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Chairman, 1955

BRITISH PHARMACEUTICAL CONFERENCE ABERDEEN, 1955

Chairman: J. P. TODD

CHAIRMAN'S ADDRESS

BACTERIAL PYROGENS

FOREWORD

DR. SEIBERT: "I started to work with the pyrogen back in 1923 and I can really say that I have never found any more difficult work than the work with pyrogen. In fact, I used to call it my little blue devil because it was there and wasn't there. I was impressed with the elusiveness of it, and the fact that it might be everywhere. It appears in all your flasks, all your water and in everything you work with. I am wary of the possibility of contaminating what I am working with, with a pyrogen. I have to wash all my glassware with freshly distilled water, make all my chemical reagents up with freshly distilled water, and I have to use special filters in order to eliminate the pyrogen.

Pyrogens exist in very small concentration and give such a tremendous reaction. I am so much impressed with all this work that is being done, but I wonder, has it been done that carefully? Are some of these pictures that you get mixtures, due partly to what you are giving but also due to contaminants?"

Proceedings Research Conference on Activities of Bacterial Fyrogens at the University of Pennsylvania March 2, 1951, S. 58.

I AM going to speak to-day about bacterial pyrogens. I am doing so because recently they have attracted renewed interest as therapeutic agents. I have said "renewed" because in different forms they were used with some success earlier in this century for the treatment of a number of disorders. I would also like to speak about them because I have had many tussles with them, especially in blood transfusion work, and many of the problems encountered then are still not solved. I feel too that in this country we are thinking too much about their nuisance value and not enough about their potentialities in the treatment of disease. I would like to discuss their potential value as non-specific therapeutic agents and to show that we have now reached the stage where bacterial pyrogens in pure form can, with advantage, replace the older materials and methods for producing a general stimulation of the defence mechanisms of the body.

In this country great interest used to be taken in the therapeutic uses of materials such as typhoid vaccine, used non-specifically, which were of value in the treatment of certain diseases although they had some disadvantages such as uncertainty of action and undesirable side effects. But during the last 30 years the older preparations have gradually been falling out of use and the flood of modern chemotherapeutic agents has hastened this process. Like many another old remedy they are again exciting interest, especially in the United States of America, and on the Continent, since their active principles have recently been isolated and purified and their effects and dose can now be readily controlled. The undesirable side effects are almost entirely absent in the new forms.

I should like also to consider the nature and chemical structure of these purified pyrogens so far as it is known and their behaviour when injected into the body and then to make brief reference to their uses in the treatment and amelioration of a variety of disorders.

I shall not be able to devote any time, to the purely pharmaceutical problems which these substances present as contaminants in parenteral preparations. This was the subject of a recent Symposium on Pyrogens[1].

Bacterial pyrogen appears to be capable of effecting, safely and rapidly, a general mobilisation of the body defences to an extent seen only when the body has been insulted by the harmful effects of trauma, infection and other forms of injury.

The stimulation and mobilisation of body defences which follows bacterial infections or trauma, or the injection of irritant substances, or excessive heat or cold has for long been known to produce a state of alarm and stress in the animal body. No complete understanding has yet emerged of the complicated "chain-reactions" which Selye has, perhaps too simply, called the "alarm reaction[2]." The substance or condition which produces it has been called the "stressor[2]." The same or similar effects to those produced by the injection of bacterial pyrogen are produced by a variety of stressors. Pyrogen differs from the others in that its effects are produced without disagreeable or unpleasant aspects such as being ill or injured. One would hesitate to induce a general stimulation by infecting a sick person with an organism causing malaria or other disease to alleviate the patient's sickness if another less drastic method were available, or to inject an intensely painful and irritating substance like turpentine or sulphur deep into a muscle, but this is still practised in certain places for the benefit to the patient which ensues in certain disorders[3]. These general methods of stimulating the body's defensive mechanism, including the injection of bacterial pyrogen, are forms of nonspecific therapy as opposed to specific therapy seen in the use of diphtheria antitoxin to treat diphtheria.

One of the forms of non-specific therapy practised shortly after the beginning of the century was called "protein shock," because it was believed that protein when injected was capable of acting as a non-specific stimulating agent or stressor. I first became acquainted with this form of therapy when working with the late Professor Ralph Stockman in the 1920's. Stockman was a great clinician and research worker, and many people besides myself are grateful for his influence at the formative period of our lives. Professor Sir David Campbell of Aberdeen University, one of our guests at this Aberdeen Conference and the President of The General Medical Council, was one of Stockman's lecturers at that time, and he also was interested in protein shock therapy and published at least one paper on its use in the treatment of rheumatoid arthritis.

At that time it was believed that almost any protein from almost any source was effective, and this led to the injection of milk protein, tumour extracts, horse serum and many others. It is believed now that the stimulating effect of the injection of protein from many different sources was really the result of contamination with bacteria or their metabolites

and was, in fact, a reaction caused by bacterial pyrogen. Ordinary household milk was a popular source of protein for shock therapy at that time as it was easily available, and one writer[4] describes how he obtained better effects by the injection of "market" milk. This supports the view which was even then gaining favour, that the effect was the result of bacterial contamination, especially when it was later shown that protein from milk obtained aseptically was not pyrogenic and was not effective.

It is not easy to prepare protein material or derivatives such as blood plasma or protein hydrolysates or even milk for intravenous injection without bacterial contamination and it is no reflection on the competence of the earlier workers to say they were mistaken as to the agent causing the reaction. Simple protein as such does not produce a specific stimulation although it does possess its own special effects. But we must not dismiss protein altogether from our picture since some bacterial proteins may function as carriers of the pyrogenic grouping under certain conditions which we shall discuss later. Anyone interested in this period when protein shock treatment was at its zenith can read about it in a book published by Petersen[4].

Bacterial vaccines, notably typhoid and TAB, were also used in this connection as a protein source with surprisingly good results; but it was not then suspected that what is now believed to be the active component of the vaccines, namely bacterial pyrogen, belonged to the same group of substances as those which at that time were causing trouble in injection fluids—substances to which Hort and Penfold had drawn attention in 1912[5–7], and which Seibert[8–11] was investigating in the early 1920's. It is now tolerably certain that the active substance in our vaccines and pharmaceutical injections and the very active substance now being supplied for clinical trials are the same, or differ in minor characters only.

By means of any of the agencies we have mentioned as well as by physical methods and tissue injury, many bodily changes including high fever can be produced. These are accompanied first by a fall in the white blood cells which is called a leucopenia, then by an increase in the white cells called a leucocytosis, and by other changes which are characteristic of the "alarm reaction" of Selye. We are chiefly concerned to-day with bacterial pyrogen and its various properties, but before finally leaving these other methods of stimulation I would like to refer again to the use of sulphur or turpentine injections.

Menkin[12–14] and Abderhalden[15,16] have shown that there exist in body tissues and cells, endogenous substances which are capable of causing the characteristic fever and white blood cell changes produced by bacterial pyrogen. To distinguish our bacterial pyrogen from the endogenous pyrogen of the body tissues, we usually refer to it as "exogenous pyrogen." We do not know what relation, if any, exists between our exogenous bacterial pyrogen and the endogenous factors of the body as described by Menkin and Abderhalden but it is widely felt that either they or other endogenous substances must be concerned.

When, for example, sulphur is injected into a muscle a great deal of local

inflammation and œdema is produced and local cell damage is caused resulting in a high and prolonged fever accompanied by the blood cell changes already mentioned. A distressing feature of this method is the great pain and discomfort caused. It appears here as if some endogenous pyrogen arising from the damaged tissue cells or white blood cells had been liberated after the injection. It is possible, therefore, to inject an extremely irritating substance either intravenously or intramuscularly, and so to reproduce the effects characteristic of a highly pyrogenic reaction when in fact no pyrogen is injected at all. How many of the reported reactions which had stimulated Hort and Penfold and Florence Seibert to study pyrogenic reactions in injections and which led to the conception of bacterial pyrogen, previously described by various names such as "injection" fever, "salvarsan fever" and so on, were in fact due entirely to pyrogen and how many to the irritation of the medicament or the method of injection. We must not, of course, exaggerate this point, but it is perhaps worth remembering as it may sometimes explain an unexpected reaction.

In this connection Dr. Favez[17], head of a large tuberculosis clinic in Lausanne, has described the effect of PAS when given in massive doses by vein, as is the practice in Switzerland and in the west of Scotland in the treatment of tuberculosis. Favez's patients were so much benefited by a stimulating side effect of his undoubtedly non-pyrogenic material that he conceived the idea of the simultaneous administration of a purified pyrogen to increase and extend even further this effect. In certain types of tuberculosis he obtained highly beneficial results.

It is generally held that stimulation therapy is contra-indicated in tuberculosis patients since it often liberates dormant organisms from resistant foci which can be a dangerous procedure. This view, however, belongs to the period when the chemotherapy of tuberculosis was much less advanced that it is to-day. In any case, Favez is convinced of the value of the method and has much evidence to support his view. It is known that bacterial pyrogen has a fibrinolytic action[18] and Favez is of the opinion that this fibrinolytic effect may bring about the liberation of the tubercle bacilli from resistant foci, so exposing them to attack by chemotherapeutic agents which otherwise would be ineffective.

Pyrogen has effects other than the production of fever. In fact for therapeutic purposes the title is no longer very suitable, and its retention is justified only because any change would cause confusion. Westphal in Germany calls it "Reizstoffe" or "irritating substance". In fact, from recent clinical reports it appears that in a great many cases the pyrogenic (thermal) effect is unnecessary and undesirable, and it has become the custom either to suppress the fever by the administration of antipyretics or by using a smaller dose. In any case fever is only one of the effects produced. But perhaps if we do not take the name too literally it is on the whole better to retain it if only for the sake of tradition.

Before going on to consider the source and nature of bacterial pyrogen let me conclude this section by saying that there is a great deal of published evidence to support the view that the older methods and materials used in

non-specific therapy had many virtues. Now, with the advent of the purified active principles capable of exact dosage and predictable effect, it is possible to reassess the value of pyrogen in medical treatment. I do not think we can ignore its possibilities.

The Source and Nature of Bacterial Pyrogen

All the evidence suggests that only the Gram-negative organisms need be considered as fruitful sources of the pyrogenic and stimulating substances we have been discussing and that the pyrogen is associated with the endotoxin. If Gram-positive organisms are killed by heat they exert little or no pyrogenic action, whereas either alive or dead the Gram-negative bacteria have a powerful action when injected[19]. In general the Gram-positive types allow soluble exotoxins to pass into the medium whereas the Gram-negative types retain the complete endotoxic principles in or on the cell surface and only soluble fractions including pyrogen are found in the medium[20].

The endotoxin was first extracted in undegraded form by Boivin and his colleagues[21–24]. Since then it has been further studied by many workers who approached the problem chiefly from the immunological and biochemical aspects and were not concerned with these substances as sources of pyrogen. Later, groups of workers examined the water-soluble fraction to study another curious property, that of causing necrosis or break-down in tumour tissues, a property of bacterial extracts which had been known for many years. The endotoxin exists in all types of Gram-negative organisms so far investigated and, in practically all, the general structure and properties are very much the same.

Its characteristic properties are not destroyed by heating in water at 100° C., and it thus differs sharply from the exotoxins of the Gram-positive forms which, with few exceptions, are quickly inactivated by heat.

In most Gram-positive organisms the exotoxins are largely composed of protein, which readily suffers denaturation, whereas Boivin found that the Gram-negative endotoxins are complexes of polysaccharides and other constituents. Immunologically they behave as the dominant O-somatic antigens and because of their toxicity they were originally called bacterial endotoxins, so that either name may be met. In far-reaching researches into the nature of this antigenic complex, Morgan and Partridge[27] showed that it consists of a complex of protein, active lipopolysaccharide and inert lipid.

Goebel and others[30] had found that Flexner dysentery organisms, which are also Gram-negative, yielded a strongly antigenic and toxic endotoxin which went into solution in pyridine and water. Palmer and Gerlough[25] devised the useful phenol process of deproteinisation which, in modified form, has in recent times yielded such valuable results in the hands of Westphal, Luderitz and their colleagues in Germany.

The pioneer endotoxin work of Boivin[21–24], Morgan and Partridge[27], Miles and Pirie[28,29], Goebel[30] and many others, paved the way for recent workers such as Westphal, who studied these substances mainly as sources of pyrogen, and for others such as Shear[32–34], who studied them because

of their tumour-necrotising action. The result of the work of this group made it clear that most Gram-negative organisms contain a similar complex made up of a protein, a toxic factor bound to a polysaccharide and an inert lipid of the cephalin type. The toxic factor, which appears also to contain phosphorus, is the factor in which pharmacists are chiefly interested, as it appears that this substance is mainly responsible for the pyrogenic and the other related effects. The toxic factor when isolated from the bacterial complex seems to be attached firmly to the polysaccharide, which is therefore described as a lipopolysaccharide. The toxic lipid, usually found firmly bound to the polysaccharide, is different in structure and properties from the inert lipid previously mentioned which is not at all toxic, and it is also a more complex substance. The whole endotoxic complex appears to constitute, or to be closely connected with, the surface of the bacterial cell in smooth varieties, the polysaccharide moiety resembling the capsular membrane of the pneumococcus in this respect. The amount of the lipopolysaccharide appears to vary in R-forms of the organism and this seems to be devoid of the O-specific characteristics. These lipopolysaccharides from the R-forms are almost as pyrogenic as those from smooth forms but are devoid of some sugars especially the chromatographically quickly moving desoxy-sugars. It appears that a certain amount of toxic lipid is synthesised which is bound to the polysaccharide; if this is not synthesised in sufficient amount some is bound to the protein instead, giving lipoprotein. Smooth forms have been found which contained toxic protein as well as lipopolysaccharide and on the other hand R forms have been examined which contained besides toxic and pyrogenic protein, variable amounts of lipopolysaccharide[57].

It is an oversimplification therefore to say, as is often done, that pyrogens are lipopolysaccharide, since in rough forms of the organisms the pyrogenic constituent can be separated along with the protein and the polysaccharide constituent is present only in small amount. We can, in fact, extract from R-forms a pyrogen which is associated with the protein of the R-types of organisms. This pyrogenic protein is, however, much less active than the pyrogenic lipopolysaccharide, suggesting that the degree of activity is related to the particle structure and that protein is a less suitable carrier for the activity-conferring lipid than is polysaccharide.

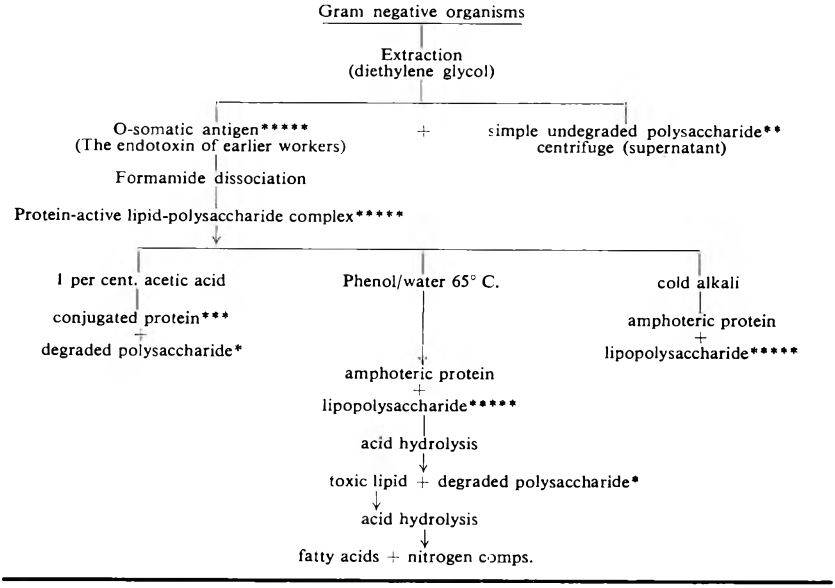
This is also shown if the toxic lipid is separated by acid hydrolysis from either the protein or the polysaccharide when it occurs as a fatty or waxy substance very insoluble in water but soluble in chloroform, and, is not active for the purely physical reason of insolubility. Westphal[35] has shown that if the separated lipid is dispersed by means of a surface-active substance such as Tween, it regains some, but not all, of its activity. The removal of this lipid from the bacterial complex removes also the toxic and pyrogenic properties leaving either degraded polysaccharide or simple amphoteric protein.

If the bacterial endotoxin, which is composed of a complex of bacterial protein, lipopolysaccharide and inert lipid, is split by the method of Goebel by hydrolysis in alkaline alcoholic solution, we obtain products, one of which is a toxic lipopolysaccharide and the other a non-toxic

protein; whereas with gentle acid hydrolysis we obtain a toxic protein and a degraded non-toxic polysaccharide. We see from this that the toxic pyrogenic factor may occur along with a polysaccharide or a protein carrier, according to conditions. These reactions are summarised in Table I.

TABLE I

THE RELATION BETWEEN THE PROPERTIES AND ORIGINS OF BACTERIAL PYROGEN



Increase in pyrogenic potency expressed by increased number of asterisks.

TABLE II

THE RELATIVE TOXIC, ANTIGENIC AND PYROGENIC PROPERTIES OF THREE POLYSACCHARIDES FROM *Shigella dysenteriæ*

Degraded polysaccharide	..	Non-toxic, non-antigenic	Pyrogenic, 2–5 μg./kg.
Undegraded polysaccharide	..	Poorly toxic, weakly antigenic	Pyrogenic, 0·05 μg./kg.
Lipopolysaccharide	.. .	Toxic, weakly active in producing agglutinins or precipitins in rabbits. Strong heterophile (Forssman), antigen	Strongly pyrogenic, 0·002 μg./kg.

Davies, Morgan and Record[106].

Workers who have investigated the problem with a view to isolating a pyrogenic factor generally isolate the toxic lipopolysaccharide, largely because of the methods adopted and the fact that the lipid fraction is firmly bound to the polysaccharide. Recently (June, 1955), Davies, Morgan and Record have separated from *Shigella dysenteriæ* a polysaccharide in three forms (Table II). The first, a degraded form with a molecular weight of about 25,000, which proved to be non-toxic, non-antigenic but was pyrogenic in relatively large doses. The second, an undegraded

polysaccharide, extracted from the organism with diethylene glycol, with a molecular weight of the order of one million. This material was poorly antigenic but was pyrogenic in doses of 0·05 μg./kg. The third was a lipopolysaccharide isolated from the protein-polysaccharide complex with phenol. The lipopolysaccharide was of very large particle size and was a powerful heterophile (Forssman) antigen, but was only weakly active in the production of specific agglutinins and precipitins in rabbits. This lipopolysaccharide appears to be of the same order of pyrogenic activity as the pure lipopolysaccharides of Westphal.

It is possible to transfer the lipid to other carriers by a method devised by Morgan[36,37], who showed that artificial complexes could be made by coupling the active lipopolysaccharide or the conjugated protein of dysentery or typhoid organisms to a variety of substrates such as agar or mucin or to proteins such as vitellin or serum globulin. Westphal[35] finds that such coupling only occurs when the toxic lipid is present, and has succeeded in transferring the lipid to a casein carrier, so producing a highly active artificial pyrogen.

Little is known about the nature of the pyrogenic substance in pharmaceutical solutions. The substance must be present in a very active form since the few bacteria originally present are represented only by their soluble by-products, usually much diluted. The active substance may be the lipopolysaccharide already described, or perhaps a more active form containing the active grouping favourably presented by a suitable carrier.

Co Tui[38] was the first worker to attack this problem from the angle of pyrogenic activity. He succeeded in isolating from aqueous cultures of the dead bacterial bodies, a polysaccharide substance which was free from protein and had high activity; but the greatest amount of light yet thrown on this subject has undoubtedly come from the work of Westphal and his colleagues in Germany and Switzerland during the last year or two. They improved Palmer and Gerlough's phenol process by extraction with water and phenol at the high temperatures when they are miscible; these separated on cooling to an aqueous phase containing lipopolysaccharide and nucleic acid and a phenol phase containing bacterial protein and inert lipid. Westphal has in this way produced what is probably the purest and most pyrogenically-active polysaccharide yet extracted[39–42] From the material obtained by the phenol extraction of the acetone-dried bacterial bodies he separated the active substance from the nucleic acid by fractional precipitation with alcohol, taking advantage of the nucleic acid absorption at 258 to 260 mμ, and finally by purification using the preparative ultracentrifuge. He found that this method was applicable to all the Gram-negative organisms he examined.

He and his colleagues were able to show that the lipopolysaccharide is electrophoretically homogeneous with a molecular weight of about one million. Other workers examining active polysaccharides from similar organisms have given values of up to 10 million. It is probable that molecular weight is largely influenced by the condition of extraction and degree of polymerisation which has occurred.

There is a need for a standard pyrogen, and at present Westphal's

preparation is probably the most suitable for this purpose and could well be adopted until further knowledge is forthcoming about the nature of the active fraction or active grouping. Workers in America[43,44] have also produced highly purified preparations, but according to our measurements of relative potency it is doubtful if those we have been privileged to examine approach the activity of Westphal's preparation. The great difficulty lies in separating the nucleic acid. It has been shown that Westphal's *E.coli* lipopolysaccharide contains about 40 per cent. of active lipid tightly bound to the polysaccharide carrier. The polysaccharide portion from the lipopolysaccharides of Gram-negative bacteria is composed of amino-sugars including glucosamine and chondrosamine with pentoses and methyl pentoses. The amino-sugars are acetylated and phosphorus is bound to the toxic lipid in small amount, and to the polysaccharide in greater amount, probably in the form of esters. The complete structure of the active lipid is still unknown, but it contains phosphorus and is a phospholipid. The active lipid can be separated from the undegraded polysaccharide by hydrolysis with dilute mineral acid as was shown by Miles and Pirie and other workers with the O-antigen of brucella some years ago.

Niemann[45–48] has examined a pyrogenic lipopolysaccharide isolated in a study of the tumour-necrotising action of a strain of *E. coli*, and has shown the presence of a number of fatty acids such as lauric and myristic along with glucosamine, ethanolamine, phosphoric acid and a curious substance not hitherto reported which consists of a paraffinoid chain with two substituting amino groups. He calls this substance "necrosamine" and this may prove to be of great pharmacological interest. It is seen that we are on the verge of interesting discoveries and that there remains a great deal of work still to be done.

Westphal has endeavoured to produce active preparations for sub-cutaneous use by acetylation of the hydroxyl groups of the polysaccharide, and many other interesting attempts to modify the molecule with a view to modification of the pharmacological characters have also been made with some success. Acetylation of the sugars reduces the toxicity and modifies some of the stimulating actions, but the acetylated pyrogen prepared from *E. coli* is still undergoing clinical trials.

Mode of Action

The mode of action of bacterial pyrogen is not yet completely understood but it is believed that the fever and other effects produced as a result of the injection of bacterial (i.e., exogenous) pyrogen, are largely due to a stimulation of the central nervous and other systems by an endogenous factor or factors liberated into the bloodstream. Rather surprisingly, evidence has been produced to show that the hypothalamus is not necessarily involved in this reaction[49,50]. After injection and before the rise in temperature takes place there is a great increase in the intake of oxygen; but the rise in temperature which follows is produced by conservation of heat by the constriction of surface blood vessels rather than by an increased heat production by shivering[51,52].

During a pyrogen test most workers have noticed that the rabbits' ears and pads become very cold as a result of this local vasoconstriction which it is said, is mediated by the sympathetic nerves. Anti-pyretics will abolish the temperature response without interfering with the other effects of pyrogen and this offers some therapeutic advantages but dictates in these instances the use of some other index of pyrogenic activity, (e.g., a white blood cell method). This is important also in the testing of anti-pyretic substances for the presence of pyrogen.

The rise in temperature after intravenous injection of pyrogen is preceded by a latent period of no temperature rise of up to 90 minutes in man and rather less in rabbits, after which there is a fairly sharp rise in temperature. As was stated this is proportional to the dose given within a limited dose range. Pre-injection temperature is gradually reached again after several hours.

The explanation of the latent period is still a matter for some speculation, but it has been shown that if plasma is taken from a normal animal and incubated with pyrogen and then reinjected, the ensuing latent period is shortened[53,54]. This, together with the work of Grant[55] suggested that during the latent period a new substance is being formed in the body or that the bacterial pyrogen is being modified in some way to produce an endogenous substance which if re-injected acts more rapidly. Other workers believe that plasma alone will not transform exogenous to endogenous pyrogen. It seems more likely that the exogenous pyrogen is first phagocytosed by leucocytes which later release the endogenous pyretic mediator[107]. The important role of the leucocytes for the initial phase of pyrogenic action in higher animals has also been impressively shown by Braude and his colleagues[108] who by using ^{51}Cr-labelled endotoxin from *E. coli* found that more than 90 per cent. of the injected endotoxin is very quickly taken up by the buffy coat layer.

We already have mentioned that Menkin[12–14] and also Abderhalden[15–16] have described substances occurring in body fluids which display some of the properties of endogenous pyrogens and which are liberated more abundantly from tissues and cells on injury; it has not been proved that the reaction following the injection of exogenous (bacterial) pyrogen is due to the liberation of endogenous pyrogen from the tissues although some workers believe this to be the case.

If repeated daily injections of pyrogen are given to experimental animals the dose has to be increased in order to maintain the same level of response because the body seems quickly to become tolerant to the effects of pyrogen. This tolerance, however, disappears in 2 or 3 weeks in rabbits[56] and this has to be remembered in carrying out routine British Pharmacopœial limit tests. Man also becomes tolerant to the effects of injected pyrogen and this was a major difficulty in using vaccines as sources of pyrogen.

The mechanism of the production of tolerance is not clearly understood, but it is fairly well agreed that it is not directly related to the production of antibodies. This does not mean that the pyrogenic lipopolysaccharide is completely non-antigenic; it appears to be a hapten or incomplete

antigen and it seems to be more antigenic in some animals than in others[57] It is, however, a potent heterophile (Forssman) antigen[106]. Even in those instances in which the presence of circulating antibodies to pyrogen have been claimed, no diminution in the pyrogenic response was found; and where pyrogens have been administered together with antigens the appearance and disappearance of tolerance of pyrogen did not parallel the appearance and disappearance of antibodies[58–61] whose production was stimulated by the antigens. Barry Wood and Atkins[109] have now shown that specific bacterial immunity does not diminish pyrogen activity. It does not seem that production of antibodies is involved in the phenomenon of pyrogen tolerance.

