin sensitivity test for cortisone. It was injected intraperitoneally, whereas cortisone was injected subcutaneously. The substance was without effect on the cortisone action on the tuberculin insensitivity. It was however still permissible to speculate, and although cortisone was usually thought of in terms of its anti-inflammatory activity, and more popularly its antirheumatoid value, it was as well to recognise its other properties such as stimulation of dormant tuberculosis. Steroids with anticortisone properties might therefore open up a new picture in chemotherapy. It was conceivable that among the steroids there would be found a material not only capable of combating the spread of infection but perhaps even of acting prophylactically.

ASCARIDOLE STUDIES

PART II*. AN EXAMINATION OF THE IODIMETRIC AND POLAROGRAPHIC METHODS OF DETERMINATION OF ASCARIDOLE

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THE iodimetric method for the determination of ascaridole described in the B.P. 1953 and U.S.P. (XII) is based on the work of Cocking and Hymas[1]. We found that the results obtained by the official process in the assay of both commercial samples of "ascaridole" and oils of chenopodium differed greatly from the results obtained by the polarographic method[2,3] (see Table I). The two methods have therefore been investigated in some detail in an attempt to account for the observed discrepancies.

In the official method it is necessary to adhere rigidly to the conditions laid down if reproducible results are to be obtained. Furthermore, the factor given for the calculation of results is an empirical one based on the titration of a certain sample of ascaridole stated to contain 96 per cent. of ascaridole. However, this figure had been obtained by Paget's method of assay[4], using titanous chloride, where again an empirical factor based upon the reaction of titanous chloride with pure ascaridole was used. The physical constants quoted for this sample of "pure" ascaridole do not agree with the values published in more recent work. The factor adopted by the B.P. therefore seems to require further investigation. Since the factor was obtained on the basis of the reaction of a definite weight (0·25 g.) of ascaridole with acidified potassium iodide solution under rigid conditions, the use of the same factor (as in B.P.) is questionable when 0·25 g. of oil of chenopodium is used, because of the change in the ratio of ascaridole to the acidified potassium iodide solution.

In another communication[3] we have described the preparation of a product which we consider to be 100 per cent. ascaridole. The analysis of the sample by the B.P. method and using the B.P. factor gave results equivalent to 110·4 per cent. w/w of ascaridole. These results support those of Böhme and van Emster[5] who recently obtained results above 100 per cent. using the B.P. method with purified ascaridole. This incorrect factor, however, does not account for the whole of the difference in the results obtained by the polarographic and B.P. methods of assay of various oils of chenopodium (see Table I). In order to investigate the dependence of the observed percentage of ascaridole on the weight of sample used in the iodimetric method of determination, various oils of chenopodium and ascaridole were examined under experimental conditions which were constant except for the weight of sample used. These results (Table II and Fig. 1) indicate that the amount of iodine liberated is not

*The paper in *J. Pharm. Pharmacol.*, 1952, **4**, 738, is regarded as Part I.

directly proportional to the amount of sample present in the reaction mixture, and consequently the use of a constant factor for oils of differing ascaridole content must yield incorrect results.

TABLE I

Sample	Percentage w/w of ascaridole			
	B.P. method and factor	Polarographic method	B.P. method (adjusted)	B.P. method (quadratic expression)
Pure ascaridole	110·4	100·0	100·0	100·0
"Ascaridole" 1	95·1	84·4	83·9	83·6
"Ascaridole" 2	105·5	95·0	94·7	94·9
*Oil of chenopodium 1	79·8	64·0	68·0	68·3
" " 2	65·1	54·3	54·6	54·3
" " 3	68·1	57·3	58·3	57·6
" " 4	68·7	57·4	—	57·8
" " 5	77·8	64·2	—	66·7
" " 6	79·5	68·3	69·2	68·2
" " 7	74·2	63·4	—	63·1
" " 8	75·3	64·3	65·0	64·1

* This sample gives an anomalous result. We presume that it contains another substance, apart from ascaridole, which liberates iodine from acidified potassium iodide solution but does not give a reduction step in the polarographic method.

To eliminate the effect of the change in the ratio between ascaridole and acidified potassium iodide solution on observed percentages, when constant amounts of various oils of chenopodium were used, the results were corrected in the following manner. The weights of the various oils containing 0·25 g. of ascaridole were calculated from the polarographic results. The percentages of ascaridole corresponding to the use of these weights of the various oils in the iodimetric method were read off from the graphs of weight of sample against observed percentage. A further correction was applied because the B.P. factor is approximately 10 per cent. high. The resultant values for ascaridole content of the various oils (Table I, B.P. adjusted method) then corresponded with the values obtained by the polarographic method.

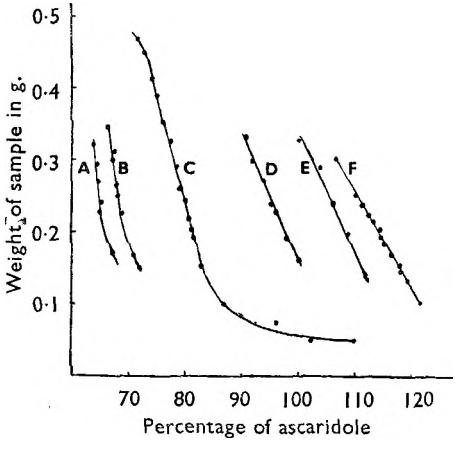

FIG. 1. The effect of the variation of weight of sample upon the observed ascaridole percentage determined by the B.P. procedure.

A. Oil of chenopodium 2.
B. Oil of chenopodium 3.
C. Oil of chenopodium 1.
D. "Ascaridole" 1.
E. "Ascaridole" 2.
F. Pure ascaridole.

It was apparent that the iodimetric method could therefore still constitute a convenient and accurate method for the analysis of oils of chenopodium if the B.P. factor were replaced by a conversion factor which varied with the amount of ascaridole coming into contact with the

TABLE II

Sample	Weight of sample g.	0·1N $Na_2S_2O_3$ (n) ml.	Percentage w/w of ascaridole	
			B.P. method and factor	B.P. method and quadratic expression
Pure ascaridole	0·3071	49·38	106·9	100·7
	0·3006	47·38	106·4	97·8
	0·2510	41·60	110·2	100·0
	0·2383	39·84	111·2	100·1
	0·2227	37·69	112·6	100·4
	0·2196	37·15	112·5	100·0
	0·2142	36·55	113·4	100·6
	0·2123	36·28	113·6	100·5
	0·2040	35·14	114·5	100·8
	0·1941	33·47	114·7	100·2
	0·1842	31·81	114·9	99·5
	0·1705	29·85	116·4	99·9
	0·1551	27·48	117·8	100·0
	0·1465	25·94	117·8	99·2
	0·1319	23·68	119·4	99·5
	0·1027	18·78	121·6	98·5
"Ascaridole" 1	0·3323	45·27	90·6	83·5
	0·2992	41·44	92·1	83·5
	0·2728	38·54	93·8	84·1
	0·2375	34·02	95·3	83·4
	0·2304	33·28	96·0	83·7
	0·1934	28·48	98·1	83·4
	0·1611	24·23	100·1	83·6
"Ascaridole" 2	0·3281	49·71	100·8	94·8
	0·2876	44·90	103·8	95·6
	0·2415	38·50	106·0	94·8
	0·1974	31·29	108·7	91·1
	0·1408	23·75	112·2	93·4
Oil of chenopodium 1	0·4661	50·14	71·5	67·6
	0·4478	48·93	72·6	67·1
	0·4123	45·82	73·9	68·3
	0·3918	44·22	75·0	68·7
	0·3548	40·60	76·1	68·8
	0·3273	38·08	77·4	69·0
	0·2901	34·28	78·6	68·9
	0·2615	31·12	79·1	68·3
	0·2471	29·67	79·9	68·7
	0·2449	29·41	79·8	68·4
	0·2187	26·54	80·6	68·2
	0·2034	24·78	81·0	67·9
	0·1926	23·60	81·5	67·9
	0·1530	19·04	82·8	67·4
	0·1015	13·26	86·9	68·6
	0·0758	10·98	96·2	75·3
	0·0496	8·21	110·0	84·5
	0·0490	7·56	102·5	78·5
Oil of chenopodium 2	0·6670	53·62	53·4	51·1
	0·6372	51·32	53·5	50·6
	0·3220	30·80	63·6	54·9
	0·2947	28·54	64·4	54·9
	0·2708	26·29	64·5	54·4
	0·2399	23·48	65·0	54·2
	0·2295	22·39	64·9	53·7
	0·1710	17·26	67·1	54·1
	0·0634	8·64	90·6	69·9
Oil of chenopodium 3	0·6132	51·05	55·4	52·2
	0·3467	34·48	66·1	58·0
	0·3110	31·51	67·4	58·3
	0·2987	30·11	67·0	57·6
	0·2641	26·92	67·8	57·4
	0·2486	25·50	68·2	57·3
	0·2276	23·53	68·8	57·2
	0·1680	17·86	70·7	57·2
	0·1498	16·25	72·1	57·8
	0·0578	8·11	93·2	71·7

acidified potassium iodide solution. A suitable expression was derived as follows:—

Let m = g. of ascaridole reacting with the acidified potassium iodide solution.

Let n = ml. of 0·1N sodium thiosulphate solution required (less blank titration).

Let x = variable conversion factor.

Then $m = xn$ .. .. (1)

$\therefore \quad x = m/n$

Furthermore $x = f(n)$ or (m) .. .. (2)

When m/n was plotted against n for pure ascaridole a straight line was obtained (Fig. 2) for values of n between 20 and 40 (corresponding to a weight of 0·11 to 0·24 g. of ascaridole).

$\therefore$ (2) becomes $x = Bn + A$ .. .. (3)

Where B and A are constants.

(The linear relationship between m and n holds beyond the values of n = 20 to 40 when pure ascaridole is used, but these seem to be about the safe limits for samples of oil of chenopodium. See Fig. 2).

Substitute for x in (1)

$\therefore \quad m = An + Bn^2$ .. .. (4)

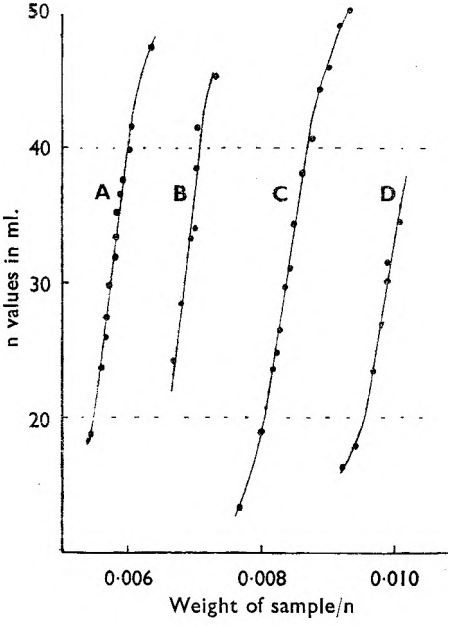

FIG. 2.

A. Pure ascaridole.
B. "Ascaridole" 1.
C. Oil of chenopodium 1.
D. Oil of chenopodium 3.

The values for A (0·00489) and B (0·0000275) for pure ascaridole were obtained graphically (Fig. 2). When the weights of commercial samples of ascaridole and oils of chenopodium were divided by their corresponding 0·1 N sodium thiosulphate figures (n) and plotted against n (Fig. 2) straight lines were also obtained for values of n between 20 and 40. The gradient of these lines increased with decreasing sodium thiosulphate titration per g. of sample as expected from the work already described, but it was necessary to establish that the constants A = 0·00489 and B = 0·0000275 obtained for pure ascaridole applied equally to the ascaridole content of oils of chenopodium before the method could be considered to be generally applicable. As a

first approximation, we applied equation (4) where A = 0·00489 and B × 0·0000275 to obtain a figure for the ascaridole content of the various oils and the weights of the various samples in Table I corresponding to titration figures (n) between 20 and 40 were then converted into weights of ascaridole. These values were divided by their corresponding titration figures (n) and plotted against n values to give straight lines which were found to be superimposable upon the m/n against n line for pure ascaridole. Thus the formula

$$m = 0{\cdot}00489\,n + 0{\cdot}0000275\,n^2 \quad \ldots\ldots\ldots\ldots\ldots \quad (5)$$

is applicable to the calculation of the weight of ascaridole from the titration figure obtained in the B.P. assay process of oil of chenopodium provided the number of ml. of 0·1N sodium thiosulphate required (minus blank) is within the limits of 20 to 40. Böhme and van Emster[5] have also shown recently that, for pure ascaridole, the relation between weight of ascaridole and number of ml. of sodium thiosulphate solution can be expressed with satisfactory accuracy by means of a similar quadratic expression, but they do not indicate that the expression can only give the correct results between certain values of thiosulphate readings. The values obtained by the use of the formula (5) for the calculation of percentages [Table II—B.P. method (quadratic expression)] from the experimental results were constant (for a given oil) even if varying weights of sample were used, and these percentages agreed with the values obtained by the polarographic method. For titration figures below 20 ml. the values obtained for the percentage of ascaridole were slightly too high and above 40 ml. the values were too low. The use of the B.P. factor for the calculation of ascaridole percentages from the same experimental results gave values which varied greatly with the weight of sample used [Table II—B.P. method and factor]. A sample of oil of chenopodium which analysed at 65 per cent. w/w of ascaridole by the present B.P. method would only contain, in fact, 54 per cent. w/w (a 20 per cent. error) whereas an oil giving a result of 80 per cent. w/w would really contain 68·5 per cent. w/w (a 14·5 per cent. error).

Although it is unlikely that any of the constituents of oil of chenopodium, other than ascaridole, will give a reduction step in the polarographic method, the application of this assay process to the determination of ascaridole in oil of chenopodium has now been studied in more detail. When various mixtures of pure ascaridole with *p*-cymene (the chief constituent of oil of chenopodium other than ascaridole) were determined polarographically, the results corresponded with the amount of ascaridole added (Table III). A good correspondence of polarographic results with the theoretical values was also obtained with mixtures of pure ascaridole with oils of chenopodium of low ascaridole content, and with oil of chenopodium heated to decompose the ascaridole. The polarographic method will therefore give correct values for the ascaridole content of oil of chenopodium if the calibration curve is based upon 100 per cent. ascaridole.

The very good agreement between the results obtained by the polaro-

graphic method and the iodimetric procedure adopting the quadratic expression for the calculation of results ensures that the latter method can be regarded as suitable for simple routine analysis. We have therefore outlined the suggested iodimetric method below. It is recognised that,

TABLE III

Pure ascaridole mg.	Diluent added mg.	Total ascaridole present mg.	Ascaridole found by polarographic method mg.
11·0	A 9·6	11·0	10·8
10·2	A 5·3	10·2	10·0
15·3	A 20·0	15·3	15·3
11·3	B 30·6	11·6	11·8
16·2	B 48·8	16·7	16·4
13·3	C 9·4	18·3	18·5
15·2	C 8·4	19·7	19·7

A = *p*-cymene.
B = *Oil of Chenopodium [54·2 per cent.]. heated at 130° C. for 8 hours. It was then found to contain* about 1 per cent. of ascaridole [determined polarographically].
C = Low grade Oil of Chenopodium containing 54·2 per cent. of ascaridole [determined polarographically].

in presuming the two methods give correct figures for ascaridole content, we are assuming that the sample of ascaridole upon which this work is based is 100 per cent. pure. We have taken many precautions to ensure the purity of our sample, and the details of this work are published elsewhere[3].

Proposed Iodimetric Method

Perform the determination as described under chenopodium oil B.P. If the number of ml. (n) of 0·1N sodium thiosulphate required (after deduction of the blank titration) is within the limits of 20 to 40, then calculate the weight of ascaridole (m) from the formula

$$m = 0{\cdot}00489n + 0{\cdot}0000275n^2$$

If the titration is outside the stated limits, repeat the determination using more or less than the stated 5 ml. of the acetic acid solution of the oil in order to give a titration of between 20 and 40 ml. of 0·1N sodium thiosulphate, and apply the above equation.

Experimental

Pure ascaridole. Samples were prepared according to the methods to be described[3].

Polarographic method of determination of ascaridole. The details of the method have already been published[2].

Iodimetric method of determination of ascaridole. The B.P. method was used with the following minor modifications which led to an improved reproducibility of results:—(*a*) A w/w solution of ascaridole (or oil of chenopodium) was made in acetic acid (90 per cent.) and weighed amounts of this solution added to the acidified potassium iodide solution.

(*b*) After the rapid addition of the ascaridole solution and immediate thorough mixing, the mixture was set aside for 5 minutes in a cold water-bath at 5° C.

SUMMARY

1. The discrepancies between the percentage values of ascaridole in oils of chenopodium obtained by the B.P. and polarographic methods of analysis have been shown to arise because (*a*) the factor used in the B.P. is incorrect and (*b*) the amount of iodine in the B.P. method is not directly proportional to the weight of ascaridole added to the acidified potassium iodide solution.

2. A suitable quadratic expression is derived for the conversion of the titration figures obtained by the B.P. procedure into correct figures of ascaridole percentages.

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DISCUSSION

DR. A. H. BECKETT, in presenting the paper, explained that it should be considered in conjunction with a complementary paper, the publication of which had been delayed.

THE CHAIRMAN asked whether in view of the dosage range the differences in the results obtained by the different methods were of practical significance, although of course, from the analytical point of view they were very important.

DR. G. E. FOSTER (Dartford) said that it appeared that the B.P. method gave values which varied with the weight of sample taken. However, Dr. Beckett's method was still empirical, as it was based upon a sample of ascaridole which had not been proved 100 per cent. pure. He considered that the B.P. method was satisfactory as a comparative method and pointed out that infra-red absorption analysis results reported from Japan gave comparable results.

DR. A. H. BECKETT, in reply, said that veterinary surgeons desired accurate figures for the ascaridole content of oils of chenopodium and mixtures of oils of chenopodium with castor oil. He considered that the B.P. method gave results which were 20 per cent. too high in a reputed 65 per cent. oil and 14·5 per cent. too high in an 80 per cent. oil. The observations were therefore important from a dosage point of view as well as from the analytical standpoint. The sample of ascaridole they had prepared assayed 110·4 per cent. by the B.P. method. Furthermore, because results varied with the weight of ascaridole used in the test, B.P. values for oils of differing ascaridole content were not comparable. The proposed quadratic function eliminated the effect of variations in the weight of ascaridole used. Infra-red details would be published in the near future. Here, too, the result depended upon the purity of the standard and the same was true of all methods in the absence of any stoichiometric reaction of ascaridole.

THE ASSAY OF TINCTURE OF DIGITALIS AND OF THE GLYCOSIDES OF *DIGITALIS PURPUREA*

By Harry Brindle, Gerald Rigby and Shri Nath Sharma
From the Pharmacy Department, University of Manchester

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Previous work carried out in this department (Brindle and Rigby[1]), has established that the removal of saponin from a saponin containing tincture of digitalis causes no significant change either in its potency to frogs or in the intensity of its reaction with the Baljet alkaline picrate reagent. This has been confirmed by Neuwald and Zöllner[2]. It was also shown that the Knudson and Dresbach colorimetric assay of tinctures prepared from the same sample of leaf, yields results which are reasonably comparable with those obtained by an 18-hour frog bioassay. Finally, previous work indicated that the Knudson and Dresbach assay of two glycosides—gitoxin and digitoxin—gave a false estimate of their potencies.

Comparative chemical and biological assays have been carried out on several tinctures of different origin, using the frog bioassay, and chemical assays based on the Baljet reaction and on the Kedde reaction.[3] No correlation between the results of the chemical and biological assays was obtained. This led to an investigation of the individual primary and secondary glycosides, and the aglycones of *D. purpurea*, using the 18-hour frog assay and a colorimetric assay based on the Kedde reaction. The results of this work are published below, and show the inadvisability of such a chemical assay in standardising preparations of digitalis containing mixtures of these constituents in unknown concentration. The results also indicate that 3 glycosides have relatively little potency to frogs. The chemical evaluation of tinctures of digitalis should therefore be based on a reagent which estimates only the active constituents. The reaction of each constituent with the reagent should be proportional to its potency, otherwise the active constituents must be isolated and their individual concentrations estimated.

Experimental

The Glycosides and Aglycones of D. purpurea

Small quantities of about 25 mg. of several glycosides were very kindly supplied by Professor Arthur Stoll and standard solutions in ethanol were prepared. Similar solutions of commercial digitoxin and gitoxin were also prepared.

Bioassays. After obtaining approximate values for the LD50 of each glycoside, groups of frogs were injected using from 3 to 5 dose values, and 20 frogs for each dose value where the LD50 was low and 10 frogs where it was high, so that sufficient of each glycoside remained for future work. The large LD50 for gitoxin was determined by using a 100 mg./100 ml. solution in polyethylene glycol, diluted appropriately with saline solution immediately before injection, as a solution of gitoxin in the concentration

required could not be obtained in the usual non-toxic solvents. Polyethylene glycol alone was injected into a control group of frogs. From the results of these bioassays regression equations have been calculated for each glycoside and the experimental data are shown in Table I. Desacetyldigilanids A and B (purpurea glycosides A and B) and digitoxin are the most potent of these 6 compounds. Gitoxin, gitoxigenin and digitoxigenin can be regarded as having a very low potency to frogs. No sample of gitalin was available, but in view of the report by Merz[4] that its potency is slightly less than that of gitoxin, it may also be regarded as having little potency.

TABLE I

DATA COMPUTED FROM THE RESULTS OF THE BIOASSAYS OF SEVERAL OF THE CONSTITUENTS OF *D. purpurea*

Glycoside	Number of frogs used	Number of dose values employed	Regression Equation	Regression coefficient (b) ± σ (b)	Approx. LD50 mg./kg.	Fiducial limits (P = 0·05)	X^2
'A" Series							
Purpurea Glycoside A. ..	100	5	Y = 5·15 + 3·15 (X − 0·386)	3·15 ± 0·93	2·2	1·8 – 2·7	0·88
Digitoxin (Stoll)	80	4	Y = 4·88 + 7·46 (X − 0·556)	7·46 ± 1·51	3·7	3·4 – 4·1	2·34
Digitoxin (commercial) ..	80	4	Y = 4·83 + 4·8 (X − 0·585)	4·8 ± 1·45	4·2	3·6 – 4·8	0·72
Digitoxigenin	40	4	Y = 5·00 + 4·97 (X − 1·241)	4·97 ± 2·23	17·4	14·4–21·0	1·30
'B" Series							
Purpurea Glycoside B. ..	80	4	Y = 4·69 + 4·74 (X − 0·691)	4·74 ± 1·28	5·7	4·8 – 6·75	1·10
Gitoxin (Stoll)	30	3	Y = 5·25 + 4·82 X − 1·265	4·82 ± 2·7	16·3	12·6 – 21·2	0·152
Gitoxin (commercial) ..	40	4	Y = 5·11 + 5·48 (X − 1·19)	5·48 ± 1·97	13·1	10·7–16·0	0·69
Gitoxigenin	30	5	No deaths were observed with doses up to 40 mg./kg.		40·0		
Polyethylene Glycol 20 ml./kg.	40	No deaths were observed					
Polyethylene Glycol 10 ml./kg. and Saline 10 ml./kg. ..	40	No deaths were observed					

Chemical Assays. Different volumes of each glycoside solution were added to sufficient distilled water or ethanol (30 per cent.) to produce 7·0 ml. To each 7·0 ml. were added 2·0 ml. of a 2·0 per cent. w/v solution of 3:5-dinitrobenzoic acid in ethanol (90 per cent.), and 1·0 ml. of N sodium hydroxide solution. The density of the mauve colour developed, was determined by comparison with a control containing no glycoside, at one-minute intervals. The maximum intensity was usually reached in 9 to 12 minutes. A photoelectric absorptiometer was used to make these determinations. Distilled water was used when the volume of the glycoside solution was 5·0 ml. or more since sodium 3:5-dinitrobenzoate is insoluble in concentrated ethanolic solutions. From the density readings obtained, those for 0·5 mg. of each glycoside in 10 ml. of the final coloured solution have been selected and are recorded in column 3 of Table II.

In column 4 of Table II the potency of each glycoside to frogs is expressed as the number of LD50 doses per g. of glycoside using the data of Table I. Comparison of the figures in columns 3 and 4 shows that the relative potency of each glycoside is not proportional to its Kedde colour density. The Kedde reagent is reported to react with the unsaturated lactone grouping in the aglycone portion of the molecule. Since each molecule

TABLE II

COMPARISON OF THE POTENCY TO FROGS OF THE DIGITALIS GLYCOSIDES, WITH THEIR DENSITY READINGS WHEN ASSAYED BY TWO COLORIMETRIC METHODS

1 Glycoside	2 Molecular weight	3 Density readings for 0·5 mg. of glycoside using the Kedde reagent	4 Number of frog LD50 doses/kg. in 1 g. of glycoside	5 "Density" of glycoside using the Kedde reagent calculated on a molar basis (2xM.W. × reading in column 3)	6 Density reading for 0·3 mg. of glycoside using hydrochloric acid
"A" Series					
Purpurea Glycoside A ..	926	0·270	455	500	0·216
Digitoxin (Stoll) ..	764	0·330	270	504	0·300
Digitoxin (commercial) ..	764	0·311	238	476	0·365
Digitoxigenin	374	0·635	57	475	Nil
"B" Series					
Purpurea Glycoside B ..	942	0·186	175	350	0·245
Gitoxin (Stoll)	780	0·266	61	415	0·307
Gitoxin (commercial) ..	780	0·240	77	374	0·360
Gitoxigenin	390	0·389	<25	303	Nil

contains only one lactone grouping, one would expect that the product of the molecular weight and the colour density per mg. for each glycoside would be constant. Reference to column 5 of Table II, shows that this is approximately so in the case of the "A" series of glycosides, but not in the case of the "B" series, which are unusual in other ways—for example in their reaction with sulphuric acid and the Keller-Kiliani reagent, in their relatively low potency and in the insolubility of the second member, gitoxin, in ethanol.

It is possible that this anomalous behaviour is due to the hydroxyl group in the C_{16} position in the molecule of gitoxigenin and its glycosides, so that in the presence of ethanol and alkali, there is the possibility of isomerisation with the formation of an isogenin containing a 5-membered ring. This ring would be more stable than the 6-membered rings formed by isomerisation involving the hydroxyl group in the C_{14} position, which is present in both digitoxigenin and gitoxigenin and in their glycosides. This theory was put forward by Bell and Krantz[5] in their investigations with the Baljet reagent.

The Chromatographic Analysis of Some of the Constituents of D. purpurea

Ascending and descending paper partition chromatographic techniques have been employed using a variety of solvent mixtures. The spots were detected by spraying the developed chromatograms with a solution of

trichloracetic acid in chloroform or with a solution of antimony trichloride in chloroform, and heating, as described by Heftmann and Levant[6] and by Jaminet.[7]. The R_F values of the glycosides tested are shown in Table III.

The chloroform, methanol and water solvent mixtures used by Svendsen and Jensen[8] were preferred and using these systems, the effect of temperature on the R_F values was investigated. Variation in temperature during development of the chromatograms produced no significant change in the R_F values.

The method was applied to the analysis of tinctures of digitalis. 6 samples of tincture of digitalis were decolourised by standard methods, and volumes of the decolourised products equivalent to 1 ml. of tincture were applied to filter paper sheets and chromatograms were developed using the chloroform, methanol, water system A, of Table III.

TABLE III

THE R_F VALUES OF SEVERAL OF THE CONSTITUENTS OF *D. purpurea*

	R_F values for digitalis glycosides using the following phase systems							
Glycoside	Chloroform 10 Methanol 2 Water 5 A	Chloroform 10 Methanol 4 Water 5	Chloroform 10 Methanol 8 Water 5	Ethyl acetate 2 Pyridine 1 Water 2	Ethyl acetate 1 Water 1	Ether 2 Methanol 1 Water 1	Butanol 1 Water 1	Methyl ethyl Ketone 1 Water 1
Purpurea Glycoside A	0·08	0·11	0·13	0·80	0·06	0·12	0·70	0·86
Digitoxin (Stoll)	0·91	—	—	0·90	—	—	0·88	—
Digitoxin (commercial)	0·89	0·92	0·91	0·92	0·70	0·88	0·86	0·81
Digitoxigenin	0·92	0·93	0·91	0·90	0·71	0·89	0·86	0·80
Purpurea Glycoside B	0·02	0·04	0·06	0·73	—	0·18	0·62	0·80
Gitoxin (Stoll)	0·78	0·82	0·82	0·90	0·67	0·85	0·81	0·80
Gitoxin (commercial)	0·83	—	—	0·86	—	—	0·84	—
Gitoxigenin	0·81	0·84	0·85	0·92	0·66	0·86	0·82	0·82
Digitoxose	0·52	0·55	0·55	0·44	0·09	0·54	0·40	0·78

Chromatograms of all 6 tinctures showed 4 bands with R_F values corresponding with those of purpurea glycosides A and B, digitoxin and gitoxin respectively. Two of the chromatograms showed an extra band, the R_F of which corresponded with that of digitoxose. Other constituents, present in concentrations below the sensitivity of the reagents used, may have been undetected by this method. Furthermore, the R_F values of digitoxin and gitoxin are almost indentical with those of digitoxigenin and gitoxigenin respectively so that it was not possible to determine whether or not these two aglycones were also present. This last point, considered along with the very low potency of these aglycones towards frogs, led to the development of the hydrochloric acid assay, described below, which was found to estimate primary and secondary glycosides but not aglycones.

Colorimetric Assays of Glycosides in the Presence of Aglycones

Such assays are based on reactions involving the pentose portion of the molecule (digitoxose). We have investigated the action of phosphoric

acid, Bial's reagent, phloroglucinol in hydrochloric acid, and 33 per cent. hydrochloric acid. Bellet[9] has shown that phosphoric acid produces a yellow colour with digitoxin and a greenish-yellow colour with gitoxin; using 66 per cent. acid, we have verified this and have employed also 90 per cent. phosphoric acid which produces a greyish-brown colour and a strong mauve colour respectively with these glycosides. All the colours deepened or altered on heating and the results were not reproducible quantitatively. Militzer[10], Fernell and King[11] and others have described the use of Bials' reagent for the quantitative estimation of pentoses. These methods and numerous modifications of Bial's method have been applied by us to the glycosides of digitalis, but the results obtained could not be reproduced very accurately. Phloroglucinol in hydrochloric acid reacted with the glycosides, but again the results obtained were not constant or reproducible. The estimation of the density of the yellow colour which develops when the glycosides are heated with concentrated (33 per cent.) hydrochloric acid has proved the most satisfactory method of assaying the glycosides in the presence of their aglycones. The colour produced is stable and the readings are reproducible.

Varying volumes of the standard alcoholic glycoside solutions were placed in Folin-Wu tubes which were immersed in a boiling water bath until each glycoside solution had been evaporated to dryness. 5 ml. of 33 per cent. hydrochloric acid was added and the tubes immersed in the bath for 3 minutes, cooled and the volume in each adjusted to 10 ml. with 33 per cent. hydrochloric acid. The results obtained for 0·3 mg. of each glycoside are shown in column 6 of Table II, and it is apparent that the relative colour density of each glycoside is not proportional to its potency to frogs. The method would appear to have promise in investigating the activity of tinctures of digitalis if used in conjunction with a method of separating the glycosides quantitatively.

Summary

1. No correlation has been found between the potency to frogs of the glycosides of *D. purpurea*, and the intensity of their reaction with the Kedde 3:5-dinitrobenzoic acid reagent or with hydrochloric acid.

2. Purpurea glycosides A and B, and digitoxin are regarded as being mainly responsible for the potency of tinctures of digitalis.

3. Equimolar concentrations of the glycosides of the "A" series—i.e., purpurea glycoside A, digitoxin, and digitoxigenin have been shown to produce approximately equal colour densities with the Kedde reagent—but the same consideration does not apply in the case of the "B" glycosides.

4. The R_F values of several of the constituents of *D. purpurea* have been determined by paper partition chromatography.

5. The constituents of 6 tinctures of digitalis have been investigated.

6. 33 per cent. hydrochloric acid is shown to be a useful reagent for the estimation of primary and secondary glycosides as it does not react with the aglycones.

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DISCUSSION

The paper was presented by MR. G. RIGBY.

DR. J. M. ROWSON said that the colorimetric assay could not be expected to give a true picture of the glycosidal activity of every tincture of digitalis. Nevertheless, in view of the difficulty and cost of biological estimation, many would entertain some hopes for the chemical assay. It was a pity that Mr. Rigby had not given more details about the tinctures which he mentioned. It was important that there should be some idea of the range of the primary glycosides, secondary glycosides and their degradation products in digitalis preparations. It was only when there was that true pattern in mind that one was in a position really to tell whether the colorimetric method would give the same sort of results as the biological. The author had not presented any comparative figures for colorimetric and biological estimations of any digitalis tinctures. The authors were tying themselves too much to tinctures; they rapidly deteriorated on storage, and in his view it would be preferable if the dried leaf stored in an atmosphere of low moisture content were used, and the tinctures prepared rapidly and examined immediately. Using both the chemical and biological methods of estimation it had been proved conclusively that a sample of digitalis leaf in moderately fine powder was completely extracted by 70 per cent. alcohol after maceration and agitation over a period of one hour. This procedure, by reducing deterioration of tinctures to a minimum, was very useful. He gave results of work as yet unpublished, on the rate of degradation of tincture of digitalis on the shelf and in the refrigerator, taking samples over a period from a few days up to 6 months. The samples were assayed by guinea-pig and chemical methods. By the former method, no change was found in the refrigerated sample of the tincture, but there was a steady decline in the shelf sample. The chemical method showed each tincture to have approximately the same activity with only slight degradation.

DR. S. ROHATGI (India) pointed out that after the publication of their original paper in 1950, Svendsen and Jensen found that the method described therein did not give satisfactory results. He wondered whether the authors obtained consistent results with the use of that method, particularly with the use of trichloroacetic acid.

MR. C. J. EASTLAND (London) drew attention to the authors' statement that gitoxin and digitoxin had very low potency, and to their subsequent

similar remark about gitalin. He wondered whether the low potency recorded was a function of the test animal, because, using guinea-pigs for the assay, he had found that gitoxin possessed something like 50 per cent. of the potency of digitoxin. Of course, there was always the difficulty inherent in the low solubility of gitoxin and the fact also that it was less cumulative than digitoxin. Recent evidence had suggested that gitalin had considerable potency although, again, it was less cumulative than digitalin.

MR. G. RIGBY, in reply, said he recognised Dr. Rowson's preference for dealing with dried leaf and the rapid preparation of tinctures. He agreed with his comments on their rate of deterioration. The one-hour maceration period for the leaf was an interesting suggestion. Although Svendsen and Jensen modified their method in 1952, since then the authors and Silberman in Australia had confirmed Svendsen's original results, so it was a question of choice. The authors' aim had been to compare various chromatographic solvent mixtures to see which was preferable for the work. So far the chloroform-ethanol-water system had been perfectly satisfactory, although it was necessary to modify the method for the separation of certain glycosides. With regard to the use of trichloroacetic acid, it was well known that the reagent did not react very well with aglycones. There was no reaction with digitoxigenin or gitoxigenin and for them antimony trichloride was preferable. There were many reports in the literature that gitoxin and gitalin possessed higher potency than that reported by the authors, but they were usually in respect to the guinea-pig; the frog potency of the glycosides was very low.

THE SEPARATION OF ALKALOIDS BY PAPER PARTITION CHROMATOGRAPHY

PART III. THE ASSAY OF ERGOT

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THE separation of certain water-insoluble alkaloids by partition paper chromatography has been described in a previous paper in this series[1], and this present communication describes an extension of the method to determine quantitatively both the water-insoluble and water-soluble alkaloids of ergot in the crude drug.

Up to the present time there have been no assay methods available for the determination of the individual alkaloids of ergot, with the exception of ergometrine which is referred to later. The physiologically active and inactive alkaloids and also some deterioration products such as the lysergic acids give the same colour with *p*-dimethylaminobenzaldehyde reagent used for the colorimetric assay in the British Pharmacopœia, 1948. This may be satisfactory for the estimation of total water-soluble and water-insoluble alkaloids, but does not give a true indication of the physiological activity. The object of this present paper was to show how the active and inactive alkaloids may be separated from each other by partitition chromatography and then assayed individually.

This is, of course, the ideal case and although some difficulties still remain, the author claims that the method in this paper is a considerable advance on previous assay methods. Ergots from different sources vary greatly in their alkaloidal content—both qualitatively and quantitatively; for instance Spanish and Russian ergots contain the ergotoxine group of alkaloids together with ergometrine, whilst Central European ergots are often rich in ergotamine[2]. The author's method of assay determines these individual alkaloids and also ergometrine and should be of importance to the manufacturer who wishes to purchase ergot for the preparation of ergotamine or ergometrine.

Chemical assay methods which have been devised for the physiologically active alkaloids in ergot have been limited to ergometrine, which is perhaps the most important ergot alkaloid. Grove[3] and Powell *et al*[4] devised methods by which the ergometrine was extracted and determined directly by colorimetric assay. Further collaborative work in the United States extending over several years has been published in a report on the assay of ergot issued by the American National Formulary Committee[5]. It is interesting to note that the specific assay for ergometrine devised by the American workers was finally not adopted for ergot itself owing to its complexity, and thus the final assay of the crude drug determines ergometrine together with ergometrinine. Foster *et al*[6], separated ergometrine in ergot-by paper chromatography and obtained semi-quantitative results, the error being approximately 20 per cent. Ergotamine has been separated

from its inactive isomer ergotaminine by counter current extraction[7], but this has not yet been applied to the crude drug. Circular[8,9] and "reversed phase"[10], paper chromatographic methods for the qualitative separation of ergot alkaloids have been described.

For the quantitative determinations of individual alkaloids on filter paper chromatograms the alkaloidal spots have been extracted and assayed colorimetrically[1]. When the number of alkaloids in a mixture is small this may be satisfactory, but for more complex mixtures the methods becomes extremely tedious. Ether when used as a developing solvent for paper chromatograms involves difficulties such as maintaining saturated ether vapour in the chromatographic chamber, and incomplete saturation may lead to badly tailing spots and variable R_F values.

The extension of filter paper chromatography to columns was briefly discussed in a previous paper[1]. The column technique is more suitable for use with volatile solvents than is the filter paper strip method where saturation of the chromatographic chamber with vapour is difficult. In the following work buffered filter paper colums using Whatman ashless cellulose have been used throughout and with care give easily reproducible results.

Partition Chromatography of Pure Alkaloids—Qualitative

Details have been given for the separation of ergocristine and ergocristinine on buffered cellulose using Solka Floc cellulose[1]. Solka Floc is a finely-powdered cellulose and rate of solvent flow through it tends to be slow. In subsequent work Whatman ashless cellulose powder which became available was found to be more suitable.

For the preparation of columns 8 g. of Whatman ashless cellulose (standard grade) was mixed with 2 ml. of citrate-phosphate buffer (McIlvaine) *p*H 3·0 and packed dry into a glass tube of 1 cm. bore, 30 cm. long, into the lower end of which was fused a sintered glass filter (No. 3). The alkaloid in chloroform or ethanol solution was added to the column by first absorbing the solution on 0·3 g. of cellulose powder, evaporating off the solvent, and then mixing this powder with an equal weight of cellulose containing 40 per cent. water; the cellulose alkaloid mixture thus contained the same proportion of water as did the rest of the column. This powder was added to the top of the column, pressed down and covered with a layer of washed sand. The column was developed with anæsthetic ether saturated with water, and the alkaloids moved down the column in the following order—ergocristinine, ergocristine, ergosine and ergotamine; the water-soluble alkaloids ergometrinine and ergometrine remained stationary at the top of the column. From preliminary experiments it became apparent that by using a cellulose column buffered at *p*H 3·0 and ether as the developing solvent the slowly moving zones of ergosine and ergotamine would only be eluted by relatively large volumes of solvent. Raising the *p*H of the column resulted in more rapid movement of these zones, but in this case there was incomplete separation of the quickly moving ergocristinine and ergocristine. The difficulty was overcome by the addition of 0·1 per cent. of pyridine to the developing solvent, so that

as development of the chromatogram proceeded, the *p*H of the buffer on the column gradually increased from *p*H 3 to a value of *p*H 4·8 after the passage of 100 ml. of solvent. Under these conditions the slowly moving alkaloids were eluted more rapidly and the zones were considerably compacted. This may be considered as being analagous to gradient elution methods[11] since a *p*H gradient exists down the column. The eluate was collected in 2·5-ml. fractions by means of an automatic fraction collector, and each fraction was assayed colorimetrically using dimethylaminobenzaldehyde reagent. The first fraction collected always contained a considerable amount of cellulose extractive which gave a turbid solution on adding water and colour reagent. A slight amount of extractive also appeared in fractions 2 and 3, and these three solutions were filtered through No. 3 sintered glass filters for clarification.

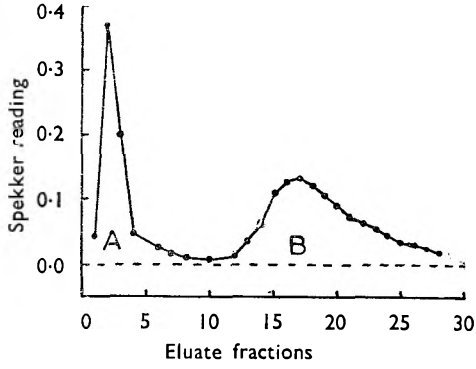

FIG. 1. Elution curve of ergocristinine (A) and ergocristine (B). Buffer *p*H 3·0. Anæsthetic ether saturated with water used as developing solvent.

The elution curves of ergocristinine and ergocristine when anæsthetic ether (saturated with water) was used are shown in Figure 1, and should be compared with Figure 2 where 0·1 per cent. of pyridine has been added to the developing solvent. The ergocristine was considerably compacted but tended to overlap the ergocristinine which appears in fraction 1 together with the cellulose extractive. To improve the separation the pyridine was not added until fraction 1 was collected. A better separation was thus achieved (as shown in Fig. 3) with the added advantage that the alkaloid did not break through until fraction 2, so that the cellulose extractive did not interfere. Figure 4 (*a*) shows typical elution curves for mixtures of pure alkaloids using this improved method. The amounts of alkaloids required for determination by colorimetric assay are so small that there is no danger of overloading the buffer on the column so that the shape and position of the elution curves are an indication of alkaloid identity. The positions of the alkaloidal peaks are summarised in Table IV. The slight variation in the position of the peaks is probably due to differences in packing the column, although this was standardised as far as possible. During hot weather increased evaporation of the eluate from the outlet of the column will also be another source of variation.

After the passage through the column of 100 ml. of anæsthetic ether (saturated with water) containing 0·1 per cent. of pyridine the *p*H of the column had risen from the original value of *p*H 3 to *p*H 4·8, and the water-insoluble alkaloids were completely eluted. The water-soluble alkaloids remained at the top of the column and were later eluted by making the column alkaline, when a complete separation of ergometrine and ergo-

metrinine was obtained. The column was made alkaline by passing through 10 ml. of developing solvent containing 0·1 ml. of diethylamine, and development was continued with anæsthetic ether saturated with water. Figure 4 (*b*) shows a typical elution curve of the water-soluble alkaloids after the elution of the water-insoluble alkaloids.

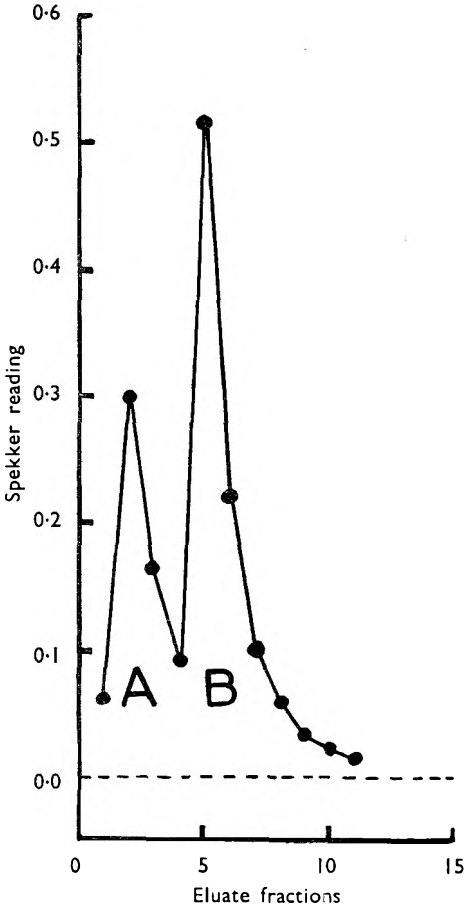

FIG. 2. Elution curve of ergocristinine (A) and ergocristine (B). Buffer *p*H 3·0. 0·1 per cent. pyridine added to developing solvent.

Absorptiometric Assay of the Fractions. Assays were carried out on the individual 2·5 ml. fractions after the solvent had evaporated off at room temperature. In some cases low recovery figures were observed especially when the alkaloidal residues had dried overnight. The fractions were collected in 50-ml. Pyrex beakers and a considerable residue of undissolved alkaloid was apparent on the sides of the beakers, even after the development of the colour reaction. This was finally overcome by the addition of a small volume of lactic acid to each beaker before collecting the fractions, the acid dissolved in the ether fraction and on evaporation of the ether a thin film of lactic acid and alkaloidal lactate was left, which was readily soluble in 1 per cent. tartaric acid solution or water, before the addition of the dimethylaminobenzaldehyde reagent. The lactic acid was most conveniently added to the beakers in the form of an ethereal solution—1 ml. of 5 per cent. solution in anæsthetic ether was added to each beaker. The volume of water or tartaric acid solution used was 1·95 ml., and 4 ml. of dimethylaminobenzaldehyde reagent was added. The developed colour was measured in the usual way in a Spekker absorptiometer using 1 cm. path cell and filters with maximum transmission at 590 mμ.

PARTITION CHROMATOGRAPHY OF PURE ALKALOIDS—QUANTITATIVE

Solutions of pure alkaloids in ethanol (50 to 80 per cent.) were freshly prepared and assayed absorptiometrically after dilution with 1 per cent.

TABLE I

SEPARATION OF ERGOT ALKALOIDS ON BUFFERED CELLULOSE COLUMNS

(Alkaloid calculated as ergotoxine and expressed in μg.)

Ergocristinine		Ergocristine		Ergosine		Ergotamine		Ergometrinine		Ergometrine	
Taken	Found	Taken	Found	Taken	Found	Taken	Found	Taken	Found	Taken	Found
	13	247	229								
	15	247	234								
366	370	296	264								
296	266	234	206	152	138						
										180	180
						88	72			270	235
								95	84	135	141
						262	207				
						262	215				

TABLE II

EXTRACTION OF ALKALOIDS FROM ERGOT BY MICROPERCOLATION WITH VARYING VOLUMES OF CHLOROFORM (5 per cent. ETHANOL)

Weight of ergot g.	Vol. of percolate ml. *	Alkaloid found (calculated as ergotoxine) per cent.	Time of percolation
0·5168	25	0·324	4½ hours
0·5022	25	0·329	1½ hours
0·5780	30	0·320	overnight
0·5030	10	0·338	1½ hours
0·5180	5	0·319	¾ hour
0·5154	5	0·320	1 hour

* Percolate evaporated, if necessary, to 10 ml., 50 ml. of anæsthetic ether and 5 ml. of acetone added and extracted with 4 quantities, each of 10 ml., of 1 per cent. sulphuric acid; mixed acid solutions made up to 50 ml. and assayed colorimetrically.

TABLE III

COMPARISON OF B.P. METHOD AND MICROPERCOLATION METHOD FOR THE TOTAL ALKALOIDS OF ERGOT

Sample of Ergot	Total Alkaloids expressed as Ergotoxine	
	B.P. Assay per cent.	Micropercolation* per cent.
AW	0·320	0·320
B	0·157	0·168
C	0·255	0·231
E	0·442	0·476
F	0·169	0·163
H	0·390	
JW	0·346	0·351

* Ergot percolated with 6 ml. of chloroform (5 per cent. of ethanol).

tartaric acid solution. Suitable volumes of the solutions (usually 0·3 to 1·0 ml.) were added to the column by first absorbing on cellulose as described previously. The dish containing the alkaloid cellulose mixture was carefully cleaned out with washed sand which was added to the column, and was also rinsed out with some of the developing solvent.

Water-insoluble Alkaloids. Column—8 g. of cellulose plus 2 ml. of citrate-phosphate buffer *p*H 3·0; developing solvent—anæsthetic ether saturated with water. 0·1 per cent. of pyridine A.R. was added after fraction 1 collected. Assay of the fractions—as described in previous section.

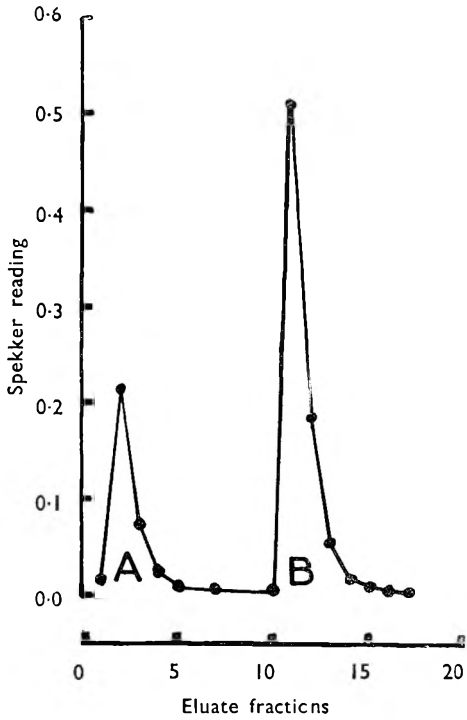

FIG. 3. Elution curve of ergocristinine (A) and ergocristine (B). Buffer *p*H 3·0. 0·1 per cent. pyridine added to developing solvent after fraction 1 collected.

Water-soluble Alkaloids. Developing solvent—0·1 ml. of diethylamine in 10 ml. of water-saturated ether was passed through the column and then development continued with water-saturated ether. 18 ml. of eluate was collected and discarded before the column was connected to the automatic fraction collector. In most cases 10 per cent. of ethanol was used to speed up the elution of ergometrine. (*See* individual graphs.)

The quantitative recoveries are shown in Table II. The average recoveries of the alkaloids with the exception of ergotamine, were about 90 to 95 per cent. The low recovery of ergotamine may be due to its slow elution from the column and the recovery figure is the summation of about eighteen assays. A total volume of 120 ml. of solvent was used to develop the column and 40 fractions collected. The rest of the eluate was collected as a separate fraction and was usually found to contain a small amount of ergotamine, which was included in the recovery figure. Lysergic and *iso*lysergic acids do not interfere with this assay since they remain at the top of the column after the elution of the total alkaloids. Extracted colouring matter from the ergot also remains at the top of the column.

THE ASSAY OF ERGOT

The previous part of this paper gives the method adopted for separations of the pure alkaloids, and in order to apply this to ergot itself it is necessary

to consider the following operations. 1. Extraction of the alkaloids from the drug. 2. Transfer of the alkaloids to the chromatographic column. 3. Partitition chromatography of the alkaloids.

Extraction of Ergot. The B.P. method of continuous extraction with ether was not considered suitable since hydrolysis and interconversion of of the alkaloids may occur in hot alkaline ether. Only semimicro quantities of ergot (0·5 to 1·0 g.) are required for the chromatographic assay and a rapid cold extraction method was devised. The alkaloids were liberated by mixing a solution of sodium bicarbonate with the powdered ergot, which was then ground thoroughly with sand before percolation with chloroform containing 5 per cent. of ethanol. Chloroform has been found to give the highest assay figures for total and water-soluble alkaloids.[5] The details of the extraction method are as follows. About 0·5 g. of defatted ergot was accurately weighed and mixed thoroughly with 0·2 ml. of water and 0·03 g. of sodium bicarbonate in a small mortar. 1 g. of neutral acid-washed sand was added and the mixture ground for a few minutes. The powder was transferred to a micropercolator which consisted of a glass tube 10 cm. long, 1 cm. bore and closed near one end with a No. 2 sintered glass filter (a Pyrex micro filter SF. 8 was found very suitable.) The powder was added to the percolator in 3 approximately equal amounts and pressed firmly down after each addition with a glass rod. The mortar was cleaned out with 2 successive 1 g. amounts of sand to remove any residual ergot and the sand placed in the percolator. The drug was then percolated with chloroform to which 5 per cent. by volume of ethanol had been added, and was found to be exhausted after the collection of 5 to 6 ml. of percolate (*see* Table II). The rate of percolation was approximately 1 drop every 1 to 3 seconds. A comparison of this micro method with the B.P. method of extraction is shown in Table III and a good agreement between the two methods was obtained. The micro percolation method was thus considered a suitable extraction process for the total alkaloids.

TABLE IV

Alkaloid	Position of maximum on elution curve Fraction number
Ergotinine	2 or 3
Ergotoxine	11 - 13
Ergosinine	11 - 13
Ergosine	18 - 21
Ergotaminine	11 - 13
Ergotamine	26 - 29

Transfer of the Alkaloids to the Chromatographic Column. This was most conveniently done by absorbing the chloroform as it dripped from the percolator on warmed cellulose and this was later transferred to the column. The details are as follows.

0·3 g. of cellulose in a small Pyrex evaporating dish was warmed to 50° to 60° C. and the micropercolator containing the ergot was mounted above it, the outlet tip practically touching the cellulose. 6 to 8 ml. of chloroform (5 per cent. ethanol) was added to the percolator and, towards the end of the percolation, slight positive pressure was applied to displace the solvent. To ensure rapid evaporation of the chloroform a gentle current of air was passed over the dish. After the evaporation of the

chloroform an equal weight of cellulose containing 40 per cent. water was added and well mixed with a glass rod. The powder was transferred to the top of the column and the dish cleaned with two successive 1 g. amounts of sand which was transferred to the column. The dish was also rinsed out with some of the developing solvent.

TABLE V

COMPARISON OF THE PARTITION CHROMATOGRAPHIC METHOD AND THE B.P. ASSAY

	Chromatographic Assay								B.P. Assay	
	Water-insoluble Alkaloids				Water-soluble Alkaloids		Total water-insoluble Alkaloids	Total water-soluble Alkaloids		
Sample of Ergot	Ergotinine A	Ergotoxine B	Ergosine C	Ergotamine D	Ergometrinine E	Ergometrine F			Water insoluble	Water soluble
AW	24·8 19	125 125	37·7 45·1	— —	14·7 13	20 20·3	188 189	34·7 33·3	233	46·8
B	9·8 7·7	59·1 68·5	15·1 20·8	11·5 13·5	6·2 5·2	11·1 9·4	96 111	17·3 14·6	131	14·0
C	35·4	93	20	29·8	2·7	16·4	178	19·1	223	18·8
E	23	177	38	53	18·5	38	301	56·5	330	60·2
F	8·7	53·3	7	44·6	4·2 5·2	2·4 3·4	114	6·6	144	13·5
H	26·7	147	62·3	—	13·7	22·8	236	36·5	260	70
JW	23	173	72	—	17·3 15	35·4 31	268	52·7 47	261	45·8

Water-insoluble alkaloids calculated as ergotoxine and expressed in mg./100 g. of defatted drug.
Water-soluble alkaloids calculated as ergometrine and expressed in mg./100 g. of defatted drug.

Partition Chromatography of the Alkaloids. The method used was the same as for the quantitative separation of the pure alkaloids which has been discussed in an earlier part of this paper. The position of the peak of the elution curve for each of the alkaloids was found to be the same as in the case of the pure alkaloids, and provided a means of identification.

TABLE VI

GEOGRAPHICAL SOURCE OF THE ERGOT SAMPLES ASSAYED

Sample	Geographical Source
AW	Spain (Supplied in 1946 by the wholesaler, as defatted ergot)
B	Odenwald, Germany. 1951 crop
C	Austria
E	Alsace. 1951 crop
F	Black Forest, Germany. 1951 crop
H	Portugal. 1950 crop
JW	Spain or Portugal (Supplied in 1952 by the wholesaler, as defatted ergot)

Samples B, C, E, F, H, were kindly supplied by Professor A. Stoll.
All samples were supplied as being *Claviceps purpurea* grown on rye.

Identification by other methods, such as by melting point determinations or by preparation of crystalline derivatives is difficult. Identification of an unknown peak was also checked in most cases by the addition of the pure suspected alkaloid to the ergot extract and noting the position of the

increased peak. Further confirmation of identity was also carried out by paper chromatography on buffered filter paper[1] and on unbuffered paper[6] for the water-insoluble and water-soluble alkaloids respectively.

Fat which was always found in ergots defatted with light petroleum by the B.P. method appeared in fractions 1, 2 and 3. Fraction 1, which should be alkaloid-free, contained most of the fat and was not assayed. The

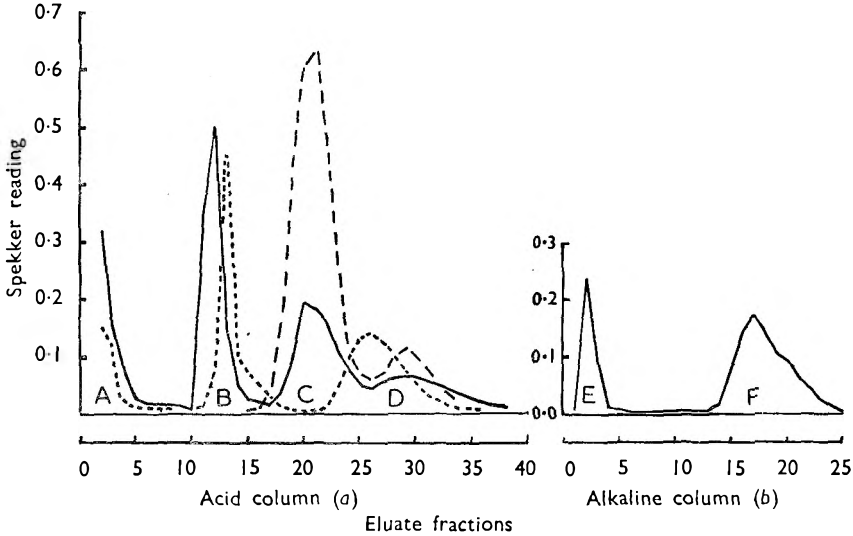

FIG. 4*a*. Elution curves of the water insoluble alkaloids on buffered column *p*H 3·0. 0·1 per cent. pyridine added after fraction 1 collected.

A. Ergocristinine.	B. Ergocristine.	C. Ergosine.	D. Ergotamine.
———— 0·15 mg. A.	0·3 mg. B.	0·25 mg. C.	0·14 mg. D.
- - - - - 0·07 mg. A.	0·25 mg. B.		0·25 mg. D.
— — —		0·8 mg. C.	0·2 mg. D.

FIG. 4*b*. Elution curve of the water soluble alkaloids on buffered column (original *p*H 3·0) made alkaline by the passage of 0·1 ml. diethylamine in 10 ml. of developing solvent.

E. Ergometrinine 0·05 mg. F. Ergometrine 0·14 mg.

Fraction 1 collected when ergometrinine 4 cm. from base of column.

estimation of alkaloid in fractions 2 and 3 was slightly modified in view of the fat content. 3·95 ml. of 1 per cent. tartaric acid solution was added to the alkaloid and fat residue, the mixture warmed to 60° C. for a few minutes, stirred, cooled and then filtered through Whatman No. 1 paper. 2 ml. of the clear filtrate was then assayed. (On the accompanying graphs, to keep the scale of the elution curves unchanged the Spekker readings for fractions 2 and 3 have been converted to values which would have been found if the normal value of 1·95 ml. of tartaric acid solution had been added.)

The geographical source of the ergots examined is shown in Table VI and the elution alkaloid curves for three samples are shown in the Figures 5 to 7. The alkaloidal content is summarised in Table V and these assay figures have been obtained by the summation of the individual colour

assays. Where incomplete separation of the two zones has occurred an arbitrary division into two zones has been made at the point of inflexion of the curve.

It is not claimed that all the active alkaloids are separated completely from the inactive ones by using this chromatographic method since the ergotoxine and ergosine fractions may contain some ergosinine and

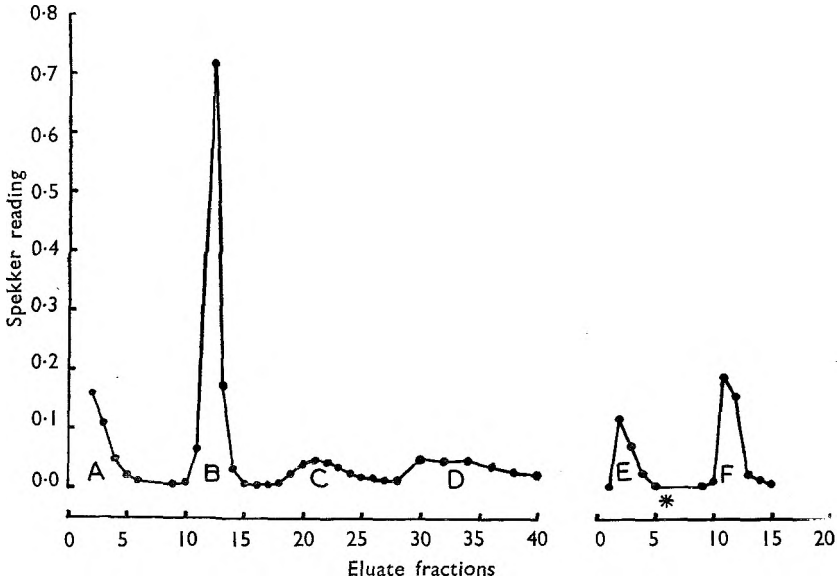

FIG. 5. Elution curves of the alkaloids of ergot. Sample B (German).
* 10 per cent. of ethanol added.

ergotaminine and it will be necessary to chromatograph these fractions again using a different solvent system in order to obtain more information about their relative proportion. The separation of ergometrinine from ergometrine is much more distinct.