Beeson[58] has shown that an induced tolerance to pyrogen may be broken down by blocking the reticuloendothelial system with colloidal thorium dioxide. This suggests that in the tolerant animal it is the reticulo-endothelial system which has developed enhanced ability to eliminate or destroy endogenous pyrogen. This rapidly acquired tolerance, necessitating an increased dose to maintain the level of response, was one of the factors which discouraged the use of vaccines to produce a pyrogenic reaction which would stimulate the body defences. The dosage of successive injections had to be rapidly stepped up in order to maintain an effective response. One worker writes of administering a milky fluid[62] heavily loaded with bacterial bodies in an attempt to maintain the same level of reaction. As vaccines are not without other toxic components this incidental increase in the toxic substances also administered, had undesirable side effects. The new highly purified pyrogens are free from side effects of this kind because they are free from impurities, and while increasing dosage must be given to offset induced tolerance the initial dosage is so minute, (of the order of 0·1 to 0·2 μg. for Westphal's pyrogen, "Pyrexal," prepared from *Salmonella abortus equi*, and 1 to 2 μg. for "Piromen," the American preparation,) that even the largest dose given contains little, if any, extraneous toxic material.

While on the subject of tolerance it is appropriate to mention our own experience with purified pyrogens. We have found that the tolerance developed with a pyrogen preparation from *Proteus vulgaris* is also valid for a salmonella and a pseudomonas pyrogen, indicating a high degree of cross tolerance and a common active component in all three.

The rise in temperature which occurs is always accompanied by changes in the white blood cells, although the reverse is not always true[63–65]. With an ordinary pyrogenic dose there is first a disappearance of the white blood cells, that is leucopenia, followed by a rapid increase in the total number of white cells, a leucocytosis, especially of young polymorphonuclears leucocytes with undivided nuclei. This is accompanied by an eosinopenia and a sustained lymphopenia. This appearance of the young polymorphs giving what is called a "shift to the left" is also seen in many conditions of stress, for example bacterial infections, and injury. With pyrogenic stimulation the degree of shift to the left is proportional to the dose of pyrogen[66].

It is believed that this white blood cell effect is at least in part mediated

by the liberation of ACTH from the hypophysis with subsequent increased secretion of the adrenal cortical hormones[63,67,68]. It is interesting to speculate to what extent a course of pyrogen injections could replace a course of injections of either ACTH or cortisone in certain conditions. Several of the effects of ACTH, for example white blood cell effect, are similar to the effects of pyrogen and it was this finding which first prompted the replacement of ACTH by pyrogen in allergies. Nevertheless one very important difference exists: ACTH causes a profound temperature-fall in normal animals (hypothermia) when injected at the rate of 1 unit per kg. We have found that added pyrogen will reduce this hypothermia, and will elicit the typical pyrogenic response with a delayed peak if large amounts are present. If smaller amounts are present the response does not correspond to the amount added.

Some rabbits appear to be unduly sensitive to the action of ACTH and a state of semi-collapse follows the injection of even 1 unit/kg. In these the hypothermia is so severe and prolonged that no pyrexia occurs and the test fails to detect the presence of added pyrogen. In our experience with rabbits in testing ACTH for pyrogen, the test which uses fever as an index is extremely unsatisfactory, but we can as yet suggest no alternative. ACTH also interferes with the white cell response to pyrogen, so that this too is rendered unsuitable as an index of pyrogen present in the ACTH.

It is believed that the white blood cell changes following injection of pyrogen are largely mediated by the adrenal cortex as they are substantially altered in adrenalectomised animals[69–71]. This stimulation of the adrenal cortex appears to be in response to the increased secretion of ACTH which may in turn be the result of a fall in circulating corticoids which may follow the demands of the tissues after injury or stimulation of the tissue cells by pyrogen. Evidence of pituitary adrenal stimulation by pyrogen is also found in the increased urinary excretion of corticoids[72] and in the fluctuation of plasma ascorbic acid levels[73]. Whatever the mechanics of the process there is adequate evidence of the activation of the pituitary adrenal cortex cycle.

Clinical experience has shown that long-continued administration of cortisone or ACTH has disadvantages; the withdrawal effects of these hormones are also common and are sometimes evidenced by resistant exacerbations[72]. The advantages of stimulating the adrenal cortex with pyrogen thus becomes apparent. The withdrawal effects and endocrine disturbances seen with ACTH and cortisone do not occur in pyrogen therapy.

Besides a well marked stimulation of the pituitary adrenal systems the influence of injected pyrogen is seen on connective tissue and on the reticulo-endothelial system. The dermis is an essential part of the reticulo-endothelial system, and the stimulation of this system by pyrogen has been shown dramatically in the healing effect of pyrogen on burned, wounded and frostbitten skin[58,73–77]. In experiments on the regeneration in the central nervous system it has been shown that by enhancing vascularity of the area, pyrogen inhibits the process of gliosis and so facilitates

regeneration of nerve fibre. Evidence for structural regeneration has been supplied and in laboratory studies some functional regeneration has been reported[78–82]. This is new evidence in a problem which has been perplexing neurologists for years.

It has been known for many years that Gram-negative bacterial extracts when injected have the power to cause breakdown and necrosis in tumour tissue. A preparation known as Coley's fluid was in use for some time for this purpose. Dr. Shear in America has investigated the purified lipopolysaccharide which he has obtained from *Serratia marcescens*[32,34] (*Chr. prodigiosum*). In addition to its ability to cause breakdown of neoplastic tissue this substance also proved to be a potent pyrogen[83] and there is evidence of a common tumour—necrotising activity in many of the Gram-negative endotoxins.

We have seen in this section that apart from being a potentially useful remedy with remarkable effects, the lipopolysaccharide is proving to be a useful tool in revealing how many of the mechanisms of the various body systems function. Much work remains to be done to explain these actions fully and to decide, for example, whether there exist substances in the body each separately capable of stimulating one or other of the actions discussed.

Pyrogens as Medicaments

I cannot close without some reference to the various disorders which have been successfully treated by means of either vaccines or pure pyrogen. I shall mention only a few, but there is much published evidence concerning favourable effects in many disorders.

Let us be perfectly clear about one point: pyrogen is not a specific therapeutic agent like an antibiotic which has its own bacterial spectrum nor for that matter is it like an antitoxin. Its action is not an attack but a vigorous stimulation of the natural defences of the patient; and this is the rationale for its use in so many diseases. It may not be too bold to say that it has been the absence of such a defensive agent which has directed medical research towards finding attacking agents such as serologicals and antibiotics. Now that we have both there is no reason why they should not be used together—the one to help the other.

Some of the recent reports on the newer preparations advocate their use in sub-febrile doses; in others, febrile doses have been recommended but the fever has been suppressed with antipyretics. On the other hand some workers believe that the actual production of fever is necessary for the full beneficial effects to be produced. There is little doubt that febrile doses are essential in certain conditions. In general, pyrogen appears to be most usefully used in conjunction with other specific therapies when such a combination appears rational.

The similarity of the effects of pyrogen, ACTH and cortisone on the white blood cell picture pointed to involvement of the pituitary-adrenal system in the pyrogen reaction, and the known beneficial effects of ACTH and cortisone in the treatment of allergic conditions suggested the use of pyrogen in a variety of conditions[63]. It seems that pyrogen is capable of filling a role similar to that filled by these hormones in allergic treatment

but without their dangers. And this is important; for dangers such as resistant exacerbations on withdrawal and endocrine imbalance often follow their use, while pyrogen therapy has, so far as is known, neither withdrawal symptoms nor long-term side effects[84].

It is reported that sub-febrile doses are no less effective in giving relief in many conditions[84–88], and moreover some patients report a sensation of relaxation and well-being after pyrogen treatment. It has since been found that sub-febrile doses of pyrogen are particularly useful in relieving the depression which often accompanies the allergic state[87]. It should be remembered however that pyrogen, like ACTH or cortisone, can act only as an adjunct to specific diagnosis and therapy in allergy. In this role pyrogen has been most useful especially in stubborn cases of multiple allergies.

Several authors have reported favourably on pyrogen in dermatological practice using febrile and sub-febrile doses, and febrile doses with antipyretics. Superiority over vaccine therapy in this field has been well established[90] when febrile doses are administered. Fever can be avoided by utilising the subcutaneous or intra-muscular routes of administration and pyrogen has been used successfully in this manner in various dermatological conditions as an adjunct to topical therapy[88]. More than one author has been impressed with the striking results obtained in the pyrogen treatment of otitis externa[88–91]. Complaining of the dangers and disadvantages attendant on the use of ACTH and cortisone in dermatological practice, Guerrieri, reporting a successful trial of pyrogen alone (Piromen) (alone) in neurodermatitis[84], comments on its safety in use and the absence of any "post-treatment rebound." Other workers[92–94] have confirmed the beneficial effects in varied dermatoses.

The findings that in cats and dogs pyrogen aids nerve regeneration and that in transected spinal cords good anatomical regeneration takes place with enhanced vascularisation and absence of glial scarring are interesting[78–82], and prompted one author[95] to use it (Piromen) in 118 cases of spinal cord injury and disease. Beneficial results were obtained in 10 per cent. of the patients, but he felt that further investigation was warranted as the dosage and duration of treatment had been inadequate. Improvement with pyrogen therapy has also been reported in other diseases involving the nervous system[96–100].

The effect of pyrogen in stimulating the repair of damaged tissue has been investigated in the treatment of duodenal ulcers. The value of this treatment can easily be assessed from the results which are reported[101] as relief in 19 out of 25 cases with complete healing of the crater in 18 out of the 19. Recurrence was less than one-third.

The fibrinolytic action of pyrogen[18] has already been mentioned, but this localised action of pyrogen has another interesting application which has been known for many years. This concerns the use of pyrogen[102] for the detection of unsuspected foci in, for example, an infected gall bladder or other organ or tissue. Frequently, patients have complained of localised pain while undergoing pyrogen therapy and this has led to the detection of a septic focus at the site of pain.

One of the oldest uses of pyrogen has been the non-specific antibacterial effect; diptheria carriers[103] have been cleared and other infections such as typhoid have been aided by fever therapy. The action of pyrogen as an adjuvant in sulphonamide and chloramphenicol treatment has also been reported[105]. Cases of agranulocytosis[104] caused by drug treatment have been successfully treated and it has been assumed that the leucocytosis which follows the administration of pyrogen is responsible for this success.

This very brief review of the clinical applications of pyrogen will enable my audience to appreciate how the physiological effects of this remarkable substance have been systematically applied to the treatment of various disease states with considerable success.

We have reviewed the properties of bacterial pyrogens and have shown that they bring about a stimulation of the body's natural defences which are immediately mobilised when the body is attacked. Although our description of this attack and this mobilisation of defences is perhaps inadequate, and although we speak of stimuli, injury and irritation, which barely express the full meaning, it is clear that in pyrogen we have a tool which, if used intelligently has great powers for good, not in one, but in a wide variety of conditions. It would be foolish to suggest that pyrogen therapy is a panacea or will render any existing useful drug unnecessary, but it is suggested that it will act as a vigorous adjuvant to the well-tried medicament. Pyrogen therapy, I feel, is an ally and not a substitute for specific therapy.

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SCIENCE PAPERS AND DISCUSSIONS

PREPARATION OF AMINOPHYLLINE TABLETS

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Aminophylline was first included in the 7th Addendum to the 1932 British Pharmacopœia under the name of Theophylline with Ethylenediamine. Tablets of Theophylline with Ethylenediamine were included in the 1951 Addendum to the 1948 Pharmacopœia and are retained in the 1953 edition under the name of Aminophylline Tablets.

The pharmacopœial method of preparation is moist granulation and compression. Tablets made by this method become discoloured and develop an odour of ammonia within a few weeks of preparation. During discussion at the British Pharmaceutical Conference, 1954[1], Denston[2] suggested that the moist granulation method had been found to be satisfactory if drying is carried out thoroughly. However, discussion with several pharmacists reveals the widespread observation of the rapid deterioration of tablets prepared by the official method, and also, commercially available tablets vary appreciably in appearance and odour. For example, of six samples of tablets purchased on the open market from different sources, the colour varied from white to pale yellow and the odour from practically none to a marked odour of ammonia. It is noteworthy that no positive correlation exists between colour and odour. The properties of these tablets are shown in Table I.

TABLE I

Properties of commercial samples of aminophylline tablets

Appearance	Odour
Pale cream colour with a few darker patches	Practically none
White	Faint odour of ammonia
Pale yellow colour	Faint odour of ammonia
White	Very faint odour of ammonia
Cream colour	Marked odour of ammonia
Very pale cream colour	Marked odour of ammonia

These six samples were purchased from different sources on the open market. It will be seen that there is no correlation between colour and odour.

Experimental

Samples of aminophylline powder were purchased from each of six manufacturers and their physical properties were noted. The colour varied from white to pale yellow and the odour from practically none to a marked odour of ammonia or acetamide. Again there was no correlation between colour and odour. The properties of these powders are shown in Table II. Batches of tablets were prepared from all of these samples of powder by each of three different methods.

Method A. Starch, talc and magnesium stearate were mixed with the

aminophylline in suitable proportions and granules were made by the moist granulation method. The granules were dried carefully and then compressed into tablets.

Method B. A base consisting of talc and starch was first prepared by moist granulation and subsequent drying, and the dry base was then mixed with the aminophylline and a small amount of magnesium stearate and the mixture was compressed into tablets.

Method C. Aminophylline, starch, talc and magnesium stearate were mixed in suitable proportions and the mixture was compressed into "slugs". These were broken up through a 16 mesh sieve and the 36 mesh dust was recompressed. The dry granules obtained were mixed with more starch and magnesium stearate and the mixture was then compressed to give the finished tablets. Recompression of the dust was necessary because the formula does not bind well and much "fines" are produced.

TABLE II

PROPERTIES OF BULK AMINOPHYLLINE POWDER

Colour	Odour
Cream	Marked odour of ammonia
White	Faint odour of ammonia
Very pale cream	Practically none
Cream	Faint odour of ammonia
Pale yellow	Strong odour of acetamide
Cream	Odour of acetamide

These six samples were also purchased from different suppliers on the open market. Again there is no correlation between colour and odour.

The tablets thus prepared were examined on preparation and at the end of storage for six months and one year in glass containers with metal screw caps and waxed wads which were stored at room temperature in the dark. When freshly prepared the tablets were all practically white or pale cream in colour and were almost odourless or did not have more than a faint odour. This was surprising, in view of the properties noted in Table II, but may be due to some of the odour dissipating during preparation and the colour may be masked by the addition of other ingredients.

TABLE III

PROPERTIES OF SAMPLES OF AMINOPHYLLINE TABLETS AFTER STORAGE

Sample	After storage for 6 months	After storage for 1 year
A 1	Deep cream colour. Strong odour of ammonia	Brown colour. Strong odour of ammonia
A 2	Pale cream colour. Strong odour of ammonia.	Brown colour. Strong odour of ammonia
A 3	Deep cream colour. Strong odour of ammonia.	Brown colour. Strong odour of ammonia
A 4	Very deep cream colour. Strong odour of ammonia	Brown colour. Strong odour of ammonia
A 5	Deep cream colour. Strong odour of ammonia	Brown colour. Strong odour of ammonia
A 6	Pale cream colour. Strong odour of ammonia	Brown colour. Strong odour of ammonia
B 1	White. Practically no odour	White. Practically no odour
B 2	White. Practically no odour	White. Practically no odour
B 3	White. Practically no odour	White. Practically no odour
B 4	White. Practically no odour	White. Practically no odour
B 5	Slightly mottled. Practically no odour	Slightly mottled. Practically no odour
B 6	Pale cream. Practically no odour	Pale cream. Practically no odour
C 1	White. Practically no odour	White. Practically no odour
C 2	White. Practically no odour	White. Practically no odour
C 3	White. Practically no odour	White. Practically no odour
C 4	White. Practically no odour	White. Practically no odour
C 5	Very mottled. Strong odour of acetamide	Very mottled. Strong odour of acetamide
C 6	White. Practically no odour	White. Practically no odour

Samples A, 1–6 were made by Method A from the different samples of aminophylline powder listed in Table II.
Samples B, 1–6 were made by Method B.
Samples C, 1–6 were made by Method C.

The properties of these tablets after storage for six months and one year are given in Table III. It will be seen that, after six months, those made by moist granulation varied in colour from pale cream to very deep cream and all smelled strongly of ammonia. Most of the tablets prepared by the other two methods were white in colour and practically odourless. One was pale cream and another slightly mottled, these two samples being practically odourless. One sample (C 5), however, had become very mottled and smelled strongly of acetamide. The cause of this is unknown.

After one year's storage the colour of the tablets made by moist granulation had become brown, whereas those made by the other two methods remained as they had appeared after 6 months.

Samples of all these tablets after 6 months' storage were assayed by Dr. G. E. Foster and the results are listed in Table IV. These show

TABLE IV

ANALYSIS OF AMINOPHYLLINE TABLETS AFTER SIX MONTHS' STORAGE AT ROOM TEMPERATURE

Sample	Time of disintegration at 37° C. (min.)	Average weight (g.)	Ethylenediamine per cent.		Theophylline per cent.	
			In products	Label strength	In products	Label strength
A 1	13	0·2438	5·53	108·0	31·5	102·5
A 2	9	0·2464	6·16	121·5	33·9	111·5
A 3	12	0·2374	6·1	116·0	36·5	115·5
A 4	16	0·2309	5·11	94·5	32·0	98·5
A 5	11	0·2436	5·59	109·0	30·2	98·0
A 6	12·5	0·2377	5·50	104·5	31·1	98·5
B 1	5	0·1313	10·1	106·0	55·2	96·5
B 2	3	0·1390	9·36	104·0	54·5	101·0
B 3	4	0·1318	10·75	114·0	63·0	110·5
B 4	3·5	0·1318	11·1	109·5	60·5	99·5
B 5	2·5	0·1280	10·5	108·0	57·0	97·0
B 6	2·25	0·1295	11·5	119·0	66·0	114·0
C 1	4	0·1327	10·2	108·0	55·2	98·0
C 2	2	0·1229	9·84	97·0	56·1	92·0
C 3	1·25	0·1356	6·75	73·0	41·1	74·0
C 4	3	0·1336	10·2	109·0	54·5	97·0
C 5	6	0·1308	0·72	7·9	5·7	10·4
C 6	3·5	0·1339	10·3	109·0	60·0	107·0

Samples A, 1–6 were made by Method A from the different samples of aminophylline powder mentioned in Table II. Samples B, 1–6 were made by Method B. Samples C, 1–6 were made by Method C. Label strengths have been calculated on the basis of an average B.P. tablet containing ethylenediamine 0·0125 g. and theophylline 0·075 g.

that the samples prepared by moist granulation were unfit for use after six months because of their physical properties although they still complied with the pharmacopœial requirements for theophylline and for ethylenediamine content. Those made by method B were all reasonably satisfactory in physical properties, after one year and complied with the requirements for strength of active ingredients. With the exception of sample C 5, the tablets made by method C were satisfactory in physical properties after storage for one year. The faulty sample was also very low in strength and sample C 3 was also low in strength when assayed after 6 months. The cause of this is unknown. A sample of tablets made by the dry granulation process (method C) sent to me by I. C. Edmundson is in perfect condition over two years after the date of their preparation.

Effect of Various Conditions of Storage

It is stated in the International Pharmacopœia that aminophylline should be stored protected from light, and Bull[3] also recommended that aminophylline tablets should be protected from light. Since many compounds containing amino groups darken in colour on exposure to light, six different samples of tablets enclosed in screw-capped white glass bottles were exposed to direct sunlight for three months. At the end of this time their appearance was compared with samples from the same batches which had not been exposed to sunlight and no darkening was noted. Some of each sample were assayed by Dr. G. E. Foster and the results are given in Table V. The results show that within the experimental error of the assay, no loss of strength had occurred.

TABLE V

ANALYSIS OF AMINOPHYLLINE TABLETS BEFORE AND AFTER EXPOSURE TO SUNLIGHT

Sample		Average weight (g.)	Ethylenediamine per cent.		Theophylline per cent.	
			In products	Label strength	In products	Label strength
1	Before exposure ..	0·2235	7·47	133·6	32·7	97·4
	After " ..	0·2235	7·80	139·5	31·4	93·6
2	Before " ..	0·2293	5·74	105·3	35·0	107·0
	After "	0·2293	5·74	105·3	34·8	106·4
3	Before " ..	0·2294	5·45	99·99	36·5	111·6
	After " ..	0·2294	5·51	100·1	35·7	109·1
4	Before " ..	0·2503	6·26	125·5	42·0	140·1
	After " ..	0·2503	6·20	124·4	41·1	138·8
5	Before " ..	0·2330	6·82	127·2	30·7	95·4
	After " ..	0·2330	6·87	131·1	32·7	101·6
6	Before " ..	0·2065	6·10	100·8	35·7	98·3
	After " ..	0·2065	6·07	100·3	36·1	97·1

Five of these samples were purchased on the open market and the sixth was manufactured. Label strength have been calculated on the basis of an average B.P. tablet containing ethylenediamine 0·0125g.and theophylline 0·075 g. It appears that, having regard to the experimental error of the assays, exposure to light has not had any appreciable effect on the composition of the tablets.

One sample prepared by moist granulation and one by preliminary compression were stored in screw-capped bottles with cork wads at 37° C. in an electrically-heated oven for 6 months. The former had become pale cream in colour with brown spots and had a faint odour of ammonia. The latter had remained white and had a faint odour of ammonia. Similar samples were stored in loosely closed bottles over water to give an atmosphere with maximum moisture content. At the end of 7 months the tablets made by moist compression had become deep brown in colour and had a strong odour of ammonia. Those made by preliminary compression were still white and practically odourless. This is surprising since both samples had absorbed sufficient water to become very soft.

DISCUSSION

Aminophylline obtained from different commercial sources varies in colour and odour. The pharmacopœial description states that the colour is either white or yellowish-white. The colour darkens on storage but the rate of darkening does not appear to be increased by direct sunlight.

The Extra Pharmacopœia (Martindale) Vol. I, 1952, and the United States Dispensatory, Vol. I, 1950, state that aminophylline develops a yellow or brown colour on contact with lactose. Bull[3] (British Pharmaceutical Conference, 1954) states that sugars should be avoided in the formulation of aminophylline tablets and our experience confirms this, as does that of I. C. Edmundson of Dunedin, New Zealand, and N. J. Van Abbé of Loughborough, with whom I have corresponded during this work.

If granules containing lactose are made by the moist process a yellow colour develops within an hour or so of preparation and with dextrose a deep brown colour appears in about the same time In addition, tablets containing glucose prepared by granulation by preliminary compression become lemon yellow in colour within three days of preparation. With sucrose a yellow colour develops within about 12 hours of the preparation of moist granules. Discoloration also occurs in tablets made by moist granulation containing a high proportion of starch but is slower in developing. Edmundson suggested that the colour may be due to caramelisation of the carbohydrates in the presence of the alkaline ethylenediamine, but we have found some discoloration in tablets entirely free from carbohydrates. Another possible source of discoloration is contamination with metals. Ethylenediamine is, of course, a chelating agent and readily reacts with metals. For example, aminophylline gives a yellowish-brown colour with ferrous iron, a chocolate colour with ferric iron and a vivid purple colour with cupric salts. In each case the theophylline is precipitated. Yet another cause of discoloration may be oxidation. If 100 volumes hydrogen peroxide is added to aminophylline a yellow colour develops within about 10 minutes.

Tablets of aminophylline prepared by the official method of moist granulation have always, in our experience, deteriorated fairly rapidly, often becoming unfit for use in a few months or even weeks. Tablets which have become markedly discoloured and strongly odorous may still comply with the official assay for theophylline and for ethylenediamine content. Van Abbé, however, has told me that in his experience the discoloration is usually, if not always, accompanied by a fall in ethylenediamine content.

Satisfactory tablets can be prepared by two methods in which moistening of the aminophylline is avoided. Samples made by these two methods have remained in perfect condition on storage for at least a year.

SUMMARY

1. The discoloration of aminophylline tablets is discussed and the official method of moist granulation is criticised.

2. Two methods of preparation in which the aminophylline is not moistened are suggested.

I wish to thank Mr. I. C. Edmundson for many helpful suggestions, including methods B and C; Dr. G. E. Foster for carrying out assays on several samples; Mr. N. J. Van Abbé for exchange of information and Mr. A. J. Pearson for technical assistance.

REFERENCES

1. Whittet, *J. Pharm. Pharmacol.*, 1954, **6**, 1074.
2. Denston, *ibid.*, 1954, **6**, 1080.
3. Bull, *ibid.*, 1954, **6**, 1077.

DISCUSSION

The paper was presented by the AUTHOR.

PROFESSOR K. BULLOCK (Manchester) said that the figures in Tables IV and V would be much more valuable if some information had been given about the errors and their possible sources particularly in sampling.

Dr. G. E. FOSTER (Dartford) said that Mr. Whittet had purchased samples of aminophylline and compressed them. If he had taken theophylline and ethylenediamine and purified them, he might have found that another factor involved was some impurity in the material used. The samples tested in his laboratory consisted of about 25 tablets each, and it had been assumed that the author had sent a representative sample.

MR. H. GRAINGER (London) pointed out that aminophylline was a sequestering agent, for metallic ions which might be the cause of discoloration rather than the sugars.

MR. V. REED (London) asked whether tablets with an odour of ammonia should be used.

MR. N. J. VAN ABBÉ (Loughborough) said he had found that when compressed dry, aminophylline often caused binding in the dies and mechanical damage. Had the author experienced this difficulty?

MR. T. C. DENSTON (London) said that as the official moist granulation process was a general method which permitted a great deal of latitude to the operator, precise details of the method used by the author should be stated.

DR. F. HARTLEY (London) said that although aminophylline was an established drug it was difficult to understand why the ethylenediamine compound of theophylline should be used in tablets since it was the theophylline which exerted the therapeutic action. Did the discoloration reduce the therapeutic effect of the tablets? He suggested that a critical therapeutic evaluation of theophylline and of ethylenediamine was desirable.

MR. WHITTET, in reply, agreed that samples of aminophylline varied greatly. Because ethylenediamine was a sequestering agent, moist granules put through a metal sieve became discoloured.

He did not think that an odour of ammonia indicated any great deterioration in the tablets. He had had no damaged punches and dies.

In regard to incompatibility with sugars, he had found that it was the ethylenediamine which reacted, samples of theophylline with sugars not being discoloured after several months.

He appreciated that it might be possible to obtain a better solubilising agent for theophylline. There was no evidence of great loss of activity in discoloured tablets, but pharmaceutically they looked bad.

AN EXAMINATION OF RUBBER USED AS A CLOSURE FOR CONTAINERS OF INJECTABLE SOLUTIONS

PART I. FACTORS AFFECTING THE ABSORPTION OF PHENOL

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INTRODUCTION

RUBBER closures are used extensively to seal containers of a variety of injectable solutions and these are composed of rubber of widely differing compositions. The British Pharmacopœia 1953 states simply that rubber closures are made from suitable heat vulcanised rubber of good quality, and does not define any controlling chemical tests or physical standards. It does recognise, however, the property of rubber in absorbing sulphite and bacteriostatic substances. It directs that closures to be used for sealing containers, in which the injection containing these substances is enclosed, shall be subject to refluxing or contact with solutions equal to or double the strength of the sulphite or bacteriostatic substance concentration in the final injection. These requirements in no way standardise the type of rubber, particularly in limiting its physical variation or chemical reactivity with the ingredients of the injections into which it comes into contact. In a recent paper Wiener[1] has shown the ability of rubber to absorb thiomersalate from solution. Burrell[2] mentions the absorption of phenol, chlorbutol, chlorocresol and phenylmercuric nitrate by pure latex rubber. McGuire and Falk[3] have shown that phenol is absorbed by rubber caps on prolonged storage and Berry[4] has noted the amount of chlorocresol withdrawn from solution by rubber caps, and has referred to the absence of detailed evidence about this subject.