The British Pharmacopœia assay has been criticised by Foster *et al.*[6] since it gave high figures for water-soluble alkaloids when compared with the U.S. National Formulary assay and a paper chromatographic assay method described by them. The high figure will be partly due to ergometrinine being estimated as ergometrine, but in addition it was suggested that hydrolysis of the alkaloids to the lysergic acids during the B.P. extraction resulted in these being estimated as water-soluble alkaloids. The chromatographic assay of the present author excludes the lysergic acids since these are retained on the column. A comparison of the chromatographic and B.P. assays of 7 samples of ergot is shown in Table V. The B.P. assay figures for total water-soluble alkaloids in ergots AW, F and H were considerably higher than those found by the chromatographic method, but there was reasonable agreement between the assays of the remaining samples. The inactive ergometrinine constituted about one-third of the total water-insoluble alkaloids. In all the B.P. assays the

extraction flask was heated on a water bath, the ether level being well above the heating surface, and the flask was covered with cotton wool to exclude light. If the extraction flask is deeply immersed in a hot water bath then any alkaloid deposited on the sides of the flask due to loss of solvent, may be considerably overheated. Such overheating was found to

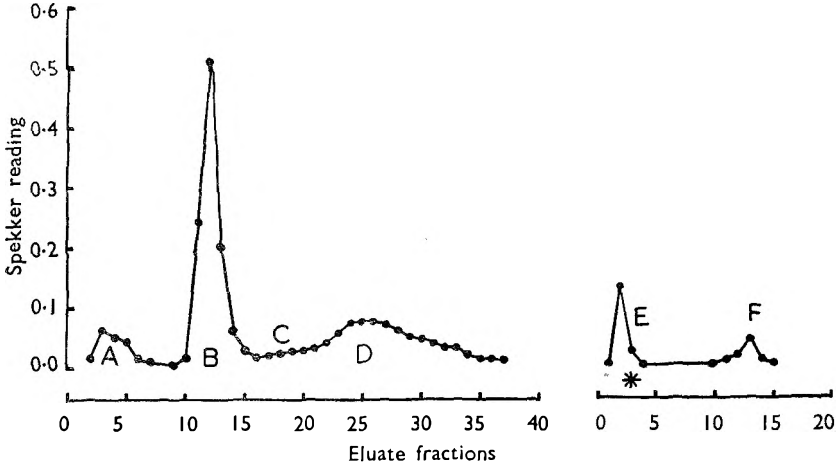

FIG. 6. Elution curves of the alkaloids of ergot. Sample F (German).
* 10 per cent. of ethanol added.

give a high figure for water-soluble alkaloid presumably due to decomposition. The adverse effect of exposing alkaline solutions of ergot alkaloids to bright daylight has been emphasised by the N.F. Committee[5]. During the B.P. assays alkaline solution of ergot alkaloids were exposed only to diffused daylight and the transfer of the alkaloids to the acid solution was

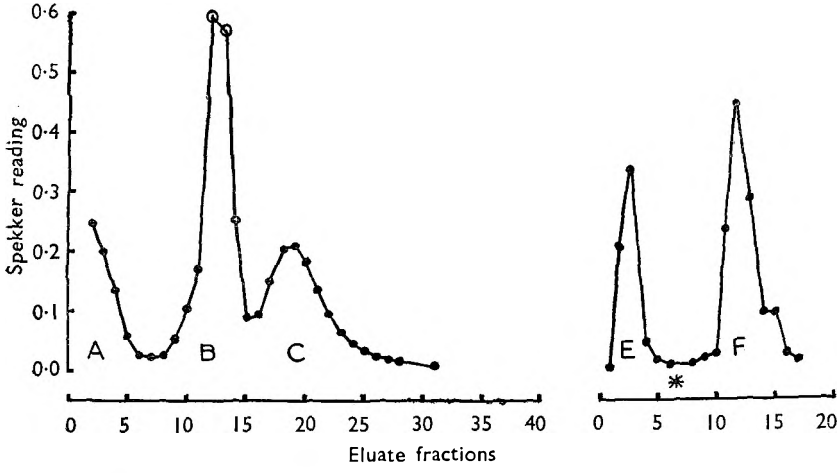

FIG. 7. Elution curves of the alkaloids of ergot. Sample JW (Spanish).
⊙ 3·95 ml. of water and 8 ml. of colour reagent.
* 10 per cent. of ethanol added.

carried out as quickly as possible. It appears advisable that the B.P. should include the above-mentioned precautions in the directions for the official assay to ensure uniformity.

During the examination of an alkaline hydrolysate of ergotamine in methanol, the column partition chromatogram revealed a zone at the same position that ergometrinine occupies. This could not be due to the lysergic acids since these remain at the top of the column. The presence of ergine (the amide of lysergic acid) was suspected since this has been prepared by alkaline hydrolysis of ergotamine and ergotoxine in methanol solution[12]. This was later confirmed by chromatographing a sample of pure ergine. Paper chromatography of the ergometrine and ergometrinine eluate fractions from the ergot samples using Whatman No. 1 paper with butanol-acetic acid-water[6], gave only the characteristic spots of ergometrine and ergometrinine. Ergine which moves more slowly than the water-soluble alkaloids in this solvent system was not detected. Thus there was no evidence of ergine being present in the original ergots, nor of its being produced during the chromatographic process.

Discussion

The separation of the water-soluble alkaloids from the water-insoluble alkaloids before colorimetric assay of these two groups, has been the basis of most of the published assay methods of ergot. The determination of the individual alkaloids within each group is complicated by their closely related chemical and physical properties. Partition chromatography offers a partial solution to these difficulties, and alkaloids whose pK_b and/or solubility values differ slightly from one another may be completely separated on buffered columns. Thus the *l*-and *d*-pairs of the ergot alkaloids may be separated from each other, the physiologically inactive *d*-form moving faster than the *l*-form. The problem is not so easy when several alkaloids are present together since the *l*-form of one alkaloid may overlap the *d*-form of another alkaloid; for instance, ergotoxine and ergotaminine are not separated in the system used. In such cases it is necessary to run additional chromatograms on paper to check the purity of the fraction.

Alkaline solutions of ergot alkaloids, especially if exposed to light undergo rapid decomposition[5]. Acid aqueous solutions are much more stable. The conditions under which the alkaloids are separated in this chromatographic method are unlikely to result in their decomposition, since the water-insoluble alkaloids are separated under acid conditions (*p*H 3 to 5) and the water-soluble alkaloids are only in alkaline solution for a relatively short time, during their passage down the column, the fractions being acidified on collection, with lactic acid. The chromatographic method also has the added advantage that the bulk of the alkaloids are protected from light, so that beyond covering the chromatographic apparatus to exclude bright daylight no other precautions were taken. In the recovery experiments using pure alkaloids 90 to 95 per cent. of the original alkaloid added was recovered, with the exception of ergotamine of which about 80 per cent. was recovered. This low figure may be

partially due to the wide spread of the elution curve and might be improved by bulking together the fractions. This has not been adopted so far since the shape of the elution curve has been used to predict the identity of the alkaloid. Recoveries of the water-soluble alkaloids appear to be a little higher than those of the water-insoluble alkaloics and this may be due to the increased purity of the cellulose after the passage of a large volume of solvent. It should not prove difficult to simplify this method for routine assay work if the determination of the individual water-insoluble alkaloids is not required. The first 5 to 10 ml. of eluate containing the inactive ergotinine could be discarded, and the next 120 to 150 ml. containing the water-insoluble alkaloids collected, the alkaloid being shaken out into acid solution and assayed. The water-soluble alkaloids could then be collected in fractions, either by means of a fraction collector or guided by inspection of the column under ultra-violet light to show up the position of the fluorescent alkaloid.

This chromatographic method offers many advantages over the present methods of evaluating ergot. Only a small sample is necessary and the assay can be completed within 2 days. The practical uses are not confined to the manufacturer who wishes to determine the proportion of individual alkaloids, but the method should be of value in assessing the physiological activity of the drug itself.

Summary

1. A method for separating the water-insoluble alkaloids ergotinine, ergotoxine, ergosine, ergotamine and the water-soluble alkaloids ergometrine and ergometrinine by partition chromatography on buffered cellulose columns has been described.

2. The recoveries of pure individual alkaloids were consistent, being about 90 per cent., with the exception of ergotamine of which about 80 per cent. was recovered.

3. The method has been applied successfully to defatted ergot, of which only 0·5 g. was required for the assay.

4. 7 samples of ergot were assayed by the partition chromatographic method and the British Pharmacopœia, 1948, assay method and a comparison of the two methods was made.

5. 3 samples of Spanish Portugese ergots were found to contain ergotinine, ergotoxine, ergosine, ergometrine and ergometrinine. Ergotamine was not detected. 4 samples of Central European ergots differed from the Spanish ergots since they contained ergotamine and generally smaller amounts of ergosine.

In conclusion I wish to express my thanks, to Professor A. Stoll for supplying samples of commercial ergots, ergotamine tartrate, ergosine, ergosinine, and ergine, to Dr. G. E. Foster for supplying samples of ergotoxine, ergosine and ergosinine and to Professor H. Brindle, under whose direction this work has been carried out.

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DISCUSSION

The paper was presented by MR. J. E. CARLESS.

DR. G. E. FOSTER (Dartford) said that to carry out a large number of colorimetric estimations would be rather time-consuming, and he asked whether, if it became a general routine method, some electronic device could not be constructed for carrying out the assay. There was no doubt that ergotoxine and ergotamine could co-exist in the same ergot. With regard to nomenclature, he pointed out that the authors referred to ergotinine and ergocristine, and it would be better to use the same name for the alkaloid. In the case of the water-soluble alkaloids, he noticed that the authors raised the *p*H in order to elute them from the cellulose, and he wondered whether they had considered the possibility of ergometrine being converted into ergometrinine.

MR. H. B. HEATH (Sudbury) said it was interesting to note that the N.F.IX used filter 590 mμ for ergot, whereas the U.S.P. XIV filtered at 540 to 560 mμ. He desired to know whether Beer's Law was more accurately followed at 550 than at 590 mμ. He had found no difference in peaks between ergometrine or ergotoxine in tartaric acid solution as obtained in an assay of ordinary ergot.

DR. W. MITCHELL (London) asked whether the authors had any information as to the relative stability of the alkaloids in different ergots. He had some reason to believe that the stability of alkaloids in liquid extract of ergot B.P. varied with different batches of ergot.

PROFESSOR H. BRINDLE (Manchester) said that he and his co-workers were comparatively satisfied with the assay of ergot after some 5 or 6 years intensive work. The apparatus used for the assay included a mechanical device which moved when a certain amount of eluate had been collected, and prepared for the next portion of eluate. It was not time-consuming at all.

MR. J. E. CARLESS, in reply, referring to the time taken in assays said that the initial extraction of the defatted ergot, could be completed in about 1 hour, the transfer of the alkaloids to the column was easily carried out, and then the use of a siphon escapement mechanism made the fractionation automatic. He had considered the use of radio frequency methods for detecting the presence of alkaloids in the eluate as it came from the column. He admitted that the terminology he had used was not as clear as it might be. Where he referred to the ergotoxine group of alkaloids this included ergocornine, ergocristine and ergokryptine. Where ergocristine was mentioned it was a pure sample of ergocristine

which had been used and, similarly, with ergocristinine. There was no separation of the individual members of the ergotoxine group or the ergotinine group. The conversion of active ergometrine into inactive ergometrinine was a possibility, especially if the alkaline solution of ergometrine was stored for any length of time, but there was no evidence of that having occurred on the column. He had followed the N.F. recommendation of using filters of 590 $m\mu$ which gave a straight line calibration curve up to about 0·006 or 0·008. After that it tended to diverge. He had little information concerning the stability of the alkaloids in different ergots but from earlier work he had concluded that liquid preparations of ergot were most stable at *p*H 3. While Spanish and Portugese ergots showed little difference in alkaloidal content, Central European ergots varied considerably from batch to batch.

SOME OBSERVATIONS ON THE B.P. AND U.S.P. TESTS FOR PYROGENS

By J. G. Dare

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The B.P. and U.S.P. tests for pyrogens depend on the temperature response of rabbits to intravenous administration of the test material. The methods of interpreting the data differ in the two tests but, in each, the material fails to pass the test if it produces a temperature rise greater than that prescribed. The maximum permissible response, in both the B.P. and the U.S.P., is a specified temperature rise, which is not related to any comparison with a standard preparation. Therefore, any factor which affects the magnitude of the temperature response will affect the sensitivity of the tests.

The writer has already shown that the degree of restraint imposed on the rabbits, the type of thermometer used, and the frequency of administration of pyrogen, all affect the magnitude of the rabbit's temperature response to bacterial pyrogen[1]. Thus when the degree of restraint imposed on the rabbits is severe and clinical thermometers are used, the magnitude of the response to pyrogens is much less than when the degree of restraint is slight and electrical thermometers are used. Furthermore, there is a progressive reduction in response when pyrogen is given repeatedly. The B.P. and U.S.P. specify neither the degree of restraint nor the type of thermometer to be used. Both pharmacopœias permit the repeated use of the same animals.

Two questions of practical importance arise from these considerations. The first and more obvious is; how far may the reliability of the tests be affected by failure to take these sources of variation into account? The second, which derives from the different methods of interpreting the data in the B.P. and U.S.P. tests, is; how do the relative efficiencies of the two tests compare when conducted in similar conditions? This paper seeks to provide an answer to these two questions.

Methods and Results

The data used are drawn from a large series of quantitative studies on a dry powdered bacterial pyrogen preparation from *Proteus vulgaris*. Some of these studies have been described in a previous paper[1], where details of the preparation of Pyrogen Test Preparation No. 1, and the two experimental methods used in investigating it, are given. Method 1 consists of determining the difference between the pre-injection normal and the post-injection maximum temperatures of rigidly restrained fasting rabbits weighing more than 1·5 kg., the temperatures being taken with clinical thermometers. A known dose of Pyrogen Test Preparation No. 1 is given intravenously in 5 ml. of pyrogen-free saline solution. Method 2 differs from Method 1 only in that the degree of restraint is very much

less and that electrical thermometers are used. In many experiments the rabbits were used repeatedly, an interval of at least 3 days being allowed to elapse between experiments. Thus both experimental methods meet the requirements of the B.P. and U.S.P. tests, except for the injection volume. Control experiments, however, established that this difference in injection volume did not affect the response to a standard dose of Pyrogen Test Preparation No. 1.

The mass of data available is so extensive that it is not feasible to present an analysis of all of it in this paper. Bias in selecting the data presented has been avoided, by specifying a series of typical sets of conditions under which data had been obtained, and then collecting, into groups, all data obtained under those conditions. All the groups obtained in this way are presented and analysed here, except where the total number of observations in a group happened to be less than ten. The conditions specified for each group were:—

(i) dose of Pyrogen Test Preparation No. 1;

(ii) the number of occasions on which pyrogen had been given previously, at the same dose level, at 3 or 4 day intervals;

(iii) the experimental method.

18 groups of data from experiments in which pyrogen was given and 2 groups in which pyrogen-free saline solution was given have been collected in this manner. Details for each group are given in the tables.

The variance of all observations from experiments in which pyrogen was given was analysed, and the "within groups" variance used as the variance for each group. In experiments where no pyrogen was given the data were collected in sets by experimental days, and the "within days" variance was used as the variance for each group. Given the mean and variance of each group of data, and assuming that the individual observations within a group are a random sample from a normally distributed population, the frequency with which the presence of bacterial pyrogen may be expected to be correctly detected by the two tests can be calculated as described below.

(a) *Evaluation of the data on the basis of the B.P. test*

3 rabbits are used in a test, and pyrogens are judged to be absent if the mean temperature rise above normal does not exceed 0·6° C. Now 0·6° C. in standard measure (x) for the distribution of means of groups of 3 observations is:—

$$x = \frac{0{\cdot}6 - \mu}{\sqrt{\frac{\sigma^2}{3}}}$$

when μ = mean of the distribution, and

σ^2 = variance of a single observation.

The percentage frequency with which mean values less than 0·6° C. will occur on such a distribution can be conveniently found from the marginal figures in a table of probits[2] corresponding to the location of the value

$(x + 5)$ in the body of the table. This figure is the percentage of occasions on which tests carried out under the same conditions will be judged to indicate that pyrogens are absent. The percentage of occasions on which pyrogens will be judged to be present is given by subtracting this figure from 100.

The results for the B.P. test given in the tables have been calculated in this way.

(b) *Evaluation of the data on the basis of the U.S.P. test*

3 rabbits are used in a test and a preliminary assessment of the data is made as follows:—

(i) If 0 rabbit exhibit a temperature rise above 0·6° C.—pyrogens judged absent.

(ii) If 1 rabbit exhibits a temperature rise above 0·6° C.—doubtful result, test must be repeated on 5 rabbits.

(iii) If 2 or 3 rabbits exhibit a temperature rise above 0·6° C.—pyrogens judged present.

In order to ascertain the proportions in which the above results occur in any particular set of conditions it is first necessary to determine the proportions of individual observations in which the temperature rise is (*a*) less than 0·6° C., and (*b*) equal to, or greater than, 0·6° C. These values, subsequently referred to as "a" and "b," respectively, are found in the same manner as for the B.P. test, except that the standard deviation of a single observation is used instead of that for the mean of three, and the values for "a" and "b" are expressed as proportions of unity instead of percentages. Thus:—

$$x = \frac{0{\cdot}6 - \mu}{\sigma}$$

then a = value from Probit table for $(x + 5)$, divided by 100, and $b = 1 - a$.

As 3 rabbits are used in a test there are 4 possible combinations of results when the individual increases in temperature are classified as greater or less than 0·6° C. The proportion of test results which will occur in each of the combinations is given by the terms of the expansion of $(a + b)^3$—"a" and "b" having the values previously determined. Hence:

a^3 = the proportion of tests which indicate that pyrogens are absent.

$3a^2b$ = the proportion of tests which have to be repeated.

$3ab^2 + b^3$ = the proportion of tests which indicate that pyrogens are present.

There is, however, a further condition attached to tests in which all three observations are less than 0·6° C., namely, that if the sum of the temperature rises exceeds 1·4° C.—that is if the mean exceeds 0·46° C.—the test must be repeated. Thus the proportion of first tests in which pyrogens will actually be found absent is less than "a^3," and the proportion

of tests which must be repeated is larger than "$3a^2b$" by the amount by which "a^3" must be diminished. To determine what proportion must be transferred it is first necessary to find what proportion of groups of three rabbits exhibit a mean temperature greater than $0{\cdot}46\dot{6}°$ C. when no animal in the group shows an individual rise greater than 0·6° C.

Pearson[3,4] gives tables for the calculation of the mean and standard deviation of a normal distribution when only the moments of its truncated tail are known. These tables can also be used in the reverse manner. Here the standard deviation and the mean of the whole distribution are known, and those for the tail with its stump at 0·6° C. are required. The mean (m) and standard deviation (Σ) of the tail are calculated as shown below. All symbols have the same meaning as is attached to them in Pearson's Tables.

$$\Sigma^2 = \psi_1 \times d^2$$
$$m = 0{\cdot}6 - d$$
$$d = \frac{\sigma}{\psi_2}$$
$$h' = \frac{h}{\sigma} = \frac{0{\cdot}6 - \mu}{\sigma}$$

Where $\psi_1 + \psi_2$ are found from the tables by entering with h',

μ = mean of whole distribution,

σ = standard deviation of whole distribution,

d = distance from stump to mean of tail,

m = mean of tail,

and Σ = standard deviation of tail.

The proportion of tests "a'" in which the mean is less than $0{\cdot}46\dot{6}°$ C. and in which no individual rabbit exhibits an increase in temperature greater than 0·6° C. can now be calculated in a manner similar to that used for the B.P. test:—

$$x' = \frac{0{\cdot}46\dot{6} - m}{\sqrt{\frac{\Sigma^2}{3}}}$$

then a' = value from Probit table of (x' + 5), divided by 100; and b', the proportion of tests in which the mean is greater than $0{\cdot}46\dot{6}° = 1 - a'$. In arriving at "a'" and "b'" it has been assumed that the means of groups of three from the tail are normally distributed; this is not true, but the error introduced is small.

Now the proportion of all tests in which no rabbit exhibits a temperature increase of more than 0·6° is a^3. Hence the proportion of all tests in which no rabbit shows a temperature rise of more than 0·6° C. whilst at the same time the mean rise is greater than $0{\cdot}46\dot{6}°$ C., is

$$a^3 \times b'.$$

This is the proportion of test results which must be transferred, from those

previously interpreted as establishing that pyrogens are absent, to those which have to be repeated. These proportions now become:—

$$\text{pyrogens absent} = a^3 - (a^3 \times b')$$
$$\text{repeat test required} = 3a^2b + (a^3 \times b').$$

The U.S.P. requires that repeat tests shall be carried out on 5 rabbits, and the repeat test is considered as establishing the absence of pyrogens if not more than 1 rabbit exhibits a temperature rise of 0·6° C. or more. The proportion of tests in which 0, 1, 2, etc., rabbits show a rise of 0·6° C. or more, when 5 rabbits are used, is given by the terms of the expansion of $(a + b)^5$—where "a" and "b" have the values previously determined. Reasoning as before, there are 6 possible combinations of results from 5 rabbits, of which the sum of the terms $a^5 + 5a^4b$ gives the proportion of tests in which pyrogens will be found absent. This is the proportion of tests using 5 rabbits, in which 0 or 1 rabbit will exhibit a temperature rise of 0·6° C. or greater. Conversely, $1 - (a^5 + 5a^4b)$ gives the proportion of tests in which 2 or more rabbits will exhibit a temperature rise of 0·6° C. or greater. Now the probability of two independent events occurring simultaneously is the product of their individual probabilities. Hence the proportion of all tests in which pyrogens will be found absent, as the result of a repeat test, will be the product of (i) the proportion of tests which have to be repeated, and (ii) the proportion of tests, using 5 rabbits, in which not more than 1 rabbit exhibits a temperature rise of 0·6° C. or greater, i.e.,

$$[3a^2b - (a^3 \times b')] \times (a^5 + 5a^4b).$$

The probability of either of two events occuring on a particular occasion is the sum of their probabilities. Hence the proportion of all tests under a particular set of conditions in which pyrogens will be found absent, is the sum of the proportions of tests in which pyrogens will be found absent in either (i) the first test, or (ii) the repeat test, i.e.,

$$a^3 - (a^3 \times b') + (3a^2b + (a^3 \times b')) \times (a^5 + 5a^4b)$$

By a similar argument it can be shown that the proportion of all tests in which pyrogens will be found present is:—

$$3ab^2 + b^3 + [3a^2b + (a^3 \times b')] \times [1 - (a^5 + 5a^4b)].$$

Multiplication of the two probabilities by a 100 gives the results in expected percentage frequencies.

The results for the U.S.P. test given in the tables have been calculated in this way.

Discussion

The data given in Table I are from groups of rabbits experimented upon for the first time, and where method 1 was used. The data given in Table II, columns 2, 4, 6, 9 and 12, are also from groups of new rabbits, but where method 2 was used. It is evident from the tables, and from Figure 1, that the frequency with which pyrogen will escape detection by either the B.P. or the U.S.P. test is much greater with method 1 than with method 2. From Figure 1 it can also be seen that when method 2 is

used, pyrogen can be detected with certainty (P = 0·99), when it is present in only about one fifteenth the amount that can be detected with method 1. These results also show that the magnitude of the response, previously shown[1] to depend on the experimental technique for doses of 1 and 2 μg./kg. of body weight is equally affected by differences in technique for

TABLE I

EXPECTED EFFICIENCY OF THE B.P. AND U.S.P. TESTS FOR PYROGENS USING METHOD 1 WITH NEW RABBITS

		Dose of Pyrogen Test Preparation No. 1 in μg./kg. of rabbit's body weight				
		0·3	0·6	1·0	2·0	6·0
Number of observations in each group ..		10	11	12	16	11
Mean response in °C.		0·81	1·06	1·02	1·10	1·26
Within Group Variance = 0·1273						
Percentage of tests in which pyrogen will escape detection	B.P.	15·98	1·28	2·07	0·77	0·07
	U.S.P.	2·25	0·06	0·12	0·04	<0·01
Percentage of tests in which pyrogen will be detected	B.P.	84·02	98·72	97·93	99·23	99·93
	U.S.P.	97·75	99·94	99·88	99·96	>99·99

lower doses. Hence, to achieve uniform sensitivity in the pharmacopœial tests, the degree of restraint which it is permissible to impose upon the animals, and the type of thermometer used to measure the temperature, must be specified. When the degree of restraint is minimal, and electrical thermometers are used, the efficiency of both tests is maximal.

The upper part of Table II gives data from experiments in which groups of rabbits were given pyrogen repeatedly at three or four day intervals: all the data for each dose level were collected from the same rabbits. The

TABLE II

EXPECTED EFFICIENCY OF THE B.P. AND U.S.P. TESTS FOR PYROGENS USING METHOD 2 AND SHOWING THE EFFECT OF REPEATED ADMINISTRATION OF PYROGEN

		Dose of Pyrogen Test Preparation No. 1 in μg./kg. of rabbit's body weight												
		0·02		0·08		0·40			1·00			2·00		
Number of previous administrations of pyrogen		0	2	0	2	0	2	4	0	2	4	0	2	4
Number of observations in each group		18	18	18	18	12	12	12	24	24	24	16	16	16
Mean response in °C.		0·74	0·61	1·15	0·83	1·30	1·07	0·61	1·46	1·15	0·89	1·83	1·35	1·22
Within Group Variance = 0·1273														
Percentage of tests in which pyrogen will escape detection	B.P.	19·91	47·29	0·36	12·21	0·03	1·11	47·29	<0·01	0·38	7·67	<0·01	0·01	0·14
	U.S.P.	4·50	14·15	0·02	1·80	<0·01	0·05	14·15	<0·01	0·01	0·53	<0·01	<0·01	<0·01
Percentage of tests in which pyrogen will be detected	B.P.	80·09	52·71	99·64	87·79	99·97	98·89	52·71	>99·99	99·62	92·33	>99·99	99·99	99·86
	U.S.P.	95·40	85·85	99·98	98·20	>99·99	99·95	85·85	>99·99	99·99	99·47	>99·99	>99·99	>99·99

results are given in the lower part of Table II, and are illustrated graphically in Figure 2. They reveal, very clearly, the effect of repeated administration on the sensitivity of the tests. They show, indeed, that the minimum dose, detectable with certainty (P = 0·99), by either pharmacopœial test, suffers a 27-fold increase by the fifth successive administration of pyrogen. This is the situation which obtains when the same dose of pyrogen is given on successive occasions.

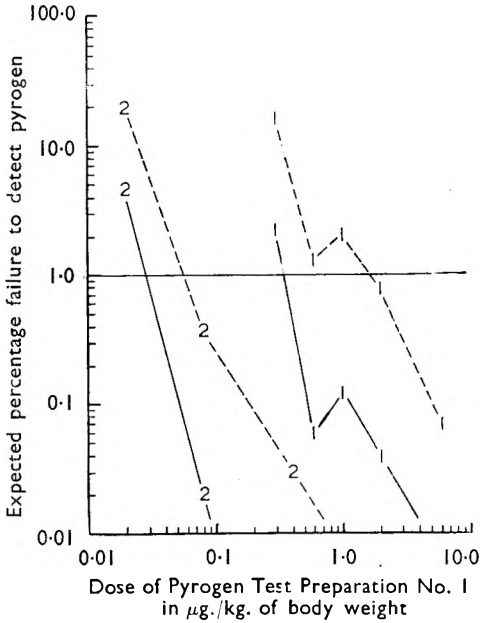

FIG. 1. Showing the percentage frequency of failure to detect pyrogen in the B.P. and the U.S.P. tests, with experimental methods 1 and 2, and with rabbits given pyrogen for the first time. Broken lines, B.P. tests; solid lines, U.S.P. tests. Points indicated by numerals 1 and 2 are from experiments with methods 1 and 2, respectively. The dose corresponding to the point at which each line intersects the ordinate at the 1 per cent. level is the smallest dose that can be detected with certainty (P = 0·99).

The question immediately arises as to what happens when the successive doses are varied. To determine this, a first large dose of 10 μg./kg. of Pyrogen Test Preparation No. 1 was given to each of several groups of 12 rabbits. A second smaller, but effective dose of 0·2 μg./kg. was given to each of the groups of animals, after a different interval of time for each group. Even after the longest interval of 17 days, on giving the smaller dose the temperature increase was barely significant, and both the B.P. and U.S.P. tests consequently failed to detect the pyrogen.

Thus the repeated administration of small doses of pyrogen greatly reduces the sensitivity of the tests, whilst a single administration of a large dose will render invalid a subsequent test in which the same rabbits are used. It was established, in another series of experiments, that repeated administration of pyrogen-free saline had no effect on the esponse to a dose of pyrogen given subsequently. Thus to maintain the sensitivity of tests it is necessary to exclude only rabbits which have previously been given pyrogen. It is, therefore, suggested that the repeated use of the same rabbits be permitted, provided that no rabbit used in a test where pyrogen was judged to be present be used again.

From the results illustrated in Figures 1 and 2 it is clear that the frequency with which pyrogen will be detected by the U.S.P. test is always greater than by the B.P. test. This is owing to the different methods of interpreting the data. The frequency with which pyrogen, at any stated

dose level, will escape detection, can be reduced to any desired value by suitably reducing the permissible temperature rise. But if the permissible temperature rise is reduced too far, the normal fluctuations in temperature may be large enough to be mistaken for pyrogen responses. The advantage gained by the increase in frequency with which pyrogen is detected, when present in small amounts, will then be more than offset by the frequency with which it will be judged to be present when, in fact, absent. It is therefore pertinent to determine whether the greater efficiency of the U.S.P. test—in which lower total temperature increases than those required by the B.P. test often result in pyrogen being judged present—is achieved only by a corresponding increase in the number of pyrogen-free samples which are declared by the test to contain pyrogen. To determine this point data from experiments in which pyrogen-free saline was given were analysed. It was not known whether both methods would lead to the same results, so two groups of data were gathered together, in one of which method 1 was used and in the other method 2. These results are given in Table III.

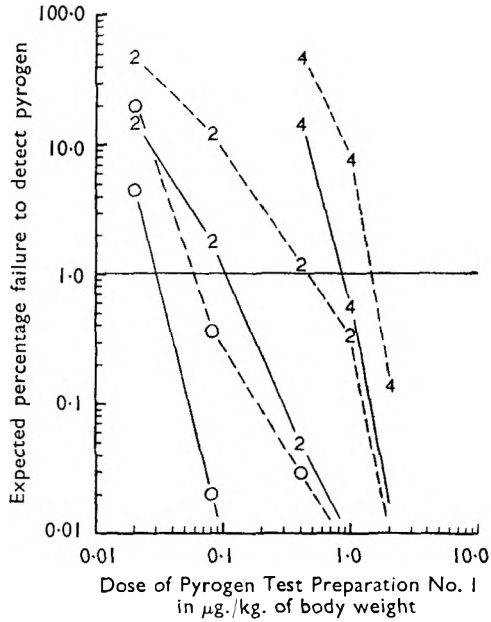

FIG. 2. Showing, with method 2, the reduction in efficiency in the B.P. and the U.S.P. pyrogen tests, when pyrogen is given repeatedly to the same rabbits at 3 or 4 day intervals. Broken lines, B.P. tests: solid lines, U.S.P. tests. Numerals indicating points on the graphs give the number of previous occasions on which the same dose of pyrogen had been given. The progressively higher dose levels at which the succeeding lines cut the ordinate at the 1 per cent. level shows the progressive reduction in efficiency of the tests as the number of previous administrations of pyrogen increases.

There is a slight diurnal variation in the normal temperature of the rabbit under the experimental conditions, the temperature being minimal at noon and rising by an average of 0·15° C. by 3 p.m. The temperature of each rabbit also exhibits random fluctuations. Hence, as in all cases the difference between the pre-injection normal and the post-injection maximum temperatures is the measure of the response, and as all injections were given at noon, the mean temperature change in each group is positive. The means for each group are not very different, but the "within days" variance for experiments in which method 1 was used is greater than that for method 2. Despite this, the proportion of test results indicating the presence of pyrogen when it is in fact absent, do not

differ materially for the two methods. The results thus show that neither the B.P. nor the U.S.P. tests are likely, with either experimental method, to result in the condemnation of pyrogen-free preparations.

TABLE III

EXPECTED EFFICIENCY OF THE B.P. AND U.S.P. TESTS FOR PYROGENS USING METHODS 1 AND 2 WHEN PYROGEN-FREE SALINE IS GIVEN

		Method 1	Method 2
Number of observations in group		55	84
Mean response in °C.		0·20	0·22
Within day variance		0·0282	0·3094
Percentage of tests in which pyrogen will be found present	B.P.	0·01	<0·01
	U.S.P.	0·02	<0·01
Percentage of tests in which pyrogen will be found absent	B.P.	99·99	>99·99
	U.S.P.	99·98	>99·99

SUMMARY

1. The effect of varying the experimental conditions upon the sensitivity of the B.P. and the U.S.P. pyrogen tests has been examined, and a comparison of the efficiency of the two tests has been made.

2. It is found that the sensitivity of both tests is maximal, (*a*) when the rabbits have not previously been given pyrogen, (*b*) when the degree of restraint is minimal, and (*c*) when electrical thermometers are used.

3. It is, therefore, recommended that in pharmacopœial descriptions of pyrogen tests, (*a*) the further use of rabbits which have once been used in a test in which pyrogen has been found present be forbidden, (*b*) the maximum degree of restraint which may be imposed on the rabbits be specified, and (*c*) the use of electrical thermometers should be required.

4. The U.S.P. test detects smaller amounts of pyrogen than does the B.P. test.

5. The frequency with which pyrogens will be judged present when they are, in fact, absent, by either the B.P. or the U.S.P. test, is so small as to be negligible.

6. It is suggested that the U.S.P. method of interpreting the test data should replace the method used in the B.P.

I wish to thank Professor W. A. Bain for advice, and the Medical Research Council for a grant towards part of the expenses incurred, during this work.

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DISCUSSION

The paper was presented by THE AUTHOR.

MR. W. P. LEGGETT (Liverpool) asked whether the author could offer an alternative method which did not involve the use of rabbits, because if manufacturers used animals once only many thousands more rabbits would be needed than were obtainable. Was the decline in response associated with any particular pyrogen? It was well established that there were many different pyrogens derived from different bacterial species. He also asked whether the author agreed that there were occasions when rabbits did not respond to pyrogens and, if so, whether the percentage of rabbits which did not respond was significant.

MR. G. A. STEWART (Dartford) said that he had used the author's pyrogen preparation in a number of studies, and had found a variation in sensitivity between sexes and breeds. Male rabbits were more sensitive than female rabbits to pyrogen, and Flemish rabbits were found to be most sensitive. When repeated doses of pyrogens were given to female rabbits (the experiment had not been carried out with male rabbits), he had found an increase in sensitivity to the pyrogens on days 2 to 6, and than it began to fall off; this was contrary to the author's report. Eventually a slight tolerance was produced, but after a few weeks rest sensitivity returned. He agreed with the idea of using standard pyrogens on test rabbits. Some rabbits were initially very insensitive to pyrogens so if they were not tested initially, rabbits could be introduced into the test which would bias the results. He asked whether the author had noticed any difference between sex and breed, and on what diet he kept his rabbits. Also, what was the relationship between the animal tests and clinical findings? He wondered whether the author had tested his own pyrogen preparation in humans.

DR. F. HARTLEY (London) said that the author had led himself to the conclusion that every rabbit must be a detector of pyrogens. That might be true for his particular standard pyrogen preparation, but if no rabbits could be used a second time, it had to be assumed that every one was a perfect detector of all types of pyrogen. That was not true in the experience of those who had examined a whole range of unknown types of pyrogenic materials rather than specially prepared standard preparations of particular pyrogens. Before the author could go so far as to express any comparison between sensitivities and particular testing methods, it was necessary for the study to be repeated with different pyrogens. He was not convinced that the author had a basis for any recommendation of switching from the present qualitative type of test in the B.P. In laboratories where it was common practice to establish first the sensitivity of the animals used, a resettling period of 4 to 5 weeks was adequate.

MR. E. ADAMS (Plymouth) said that the paper indicated one disadvantage of the fever test not apparent with the leucocyte method. In a paper published in the Transactions of the New York Academy of Science, it was stated that the response in the leucocyte method could be additive with further doses. It was possible to superimpose one response

upon another and obtain a rise in white blood cell count of from 16,000 to 67,000 per cu. mm.

PROFESSOR J. P. TODD (Glasgow) said that the author had carried out a series of tests which he had repeated at intervals of 3 or 4 days, and he had given one series of figures. Those figures he interpreted as indicating a diminution of sensitivity to the pyrogenic substances. He (Professor Todd) and his colleagues had also carried out over the last 6 or 7 years a series of tests including such tests as those described. It had, for example, in the last year been found necessary to repeat the doses at 3 or 4 day intervals, and that was coupled with the leucocyte response. The temperature response in no way agreed with the author's results. There were the usual fluctuations, but they did not, in his opinion, indicate any diminution of the sensitivity. In 1948 Wylie and himself published a paper in which that point was investigated. A crude complex mixture of pyrogens was used in order to get some increase in temperature by sensitisation of the rabbit, but that did not happen. The leucocyte response appeared to him to show possibilities of providing in the near future a test which was very much more accurate than the temperature test. Of course, it was necessary to be satisfied that a high leucocyte response was indicative of high pyrogenic activity. He asked what the author meant by an "electrical thermometer," pointing out that for many years he had been satisfied with the simple thermocouple. The B.P. test for pyrogens was, in his view, possibly the crudest test which ever got into the B.P.

MR. J. W. LIGHTBOWN (Mill Hill) said that an expert committee of the World Health Organisation were instigating an international investigation into the assessment of pyrogens, and they had obtained two pyrogen samples which they intended to distribute for examination. One was a preparation of *proteus* and the other a polysaccharide, more highly purified, prepared from *Chromobacterium prodigiosum*. Some years ago he was concerned in some experiments with pure polysaccharide in an investigation of the Schwartzman reaction, which depended on repeated injection of bacterial products into an animal. If pure polysaccharide were injected into the skin and 24 hours later a second dose were given there was necrosis at the site of injection. If both injections were given intravenously there was diffuse hæmorrhage throughout the organs, and on the second injection there was also a fall in temperature. If the polysaccharide were used in a dose below about 1 μg./kg. there was a strong pyrogen response. If a dose above 10 μg./kg. were used there was a decrease in temperature. It would be interesting to know whether the author had observed anything like that with *proteus*, and whether high doses gave no response at all or decreased responses. There was a possibility of antibody production due to repeated doses of the *proteus* preparation. With the polysaccharide, which was free from protein, after frequent injections precipitins were detected in the plasma which would eliminate the pyrogenic effect. It would be interesting to know whether the author's animals had produced any antibody which might account for a decrease in response.

MR. K. L. SMITH (Nottingham) said it was a pity that the author had applied so much restraint to the animals. In his laboratory when a clinical thermometer was used the operators nursed the animals in their laps. He was surprised that the variance of the rise in temperature in the method using more restraint was the same as when less restraint was applied. There was some justification for reducing the critical temperature of the author's pyrogen test. It was difficult to increase the dose of distilled water and the critical level could be reduced, but he did not suggest that the U.S.P. method should be accepted *in toto*. Before the test was made more restrictive, it was desirable to consider its object. There must be a great deal of water used with intravenous injections on a large scale which was never submitted to a pyrogen test at all, but he did not think that the incidence of pyrogenic response was great. It was impossible to decide in manufacture, where distilled water was being continuously produced, what constituted a batch. The B.P. test could not be followed exactly.

DR. G. SOMERS (London) agreed that large amounts of water were injected which had never been submitted to the pyrogen test. Although water for injection should be pyrogen free when being placed into ampoules and subsequently sterilised, the material was able to derive pyrogens from the glass container itself. Therefore, some requirement should be made, as in the sterility test, that a percentage of the final product should be tested. In the design of pyrogen tests it was necessary to take a practical view; large batches of material could not be rejected because the temperature of 3 rabbits went up. It was not unknown for rabbits to show false rises due to excitement and similar causes.

MR. T. D. WHITTET (London) supported the suggestion that the B.P. should be more specific in regard to the restraining apparatus in the pyrogen test. In a recent paper it had been shown that not only did severe immobilisation cause hyperthermia, but that rabbits having unrestricted freedom could show a rise in temperature of 1 to $1\frac{1}{2}^{\circ}$ in an hour.

MR. BROOM (Nottingham) said there was no evidence whether the permitted temperature rise in the B.P. or U.S.P. was right or not. There was real need of collaboration between pharmacologist and clinician to ascertain the correlation, if any, between the response in animals and in humans.

DR. G. E. FOSTER (Dartford) pointed out that the fundamental requirement of a biological test was that the standard preparation should be the same as the test preparation, but he was not sure that that had been fulfilled in the present case.

DR. J. G. DARE, in reply, said he knew of no more convenient animal than the rabbit and it was not suggested that, in routine testing, animals should be used only once. In the literature there were now reports of pyrogens from 4 different bacterial species having been examined for tachyphylaxis and it was observed in each case. In his experience only very rarely did rabbits fail to respond to doses of pyrogen within the normal effective range, and no rabbit has been found which failed to respond more than once, but the minimum effective dose varied widely. Part of the superiority of the U.S.P. test was due to the fact that when a

threshold dose of pyrogen is given it will be discovered even when a relatively insensitive rabbit is included, because the data are assessed by the number of individual responses.

The dose response curve for males appeared to be steeper than for females, but the minimum effective dose for each appeared to be similar, so that for qualitative tests either sex is suitable. He had made no comparison of different breeds of rabbits. An ordinary standard diet with greens and oats had been used. He had not given pyrogens day after day, but Beeson reported that when this was done there was a progressive reduction in response. Studies on human beings were in progress, and it was hoped to report on these shortly.

He was not clear what Dr. Hartley meant by a "perfect detector." But it would seem that he wished to specify that particular rabbits must only be used for pyrogen testing after they have been shown to be sensitive to pyrogens from all sources. This was clearly impossible. For practical purposes it had to be assumed that any rabbit will react to pyrogen if the dose is sufficient: nor is there any evidence to the contrary. The data in the paper showed, however, that when pyrogen had been given previously the "sufficient" dose may be very much greater than if no pyrogen had been given before. Dr. Dare said he made no recommendation to change from the qualitative type of test in the B.P.; his recommendations were still for a purely qualitative type of test and they would not make the test any more stringent than the present U.S.P. test.

He wished to draw Professor Todd's attention to the references, given in his earlier paper, to other work in which a diminution in sensitivity to pyrogens was reported. Favorite and Morgan have also reported that effect in man. As a generally applicable qualitative test, leucocyte changes could have only a limited value, because many of the preparations tested would contain substances other than pyrogens which had profound effects on leucocytes. Furthermore, Favorite and Morgan observed that to produce the same degree of leucopœnia the dose of pyrogen had to be increased on each occasion. The thermocouple was, of course, only one of many electrical devices that might be used as thermometers.

A purified polysaccharide from *Chr. prodigiosum* is one of the preparations which have been shown by others to exhibit tachyphylaxis in rabbits. With greater than maximum effective doses of his preparation serious distortion of the normal response pattern occurred.

In the U.S.P. test no sample passes without further examination if the mean is greater than 0·46° C. Thus Mr. Smith's suggestion that a lower temperature could be used is clearly included, whilst the advantage of making use of individual temperature changes is retained. He agreed that tests on the final product should be required, especially if it was to be given intravenously.

With regard to the permitted rise of temperature, it was hoped to obtain information on this point when the results from the human studies were related to those from rabbits.

The B.P. and U.S.P tests were qualitative tests which did not involve any comparison with a standard preparation.

A NOTE ON SURFACE-ACTIVE AGENTS AND SURGICAL DRESSINGS

By D. Maxwell Bryce and R. Maxwell Savage

From the Laboratories of S. Maw Son and Sons Ltd., Barnet

Received July 15, 1953

Introduction

There are two properties of a surgical dressing which are important as measures of its ability to absorb water or secretions in a satisfactory manner. One is the absorbency, that is to say, the rate at which the liquid is absorbed and the other is the water retention coefficient, that is to say, the quantity of liquid which the dressing can retain. In this paper, the absorbency is considered as the main subject, but water retention coefficient has been examined because the possibility exists that there is sometimes an inverse relationship between the two properties. It is unfortunately true that absorbency is not always a permanent property of a surgical dressing. Sometimes a dressing will remain highly absorbent for years, but at other times, one similar in all apparent respects will lose its absorbency, and may in time, usually after some years, become so non-absorbent that it will float on water indefinitely. Two theories have been proposed to account for this change. One (Savage[1]) is based on the fact that by the empirical practice of the trade, absorbent cotton of commerce has upon the surface of the fibres a layer of material largely composed of fatty acids. It is known that such a layer can exist in two orientated forms, in one of which it is the carboxyl group which faces outwards, and in the other the non-wettable terminal methyl group. It would be expected by this theory that absorbent cotton would normally exist in wettable or non-wettable forms, irrespective of the quantitative analysis of the material, that reversal of orientation could occur after various treatments and that cotton with very low quantities of fatty extract (lower than obtainable by normal commercial methods) would be permanently absorbent, because there would then be insufficient material to cover the fibres. These conditions were in fact reproduced experimentally. The other theory, which has some general currency, but has perhaps not been established, attributes loss of absorbency to the slow passage of fatty material from the lumen of the fibre to the outside. It does not, however, fit the facts very well, for high absorbencies and good keeping properties occur in samples with a high fatty content, and poor samples may have a low fatty content. Moreover, the reversal of absorbent properties by treatment with heat and with steam cannot be explained, for it is hardly likely that fatty material would return to the lumen. The paper cited contains more details.

The layer of fatty material is of universal occurrence, and the usual range of fatty extractive is from 0·15 to 0·5 per cent., more than enough completely to coat the fibres. Some of this material is there because by commercial means, it cannot be removed, but in many cases the rest is

intentionally added. If there must be fatty material present, it is apparently better that fatty acids should be present as well as the unwettable residue of cotton waxes, rather than to have these alone. In a sense, therefore, it is already the fact that "wetting agents" are used in surgical dressings, and have been so used for very many years.

It is clear from what has already been said that these traditional wetting agents are not entirely efficient. Loss of absorbency continues to occur, and there is an element of chance in the behaviour of a dressing. It cannot be said that trouble is very widespread—loss of absorbency is perhaps best regarded as a sporadic nuisance, but on occasion, it can be quite a serious matter. Ships serving in tropical waters for long periods are cases in point—the dressings they carry can become non-absorbent and useless, and specifications are in force which require very low values for extractive, a method which does achieve a certain amount of success, for bare cellulose is always wettable, and if the fatty content is kept low enough, there must be areas of bare cellulose. It is unfortunate that this ideal method is so difficult to use in practice that the specification limits cannot be set low enough for complete safety. Moreover, the mechanical properties of the cotton are changed by such complete extraction, and it is doubtful if the users would appreciate the handle of the material if they had it. The price of a dressing is one of its important properties, and solutions of this problem which are scientifically possible are generally commercially impossible if they involve an increase in price.

There are nowadays many other wetting agents. Some of these, for example, sodium lauryl sulphate, have passed into use in medicine, and it seemed of interest to examine the effect of using materials of this type in dressings.

Experimental

Some preliminary experiments were made in which quantities of cotton wool, bleached and finished by conventional methods, were treated with

TABLE I

Sinking times of cotton wool after treatment with various wetting agents

Treatment									
		Heated for 1 hour at			The 150° C. samples 4 days later, seconds	The 110° C. samples 5 days later, seconds	Unheated		
	Initial seconds	170° C. seconds	150° C. seconds	110° C. seconds			38 days, seconds	98 days, seconds	204 days, seconds
A	2·1	45	5·5	3·1	4·6	5·3	2·2	2·5	2·3
B	3·3	∞	80	4·7	33·6	4·3	2·6	3·1	4·8
C	3·6	∞	46	3·6	40	4·8	3·2	2·6	4·6
D	2·8	∞	13	2·7	26·7	5·2	3·3	6·4	4·4
E	1·8	12	3·1	1·3	2·7	2·0	1·5	1·8	2·0
F	4·0	∞	11·2	3·3	75	5·8	7·8	6·4	7·4
G	2·4	29	3·2	1·7	2·2	1·9	2·2	2·4	1·8
H	∞	∞	∞	∞	∞	∞	∞	∞	∞
I	2·8	∞	13	3·6	11·7	4·1	3·9	5·0	5
J	6·1	∞	25	7·9	38	16·6	6·7	9·0	12
K	2·7	∞	14	3·8	13	7·8	2·3	3·8	7
L	∞	∞	∞	∞	∞	∞	∞	∞	∞
M	—	∞	∞	18·4	∞	26·6	20·4	26·3	39
N	21·5	∞	∞	25	∞	75	12·0	23·2	36

∞ means that the sample did not sink during the period of observation. The wetting agents used were:—A, B and C—different grades of sodium lauryl sulphate; others—various commercial wetting agents H—a non-ionic material; G—a cationic amine; the remainder—uncertain composition; N—contol.

0·5 per cent. of the wetting agent dissolved in a sufficient quantity of water. The cotton wool was then dried, and mixed by passing through a Shirley analyser. The cotton wool was then subjected to dry heat, and other samples were stored for various periods with and without previous heat treatment. These experiments showed (Table I) that most of the wetting agents did in fact produce cotton wool whose absorbency withstood drastic treatments which altogether destroyed the absorbency of the untreated material. The untreated material, poor at all times, became less absorbent on simple storage and failed to comply with the B.P.C. requirements.

There seems therefore good reason for the opinion that by the use of these reagents cotton wool can be produced which is much less likely to lose absorbency than the existing B.P.C. material, even when drastically treated, or stored for a long time. There are, however, other considerations which must be examined before such a material could be considered suitable as a dressing.

Effect of the Treatment on the Water Retention Coefficient. In a previous paper (Savage, Bryce and Elliott[2]), it was suggested that the use of a wetting agent in the water tended to lower the water retention coefficient If the effect were large, it would be a disadvantage. Two wetting agents (Cithrol A and sodium lauryl sulphate) were used for detailed investigation (Table II). The results were subjected to statistical analysis, and

TABLE II

EFFECT OF SODIUM LAURYL SULPHATE* ON THE WATER RETENTION COEFFICIENT† OF COTTON WOOL AND VISCOSE RAYON WOOL

Series	Cotton wool pressure 3 cm. Hg.						Viscose rayon wool pressure 3 cm. Hg.					
	percentage of wetting agent					Water	percentage of wetting agent					Water
	2	0·4	0·08	0·016	0·0032		2	0·4	0·08	0·016	0·0032	
1	9·7	11·2	11·0	11·2	11·5	12·0	8·9	10·0	9·9	10·9	10·5	10·6
2	10·8	10·8	12·0	11·7	11·0	11·7	10·0	9·3	10·8	10·1	9·9	10·9
3	11·3	11·6	11·7	11·3	11·3	11·6	10·1	10·1	11·0	10·0	10·2	11·1
4	11·7	11·1	11·5	11·4	11·7	11·5	9·3	10·3	10·6	10·5	10·7	10·1
5	11·3	11·2	11·6	11·4	11·8	11·6	9·7	10·4	10·0	10·2	9·2	10·3
Means	10·9	11·1	11·5	11·4	11·4	11·6	9·6	10·0	10·4	10·3	10·1	10·6

* 40 per cent. sulphonated lauryl alcohol powder + sodium sulphate filler.
† Piston type apparatus 3·9 cm. diameter with 1g. of sample.

showed that there was a significant decrease in the water retention coefficient, but it was not a large effect, and is probably of no practical importance, being less than 10 per cent., even at high concentrations much beyond those likely in practice.

Physiological Effects. At one time, such materials were not used in medicine, and there was no information on their effect when used in a wound. But nowadays, ointment bases frequently contain sodium lauryl sulphate, and there seems no reason for anticipating adverse physiological effects. It would, however, seem preferable not to use wetting agents of the industrial type, of unknown composition, and any specification should, we consider, require the use of a material such as the B.P. product. It must here be pointed out (following suggestions by Mr. J. R. Elliott) that

cotton wool is not always used for absorbing secretions and liquids, but may be used for bacteriological swabs and for filtering liquids. For both these purposes the presence of a soluble wetting agent is probably undesirable and if such dressings do come into use, a small demand may still justifiably exist for the present product. This fact emphasises our belief that soluble wetting agents should not be used until fully investigated and officially approved, notwithstanding the fact that the B.P.C. limits for water extract would not necessarily be exceeded if they were used, and treated material might be considered as complying with the B.P.C. specification.

TABLE III

SURFACE TENSIONS OF EXTRACTS OF COTTON WOOL

	Aqueous extract*	Surface tension, dynes per cm. (extract evaporated to 5 ml.)
1	Clear	72
2	"	52
3	"	48
4	"	59
5	"	63
6	"	67·5
7	Opal	70
8	Clear	67·5
* 9	"	50
†10	"	59

* 1 g. extracted with 100 ml. cold distilled water.
† 1 to 8 were samples of commercial cotton wool, 9 and 10 were the samples treated with sodium lauryl sulphate and with Cithrol A respectively.

Detection of Wetting Agents. Since quite small quantities of wetting agents are effective, and the extraction of cotton wool requires large quantities of liquid, which may also extract other water-soluble material, the detection and estimation of wetting agents is not chemically simple. We have found that by extracting with water and concentrating the solution, wetting agents may be detected by measuring the surface tension of the extract, using the method of capillary rise. By comparison with solutions of known composition, some idea of the quantity can be obtained, but further work would be needed before the method could be accepted as quantitative. It has, however, enabled us to carry out a survey of cotton wools purchased by retail, and as a result, we conclude that the products of one maker sometimes contain soluble wetting agents of the type discussed here.

TABLE IV

SURFACE TENSIONS OF SOLUTIONS OF WETTING AGENTS

		Sodium lauryl sulphate	
Concentration, per cent.	Cithrol A, surface tension	Concentration, per cent.	Surface tension
10	35	2	29
2	38	0·4	35
0·4	43	0·08	45
0·2	46	0·016	60·5
0·08	57	0·0032	71
0·02	71·5	Water	73
0·016	70	—	—
0·0032	73	—	—
0·00064	73	—	—
0·00013	73	—	—
Water	73	—	—

After some preliminary experiments, the general method adopted was to percolate 1 g. of cotton wool with 100 ml. of hot water, and concentrate to 5 ml. The capillary rise of this solution was then measured by the

usual method and the surface tension calculated. Water gave a result of 73 dynes per cm., and the extracts from treated cotton gave results of 50 and 59 (Table III). Of the purchased samples, 2 gave results of 48 and 52, and the solutions from these frothed on shaking. No alteration of surface tension or other properties occurred when calcium chloride solution was added so that soap residues were not responsible for the lowering of surface tension.

SUMMARY

1. Theories of absorbency are discussed, and it is pointed out that normal surgical cotton absorbent dressings do not consist of pure cellulose, but have a wettable surface of other materials.

2. By using soluble surface active agents in small amounts cotton dressings can be made which retain absorbency better than the conventional types.

3. The ethics of treating dressings in this way are discussed, and it is concluded that such agents should not be used unless officially recognised because they would not be suitable for certain minor uses.

4. Methods of detecting and roughly estimating soluble wetting agents are given.

We acknowledge the information and samples supplied by Watford Chemicals, Ltd., Glover's Chemicals, Ltd., and Croda, Ltd., and technical assistance by Miss F. Wilbraham.

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1. Savage, *J. Soc. chem. Ind.*, 1934, **53**, 3797.
2. Savage, Bryce and Elliott, *J. Pharm. Pharmacol.*, 1952, **4**, 944.

DISCUSSION

The paper was presented by DR. R. MAXWELL SAVAGE.

MR. J. R. ELLIOTT (London) asked whether the experiments at 110° C. were carried out in hot air or by autoclaving, and also whether the author had any experience of the effect of repeated autoclaving of dressings treated with wetting agents. If wetting agents were incorporated with dressings, in his view, labels should carry information to that effect.

DR. R. RUYSSEN (Belgium) suggested that water-soluble saponins when used as wetting agents could not be detected by the method of capillary rise, because the surface tension of the saponin solution was similar to that of pure water.

MR. R. L. STEPHENS (Brighton) referred to the antagonism between anionic types of wetting agent and the quaternary ammonium and acridine type of antiseptics, and said that it would obviously be a serious matter for the user of a dressing if such wetting agents were present.

DR. R. MAXWELL SAVAGE, in reply, said that the experiments were carried out in hot air. He had no information as to the effect of repeated

autoclaving but thought it would not have such a serious effect in diminishing absorbency as the hot air treatment. In fact, it could improve it. He agreed that there should be some means of indicating to a user what he was getting. He was surprised that the method of capillary rise was not suitable for such preparations because he had used it to a considerable extent quite successfully. It might be a matter of relative concentrations. The quantity of wetting agent on fibres would be very small. It might be insufficient to offer antagonism to quaternary ammonium compounds and antiseptics of the acridine type.

STUDIES IN THE DETERIORATION OF AQUEOUS SOLUTIONS AND DISPERSIONS OF PROCAINE BENZYLPENICILLIN

By R. Levin

From the Laboratories of The Distillers Company (Biochemicals), Ltd.

Received July 25, 1953

Among the more widely known properties of benzylpenicillin is its characteristic instability in the presence of water. This unfortunate feature gave rise to many problems in the course of its isolation and development, which were accentuated by the later production of a highly purified crystalline material. In addition to instability in solution, pharmacological examination revealed that benzylpenicillin was rapidly absorbed and excreted, thus necessitating administration by frequent injection or continuous drip. Many methods were attempted which were designed to prolong the therapeutic effect within the body, for example by dispersing the benzylpenicillin in an oleaginous, slowly diffusing vehicle, or by delaying renal excretion. Whilst some success was being achieved in the prolongation of benzylpenicillin blood levels, these techniques were quickly superseded when, in 1948, Sullivan *et al.*[1] first described an insoluble salt of benzylpenicillin formed by simple admixture in solution of procaine hydrochloride and sodium benzylpenicillin which, when injected, produced a prolonged blood level. By injecting a sparingly soluble salt in the form of an aqueous or oily suspension of fine crystals the need for frequent injection is reduced, and in most cases a single injection of 300,000 units of the procaine salt will provide a demonstrable serum level for 24 hours[2,3,4].

The procaine salt of benzylpenicillin is stable in aqueous media and it has been found possible to prepare an aqueous suspension of this material which, whilst in a form suitable for immediate injection, can be stored as a suspension for periods of 12 months or longer. Although it is thus known that procaine benzylpenicillin is relatively stable in aqueous suspension, it is also known that this stability is dependent on certain factors, including temperature of storage, *p*H of the suspension, presence of buffers and other agents, etc., and observations relating to studies on these aspects are described.

Part I

Deterioration in Dispersions of Procaine Benzylpenicillin

Procaine benzylpenicillin is frequently administered in the form of a ready prepared aqueous dispersion containing 300,000 I.U./ml., and it is the main purpose of this investigation to assess the stability of this form of the material. Benzylpenicillin, the product of a biosynthesis may show small batch to batch variations, in spite of careful efforts during the course of its manufacture to obtain consistently uniform material. The B.P. and T.S.A. Regulations lay down certain minimal standards of purity, e.g., moisture, toxicity, potency, etc., to which all batches must conform if they are to be suitable for clinical use. Beyond the scope of

these standards however, are small variations, including the presence of traces of iron or other metals, pigments, solvents, etc., any of which may influence the stability of the final product.

It is common practice in the manufacture of many pharmaceutical products, to select at random a number of samples from each batch, other than those which may be required for statutory or other tests, to be subjected to a range of storage conditions, so that observations may be made on their keeping properties. From a series of sterile filled, silicone-treated vials of suspension set aside at normal room temperature, i.e., 20° ± 5° C., samples were withdrawn and subjected to the tests described. Each sample tested was representative of a single batch of procaine benzylpenicillin and therefore factors other than time and temperature alone may account for the course of deterioration. The samples had been stored for periods varying from 1 to 46 weeks.

Formulation of Test Suspensions

Test Vehicle:

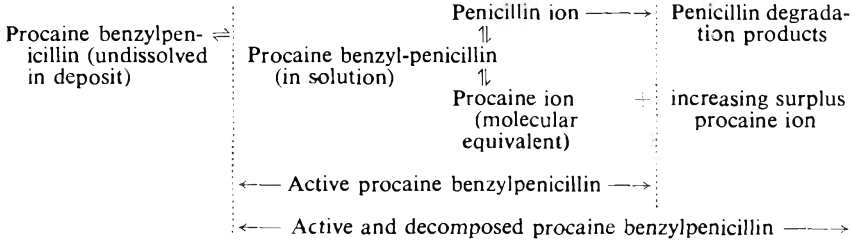
Sodium citrate, tribasic, anhydrous	1·44 g.
Sodium carboxymethylcellulose	0·481 g.
Polyoxyalkylene sorbitan mono-oleate	0·086 g.
Phenylmercuric nitrate	0·137 mg.
Water to 100 ml.	

Test Suspension:

Procaine benzylpenicillin	300,000 I.U.
Test vehicle to 1 ml.	

Theoretical Considerations

Procaine benzylpenicillin has been found to be unstable in aqueous solution (see Part II), the instability being partially mitigated by the inclusion of a buffering agent, e.g., tribasic sodium citrate. The pattern of degradation in a suspension is thought to be—



Thus on admixture of procaine benzylpenicillin with water, a small amount dissolves (solubility about 1 in 200), the remainder settling out as a deposit. The procaine benzylpenicillin molecule in solution may be expected to dissociate, giving rise to a procaine fraction which will remain in solution within the limit of its solubility, or may react with benzylpenicillin decomposition products; the benzylpenicillin moiety, being unstable, undergoes degradation. Therefore, the procaine fraction in

solution at any one time is made up of (*a*) procaine from decomposed procaine benzylpenicillin, as procaine ion or salt, and (*b*) procaine fraction of dissociated procaine benzylpenicillin in solution as such.

Practical Considerations

Since one may deduce the concentration of active procaine benzylpenicillin in solution at a given time from a microbiological assay, and that of total procaine benzylpenicillin (active and decomposed) from a spectrophotometric assay, a simple calculation will give the amount of procaine benzylpenicillin which has decomposed at that time. The samples of dispersions were separately centrifuged, the clear supernatant liquid removed and examined as follows:

(*a*) *Active and decomposed procaine benzylpenicillin concentration, from total procaine base.* Using the Unicam SP.500 Spectrophotometer, a purified sample of procaine benzylpenicillin had been found to show maximum absorption at 289·5 mμ, and to obey Beer's law in concentrations up to 80 μg./ml., although subsequent readings were not made on solutions in excess of 60 μg./ml. The specific extinction coefficient $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 289·5 mμ was found to be 316. Supernatant liquids removed from test samples were diluted to less than the equivalent of 60 μg./ml. procaine as procaine benzylpenicillin and were assayed by measuring the optical density $E_{289\cdot5}$ and calculating:

$$\mu\text{g./ml.} = \frac{E_{289\cdot5}\text{ observed.}}{0\cdot0316}$$

Thus, assuming a potency of 1000 I.U./mg.

$$\mu\text{g./ml.} = \text{I.U./ml.}$$

The figures obtained for procaine ion were thereby translated in terms of the stoichiometric equivalent procaine benzylpenicillin (100 mg. of procaine benzylpenicillin contains 40·2 mg. of procaine ion), and are quoted as such in Table I, Column I.