ESTIMATION OF PHENOL

The writer has employed a method for the estimation of phenol using Folin-Ciocalteau reagent. Initially a calibration curve was produced using dilutions of a stock standard solution of phenol containing 0·03, 0·06, 0·09 and 0·12 mg. of phenol per 4 ml. 0·6 ml. of Folin-Ciocalteau reagent and 1 ml. of 25 per cent. solution of sodium carbonate was added to each. After shaking to mix and heating for 15 minutes in a water bath at 37° C. readings were obtained in a Spekker absorptiometer using filter number 608 and with the test and blank solutions in 1 cm. cells.

When determining the solutions which had been in contact with rubber, suitable dilutions were made to contain approximately 0·05 mg. per 4 ml. or 0·1 mg. per 4 ml. when using 0·5 cm. cells.

4 ml. of this solution was treated as above and the readings made in the Spekker absorptiometer. The concentration of phenol was read from the calibration curve and the strength of the original solution obtained from the following equation.

$$\text{Phenol concentration (Percentage w/v)} = \frac{\text{No. of mg. per 4 ml. of dilution} \times 25 \times \text{dilution.}}{\text{Thickness of cell in cm.}}$$

Composition of Rubber

Rubber is a complex mixture in which latex or acid precipitated latex dried in smoke houses or the air is combined with a number of chemical substances. Sulphur is usually included as the vulcanising agent, zinc oxide as the activator and such organic substances as aldehyde amines, guanidines, dithiocarbamates and benzthiazoles as accelerators. Fillers are also added; these including such substances as chalk, magnesium carbonate, zinc oxide, china clay, silica and carbon. Latex contains proteins, resins, querbrachit and sugars, and also in its preservation, ammonia is commonly added.

In this investigation a number of rubbers have been chosen mainly from commercial sources. Particulars of these tubings are given in Table I.

TABLE I

Particulars of rubber samples employed

Sample number	Type of tubing	Rubber content, per cent.	Main filler	Other details
1	Red	33	Calcium carbonate	Laboratory tubing
2	Red	50	Calcium and magnesium carbonates	Drainage tubing
3	Latex	90	Magnesium carbonate	Transfusion tubing
4	Black	50	Carbon Black	Anti-static tubing
5	Red	—	—	General purpose tubing
6	Red	—	—	〃
7	Latex	—	—	Transfusion tubing
8	Latex-silicone	—	None	Vulcanising ingredients:—sulphur, zinc oxide and organic accelerator of dithio-carbamate series. Latex is pre-vulcanised and afterwards centrifuged to remove surplus vulcanising ingredients
9	Silicone	—	Silica	Transfusion tubing
10	Red	50	Calcium and magnesium carbonates	Drainage tubing

Rate and Extent of the Absorption of Phenol

Apart from the different compositions of rubber, additional factors involved in the absorption of phenol are time, temperature and the concentration of the phenol solution in contact. Rubber sample No. 10 was used to investigate these factors, two series of tubes being stored at 2° C. and 37° C.

In the first series, four tubes containing 7·5 cm. of rubber tubing were immersed in 15 ml. of 0·5, 1, 2 and 4 per cent. solutions of phenol together with four tubes containing phenol solutions only. These were placed in a refrigerator at 2° C. In the second series five sets of eight tubes were set up, each containing 5 cm. of rubber tubing and immersed in 10 ml. of 0·5, 1, 2, 4, and 7·5 per cent. solutions of phenol. Five sets of each of the phenol solutions were similarly prepared. All tubes were flame-sealed and placed in an incubator at 37° C.

Measured volumes were removed at intervals from the first series, and the phenol content of the solution determined. Similarly, the tubes of

the second series were taken at intervals, opened and the contents analysed for phenol.

Table II shows the amount of phenol which was absorbed by the rubber after increasing periods of time in the phenol solution at 2° C., and for comparison is expressed as mg. of phenol absorbed per g. of rubber. This

TABLE II

PHENOL CONTENT OF RUBBER AND IMMERSING SOLUTIONS AFTER INTERVALS OF STORAGE AT 2° C.

Phenol content of original solution per cent. w/v	Storage time	Total phenol absorbed in mg.	Amount of phenol absorbed per g. of rubber	No. of mg. of phenol in solution	Volume of solution in ml.
0·5	7 hours	4·5	1·50	70·5	15·0
	26 ,,	4·8	1·60	67·9	14·5
	170 ,,	4·8	1·60	65·5	14·0
	3 weeks	13·0	4·25	62·0	15·0
	6 ,,	16·9	5·53	54·6	14·3
	24 ,,	18·4	6·02	45·4	13·6
0·98	7 hours	2·0	0·65	145·0	15·0
	26 ,,	6·2	2·00	136·0	14·5
	170 ,,	14·5	4·71	123·0	14·0
	3 weeks	25·9	8·41	121·0	15·0
	6 ,,	25·9	8·41	112·6	14·4
	24 ,,	32·0	10·40	95·1	13·6
1·95	7 hours	5·25	1·70	287·3	15·0
	26 ,,	13·0	4·13	270·0	14·5
	170 ,,	35·0	11·10	238·0	14·0
	3 weeks	44·4	14·10	247·5	15·0
	6 ,,	44·2	14·10	240·0	14·6
	24 ,,	66·8	21·20	194·4	13·8
3·90	7 hours	15·0	4·76	570·0	15·0
	26 ,,	26·1	8·29	539·0	14·5
	170 ,,	71·4	22·70	474·6	14·0
	3 weeks	91·2	28·90	494·0	15·0
	6 ,,	90·2	28·60	463·5	14·6
	24 ,,	124·4	39·50	412·4	13·8

TABLE III

PHENOL CONTENT OF RUBBER AFTER INTERVALS OF IMMERSION AT 37° C. IN DIFFERENT STRENGTHS OF PHENOL

Approximate strength of phenol solution, per cent.	Period of storage	Total phenol absorbed in mg. (average of two determinations)	Amount of phenol (mg.) absorbed per g. of rubber
0·5	7 hours	3·55	1·71
	26 ,,	9·85	4·76
	7 days	11·85	5·72
	21 ,,	12·15	5·87
1	7 hours	13·50	6·52
	26 ,,	20·35	9·83
	7 days	22·00	10·62
	21 ,,	23·00	11·11
2	7 hours	21·70	10·48
	26 ,,	30·60	14·78
	7 days	45·70	22·08
	21 ,,	44·10	21·30
4	7 hours	35·40	17·10
	26 ,,	60·45	29·20
	7 days	84·00	40·60
	21 ,,	83·70	40·43
7·5	7 hours	94·50	45·65
	26 ,,	127·80	61·73
	7 days	145·60	70·30
	21 ,,	145·00	70·10

is also shown more clearly in Figure 1. Similarly Table III and Figure 2 show the amount of phenol absorbed during storage at 37° C.

It is seen that a similar state of equilibrium is eventually reached, but more slowly, at the lower temperature. At 37° C. the absorption is rapid

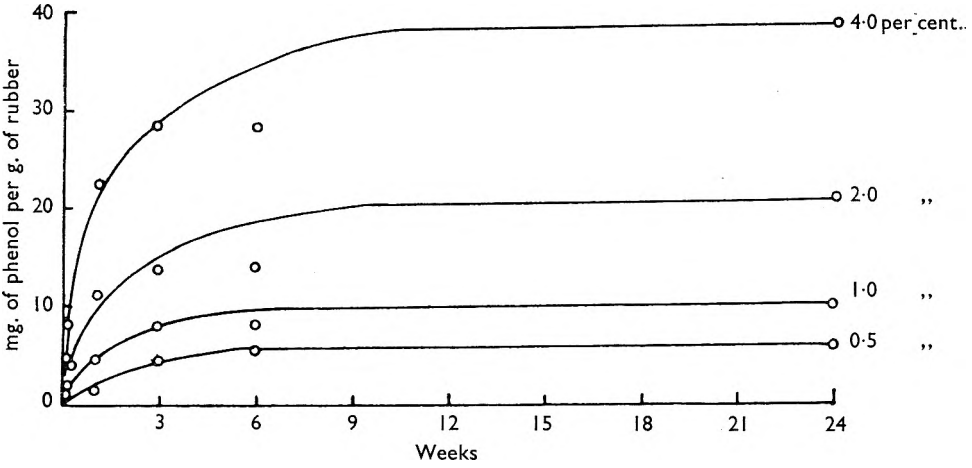

FIG. 1. Rate and amount of phenol absorbed by rubber sample 10 when immersed in phenol solutions at 2° C. of the strength indicated.

during the first day and equilibrium is reached about the sixth day of contact. At 2° C. an appreciable proportion of phenol is absorbed after 3 days but equilibrium seems to be delayed for 7 to 15 weeks depending on the concentration of phenol solution. It also appears that the amount of phenol absorbed is almost directly proportional to the concentration of the original phenol solution. After equilibrium is reached this sample of rubber did not continue to absorb phenol, contrary to the inference of the Pharmacopœia.

In order to determine to what extent this process was reversible the final samples under examination at 37° C. were transferred, after the sealed tubes had been opened for analysis, and after solution had been

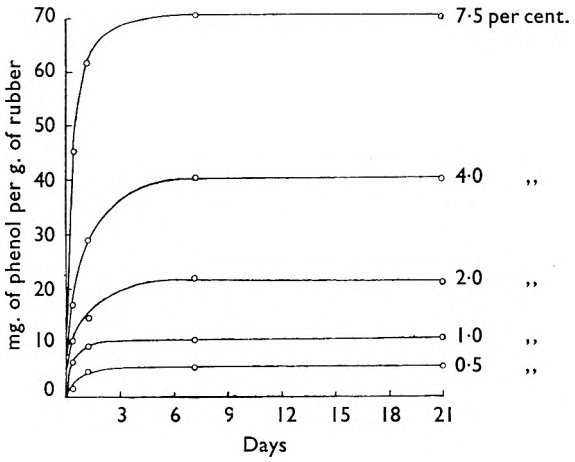

FIG. 2. Rate and amount of phenol absorbed by rubber sample 10 when immersed in phenol solutions at 37° C. of the strength indicated.

removed from the rubber with a dry cloth, to 10 ml. of water in test tubes. These were sealed by heating the glass and replaced in the incubator at 37° C. After 10 days the solution in the tubes was analysed. The results of this are shown in Table IV.

TABLE IV

THE PHENOL CONTENT OF A SAMPLE OF RUBBER AFTER IMMERSION IN PHENOL SOLUTIONS AND AFTER THE SAME SAMPLE HAD SUBSEQUENTLY BEEN PLACED IN WATER FOR 12 DAYS AT 37° C.

A. Rubber immersed in phenol solutions		B. Rubber containing phenol immersed in water	
Amount of phenol in rubber in mg.	Concentration of phenol in water as mg. per 10 ml.	Amount of pheno. in rubber in mg.	Concentration of phenol in water as mg. per 10 ml.
12·15	37·15	2·85	9·30
23·00	75·60	6·25	16·75
44·10	153·40	11·90	32·20
83·70	313·40	18·60	64·60

When one divides the amount of phenol, expressed as mg. in the 10 ml. of aqueous phenol solution in contact with the rubber, by the phenol content of the rubber at the points of equilibrium shown in Table III and Table IV the figures obtained point to a similar relationship in the distribution of phenol. One can thus conclude that the reaction is reversible. The figures for comparison are given in Table V.

TABLE V

Strength of original phenol solution (approx.), per cent.	Concentration of phenol solution at equilibrium in mg. per 10 ml. divided by total amount of phenol in rubber in mg.	
	A. Rubber immersed in phenol solution	B. Rubber containing phenol immersed in water
0·5	3·06	3·36
1·0	3·29	4·25
2·0	3·49	2·71
4·0	3·75	3·47

PHENOL ABSORPTION BY DIFFERENT RUBBERS

It was felt that the results obtained in the examination of one sample of rubber tubing should be extended to other rubbers to see whether the absorption process followed a common pattern.

Nine rubber tubings, samples 1 to 9, were taken and ten 5 cm. lengths of each were weighed. Four of each sample were placed in tubes with 10 ml. of 0·5 per cent. w/v phenol solution. Two of each were stored respectively at 2° C. for 44 days and 37° C. for 7 days. Six of each sample were similarly placed in 10 ml. of 4 per cent. w/v phenol solution and two of each were stored for 16 hours, 64 hours and 10 days respectively. All tubes during storage were sealed by flame sealing of glass. At the end of the period of storage all were opened and the phenol content of the solution determined. At the same time the strength of solutions containing approximately 0·5 per cent. and 4 per cent. of phenol were similarly determined and the *p*H value of all solutions from the opened tubes was determined electrometrically. In addition, a further duplicate set of

ten tubings including sample 10 were placed in 4 per cent. w/v phenol solution at 37° C. for 12 days.

In order to see whether the process was reversible, the tubings which had been stored at 2° C., in 0·5 per cent. w/v phenol solution after removal of the phenol solution in which they had been immersed, were transferred to 10 ml. of water and stored for 13 days at 37° C. in sealed tubes. Similarly the second series which had been stored at 37° C. in 4 per cent. w/v phenol solution were subject to 7 weeks storage at 37° C. in 10 ml. of water. All were examined at the end of storage for both phenol content and *p*H value.

From an examination of the amounts absorbed by the sample tubings 1 to 9 stored for 16 hours, 64 hours and 10 days, it can be seen that the results show a close comparison with the rate of phenol absorption by sample 10 as illustrated in Figure 1, namely, that at 37° C. roughly 75 per cent. of the total absorption of phenol is completed in 16 hours and 95 per cent. and upwards of total absorption is reached after 64 hours storage. The results are shown in Table VI.

TABLE VI

SHOWING THE AMOUNT OF PHENOL IN MG. ABSORBED BY 5 CM. OF TUBING FROM 4 PER CENT. W/V PHENOL SOLUTION AT 37° C.

Sample	Time of storage		
	16 hours	64 hours	10 days
1	48, 55	66, 59	72, 63
2	69, 57	77, 78	83, 79
3	—, 38	50, 46	56, 51
4	52, 47	49, 44	56, 54
5	53, 51	53, 57	—, 64
6	44, 55	53, —	54, 63
7	29, 38	46, 38	47, 39
8	43, 37	42, 44	51, 41
9	23, 21	24, 21	29, 18

When the rubber tubings had been in contact with the phenol solutions for periods of time which had been shown from the previous results to be sufficient for the establishment of equilibrium, it became apparent that in the case of each rubber,

(1) the amount of phenol absorbed was constant when in contact with the same strength of phenol solution,

(2) the amount of phenol absorbed was almost directly related to the concentration of the phenol solution,

(3) the process was reversible and proceeded to a point of equilibrium whether the rubber was receiving phenol from phenol solution or transmitting it to water, and

(4) the rate of absorption of phenol increased with temperature.

In this process it appeared that rubber acted as a solvent.

Tables VII and VIII show the results upon which these conclusions are based.

It can be seen from Table VIII that the phenol content of the aqueous solution in contact with each rubber shows a high degree of uniformity between the two samples examined, thus pointing to a uniform distribution of phenol between water and rubber when the conditions of storage remain constant. The larger differences occurring in the phenol content of rubber are explained by normal limits of error which result from the fact that these calculations are obtained by difference and not by direct analysis as in the solutions.

TABLE VII

PHENOL DISTRIBUTION BETWEEN RUBBER AND WATER AT POINTS OF EQUILIBRIUM

Concentration of original phenol solution = 0·5 per cent. w/v approx

	Storage time in days							
	7 (37° C.)		7 (37° C.)		44 (2° C.)		44 (2° C.)	
Sample No.	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
1	9·4	3·89	8·7	3·96	8·2	4·07	7·6	4·20
2	10·8	3·75	11·7	3·68	11·2	3·77	12·1	3·75
3	6·3	4·20	6·6	4·17	6·1	4·28	6·8	4·28
4	6·1	4·22	6·2	4·21	6·8	4·21	7·1	4·25
5	9·3	3·90	8·7	3·96	9·0	3·99	9·6	4·00
6	9·4	3·89	8·7	3·96	9·6	3·93	11·1	3·95
7	3·6	4·47	4·1	4·42	3·9	4·50	5·1	4·45
8	5·0	4·33	4·5	4·38	6·3	4·26	6·4	4·32
9	2·5	4·58	2·8	4·55	2·8	4·61	3·3	4·63

Concentration of original phenol solution = 4 per cent. w/v approx.

	Storage time in days							
	12 (37° C.)		12 (37° C.)		10 (37° C.)		10 (37° C.)	
Sample No.	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*
1	76·0	31·7	78·0	31·5	63·0	33·7	72·0	32·8
2	86·0	30·8	82·0	31·1	79·0	32·1	83·0	31·7
3	52·0	34·1	45·0	34·8	51·0	34·9	56·0	34·4
4	53·0	34·1	50·0	34·3	54·0	34·6	56·0	34·4
5	66·0	32·7	64·0	32·9	64·0	33·6	—	—
6	68·0	32·5	71·0	32·2	63·0	33·7	54·0	34·5
7	42·0	35·1	35·0	35·8	39·0	36·1	47·0	35·3
8	55·0	34·9	38·0	35·5	41·0	35·9	51·0	34·9
9	11·0	38·2	17·0	37·6	18·0	38·2	29·0	37·1
10	75·0	31·8	82·0	31·2	—	—	—	—

a = mg. of phenol in rubber.
b = mg. of phenol per ml. of solution of phenol.

TABLE VIII

PHENOL DISTRIBUTION BETWEEN RUBBER AND WATER AT EQUILIBRIUM AFTER RUBBER SAMPLES 1 TO 9 CONTAINING PHENOL HAD BEEN PLACED IN WATER AND STORED AT 37° C.

	Stored in 0·5 w/v phenol solution before transfer to water		Stored in 4 w/v phenol solution before transfer to water	
	Phenol distribution		Phenol distribution	
Sample No.	Rubber, mg.	Water, mg. per ml.	Rubber, mg.	Water, mg. per ml.
1	1·7, 1·2	0·65, 0·64	14·4, 14·6	6·21, 6·37
2	2·6, 2·9	0·86, 0·92	18·8, 15·6	6·68, 6·66
3	0·5, 1·2	0·56, 0·56	9·3, 3·6	4·28, 4·16
4	1·1, 1·1	0·57, 0·60	10·2, 7·7	4·25, 4·25
5	1·7, 2·1	0·73, 0·75	15·8, 14·0	5·05, 5·28
6	2·2, 2·2	0·74, 0·77	17·8, 18·7	5·05, 5·28
7	0·3, 1·3	0·36, 0·38	6·2, 1·8	3·59, 3·34
8	1·0, 0·8	0·53, 0·56	17·6, 1·5	3·70, 3·62
9	— —	0·37, 0·37	— —	2·78, 2·81

THE RELATIONSHIP OF ALKALINITY TO PHENOL ABSORPTION

Phenol under some circumstances acts as an acid, and rubber often contains alkaline fillers such as zinc oxide and alkali carbonates. In addition, ammonia is often added to latex. For this reason it was decided to examine all solutions, after being stored in contact with rubber to determine whether phenol absorption could be related to the alkaline reaction of the rubbers examined. From Table IX it is seen that, although there appeared a certain uniform variation between each series of

rubber examined, and the *p*H alteration of solution to the alkaline side in some cases bore a relation to phenol absorption, this was by no means consistent. The alkaline nature of the tubings might be interpreted to being one minor factor, out of several, which possibly influenced the main process of absorption of phenol.

TABLE IX

THE *p*H OF PHENOL SOLUTIONS AFTER RUBBER HAD BEEN STORED IN THEM

Strength of phenol solutions, per cent. / storage time	4 / 16 hours	4 / 64 hours	4 / 10 days	4 / 10 days	4 / 10 days	0·5 / 7 weeks	0·5 / 7 weeks	A / 13 days	B / 13 days	C / 14 days
Sample										
1	—	7·66	7·8	7·75	7·85	7·14	—	6·67	7·4	6·67
2	7·13	7·3	7·58	7·70	7·73	6·96	6·68	8·1	7·28	6·72
3	7·20	7·12	7·42	7·38	7·46	6·47	6·53	7·78	7·16	6·32
4	6·86	6·91	7·22	7·12	7·22	6·08	6·42	7·33	7·70	6·05
5	6·86	6·96	7·18	6·90	7·00	6·16	6·00	7·12	6·32	5·92
6	6·82	6·83	7·20	6·81	6·88	6·15	6·04	6·93	6·40	5·84
7	6·63	6·45	6·83	6·38	6·81	5·44	5·74	6·46	6·20	5·78
8	7·12	7·14	7·24	7·11	7·18	6·84	6·57	6·32	6·36	6·55
9	6·4	6·23	6·71	6·29	6·82	5·47	5·76	6·67	6·12	5·42
Phenol solution	6·45	5·16	6·42	6·66	6·77	5·48	5·48			

A and B = Water into which phenol had passed from rubber containing phenol.
C = Rubber immersed in water.

PHENOLIC SUBSTANCES IN RUBBER

Rubber samples were autoclaved in 10 ml. of water for 1 hour, autoclaved a second time, and again examined after 14 days storage at 37° C. to determine whether phenolic substances were transferred to the water, and whether that had any direct relationship to phenol absorption. The amounts, calculated as phenol, were very small but did indicate the possibility that phenolic substances used in manufacture had increased the phenolic absorption. On the other hand, this could not be regarded as the main factor. Also, it is noted that these substances can be removed by repeated treatment as shown in Table X.

TABLE X

THE AMOUNT OF PHENOLIC SUBSTANCE, CALCULATED AS MG. OF PHENOL, TRANSMITTED TO 10 ML. OF WATER BY 5 CM. OF EACH RUBBER TUBING

Sample	Autoclaved 1 hour	Again autoclaved 1 hour	14 days at 37° C.
1	0·210	0·104	0·128
2	0·246	0·065	0·155
3	0·055	Less than 0·020	0·078
4	0·103	0·040	0·061
5	0·261	—	0·156
6	0·280	—	0·167
7	0·090	—	0·045
8	—	—	0·186
9	—	—	Less than 0·020
10	0·125	—	0·137

A PARTITION COEFFICIENT FOR RUBBER

From the examination of the rubber specimens, it seemed that rubber acted as a solvent for phenol. Owing to the consistent results obtained by the examination of different samples of each specimen of rubber when

stored at the same temperature in the same strength of phenol solution, the conclusion was reached that as with other immiscible solvents, a partition coefficient of phenol between rubber and water was established. The results referred to were those obtained after long enough storage to ensure that a point of equilibrium had been reached.

The partition coefficients were calculated by dividing the concentration of phenol in rubber expressed as mg./ml. by the concentration of phenol in water expressed as mg./ml.

$K_c^t = \frac{Cr}{Cw}$ where K = partition coefficient at temperature t determined for rubber immersed in phenol solution of strength c expressed as percentage phenol w/v; Cr = concentration of phenol in rubber expressed as mg./ml.; Cw = concentration of phenol in water expressed as mg./ml.

TABLE XI

PARTITION COEFFICIENTS FOR SAMPLE 10 RUBBER FOR DIFFERENT CONCENTRATIONS OF IMMERSING SOLUTION AT 2° C. AND 37° C.

Initial strength per cent. of phenol solution w/v	Partition coefficient at	
	2° C.	37° C.
0·5	2·16	1·87
1·0	1·78	1·70
2·0	1·81	1·70
4·0	1·62	1·52

From Table XI it will be seen that a slightly higher concentration of phenol in rubber is obtained at lower temperatures, and a slightly lower concentration of phenol in rubber compared with that in water is obtained as the concentration of immersing solution increases. Thus the partition coefficient decreases with rise of temperature and strength of phenol solution used.

In Table XII are given the partition coefficients calculated for rubber samples 1 to 9. These show the same general picture, at the same time providing the general impression that a low value of K links lower phenol absorption with the more desirable type of rubbers for use with injections. This combination of properties is desirable in the making of suitable closures for containers of injectable solutions.

It is seen from Table XII that samples 3 and 7 which are latex tubings intended for intravenous infusions, sample 8 which is a mixed latex and silicone tubing and sample 9 which is a silicone tubing all show K values of less than 1. All the red rubber tubings show K values greater than 1. Sample 4 which is an anti-static tubing containing carbon has a relatively low K value.

DISCUSSION

The problem of the removal of certain substances from injections, particularly the bactericides employed in multiple dose containers, appears to be mainly physical in nature. Although certain rubbers are alkaline in reaction or contain certain basic or basic carbonate substances as fillers it does not appear that there is any chemical interaction between them and phenol, at least, not sufficient to explain the process involved. It appears that rubber acts as a solvent and that phenol divides itself between water and rubber, which act essentially as two immiscible solvents.

The reactions have also been considered in relation to the amount of

TABLE XII

PARTITION COEFFICIENTS FOR RUBBER SAMPLES 1 TO 9

Sample	Specific gravity of rubber	K 2° C. 0·5		K 37° C. 0·5		K 37° C. 4	
		Readings	Mean	Readings	Mean	Readings	Mean
1	1·37	1·16 1·06	1·11	1·41 1·27	1·34	1·36 1·41 1·09 1·28	1·28
2	1·22	1·70 1·83	1·76	1·62 1·78	1·70	1·58 1·49 1·41 1·48	1·49
3	0·994	0·93 1·03	0·98	0·96 1·01	0·98	0·98 0·84 0·95 1·06	0·96
4	1·176	0·87 0·90	0·88	0·76 0·81	0·79	0·82 0·78 0·84 0·92	0·84
5	1·112	1·21 1·26	1·23	1·22 1·16	1·19	1·10 1·07 0·99	1·05
6	1·125	1·31 1·48	1·39	1·36 1·17	1·27	1·12 1·13 0·99 0·84	1·02
7	0·923	0·52 0·69	0·61	0·48 0·55	0·52	0·73 0·59 0·64 0·80	0·69
8	0·915	0·95 0·97	0·96	0·75 0·66	0·71	1·04 0·71 0·79 1·01	0·89
9	1·176	0·38 0·46	0·42	0·35 0·39	0·37	0·18 0·29 0·29 0·50	0·31

rubber in the rubber mixes. The results do not show any consistent agreement between phenol absorption and the rubber content of the rubber mix. Neither is there any clear relation between the amount of phenol absorbed and the filler content. The physical state of the rubber mix possibly explains the difference between the various samples in phenol absorption. There are several factors which may cause this, such as the influence of fillers, activators and accelerators in altering the physical state of the rubber. However, it does appear that whatever the explanation, the process is consistent for particular rubber mixes, to the extent that a partition coefficient can be determined which is capable of being used as a standard for determining the suitability of certain rubbers for making closures of multiple dose containers of injections. It must not be presumed that the results obtained for phenol are equally applicable to other substances employed as bacteriostatic agents, and that aspect requires further investigation. Indeed the work of Wiener, which was mentioned earlier indicates the possibility of a different type of absorption when dealing with thiomersalate.