(*b*) *Active procaine benzylpenicillin concentration.* This was obtained by microbiological assay of the supernatant liquid, using a cavity plate diffusion technique, with *Staphylococcus aureus* as the test organism. Results obtained are quoted as I.U./ml., in Table I, Column II.

(*c*) *Apparent loss of procaine benzylpenicillin.* Decomposed and active procaine benzylpenicillin concentration = I. Active procaine benzylpenicillin concentration = II. I − II = apparent loss of procaine benzylpenicillin, in I.U./ml. of supernatant liquid. Since the supernatant liquid represents about 65 per cent. of the total volume of the suspension, the actual loss in I.U./ml. of suspension is approximately two-thirds of the apparent loss I.U./ml. of supernatant liquid.

Samples were frequently taken which whilst incorporating different batches of procaine benzylpenicillin, were, nevertheless prepared during the same week and stored under identical conditions. Such results have been averaged for the particular week, the average, together with an indication of the number of samples involved being shown in Table I.

Assessment of Deterioration

When the results shown in Table I were plotted as graphs — (a) total procaine ion as procaine benzylpenicillin *v.* time, (b) active procaine benzylpenicillin *v.* time and (c) apparent loss of procaine benzylpenicillin *v.* time a linear relation was found to obtain in all cases, thus illustrating the regularity of the procaine penicillin deterioration.

TABLE I

PROCAINE BENZYLPENICILLIN—DETERIORATION IN TEST SUSPENSIONS

Period of storage (weeks)	Number of samples averaged	Total procaine ion as procaine benzylpenicillin* Spectrophotometric assay (I.U./ml.) *I*	Procaine benzylpenicillin concentration Bioassay (I.U./ml.) *II*	Apparent loss procaine benzylpenicillin (I.U./ml. of supernatant liquid) *III*
46	3	15,100	—	—
45	2	14,500	—	—
44	1	15,500	—	—
43	3	15,300	—	—
42	2	17,000	—	—
41	3	15,800	—	—
40	2	17,000	—	—
39	4	15,600	—	—
37	1	16,000	3,000	13,000
35	2	14,450	2,600	11,850
34	3	15,200	2,300	12,900
33	4	15,500	2,500	13,000
32	1	12,700	2,700	10,000
31	5	12,400	2,400	10,000
30	4	12,000	2,500	9,500
28	2	13,000	3,200	9,800
24	2	11,700	4,000	7,700
19	1	11,600	3,800	7,800
18	3	10,600	3,300	7,300
17	1	10,800	2,950	7,850
16	6	11,200	3,450	7,750
15	3	10,300	3,650	6,650
14	2	6,500	3,000	3,500
13	4	10,300	3,650	6,650
11	4	9,100	3,400	5,700
10	3	8,300	3,300	5,000
8	3	8,300	3,600	4,700
7	4	7,750	3,600	4,150
6	4	7,400	3,500	3,900
5	2	7,550	3,500	4,050
4	3	7,700	4,400	3,300
3	4	7,500	1,600	5,900
2	3	7,000	2,400	4,600
1	4	6,750	3,500	3,250

* Procaine benzylpenicillin activity = 1000 I.U./mg.

CONCLUSIONS

1. *Potency Stability*

When stored at normal room temperature (20° ± 5° C.) in silicone-coated, rubber capped vials, the rate of deterioration of procaine benzylpenicillin in dispersions is regular. After 35 to 40 weeks storage the rate of decomposition was seen to decrease, probably because of the gradually increasing procaine ion concentration which, by exerting a common ion effect, depresses the solubility of the procaine benzylpenicillin. The procaine ion concentration of the supernatant liquid was found to increase at a steady weekly rate equivalent to 0·2 to 0·3 mg. of procaine benzylpenicillin/ml. for the first 35 to 40 weeks, thence falling to 0·1 to 0·15 mg./ml. per week. That is to say, the supernatant demonstrates a weekly loss of procaine benzylpenicillin of about 200

to 300 I.U./ml. per week. Since the supernatant represents some 65 per cent. of the total volume of suspension, the actual loss in suspension would appear to be at the rate of 120 to 180 I.U./ml. per week, or 6 to 9000 I.U./ml. per annum, i.e., <3 per cent. of the activity.

2. p*H Changes*

Readings were made on 10 samples of suspension stored for 1 to 3 weeks, in addition to a further 10 samples stored for 11 to 14 weeks, both at room temperature. The readings were compared with those obtained at the time of preparation, and changes were seen to have occurred. Most samples showed a drop in *p*H (see Table II), the greatest drop occurring in the older preparations. This would suggest that the penicillin fraction decomposition gives rise to breakdown products with acidic properties, which is consistent with the accepted sequence of penicillin degradation.

TABLE II

PROCAINE BENZYLPENICILLIN—CHANGES IN *p*H OF TEST SUSPENSIONS

After 1 to 3 weeks	After 11 to 14 weeks
−0·11	−0·86
−0·06	−0·77
−0·23	−0·67
−0·12	−0·82
−0·42	−0·74
+0·07	−0·67
+0·05	−0·59
−0·10	−0·43
−0·07	−0·67
−0·00	−0·56
Average = −0·10	Average = −0·68

PART II

DETERIORATION OF SATURATED SOLUTIONS OF PROCAINE BENZYLPENICILLIN IN PRESENCE OF SPECIFIC STABILISING AGENTS

Since the supernatant liquid used for the determinations described in Part I represented a saturated solution of procaine benzylpenicillin in the presence of excess of procaine benzylpenicillin, it was considered of interest to study the stability of saturated solutions of procaine benzylpenicillin in the absence of excess of procaine benzylpenicillin, but instead with and without specific stabilising agents. The samples were subjected to various storage temperatures (i.e., 8° C. and 24° C.). The following agents were chosen.

(*a*) *Sodium citrate.* The value of this salt is associated with its buffering action for which purpose it is widely used in pharmaceutical products, especially in solutions of antibiotics.

(*b*) *Procaine hydrochloride.* The addition of a salt with a common ion decreases the concentration of the other ions of a sparingly soluble salt. Procaine hydrochloride, therefore, was selected to provide a source of procaine ion, and was confirmed experimentally by the author to depress the solubility of procaine benzylpenicillin appreciably when present in the solution in a concentration of 2 per cent. w/v. The lower concentration of procaine benzylpenicillin soluble in the presence of procaine hydrochloride proportionately reduces the quantity of the former available for decomposition.

(*c*) *Ethylenediamine tetra-acetic acid.* This material is a sequestering agent capable of removing from solution traces of metals such as iron,

considered to be a potential activator in the chemical decomposition of the dissolved procaine benzylpenicillin. It has been found by Swallow[5] to exhibit stabilising activity in solutions of sodium benzylpenicillin. The material was dissolved in a solution of sodium citrate during preparation, to form the sodium salt.

(*d*) *Hexamine.* Hobbs *et al.*[6] observed the specific stabilising action of hexamine in aqueous solutions of the soluble salts of benzylpenicillin. It was decided to investigate whether similar properties would be exhibited in favour of the procaine salt.

Method of Preparation of Samples

Saturated solutions of procaine benzylpenicillin were prepared in water as a control, or in aqueous solutions of the agents, as described in Table III. The solutions were prepared and filled 10 ml. each into silicone-treated vials of 26 ml. capacity, finally closed with white rubber caps,

TABLE III

SATURATED SOLUTIONS PROCAINE BENZYLPENICILLIN—FORMULATIONS (PER CENT. W/V)

	1	2	3	4	5	6	7	8	9	10	11	12
Procaine hydrochloride B.P.	—	1·0	2·0	3·0	—	—	1·0	2·0	3·0	2·0	—	—
Sodium citrate anhydrous	—	—	—	—	2·1	0·02	2·1	2·1	2·1	2·1	—	2·1
Ethylenediamine tetra-acetic acid	—	—	—	—	—	—	—	—	—	0·1	—	—
Hexamine B.P.C.	—	—	—	—	—	—	—	—	—	—	0·5	0·5

Procaine benzylpenicillin was added in excess to saturate the solution, stirred vigorously for 3 hours and finally filtered free from excess of procaine benzylpenicillin before filling for storage.

clamped firmly in position by means of aluminium seals of a type commonly in use for this purpose. Sufficient vials were prepared to permit one of each formulation to be withdrawn twice weekly from each storage temperature over a period of 13 weeks. The average for each weekly set of two results was used in the preparation of the Tables, which illustrate the course of deterioration (Tables IV and V). Assays were carried out microbiologically using a cavity plate diffusion technique, with *Staphylococcus aureus* as the test organism.

TABLE IV

STABILITY OF SATURATED SOLUTIONS OF PROCAINE BENZYLPENICILLIN

Storage temperature 8° C. I.U./ml.

Formulation number	1	2	3	4	5	6	7	8	9	10	11	12
Initial	5100	1450	1100	900	4800	4800	1000	820	650	700	6850	6500
After 1 week	5200	1500	1040	940	6200	4900	1100	1100	730	850	5650	5550
" 2 weeks	3900	1240	1070	840	4750	4800	1020	900	680	790	4400	4250
" 3 "	2450	1330	880	760	4550	4500	690	780	860	850	3850	4100
" 4 "	3000	1250	950	740	4850	4750	960	870	710	840	4200	4450
" 5 "	2450	1350	990	850	3450	3350	1060	680	620	735	4000	4200
" 6 "	2150	1320	1010	930	4750	4600	940	810	600	680	4100	4300
" 7 "	2000	1210	920	860	4700	4450	820	760	610	660	3700	3100
" 8 "	450	1260	920	720	4350	4550	960	750	480	530	3900	4300
" 9 "	720	1210	900	830	4900	4650	970	760	550	600	3900	4500
" 10 "	240	1200	890	850	4850	4650	1120	770	790	595	3700	4350
" 11 "	< 100	1025	830	840	4450	4150	1000	820	680	620	4050	4300
" 12 "		1040	870	750	4500	4450	1090	720	550	610	3650	3900
" 13 "		1160	900	780	4750	4700	800	630	480	540	3700	2200

* Procaine penicillin activity = 1000 I.U./mg. † Not available.

TABLE V

STABILITY OF SATURATED SOLUTIONS OF PROCAINE BENZYLPENICILLIN

Storage temperature 24° C. I.U/ml.

Formulation number	1	2	3	4	5	6	7	8	9	10	11	12
Initial	5200	1500	1000	950	4800	4900	1650	1050	900	1120	6850	6500
After 1 week	1700	1000	840	780	5100	4400	1500	1080	980	1200	5250	5200
" 2 weeks	500	750	*	640	4550	*	1740	960	890	1080	3450	4000
" 3 "	100	630	200	440	3950	500	1710	990	930	1200	3300	3700
" 4 "	<100	250	500	180	4500	*	1650	890	870	960	3050	3800
" 5 "		<100	400	180	3700	140	1300	680	740	820	2400	3700
" 6 "			350	<100	3650	<100	1400	640	710	800	2200	3650
" 7 "			<100		2750		1500	770	760	890	2000	2900
" 8 "					3750		1680	810	770	770	2350	3100
" 9 "					3750		1650	710	590	590	2150	3550
" 10 "					3850		1250	670	660	790	650	3400
" 11 "					2750		1070	580	640	590	*	3700
" 12 "					3300		1310	620	710	870	850	2900
" 13 "					*		1170	640	690	890	790	3000

* Sample not available.

Observations

A. 8° C. Storage

(i) *Control*: A slow and steady deterioration, with complete loss after 10 weeks.

(ii) *Added procaine hydrochloride*: The addition of 1 to 3 per cent. of procaine hydrochloride effects a marked depression in the solubility of procaine benzylpenicillin. The reduction of solubility brought about by 1 per cent. of procaine hydrochloride is but slightly enhanced when the proportion is increased to 3 per cent. When sodium citrate is also present, the solubility is further depressed. The total loss of activity in solutions containing added procaine hydrochloride only, was less than 5 per cent. of that of the control, whilst on the further addition of sodium citrate, this loss is reduced to less than 1 per cent. of that of the control.

(iii) *Added sodium citrate*: (*a*) 2·1 per cent. exerts a marked stabilising action, resulting in very slight loss of penicillin activity after 13 weeks. (*b*) 0·02 per cent. (≡ 4 per cent. of the procaine benzylpenicillin content) exerts a marked stabilising effect, with a slight loss apparent after 13 weeks.

(iv) *Citrate/procaine hydrochloride*: The loss of activity in a combination of procaine hydrochloride and citrate is lower than that of either of the agents used alone.

(v) *Combined procaine hydrochloride/citrate with sodium ethylenediamine tetra-acetate*. The additions of sodium ethylenediamine tetra-acetate was found to enhance the stabilising action of the two agents.

(vi) *Hexamine*: This substance apparently increases the solubility of procaine benzylpenicillin, the initial concentration being 6000 to 7000 I.U./ml. A rapid loss of procaine penicillin occurs within the first 3 weeks, with or without citrate, diminishing to a slow deterioration in the absence of citrate, or very slight further losses if citrate is present.

B. 24° C. Storage

(i) *Control*: Very rapid loss of procaine benzylpenicillin, decomposition being complete within 2 weeks.

(ii) *Added procaine hydrochloride*: Deterioration is quite rapid, the whole of the material in solution being decomposed within 5 weeks.

(iii) *Added sodium citrate*: (*a*) 2·1 per cent. exerts a significant stabilising effect, but the sample shows steady and continuous decomposition until at the end of the 13 weeks test period it was approaching its half life, i.e., loss of one half of its original potency. (*b*) 0·02 per cent. shows only very slight stabilising activity; steady deterioration brings about complete loss after 5 weeks.

(iv) *Citrate/procaine hydrochloride*: Only slight losses were recorded after the test period. No significant difference between 1 and 3 per cent. of procaine hydrochloride.

(*v*) *Citrate/procaine hydrochloride/sodium ethylenediamine tetra-acetate*: The presence of the sequestering agent did not materially enhance the effect of the other two agents.

(vi) *Hexamine*: Very rapid loss observed over the first 3 weeks, once again diminishing to a steady loss in the absence of citrate, and a slower loss when citrate was present.

Conclusions

1. Procaine hydrochloride has been found to depress the solubility of procaine benzylpenicillin. Sodium citrate exerts a stabilising effect on solutions of procaine benzylpenicillin. Using a combination of the two agents, a reduction in the loss of procaine benzylpenicillin in aqueous solution is obtained. The best results were obtained with the following combinations—

Procaine hydrochloride	1 to 3 per cent.
Anhydrous sodium citrate	2·1 per cent.

2. Excepting in solutions containing hexamine, it has been found that deterioration in saturated solutions of procaine benzylpenicillin is regular, and may be represented by a linear graph.

3. Hexamine appears to increase the solubility of procaine benzylpenicillin thus making a higher concentration available for decomposition. Its incorporation in a dispersion of procaine benzylpenicillin may be expected to bring about a deterioration in the keeping properties.

Summary

1. Methods of assessing the chemical stability of procaine benzylpenicillin both in aqueous solution and in aqueous dispersion are described. The effect of the addition to solutions of specific stabilising agents are examined.

2. The addition of procaine hydrochloride 1 to 3 per cent. to solutions of procaine benzylpenicillin will depress the solubility of the latter from 5000 I.U./ml. to *ca.* 1000 I.U./ml.

3. Under these conditions, and especially in the presence of a buffer, procaine benzylpenicillin solutions have been found to retain their potency for prolonged periods. For the same reasons, the addition of a suitable

procaine salt to dispersions of procaine benzylpenicillin may be expected to lengthen their storage life[7].

4. Aqueous dispersions of procaine benzylpenicillin 300,000 I.U./ml., when suspended in the test vehicle and stored at normal room temperature (20° ± 5° C.) for a period of 46 weeks, have been found to show a loss of activity of about 6000 I.U./ml.

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DISCUSSION

The paper was presented by the Author.

Professor E. H. VOGELENZANG (Netherlands) asked how the author distinguished in the spectrophotometric assay between the procaine derived from the procaine benzylpenicillin in solution, that derived from the added procaine hydrochloride and that derived from decomposed procaine benzylpenicillin.

MR. J. L. LIVINGSTONE (London) said that citrate was by far the most potent stabiliser, and with other additions, particularly excess of procaine, it was not possible to detect any advantage. A disadvantage of adding excess of procaine was that owing to oxidative colour changes the appearance of the suspension might be adversely affected towards the end of its life.

MR. G. SYKES (Nottingham) said that the paper contained useful information concerning the solubility of procaine penicillin in the presence of other agents. It would seem that the substance achieved a certain balance. On the one hand the change of *p*H recorded by the author towards the acid state accelerated decomposition. On the other hand, it was known from experience that the addition of procaine retarded decomposition. He wondered whether the author had any information on the effect of phenol as an antiseptic in the preparation in place of phenylmercuric nitrate. What was the effect of ethylenediamine tetra-acetic acid in the presence of procaine?

MR. F. TAYLOR (London) expressed surprise at the figures for the solubility of the saturated solution at 24° C. in water. He would have expected it to be higher, and certainly a 1000 units difference for a 16° C.

rise in temperature. The figures appeared to show the same solubility at 8° C. as at 24° C. There appeared to be no improvement in stabilisation with a procaine hydrochloride content of between 1 and 3 per cent. Might it not be that the stabilising concentration of the soluble procaine salt was even less than 1 per cent.? Had the author any information about the effect of the preparation in causing a flaking effect on the side of silicone-treated vials? He believed that the effect was due to the breakdown of procaine and not to procaine itself.

DR. K. BULLOCK (Manchester) said that procaine in aqueous solution would break down rapidly at *p*H 7 and over. The process would continue until the *p*H fell to about 4·5, and then there would not be much subsequent decomposition.

MR. J. W. LIGHTBOWN (Mill Hill) referred to Table I, and said it seemed rather peculiar that the procaine benzylpenicillin concentration did not change significantly between the first week and the last recorded results. That might indicate that procaine was being decomposed, in which case the conclusions drawn as to the amount of procaine benzylpenicillin decomposition might be faulty? If procaine were to be built up in the supernatant liquid, it would seem desirable, in choosing the bacteriostatic, to ensure that it was compatible with the procaine. Would procaine be incompatible with the *p*-hydroxybenzoic acid esters?

MR. W. F. HARTE (Nottingham) asked the author to comment further on the point that the value of sodium citrate was associated with its buffering action.

MR. R. LEVIN, in reply, said that the question asked by Professor Vogelenzang did not arise, because where procaine hydrochloride was used the assays were all done microbiologically. The procaine ion could give rise to colour changes following oxidation. Small variations in the iron content of the procaine hydrochloride used in the manufacture of procaine penicillin or hydrochloride would cause variation in the extent of the discoloration. He had no experience of phenol as a bacteriostatic in the product, but he believed that it was not very satisfactory. Procaine hydrochloride when present in a concentration less than 1 per cent. would be effective in stabilising procaine penicillin, and he agreed that in the later stages of the deterioration which he had reported procaine ion would be present in a concentration of something like 0·5 per cent. At that concentration he believed it had exerted a stabilising effect on the suspension without having added procaine hydrochloride. The original *p*H of the suspension was in the region of 7 to 7·3, and no suspension was below *p*H 6·5; this showed the buffering action of sodium citrate. If the figures for the microbiological assay of penicillin in Table I were subjected to statistical analysis, it would be found that the penicillin content actually went down.

THE ORIGIN OF STIMULATION ZONES ON PENICILLIN ASSAY PLATES

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Numerous authors have observed the occurrence of zones of stimulation at the peripheries of inhibition zones on assay plates of penicillin and other antibacterial substances. Pratt and Dufrenoy[1,2] give many references. The phenomenon was ascribed by Ingram[3] to unused nutrients within the inhibition zones being available to the marginal cells. Pratt and Dufrenoy[2] expressed a different view, that "the enhanced growth evidenced by bacteria exposed to sublethal concentrations of penicillin may be ascribed to the action of penicillin *per se*, to degradation products of penicillin or to the release of metabolites by some of the organisms affected by penicillin. It is difficult at the present time to evaluate the extent to which each of these factors contributes to the enhancing action".

The experiments described below were undertaken in an attempt to demonstrate that stimulation may be caused by additional nutrients diffusing from within the zones as well as by a direct or indirect action of the penicillin itself.

Experimental and Results

The organisms used were: *Staphylococcus aureus* N.C.T.C. 4736 and a freshly isolated *Bacterium coli* Type I (44° positive) the latter being relatively resistant to penicillin. The medium generally used was a nutrient broth (oxoid peptone 1 g., Lab-Lemco 1 g., sodium chloride 0·5 g., tap water 100 ml., *p*H 7·4) solidified with 1·5 per cent. of agar, and it was bulk-seeded before pouring with about 10^7 organisms/ml. from a 24-hour agar slope culture. The mean zone diameters were established using 10 I.U./ml. for the staphylococcus and 10^4 I.U./ml. for the *Bact. coli*.

The fact that diffusion of nutrients from within a zone not occupied by bacterial growth can cause stimulation at the zone margin was demonstrated by removing a disc of medium 20 mm. in diameter from a seeded plate with sterile cork borer and filling the hole with sterile melted nutrient agar. After incubation for 18 hours at 37° C. there was a ring of denser growth at the circumference of the circle of sterile agar. The effect was slight with the staphylococcus, but with *Bact. coli* it was quite marked. It was not possible to prevent some organisms spreading on to the sterile agar during filling and scattered colonies always appeared within the "zone". A difficulty was that if the filling did not reach the level of the original surface a spurious effect was obtained, due to the reflection of light at the "step". On the other hand, if overfilling occurred the spreading agar covered organisms which would otherwise have been on the surface,

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with the result that their growth was restricted and the "zone" margin appeared ragged. By studying the reflection of light from the plates immediately after filling the holes, unsatisfactory plates could be rejected.

The next step was to determine whether this nutrient effect was sufficient to account for the growth stimulation observed around zones produced by penicillin, and for this it was necessary to prepare plates containing sterile agar circles of the same size as the zones which would be formed by the stated concentration of penicillin placed in cups in the same plate. The procedure adopted was to pour plates from bulk-seeded medium and when set divide them into 3 equal segments, from 2 of which discs were removed and the holes refilled with sterile medium. Then two 8 mm. cups were made, one in the centre of a refilled hole and the other in the third segment. Penicillin in *p*H 7·0 phosphate buffer solution was then placed in these cups and the plates incubated for 18 hours at 37° C. The diameter of the discs removed was as close as possible to the mean zone diameters in tests with the stated concentrations of penicillin against both organisms. However, as is well known, the individual replicate zones of an assay of a single concentration differ in size and this was especially so with *Bact. coli*. It was therefore necessary to prepare a considerable number of replicate plates in order to obtain sufficient cases where the zone formed by the penicillin was of exactly the same diameter as the sterile agar circles and which were suitable for photographic reproduction.

With *Bact. coli* rings of slight stimulation were formed round the circles of sterile medium (Fig. 1.C). Around the circles of sterile medium containing penicillin the rings were more strongly marked (Fig. 1.A), and were similar in density to those obtained with penicillin used in the normal way (Fig. 1.B). The additional stimulation in the presence of penicillin was clearly visible. With *Staph. aureus*, the contrast between stimulation and background growth was slight in all cases but the results were qualitatively the same. The rings were hardly discernible around the circles of sterile medium and somewhat more strongly marked around the penicillin cups, but plates suitable for photographic reproduction could not be obtained with the nutrient agar. In order to obtain enhanced differences between the degrees of stimulation the experiments were carried out using other media and it was found that enrichment of the medium with yeast extract gave more pronounced stimulations while a less nutrient medium gave smaller stimulations. Thus heavier growth and more pronounced stimulations were obtained with *Bact. coli* on a medium containing 0·5 per cent. each of peptone, beef extract and yeast extract (all Difco) (Fig. 2) than on the nutrient agar (Fig. 1). With *Staph. aureus* poor growth and slight stimulations were obtained on 0·5 per cent. each of peptone and beef extract (both Difco) (Fig. 3) but a richer growth with heavier stimulations on the nutrient agar fortified with 0· 5 per cent. of Difco yeast extract (Fig. 4).

Circles of sterile medium do not exactly represent the nutrient conditions inside a penicillin inhibition zone, which must contain dead and lysed cells. Products from these might be responsible for additional stimulation, supposing they were more readily available to the growing bacteria than

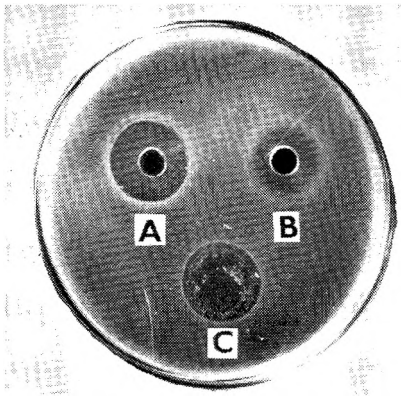

FIG. 1. *Bact. coli* grown on a medium of nutrient agar.

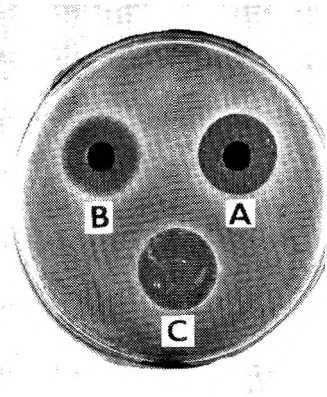

FIG. 2. *Bact. coli* grown on a medium containing 0·5 per cent. each of peptone, beef extract and yeast extract (all Difco).

A. Penicillin cup in unseeded agar disc.
B. Normal penicillin zone in seeded agar.
C. Unseeded agar disc.

(Approximately half natural size.)

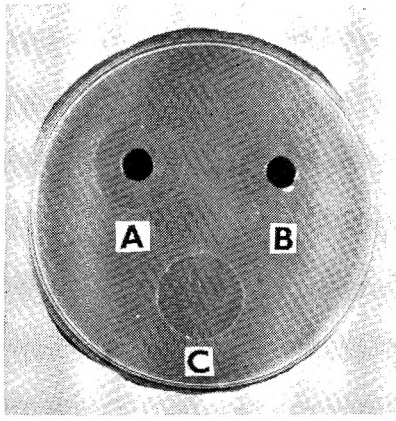

FIG. 3. *Staph. aureus* grown on a medium containing 0·5 per cent. each of peptone and beef extract (both Difco).

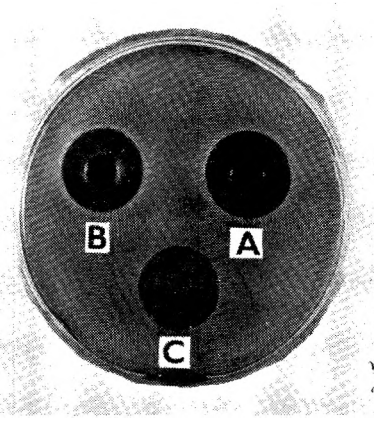

FIG. 4. *Staph. aureus* grown on a medium of nutrient agar fortified with 0·5 per cent. of yeast extract (Difco).

A. Penicillin cup in unseeded agar disc.
B. Normal penicillin zone in seeded agar.
C. Unseeded agar disc.

(Approximately half natural size.)

the nutrients of the sterile medium, so trials were made to investigate this point. The growth on a 24 hours slant culture of *Staph. aureus* 4736 was washed off in 5 ml. of nutrient broth. After incubation for 1 hour at 37° C. penicillin was added to a concentration of 0·02 I.U./ml. and the suspension incubated for a further 5 hours, by which time considerable lysis had occurred. The suspension was then centrifuged and penicillinase was added to the supernatant fluid and allowed to act for 1 hour at 37° C. Drops of the final product were then put into cups in nutrient agar plates seeded with the two organisms, but in no case was there any sign of increased growth around the cups. A control fluid was prepared in exactly the same way except that the bacteria were omitted. A staphylococcus autolysate prepared according to the method given by Todd[4] also gave negative results. It was thought that the lack of growth response to the staphylococcus products might have been due to the use of too rich a medium, so the lysates were tested against both organisms in a synthetic medium (mannitol 1 g., ammonium chloride 0·5 g. in 100 ml. of a mineral salt solution with 1·5 per cent. agar). Stimulation around cups due to lytic products was very weak or completely absent. The slight growth of *Staph. aureus* on this medium without added aneurine was probably due to the presence of small amounts of the vitamin in the mannitol.

Finally, penicillin zones were formed in the synthetic medium, using two seeding rates of 10^6 and 10^9 cells/ml. to determine whether the products from a large number of lysed cells could exert a significant nutrient effect in the poorer medium. It appeared that there might have been a more marked stimulation with the higher seeding rate, but owing to the great differences in zone size at different inoculum levels no reliable conclusions could be formed.

Discussion

It has been shown that nutrients diffusing from an uninoculated zone may cause stimulation at the periphery. The extent of the response was different with the two organisms tested, and this may be a reflection of their nutritional requirements; the non-exacting *Bact. coli* may be capable of greater and more regular stimulation where the exacting *Staph. aureus* may not respond, because growth of the latter is normally limited by the supply of various factors, a slight additional availability of which might be insufficient to produce a marked response. It has also been shown that the stimulation rings round penicillin zones are more marked than around circles of uninoculated agar. There would thus seem to be two effects, one due to increased amounts of nutrients which may be regarded as prolonging the period of active growth[5], and the other due to penicillin, a stimulation which may be regarded as an increase in metabolic rate[6]. But, for an increased metabolic rate to manifest itself in increased growth, additional nutrients must be available. The supply of nutrients from within either a penicillin zone or a disc of sterile medium to the periphery is limited by rates of diffusion, and it is difficult to see why these should be different in the two cases. The extra growth around the penicillin zone might be due to a more effective utilisation of the nutrients, but on this

point no evidence is available. There is, however, an important difference between a penicillin zone and a disc of sterile medium; the former contains dead, possibly lysed, bacteria and products diffusing from these may contribute to the nutrient status at the periphery of the zone. *Bact. coli* and *Staph. aureus* must be considered separately in this connexion, for while Pratt and Dufrenoy[7] have shown that penicillin acts on these species in essentially the same way, *Staph. aureus* is readily lysed by penicillin, possibly by accelerated autolysis[4], while *Bact. coli*, because it is not subject to autolysis[8], probably does not lyse in the presence of penicillin.

Pratt and Dufrenoy[1] have interpreted the waves of post-lytic growth observed in cultures of staphylococci by various workers, e.g., Bonét-Maury[9], in terms of release of nutrients from cells whose permeability has been increased by the action of penicillin, but since the experiments in question were all conducted in media containing abundant nutrients there is no justification for concluding that in the waves of growth nutrients derived from other bacterial cells were being used, especially since increased cell permeability could not be demonstrated by Gale and Taylor[10] with penicillin. There would, however, seem to be no reason why such nutrients should not be utilisable, and indeed it is possible that they may be of especial value, the argument being that they might be structurally very similar to units in living cells and hence easily synthesised into cell substance. Indirect evidence on this point is found in the work of Lesfargues and Delaury[11] on the elaboration by bacteria of substances capable of stimulating the growth of isolated animal tissues, while yeast cells killed by ultra-violet radiations yield materials which increase the growth rate of yeast[12,13,14,15]. The possibility of a similar action in the case of *Bact. coli* is not so well founded.

The presence or absence of stimulation due to bacterial products must, however, depend on the quantity available, and calculation shows that, considering *Bact. coli* cells to have a volume of $2\mu^3$, a specific gravity of unity and a water content of 80 per cent., the seeding rate of 10^7 cells/ml. would provide only about 0·004 mg./ml. of dry matter in the medium of which only part could be of high value. Of this 0·004 mg., nucleic acids might amount to 0·0005 mg.[16] and amino-acids might total 0·0013 mg.[17] Growth prior to lysis might increase these amounts 2 to 4 fold, but even so they are still small compared with the 15 mg. or so of nitrogenous organic nutrients/ml. of the original medium, and evidently, unless the bacterially-derived nutrients have a very high food value indeed, their influence could be only slight. This is supported by the present finding that the amount of stimulation depends on the nutrient value of the medium and by the work of Miller, Green and Kitchen[18], who found stimulatory effects to be produced most readily in a medium fortified with yeast extract. If nutrients from bacteria were of especial value it might be expected that growth rates in the post-lytic waves would be increased, but in fact these growth rates seem to be the same as, or lower than, those recorded prior to lysis[9,19,20,21].

The work of Ingram[3] and of Mitchison and Spicer[22] seems to indicate that stimulation zones are the result of greater development of individual

colonies rather than of a greater number of colonies. This would seem to indicate a direct stimulatory action of penicillin on the cells and this is also suggested by the development of complex ring patterns around assay cups, the most likely explanation for which is that the penicillin diffuses in Liesegang ring patterns[23,24]. Moreover Ericksen[25] found that staphylococci trained to penicillin resistance showed stimulation zones whose widths varied directly with the penicillin concentration.

Summary

The peripheral stimulation around penicillin inhibition zones is due in part to additional nutrients which have diffused from the zones; there is also a stimulation attributable to the penicillin itself or to products of its degradation.

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DISCUSSION

The paper was read by MR. N. D. HARRIS.

MR. N. L. ALLPORT (London) referred to a note in *Nature* by Professor A. D. Gardner in 1940 in which he attributed the spherical enlargement of the organisms to possible imperfect fission. *Streptococcus pyogenes* growing at the edge of the penicillin zones were very much bigger than organisms growing normally. He also commented that in the case of some Gram-negative organisms such as *Bact. coli.* the enlargement of the bacteria may become so great that they even burst. Mr. Allport asked whether the author could throw any light on that.

MR. E. ADAMS (Plymouth) said that he had always thought that penicillin was one of the few antibacterial substances which were relatively insensitive

to differences in inoculum. Burkholder had found that a small concentration of penicillin did not stimulate the growth of organisms.

MR. G. SYKES (Nottingham) said there was no doubt that the basic medium itself was of high significance. With regard to the authors' suggestion that penicillin acted as a stimulant to the growth of the organisms, it was realised that in a diffusion plate assay a dynamic state was being dealt with, and that whilst growth was taking place there was also a potential of diffusion from the cup into the agar. In contrast to that in the turbidimetric assay there was certainly a growth of organisms. There was a definite relationship between the amount of growth and the concentration of organisms and this did not suggest that a small amount of penicillin could possibly give any stimulation.

MR. J. S. CANNELL (Ashton-under-Lyne) said that difficulty had been experienced in obtaining sharp zones when certain suspensions of *B. subtilis* spores were employed. There had been a tendency to obtain small sized zones with rather diffused edges, and it had been found that the addition of *p*-aminobenzoic acid to the medium tended to increase the sharpness of the zone as well as its diameter.

MR. R. L. STEPHENS (Brighton) asked whether it was possible that the stimulation might be due to the diffusion of inhibitory metabolites from the bacteria at the edge of the zone. The author had considered the diffusion of materials from the zone outwards, but what of the self-inhibitory substances which were produced by most micro-organisms during their own growth?

MR. N. D. HARRIS, in reply, said that the enlargement of the cells was not due solely to penicillin or other antibiotics. On enlargement the cells became more transparent. It was to be doubted whether such cells would give the same appearance of intense growth as in fact obtained at the margins of the zones. Although it had been reported that penicillin activity was independent of inoculum size, if the same experiments were carried out using different inocula, considerable differences in the zone sizes would be obtained. He was not very surprised that Burkholder did not obtain stimulation by penicillin. One did not always obtain stimulation with one particular strain of organism on different occasions. The problem of direct stimulation was complex. Even in dilution assays, as the concentration of penicillin increased the density of growth did not necessarily fall. There was a zone phenomenon whereby as the concentration increased there was decreasing growth, then increased growth followed by decreasing growth again. If *p*-aminobenzoic acid was acting as a growth factor then he would have expected smaller zones, but that did not seem to be the case. It might be that *p*-aminobenzoic acid inhibited substances in the medium which were known to inhibit the germination of the *B. subtilis* spores. The appearance of stimulation could be obtained as the result of diffusion of inhibitory substances away from the cells.

THE MICROBIOLOGICAL ASSAY OF MIXTURES OF PENICILLIN AND DIHYDROSTREPTOMYCIN

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Considerable interest has recently been aroused by reports of the superiority of preparations of mixed antibiotics over single substances in the treatment of certain infections. Not only do these preparations often yield a wider antibacterial activity but also, with certain combinations, enhanced activity over that of the individual components. Jawetz and Gunnison[1] divided the commoner antibiotics into two groups, those which were mutually synergistic with each other and never antagonistic, and those which were either indifferent or demonstrated simple additive effects. The first group comprised penicillin, streptomycin, bacitracin and neomycin, and the second aureomycin, chloramphenicol and terramycin. The effects of members of one group with those of the other depended largely on the relative susceptibility of the micro-organism. The reliability of *in vitro* assays of these various combinations might also be expected to be conditioned by similar considerations.

Using a serial dilution method of assay with a *Staphylococcus aureus*, Chopra and Gupta[2] found the minimal inhibitory concentration of penicillin reduced to one-tenth by the addition of 0·002 mg. of streptomycin per ml. and to one-thousandth by 0·007 mg. of streptomycin per ml. In these experiments the ratio of penicillin to streptomycin was of the order of 1 : 50, whereas therapeutic mixtures are generally in the ratio of about 1 : 2. These findings, therefore, may not be relevant to the present problem, but they suggest a possible interference in the assay of the penicillin. Using a plate diffusion technique, Uraya[3] successfully assayed penicillin in the presence of streptomycin by using organisms such as a staphylococcus or "dried grass spores". The method using the "dried grass spores" was not only said to be the simpler method to carry out but also to be capable of assaying low concentrations of penicillin in the presence of high concentrations of streptomycin and to be applicable in assaying blood serum.

Peyré and Velu[4], employing both plate and linear diffusion methods, reported values for mixtures of penicillin and streptomycin using a *Staph. aureus*, and of chloramphenicol and neomycin using *Bacterium coli*, which were practically identical with those of the more rapidly diffusing components. These observations pointed to the absence of interference, stimulatory or otherwise, between the pairs of antibiotics. The American Food and Drug Regulations[5] avoid microbiological estimations and rely on physical methods of assay.

Since mixed antibiotics are administered in previously determined proportions, it is essential that the concentrations of both components

should be accurately known; it is not enough to determine the *in vitro* activity of the mixture and from it assume that the correct proportions are present. With the mixture at present under consideration, the two antibiotics concerned exhibit different antibacterial activities, penicillin being selective against Gram-positive and dihydrostreptomycin active against both Gram-positive and Gram-negative organisms. It would seem reasonable,, therefore, to assay the dihydrostreptomycin component by means of a suitably sensitive Gram-negative organism. Alternatively, should the mixture how a synergistic effect, penicillinase could always be used to inactivate the penicillin, after which treatment any streptomycin-sensitive organism should be effective.

The problem with the assay of the penicillin component appeared to be somewhat more difficult at the outset, because both penicillin and dihydrostreptomycin aré sensitive to the same groups of organisms. Arquie *et al.*[6] have described a "streptomycinase" derived from several organisms including *Pseudomonas pyocyanea*, and Lightbown[7], whilst not agreeing that it is an enzyme, states that it is equally effective against dihydrostreptomycin; unfortunately it is also inhibitory to bacterial growth. Several other substances, such as hydroxylamine, semicarbazide and cysteine, known to inhibit the activity of streptomycin, are either ineffective against dihydrostreptomycin or are also active to some degree against penicillin. It was eventually established that the success of any microbiological method for the assay of penicillin in the presence of dihydrostreptomycin must depend on the absence of interference, synergistic or otherwise, of the dihydrostreptomycin.

Experimental

Assay of Dihydrostreptomycin. Weighed amounts of dihydrostreptomycin sulphate with a potency of 745μg/mg. and of sodium benzylpenicillin with a potency of 1690 I.U./mg. were mixed intimately and

TABLE I

Results of assays of dihydrostreptomycin when mixed with penicillin

Test material	Assay values for dihydrostreptomycin using	
	B. subtilis	*Bact. coli*
Dihydrostreptomycin	745 μ/mg.	745 μ/mg.
Dihydrostreptomycin + penicillin ..	734, 776 } = 755 μ/mg.	752, 758 } = 755 μmg.

submitted to assay by the cylinder-plate method. Two test organisms were used, *Bacillus subtilis* (N.C.T.C. 6752) and *Bacterium coli* (N.C.T.C. 86). Since penicillin is known to be more active, weight for weight, than dihydrostreptomycin against *B. subtilis* it was obvious that in carrying out assays with this organism, the penicillin should be first inactivated. Accordingly, a known amount of the mixed solid was dissolved in water and treated with excess penicillinase for 30 minutes. The solution was then diluted further with appropriate amounts of buffer solution and the

assay continued in the usual manner. The results obtained are given in Table I.

In carrying out assays with *Bact. coli* it did not seem necessary to inactivate the penicillin, consequently the solutions were made in standard buffer and the plate assay continued in the usual manner. The medium used in this case was a modified McConkey's agar as suggested by Sykes and Lumb[6]. Results of these assays are also given in Table I. Both methods yielded satisfactory values, hence either can be considered suitable for the assay of the dihydrostreptomycin component.

Assay of Penicillin. From previous experience, the zones of inhibition produced in the plate assay are much smaller with dihydrostreptomycin than with the same amount of penicillin. The ratio varies with the *p*H of the test solutions, the culture medium used and the test organism, the value being greater with *Staph. aureus* than with *B. subtilis*. *Staph. aureus* (N.C.T.C. 6571) was therefore chosen as the test organism using a nutrient agar made from the tryptic digestion of ox heart. In the first trials simple assays of the penicillin-dihydrostreptomycin mixtures were made against pure penicillin standard. These consistently revealed fluctuations in the slope of the log dose-response curve which were sufficiently great to render any attempt at assessing potencies valueless (Fig. 1). In an attempt to overcome this, certain substances believed to inhibit, but not to destroy, the dihydrostreptomycin were added to the assay medium. Solutions of penicillin and of the mixture were assayed over the range 20 I.U./ml. to 0·2 I.U./ml. using nutrient agar containing either 0·03 per cent. of sodium thioglycollate or 0·03 per cent. of semicarbazide

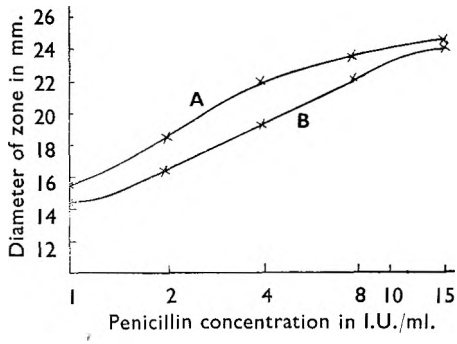

FIG. 1. Cylinder-plate assay responses of penicillin and of penicillin-dihydrostreptomycin mixtures with trypsin broth agar medium.

A. Penicillin with dihydrostreptomycin.
B. Penicillin.

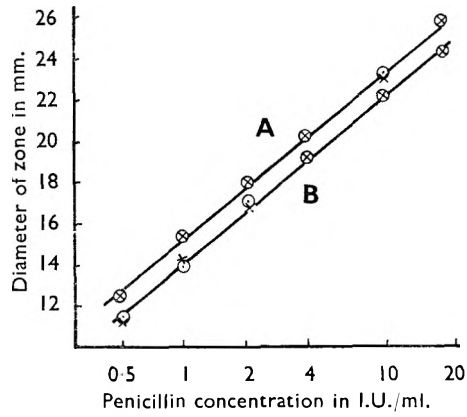

FIG. 2. Cylinder-plate assay responses of penicillin and of penicillin-dihydrostreptomycin mixtures with different agar media.

A. Using B.P. assay medium.
B. Using peptone-yeast extract medium.
⊗ Penicillin.
⊙ Penicillin dihydrostreptomycin mixture.

hydrochloride. In both cases the relationships between the two slopes were improved, but were not satisfactory. With thioglycollate they still lacked consistent parallelism and with semicarbazide they remained parallel but did not coincide, giving an average of 10 per cent. apparent stimulation of the penicillin activity. The zone edges were also rather diffuse, making the diameters difficult to measure.

A more important factor in countering the stimulatory or other influence of the mixture appeared to be the assay medium. When tryptic digest agar was replaced with that prescribed for the assay of aureomycin in the British Pharmacopœia, 1953, or with a simple peptone-yeast extract medium, (peptone 2 per cent., yeast extract 0·2 per cent., sodium chloride 1 per cent., agar 1·5 per cent., adjusted to *p*H 7·0), the log dose-response slopes for penicillin and for an equi-unit penicillin-dihydrostreptomycin mixture were found to coincide exactly, and the slope over the range 0·5 I.U./ml. to 20 I.U./ml. was almost a straight line (Fig. 2). The aureomycin assay medium, however, yielded zones with large halos which made their measurement difficult and tedious, whereas those from the peptone-yeast extract medium were clearly defined.

Increase in the unit concentration of the dihydrostreptomycin component to twice that of the penicillin or decrease to one half did not affect the results beyond the normal limits of error of the assay.

Suggested Methods of Assay

From the foregoing experimental observations the following methods for the assay of the components of a penicillin-dihydrostreptomycin mixture are suggested.

Dihydrostreptomycin

(*a*) *Using Bact. coli.* Dissolve a weighed quantity of the mixture in 0·05 M phosphate buffer, *p*H 7·8, and from this prepare dilutions containing approximately 10 and 100μg. per ml. Prepare similar dilutions of the Standard Preparation of Dihydrostreptomycin. Carry out a normal cup-plate assay with a 24 hour broth culture of *Bact. coli* (N.C.T.C. 86) and using a medium containing peptone 2 g., bile salts 0·5 g., neutral red (1 per cent. solution) 0·5 ml., agar 1·5 g., made up to 100 ml. with water and adjusted to *p*H 7·8 to 8·0.

(*b*) *Using B. subtilis.* Prepare a solution of the mixture in 0·05 M phosphate buffer, *p*H 7·8, add sufficient penicillinase to inactivate the penicillin, allow to stand for 30 minutes and then prepare further dilutions to contain 1 and 10μg. of dihydrostreptomycin per ml. Carry out a normal cup-plate assay with a spore-suspension of *B. subtilis* (N.C.T.C. 6752) using a medium containing peptone 0·5 g., meat extract 0·3 g., agar 1·5 g., made up to 100 ml. with water and adjusted to *p*H 7·8 to 8·0.

Penicillin

Proceed with the assay of penicillin as described in the British Pharmacopœia, 1953, p. 796, but use the Oxford strain of *Staph. aureus* (N.C.T.C. 6571) with a medium containing peptone 2 g., yeast extract 0·2 g.,

sodium chloride 1 g., agar 1·5 g., made up to 100 ml. with water and adjusted to *p*H 7·0.

If the preparation is in an oily base, first suspend 1 ml. of the preparation in 10 ml. of chloroform and extract with 3 successive quantities of 20 ml. of phosphate buffer. Bulk the extracts, dilute further as required with the appropriate phosphate buffer and complete the assays described above.

To illustrate the reliability of these methods, a suspension of sodium benzylpenicillin and dihydrostreptomycin sulphate each at a nominal concentration of 100,000 units per ml. in a liquid paraffin base containing 0·75 per cent. aluminium stearate was assayed against the appropriate standards with the following results:—

Dihydrostreptomycin

Nominal value 100,000 U./ml.
Values found with *Bact. coli* (*a*) 96,000 U./ml.
(*b*) 103,000 U./ml.
„ „ „ *B. subtilis* 95,500 U./ml.

Penicillin

Nominal value 100,000 I.U/ml.
Value found with B.P. aureomycin assay medium 96,000 I.U./ml.
„ „ „ peptone-yeast extract medium 93,000 I.U./ml.

SUMMARY

1. Methods have been described for the microbiological assay of dihydrostreptomycin and of penicillin in mixed preparations.
2. The assay of dihydrostreptomycin was carried out with *B. subtilis* or *Bact. coli* and presented no difficulty.
3. The assay of penicillin was carried out with *Staph. aureus* and its reliability was found to be dependent on the assay medium used, satisfactory results being obtained with a peptone-yeast extract medium.

We wish to thank Mrs. H. M. Payne, B.Sc., for her assistance in carrying out many of these assays.

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DISCUSSION

The paper was presented by MR. D. G. LEWIS.

MR. J. W. LIGHTBOWN (Mill Hill) said that one of the most important principles in biological assay was that both standard and the test should contain the same biologically active substances. That was not always

possible, and then any biological changes would affect the test and the standard to a different extent. In the assay under discussion the results depended upon the medium. They would also be dependent on the *p*H, which was controlled by many other factors. Therefore, although it was quite likely that the authors could carry out an assay in their own laboratory and obtain good results, it was probable that if others tried to use it in other laboratories they would run into difficulty. It was difficult to obtain standardised media, and, using the same ingredients, even different batches in the same laboratory varied significantly. Had the authors tried the assay with a number of batches, and had they always obtained satisfactory results? He felt that in the assay of penicillin and dihydrostreptomycin mixtures it would be more satisfactory to isolate penicillin chemically or physically and assay it separately.

MR. H. P. LEGGETT (Liverpool) said that a more satisfactory process in his view would be to remove dihydrostreptomycin by precipitating with silicotungstic acid. With a mixture containing procaine penicillin as well as a soluble salt, precipitation would take out the procaine penicillin as well, enabling one to work through the components individually.

MR. D. G. LEWIS, in reply, said that various manufacturers' peptones had been tried. They all gave satisfactory assays and no difference was found in the laboratory between the batches. The suggestion for removing the penicillin and other ingredients was a good one.

THE DEMONSTRATION OF PROLONGED ACTION OF LONG-ACTING INSULIN PREPARATIONS IN THE GUINEA-PIG

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Since Reiner, Searle and Lang[1] first prepared globin insulin and described its prolonged action in rabbits, the preparation has been used with success by diabetics throughout the world in the control of hyperglycæmia. When the Provisional British Standard for Globin Zinc Insulin was examined for prolonged action in rabbits against the International Standard for Soluble Insulin, Emmens *et al.*[2] concluded that "no routine method of estimating the delayed action of globin zinc insulin can be recommended since a detailed investigation revealed that the rate of recovery of rabbit blood sugar following the decrease induced by globin zinc insulin is biometrically indistinguishable from that induced by soluble insulin unless an impracticably large number of animals is used in a cross-over test".

The United States Pharmacopeia XIV, however, outlines a test for the demonstration of prolonged action of globin zinc insulin in rabbits using as a standard a preparation of U.S.P. Zinc-Insulin Crystals Reference Standard combined extemporaneously with the U.S.P. Globin Reference Standard prior to the test. Dr. Ferry[3] of Burroughs Wellcome and Co. (U.S.A.) Inc., has provided data which show the prolonged action of globin zinc insulin when compared with soluble insulin in rabbits.

The success of American workers in demonstrating a prolonged action with globin zinc insulin in rabbits may in part be due to the type of rabbit used or, in part, to the diet which is fed to the animals.

The details and data which follow describe the use of the guinea-pig for the demonstration of prolonged action of long-lasting insulin preparations.

Method

All guinea-pigs used were albinos, either home-bred or obtained from dealers. They were separated as regards sex and given an *ad lib.* diet of greens, diet 18[4] and water. 16 hours prior to the commencement of the test the animals were taken into the laboratory and food and water removed from their cages. For any one test the animals were either all males or all females. On the day of the test the guinea-pigs were weighed to the nearest g. and placed in individual cages. The weight range of the guinea-pigs has been from 200 to 500 g. though for any one test the heaviest has not exceeded the lightest by more than 160 g. Immediately before the injection of the insulin preparation a little over 0·1 ml. of blood was removed from the heart using a 23 S.W.G. $\times$ $\frac{5}{8}$ needle fitted to a 1 ml. tuberculin syringe. The blood sample was straightway fed into an 0·1 ml. graduated micropipette, and the accurately measured 0·1 ml. of blood passed into the deproteinising suspension of Somogyi[5]. Individual reducing sugar con-

centrations were determined by the method of Hagedorn and Jensen[6]. After the collection of the initial blood sample the undiluted insulin preparation was injected subcutaneously above the pelvic girdle using a micrometer syringe. The absolute sensitivity of guinea-pigs is less than that of rabbits and the doses used have ranged from 1·5 to 3·0 I.U./kg. depending upon environmental temperature and weight of animals, though kept constant for any one experiment. Following each injection the time was noted, and at hourly intervals thereafter 0·1 ml. samples of blood were collected in a similar manner.

Since we wished the blood sugar concentrations of guinea-pigs receiving soluble insulin to return at the 6th hour to 90 per cent., or greater, of the initial value it was necessary to exercise each guinea-pig 15 minutes prior to the collection of the blood sample by holding the hind limbs off the bench and allowing the animal to exercise itself with its fore limbs for 30 seconds. This exercising was carried out at each hour whether a blood sample was collected or not. Guinea-pigs ear-marked at the end of the test could be used for a reverse test after a rest period of one week.

The data which follow are derived from tests carried out from January to June, 1953 in a laboratory where the environmental temperature was from 50° to 78° F., though on any one day it never ranged more than 10° F.

Results

The results are presented as mean blood sugar values (as a percentage of the initial) at each hour for which determinations were made.

All data were submitted to variance analyses, and the significance of differences between mean blood sugar values for prolonged action and soluble insulins at each hour was determined by means of Student's t-test[7].

The soluble insulin used in all the experiments which follow was the British Insulin Manufacturers' Sub-standard of Crystalline Insulin having a potency of 23·35 I.U./mg.

Typical Analysis

The Provisional British Standard for Globin Zinc Insulin was tested against soluble insulin in a 1-day experiment. The animals were virgin albino females. Table I shows the mean blood sugar values for the globin and soluble insulins, and the significance of the differences.

Table II gives the analysis of variance of the data.

TABLE I

Blood sugars as a percentage of initial values

Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
British Globin Standard ..	79·1	60·2	56·0	60·1	59·5	60·2	8
Soluble insulin	72·1	59·0	62·6	66·0	81·1	96·3	8
P difference	*—	—	—	—	<0·1	<0·01	

Weight range: 230 to 292 g. Dose: 2·5 I.U./kg.

* In this table, and all subsequent tables — indicates that the P difference is >0·2.

Differences between insulins must be tested against the mean square for "between animals within treatments", in this instance 473·20. This value is therefore used as the estimate of the error variance for testing significance of differences between means. Since each mean in Table I is based upon 8 observations the variance of any mean is $\frac{473{\cdot}20}{8} = 59{\cdot}15$. As all means in the table have the same variance, the variance of the difference between any two means is twice the variance of a single mean, i.e., $59{\cdot}15 \times 2 = 118{\cdot}30$. The standard error of the difference is therefore $\sqrt{118{\cdot}30} = 10{\cdot}88$, which is used to calculate "t", for the significance of the differences *P* given in Table I. It will be noted that in order to perform the "t" test it is only necessary to make part of the detailed analysis of variance. Provided the mean squares for between treatments and between animals within treatments are available, the remainder of the analysis is unnecessary for routine purposes.

TABLE II

ANALYSIS OF VARIANCE

Source of variance	Degrees of freedom	Sums of squares	Mean square	P
Between insulins	1	2565·77	2565·77	<0·05 >0·01
Between hours	5	5415·54	1083·11	
Hours × insulins	5	5028·23	1005·65	
Between animals within treatment ..	14	6624·82	473·20	
Within animals	70	4845·21	69·22	

COMPARISON OF THE "CROSS-OVER" TEST WITH ONE-DAY TESTS

A "cross-over" test on a routine production batch of globin zinc insulin (G.Z.I.) was carried out in male albino guinea-pigs. Table III gives the mean percentage blood sugar values, averaged over the two days, and the significance of differences as before.

TABLE III

BLOOD SUGARS AS A PERCENTAGE OF INITIAL VALUES

Hours after injection	1	2	3	4	5	6	Number of animal observations/ preparation
G.Z.I.	70·1	47·7	49·9	56·7	61·8	68·5	16
Soluble insulin ..	68·8	55·6	71·6	92·2	97·7	101·5	16
P difference	—	—	<0·01	<0·001	<0·001	<0·001	

Weight range: 256 to 351 g. Dose: 2·0 I.U./kg.

The "cross-over" test therefore demonstrates satisfactorily the prolonged action of globin zinc insulin in guinea-pigs. Since it is normally necessary to wait one week before performing the second half of a "cross-over" test (as with rabbits) several one-day runs were carried out to find out whether they were satisfactory for routine testing. Tables IV (*a*), (*b*), and (*c*) show the results of 3 such trials with 3 different routine production batches of globin zinc insulin.

The 1-day test is therefore quite reliable for the demonstration of prolonged activity.

TABLE IV

BLOOD SUGARS AS A PERCENTAGE OF INITIAL VALUES

(*a*) Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
G.Z.I.—A	77·0	43·0	43·4	54·6	71·0	78·0	7
Soluble insulin	80·0	57·7	69·7	103·9	107·1	108·2	7
P difference	—	—	<0·01	<0·001	<0·001	<0·01	

Weight range: 303 to 363 g. Dose: 2·0 I.U./kg.

(*b*) Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
G.Z.I.—B	70·6	63·6	49·2	55·3	55·7	72·3	8
Soluble insulin	66·8	64·7	63·9	86·1	94·2	112·7	8
P difference	—	—	—	<0·01	<0·001	<0·001	

Weight range: 232 to 301 g. Dose: 2·5 I.U./kg.

(*c*) Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
G.Z.I.—C	69·6	46·1	52·3	43·5	52·4	55·8	7
Soluble insulin	59·0	44·5	53·4	80·5	93·1	100·0	7
P difference	—	—	—	<0·01	<0·01	<0·01	

Weight range: 223 to 303 g. Dose: 2·0 I.U./kg.

THE PROLONGED ACTION OF PROTAMINE ZINC INSULIN

Table V shows the prolonged action in guinea-pigs of a commercial sample of protamine zinc insulin.

TABLE V

BLOOD SUGARS AS A PERCENTAGE OF INITIAL VALUES

Protamine Zinc Insulin. (P.Z.I.)

Hours after injection	1	2	3	4	5	6	Number of animals/ preparation
P.Z.I.	114·9	92·5	71·0	59·7	56·2	62·3	5
Soluble insulin	82·5	63·2	66·8	79·4	97·6	108·8	5
P difference	<0·05	<0·1	—	—	<0·02	<0·01	

Weight range: 258 to 366 g. Dose: 2·5 I.U./kg.

DISCUSSION

The above results clearly indicate that the guinea-pig is a satisfactory test animal for the demonstration of the prolonged action of long-acting insulin preparations, whereas in the rabbit[2], the prolonged action of globin zinc insulin cannot be demonstrated. This difference can be either one of species or of sensitivity to soluble insulin. The latter reason is more probably correct for Ferry[3] believes that the retardation effect is less evident in rabbits when a small dose is given than when a large dose is given. Since the guinea-pig is less sensitive than the rabbit, weight for weight, in its response to soluble insulin a much larger dose of the long-acting insulin has to be given to produce a response, thus increasing the

amount given of the agent producing the prolonged action. The ratio of this agent to the dose of insulin is, however, always the same whether a large or small dose is given. Experiments with globin zinc insulin have been conducted recently in our laboratory on fed rabbits where it has been necessary to increase the dose of insulin given to produce a response. A significant prolonged action has been demonstrated. It thus seems probable that a minimum amount of the prolonging agent, not directly dependent on the amount of insulin injected, has to be present in the tissue fluid at the site of injection, or in the serum, to produce a satisfactory prolonged action. Further experiments are in progress to clarify this problem.

It would be valuable to conduct parallel trials in diabetics and the guinea-pig and rabbit with all the prolonged-acting insulins to ascertain the most satisfactory animal test for the control of insulin preparations used clinically.

SUMMARY

1. A method is described using small groups of guinea-pigs for the demonstration of the prolonged action of globin insulin and protamine zinc insulin.

2. A 1-day test gives results which compare favourably with those of a 2-day "cross-over" test.

The authors wish to thank Mr. D. Hunt and Mr. K. Rivett for valuable technical assistance, Dr. M. R. Sampford and Mr. P. A. Young for advice on the statistical analysis, and the Directors of the Wellcome Foundation for permission to publish this paper.

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DISCUSSION

The paper was presented by MR. G. A. STEWART.

DR. F. HARTLEY (London) said that hitherto workers in this country had failed to demonstrate prolongation of action in rabbits, and, as the authors pointed out, that differed from American experience. Even clinically, experience in this country by no means confirmed the extent of the prolongation which seemed to have been found in humans in the United States. The author had only recorded one set of results with protamine zinc insulin in guinea-pigs and the impression might be given that globin zinc insulin gave a similar prolongation to that given by protamine zinc insulin. That was contrary to clinical experience, and amplification of Table 5 by the inclusion of results beyond the 6-hour

period might well have brought out the more prolonged action of protamine zinc insulin. He would like to have seen tests carried out in guinea-pigs with soluble insulin. Was it certain that a delayed action would not have been demonstrated in some of the animals?

MR. G. A. STEWART, in reply, said that soluble insulins had been tried in guinea-pigs. They had not demonstrated any delay action, nor did the curves differ. The test described was only the demonstration of the prolonged action of globin zinc solution and was not a quantitative assay. He agreed that if the tests reported in Table V were continued for a longer period protamine zinc insulin would show a greater prolongation of the effect than globin zinc insulin.

AIR FILTERS FOR SMALL-SCALE ASEPTIC UNITS

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In the last few years the importance of air hygiene has become increasingly recognised; many procedures now need to be carried out in dust-free atmospheres and in some cases in air completely freed from micro-organisms. This importance is reflected in numerous publications, in particular in the Medical Research Council Special Report, "Studies in Air Hygiene[1]," in the American Association for the Advancement of Science monograph, "Aeriobology[2]," and in the symposium "Air Disinfection and Sterilisation" organised by the Microbiology Group of the Society of Chemical Industry[3]. Although much attention has been given to other aspects of sterilisation procedures, relatively little work seems to have been done on the performance of filters for supplying bacteria-free air and it is the purpose of this paper to consider the efficiencies of some available commercially, particularly of those used in small units, e.g., for the single unit "sterile" room.

Filtration is one of the several methods which might be considered and is the one which has proved most practicable for this purpose. Other methods include washing with water containing a germicide, electrostatic precipitation, high pressure compression with filtration and treatment with ultra-violet radiations or with a bactericidal aerosol or vapour. The first three of these require large and expensive installations; ultra-violet light treatment is unsuitable because of its relative slowness in action, its limited range of activity and ineffectiveness against bacterial spores and other protected organisms (see, for example, Bourdillon *et al.*[4] and Mellors[5]), and bactericidal aerosols are impracticable when required for continuous use.