It is suggested that the partition coefficient of phenol, for rubber in contact with aqueous solutions, under definite conditions of concentration and temperature can be used as a basis for the selection of rubber used for pharmaceutical purposes.

SUMMARY

1. The absorption of phenol from aqueous solutions by rubber has been found to proceed to a state of equilibrium under controlled conditions of storage, the process being reversible.
2. The amount of phenol absorbed has been shown to bear a direct relation to the concentration of phenol in the solution in contact with the rubber.
3. The rate of absorption was found to increase with rise of temperature up to the point of equilibrium.
4. The amount of phenol absorbed varies with the type of rubber.
5. A partition coefficient for rubber may be calculated which is suitable for use as a method of controlling the standard of rubber for pharmaceutical purposes.

In conclusion the writer wishes to thank Messrs. J. C. Franklin and Son, Ltd., Veedip, Ltd., William Warne and Co., Ltd., and the Dunlop Rubber Co., Ltd., for samples of rubbers, Dr. R. H. Muller, Mr. G. B. Pendleton, Mr. J. T. Ogden and Mr. S. D. Sutton for valuable information, Dr. B. E. Tomlinson for the use of certain equipment and to Mr. A. Turnbull and Mr. J. Watson who assisted in certain of the determinations.

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DISCUSSION

The paper was presented by the AUTHOR.

MR. G. SYKES (Nottingham) said it seemed that absorption of a preservative by rubber was a two-stage process, adsorption on the surface followed by diffusion through the body of the rubber. In view of statements made at the London conference two years ago he doubted whether any one rubber could be used as a standard, as it was not possible to guarantee that the mix of two batches of a rubber would be identical.

Would the author comment on the pharmacopœial method of preparing rubber caps for use? On page 651 of the paper it was stated that "After equilibrium is reached this sample of rubber did not continue to absorb phenol, contrary to the inference of the Pharmacopœia". However, the conditions were different. In the case of a rubber closure a continuous diffusion of the phenol could take place, first into the rubber from the solution and, then by volatilisation to the air from the outer surface.

MR. F. G. FARRELL (London) said he had been studying the same problem using chlorocresol, and employing a spectrophotometric method of assay. Using immersed silicone rubber strips the results were similar to those obtained by the author, equilibrium being reached in a few days. With rubber caps on multidose containers where only one surface was in contact with the solution, equilibrium was not obtained for many weeks. Rubber caps subjected to the B.P. treatment of storing in 0·2 per cent. chlorocresol solution for 48 hours when used on multidose containers containing 0·1 per cent. chlorocresol solution, continued to take up chlorocresol after four months. Two samples of preserved physiological saline solution lost over 50 per cent. of chlorocresol in one year. It would seem that the surface area exposed was a very important factor and it would be interesting to know the diameter and thickness of the rubber tubes used by the author. The concept of a partition coefficient was very interesting and implied that the phenol was distributed in a homogenous manner. Had the author cut the rubber tubing and examined the innermost and outermost parts for phenol content?

PROFESSOR H. BERRY (London) said that the Pharmacopœia was interested only in the rubber used for closures for multidose containers, and such rubber should not contain fillers. Alkaline fillers were a source of danger if the solution in the container had an acid reaction. The author referred to a carbon filler, but he did not consider that a carbon filler should be used in rubber caps because of the difficulty of piercing such caps with a needle. Factors affecting the absorption of phenols into rubber were the area exposed and the presence of other substances in the solution which would alter the partition coefficient. If the medicament were a salt, the solubility of the phenol in the solution would be less and more phenol would go into the rubber. If the medicament was a substance which solubilised phenol such as soap, then less phenol would go into the rubber. The suggestion that if silicone rubber were used all the hazards in connection with rubber would disappear was not true from his own experience, and the author had confirmed that silicone rubber did absorb phenol. Further, when a closure of silicone rubber was pierced with a needle the hole remained wide open, and it was therefore useless as a material for closing multidose containers. He suggested that the author should extend his experiments with a view to obtaining information on the effect of medicaments on rubber.

MR. T. D. WHITTET (London) said that in contrast to the experience of the author, who found that phenols were distributed throughout the rubber he had found that when caps were soaked in metabisulphite solution they became bleached; but if cut open the inside of the rubber did not appear to be affected at all.

DR. L. SAUNDERS (London) commented on the rather loose use of the word "equilibrium" by the author in connection with the results at 2° C. Table II did not show that any equilibrium was reached. The curves in Fig. 1 did not appear to be the curves that one would draw from the results given without assuming that equilibrium was being reached. Had

the author made any attempt to calculate the diffusion coefficients for phenol in the rubber?

MR. W. T. WING, in reply, agreed with the remarks made by Mr. Sykes concerning absorption and diffusion. In the first instance his intention was to establish the conditions under which phenol was absorbed. He agreed that, in the case of a closure, phenol was absorbed and could diffuse through the rubber into the air. He had not intended to imply that one rubber should be used as a standard.

He was about to examine the British Pharmacopœia method of boiling in twice the strength of phenol more closely. Rubber might absorb too much phenol by boiling it in a double strength solution, it was possible that it should be boiled only in the strength of phenol to be used in the injection. He had done no work on chlorocresol. There was a possibility of chlorocresol and phenol behaving differently. Rubber would react with chlorine and form a definite chemical compound It would also react with sulphites although probably only on the surface of the rubber. The tubings used were roughly $\frac{1}{4}$ in. in diameter and of 1/16 in. thickness. He had not examined the inside and outside of the tubings for their phenol content. He agreed with Professor Berry that fillers should not be present in the rubbers which were used as closures for containers. The purpose of his examination of several rubbers was to establish the differences between rubbers of varying composition. It would be seen from the tables that rubbers which were free from fillers absorbed less phenol than other types. Silicone rubber absorbed phenol but it had been shown to absorb less than any other type of rubber. It appeared that after sterilising the silicones gave off toxic substances and the dangers in its use had resulted in the withdrawal of silicone tubing for use in transfusions of electrolyte solutions. He had not dealt with diffusion coefficients.

STUDIES ON BACTERIAL POPULATIONS IN SOLUTIONS OF PHENOLS

PART I. THE VIABILITY OF *Bacterium coli* IN AQUEOUS SOLUTIONS OF BENZYLCHLOROPHENOL

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ANALYSES of the mortality of bacteria in aqueous solutions of bactericides have produced two major theories about the nature of the bactericidal reaction. The earlier theory, which has been referred to as the "mechanistic theory" likened the bactericidal reaction to a chemical reaction. Madsen and Nyman[1] and Chick[2] postulated that the mortality rate of organisms in a bactericidal system approximated closely to that which would be expected if the bactericidal reaction was a monomolecular one. This would imply a constant death-rate throughout the entire course of the reaction. The experiments of Chick show, however, that there is often a marked variation of the death rate during the reaction. Further, the majority of a large number of experiments described in the literature and analysed by Rahn[3] show a departure from the monomolecular rate. The alternative theory—the "vitalistic theory"—is based on the distribution of resistances of the individual organisms. The theory was supported by Hewlett[4], Reichel[5], Reichenbach[6] and by Henderson Smith[7,8]. Withell[9] demonstrated the existence of a rectilinear relation between probit-mortality and a simple transformation of contact time, such as the logarithm of the contact time. Neither Berry and Michaels[10,11], nor Jordan and Jacobs[12] accepted unconditionally the relation suggested by Withell, and have discussed the significance of the shape of the probit-mortality log-time curve.

The mechanistic and vitalistic theories offer different explanations for the course of the bactericidal reaction, but they have in common the axiomatic concept that the longer the time of contact between bacteria and bactericide the smaller the number of living organisms in the system. It is partly the intention of the present communication to demonstrate that, under appropriate conditions, there may be marked departures from the anticipated course of the bactericidal reaction and therefore, in the number of living organisms in the system.

It has been shown by several workers[13,14,15,16] that when bacteria are treated with certain bactericides, there is a leakage into the surrounding medium of nitrogenous materials, such as purines and pyrimidines which are characterised by having a maximal ultra-violet absorption at a wavelength of 260 mμ. Salton and Alexander[13] and Salton[14] have indicated that there is a relation between the amount of cetrimide added to a suspension of *Staphylococcus aureus* and the amount of 260 mμ absorbing material released from the cells, the latter also being increased by an

elevation of temperature. Newton[15] reported that the antibiotic polymyxin E produced a release of 260 mμ absorbing material from washed cells of *Pseudomonas aeruginosa*. Few and Schulman[16] found a release of similar material from a number of species of bacteria when they were treated with polymyxin E. The results of the experiments published by Salton[14], Few and Schulman[16] and by Newton[15] indicate that an increase in the severity of the bactericidal conditions leads to an increase in the amount of 260 mμ absorbing material released from the cells. The present communication examines whether a correlation exists between the mortality of certain bacteria in an aqueous system and the amount of 260 mμ-absorbing material released from the cells.

Materials and Methods

The Organism

Bacterium coli (N.C.T.C. No. 5933) was selected for the present study since it had been used satisfactorily in earlier disinfection studies by Berry and Michaels[17] and Berry and Bean[18]. Further, the latter authors used it in conjunction with the same bactericide that was selected for the present experiments. The organism was maintained by freeze-drying from nutrient broth.

A tube of freeze-dried material was reconstitued at monthly intervals by suspension in horse meat infusion broth and the suspension used in a manner similar to that described in the British Standard Specification for the Rideal Walker Test (B.S.S. No. 541: 1934—amended May 1951). Three stock agar slope cultures were prepared from the reconstitued material, 2 of them were reserved and the third used for the preparation of daily slopes during the first part of a month. Subcultures were made from the stock slope into broth at 24-hour intervals for 3 days, 2 ml. of the third subculture being distributed over the surface of an agar slope and incubated at 37° C. for 16 hours. This procedure was repeated until the fourteenth subculture, when the cycle was recommenced from one of the reserved stock slopes.

Preparation of the Bacterial Suspensions

The 16-hour growth was washed from the slope with quarter-strength Ringer's solution and the resulting suspension centrifuged at about 1000 r.p.m. to remove any small lumps of agar removed with the organisms. The supernatant was removed and the organisms washed 3 times in quarter-strength Ringer's solution using about 100 ml. solution in all. After the third washing the supernatant was removed, and the closely packed wet cells resuspended to produce a concentration of approximately 2000×10^6 per ml. in the solution under test. The cell concentration was adjusted with the aid of an Eel nephelometer previously standardised against suspensions of the same organism which were counted using darkground illumination in a Thoma hæmocytometer cell (depth 1/50 mm.).

The Bactericide

The bactericide used in the experiments was benzylchlorophenol (5-chloro-2-hydroxy diphenylmethane). It had a m.pt. of 48·5° C. and

its molecular extinction $\epsilon_{max.}$ at $\lambda_{max.}$ 282 mμ in water was found to be 2310. The water solubility was 1 in 6380, determined by means of a Unicam S.P. 500 spectrophotometer. (Allawala and Riegelman[19] give $\epsilon_{max.}$ 2360 at λ 282 mμ in water and a water solubility of 1 in 6300). Figure 1 shows the compliance of aqueous solutions of benzylchlorophenol with the Beer-Lambert law, and provides a reference graph from which the concentrations of unknown solutions can be read.

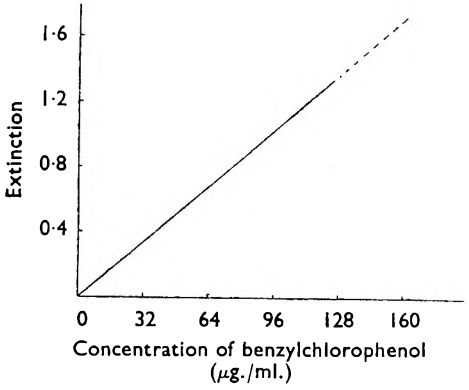

FIG. 1. Relationship of the extinction at 282 mμ to benzylchlorophenol concentration.

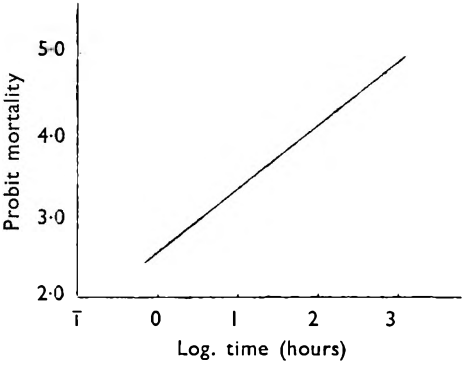

FIG. 2. Relationship of probit mortality to log contact time of *Bact. coli* with distilled water.

The Bacteriological Method

The roll-tube capillary dropping pipette technique described by Wilson[20], Withell[21] and by Berry and Michaels[17] was used for all viable counts. The method is economical both of materials and space and, with experience, it yields accurate results[17,22].

For each viable count five roll-tubes were prepared and the mean count calculated.

Estimation of the soluble cell constituents or Benzylchlorophenol in the supernatants from bacterial suspensions

8 to 10 ml. samples of the cell suspension were taken at the time of making viable counts and the majority of the cells removed by centrifugation at x 8500 *g* for 3 minutes. The supernatant was then subjected to a further centrifugation at x 8500 *g* for 3 minutes. The ultra-violet absorption spectra of the cell-free supernatant fluids were examined using a Unicam S.P. 500 spectrophotometer. Where necessary corrections were made for the absorption of the suspending fluids.

ESTIMATES OF EXPERIMENTAL ERRORS

The sources of error in viable count techniques have been discussed by Berry and Michaels[17] and by Bullock, Keepe and Rawlins[22]. The major errors may be assumed to be due to variations in the capillary pipettes, variations in the volume of bacterial suspension measured by the pipettes, the non-homogeneity of the suspension, batch to batch variation in the

nutrient medium, and to personal errors in counting the colonies in the roll tubes after incubation.

Personal Error due to Counting

The causes of errors in counting bacterial colonies have been discussed by Wilson[23]. The magnitude of the error was estimated by the same author and by Berry and Michaels[17] and by Bullock, Keepe and Rawlins[22]. Table I shows the results during the present experiments when the colonies in each of 10 roll tubes were counted on each of 3 different occasions.

The standard error of the mean of 3 counts is 1·90, compared with the value of 1·65 obtained by Bullock, Keepe and Rawlins[22] and of 2·41 obtained by Berry and Michaels[17]. This value may be regarded as satisfactory.

TABLE I

ESTIMATION OF THE PERSONAL ERROR DUE TO COUNTING COLONIES IN 10 ROLL TUBES ON EACH OF THREE OCCASIONS

Roll tube No.	Counts (x) $n = 3$	Total of three counts $\Sigma(x)$	Mean $\bar{x}$	Corrected sum of squares (SS) $\Sigma(x-\bar{x})^2 = \Sigma(x^2) - \frac{\Sigma^2 x}{n}$			Variance = SS/N where N = 2
				$\Sigma(x^2)$	$\Sigma^2(x)/n$	SS	
1	137,135,141	413	137·7	56875	56856	19	9·5
2	146,150,147	443	147·7	65425	65416	9	4·5
3	252,247,246	745	248·3	185029	185008	21	10·5
4	195,190,188	573	191	109469	109443	26	13
5	237,230,231	698	232·7	162430	162401	29	14·5
6	182,188,190	560	186·6	104568	104533	35	17·5
7	99,102,100	301	100·3	30205	30200	5	2·5
8	127,129,133	389	129·6	50459	50440	19	9·5
9	135,129,132	396	132	52290	52272	18	9
10	131,129,137	397	132·6	52571	52536	35	17·5
	Totals	4915				216	

Total sum of squares for 30 observations = 216. Sum of squares for count variation $\frac{\Sigma SS}{\Sigma N} = \frac{216}{20} = 10{\cdot}8$.

Standard deviation of count variation = $\sqrt{10{\cdot}8} = 3{\cdot}286$. Standard error of mean of 3 counts = $\sqrt{\frac{10{\cdot}8}{3}}$ = 1·897.

Errors due to Batch to Batch Variation in the Agar Medium

The influence of batch to batch variation in the agar has been examined by other workers[17,22], who have concluded that, provided the medium is prepared in a satisfactory manner, batches of reproducible sensitivity are obtained. The problem was not reinvestigated during the present experiments.

Suitability of the Viable Count Technique

In accordance with the recommendations of Thornton[24], the suitability of the nutrient medium employed for a viable count is usually expressed by calculating the Index of Dispersion for the colonies in a number of roll tubes. Table II shows the Index of Dispersion for 20 roll tubes for a viable count on a suspension of *Bact. coli* in quarter strength Ringer's solution. Reference to tables of χ^2 indicates a probability of 0·9 to 0·8, and thus the medium may be regarded as satisfactory.

The Index of Dispersion examines rather more than the suitability of the medium and includes the whole of the technique involved in making

20 replicate counts on a suspension of organisms. Thus the high value of P obtained is a favourable comment on the technique as a whole.

The overall errors involved in performing replicate counts on a series of bacterial suspensions are tested by a rather more comprehensive test, the basis of which is described by Berry and Michaels[17]. Table III records the Goodness of Fit of χ^2 values for 50 sets of five replicate roll tubes for counts of suspensions of *Bact. coli* which have not been in contact with a bactericide.

TABLE II

GOODNESS OF FIT OF χ^2 FOR 20 ROLL-TUBES USED IN ESTIMATING THE SUITABILITY OF A BATCH OF NUTRIENT AGAR

Counts (x)	Mean count ($\bar{x}$)	Deviations from mean ($x - \bar{x}$)	$(x - \bar{x})^2$
100		−14·75	217·56
99		−15·75	248·06
126		11·25	126·56
117		2·25	5·06
109		−5·75	33·06
120		5·25	27·56
123		8·25	68·06
118		3·25	10·56
117	114·75	2·25	5·06
106		−8·75	76·56
125		10·25	105·06
121		6·25	39·06
118		3·25	10·56
114		−0·75	0·56
122		7·25	52·56
121		6·25	39·06
105		−9·75	95·06
120		5·25	27·56
100		−14·75	217·56
114		−0·75	0·56
$\Sigma x =$ 2295		$\Sigma(x - \bar{x}) =$ 0·00	$\Sigma(x - \bar{x})^2 =$ 1405·7

$$= \frac{\Sigma(x - \bar{x})^2}{\bar{x}} = \frac{1405{\cdot}7}{114{\cdot}75} = 12{\cdot}25;\ N = 19;\ P = 0{\cdot}9\text{–}0{\cdot}8$$

TABLE III

GOODNESS OF FIT OF THE DISTRIBUTION OF χ^2 VALUES FOR VIABLE COUNTS ON 50 SETS OF FIVE REPLICATE TUBES, USING *Bact. coli* SUSPENDED IN DISTILLED WATER OR PHOSPHATE BUFFER

Value of χ^2	Expected frequency (m)	Observed frequency ($m + x$)	Difference (x)	x^2	$\frac{x^2}{m}$
Under 1·00	4·51	6	+1·49	2·25	0·50
1·00–1·99	8·70	15	+6·30	39·69	4·56
2·00–2·99	8·90	12	+3·10	9·61	1·10
3·00–3·99	7·59	7	−0·59	0·35	0·05
4·00–4·99	5·94	4	−1·94	3·76	0·63
5·00–5·99	4·41	2	−2·21	4·88	1·11
6·00–6·99	3·16	2	−1·16	1·35	0·43
7·00–8·99	3·74	1	−2·74	7·51	2·00
Over 9·0	3·05	1	−2·05	4·20	1·38
Total	50·00	50·00			11·76

$\chi^2 = 11{\cdot}76$; n = 8; P = 0·1–0·2

EXPERIMENTAL RESULTS

The Viability of Bacterium coli *in Distilled Water at* 20° C., *and the Release of Soluble Cell Constituents from the Organsims during Incubation*

Viable counts made at intervals on suspensions of *Bact. coli* stored at 20° C. in water from a heavily tinned still fitted with an all-glass condensing system are recorded in Table IV, and the corresponding log-time probit-mortality curve is shown in Figure 2.

The ultra-violet absorption spectra of cell-free supernatants from the bacterial suspension were examined at the time of making the viable counts and are plotted in Figure 3. Figure 4 shows the release of 260 mμ-absorbing material during storage of the cells in the distilled water, and

TABLE IV

VIABILITY OF *Bact. coli* IN DISTILLED WATER

Time (hours)	Log time	Survivors (per cent.)	Mortality (per cent.)	Probit mortality	Extinction 260 mμ
0	—	100	0	—	—
1	—	99·5	0·5	2·4242	0·07
24	1·3802	88·4	11·6	3·8048	0·117
100	2·0000	83·5	16·5	4·0259	0·158
187½	2·2730	73·4	26·6	4·3750	0·179
291½	2·4646	69·4	30·6	4·4928	0·195
528	2·7226	60·7	39·3	4·7285	0·246
695½	2·8423	58·6	41·4	4·7827	0·243
936	2·9713	55·1	44·9	4·8718	0·286
1345	3·1287	54·8	45·2	4·8794	0·326

Figure 5 the relation between the appearance of 260 mμ-absorbing material and the viability of the organisms in the distilled water.

Figure 5 shows that there is a gradual decline in the number of living organisms when *Bact. coli* is incubated at 20° C. in distilled water, and reveals that there is a simultaneous gradual appearance in the supernatant of material which has a maximal ultra-violet absorption at a wavelength of 260 mμ. The appearance of material having a similar ultra-violet absorption has previously been reported[14,15,16] in suspensions of bacteria in distilled water, 1 per cent. w/v sodium chloride solution and in phosphate buffer. Salton and Alexander[13] and Salton[14] have shown that free purines and pyrimidines are present in the released material.

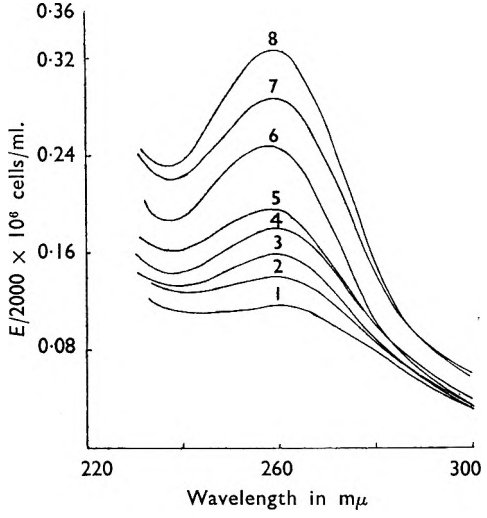

FIG. 3. Ultra-violet absorption spectra of cell-free supernatants from suspensions of *Bact. coli* in distilled water after varying contact times at 20° C. Curves 1 to 8 after 24, 48 100, 187·5, 291·5, 528, 936, 1345 hours respectively.

Salton[14] found that the amount of 260 mμ-absorbing material released from bacterial cells treated with cetrimide was proportional to the number of cells killed. Neither our own results nor those of Few and Schulman[16] support this finding. An examination of Figure 5, suggests that there may be a rectilinear relation between certain mortality ranges, but when the curve as a whole is examined no such rectilinear relation is apparent.

The Absorption of Benzylchlorophenol by Bact. coli

Suspensions of *Bact. coli* at a concentration of 2000 × 10^6 organisms per ml. were prepared in phosphate buffer solutions (*p*H 7·0) containing

10, 25, 50 and 100 μg. of benzylchlorophenol per ml. respectively. Immediately after preparation of the suspensions, the organisms were spun down at 8500 g. and the ultra-violet absorption at 282 mμ of the cell-free supernatant was examined. The extinction of the solutions before and after contact with the organisms is recorded in Table V where the "after contact" extinction has been corrected for the absorption at 282 mμ which is

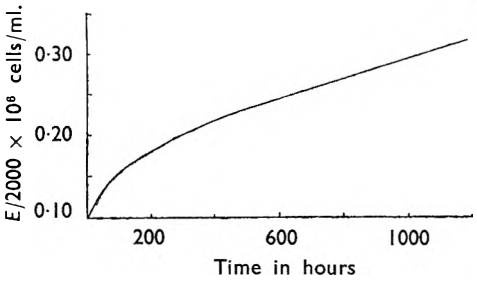

FIG. 4. Release of 260 mμ absorbing material from *Bact. coli* suspended in distilled water at 20° C.

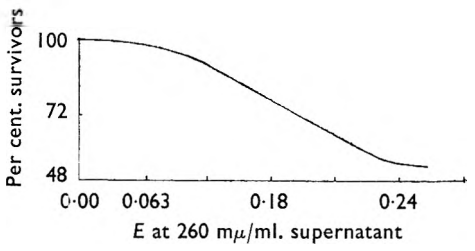

FIG. 5. Relationship between release of 260 mμ absorbing material and viability of *Bact. coli* suspended in distilled water at 20° C. over a period of 56 days.

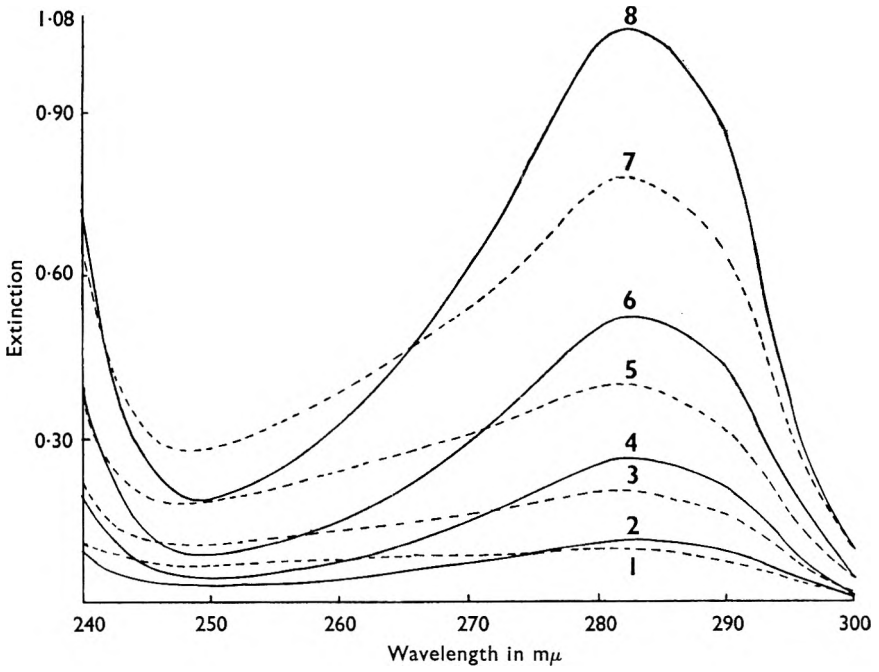

FIG. 6. Ultra-violet absorption spectra of benzylchlorophenol solutions (unbroken lines) and of the cell-free supernatants after contact with *Bact. coli* 2000 × 10^6 cells/ml. (broken lines).