A filter for the purpose under consideration should have a high filtration efficiency combined with an adequate air flow capacity and low back pressure, and those most generally used are of a fibrous construction. Glass wool, cotton fibres and slag wool are the materials usually used, and there is little doubt from the published work of Terjesen and Cherry[6] and later of Cherry, McCann and Parker[7] that slag wool is the most effective. However, it suffers the considerable disadvantage of having a high resistance to air flow, and is thus unsuitable for many installations and is precluded from the present discussion.

The interstices of all fibrous filters are relatively coarse in terms of the sizes of particles to be filtered and it follows, therefore, that the mechanism of filtration is not dependent simply on mechanical sieving. Considerable theory on the factors governing the efficacy of filtration of aerosol particles has been proposed in recent years. Of all those considered, the most important according to Stairmand[8] are the impingement and

diffusion factors. Efficiency of filtration may be expected, therefore, to vary with rate of air flow as well as with mass or size of particles to be filtered. The published experimental evidence in support of the theories, particularly with bacteria used as test particles, is unfortunately scanty. Phillips[9] claimed that bacteria-free air supplies could be obtained continuously over periods of several months with glass-wool filters with flow capacities of from 1 to 600 cu. ft./minute; Yaglou and Wilson[22] claimed an efficiency of 40 to 60 per cent. with oiled glass or steel wool filters against normal air-borne organisms; DallaValle and Hollaender[11] with several commercial filters found efficiencies of 65 to 80 per cent. against spores of the hay bacillus, and Decker *et al.*[12] obtained a 98 per cent. efficiency with a special spun glass filter and later claimed an efficiency of over 99 per cent. with a similar filter with a flow capacity of 250 cu. ft./minute against *Serratia indica* and *Bacterium coli* bacteriophage. The considerable discordance between these results arises probably because they were obtained somewhat empirically under fixed conditions of air flow, bacterial infection, etc., and no attempt was made to examine the filters systematically. On the other hand, Terjesen and Cherry[6], working with a specially constructed oiled glass silk filter, and Kluyver and Visser[13], working with carbon granule filters, both found variation in efficiency with rate of air flow.

Experimental

The filters examined in these experiments were confined to those available commercially, and were constructed of oil-treated glass fibres, oil-treated fine wire gauze or cotton fibres. Tests were carried out with each filter with different sources of infection and over a wide range of air flows. The apparatus used was the same in principle as that described by Terjesen and Cherry[6] and is shown diagrammatically in Figure 1.

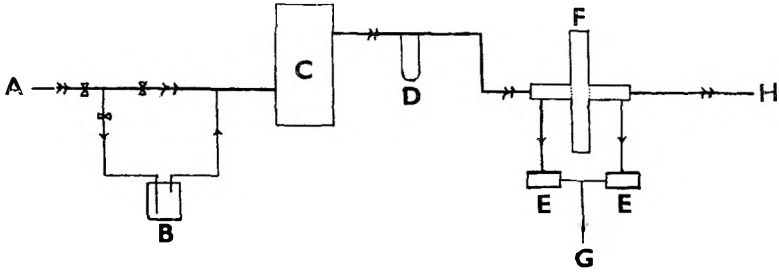

Fig. 1. Arrangement of apparatus for testing air filters.

A. Air input.
B. Source of infection.
C. Mixing tank.
D. Flow meter.
E. Slit sampler.
F. Test filter.
G. Vacuum.
H. Exhaust.

It consisted essentially of a controlled source of air and of bacterial infection, a mixing tank, the test filter, and air sampling devices immediately before and after the filter. The air supply was taken from a service main and was practically free from bacteria. The bacterial infection was introduced into the main air stream through a venturi throat system

from either a sprayed aerosol of a culture of *Chromobacterium prodigiosum*, or a spore-infected dust maintained in a state of constant but gentle agitation to ensure evenness of infection during an experiment. The bacterial cloud density of the sprayed culture varied between 100 and 500 cells per cu. ft. and of the dust mixture between 50 and 500 cells per cu. ft., but the level was maintained constant for any one experiment. Two sources of infected dust were used, (1) from pellicle cultures, and (2) from centrifuged shaken cultures of *Bacillus subtilis*, ground and diluted with French chalk. These mixtures gave different responses in the filters, as will be seen from Table I, due to the different state of separation of the organisms. Standard Bourdillon-type slit-samplers were used for testing the air, samples of 1 cu. ft. being taken before, and up to 15 cu. ft. after, filtration.

TABLE I

VARIATIONS IN EFFICIENCIES OF FIBRE FILTERS WITH AIR FLOW RATE

	Percentage filtration efficiency with								
	Glass fibre filter			Wire gauze filter			Cotton fibre filter		
Air flow rate (ft./minute)	Aerosol	Dust 1	Dust 2	Aerosol	Dust 1	Dust 2	Aerosol	Dust 1	Dust 2
864	40	96							
665			92	34	84		55		95
576	19	92		29	75		45		
475			82	20	72		45		92
380		79	63	14	64		41		85
288	7		58	10	59		45	99	90
184	1	82	52	19	54		46		88
123			48	20	43		48		82
92	15	70						97	
60			49	25	46		56		81
30	46	74	40						79
18			46					97	84
12		71							88
6			53						90
2		85							

Each individual filtration experiment was run for a period of at least 3 hours, but in some cases it was continued for several days; this did not seem to affect any of the results obtained. At intervals during each experiment, slit sampler counts were taken of the air before and after filtration. At least 3 such observations were made and from their means the percentage efficiency of the filters were calculated.

The smallest filter obtainable commercially was 10″ × 10″ × 2″ deep, and as this was larger than was convenient for much of our experimental work, the majority of the filter was blanked off on both sides leaving a test area near the middle of only $4\frac{1}{2}$ sq. in. This enabled considerable air velocities to be employed without demanding abnormally large volumes of air. Air flows were always measured in terms of velocities in preference to volumes per unit of time.

RESULTS

The standard glass wool filters examined were made from long glass fibres treated with a light adhesive oil. Their mean diameter was 180 μ, but ranged from 92 μ to 360 μ. In addition, 3 others supplied specially

by one of the makers of these filters were also examined. The cotton fibre filters consisted of a thin layer of cotton wool spread evenly over a cotton gauze and held rigid on a wire gauze mount. This mount was bent in the filter frame into a multi-"V" form, presumably to afford a greater filtration area within the frame of the filter. On close examination, both of these types of filter exhibited small variations in evenness and density of packing, a factor which was manifested in differences between the performances of filters of the same type or of areas of the same filter. The wire filter was built up of several layers of 18 mesh, oil-treated wire gauze, alternate layers being bent in multi-"V" form. The pressure differences across all of these filters were negligibly small, observed values being less than 0·2 in. except at very high air-flow rates.

The first experiments were of a preliminary nature to determine whether a bacterial aerosol gave the same responses as dust-borne bacteria. The aerosol of *Chr. prodigiosum* had a particle size of about 1 μ and the spore-infected dust from 2 μ to 30 μ, the majority lying between 2 μ and 15 μ. Typical comparative results from the same filter under otherwise identical conditions were:—

Glass fibre filter—14·7 per cent. efficient against the aerosol, 70 per cent. efficient against infected dust.

Cotton fibre filter—45 per cent. efficient against the aerosol, 92 per cent. efficient against infected dust.

Wire gauze filter—19 per cent. efficient against the aerosol, 54 per cent. efficient against infected dust.

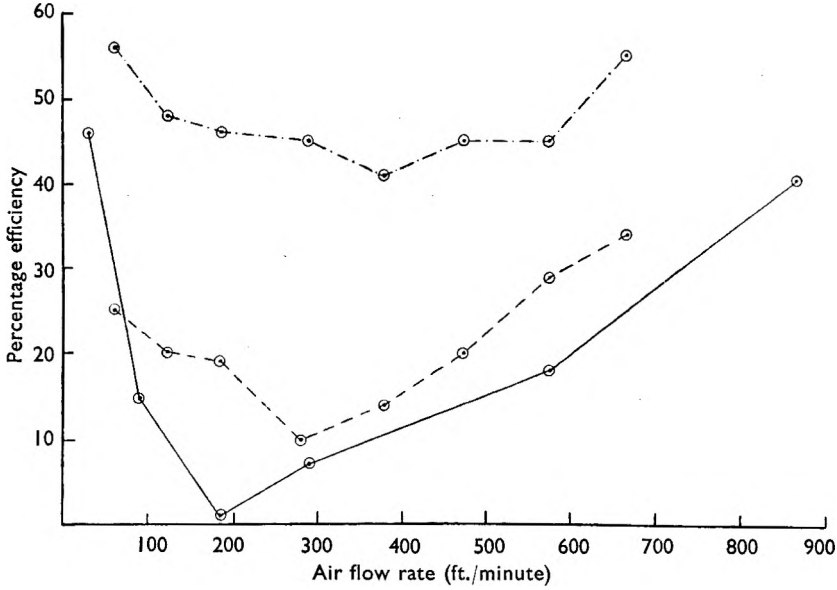

FIG. 2. Variation in efficiencies of filters with air flow rate using a bacterial aerosol.
—— Glass fibre filter.
– – – Wire gauze filter.
–·–·– Cotton fibre filter.

The significance of the differences in particle size was immediately evident, thus making it imperative that both types of infection should be included.

Examination of the influence of air flow rate on the efficiency of filtration showed it to be highly significant. With each type of filter the pattern of response was similar but was operative at different levels. Typical results are quoted in Table I and illustrated in Figures 2 and 3

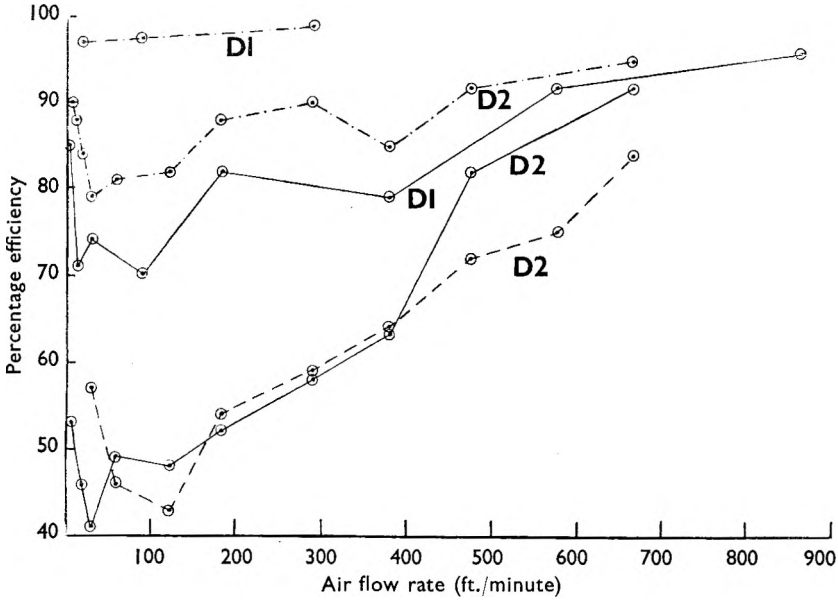

FIG. 3. Variation in efficiencies of filters with air flow rate using spore-infected dusts.

——— Glass fibre filter
– – – – Wire gauze filter
–·–·– Cotton fibre alter.

D1. Dust 1.
D2. Dust 2.

showing that, starting with low initial velocities, increasing air speeds resulted in a fall in efficiency followed by a continuous rise which was maintained over the remainder of the range examined. It is of interest to note that one manufacturer recommends that glass fibre filters be used at an air velocity of about 300 ft. per minute (equivalent to 200 cu. ft. per 10 in. square filter) at which rate the efficiency of filtration against bacteria is near its minimum. The tests also illustrate the anticipated different responses of the relatively coarse glass fibre or wire gauze filter materials against the finer cotton fibre filter.

One disadvantage found with the cotton fibre filter was the rapidity with which it became clogged when used against dust. For this reason, and also because the use of two filters in series is often recommended, experiments with more than one filter were carried out. Table II shows the results of tests using two filters with air flows of about 300 ft. per minute against spore-infected dust 1. With two glass fibre filters the response showed practically no improvement over that from one; with

a glass fibre filter followed by a cotton one, the response was the same as that from the cotton filter alone. In this case, the cotton filter showed no sign of clogging even after several days continuous use.

TABLE II

EFFICIENCIES OF TWO FILTERS IN SERIES

Filters used	Percentage efficiency of filtration	
	Test 1	Test 2
One glass fibre	89	84
Two glass fibres..	90	90
One glass fibre and one cotton fibre ..	98·5	98
One cotton fibre	99	—

Tests against spore-infected dust

Further evidence of the lack of improvement with multiple glass filters is shown in Table III, when three filters were tested in series against the aerosol and against spore-infected dust 2. Air samples were taken after each filter and in every case the majority of the infection removable by these filters under the conditions given was taken out by the first one, the effect of the second and third filters being negligible or only small.

TABLE III

EFFICIENCIES OF THREE GLASS FIBRE FILTERS IN SERIES

Air flow rate (ft./minute)	Bacterial infection	Percentage efficiency of filtration after		
		1 filter	2 filters	3 filters
864	Aerosol	52	50	44
576	"	35	44	46
465	Dust 2	49	61	66
288	" "	40	45	54
184	" "	32	30	33

In Table IV are shown the results of tests with the 3 specially made filters against spore-infected dust at 3 different air flows. Filter A was more densely packed with a normal grade of glass fibre; Filter B had graded fibres, coarse ones on the input side graduating to finer ones on the outlet; filter C was a normal filter but had been treated with a different adhesive oil. No great differences in performance were evident under the conditions chosen, but it appeared that each of the specially made filters was slightly better than a normal one taken from stock.

TABLE IV

EFFICIENCIES OF DIFFERENT GLASS FIBRE FILTERS

Air flow rate (ft./minute)	Percentage efficiency of filtration of filter			
	A	B	C	Normal
360	95	95	94	88
270	86	89	89	86
180	82	87	81	80

A = more densely packed filter.
B = graded fibre filter.
C = normal filter with different adhesive oil.

Filters installed in aseptic rooms. A few experiments with some of the filters were carried out under actual working conditions in an aseptic filling room. This room measured about 12 feet cube and was fitted high in one wall with a fan supplying controlled normal air through the test filters. The air escaped naturally through door gaps, etc., thus giving a transverse-downward air flow. In these experiments a normal air contamination was allowed to develop with two operators moving about freely for some time. The room was then vacated, and flushed with an air flow equivalent to 8 changes per hour, the air being sampled at intervals by means of the slit sampler. With either a glass fibre, cotton fibre, or a glass fibre followed by cotton fibre filter, the bacterial content was reduced to 3 to 6 organisms per cu. ft. in about 30 minutes and this level remained practically unchanged after further prolonged flushing. This order of result is borne out in the very many observations taken over a number of years in aseptic filling rooms whilst normal activities were in progress, when counts of 5 to 10 organisms per cu. ft. have been regularly obtained. They support the findings of Yaglou and Wilson[10] and of Coulthard[14], who reported low bacterial counts in rooms flushed with filtered air.

For many operations the screen has become an indispensable part of the sterile room equipment. A logical development of the foregoing observations was to apply it to the screen itself and flush it with a continuous supply of sterile filtered air. Since the volume of the screen is comparatively small, it is not difficult to provide a much faster air flow rate than in the whole room. In practice a flow rate of one to two changes per minute has been found most satisfactory and has proved to be the most efficient way of using the screen. Many hundreds of bacterial air counts taken under such screens have always shown the superiority of this adaptation over the normal method.

Discussion

Although the manufacturers do not claim that their various filters are completely effective in removing bacteria from the air, nevertheless several types are used extensively for this purpose. Our experiments have shown their efficacy as bacterial filters to be dependent on the nature of the filter material, on the size of particles to be filtered and on the rate of air flow through the filter; packing density and depth of filter must also play a significant rôle. In particular, each type of filter appears to possess a minimal efficiency at an air flow rate which varies with particle size and according to the nature of the filter bed. These observations find a ready explanation in Stairmand's theory of filtration[8] in which he postulates that impingement forces exert the greatest effect at high velocities and diffusion forces at low velocities.

Efficiency due to impingement, or inertial, forces will naturally increase with velocity since the faster the speed of a particle the more likely it is to strike a fibre of the filter and be held there. Again, the greater the mass of a particle the more likely it is to strike against a fibre and be held, hence the greater efficiences of these filters against dust-borne infection

than against bacterial aerosols. With diminishing air flow rates the impingement effects must decrease so that, if no other effects were operative, efficiency would reach zero. However, diffusion effects become more significant with lower flow rates and thus retard the decline in efficiency, and eventually reverse it. In practice, as is seen from experimental results, this does not occur until well below the normal working range of the filters. It is not improbable that forces such as electrostatic attraction, humidity and the condition of the particle also play a significant part in determining filtration efficiency. These aspects do not as yet seem to have been investigated.

Throughout the tests, greater variation in efficiencies with changes in flow rate was found with the larger fibre filters, and the greatest was obtained with the smallest particles. Again applying Stairmand's theory, the impingement efficiency of a single fibre is shown to be a function of the expression $\frac{Dg}{Vf}$ (where D = diameter of the fibre, g = gravitational constant, V = velocity of approach and f = free falling speed of the particles). When D is large, as with the glass fibres, the variation in efficiency with flow rate, V, will be greater than when D is small, as with cotton fibres. Similarly, if f is small, as in the case of an aerosol, greater variation will be found than with a dust where f is greater. This explains, for example, why steeper slopes were obtained with the coarser fibre filters. From this theory can also be deduced why two or more filters of the same type used in series did not give any significantly enhanced efficiency over a single filter; the first filter would appear to remove all particles down to a certain size, the residue of which then pass practically unchecked through the subsequent filters.

In practice, nearly all air-borne bacteria are carried on dust particles. Hence a filter is not generally called upon to remove the smaller bacterial particles of 1 μ or less. Under these conditions, a glass fibre filter is adequate for many purposes, but where a higher efficiency of filtration is necessary, the cotton fibre filter is superior. Unfortunately, this type of filter becomes fairly rapidly choked with dust, hence a combination of a glass fibre filter followed by a cotton one would seem to be the choice, the glass filter to remove the larger dust particles and the cotton filter to remove the finer ones. Such an arrangement has been run under experimental conditions with a heavily dust-laden air flow for several days without any sign of choking and has maintained an efficiency of 98 to 99 per cent.

Summary

1. Comparisons of commercially available glass fibre, wire gauze and cotton fibre filters against a bacterial aerosol and spore-infected dusts have shown the cotton fibre filter to be the most efficient.

2. Variation in efficiencies of all filters with air flow rate and with size of particles to be filtered has been found. Previously proposed theories have been used to explain this.

3. No advantage was gained from using two or more glass fibre

filters in series, but a glass fibre followed by a cotton fibre filter had certain advantages.

4. Under working conditions, many observations have proved the glass filter effective in maintaining a satisfactory low level of bacterial air infection in aseptic filling units.

5. Screens flushed with sterile filtered air are recommended for small-scale sterile operations.

Thanks are due to Mr. G. A. B. Maxfield for his technical assistance.

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DISCUSSION

The paper was presented by MR. G. SYKES.

DR. H. S. BEAN (London), referring to Figure 3, asked whether it meant that at a low air flow rate, for every 100 bacteria-carrying particles per unit volume of air before the filter, there were only 50 in the same volume of air after passing through the filter. The graph showed further that when the airflow was increased to 600 cu. ft. per minute, the efficiency of the filter increased to something like 90 per cent. By increasing the airflow there was a greater number of bacteria impinging on the filter but, at the same time, the increased volume of air passing into the room carried an increased number of bacteria-carrying particles. If that were the case, it would appear that the logical step was to take every precaution to prevent the air from the duct entering the screen.

MR. E. ADAMS (Plymouth) said that filtration alone was not completely satisfactory. If the requirement was to remove particles down to, say, 5 μ, it was fairly satisfactory, but if the requirement was to remove still finer particles it meant that the number of air changes had to be increased from, say, 10 to 20, in which case the draught from the fans and the noise became appreciable. Consequently, he found it difficult to appreciate the very great difference in efficiency at the different rates (Table I). He wondered how the results would appear if the determinations were carried out in an aseptic room, and whether there would be a similar divergence with different flow rates.

MR. N. D. HARRIS (London) said that there was an impregnated resin filter available which the manufacturers claimed would remove 99·9 per cent. of all particles down to 0·2 μ. If that were so it would seem to be very satisfactory

MR. D. N. GORE (Dorking) described a large filter which consisted of banks of undulating plates supporting a film of oil, the plates being arranged so that a large surface was produced upon which the air impinged. The filter was satisfactory except under conditions of high humidity, when the oil film seemed to be disturbed.

DR. W. P. KENNEDY (London) said that, with regard to impaction surface, one device which had given satisfactory results consisted of placing a flat tray about $1\frac{1}{2}$ ft. square below the outlet from the filter and fan. The tray was filled with water to a depth of about $\frac{1}{2}$ in. There was an increased efficiency of the sterilisation as judged by plate counts. He also referred to a circular container holding a filter paper about 1 ft. in diameter. The air impinged upon the paper, and on passing through flowed by an ultra-violet lamp.

MR. J. H. OAKLEY (London) described an apparatus for removing water from compressed air, in which droplets of moisture were continuously removed by centrifugal action. As the droplets would carry with them the bacteria-laden dust particles the apparatus seemed to have possibilities for clearing air and for reducing its bacterial content.

MR. E. W. SIMPSON (London) said that insufficient attention was given to the air after it had passed through the filter. Very often there was a long length of trunking which accumulated a great deal of fine particles, and if the rate of flow changed, those particles were carried through into the presumably sterile atmosphere. It was usual in a filtration system to operate the optimum rate of air flow. With a large filter a high rate of air flow produced the best results, but also produced a gale in the sterile room. This could be obviated and efficiency remain unimpaired if the frontage area of the filter were reduced. Had any work been done on the nature of the surface of the filtering material?

MR. G. SYKES, in reply, pointed out that to increase the air flow rate through the filter did not necessarily produce a gale, but there was an optimum rate of flow. Also it did not necessarily follow that every particle impinging on the filter carried bacteria. It had to be borne in mind in designing sterile rooms that the points of ingress of the air and extraction were both important, and that infection could come from various sources, particularly from personnel inside the room. Adequate treatment of the air was only one of the factors in the design of a sterile room. He had recently seen published the statement that particles in the atmosphere of London average 0·6 μ whereas in the provinces the particle size was 1 μ or greater. There was no doubt that resin-treated filters were more effective than glass wool and similar filters. The device described by Mr. Oakley was useful, and appeared to be in effect another impingement apparatus. There was no doubt that the surface of an impingement unit played a very important part. Further information on the effect of different oils in filters would be desirable. The problem

of high humidities was scheduled for investigation. He did not think that an ultra-violet lamp was of any use in sterilisation.

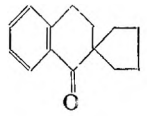
The CHAIRMAN, in closing the science sessions, thanked the authors and those who had contributed to the discussions. He thanked particularly the Editor of the *Journal of Pharmacy and Pharmacology* for having made preprints of the papers available.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

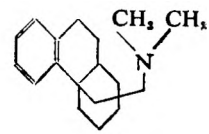
CHEMISTRY

ALKALOIDS

Morphine, Synthetic Approach to Structure of. B. Belleau. (*J. Amer. chem. Soc.*, 1953, **75**, 1159.) The spiroketone, 2:2-tetramethylene-1-tetralone (I) was



(I)



(II)

condensed with methyl bromoacetate to yield a substance which underwent Wagner rearrangement to yield a hydrophenanthroid lactone which, after lithium aluminium hydride reduction followed by dehydration, catalytic hydrogenation, reaction with phosphorus tribromide and subsequent reaction with dimethylamine, was converted to the dihydro desbase of *N*-methyl*iso*morphinane (II). The possible reaction mechanisms involved in the formation of the various intermediates are briefly presented. A. H. B.

ANALYTICAL

Aluminium, Fluorimetric Determination of. E. Goon, J. E. Petley, W. H. McMullen and S. E. Wiberley. (*Analyt. Chem.*, 1953, **25**, 608.) Interference in the colorimetric determination of aluminium in steel using the aluminium salt of 8-hydroxyquinoline in chloroform solution is caused by titanium and vanadium 8-hydroxyquinolines which yield absorption bands in chloroform solution which overlap the band of the aluminium complex; as the aluminium complex fluoresces, an investigation was undertaken to see if the fluorescence could be used for a quantitative method. Details are given of a method by which aluminium can be determined by quantitative fluorimetry using prepared calibration curves. Extraction of the aluminium 8-hydroxyquinolate into chloroform was complete at *p*H values between 6·5 to 10·0 and the fluorescence did not vary critically with time. Of the anions acetate, chloride, citrate, nitrate, perchlorate, sulphate, and tartrate, only the citrate and tartrate caused interference, the recovery of aluminium being only 25 per cent. in the presence of the citrate and 82 per cent. in the presence of tartrate. The presence of iron, titanium and vanadium caused quenching, but for a 1 to 1 weight ratio of titanium, vanadium, or iron to aluminium, respectively, 100, 89, and 83 per cent. recovery of aluminium was obtained; the interference from these elements is thus less serious than in the colorimetric method using 8-hydroxyquinoline.

R. E. S.

Bismuth, Lead and Thallium, Spectrophotometric Determination of. C. Merritt, H. M. Hershenson, and L. B. Rogers. (*Analyt. Chem.*, 1953, **25**, 572.) A survey of the absorption spectra of the bromo- and iodo-complexes of bismuth, lead and thallium has been made together with the absorption spectra of elements in hydrochloric acid including arsenic, copper, indium, iron,

molybdenum, selenium, tin, titanium and tungsten; the survey of cadmium and mercury has been extended to bromide and iodide solutions. The position and intensity of the absorption maxima of bismuth, lead and thallium in hydrochloric acid varied with the acid concentration; 6M hydrochloric acid was chosen as the working medium. Graphs are given of the absorption spectra of the elements studied under varying conditions and consideration is given to the possibility of interference by various cations and anions and their elimination. In the absence of interfering substances, the estimation of lead, bismuth, and thallium can be performed without prior separation. If interfering substances are present, a preliminary separation from cyanide medium with dithizone would permit extraction as a group from all other substances with the exception of tin. Sample mixtures of bismuth, lead, and thallium were analysed by means of multicomponent analysis technique, the optical density indexes being evaluated from measurements of the optical density at each of three wavelengths, using known concentrations and cell thicknesses, with satisfactory results. The accuracy with which thallium can be determined in the presence of bismuth may be somewhat less owing to the uncertainty in evaluating the optical density index for bismuth at 245 mμ. R. E. S.

Digitoxigenin, Fluorimetric Determination of. K. B. Jensen. (*Acta pharm. tox. Kbh.*, 1953, **9**, 66.) A method is described for quantitative determination of digitoxigenin, digitoxin and purpurea glycoside A, based on the fluorescence of digitoxigenin. Digitoxigenin is treated with hydrogen peroxide in a solution of hydrochloric acid and methanol, to which has been added ascorbic acid. The ascorbic acid prevents the rapid splitting into non-fluorescent reaction products that otherwise takes place in presence of excess hydrogen peroxide. Maximum fluorescence is attained after about 40 minutes and the intensity then remains unchanged for nearly an hour. Exposure to irradiation by light of the wavelength producing fluorescence (about 425 mμ) causes a relatively rapid reduction of the intensity. The time of development, intensity and stability of the fluorescence depend on the relation between the amount of hydrogen peroxide, the acid concentration, the amount of ascorbic acid and the temperature. Concentrations of from 2 to 20 μg. of digitoxigenin (or equimolecular amounts of the other two A substances) per 10 ml. of test solution give a linear fluorescence curve. The maximum deviations were about ± 5 per cent. in the amounts of digitoxigenin determined. S. L. W.

Local Anæsthetics, Identification of, by Vacuum Microsublimation. J. Büchi, X. Perlia and A. Strebel. (*Pharm. Acta Helvet.*, 1953, **28**, 109.) The appearance of crystals obtained by microsublimation is not a reliable guide for identification, since, on the one hand, different substances may give similar crystals, and, on the other, the same substance may give different crystalline appearances, even in the same sublimate. The method, however, forms a valuable method of purification or extraction, and identification is then possible from the character, especially crystallographic, of the sublimate, together with microscopic reactions. For this purpose the authors suggest the following physical properties: micromelting point, extinction, interference colours, direction of polarisation, number of axes, optical character, refractive indices on different axes and crystal system. In addition reactions with picric acid, trinitroresorcinol potassium iodide, potassium bromide, potassium permanganate, potassium dichromate, hydrochloric acid, iodine solution and bromine water. These characters and reactions are tabulated for a number of local anæsthetics: benzocaine, propæsine, cycloform, scuroform, monocaine, amylocaine, larocaine, tetracaine, intracaine, surfacaine and lidocaine. G. M.

Thiopentone, Test for Chloride in. E. Kühni and G. Stierli. (*Pharm. Acta Helvet.*, 1953, **28**, 96.) When a solution of thiopentone-sodium is precipitated with dilute nitric acid, small quantities of free thiopentone are still present in the filtrate, and the precipitate given on the addition of silver nitrate appears to be thiopentone-silver. Suitable tests for chloride are with thallium nitrate or with aniline and *o*-toluidine. The latter, which is the more sensitive is recommended for official adoption. It is carried out as follows: 0·5 g. of the substance is dissolved in 10 ml. of water and precipitated with 1·5 ml. of dilute nitric acid. To 1 ml. of the filtrate, in a microtest-tube, is added a crystal of potassium permanganate and 2 drops of sulphuric acid. A test paper, soaked in a solution prepared from 100 ml. of saturated aniline solution, 20 ml. of saturated *o*-toluidine solution, and 30 ml. of glacial acetic acid, is placed on the mouth of the test-tube, which is then heated slowly. A blue coloration on the paper shows the presence of chlorine. G. M.

Vitamin A in Presence of Tocopherols, Determination of. D. T. Ewing, L. H. Sharpe and O. D. Bird. (*Analyt. Chem.*, 1953, **25**, 599.) Chromatographic separation has been investigated for the determination of vitamin A in the presence of tocopherols. Data are presented for mixtures of pure vitamin A and pure α-tocopherol, distilled natural vitamin A esters and pure α-tocopherol, and distilled natural vitamin A esters and Type IV mixed tocopherols. The vitamin A-tocopherol mixture was saponified and the two vitamins separated by chromatography on activated alumina; hexane solutions of the vitamins were used to deposit the vitamins on the column, the optimum solvent for development being a 1 : 2 v/v mixture of ether and hexane. Curves of known mixtures of α-tocopherol and vitamin A alcohol show essentially complete separation of the two components; Type IV mixed tocopherols containing a relatively high proportion of β, γ and δ tocopherols cannot be satisfactorily treated by this method. An average recovery of vitamin A, from the column, was found to be 94·4 per cent. on the basis of 4 experiments; the Morton-Stubbs correction procedure was applied to the eluted vitamin A alcohol. R. E. S.