1. Supernatant from 10 μg./ml. solution
2. 10 μg./ml. solution
3. ,, ,, 25 μg./ml. ,,
4. 25 μg./ml. ,,
5. ,, ,, 50 μg./ml. ,,
6. 50 μg./ml. ,,
7. ,, ,, 100 μg./ml. ,,
8. 100 μg./ml. ,,

TABLE V

EXTINCTION AT 282 mμ OF BENZYLCHLOROPHENOL SOLUTIONS BEFORE AND AFTER CONTACT WITH *Bact. coli*

Concentration of benzylchlorophenol (μg./ml.)	Extinction		Benzylchlorophenol remaining in supernatant (μg./ml.)	Benzylchlorophenol absorbed by *Bact. coli* (μg./ml.)
	Untreated solution	After contact with *Bact. coli*		
100	1·06	0·758	72	28
50	0·530	0·359	34	16
25	0·267	0·183	17·4	7·6
10	0·107	0·075	7·2	2·8

attributable to soluble cellular constituents released into the solutions. In these connections it is assumed that the cellular material released upon contact with the phenol is of a similar nature to that occurring when the cells are suspended in distilled water or phosphate buffer. Figure 6 shows the absorption spectra of the supernatants after immediate removal of *Bact. coli* together with the absorption spectra of the untreated solutions.

The absorption curve showing that the amount of benzylchlorophenol absorbed by the cells is proportional to the concentration in the supernatant is produced in Figure 7. The benzylchlorophenol absorbed

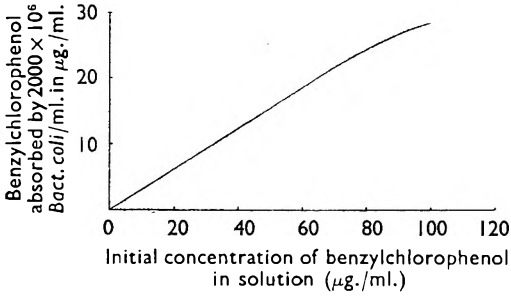

FIG. 7. Absorption of benzylchlorophenol by *Bact. coli* at 20° C.

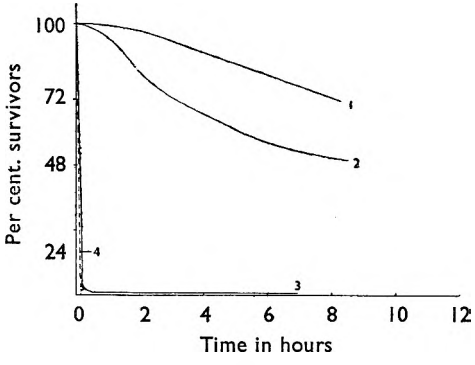

FIG. 8. Viability of *Bact. coli* in solutions of benzylchlorophenol in 0·01M phosphate buffer Curves 1 to 3, 10, 25 and 50 μg./ml. respectively Curve 4 (broken line) 100 μg./ml.

by the cells from the solutions at the 4 concentrations used viz. 10, 25, 50 and 100 μg. per ml. was 28, 30·4, 32 and 28 per cent. respectively of the amount initially present in the solutions. The shape of the absorption curve suggests that initial saturation of the organisms was not obtained in the concentrations used, and that greater quantities would have been taken up in the same time by the organisms from more concentrated solutions.

The Viability of Bact. coli *in Aqueous Solutions of Benzylchlorophenol*

Viable counts were made on suspensions of *Bact. coli* in 0·01M phosphate buffer solutions containing 10, 25, 50 and 100 μg. of benzylchlorophenol per ml. respectively. The percentage survivors at each contact time

examined are shown in Table VI, VII and VIII, and are plotted in Figure 8. In the solutions containing 10 and 25 μg. per ml. of benzylchlorophenol there was a steady decline in the number of survivors with time, and in the

TABLE VI

VIABILITY OF *Bact. coli* IN SOLUTIONS CONTAINING 10 AND 25 μG./ML. BENZYLCHLOROPHENOL AND THE EXTINCTION AT 260 AND 282 Mμ OF THE CELL-FREE SUPERNATANTS

Benzylchlorophenol concentration	Time (hours)	Survivors (per cent.)	Extinction	
			260 mμ	282 mμ
10 μg./ml.	0	100·0	0·071	0·093
	1	98·6	0·132	0·126
	2	95·3	—	—
	3	96·8	0·134	0·121
	4	89·2	—	—
	6	—	0·174	0·137
	6½	79·5	—	—
	8	72·5	0·194	0·143
25 μg./ml.	0	100·0	0·125	0·216
	1	95·7	0·169	0·226
	2⅓	79·6	0·172	0·223
	3	—	0·185	0·224
	4	67·2	0·194	0·228
	6	—	0·214	0·234
	6½	53·9	—	—
	7	—	0·227	0·237
	8	49·7	0·242	0·244
	24	35·5	0·405	0·297
	76	11·8	0·704	0·393

solution containing 100 μg. per ml. a very rapid decline in the number of survivors. The latter solution was sterile within 5 minutes. The solution containing 50 μg. per ml. of benzylchlorophenol is of special interest. In

TABLE VII

VIABILITY OF *Bact. coli* IN SOLUTIONS CONTAINING 50 μG./ML. BENZYLCHLOROPHENOL AND THE EXTINCTION AT 260 AND 282 Mμ OF THE CELL-FREE SUPERNATANTS

Experiment	Time	No. of viable organisms/ml.	Extinction	
			260 mμ	282 mμ
1	0	1,172,615,700	—	—
	5 min.	1,132,725	0·251	0·417
	1 hour	212,217	0·295	0·421
	4 ,,	20,437	0·356	0·421
	5 ,,	19,174	—	—
	5½ ,,	19,290	—	—
	19 ,,	976,860	0·505	0·465
	26½ ,,	5,858,240	0·521	0·473
	50½ ,,	5,010,480	0·629	0·507
	98¾ ,,	3,585,440	—	—
	124 ,,	3,544,330	0·859	0·587
	170½ ,,	9,682,970	—	—
	258½ ,,	10,385,220	0·984	0·582
2	0	1,172,615,700	—	—
	5 min.	29,118,620	0·230	0·416
	2½ hours	134,740	0·321	0·422
	4 ,,	82,100	—	—
	7 ,,	77,620	0·361	0·429
	8 ,,	108,430	—	—
	26½ ,,	1,287,160	0·492	0·467
	79 ,,	3,870,480	0·727	0·532
	103¼ ,,	3,542,600	—	—
	173½ ,,	3,252,750	0·978	0·615
	317½ ,,	4,283,550	—	—
	339½ ,,	4,695,870	1·136	0·665
	653 ,,	2,267,760	1·291	0·742
	987 ,,	1,639,710	—	—

the 2 experiments reported in Table VII the percentage survivors after 5 to 7 hours is between 0·002 and 0·008. During the following few hundred hours a 50 to 500-fold increase in the number of living cells was observed. The causes of the variations in the several experiments performed with this concentration of benzylchlorophenol are under investigation, but it must nevertheless be acceded that the comparatively enormous multiplications in what was initially a bactericidal system are remarkable.

It does not appear that the observed multiplication can be accounted for by adaptation as normally understood, because washed cells were used and they were suspended in 0·01M phosphate buffer solution instead of a nutrient medium as normally used in adaptation studies.

TABLE VIII

VIABILITY OF *Bact. coli* IN SOLUTIONS OF BENZYLCHLOROPHENOL 100 μG./ML. AND THE EXTINCTION AT 260 AND 282 Mμ OF THE CELL-FREE SUPERNATANT

Time	Survivors (per cent.)	Extinction	
		260 mμ	282 mμ
0 min.	100	—	—
5 ,,	0	0·402	0·808
1 hour	0	0·420	0·810
5 ,,	0	0·476	0·814
8 ,,	0	0·470	0·808
26¼ ,,	0	0·593	0·825
79½ ,,	0	0·868	0·884
128 ,,	0	1·011	0·946
272 ,,	0	1·174	0·952

The ultra-violet Absorption Spectra of Supernatants from Bact. coli *suspensions containing Benzylchlorophenol in 0·01M Phosphate Buffer Solution*

Two separate portions of the bacterial suspension were taken at each contact time. One was used for the viable count and the other was centrifuged at 8500 g. to provide a cell-free supernatant which was examined for absorption at 260 and 282 mμ. The absorptions are recorded in Tables VI, VII, VIII.

Figure 9 records the absorption spectra of supernatants after treatment of *Bact. coli* with solutions containing 50μg. per ml. of benzylchlorophenol in phosphate

TABLE IX

GROWTH OF *Bact. coli* AND THE EXTINCTION AT 260 Mμ IN CELL ELUATES OBTAINED

(*i*) *By storage of* Bact. coli *in distilled water for* 10 *weeks at* 20° *C.*

Time of incubation in cell eluate hours	Viable organisms (per ml.)	Log. viable organisms	Extinction at 260 mμ
0	205,980	5·3139	0·335
7½	209,280	5·3207	0·338
21¾	2,985,960	6·4751	0·334
76	5,047,020	6·7030	0·333
102	4,951,100	6·6947	—
150	5,289,100	6·7233	—
340½	5,216,020	6·7173	0·345

(*ii*) *By heating a suspension of* Bact. coli *in distilled water at* 100° *C. for* 1 *hour.*

0	18,270	4·2617	1·132
6½	41,470	4·6167	—
23½	8,945,060	6·9515	1·122
78	19,048,580	7·2798	1·118
126	21,022,950	7·3226	1·109
146	22,413,360	7·3504	—
269½	22,580,210	7·3537	1·104
1011	18,229,900	7·2607	1·087

(*iii*) *By heating a suspension of* Bact. coli *in 0·01M phosphate buffer* (p*H* 7·0) *at* 100° *C. for* 1 *hour*

0	162	2·2095	0·913
3¾	222	2·3464	0·906
19	10,880	4·0370	0·910
72	18,817,840	7·2746	0·853
98	19,935,850	7·2997	0·854
331	18,561,737	7·2686	0·844

buffer for contact times to up 653 hours. The peak at 282 mμ in the absorption spectrum curve shows a sharp fall immediately after the addition of the organisms to the solution due to removal of benzylchlorophenol from the solutions by the bacterial cells. With continued incubation of the cells in the solution of benzylchlorophenol the extinction at 282 mμ gradually increased and ultimately became greater than the initial density due to the benzylchlorophenol. This increase in the amount of

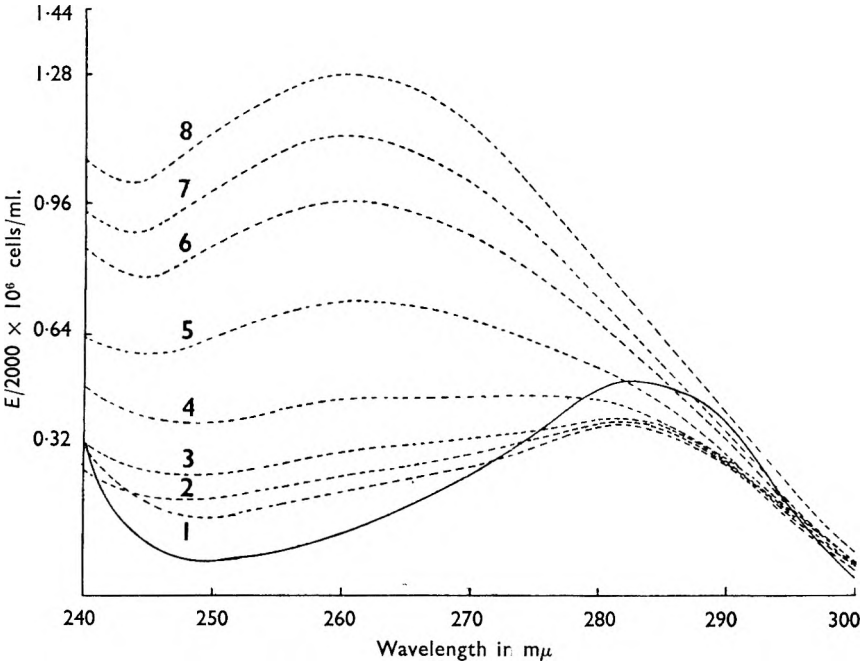

FIG. 9. Ultra-violet absorption spectra of supernatants from suspension of *Bact. coli* in solution of benzylchlorophenol 50 μg./ml. Curves 1 to 8 after zero time, 3, 7, 26·5, 79, 173·5, 339·5, 653 hours respectively. Unbroken line = untreated solution.

282 mμ-absorbing material can be attributed to material released from the bacterial cells upon treatment with benzylchlorophenol. The figure shows that simultaneously there was a marked increase in the absorption at 260 mμ due to material being released from damaged or killed bacterial cells. The release of 260 mμ-absorbing material was observed in each of the 4 concentrations of bactericide used, and in each concentration the extinction at 260 mμ was considerably higher than that obtained after similar time intervals from cells which had been suspended in distilled water. A similar pronounced absorption at 260 mμ has been noted in the supernatants of cells treated with polymyxin E and cetrimide[13,14,15,16].

Figure 10 shows that 260 mμ-absorbing material continues to be released over many days from cells treated with benzylchlorophenol even though all the cells are killed within a few minutes. This supports the suggestion made earlier in this paper that there is apparently no simple relation

between the mortality of *Bact. coli* and the amount of 260 mμ-absorbing material released from the cells.

The Viability of Bact. coli *in cell-eluates*

In view of the multiplication previously described when *Bact. coli* was treated with benzylchlorophenol 50 μg/ml. the possibility of cellular release materials functioning as a nutrient material was investigated. The cell-eluate was obtained by storage of *Bact. coli* in distilled water and also by maintaining at a temperature of 100° C. for 1 hour.

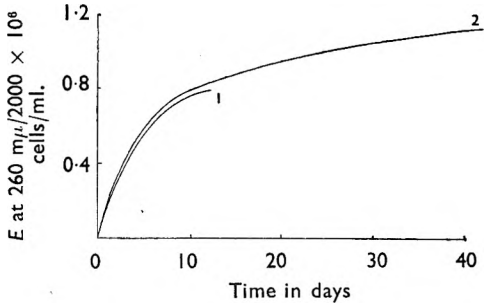

FIG. 10. Release of 260 mμ absorbing material from *Bact. coli* during incubation in benzylchlorophenol solution at 20° C. Curve 1 = 100 μg./ml., curve 2 = 50 μg./ml.

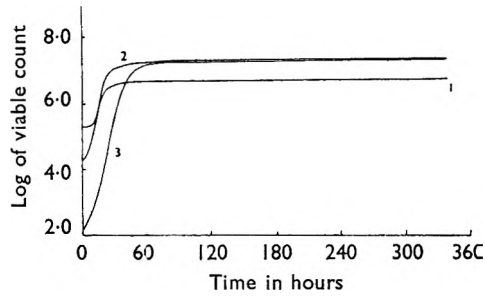

FIG. 11. Multiplication of *Bact. coli* in cell eluates.
Curve 1. Eluate obtained by storage in distilled water.
Curves 2 and 3. Eluate obtained by heating suspension of *Bact. coli* in distilled water and phosphate buffer for 1 hour respectively.

(*a*) *Cell-eluate obtained by storage*

A suspension of *Bact. coli* was prepared as usual in distilled water and left for 10 weeks at 20° C. The majority of the cells were then removed by centrifuging at 8500 g. for 3 minutes and the supernatant filtered through a 5 on 3 sintered glass filter. One ml. of the filtrate transferred to 10 ml. of nutrient broth showed no growth on incubation.

A new suspension of *Bact. coli* was prepared in distilled water and a small inoculum was added to the cell-eluate.

(*b*) *Cell-eluate obtained by heating at* 100° *C. for* 1 *hour*

Suspensions of *Bact. coli* in distilled water and 0·01M phosphate buffer were prepared by the usual method, and then heated at 100° C. for 1 hour. The bacteria were removed and the cell-free supernatants were inoculated with a freshly prepared suspension of *Bact. coli* in distilled water and 0·01M phosphate buffer respectively and incubated at 20° C. The number of viable organisms and the ultra-violet absorption spectra of the cell-free supernatants were determined at various time-intervals. Table IX tabulates the numbers of viable organisms and the ultra-violet absorption at 260 mμ, and Figure 11 relates the log of the viable count to the time of incubation.

Discussion

The results obtained indicate that materials released into the suspending fluids when *Bact. coli* die are capable of promoting cell-growth. When a small inoculum of organisms is added to the cell-eluate and incubated, a typical growth curve is obtained. It is thus possible that the leakage of material from *Bact. coli* when treated with benzylchlorophenol 50 μg./ml., is a contributory factor in the multiplication of the surviving organisms. Similarly, the reduction in mortality rate with time, of many organisms when treated with bactericides, which is frequently observed at the distal portions of the survivor-time curves, and which has been attributed to an increased resistance of the last survivors, may be in part due to cellular release materials in sub-optimal nutrient concentrations.

A recent paper by Whitehead[25], demonstrated that when a suspension of *Bact. coli* was irradiated by ultra-violet light and the cells removed, washed, and re-suspended in the original suspending fluid or in fresh phosphate buffer, the mortality of the cells returned to the original suspending fluid was only 25 per cent. of that of cells suspended in fresh buffer. Whitehead suggested that a "restorative factor" was released by the cells as a physiological response to injury of the cells by the irradiation. It would appear that his observation is analogous to that described in the present communication.

The change in ultra-violet absorption at 260 mμ shown in Table IX, which takes place simultaneously with the multiplication of the organisms is surprisingly small for the amount of growth observed. The significance of this observation is being further investigated.

Summary

1. The absorption by *Bact. coli* of benzylchlorophenol from aqueous solution has been demonstrated and an absorption isotherm plotted.

2. Soluble cellular constituents which have a maximal ultra-violet absorption at 260 mμ are released when *Bact. coli* dies in aqueous solutions of benzylchlorophenol.

3. Multiplication of *Bact. coli* survivors has been observed in a solution of benzylchlorophenol demonstrated to be initially bactericidal.

4. Water-soluble cellular constituents extracted from *Bact. coli* are capable of promoting cell growth.

5. It is suggested that soluble cellular constituents released when *Bact. coli* dies in aqueous solutions of benzylchlorophenol can influence the viability of the last survivors and alter the course of the bactericidal reaction.

The authors wish to acknowledge a generous gift of benzylchlorophenol by the Cocker Chemical Co. Ltd.; they are also indebted to Mr. G. O. Jolliffe for advice and assistance in the use of the spectrophotometer.

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DISCUSSION

The paper was presented by MR. V. WALTERS.

DR. K. R. CAPPER (London) said it would be interesting to learn something about the character of the survivors. That material which would support the growth of the organism could be released from cells was perhaps not at all surprising, but the fact that growth occurred in the presence of quantities of a phenolic substance seemed to indicate that the survivors had characteristics which were not shared by the culture as a whole. In many cultures there were organisms with abnormal resistance, but they usually had cultural characters which differed from those of the original organisms. Had any work been done on the survivors to find out whether that resistance could be inherited, and whether they were different in cultural requirements from the original *Bact. coli* strain? In any subsequent work carried out on the material absorbing at 260 mμ it would be better if a synthetic medium were used.

MR. G. SYKES (Nottingham) said he was disappointed to find that the references did not include the name of Gale because of his fundamental work on the role of glutamic acid and the prevention of the diffusion of that substance in the presence of certain surface-active agents. He did not believe that this phenomenon could be anything more than partially contributory to the problem described, because there was always a residual excessive resistance even with a relatively small bacterial population, when the concentration of the nutritive material released into the solution must be infinitely small. He also found it difficult to associate the phenomenon with the well known phenomenon of adaptation.

MR. H. D. C. RAPSON (Dorking) referred to the experimental results discussed under the heading "Absorption of Benzylchlorophenol by *Bact. coli*" and the relevant data of Fig. 7, which was presented in the form of an "isotherm" it being stated that adsorption was proportional to the

concentration. The "isotherm" of Fig. 7 was curved, consequently the amount adsorbed was not strictly proportional to the concentration. Although that did not seriously affect the substance of the paper, it tended to inhibit a fuller appraisal of the result. Since the term "isotherm" was used, the data should have been presented in a more conventional manner, that is, the amount adsorbed should have been plotted against the concentration of the solution in contact with the surface. If that procedure were adopted, the curvature would be found to be more pronounced, and to start in the 50 μg./ml. region which was interesting in view of the anomalous results. If the isotherm concept were extended and the approximate surface area of the bacterium were calculated, it could be shown that something approximating to a monomolecular layer of bactericide was present on the surface of the bacterium, suggesting that the organism might have died of "asphyxiation". It was very desirable to treat the results of such calculations with caution since a living organism was involved. If on expiration the effective area of the bacterium were suddenly to increase, then a physical explanation of the abnormal viability could be given. Adsorption techniques afforded a powerful method whereby surfaces might be studied and it should be possible thereby to differentiate between the lethal effect due to processes connected with adsorption, and that following diffusion into the organism.

DR. G. E. FOSTER (Dartford) asked the authors whether they could give further details of the chemical nature of the material absorbing at 260 mμ.

DR. A. H. BECKETT (Chelsea) said that the measurement of the absorption of phenols in the presence of ultra-violet absorbing materials released from the bacteria at present must only be looked upon as very approximate.

MR. V. WALTERS, in reply, said that they had not determined whether the residual viable organisms had developed a resistance. He agreed that the name of Gale should have been included in the references and that the absorption curve shown in the paper should have been expressed as had been suggested and not as an initial concentration. He had no personal knowledge of the chemical nature of the substances liberated though he had commenced some paper chromatographic work to elucidate the amino-acids. He could only quote from previous work that they consisted of purines and pyrimidines. With regard to the measurement of the phenol absorption, the Morton-Stubbs correction had been applied to calculate the amount of phenol absorbed, and bearing in mind the 260 mμ fraction also absorbed at 280 mμ, the values obtained for the percentage absorption using that correction were respectively: 38·5, 37, 40 and 40, roughly 10 per cent. higher. Unfortunately, it did not follow that the Morton-Stubbs correction was applicable since the absorption contribution of cellular released material appeared to be as much at 280 mμ as at 260 mμ.

SOME OBSERVATIONS ON THE ACTIVITY OF MIXTURES OF ANTIBACTERIAL SUBSTANCES

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Introduction

During an investigation of the possibility of preparing an improved general antiseptic, the use of mixtures of known antiseptic substances was considered. Little work appeared to have been previously carried out in this direction and it seemed possible that some degree of synergism might exist between certain antibacterials. Attention was first paid to combinations of phenolic substances, such as *o*-phenyl-phenol, octyl-cresol, mono- and di-chlor-*m*-xylenol and hexachlorophene. None of the mixtures had an antibacterial activity significantly greater than existing antiseptics, such as Solution of Chloroxylenol B.P., and consideration was then given to the use of quaternary ammonium compounds. Although the high antibacterial activities of quaternary ammonium compounds have been known for many years[1] and numerous quaternary salts are available as general antiseptics, yet it is surprising to find that no attempt appears to have been made to overcome the main disadvantage of this group—their inactivation by organic matter and soap. The inactivation arises from the very nature of the quaternary salts, and it was because of this fact that attention was turned to the possibility of reinforcing the activity of the salt with substances compatible with quaternary compounds and having high antibacterial powers unaffected by organic matter or by soap. Preliminary work showed that Domiphen Bromide B.P.C. (alkyl dimethyl-2-phenoxy ethyl-ammonium bromides) was the most active of the quaternary ammonium compounds tested and that 5-aminoacridine (Aminacrine Hydrochloride B.P.) was likely to prove a suitable reinforcing agent. It was found that these two substances were compatible over a wide range of concentrations and temperatures, and a solution containing 1·0 per cent. of domiphen bromide and 0·1 per cent. of 5-aminoacridine was finally selected for further investigation. The evaluation of the antiseptic activity of the mixture was attempted under conditions resembling as closely as possible those applying during everyday use. In view of the many conflicting reports in the literature on the antibacterial powers of quaternary ammonium compounds[2–5], it was decided to employ two basic tests, giving bacteriostatic and bactericidal performance. In addition other known antiseptics were compared at the same time.

Experimental Methods

(*a*) *Maintenance of Cultures*

All cultures of micro-organisms used were obtained initially from the National Collection. The stocks were maintained on nutrient agar and

day to day culturing was made in nutrient broth No. 2 (Oxo). The *Vibrio comma* fluid cultures were in peptone water (Oxo) and *Aspergillus niger* and *Candida albicans* were cultured on Sabouraud's agar and in Sabouraud's broth. Only with the last two organisms were the cultures older than 24 hours when used.

(*b*) *Bacteriostatic Tests*

The procedure adopted was that of serial dilution of the stock solution in 5 ml. quantities of broth and to each dilution 0·1 ml. of an undiluted broth culture was added. Growth was recorded after 48 hours incubation.

The effect of soap and blood on the antiseptic mixture was examined using representative organisms. Contact with soap solution (0·1 per cent. in water) or horse blood (20 per cent. in saline) with an equal volume of antiseptic was allowed for 30 minutes before dilution.

(*c*) *Bactericidal Tests*

The procedure adopted was an exposure time of 5, 10 and 15 minutes between the antiseptic solution (in 5 ml. amounts) and 0·2 ml. of broth culture (containing approx. 10 million organisms). 1 loopful was transferred into broth after the exposure times.

The organic matter used was a 5 per cent. killed yeast suspension. A contact time of 15 minutes was allowed between antiseptic and the yeast suspension before the culture was added and exposed to the action of the antiseptic.

To compensate for the alleged strong absorptive properties of the quaternary ammonium salts, a neutralising agent was incorporated in the subculture broth[6]. Lubrol W. (polyethylene oxide condensate, I.C.I.) was used as a 1 per cent. solution in broth.

Resistance Emergence

Following a report[7] that organisms rapidly become resistant to quaternary ammonium compounds, experiments were carried out to determine if the mixed antiseptic would delay the emergence of resistance in the Gram-negative organism, *Serratia marcescens*. The method used is that described in the paper quoted.

Results

Comparative values obtained with a wide range of antiseptics and a wide range of micro-organisms are shown in Table I. The effect of incorporating serum in the medium is shown in Table II. It is apparent from these Tables, (*a*) that domiphen bromide has a higher antibacterial activity than the other quaternary ammonium compounds examined, (*b*) that the quaternary compounds are more active bacteriostatically than any other group of compounds examined, and (*c*) 5-aminoacridine is a compound with a high degree of activity which is little affected by the presence of serum.

The outstanding activity of domiphen bromide as seen in the successive dilution test was also apparent in the time exposure test. The results are

TABLE I

MINIMUM INHIBITORY CONCENTRATIONS OF SOME ANTISEPTICS
(ORGANIC MATTER ABSENT)

Name of antiseptic	Minimum inhibitory concentration expressed in mg. per 100 ml.						
	Staph. aureus	*P. vulgaris*	*Salm. typhi*	*K. pneumo.*	*B. mycoides*	*Ps. aeruginosa*	*Cl. tetani*
Domiphen bromide B.P.C.	0·097	1·56	1·56	3·12	0·39	12·5	0·78
Benzalkonium chloride U.S.P.	0·097	1·56	3·12	6·25	0·39	25·0	1·56
*Octaphen	0·097	3·12	3·12	3·12	0·39	25·0	1·56
Cetrimide B.P.	0·097	1·56	3·12	12·5	0·78	50·0	1·56
†Dibromopropamidine isethionate	0·097	6·25	1·56	3·12	6·25	25·0	0·39
5-Aminoacridine	3·12	1·56	1·56	1·56	1·56	12·5	0·78
Auramine	6·25	6·25	12·5	3·12	1·56	>50	3·12
Hexyl resorcinol	0·78	1·56	6·25	3·12	1·56	>50	3·12
Di-chloro-*m*-xylenol	6·25	12·5	25·0	12·5	6·25	>50	6·25
p-Chloro-*m*-xylenol	3·12	12·5	25·0	3·12	3·12	>50	3·12
Benzyl cresol	1·56	6·25	12·5	6·25	3·12	>50	6·25
Phenol	>50	50·0	>50	>50	>50	>50	>50

* "Octaphen"—(β-*p*-Tert.-octylphenoxyethyldiethylbenzyl ammonium chloride)—Ward Blenkinsop & Co., Ltd.
† "Brulidine"—May and Baker Limited.

TABLE II

MINIMUM INHIBITORY CONCENTRATIONS OF SOME ANTISEPTICS
(10 PER CENT. SERUM PRESENT)

Name of antiseptic	Minimum inhibitory concentration expressed in mg. per 100 ml.						
	Staph. aureus	*P. vulgaris*	*Salm. typhi*	*K. pneumo.*	*B. mycoides*	*Ps. aeruginosa*	*Cl. tetani*
Domiphen bromide	1·56	6·25	12·5	6·25	1·56	25·0	1·56
Benzalkonium chloride	1·56	12·5	25·0	25·0	1·56	50·0	3·12
Octaphen	1·56	12·5	50·0	25·0	3·12	50·0	3·12
Cetrimide	1·56	62·5	50·0	50·0	3·12	>50	6·25
Dibromopropamidine isethionate	0·39	25·0	3·12	12·5	12·5	25·0	1·56
5-Aminoacridine	3·12	3·12	1·56	1·56	1·56	25·0	1·56
Auramine	12·5	12·5	25·0	12·5	6·25	>50	12·5
Hexyl resorcinol	3·12	12·5	25·0	12·5	12·5	>50	12·5
Di-chloro-*m*-xylenol	25·0	25·0	50·0	>50	25·0	>50	50·0
p-Chloro-*m*-xylenol	12·5	12·5	25·0	12·5	12·5	>50	25·0
Benzyl cresol	6·25	12·5	25·0	12·5	6·25	>50	25·0
Phenol	>50	>50	>50	>50	>50	>50	>50

shown in Table III where the effect of an inactivator is seen to have little effect on the result obtained.

As is seen from Table III, 5-aminoacridine has only a weak bactericidal power during the limited 5 minutes contact, but it is seen from Table IV that the activity is greatly increased when a longer contact time is allowed. The organism used was a *Staphylococcus aureus*.

Table IV (*a*) shows that the antibacterial activity of 5-aminoacridine is unaffected in the presence of high concentrations of soap and protein.

In the presence of blood (10 per cent.) and soap (0·05 per cent.) the results shown in Table V indicate that although the antiseptic mixture is to some extent inactivated, yet a high antibacterial activity remains, and that the product, in this respect is not inferior to others such as Solution of

TABLE III

BACTERICIDAL CONCENTRATION OF ANTISEPTIC REQUIRED TO KILL THE TEST ORGANISM IN 10 MINUTES BUT NOT IN 5 MINUTES

Name of antiseptic	Inactivator	5 per cent. yeast suspension	Bactericidal concentration expressed in mg. per 100 ml.					
			Staph. aureus	*P. vulgaris*	*Salm. typhi*	*K. pneumo.*	*S. mycoides*	*Ps. Aeruginosa*
Domiphen bromide B.P.C. ..	Absent	Absent	2·5	50	7·5	7·5	10	5
		Present	75	1000	250	250	250	100
Domiphen bromide B.P.C. ..	Present	Absent	2·5	50	7·5	7·5	10	10
		Present	100	1000	250	250	250	250
Benzalkonium chloride U.S.P.	Absent	Absent	2·5	75	10	75	10	10
		Present	100	1000	250	500	250	100
Dibromopropamidine isethionate	Not required	Absent	50	500	100	250	100	750
		Present	100	1000	1000	1000	750	1000
5-Aminoacridine	Not required	Absent	1000	1000	250	250	1000	250
		Present	1000	1000	750	500	1000	750

Chloroxylenol B.P., the results for which are also shown in Table V. The effect of varying concentrations of soap are shown in Table VI.

As has been previously reported in the literature[8] quaternary ammonium compounds are reduced in activity when in contact with "hard" water.

TABLE IV

EFFECT OF VARYING CONTACT TIMES ON THE BACTERICIDAL ACTIVITY OF 5-AMINOACRIDINE AGAINST *Staph. aureus*

	Contact time			
	5 minutes	10 minutes	30 minutes	60 minutes
Inhibition concentration in mg./100 ml. ..	1000	500	200	100

TABLE IV (*a*)

ANTI-BACTERIAL ACTIVITY OF 5-AMINOACRIDINE

Organism	Minimum inhibitory concentration in mg./100 ml. in presence of:		
	Water	0·05 per cent. soap	10·0 per cent. blood
Staph. aureus	3·12	3·12	3·12
Salm. typhi	1·56	1·56	1·56
B. mycoides	1·56	1·56	1·56
Ps. aeruginosa ..	12·5	25·0	25·0

The results of the investigation with the antiseptic mixture and Solution of Chloroxylenol B.P. when examined by the successive dilution technique diluted with hard water (290 p.p.m. Ca) are shown in Table VII.

The results obtained from the investigation of the emergence of resistance are shown in Table VIII. These are of a preliminary nature, but it seems that 5-aminoacridine delays the emergence of resistance to domiphen bromide.

TABLE V

COMPARATIVE INACTIVATION OF BACTERIOSTATIC ACTIVITY BY SOAP AND BLOOD
MINIMUM INHIBITORY DILUTIONS

Organism	Domiphen bromide 1 per cent.			Antiseptic mixture			Solution of Chloroxylenol B.P.		
	Water	0·05 per cent. soap	10 per cent. blood	Water	0·05 per cent. soap	10 per cent. blood	Water	0·05 per cent. soap	10 per cent. blood
Staph. aureus ..	1:32,000	1:2000	1:4000	1:33,000	1:8250	1:8250	1:2000	1:2000	1:1000
Strep. pyogenes ..	1:32,000	—	—	1:33,000	—	—	1:1000	—	—
B. mycoides ..	1:32,000	1:2000	1:2000	1:33,000	1:8250	1:8250	1:4000	1:2000	1:2000
Ps. aeruginosa ..	1:125	1:32	1:32	1:200	1:100	1:100	1:64	1:64	1:32
C. diphtheriæ ..	1:8000	—	—	1:8000	1:2000	1:4000	1:2000	1:2000	1:1000
Cl. welchii	1:16,000	—	—	1:16,000	—	—	1:4000	—	—
P. vulgaris	1:2000	1:250	1:500	1:2000	1:500	1:1000	1:500	1:500	1:250
E. coli	1:1000	1:125	1:125	1:1000	1:250	1:250	1:250	1:250	1:125
Salm. typhi	1:1000	1:125	1:250	1:1000	1:250	1:250	1:250	1:250	1:125
Sh. shigae	1:1000	1:125	1:250	1:2000	1:500	1:1000	1:500	1:500	1:250
V. comma	1:2000	—	—	1:2000	—	—	1:500	—	—
Asp. niger	1:8000	—	—	1:8000	1:2000	1:2000	1:2000	1:2000	1:1000
Cand. albicans ..	1:16,000	—	—	1:16,000	1:4000	1:4000	1:2000	1:2000	1:1000

TABLE VI

EFFECT OF VARYING CONCENTRATIONS OF SOAP ON BACTERIOSTATIC ACTIVITY

Antiseptic mixture control	Minimum inhibitory dilution = 1:33,000 (*Staph. aureus*)
Antiseptic mixture plus 0·05 per cent. soap ..	" " " 1:8250 (*Staph. aureus*)
Antiseptic mixture plus 0·1 per cent. soap ..	" " " 1:8250 (*Staph. aureus*)
Antiseptic mixture plus 0·5 per cent. soap ..	" " " 1:4125 (*Staph. aureus*)

TABLE VII

EFFECT OF HARD WATER ON BACTERIOSTATIC ACTIVITY
MINIMUM INHIBITORY DILUTION

Organism	Antiseptic mixture		Solution of Chloroxylenol B.P.	
	Control	Hard water	Control	Hard water
Staph. aureus	1:33000	1:8250	1:2000	1:1000
Salm. typhi	1:1000	1:250	1:250	1:125

TABLE VIII

EMERGENCE OF RESISTANCE IN *S. marcescens* TO SINGLE AND MIXED ANTISEPTICS

	No. of transfers										
	1	2	3	4	5	6	7	8	9	10	
Domiphen bromide mg./100 ml.	1	1	2	32	128	1000	—	—	—	—	Minimum inhibitory dilution
5-Aminoacridine mg./100 ml.	3	6	6	6	6	24	48	—	—	—	
Antiseptic mixture mg./100 ml.	1	1	2	2	4	4	16	—	—	—	
Solution of chloroxylenol mg./100 ml.	25	25	50	50	50	100	200	—	—	—	

DISCUSSION

The results of the investigation on a mixture of domiphen bromide and 5-aminoacridine have confirmed preliminary deductions about the complementary nature of these two antiseptics.

Experiments with the two substances separately have shown firstly,

that of a number of quaternary ammonium compounds and other antiseptics examined, domiphen bromide is the most antibacterial, and secondly, they have confirmed the stability of 5-aminoacridine in the presence of soap and protein.

The mixture of the two substances is stable over a wide range of temperatures and possesses the high bacteriostatic and bactericidal activity which is characteristic of domiphen bromide. In normal circumstances, the small amount of 5-aminoacridine present does not contribute much to the high antibacterial activity of the domiphen bromide in the mixture, but in the presence of high concentrations of soap and protein, 5-aminoacridine plays an additive role, in which it maintains the activity of the mixture at a high level by superimposing its own unimpaired activity on the reduced activity of the quaternary compound.

Many recent reports have stressed the clinical value of a mixture of two or more chemotherapeutic drugs in delaying the emergence of bacterial resistance and it is interesting that a similar action is seen with the present mixture of antiseptic substances *in vitro*. Investigation, at present has been confined to one organism, *S. marcescens*, which has permitted tests to be carried out in both acid and alkaline *p*H, but further experiments are in progress to determine whether a similar result is obtained with the common pathogens. The development of bacterial resistance is probably rare during the normal use of an antiseptic but any general delaying effect on the emergence of bacterial resistance possessed by the present mixture of antiseptics would be of value in its protracted use against wound infection.

Summary

Of a number of antiseptics examined, domiphen bromide was found to be the most active in normal media, but partial inactivation occurred in the presence of soap or protein. It was confirmed that 5-aminoacridine was not affected by either of these substances. A mixture of 1·0 per cent. of domiphen bromide and 0·1 per cent. of 5-aminoacridine possessed high antiseptic activity which was largely retained in the presence of large concentrations of soap or protein.

Preliminary results suggest that the mixture of antiseptics delays the development of bacterial resistance *in vitro* to a much greater extent than does domiphen bromide alone.

The authors wish to thank their colleagues who have assisted in many ways with the experimental work.

We would also like to thank Mr. A. R. G. Chamings for much help and advice during the progress of this work.

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DISCUSSION

The paper was presented by MR. E. KAY.

MR. G. SYKES said he found some difficulty in understanding the Tables. Some were expressed as minimum inhibitory concentration in mg. per 100 ml., others were in terms of one in so many, so that without careful arithmetic it was difficult to relate the two. Although the effect of the domiphen bromide might not be reduced by the addition of 5-aminoacridine, it would seem that the effect of 5-aminoacridine was diminished by domiphen bromide. Hence the mixture would seem to be no better than the simple acridine solution. With regard to the emergence of resistance, there seemed to be little difference. In Table VIII 5-aminoacridine started with an inhibitory concentration of 3 mg./100 ml. and rose to 48 at the seventh generation. The mixture achieved exactly the same, namely, a relative increase from 1 to 16.

MR. R. LEVIN (Liverpool) said that with the advent in recent years of several potent agents having a wide spectrum of antibacterial activity, he agreed that it was opportune to subject the commonly used antiseptic solutions to a reappraisal.

MR. E. KAY, in reply, emphasised that the work was of a preliminary nature. In regard to the emergence of resistance, it was pointed out that one did not obtain a thousandfold increase in the mixture whereas it was obtained with domiphen bromide. It was difficult to express the concentration of the mixture itself in terms of mg./ml.

THE PURIFICATION AND CHARACTERISATION OF PENICILLIN V

By G. Parker, R. J. Cox and Dorothy Richards

From The Distillers Company (Biochemicals) Limited, Speke, Liverpool

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A large number of penicillins, differing from one another solely in the nature of the amide side chain, have been reported in the past. One, penicillin V (phenoxymethylpenicillin), has long been known to be elaborated by strains of *Penicillium notatum* when cultured in the presence of suitable precursors[1]. It has recently been shown that penicillin V is resistant to inactivation by acids, and can be isolated as the free acid which is sparingly soluble in water[2]. Penicillin V has been used clinically in place of penicillin G (benzylpenicillin) preparations[3,4]. As the lability of penicillin G at low *p*H necessitates its oral application in special formulations of the soluble salts or as sparingly soluble salts, it seems that penicillin V will be particularly suitable for oral use.

Methods for the rapid assay of penicillin V, were needed in the course of purifying a sample of this material for use as a reference standard for chemical and biological assays. Suitable chemical or physical assay methods were sought and at the same time, methods were devised for the rapid differentiation of penicillin V from penicillin G.

Experimental

General Methods. Ultra-violet absorption spectra were determined in 1 cm. quartz cells in a model SP.500 Unicam Spectrophotometer.

Brominations were made by treating a neutral aqueous solution contained in a suitably closed flask with an excess of solutions of both N potassium bromate and N potassium bromide, and then making acid with a slight excess of concentrated hydrochloric acid. Bromine uptake was determined by the addition of excess solid potassium iodide, and titration with sodium thiosulphate solution.

Purification of Penicillin V

The initial work was done with a sample of penicillin V supplied by "Biochemie" Gesellschaft M.B.H., Kundl, Innsbruck, Austria. This material (sample A) contained no volatile matter, and appeared to be pure when assayed iodimetrically[13]. However, chromatographic assay[14] showed that it contained a small amount of a second biologically active component which remained at or near the point of application. Attempts to separate the two components were followed by means of iodimetric and chromatographic assays. The results are treated in detail elsewhere[13,14], but are described briefly here.

When sample A was dissolved in water by the addition of sodium bicarbonate, and was then precipitated by the slow addition of slightly less than the theoretical quantity of dilute hydrochloric acid, the product (sample B) contained somewhat less of the second component but its

purity, determined iodimetrically, rose to over 100 per cent. Crystallisation of sample B from aqueous acetone yielded a product (sample C) with an even higher purity, and containing only a trace of the second component. Solubility analyses of samples A, B and C by the method of Webb[5] using the spectrophotometric technique[13], suggested that sample C was, and sample A was not, homogeneous.

A quantity of penicillin V (sample D), supplied by the Production Division of this company, was purified by the methods described in the previous paragraph. The product (sample E) when examined chromatographically, and by solubility analysis appeared to be homogeneous. When assayed iodimetrically it appeared to have a purity greater than 100 per cent. Further attempts to purify sample E have failed to increase the iodimetric potency, and the material appears to be pure.

The Ultra-violet Absorption Spectrum of Penicillin V

Penicillin V has been reported[1] to contain two relatively sharp absorption maxima in the ultra-violet band. The spectra of purified penicillin V and of its sodium salt were therefore re-examined.

The free acid (sample E) was dissolved in water. The spectrum was found to contain maxima at 268 mμ and 274 mμ, with a minimum at 272 mμ (Fig. 1). The adsorption at each maximum obeyed Beer's Law in the range 0·00 to 0·04 per cent. w/v. Because solution of the acid is slow unless the sample is finely divided, and decomposition begins on standing for a few hours in aqueous solution, the preparation of solutions of the free acid should not be attempted at concentrations above 0·025 per cent. w/v. The molecular extinction coefficient of penicillin V is 1330 at 268 mμ and 1100 at 274 mμ.

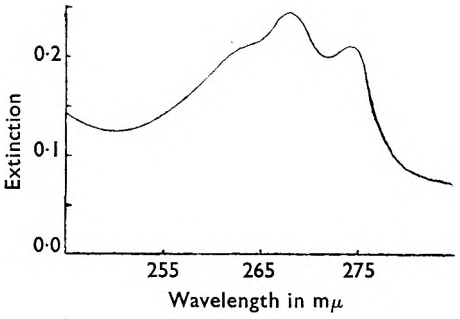

FIG. 1. The ultra-violet absorption spectrum of phenoxymethylpenicillin (0·0067 per cent. w/v in water).

Solutions of penicillin V in chloroform show a spectrum similar to that of aqueous solutions, but the positions of the maxima are shifted slightly to 270 mμ and 276 mμ. Ethanol may also be used as a solvent for the free acid, which may be determined quantitatively from the absorption at 268 mμ and 274 mμ after dilution to 5 per cent. v/v ethanol content.

The sodium salt of penicillin V, which is readily soluble in water, was prepared from the free acid and sodium bicarbonate solution; its spectrum is similar to that of the free acid, with absorption maxima at 268 mμ and 274 mμ. The absorption at each maximum obeyed Beer's Law in the range 0·00 to 0·03 per cent. w/v. Unlike the free acid, solutions of the sodium salt do not decompose on standing. There is no detectable change in the absorption intensities after ten days at room temperature.

The Ultra-violet Absorption Spectra of Alkaline and Acidic Degradation Products

Treatment of penicillin V with 0·5N sodium hydroxide solution at room temperature for 15 to 30 minutes causes complete decomposition of the penicillin V. After neutralisation, examination of the ultra-violet absorption spectrum, with an appropriate blank, reveals that it resembles penicillin V, with peaks at 268 mμ and 274 mμ. The intensity of absorption at each of these wavelengths is approximately the same as that exhibited by an equal concentration of undecomposed penicillin V.

Aqueous solutions of penicillin V are acidic, having *p*H 3–4. Examination of the spectrum of a solution of the free acid over a period of time, reveals the gradual appearance of a rather broad peak at 318 to 320 mμ. This peak first appears after 4 to 6 hours, gradually increasing in intensity to a maximum after 1 to 2 days at room temperature. The intensities of absorption at 268 mμ and 274 mμ are affected by this new peak, so that errors are caused if spectrophotometric estimations of penicillin V are made on aged solutions of the acid.

More drastic acidic treatment of penicillin V, by treatment at *p*H 1 at room temperature for 1 hour, causes an increase in the intensities of absorption at 268 mμ and 274 mμ, although the general shape of the absorption curve is similar to that of penicillin V. The peak at 318 to 320 mμ does not appear in solutions of penicillin V degraded at *p*H 1, even when neutralised after treatment at *p*H 1 for only 5 minutes.

Solubility Analyses of Penicillin V Preparations

The samples A, B, C and E described earlier, were examined for purity by the solubility analysis method of Webb[5]. Each sample was finely ground, and increasing weights were slurried in 100 ml. portions of water. The mixtures were agitated vigorously for 2 hours, filtered and diluted. The absorption at 268 mμ, 272 mμ and 274 mμ was then determined for each solution. The operations from the filtration stage onwards were made as rapidly as possible, in order to reduce to the minimum any effects caused by the decomposition of penicillin V in aqueous solution. The results obtained with samples A and C are shown in Figures 2 and 3. It seems that sample C is pure, and sample A contains more than 1 component. Sample E is similar to sample C, and also appears to be pure.

The Ultra-violet Absorption Spectra of Model Phenoxymethyl Compounds

The use of compounds containing the phenoxymethyl grouping as fermentation precursors, necessitated the examination of their ultra-violet spectra since they might interfere in the spectrophotometric assay of impure penicillin V.

The spectrum of phenoxyacetic acid has been reported previously[6]: it contains a single absorption maximum at 270 mμ in water, ethanol or ether, with a molecular extinction coefficient of approximately 1300. Phenoxyethanol was found to possess a similar spectrum with a single absorption maximum at 270 mμ, but with a much lower molecular extinction coefficient of 330.

Phenoxyacetamide was prepared from phenoxyacetic acid (3g.) by refluxing with 5 ml. of thionyl chloride for 1 hour, cooling and treating carefully with excess of concentrated aqueous ammonia. The amide was filtered, washed and crystallised from hot water. It melted at 98° C. (Fritzsche[7] reports a value of 101° C.). The spectrum of this compound

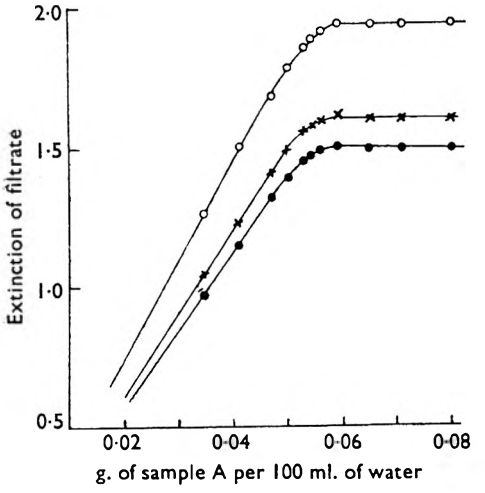

FIG. 2. The solubility analysis of phenoxymethylpenicillin sample A. Extinctions have been corrected for the dilutions made before reading.

○——○ Read at 268 mμ. ×——× Read at 274 mμ. ●——● Read at 272 mμ.

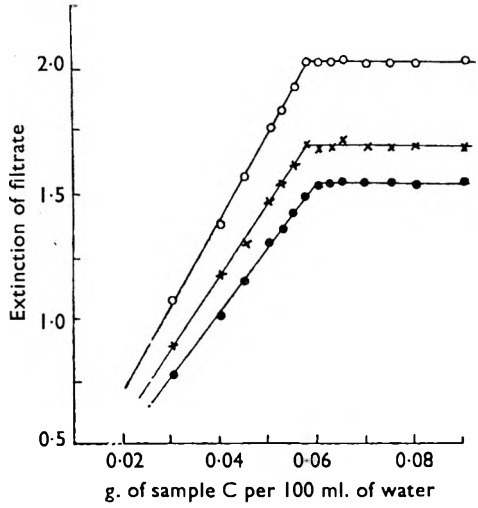

FIG. 3. The solubility analysis of phenoxymethylpenicillin sample C. Extinction have been corrected for the dilutions made before reading.

○——○ Read at 268 mμ. ×——× Read at 274 mμ. ●——● Read at 272 mμ.

had 2 absorption maxima in ethanol solution, at 269 mμ and 276 mμ *N*-Methylphenoxyacetamide was prepared in a similar fashion from the acid chloride and aqueous methylamine solution. After crystallisation from hot water, it melted at 68° C. This compound also exhibited a spectrum with absorption maxima at 269 mμ and 276 mμ in ethanol solution. The values for the molecular extinction coefficients at each peak of both the amide and the methylamide were similar to the values for the corresponding peak of the spectrum of penicillin V.

The Conversion of Pencillin V to Phenoxyacetic Acid

Treatment of penicillin V with boiling 2N sulphuric acid for 2 hours liberates phenoxyacetic acid quantitatively. The intensity of absorption of this acid at 270 mμ obeys Beer's Law in the range 0·00 to 0·01 per cent. w/v, and penicillin V can be determined by extraction of sulphuric acid hydrolysates with ether and measurement of the intensity of absorption of the ethereal extract at 270 mμ.

Attempts were made to assay the phenoxyacetic acid content of penicillin V hydrolysates bromimetrically. The ethereal extracts were washed with sodium hydroxide solution, and the washings neutralised. This

solution was then brominated for 15 minutes at room temperature in the dark, and the bromine uptake determined. Results indicated a bromine absorption of approximately 1 equivalent per mole of phenoxyacetic acid. The scatter between duplicate experiments was, however, too great to use the method for quantitative purposes.

Phenoxyacetic acid did not react with iodine solutions. The presence of this acid in crude penicillin V samples e.g. fermentation broths will thus not interfere with the determination of penicillin V by the iodimetric method[13].

Comparison of the Ultra-violet Spectra of Penicillin V and Penicillin G

The absorption peaks in the spectrum of an aqueous solution of penicillin V can be readily detected at concentrations as low as 0·002 per cent. w/v. Solutions of sodium and potassium penicillins G[8] and of benzathine dipenicillin G show markedly different spectra. At low concentrations, there is an inflection in the spectrum at 250 to 260 mμ: at higher concentrations, this develops into small peaks at 253 mμ and 257 mμ. Procaine benzylpenicillin shows a single, pronounced, absorption maximum at 290 mμ, due to the procaine component of the molecule and detectable at concentrations of about 0·0015 per cent. w/v. The various spectra are collected for comparison in Figure 4.

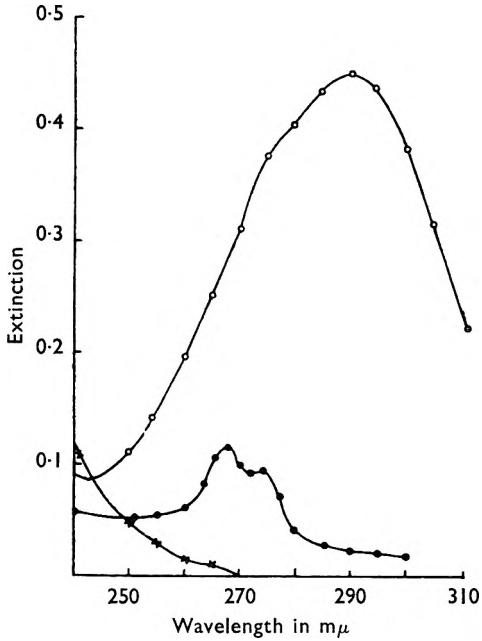

FIG. 4. The ultra-violet absorption spectrum of different penicillin preparations in aqueous solution.