Zinc, Gravimetric Methods for. J. E. Vance and R. E. Borup. (*Analyt. Chem.*, 1953, **25**, 610.) Radio-isotopic methods were used to determine the amount of zinc remaining in solution following the classical phosphate and sulphide precipitations, 3 precipitations with organic reagents, and several separations of zinc from elements with which it is commonly encountered. The ^{65}Zn isotope was used since it had decay characteristics which make its determination straightforward with a half life of about 250 days and since gamma rays of 1·11 m.e.v. energy are produced. The phosphate procedure can be modified to advantage by the use of a much smaller excess of reagent than previously suggested, adjustment of the *p*H of the wash liquid, and use of a lower ignition temperature. Zinc oxalate precipitation was an excellent process, with zinc oxide as the weighing form. Anthranilic acid and 8-hydroxyquinoline precipitate zinc quantitatively from solution, but the precipitates do not have the predicted compositions and empirical factors are needed. Zinc can be satisfactorily precipitated as sulphide from a cold sulphate-bisulphate buffer in the presence of as much as 4 parts of iron, 2 of nickel, 8 of manganese, and 4 of aluminium. The modified phosphate procedure, the zinc oxalate precipitation, and the sulphide method can all give average results within 0·1 mg. of theory. R. E. S.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

***Lactobacillus bulgaricus* Factor, Chemical Nature of.** G. M. Brown and E. E. Snell. (*J. Amer. chem. Soc.*, 1953, **75**, 1691.) The close relationship of the two growth factors *Lactobacillus bulgaricus* (LBF–1A) and pantothenic acid is indicated in the fact that, in large amounts, the latter replaces the former as a growth factor for *Lactobacillus helveticus* 80. However, LBF–1A is more than 100 times as active as pantothenic acid in promoting growth. Both pantothenic acid and LBF–1A are inactivated by acetylation and most of the activity is restored by mild hydrolysis with N potassium hydroxide in methanol, while ammoniacal methanol regenerated the activity more slowly. These data indicate the presence of one or more free hydroxyl groups. The compound is essentially neutral and is not destroyed by nitrous acid. Acid hydrolysis of LBF–1A yields β-alanine and an unidentified amine along with the disulphide of β-mercaptoethylamine. The treatment of LBF–1A with a liver enzyme liberated large amounts of pantothenic acid. Therefore, in LBF–1A, pantothenic acid must be combined with β-mercaptoethylamine by an amide linkage. The synthesis of the disulphide of *N*-(pantothenyl)-β-mercaptoethylamine gave a compound (named *pantethine*) equal to LBF–1A in growth-promoting activity for *L. helviticus* 80. Treatment of coenzyme A with intestinal phosphatase liberated a compound which is closely related to, or identical with, pantethine or LBF. A. H. B.

***Lactobacillus bulgaricus* Factor, Isolation of, from Natural Sources.** V. J. Peters, G. M. Brown, W. L. Williams and E. E. Snell. (*J. Amer. chem. Soc.*, 1953, **75**, 1688.) The procedures for the isolation of one form of the *Lactobacillus bulgaricus* factor (LBF–1A) from the culture filtrate of *Ashbya gossypii* are described. These involved adsorption and elution from activated charcoal and subsequent successive chromatography on floridin, charcoal, superfiltrol and alumina. Concentrates obtained in this way provided the material used in elucidation of the chemical nature of the growth factor.

A. H. B.

Thyroxine, Diazo Reaction of. G. Barac and H. Morran. (*Bull. Soc. Chim. biol., Paris*, 1953, **35**, 299.) Since, in thyroxine, the positions *ortho* and *para* to the hydroxyl group are occupied by iodine, the coupling of thyroxine with diazo compounds appears to be abnormal. Analysis of the products obtained by coupling with diazotised arsanilic and sulphanilic acids indicate that these are derivatives of phenyl-1-azo-3′(3:5:5′-triiodothyronine). Coupling occurs in the *ortho* position after the elimination of an atom of iodine. G. M.

BIOCHEMICAL ANALYSIS

Adrenergic Amines of Human Blood. H. Weil-Malherbe and A. D. Bone. (*Lancet*, 1953, **264**, 974.) In the authors' fluorimetric method for the determination of adrenaline plus noradrenaline in plasma, which depends on the fluorescence of condensation products of the adrenergic amines with ethylenediamine, the adrenaline condensation product gives a yellow to orange fluorescence while the fluorescence given by the noradrenaline condensation product is green. By measuring the intensity of the fluorescence, using first a yellow filter (Chance OY4) and then a blue-green filter (Ilford Bright Spectrum

Filter 623), differential determinations of adrenaline and noradrenaline in plasma and red blood cells can be made. Determinations of the amine content of red blood cells are made after hæmolysis of the cells by treating a suspension in water with cetrimide. On applying the method to the plasma and red cells of 22 males and 21 females, the average adrenaline content of the plasma, in μg./l. of whole blood was 1·18 in males and 1·46 in females; in the red cells the figure was 2·81 for males and 2·27 for females. The plasma concentrations of noradrenaline were 5·29 and 5·16 in males and females respectively, and the concentrations in red blood cells were 2·02 and 3·77 in males and females respectively. There are highly significant differences in the intracellular and the extracellular concentrations of each of the amines in the two sexes. There is also a statistically significant inverse correlation between adrenaline and noradrenaline concentrations in both red blood cells and plasma. H. T. B.

Bromide in Body Fluids, Determination of. G. Hunter. (*Biochem. J.*, 1953, **54**, 42.) Details are given for the application of the Van der Meulen reaction —the oxidation of bromide to bromate by hypochlorite—to the determination of bromide in blood, cerebrospinal fluid and urine. The biological material to be examined is first ashed in an open crucible, the ash extracted with water, and phosphate buffer and hypochlorite solution added; after heating at 100° C. for 10 minutes sodium formate solution is added followed by sulphuric acid and potassium iodide, the resulting mixture being titrated with sodium thiosulphate using starch as indicator. The presence of sodium chloride up to 90 mg. has no appreciable effect on bromine values of 10 μg. but the addition of 500 mg. sodium chloride or more lowers the values seriously even at the 50 μg. level; iodine reacts quantitatively as bromide. Determination of bromide by this method is accurate to about $\pm$ 1 per cent. when the bromine present is greater than about 10 mg./100 ml. and when 0·5 to 1·0 ml. is taken. With bromine values from 3 to 10 mg./100 ml. the error is within $\pm$ 5 per cent. A minimum of about 50 μg. of bromine is desirable with the method. R. E. S.

Citric Acid, Microdetermination of. T. G. Taylor. (*Biochem. J.*, 1953, **54**, 48.) The method of Weil-Malherbe and Bone (*Biochem. J.*, 1949, **45**, 377) was employed and found to be very reliable for 0·2 to 1·0 mg. quantities of citric acid, but with smaller amounts the results were low. A detailed study was applied to the estimation of quantities up to 100 μg. The citric acid is oxidised to acetonedicarboxylic acid in the presence of sulphuric acid followed by the conversion of this compound to pentabromoacetone; the pentabromoacetone is then extracted with light petroleum, a portion of which is shaken with sodium sulphide solution. The intensity of the yellow colour produced in the aqueous phase is measured with a suitable photoelectric instrument. Errors present in microdeterminations were eventually traced to an excess of thiosulphate and, in view of the impossibility of avoiding a local excess, it was decided to use ferrous sulphate; a bromide-bromate mixture was substituted for saturated bromine water and the ammonium vanadate incorporated in this solution. Details of procedure are given and of recovery experiments with pure citric acid. R. E. S.

Folic Acid, Determination of. K. Ilver. (*Dansk Tidsskr. Farm.*, 1953, **27**, 81.) For the assay of folic acid by the reduction and coupling method, zinc dust may be used in place of zinc amalgam, while the addition of gelatin is of no advantage. Folic acid shows an absorption peak at 298 mμ in acid

solution, while *p*-aminobenzoylglutamic acid and *p*-aminobenzoic acid show no absorption at this point, so that it would appear possible to determine folic acid directly. Experiments with a number of samples of folic acid showed that the results obtained in this way did not agree with those of the reduction and coupling method. G. M.

Progesterone, Chemical Assay of. D. G. Edgar. (*Biochem. J.*, 1953, **54**, 50.) The paper describes a method of assay of progesterone in biological material based on the extraction and purification of the hormone by partition between organic solvents, final separation by chromatographic partition on filter paper and subsequent estimation by ultra-violet absorption spectroscopy. Oxalated blood was extracted with a 3:1 (v/v) ethanol-ethyl ether mixture, the solvent being evaporated to low bulk, diluted with water and extracted with ethyl acetate. The residue from the ethyl acetate extraction after purification was taken up in water and extracted with light petroleum, the residue, dissolved in benzene, being used for chromatography. The paper chromatography of progesterone was investigated using alumina papers and an ascending technique, the paper dipping into mixtures of various solvents; a reversed phase technique on silicone treated paper using aqueous ethanol-chloroform and aqueous methanol-benzene as solvents was also used, recoveries of the order of 75 per cent. of progesterone added to blood being obtained. Reversed phase chromatography was generally the most satisfactory, an extract of progesterone-treated blood travelling with an R_F of 0·35 alongside the pure substance. With 70 per cent. aqueous methanol compact spots of both progesterone and testosterone were obtained with a complete separation of the former steroid from extracted impurities. Details of the ultra-violet absorption of progesterone are given ($E_{max.}$ 240 mμ). R. E. S.

PHARMACY

DISPENSING

Sodium Sulphonamides, a Buffer System for Ophthalmic Solutions of. H. B. Kostenbauder, F. B. Gable and A. N. Martin. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 210.) Sulphonamide salts are precipitated from 5 per cent. solutions buffered at *p*H 8·6. Precipitation is not due to chemical incompatibility, but solubility is a function of the *p*H of the solution. Using a modified buffer equation it was shown that a minimum *p*H of 8·97 is necessary to keep 5 per cent. of sulphathiazole in solution, and in practice a buffer of *p*H 9 should be used to prevent precipitation at ordinary room temperatures. The inclusion of 0·1 per cent. w/v of exsiccated sodium sulphite retards discoloration and prevents the growth of moulds in the solution. The following buffer solution is recommended for the preparation of ophthalmic solutions containing 5 per cent. of sulphathiazole sodium: boric acid, 0·043 per cent., sodium borate, 0·42 per cent., and sodium sulphite, 0·1 per cent. The calculated minimum *p*H for 5 per cent. solutions of sulphadiazine sodium and sulphamerazine sodium is close to the *p*H of a 5 per cent. solution of each substance in distilled water. A buffer is unnecessary as satisfactory solutions may be prepared by dissolving the substances in water with the addition of 0·1 per cent. of exsiccated sodium sulphite. G. B.

Phenindione (Hedulin). (*New and Nonofficial Remedies, J. Amer. med. Ass.*, 1953, **152**, 142.) Phenindione is 2-phenyl-1: 3-indandione and occurs as a pale yellow, crystalline, almost odourless substance, m.pt. 148° to 151° C., very slightly soluble in water, and soluble in ethanol (1 in 100) and ether (0·9 in 100). When treated with sulphuric acid, a deep blue to violet colour is produced, which is discharged with formation of a white precipitate on the addition of water. When refluxed for 3 hours with aniline in ethanolic solution, a red crystalline substance melting at 222° to 228° C. is obtained. A 0·0005 per cent. solution in 0·1N sodium hydroxide exhibits ultra-violet absorption maxima at about 280 mμ ($E^{1\ \text{per cent.}}_{1\ \text{cm.}}$, about 1328) and 330 mμ, and minima at about 236 and 315 mμ; the ratio of the absorptions at 280 and 330 mμ is 3·20 to 3·40. Phenindione contains not more than 20 p.p.m. of heavy metals and yields not more than 0·25 per cent. of sulphated ash. When dried at 105° C. for 4 hours, it loses not more than 1·0 per cent. in weight. It contains 95·0 to 105·0 per cent. of phenindione and is assayed spectrophotometrically by measuring the absorption at 280 mμ of a 0·0005 per cent. solution in 0·1N sodium hydroxide. Phenindione is used as a systemic anticoagulant. G. R. K.

PHARMACOGNOSY

Morphine, Production of, from Poppy Stalks. S. Biniacki and H. Ludwicki. (*Ann. pharm. franç.*, 1953, **11**, 121.) In the culture of poppies for seed and oil in Eastern Europe, the plant, deprived of the capsules, forms a waste product ("poppy straw") which is often burnt. An examination was made of the possibility of extracting alkaloids from this material. A method for the extraction of morphine has been published by Kabay, and patented in several countries. Experiments showed that this method extracts the morphine completely, but only about 60 per cent. of the non-phenolic alkaloids. To obtain complete extraction it is necessary to increase the time of extraction, or to use a different solvent. The loss of morphine in the early stages is insignificant, but about 25 per cent. of the non-phenolic alkaloids may be destroyed during the operation, owing to the decomposition of narcotine under the action of sulphur dioxide. In the final stages there may be a considerable loss of morphine, especially during its extraction and numerous crystallisations. Of the other matter present in the plant, it is especially the pentosans or the products of their decomposition which go through into the later stages of manufacture. G. M.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline Cream in Fibrositis. J. S. Lawrence and R. J. Sladden. (*Brit. med. J.*, 1953, **1**, 1085.) The value of adrenaline cream in rheumatic disorders was investigated under the two headings, palliation and recovery times, the investigation being carried out at a clinic for the treatment of rheumatism in miners. Palliation was studied in two stages. In the first, on a group of 65 patients, comparison was made between a cream containing 0·1 per cent. of adrenaline and a simple cream. In the second stage on a group of 50 patients, adrenaline cream was compared with dry massage and with no massage. The assessment was made after each patient had been treated thrice weekly for 2 weeks. Each patient in the first group was then treated in the same way with the simple cream while each patient in the second group was then treated by dry massage thrice weekly for 2 weeks and then again assessed after another

fortnight with no massage. Other physiotherapeutic procedures were continued without change during the investigation. The results obtained, as ascertained by a questionnaire, are tabulated. They show that adrenaline cream and dry massage both gave relief in a greater proportion of patients than a simple cream, and no significant difference was noted between the effects of dry massage and massage with adrenaline cream, but the difference between the adrenaline cream and the control groups was highly significant. The investigation into the effect of adrenaline cream on recovery time was carried out on 64 males, alternate patients being treated with adrenaline cream until either no further treatment was required or no improvement was shown for 4 consecutive weeks; the remainder were maintained on massage with a simple cream in the same way. The result in the two groups was identical; 7 patients in each lost all signs and symptoms; 14 and 13 respectively needed no further treatment and 7 in each group were unchanged. The results, however, show that repeated application of adrenaline cream significantly delays recovery and increases incapacity for work. While all in the control group returned to work, 3 in the treated group did not return, and 2 had to cease work after returning to it. H. T. B.

Aminoazotoluene Dermatitis. T. H. Meara and I. Martin-Scott. (*Brit. med. J.*, 1953, **1**, 1142.) 3 cases of contact dermatitis are described due to the presence of aminoazotoluene in the red and green semi-solid inks used in a popular make of ball-pointed pen. 2 of the patients had used such pens while the third was in contact with green semi-solid ink during her work. Each of the three gave a positive reaction in a patch test with 0·01 per cent. of aminoazotoluene in soft paraffin. In 2 cases the time taken to produce sensitivity was 6 to 8 weeks; in the third case it may have been longer. The dermatitis occurred on the hands and arms, and in 2 cases on the eyelids. In each case the trouble cleared up when contact with the red or green ink was avoided. In view of the absence of previous reports of this dermatitis in spite of the sale of large numbers of the pens, and the absence of dermatitis among workers in contact with these two coloured inks, it is suggested that the dye has a very low sensitivity index. H. T. B.

Antibiotics, Assay for the Histamine-like Activity of. L. W. Rowe and R. A. Brown. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 257.) In the tests for histamine-like activity of certain antibiotics in which the fall in blood pressure is observed after intraperitoneal injection into anæsthetised animals, dogs as well as cats were found to be suitable as experimental animals. Dogs anæsthetised with phenobarbitone sodium are only about 1/5 as sensitive to histamine as cats, but this is relatively unimportant since the tests are made in comparison with a histamine standard and the slope of the response of dogs to graded doses is as steep as that of cats. The standard error with either animal is about ± 20 per cent. Results show that for chemically pure substances such as crystalline penicillin or chloramphenicol, a test for histamine-like activity is unnecessary, but it is desirable for streptomycin and viomycin. A solution of chloramphenicol in *NN*-dimethylacetamide cannot be tested satisfactorily since the solvent causes an appreciable depressor action, but the test is not considered to be necessary for this preparation. The use of antihistaminic drugs to antagonise histamine-like activity in antibiotics is not feasible. G. B.

Ergot Alkaloids, Hydrogenated, Hypotensive Effect of. H. Konzett and E. Rothlin. (*Brit. J. Pharmacol.*, 1953, **8**, 201.) The hydrogenated alkaloids of ergot, dihydroergocornine, hydergine (a mixture of equal parts of dihydroergocornine, dihydroergokryptine and dihydroergocristine) and dihydroergotamine caused a fall of blood pressure when injected into the anæsthetised or decerebrated cat, mainly through an action on the vasomotor centres. After section of the spinal cord at the 7th thoracic vertebra they caused a fall in blood pressure, but after section at the 6th cervical or 1st thoracic vertebra there was either a rise or a fall. In the intact cat, after blocking the ganglia with tetraethylammonium, the alkaloids generally caused an increase in blood pressure; while in the spinal cat, even after section of the cord at the level of the 7th thoracic vertebra, or after complete destruction of the cord, they caused a fall. The main site of action is therefore the vasomotor centres and the upper part of the sympathetic outflow, as pathways conducting the impulses, have to be intact for the hydrogenated alkaloids to cause a fall of blood pressure. Without the vasomotor centres, or their efferent fibres, only the vasoconstrictor effect of these alkaloids is apparent. G. F. S.

Gentisic Acid, Toxicities of Esters of. J. F. Nash, F. W. Bope and B. V. Christensen. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 254.) The acute toxicities of 13 new esters of gentisic acid were determined in mice, the esters with amino alcohols being injected intravenously in the form of aqueous solutions. As the esters of gentisic acid with other carboxylic acids were insoluble in water, they were injected intravenously as aqueous suspensions containing not more than 5 per cent. of acacia and 5 per cent. of ethanol, prepared with the aid of tween 80. All compounds tested were more toxic than gentisic acid. The LD50 for esters with other carboxylic acids varied from 201·2 mg./kg. for the 5-phenylacetic compound to 32·5 for the 5-acetylsalicylic derivative. Esters with amino alcohols were more toxic, ranging from 3-diethylaminopropyl gentisate hydrochloride (LD50, 58·5 mg./kg.) to its 5-methoxy analogue, LD50 22·0 mg./kg. G. B.

Iron Preparations, Physico-chemical Properties and Toxicities of. J. A. Nissim. (*Brit. J. Pharmacol.*, 1953, **8**, 197.) The physico-chemical properties of a number of iron preparations have been investigated and their acute toxicities compared in mice. Of the new preparations ferric glucosate, "ferric hydroxide ferrous ascorbate," "ferrous chloride ascorbate," "ferric chloride caramelate," "ferric chloride lactate," glycine and iron and plasma and iron were all more toxic than saccharated iron oxide, although some showed better physical properties. Of the old preparations ferric chloride, because of its protein precipitating properties, was the most toxic iron preparation. Ferrous sulphate was less toxic in its immediate effect but with a more prolonged action it was as toxic as ferric chloride. Colloidal ferric hydroxide had an immediate lethal effect with few delayed deaths, and was as toxic as ferrous sulphate. Ferrous ascorbate was more toxic than "ferric hydroxide ferrous ascorbate." Iron and ammonium citrate showed a delayed effect. Iron preparations differed widely in their toxicity with LD50 ranging from 11 to 300 mg. Fe./kg. G. F. S.

Isoniazid in Treatment of Lupus Vulgaris. R. Russell, N. A. Thorne and R. V. Grange. (*Lancet*, 1953, **264**, 964.) The encouraging reports of the value of isoniazid in the treatment of pulmonary tuberculosis suggested its possible use in cases of lupus vulgaris. The authors report the results of treat-

ment in 15 cases. 5 of the patients were given tablets of isoniazid orally, 6 were treated with local injections, and the remaining 4 had both local and oral treatment. Oral dosage was 150 mg. daily at first, but later it was found that better results were obtained with 300 mg. daily and as toxic side effects were rare a daily dose of 400 mg. was sometimes given. For local injection, the dose varied between 50 and 250 mg. in 2 to 5 ml. of water administered once a week, intradermally into the whole of a lesion if it were small, or into the same part of it if it were larger. Where both methods were used, the oral dosage was 300 mg. daily and the injected dose up to 400 mg. per week. All the patients except one showed improvement, and in most this was progressive up to the time of publication of the report. None of the patients complained of any subjective symptoms. The only side-effect observed was mild urticaria in one patient treated with weekly injections but it was not certain that the urticaria was caused by the isoniazid. From the continuous improvement shown by the patients during up to 30 weeks' treatment it is concluded that drug resistance did not occur. The authors suggest that the dose by mouth should be at least 300 mg. per day but weekly injections are considered far too infrequent for maximal response. Repeated injections, however, cause considerable fibrosis which makes administration increasingly difficult. H. T. B.

Nalorphine in Racemorphan (Dromoran) Poisoning. M. Bornstein, L. Yorburg and B. Johnston. (*J. Amer. med. Ass.*, 1953, **151**, 908.) 2 cases of racemorphan poisoning resulting from the administration, in error, of 50 and 25 mg. of the drug were treated with nalorphine. 1 patient failed to respond to nikethamide and atropine and the other to caffeine. Amphetamine and oxygen treatment improved the condition for a short period, but a relapse to the comatose state occurred. Respirations in one patient were as slow as 4/minute. Slow intravenous injection of 20 mg. of nalorphine was given to this patient, who returned from a comatose state in 10 minutes; 3 hours later a further dose of 10 mg. of the drug gradually improved the condition such that by the following morning respirations were 18/minute and the mind was alert. The other patient was given the nalorphine intramuscularly in doses of 5 mg. and showed improvement after 20 minutes and reverted to the pre-methorphinan administration condition after 6 hours. It is concluded that nalorphine is a potent and rapidly acting antidote to methorphinan. J. R. F.

***p*-Nitrophenyldiethyl phosphate, Reactions of Rabbits to Poisoning by.** J. M. Barnes. (*Brit. J. Pharmacol.*, 1953, **8**, 208.) *p*-Nitrophenyldiethyl phosphate (E 600) is a potent cholinesterase inhibitor acting like tetraethyl pyrophosphate. An intravenous dose of 0·1 mg./kg. in the unanæsthetised, atropinised rabbit caused muscular fasciculations, respiratory arrest and death within 10 to 15 minutes. With a period of artificial respiration the rabbit could be saved and a series of injections could be effected. While periods of unconsciousness did not increase with successive doses the generalised fasciculations were reduced after several doses. Longer periods of artificial respiration became necessary for recovery. In rabbits anæsthetised with urethane, 1 mg./kg. produced muscular fasciculations within 1 minute and movements of the diaphragm became irregular. Cyanosis rapidly appeared and artificial respiration was necessary for recovery. With successive doses at 30- to 60-minutes intervals the diaphragm became refractory but a gradual failure of respiration and circulation occurred. Responses of the tibialis and soleus muscles to stimulation of the sciatic nerve became smaller after each dose and during the later

stages muscular fasciculations disappeared. While the drug kills the animal, due to cholinesterase inhibition, some rapid reversal of the inhibition is postulated during periods of artificial respiration. Sensitivity to acetylcholine increased after each dose and diminished but did not return to normal within a 2-hour period. It is suggested that some of the changes may be a secondary reaction to the inhibition of cholinesterase and to anoxia. G. F. S.

Sodium Aminosalicylate in Treatment of Pulmonary Tuberculosis. R. McL. Todd. (*Brit. med. J.*, 1953, **1**, 1247.) The value of sodium aminosalicylate in the treatment of primary pulmonary tuberculosis was investigated in 69 children divided into 4 age groups. Alternate patients in each group were treated for 12 weeks with 1 g./lb. daily, in divided doses, two hourly in mixture form. [Elsewhere in the paper the daily dose is stated to have been 0·5 g./lb.] The control group were given no specific chemotherapy but received the same general treatment. Most of the patients had suffered from the disease for 3 months or less. Progress was assessed by the following 6 criteria: clinical impression based on appetite, vitality, temperature and pulse rate, serial blood counts, serial sedimentation rates, weight gain, serial chest X-ray films, and incidence of complications. The clinical impression was that progress was equally satisfactory in both groups, and the other criteria selected indicated no benefit from sodium aminosalicylate. 2 children under 3 years old died of tuberculous meningitis, both in the control group, but in the treated group under 3 years old, one developed renal tuberculosis and one developed tuberculous peritonitis. The drug is most useful in acute exudative tuberculosis and since caseation in the regional lymphatic glands is the most striking feature of primary tuberculosis this conclusion was not unexpected. The large dose used was intended to give a blood level of 8 mg./100 ml. The average level 30 minutes after a dose was 12·5 mg./100 ml., while the average level just before a dose was due was 7·5 mg./100 ml. Wide variations were found in the same individual on different occasions. 1 child out of 35 in the treated group developed a generalised rash and œdema; in the others no toxic manifestations of any kind were observed. H. T. B.

Streptomycin Resistance After Previous Treatment with Salts of Aminosalicylic Acid Alone. F. W. S. Turnbull, A. T. Wallace, S. Stewart and J. W. Crofton. (*Brit. med. J.*, 1953, **1**, 1244.) The authors investigated the development of resistance to sodium or calcium aminosalicylate given alone, and the effect of such resistance on subsequent combined treatment with streptomycin and an aminosalicylate. A group of 9 patients with pulmonary tuberculosis who had been treated with an aminosalicylate alone for periods of from 6 weeks to 16 months were treated with 1 g. daily of streptomycin and 20 g. daily of sodium aminosalicylate. The dosage of the aminosalicylate when given alone was unknown; the interval between the two courses of treatment varied from a few days to 39 months. The results of serial culture and sensitivity tests of tubercle bacilli isolated from these patients were compared with similar results on a second series of 5 patients treated similarly with streptomycin and sodium aminosalicylate but who had not previously been treated with aminosalicylate alone. Of the first group, in 8 out of 9 patients bacteria resistant to aminosalicylate could be isolated at the beginning of the investigation; in some the degree of resistance increased during the combined treatment. When these 8 patients were given the combined treatment, streptomycin resistant

bacilli were obtained from 6 patients within 3 months and from all of them after 5 months. By contrast, a slightly resistant organism was isolated after 5 months from only 1 of 6 patients whose organisms were sensitive to sodium aminosalicylate at the start of combined treatment. It is concluded that the use of sodium aminosalicylate to supplement treatment with streptomycin does not prevent or diminish the emergence of resistance to streptomycin if the organisms are resistant to the aminosalicylate at the commencement of combined treatment. H. T. B.

Thyroxine, Biological Action of Substances Related to. J. H. Wilkinson, W. E. Sprott and N. F. Maclagan. (*Biochem. J.*, 1953, **54**, 16.) The thyroxine-inhibitory properties of a series of 4-hydroxy-3:5-diiodobenzoates of glycols has been studied. A series of five ω-hydroxyalkyl 4-hydroxy-3:5-diiodobenzoates (β-hydroxyethyl to ζ-hydroxyhexyl) were prepared together with the five corresponding polymethylene bis-esters and the monoesters from butane-2:3-diol and propane-1:2-diol; in addition glycerol 1-(4-hydroxy-3:5-diiodobenzoate) was examined. In general, increasing chain length of the alkyl group was paralleled by a substantial diminution in thyroxine-inhibitory activity. The hydroxyethyl and the two hydroxypropyl esters caused significant reductions in the thyroxine responses at total doses of 25 mg./kg. and were thus as effective as the *n*-butyl ester, the most potent compound observed. The substitution of a hydroxy group caused a relatively slight effect on the thyroxine-inhibitory activity of a number of alkyl 4-hydroxy-3:5-diiodobenzoates. A second hydroxyl group in the alkyl chain completely abolished the activity of the propyl ester, for the glycerol ester displayed no action at a dosage of 400 mg./kg. There was no correlation between the length of the polymethylene chain and the antithyroxine activity of the polymethylene bis-4-hydroxy-3:5-diiodobenzoates; the dimethylene compound was highly active whilst of the others only the tetramethylene and pentamethylene esters showed any activity. R. E. S.

BACTERIOLOGY AND CLINICAL TESTS

Surface-active Agents, Effect of, upon Bacterial Growth. L. Gershenfeld and G. C. Johnson. (*J. Amer. pharm. Ass. Sci. Ed.*, 1953, **42**, 187.) Rough strains of *Staphylococcus aureus*, *Streptococcus viridans* and *Neisseria catarrhalis* were grown in broth and on nutrient agar containing surface active agents. None of the organisms showed change from the rough growing habit in the presence of 0·001 to 0·0001 per cent. of sodium lauryl sulphate, and higher concentrations were toxic to the bacteria. Lecithin in concentrations from 0·03 to 0·01 per cent. caused no change in the character of the growth, but stimulated the growth of the streptococcus and staphylococcus while *Neisseria catarrhalis* was unaffected. The addition of tween 80, 0·3 to 2 per cent. to the medium caused the staphylococcus to grow in smooth suspension in broth cultures, although the colony structure on agar remained unchanged. This surface-active agent did not affect the growth of the other organisms. It is suggested that as the staphylococcus is affected by tween 80, the concentration of lipids on the cell wall is the cause of rough growth, and adsorption of the surface-active agent gives rise to a hydrophilic surface. This is evidently not the case with *Streptococcus viridans* which is unaffected by tween 80, but roughness may be the result of the interlacing chain structure of this organism. G. B.

Correction.

THE GRAPHICAL EVALUATION OF RESULTS OF SIMPLE AND MULTIPLE SLOPE-RATIO ASSAYS

BY PAMELA M. CLARKE and ZENA D. HOSKING

This Journal, 1953, **5**, 586.

Page 588, legend to Fig. 1, last line. *For* CC′ *read* BB′.

Pages 592 and 593. *For v read v.*

Page 593, last line. *For* $t_2 = t\sqrt{n(k-1)(2k+1)/k2d_n}$ *read*

$$t_2 = t\sqrt{n(k-1)(2k+1)/k}/2d_n$$

Page 594, first paragraph. *Read:*

For a multiple assay, the corresponding test for "intersections" may be made using a range test described by Cox[7]. When there is a common zero dose the range of the values of H should not be greater than $t_3 r$ where $t_3 = d_v F_{\nu_1,\nu_2}\sqrt{nk(k-1)(2k+1)/2}/(vk+1)d_n$. F is found from variance ratio tables with ν_1 and ν_2 degrees of freedom, where $\nu_1 = \nu_v$ and $\nu_2 = (vk+1)\nu_n$, using the values of ν given in Table V. When there is no common zero dose, $t_3 = d_v F_{\nu_1,\nu_2}\sqrt{n(k-1)(2k+1)/2k}/vd_n$, $\nu_1 = \nu_v$ and $\nu_2 = vk\nu_n$

Page 594, second paragraph. *For v read v.*

Correction.

A COMPARISON OF PHYSICAL AND CHEMICAL METHODS WITH BIOLOGICAL ASSAY OF VITAMIN A

BY T. K. MURRAY AND J. A. CAMPBELL.

This Journal, 1953, **5**, 596.

Page 597, the last two sentences of the first paragraph should read:—

"Unpublished results of a similar comparison conducted by an informal committee of the U.S.P.[11] indicated that the Morton and Stubbs correction procedure gave a conservative estimate of biological potency. There was, however, no indication of over-correction to the extent reported by Melnick *et al.*"

Page 599, Table I, column 5, "Potency of Concentrates" the figure 15,900 should read 159,000, and in column 6 "Confidence Limits of Concentrates" the figure 16,100 should read 161,000.

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