●——● Phenoxymethylpenicillin (0·0023 per cent. w/v).
○——○ Procaine benzylpenicillin (0·0015 per cent. w/v).
×——× Sodium benzylpenicillin (0·0047 per cent. w/v).

Conversion of Penicillin V to p-Bromophenoxyacetic Acid

Early attempts to devise a method for differentiating chemically between penicillin V and penicillin G, were based upon the hope that under suitable conditions, penicillin V could be converted by hydrobromic or hydriodic acids to phenol, for which there are sensitive, specific tests. The initial experiments were made with phenoxyacetic acid. Two grams was refluxed for 2 hours with 20 ml. of 48 per cent. hydrobromic acid. The reaction mixture was cooled and extracted three times with 20 ml. portions of ether.

The bulked extracts were washed twice with 5 ml. portions of N-sodium hydroxide solution, and these alkaline extracts were neutralised. This solution gave only a faint reaction for phenol with the Folin-Ciocalteu reagent. When the phenoxyacetic acid was brominated, a white precipitate separated immediately. Bromination was allowed to proceed for 15 minutes at room temperature in the dark. The precipitate was then filtered, well washed with water, and recrystallised from hot water. After drying, the product melted at 158° C. This product was obviously not 2:4:6-tribromophenol. Further examination showed that it was acidic, with an equivalent weight of 230. It appeared that this material might be *p*-bromophenoxyacetic acid, the initial hydrobromic acid treatment having failed to decompose the phenoxyacetic acid. Confirmation was obtained by preparation of the material from phenoxyacetic acid (1 g. in 150 ml. water), by treatment in the dark at room temperature for 15 minutes with 40 ml. of freshly prepared bromine water. After filtration, washing with water and crystallisation from hot water, the product melted at 158° C., undepressed upon admixture with the previous preparation. Final confirmation was obtained by synthesis from *p*-bromophenol by the method of Koelsch[9]. The product again melted at 158° C., undepressed upon admixture with either of the previous preparations.

These results suggested that penicillin V could be characterised by hydrolysis to phenoxyacetic acid and conversion of this to the sparingly soluble *p*-bromophenoxyacetic acid, for further work, described later, showed that under the conditions employed, phenylacetic acid did not give a sparingly soluble bromo-derivative. Accordingly, the cooled hydrolysate obtained after refluxing 1 g. penicillin V with 20 ml. 2N sulphuric acid for 2 hours, was extracted with ether. The ether extracts were in turn extracted with sodium hydroxide solution, and these extracts were neutralised and brominated in the normal manner. The product was filtered, washed and crystallised from hot water. After drying, it melted at 150° C. Surprisingly, the mixed melting point with authentic *p*-bromophenoxyacetic acid was depressed to 127° C. Examination of the degradation product showed that it contained bromine, but neither nitrogen nor sulphur, and was an acid with an equivalent weight of 220. The ultra-violet absorption spectrum in ethanol solution showed a major absorption maximum at 226 mμ, with a subsidiary peak at 278 to 280 mμ. Authentic *p*-bromophenoxyacetic acid showed a spectrum with peaks at identical wavelengths, but the intensities of absorption at each peak were some 10 per cent. higher than those of the degradation product. It was then found that recrystallisation of this product from a small volume of benzene raised the melting point to 157° C., now undepressed upon admixture with authentic *p*-bromophenoxyacetic acid. The intensities of absorption of the ultra-violet spectral peaks also now practically coincided with those of the synthetic material. The experiment was then repeated, but the crude bromination product was crystallised directly from benzene, to give a product melting at 158° C., undepressed upon admixture with the synthetic material.

Solutions of phenylacetic acid (0·2 g.) in water (20 ml.), when brominated

in the normal manner, gave no precipitate, although some uptake of bromine occurred. Similarly, 2N sulphuric acid hydrolysates of sodium penicillin G, when extracted with ether and brominated as before, also failed to give any insoluble bromo-derivative.

The Colour Reaction of Penicillin V with Chromotropic and Sulphuric Acids

Freed[10] has described a colour reaction for phenylacetic and phenoxyacetic acid derivatives. In this reaction, a small amount of the solid preparation is treated with a few crystals of chromotropic acid and 2 ml. of concentrated sulphuric acid. The mixture is immersed in a glycerol bath at 150° C. for 1 to 2 minutes, and then removed. The colour of the solution is noted, after dilution of the reaction mixture with concentrated sulphuric acid if necessary. The results of treating penicillin V and several penicillin G preparations in this manner is shown in Table I. A number of simpler compounds are also listed. It will be noted that omission of the chromotropic acid from the reaction mixture results in a marked change in the colours produced.

TABLE I

COLOUR DEVELOPMENT IN THE FREED[10] REACTION

Compound	Chromotropic acid plus sulphuric acid	Sulphuric acid alone
Blank	Pale yellow	Colourless
Phenoxyacetic acid	Deep red	Brown
p-Bromophenoxyacetic acid	Deep red	Deep brown
Penicillin V	Deep blue-purple	Orange brown
Phenylacetic acid	Pale yellow	Colourless
Sodium penicillin G	Brown	Light brown
Procaine hydrochloride	Yellow-green	Colourless
Procaine penicillin G	Red-brown	Pale yellow
Benzathine sulphate	Yellow-green	Colourless
Benzathine dipenicillin G	Deep red	Amber
Carboxymethylcellulose	Black	Black

DISCUSSION

The preparation of penicillin V samples having purities apparently greater than 100 per cent. when assayed iodimetrically, is discussed elsewhere[13], where a statistical examination of the results is presented. The purification is proved by the disappearance from chromatograms of the second biologically active component[14], and by the results of the solubility analyses. The alternative explanation, that the purification procedure actually resulted in a concentration of a lower molecular weight penicillin with similar chromatographic and ultra-violet spectral characteristics, is unlikely, in view of the solubility analysis results. Further evidence that purification has occurred, is supplied by the results obtained by Dr. G. H. Twigg (The Distillers Company Limited, Research and Development Department) during an examination of the infra-red spectra of various samples of penicillin V. Sample A was found to possess a spectrum similar to those of samples C and E, but contained in addition 2 rather weak absorption bands at 727 cm.$^{-1}$ and 846 cm.$^{-1}$: these bands occur in the spectral region containing frequencies characteristic of the out-of-plane deformation vibrations of the hydrogen atoms of the benzene

ring, and their absence from the spectra of samples C and E suggests that the second component in sample A differs from penicillin V solely in the nature of the amide side chain. The fact that the second component appears to have ultra-violet spectral characteristics practically identical with those of penicillin V also suggests that the difference in composition is probably very slight.

The ultra-violet spectrophotometric method for the determination of penicillin V is useful for checking the quality of relatively pure samples, but suffers from severe drawbacks when applied to impure materials. The close similarity of the spectra of the penicillin and its acidic and alkaline inactivation products results in inability to detect small amounts of these products in penicillin V. Large quantities of acidic inactivated material will be detectable, but since the molecular extinction coefficient of alkaline inactivated material is close to that of penicillin V, the presence of large amounts of such material will not be noted.

Application of the spectrophotometric method to crude samples, such as fermentation broths and intermediate recovery samples is similarly restricted. Such compounds as phenoxyacetic acid and phenoxyethanol, which may be present and which possess a single maximum at 270 mμ, will prevent use of the method, although the absence of such compounds could be detected from the value of the ratio of the intensities of absorption at 268 mμ and 274 mμ. The presence of the system of 2 absorption maxima at 269 mμ and 276 mμ in such simple compounds as phenoxyacetamide and *N*-methyl-phenoxyacetamide is interesting from the theoretical point of view, but illustrates yet again the unsuitability of the spectrophotometric assay procedure for impure samples.

Samples containing extraneous materials absorbing at the phenoxymethylpenicillin peaks could be assayed for their penicillin content by making use of the formation of the peak at 318 to 320 mμ under mildly acidic conditions. This peak is probably due to the formation of phenoxymethylpenicillenic acid. The formation of the corresponding acids from other penicillins has been used as the basis of a spectrophotometric assay procedure[11,12].

The spectrophotometric assay procedure does enable ready differentiation between penicillin V and penicillin G preparations. Examination of the spectrum of an approximately 0·002 per cent w/v solution will ensure identification.

The estimation of penicillin V by spectrophotometric determination of the phenoxyacetic acid produced on acidic hydrolysis, is of academic interest only, in view of the ease of the direct spectrophotometric method. Moreover, the method suffers from the same drawbacks as the direct method. However, the formation of phenoxyacetic acid can be used for chemically characterising penicillin V, because it readily yields the sparingly soluble *p*-bromophenoxyacetic acid. This is a well defined crystalline compound, which can be further characterised if necessary by means of its ultra-violet spectrum. Under similar experimental conditions, there is no precipitate from brominated hydrolysates of penicillin G preparation.

The reaction of phenoxyacetic acid derivatives, developed by Freed[10]

for the detection of 2:4-dichlorophenoxyacetic acid is sensitive, is rapid to perform, and gives characteristic colours enabling the ready differentiation between penicillin V and various preparations of penicillin G.

Summary

1. The purification of penicillin V has been studied, with the object of obtaining a pure sample for use as a reference standard for chemical and biological assays.

2. A penicillin V preparation has been obtained, which, under the experimental conditions detailed[13], absorbs 2·46 ml. 0·01N iodine solution per mg. after inactivation with penicillinase.

3. Evidence is presented to show that this material is pure.

4. The ultra-violet absorption spectra of penicillin V, its acidic and alkaline decomposition products, and a number of simpler phenoxymethyl compounds, have been determined. Pure penicillin V has a molecular extinction coefficient of 1330 at 268 mμ, and 1100 at 274 mμ.

5. An assay method for penicillin V, based upon the measurement of its absorption in the ultra-violet, is described, and the limitations of the method are discussed.

6. Methods have been found for the rapid differentiation of penicillin V from penicillin G preparations.

We are indebted to the following persons:—Dr. G. H. Twigg (The Distillers Company Limited, Research and Development Department) and Mr. R. Goodey and Mr. F. Heyworth (The Distillers Company (Biochemicals) Limited, Bromborough Research Station) for helpful discussions and for confirmation of analyses: and Miss J. Stephens (The Distillers Company Limited, Research and Development Department) for paper chromotographic assays.

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CHEMICAL AND MICROBIOLOGICAL ASSAY OF PENICILLIN V

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Chemical Assay

This account is concerned firstly with the investigation of the iodimetric assay of penicillin V (phenoxymethylpenicillin), especially of samples associated with the preparation of a pure assay standard, and secondly with a method for the assay of penicillin V in the presence of penicillin G (benzylpenicillin).

Experimental and Results

Description of Methods of Assay

Two iodimetric methods were used, one involving alkali and the other penicillinase, as inactivating agents. The reader is referred to descriptions by Alicino[1] and Ortenblad[2] for further information about iodimetric assay.

Alkali inactivation method. Approximately 100 mg. of sample were accurately weighed, dissolved in 100 ml. of 0·067M *p*H 7·0 phosphate buffer solution and made up to 500 ml. with distilled water. *Blank.* To a 20 ml. aliquot of the sample solution was added 20 ml. of 0·01N iodine in 20 per cent. w/v potassium iodide solution, and the whole was titrated immediately with 0·01N sodium thiosulphate previously standardised against potassium iodate. A 1 per cent. starch solution in 20 per cent. w/v aqueous potassium chloride solution was used as an indicator. *Test.* To a 20 ml. aliquot of the sample solution was added 5 ml. of N sodium hydroxide and the resulting mixture allowed to stand for 15 minutes at room temperature. After this period 5 ml. of 1·1N hydrochloric acid were added followed by 20 ml. of the 0·01N iodine solution. After a further period of 15 minutes the whole was titrated with the 0·01N sodium thiosulphate using the starch solution as indicator.

Penicillinase Inactivation method. A freeze dried penicillinase product was found to be non-iodine absorbing and particularly suitable for iodimetric assays. The penicillin sample solutions were prepared as described in the alkali inactivation method. *Blank.* To a 20 ml. aliquot of sample solution was added 30 ml. of 0·01N iodine solution in McIlvaine *p*H 4·4 buffer of 1·5 times the usual concentration. This was then allowed to stand for 30 minutes in the dark and titrated with the standardised 0·01N sodium thiosulphate solution using the starch solution as indicator. *Test.* To a 20 ml. aliquot of sample solution was added 1 ml. of a penicillinase solution, prepared by adding 20 ml. water to a 30 mega-unit vial

of penicillinase. This solution was allowed to stand for 15 minutes after which time 30 ml. of the 0·01N iodine solution was added. After a further 30 minutes standing in the dark, the titration was carried out with 0·01N sodium thiosulphate using starch solution as indicator.

Calculation of assay results. The difference between "blank" and "test" sodium thiosulphate titrations was taken as a measure of the penicillin content and used in terms of "ml. 0·01N iodine absorbed per mg. of sample after inactivation" in order to compare the various samples.

The Preparation of Pure Penicillin V for use as a Standard. Assay of Samples

First series. The preparation of samples A, B and C is described elsewhere[9]. From these samples duplicate assays of single solutions were obtained on five separate days. Assays were made by the two methods and are shown in Table I with a summary of the analysis of variance in Table II.

TABLE I

IODIMETRIC ASSAY OF PENICILLIN V, SAMPLES A, B, C, D, AND E

		ml. 0·01N iodine absorbed per mg. of sample after inactivation											
		Penicillinase inactivation						Alkali inactivation					
		Day						Day					
Sample	Time of assay	1	2	3	4	5	Mean	1	2	3	4	5	Mean
A	—	2·36 2·36	2·37 2·37	2·37 2·36	2·37 2·37	2·36 2·39	2·37	2·49 2·48	2·53 2·50	2·51 2·51	2·50 2·51	2·45 2·45	2·49
B	—	2·42 2·44	2·43 2·43	2·41 2·41	2·40 2·40	2·40 2·40	2·41	2·54 2·54	2·58 2·57	2·54 2·56	2·56 2·55	2·54 2·59	2·56
C	—	2·41 2·44	2·46 2·45	2·43 2·44	2·47 2·44	2·39 2·41	2·43	2·58 2·56	2·58 2·59	2·59 2·59	2·57 2·61	2·55 2·57	2·58
D	Morning	2·40 2·43	2·39 2·37	2·39 2·39	2·39 2·39	2·42 2·40	2·39	—	—	—	—	—	—
	Afternoon	2·41 2·39	2·39 2·39	2·37 2·36	2·36 2·36	2·39 2·39		—	—	—	—	—	—
E	Morning	2·49 2·48	2·44 2·44	2·47 2·46	2·47 2·47	2·48 2·46	2·46	—	—	—	—	—	—
	Afternoon	2·46 2·46	2·46 2·46	2·44 2·46	2·44 2·44	2·45 2·44		—	—	—	—	—	—

It was found that the Sample x Day interaction was significant ($p = 0·05$) using the penicillinase inactivation method, almost significant using the alkali inactivation method, and was probably due to variation in the time interval between dissolving samples and making assays. Accordingly this interaction was used in the "t" test to compare the means of sample A with sample B and sample B with sample C. By both methods of assay, sample B was shown to have a higher iodine absorption than sample A, this difference being highly significant ($p = 0·005$). Sample C was not shown to have a significantly ($p = 0·05$) higher iodine absorption than sample B, but further experimentation would possibly show a significant difference.

Second series. In a second series were sample D, a sample of Distillers Company (Biochemicals) Limited production material and E, the same

material after the purification stages described elsewhere[9]. A single solution of each was prepared on each of five days and duplicate assays were made in the morning and afternoon. The significant Sample × Day interaction shown in the first series was thought to be due to variation in the time interval between dissolving samples and making assays, therefore a time effect was included to test this possibility. Assays were carried out by the penicillinase inactivation method only (for reasons given below), and are also shown in Table I with a summary of the analysis of variance in Table II. The difference between samples was highly

TABLE II

IODIMETRIC ASSAY OF PENICILLIN V, SAMPLES A, B, C, D, AND E. SUMMARY OF ANALYSIS OF VARIANCE OF RESULTS GIVEN IN TABLE I

Samples	Method	Source of variance	Degrees of freedom	Mean square
A, B, and C	Penicillinase inactivation	Between samples (S)	2	0·010675
		Between days (D)	4	0·000604
		S × D interaction	8	0·000436
		Residual	15	0·000139
	Alkali inactivation	Between samples (S)	2	0·021841
		Between days (D)	4	0·000925
		S × D interaction	8	0·000568
		Residual	15	0·000230
D and E	Penicillinase inactivation	Between samples (S)	1	0·049070
		Between days (D)	4	0·000923
		Between times (T)	1	0·002608
		D × S interaction	4	0·000156
		T × S interaction	1	0·000002
		D × T interaction	4	0·000450
		D × T × S interaction	4	0·000030
		Residual	20	0·000066

significant ($p = 0{\cdot}001$), in addition to which the Day × Time interaction was very significant ($p = 0{\cdot}01$), when both were compared with the residual, but the Between Times effect was not significant when compared with this interaction. Inspection of the results showed that assays made in the afternoon were lower than the morning assays on four days, but that on the second day this was not the case, and probably accounted for the significant Day × Time interaction term. If the assays made on the second day were omitted, the Between Times effect would almost certainly have been significant. The appropriate interaction terms were used in the "t" test to compare the means of samples C and E and sample E was shown to have a significantly greater iodine absorption ($p = 0{\cdot}05$).

Of all the samples assayed, sample E had the highest iodine absorption after inactivation, and in conjunction with evidence given in Parts 1 and 3 of this paper was probably the purest sample. This sample E with an iodine absorption after inactivation of 2·46 ml. per mg. was therefore considered suitable as an assay standard.

Determination of Penicillin V in the Presence of Penicillin G

The investigation of the iodimetric determination of penicillin V in the presence of penicillin G was based upon the difference in the rates of inactivation of the two penicillins at *p*H 2·0.

The initial experiments involved the use of three different solutions, the

first containing penicillin G (A.S.C. III standard) only, the second containing penicillin V, and the third a mixture of the two. Each solution was adjusted to *p*H 2·0 for different periods of time, re-adjusted to *p*H 7·0 and then assayed according to the alkali inactivation method. After 1 hour at *p*H 2·0 the solution of penicillin G gave a negative assay; such a result could be explained by the difference in treatment of "blank" and "test" in this method, and was later shown to be due in particular to the differences in *p*H level of "blank" and "test" during iodine absorption. Subsequent experiments and in fact those concerning the assay of samples D and E were conducted using the penicillinase inactivation method only, where "blank" and "test" were treated identically as far as possible.

In the next series of experiments concerned with inactivation at *p*H 2·0 it was found that there was no period at which all the penicillin G was inactivated leaving the penicillin V unaffected. Therefore there appeared to be two possible ways in which the determination could be carried out. The first would consist essentially of inactivation at *p*H 2·0 for a period long enough to decompose all the penicillin G followed by a determination of the residual penicillin V, and the use of a factor to find the original content. The second would consist of a shorter period of inactivation at *p*H 2·0 to cause partial decomposition of the two penicillins, and a comparison with values obtained with mixtures of known proportions of penicillin V and penicillin G. The former method was thought to be subject to less variation and was therefore investigated further.

At *p*H 2·0 and temperatures of approximately 20° C. with solutions of about 350 units/ml. inactivation of penicillin G took 3 hours. The solutions were adjusted to *p*H 7·0 for assay. The residual penicillin V varied between 80 and 85 per cent. of the original content. Table III shows results of determinations made on solutions of penicillin G, penicillin V and mixtures of equal volumes of the two on six different days using the A.S.C. III sodium penicillin G standard and sample E. The standard deviation of the results obtained from the solutions of penicillin V only was ± 1·9 per cent. whereas the corresponding standard deviation for the mixture was ± 3·2 per cent. This method is under investigation.

The Purity and Potency of the A.S.C. III Sodium Penicillin G Standard and its Relation to Penicillin V

The A.S.C. III sodium penicillin G standard was assigned a potency of 1682 units/mg. and has been in use since October, 1953. For the purpose of control of biological assay a sample of potassium penicillin has been assayed against this standard periodically since January, 1954. A total of 35 sets of six assays of this sample was carried out using the A.S.C. III standard between the period January—June, 1954. From the mean range for this period 95 per cent. confidence limits for day ranges and means were calculated[3]. The mean assay of those falling within these limits was 1625·6 units/mg. after eliminating eight day means which were above the upper p= 0·975 limit. The 95 per cent. confidence limits of this mean were ± 6·0 units/mg. and thus its potency was significantly higher than the expected 1609 units/mg. Over the next 6 months period 36 sets of

TABLE III

IODIMETRIC ASSAY OF SOLUTIONS OF PENICILLIN G ONLY, PENICILLIN V ONLY AND OTHER MIXTURES OF EQUAL VOLUMES OF THE TWO

Solution	Penicillin estimated	Residual penicillin after 3 hours at *p*H 2·0 (per cent. of the original)					
		Day					
		1	2	3	4	5	6
Penicillin G	Penicillin G	0·2	0·1	0·3	0·0	0·0	0·0
Penicillin V	Penicillin V	82·5	85·0	80·0	84·0	82·0	84·5
Penicillin G and Penicillin V	Penicillin V	81·0	88·0	79·0	83·0	82·5	85·5

assays were made and the mean was 1624·9 $\pm$ 5·6 units/mg. ($p = 0{\cdot}05$). Over this second period therefore this sample was again shown to have a potency significantly higher than that expected. This evidence may therefore indicate that the A.S.C. III standard contains 1 per cent. of impurities.

Over a period of approximately four months the A S.C. III sodium penicillin G standard was assayed almost daily by the penicillinase inactivation method, and the mean of all these results, 734 units/ml. 0·01N iodine solution or 2·29 ml. 0·01N iodine solution per mg. were therefore suitable factors to use where comparisons were required with this standard. Using this factor, the best estimates of the assays of samples A, B, C, D, and E in terms of units/ mg. relative to the A.S.C. III standard were A 1740, B 1772, C 1785, D 1753 and E 1804.

On the basis that pure sodium penicillin G contains 1682 units/mg. these estimates may be 1 per cent. high.

Accuracy of Iodimetric Assay using the Penicillinase Inactivation Method

The mean square terms for the Sample $\times$ Day interaction and Day $\times$ Time interaction in Table II were the best estimates of assay error in the two series of assays made. Both were similar and indicate a standard deviation of 0·87 per cent. Over the same period in which these assays were made the A.S.C. III standard was also assayed daily giving a corresponding standard deviation of 0·84 per cent. over independent assays. The accuracy of the method was therefore similar for both penicillins and from the two analyses of variance it is probable that the accuracy could be improved if the interval between dissolving the sample and its assay were reduced.

MICROBIOLOGICAL ASSAY

With the renewed interest in penicillin V (phenoxymethylpenicillin) it is necessary to reconsider the terms by which potencies of penicillin preparations are expressed. The use of the term "unit" was appropriate before the chemical structure of penicillin was known, but to-day the penicillin unit refers to a given weight of a purified chemical compound. Expression of the activity of penicillin G in terms of weight has been advocated[6] and with the introduction of penicillin V this procedure is necessary to avoid confusion since penicillin V has a different activity

from penicillin G against a number of test organisms. The confusion which is likely to arise from the use of the microbiological unit is not new since the earlier known penicillins X, F, A and K have different activities from that of G. However the problem has not previously been of practical importance because these other penicillins have not been developed commercially. Already penicillin V has been given several different microbiological potencies[4,7] from which it appears that the value of penicillin V in terms of penicillin G is dependent on both the strain of assay organism and the conditions of the test.

This section deals with an examination of the activity of penicillin V in terms of penicillin G against several recognised assay organisms, and discusses the confusion which is inherent in the use of the penicillin unit. The use of a penicillin V reference standard of the highest purity is advocated in order that potencies may be expressed on a weight basis.

Experimental and Results

Penicillins. Penicillin G (benzylpenicillin) A.S.C. III sodium salt which has been assigned a potency of 1682 units per mg. Penicillin V (phenoxymethylpenicillin free acid), sample E described elsewhere[9]. Solutions of each penicillin were prepared daily by dissolving 47·6 mg. in a final volume of 100 ml. of *p*H 6·0 M/20 phosphate buffer. Further dilutions were made in *p*H 7·0 M/20 phosphate buffer.

Assay method. Assays were carried out by the cavity-plate method developed in this department[8]. The volume of medium was increased to 180 ml. per plate. The following assay organisms and inocula were used. *Staphylococcus aureus.* Inocula were broth cultures grown for 24 hours at 37° C. having opacities* between 1 and 2; the amounts were as follows. Strain 209P 0·3 ml. per plate, Oxford H 1·0 ml. per plate, NCIB 8244 0·3 ml. per plate. *Bacillus subtilis.* Inocula were spore suspensions in distilled water having opacities* between 7 and 10, prepared from nutrient agar slopes; the amounts were as follows. Strain 288 0·1 ml. per plate, ATCC 6633 0·1 ml. per plate, I.C.I. Pen D/C8 0·1 ml. per plate. The inoculated medium was held at 60° C. for 10 minutes before pouring the plates. *Sarcina lutea.* The inoculum medium was the same as for *Staph. aureus.* The inoculum was a broth culture grown for four days at 29° C. A 1 to 10 dilution had an opacity* between 2 and 3. Strain NCIB 8553 2·0 ml. per plate.

Dose-response curves. Before attempting the quantitative assessment of penicillin V in terms of penicillin G, it was necessary to examine the form of the response curves for the two substances. This was done for one strain of each of the three species of organism. The plate design was modified to accommodate the same four levels of concentration by weight of each penicillin and each level was filled into eight cavities to a randomised design. The response curves for the averages of six plates of *Staph. aureus* 209P and five each of *B. subtilis* 288 and of *Sarcina lutea*, obtained by plotting inhibition zone diameter against logarithm of concentration, are given in Figure 1. The results were analysed statistically and the main conclusions are

* Brown's scale, Burroughs Wellcome and Company, Limited opacity tubes.

summarised as follows. The curves for *B. subtilis* 288 were substantially linear and parallel. The curves for *Staph. aureus* 209P were linear and parallel over the range of the three upper levels. There was a slight deviation at the lowest level of penicillin V with the average of the six plates used, but this was unimportant since it was below the range used for the assay of penicillin V against penicillin G. For *Sarcina lutea* the response curves to penicillins G and V differed in slope and curvature and therefore in these tests one penicillin could not be expressed in terms of the other. That the response curves for *B. subtilis* and for *Staph. aureus* were parallel was confirmed by a detailed analysis of variance for one plate of each of the six organisms in the course of the following experiment on the assessment of the activity of penicillin V. A similar analysis for plates of *Sarcina lutea* confirmed the non-parallelism of the penicillin V and G response curves for this organism.

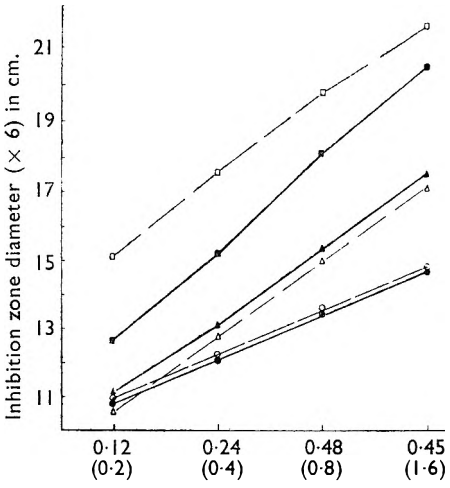

FIG. 1. Response curves for *B. subtilis* 288 ○ ●, *Staph. aureus* 209P △ ▲, and *Sarcina lutea* □ ■ against Penicillin G — — and Penicillin V ——.

Assessment of the activity of penicillin V. The activity of penicillin V was determined in terms of penicillin G for the strains of *Staph. aureus* and *B. subtilis* listed above. Solutions were prepared from two weighings of each penicillin. The four solutions were diluted to "high" and "low" concentrations and were filled in duplicate on each of two plates for each organism. Each "filling" (4 cavities "high" + 4 cavities "low") of penicillin V was calculated against the average of all the "fillings" of penicillin G on the same plate. The averages of the eight results for each organism on each of three days are given in Table IV.

A detailed statistical analysis of the results showed that the potency of penicillin V was (*a*) significantly greater than the potency of G for *Staph. aureus* strains 209P and NCIB 8244 (*b*) not significantly greater for the Oxford H strain and (*c*) significantly less for all three strains of *B. subtilis*. The relative potencies of the two penicillins for the three strains of *Staph. aureus* were different one from another and the result for *B. subtilis* ATCC 6633 was significantly lower than those for the other two strains. The activity of penicillin V against the Oxford H strain used was lower than expected from published results and this difference may have been associated with the method of maintenance. Cultures of this strain obtained from other sources are being examined. There was no difference, apart

from size, in the appearance of the zones for penicillins V and G on the same plate.

Inactivation of penicillin V by penicillinase. The inactivation of penicillin V by the penicillinase treatment described under "Chemical Assay" was examined microbiologically using *Staph. aureus* 209P and *B. subtilis* 288, and was found to be complete.

TABLE IV

ACTIVITY OF PENICILLIN V FREE ACID IN UNITS MG. AGAINST PENICILLIN G SODIUM SALT AT 1682 UNITS MG.

	Staph. aureus			*B. subtilis*		
Day	209P	Oxford H	NCIB 8244	288	ATCC 6633	ICI Pen.D/C8
1	1830	1704	1960	1514	1317	1556
2	1897	1788	2044	1578	1376	1628
3	1822	1573	1970	1642	1330	1601
Mean (±17)	1850	1688	1991	1578	1341	1595

Stability of penicillin V to acid. The stability of penicillin V to the acid treatment described under "Chemical Assay" was assessed microbiologically. On each day the following duplicate solutions were prepared: Solutions 1 and 2 of penicillin G 350 units per ml. (208 μg. per ml.); 3 and 4 of penicillin V 208 μg. per ml.; 5 and 6 of a mixture of penicillin G 175 units per ml. (104 μg. per ml.) and penicillin V 104 μg. per ml. (total 208 μg. per ml.). After treatment, the 6 solutions were assayed against *Staph. aureus* 209P and *B. subtilis* 288. The figures given in Table V show that penicillin V is more stable to acid than penicillin G confirming the results obtained under "Chemical Assay." Each figure is an average of 5 or 6 assays on different plates. The percentages given for the activity remaining for mixtures of G and V have been corrected for the residual G and therefore indicate only the residual V. Confirmation that penicillin G was not completely inactivated in solutions 1 and 5 for day 2 is presented in Figure 3 of the paper by Stephens and Grainger[10].

TABLE V

MICROBIOLOGICAL ACTIVITY AFTER 3 HOURS AT *p*H 2·0

Activity remaining as per cent. of original activity. Results for the mixtures of G and V are based on V

Day	Temperature of inactivation °C.	Assay organism	Penicillin G		Penicillin V		Penicillin G + V	
			Soln. 1	Soln. 2	Soln. 3	Soln. 4	Soln. 5	Soln. 6
1	Room temp. 20–21	*B. subtilis*	1·9	3·2	88·0	86·2	83·9	85·1
		Staph. aureus	1·8	3·3	92·8	90·0	89·6	88·5
2	Room temp. 20–21	*B. subtilis*	3·9	4·4	92·8	83·0	89·7	93·7
		Staph. aureus	4·1	4·3	90·6	82·3	81·0	85·7
3	25	*B. subtilis*	0·2	0·2	76·5	75·0	78·8	77·0
		Staph. aureus	0·1	0·2	80·5	77·8	82·8	83·8
4	25	*B. subtilis*	0·0	0·5	78·8	76·5	78·2	82·8
		Staph. aureus	0·0	0·4	82·8	77·2	80·0	81·0

Discussion

Earlier evidence[4,5] indicated that iodimetric assay of the various penicillins varied only according to the ratio of their respective molecular weights. The results of assays described in this paper do not support this theory. Sample E, for example, the purest of those prepared, assayed about 5·6 per cent. higher than anticipated from the assays of the A.S.C. III standard or 4·6 per cent. higher if the probable impurity of this standard is allowed for. However amongst other factors, iodimetric assays are known to vary with *p*H, with the time for iodine absorption, and, in the presence of much impurity, with the excess of iodine added. Thus it is equally likely that a change in the molecule could cause a change in the rate of iodination, particularly when the reaction between the penicilloic acid produced by inactivation and iodine is not stoichiometric.

When the microbiological activity of penicillin V is assessed in terms of penicillin G the result obtained depends not only on the species but also on the strain of the assay organism. A similar conclusion may be drawn from previously published work[4,7] in which activities ranging from 1670 (for the sodium salt) to 2695 units per mg. are given. It also appears that different activities in terms of penicillin G may be obtained in different laboratories using the same strain of test organism. For example, the figure of 2250 units per mg. for the Oxford H strain of *Staph. aureus* reported by Brunner[4] is appreciably higher than the activity found in the present tests. In the case of *Sarcina lutea* the potency of penicillin V in relation to G could not be expressed accurately because the response curves differed in slope.

It is evident that disagreement and confusion will occur if potencies of penicillin V preparations are expressed in terms of penicillin G by different laboratories using different methods and test organisms. It is therefore recommended that the potency of a penicillin preparation should be expressed on a weight basis in relation to a purified specimen of the same penicillin which has been established as a reference standard.

Summary

1. Iodimetric assays were made on various samples of penicillin V obtained during the purification of this material. A significant increase in purity, as judged by iodimetric assays, was obtained. The purest sample, E, was considered suitable as a reference standard and absorbed 2·46 ml. 0·01N iodine per mg. after inactivation using the penicillinase inactivation method. The difference in assay between this and the sample with the next highest purity was just significant ($p = 0·05$).

2. A method was developed for the assay of penicillin V in the presence of penicillin G based upon the difference in the rates of inactivation at *p*H 2. The greater stability of penicillin V was confirmed microbiologically.

3. A cavity-plate assay method was used to compare the activity of penicillin V in terms of penicillin G against several recognised test organisms. The potency varied according to the strain of *Staph. aureus* and *B. subtilis* used. It is concluded that penicillin V preparations should be

assayed against a pure standard of the same penicillin and the potency expressed on a weight basis.

4. Assays using *Sarcina lutea* were invalid.
5. Penicillin V was shown to be completely inactivated by penicillinase.

We are indebted to Mr. J. Ince and Mr. R. Sherlock for assistance with the design and for the statistical analyses, to Miss H. E. D. Alexander, Miss A. J. Downham and Mrs. J. M. Fawcett for technical assistance, and to Mr. L. Donegan for suggestions on the method of determination of penicillin V in the presence of penicillin G.

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PAPER CHROMATOGRAPHY OF PENICILLIN V

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The use of paper chromatography for the separation of penicillins is not new but so far as is known has not been applied previously to the examination of penicillin V. The method has been used qualitatively only, in order to demonstrate the position of penicillin V when a mixture of penicillins is chromatographed and to confirm the work described elsewhere[1,2].

Experimental

Chromatographic Method

A method of descending chromatography based on those of Goodall and Levi[3] and Glister and Grainger[4] was used. Modifications included the replacement of paper strips by sheets which simplified the technique, and gave a better comparison of samples than can be obtained on separate strips, where the distance run varies even in the same tank. Water-saturated A.R. diethyl ether was used as the developing solvent and particular attention was paid to maintaining the water-ether equilibrium. The wet ether was kept at the temperature of development for several hours before use. Glass tanks 12 in. × 8 in. × 18 in. deep with vapour-tight glass lids were used for development. Layers of water and ether were kept at the bottom of each tank and, for the purpose of maintaining the equilibrium, unbuffered filter papers were suspended from the top of the tank, close to the walls and dipping into the water layer. Halfway through the equilibration period mentioned below, 20 ml. of wet ether was added to each of these papers. The tanks were thermostatically controlled at 24 to 25° C.

Sheets of Whatman No. 4 filter paper 19 in. × 7½ in. were used on which 4 (or 5) 2·5 μl. spots of the penicillin solutions were run. The sheets were soaked in 2·5 per cent. w/v *p*H 6·6 phosphate buffer and dried. A capillary pipette was used for applying the spots to the dried buffered sheets and, after allowing the spots to dry, an equilibration period of half an hour was given before development for 3 to 4 hours in the tanks.

After removal from the tanks the sheets were dried and "biographed" in the following way. The sheets were placed on the surface of large assay plates and left for 10 minutes; the assay plates consist of glass plates 22 in. × 15 in. × 3/16 in. with aluminium frames 18½ in. × 12 in.; they were prepared as described by Goodey, Reed and Stephens[2] except that double quantities of medium inoculated with *B. subtilis* 288 were used.

The normal inoculum was twice that used for the cavity-plate assay, but when the very small zones shown in the chromatograph illustrated in Figure 2 were expected, one eighth of this amount was used in order to increase the zone size. The plates were incubated overnight at 37° C.

Methylene blue prints were taken as a record of the inhibition zones. This was done by flooding the surface with a 1·0 per cent aqueous solution of methylene blue (containing 1·0 per cent. phenol to kill the test organism), washing off the surplus stain after a minute or so, blotting with Whatman No. 1 filter paper by quickly smoothing a sheet over the surface, and finally taking several prints in a similar manner, but leaving the paper in contact with the surface until the dye had been taken up sufficiently to give a clear print. The methylene blue prints were photographed for the illustrations used in this paper.

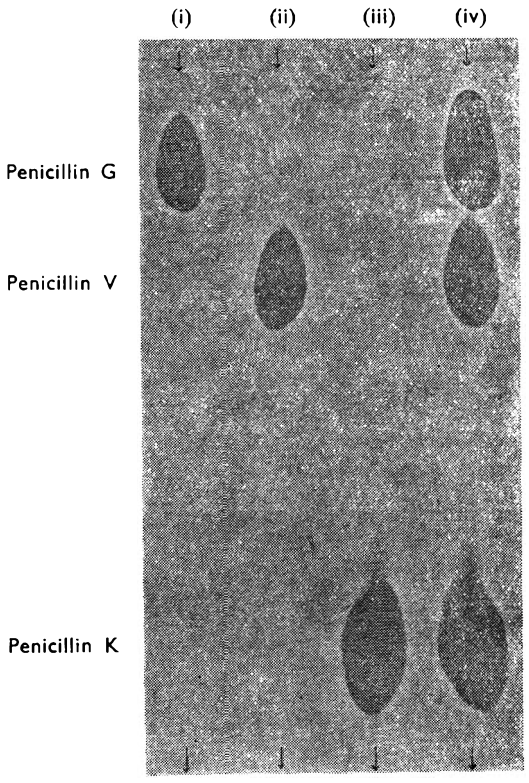

FIG. 1. The identification of penicillin V in a mixture. (i) Penicillin G. (ii) Penicillin V. (iii) Penicillin K. (iv) Mixtures of penicillins G, V and K.

Materials. Penicillin G (benzyl penicillin) A.S.C. III sodium salt which has been assigned a potency of 1682 units per mg. Penicillin V (phenoxymethyl penicillin free acid) samples A, B, C, D and E as described elsewhere[1]. The purified sample E was used for all work except where otherwise stated. Penicillin K (*n*-heptyl penicillin) ammonium salt prepared in our laboratories, to which we have assigned a potency of 995 units per mg. against *B. subtilis*.

Application. The identification of penicillin V in a mixture is demonstrated by Figure 1. Penicillins G, V and K were chromatographed both together and separately. Chromatographs of penicillin V samples A, B, C, D and E are illustrated in Figure 2. Samples A, B, C and D were shown to contain an unknown impurity which was not present in the purified sample E. The spots each contained the same weight of material.

Samples D and E were "biographed" also on plates inoculated with *B. subtilis* strains ATCC 6633 and ICI No. Pen. D/C3, *Staph, aureus* strains 209P, Oxford H and NCIB 8244 and *Sarcina lutea* strain NCIB 8553 and were found to give the same picture in each case.

Confirmation of the stability of penicillin V compared with G in the presence of acid is given by Figure 3 which shows a chromatograph of solutions 1, 3 and 5 assayed on Day 2 by Goodey, Reed and Stephens[2]. This chromatograph clearly demonstrates that the penicillin remaining after treatment is mainly V, although on this particular day there was a small amount of G as well. The zone sizes in the illustration are not strictly comparable, as the solutions used, even before treatment, contained different amounts of the penicillins. The solutions used for preparing the spots were as follows (i) was a mixture of penicillin G, 800 units per ml. (476 μg./ml.) and penicillin V, 476 μg./ml. (ii) was penicillin G, initially 350 units per ml. (208 μg./ml.), acid treated (iii) was penicillin V, initially 208 μg/ml., acid treated, and (iv) was a mixture of penicillin G, initially 175 units per ml. (104 μg./ml.) and penicillin V, 104 μg./ml., acid treated. The fact that penicillin G was present after acid treatment of the mixture, and in an amount of the same order as in the solution of acid treated G alone, supported the assumption made for calculating the penicillin V remaining in the mixtures given in Table V, of the paper by Goodey, Reed and Stephens[2].

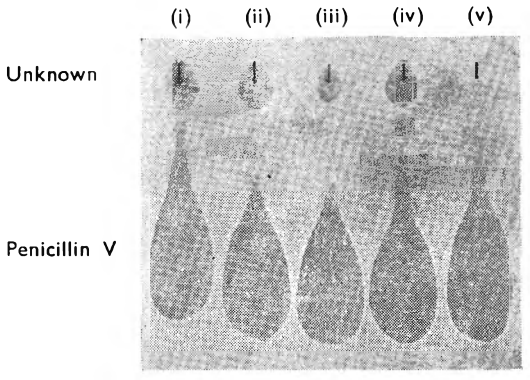

FIG. 2. The purification of penicillin V. (i) Sample A. (ii) Sample B. (iii) Sample C. (iv) Sample D. (v) Sample E.

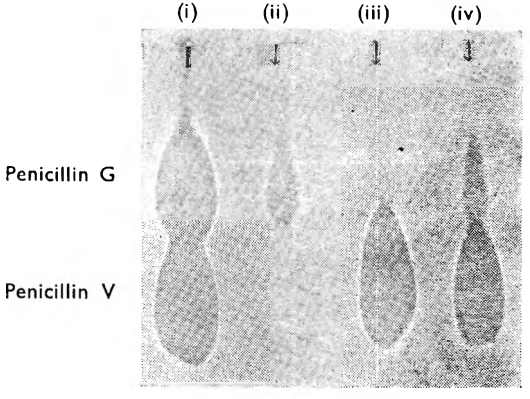

FIG. 3. Stability of penicillin V compared with penicillin G in the presence of acid. (i) Untreated mixture of G and V. (ii) Acid treated G, solution 2. (iii) Acid treated V, solution 3. (iv) Acid treated mixture of G and V, solution 5.

Discussion

Penicillin V has been shown to move faster than G on a chromatograph of a mixture of the two, taking the position normally attributed to F(Δ^2 pentenylpenicillin); it may well be that V and F chromatographed side by side would show a slight difference in position, but no specimen of pure F was available to investigate this.

The unknown impurity in the cruder penicillin V samples A, B, C and D is thought to be another acid stable penicillin since, like V, it remained after the acid treatment described by Goodey *et al.*[2] and was inactivated by penicillinase. It is hoped to extract enough of this material to make more tests.

Summary

1. The use of chromatography as a qualitative method of identifying penicillin V in a mixture is demonstrated.

2. Stages in purification of penicillin V are illustrated.

3. The much greater stability of penicillin V as compared with penicillin G in the presence of acid is confirmed by chromatography.

We wish to thank Miss P. Greenwell for technical assistance and Mr. F. Fox for preparation of the photographs.

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DISCUSSION

The three papers were presented by DR. G. PARKER.

MR. G. SYKES (Nottingham) asked the authors whether it might not be found helpful to use the penicillinase method of assay described at the 1952 Conference.

DR. G. E. FOSTER (Dartford) pointed out that the authors suggested that penicillin V was more stable to acid than penicillin G, and suggested it would be more suitable for oral administration. Had any experiments been carried out to assess the rate of absorption of the penicillin *in vivo?*

DR. F. HARTLEY (London) asked whether the authors could explain the alleged relative stability of the compound towards acid. It was difficult to see what could cause the stability of the lactam ring portion which would be broken on acid hydrolysis. Clearly it could not be solely the function of solubility.

DR. G. PARKER, in reply, said with regard to the absorption of penicillin V, he could not quote exact figures, but it had been given to a large number of volunteers and it compared very favourably indeed with penicillin G both as regards rate of absorption and blood levels obtained.

He had no suggestion as to why penicillin V should be more stable than penicillin G. The theory had been put forward in one paper that it was due to hydrogen bonding.

MR. R. GOODEY added that the penicillinase method of assay had not been tried.

THE ASSAY OF POLYMYXIN AND ITS PREPARATIONS

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In 1947 Benedict and Langlykke[1] reported the antibacterial properties of cultures of *Bacillus polymyxa* and Stansly, Shepherd and White[2] announced the isolation from such cultures of a new antibiotic, which was named polymyxin. Almost simultaneously Ainsworth, Brown and Brownlee[3], working independently, obtained aerosporin from media in which *Bacillus aerosporus* had grown. These two antibiotics proved to be polypeptides of similar composition. It was subsequently found that different strains of *B. polymyxa* gave rise to a series of closely related antibiotics and this led to a re-investigation[4] of the taxonomic derivation of *B. aerosporus* and *B. polymyxa*, the two being found identical and the latter the specific name.

Five antibiotics known as polymyxins A, B, C, D and E have now been isolated and characterised by the amino-acids formed from them on hydrolysis. Table I summarises data by Jones[5] and Catch, Jones and Wilkinson[6,7].

TABLE I

The amino-acid components of the polymyxins

Polymyxin	D-Leucine	D-Phenyl-alanine	L-Threonine	D-Serine	L-α:γDiamino-butyric acid
A	+	−	+	−	+
B	+	+	+	−	+
C	−	+	+	−	+
D	+	−	+	+	+
E	+	−	+	−	+

Chemical analysis of the hydrolysates established that in addition to the amino-acids a fatty acid, identified by Wilkinson[8] as D-6-methyl-octan-1-oic acid, is present in all polymyxins. Polymyxin A, originally known as aerosporin, has the same qualitative amino-acid composition as polymyxin E but Jones[9] was readily able to differentiate the intact antibiotics from each other by paper chromatography.

Brownlee, Bushby and Short[10,11] studied the chemotherapeutic and pharmacological properties of the polymyxins and showed that polymyxins A, C and D all cause severe proteinuria in animals when administered by injection and were too toxic for clinical use. Polymyxins B and E, however, are less nephrotoxic and their sulphates have been used in medicine. Polymyxin B sulphate is now established as the most widely used polymyxin preparation in medical practice and, on this account, its identification and assay have become of importance. In the present communication methods, which have been successfully used in our laboratories for some years, are described in the hope that the information may be of assistance to other workers in this field.

Tests for Identification and Purity

Identification of the polymyxins is best achieved by the methods of Jones[5,6,7] and his colleagues using paper partition chromatography for examination of the intact and hydrolysed antibiotics. Hydrolysis is conveniently carried out by a process subsequently employed by Foster, Macdonald and Jones[12] in their work on the ergot alkaloids. For polymyxin B sulphate the following tests are appropriate.

(*a*) Shake together four volumes of *n*-butanol, one volume of glacial acetic acid and five volumes of water, allow to separate and transfer the lower layer to a dish placed on the floor of an air tight chamber. When the atmosphere of the chamber is saturated, suspend in it a strip of Whatman No. 1 filter paper on which has been placed 0·005 ml. of solution, prepared by dissolving 5 mg. of the polymyxin sulphate in 0·5 ml. of water. Develop the chromatogram with the upper layer of the mixed solvents for about 16 hours. Dry the chromatogram in air, spray with a 0·1 per cent. solution of ninhydrin in water saturated *n*-butanol and heat in an air oven at 90° C. for about 5 minutes. The developed chromatogram shows only one spot characteristic of polymyxin B.

(*b*) Dissolve 5 mg. in 1 ml. of 5N hydrochloric acid. Transfer the solution to a small ampoule, seal and heat at 120° C. for 6 hours. Transfer the resulting solution to a small evaporating dish and evaporate to dryness on a steam bath. Continue to heat until the residue no longer gives off hydrogen chloride. Dissolve the residue in 0·5 ml. of water and place 0·005 ml. of the solution on a strip of Whatman No. 1 filter paper. As a control, prepare a solution containing 10 μg. each of leucine, phenylalanine, threonine and serine per 0·005 ml. Similarly place 0·005 ml. of this control solution on the same strip. Develop the chromatogram, as described under identification test (*a*) and locate the spots by means of the ninhydrin reagent. Definite spots for leucine, phenylalanine, threonine and $\alpha:\gamma$-diaminobutyric acid should be identified. The latter is indicated by a slow moving spot ($R_F = 0{\cdot}1$) near the starting line. No spot for serine should be present.

These tests are also applicable to polymyxins A, C, D and E, the amino-acids formed on hydrolysis being indicated in Table I. Salts of the polymyxins afford reactions characteristic of the acids present.

When the antibiotics are tested for purity by the chromatographic technique it has been found advantageous to make tests with increasing amounts (5, 10, 15 μl.) of their 1 per cent. aqueous solutions on the paper. For the examination of bacitracin in the intact state a mixture of equal volumes of acetone and water is a suitable developing solvent. The sensitivity of these tests may be increased by using the modified ninhydrin reagent proposed by Lewis[13]. Separation of phenylalanine and leucine on the chromatograms of the hydrolysates is often incomplete but they may be distinguished by the different coloured spots which they give with the ninhydrin reagent.

Biological Assay of Polymyxin B Sulphate

A plate diffusion method for the assay of polymyxin using a strain of *Escherichia coli* as test organism was first described by Stansly and

Schlosser in 1947[14]. Since *Escherichia coli* grows very rapidly and polymyxin diffuses slowly through agar it was found necessary to assay by incubating the plates for 16 to 18 hours at 25° C., followed by a further 6 hours at 37° C., in order that clearly defined zones of inhibition could be measured. Benedict and Stodola[15] found that *Brucella bronchiseptica*, NRRL Strain B-140, was a more suitable test organism since it showed the same order of sensitivity to polymyxin as *Escherichia coli* and grew more slowly. Hence it was possible to assay by incubation at one temperature only, namely, 37° C., for 14 to 16 hours. Existing methods of assay were reviewed in 1949 by Reese and Eisenberg[16]. The present United States Food and Drug Administration's (F.D.A.) schedule for the assay of polymyxin[17] is largely based on the work of Stansly and Schlosser, and of Benedict and Stodola. The assay plates contain a base layer of pancreatic digest of casein, papain digest of soybean, sodium chloride, dipotassium phosphate and dextrose in a 2 per cent. agar gel. The seed layer medium is similar to that of the base layer with a reduction in the agar content to 1·2 per cent. and with the addition of Tween 80. The *p*H of both layers is 7·3. The organism is a 24-hour culture of *Brucella bronchiseptica*, American Type Culture Collection 4617, incubated at 32 to 35° C. Whilst the original workers used a glycine-hydrochloric acid buffer at *p*H 2·0 for preparing the solutions of polymyxin to be assayed, the present official schedule requires the use of a phosphate buffer at *p*H 6·0.

The method employed in our laboratories for the assay of polymyxin is in principle similar to that described by Benedict and Stodola[15] but differs in experimental detail.

Assay Procedure

The assay plate consists of a 7 in. × 7 in. sheet of good quality window glass framed in 1 in. × ¼ in. duralumin. A loose cover of 22 S.W.G. duralumin is provided into which is inserted a sheet of blotting paper held in position by brass wires running along its length. The blotting paper absorbs any moisture from the medium during the period of incubation.

The organism used is *Brucella bronchiseptica*, Wellcome Culture No. 385, maintained on 7-ml. slopes of nutrient agar* in 20-ml. screw-capped bottles. The culture is transferred to a fresh slope once each week. For the assay a "loopful" is placed in 7 ml. of a nutrient broth† 48 hours previously and incubated at 37° C. Two ml. of this culture is added to 140 ml. of the nutrient agar at 47° C. and the bulk inoculum is then poured into a levelled assay plate. Cavities may be cut in the agar using an 8-mm. cork-borer, after which they are filled with 0·15 ml. of polymyxin solution, or "fishspine" refractory insulating beads No. 2, size 4 mm. × 3·7 mm. diameter[18], may be used to pick up by capillarity 0·017 ml. of the solution of polymyxin, and the beads containing the solution then applied to the surface of the uncut nutrient agar.

* A protein hydrolysate to which horse muscle extract, sodium chloride and 1·2 g. of New Zealand agar per litre are added.

† A papain digest of horse meat.

The polymyxin standard employed is a sub-standard assayed in terms of the proposed British standard for polymyxin B, obtainable from the Department of Biological Standards, National Institute for Medical Research, London, N.W.7. The latter has a potency of 7871 units per mg. when assayed in terms of the United States Food and Drug Administration's Standard of polymyxin B (7700 F.D.A. u/mg.).

All samples are diluted before assay with a glycine-hydrochloric acid buffer at *p*H 2·0. If the cavity-plate technique is used the standard is prepared in solution at two concentrations, namely 400 units per ml. and 100 units per ml. respectively, and the test sample is prepared at similar concentrations on the basis of the potency assumed for it. If the "fish-spine" bead method is employed then the respective concentrations of the standard and test preparations should be 1000 units per ml. and 250 units per ml.

The four solutions are applied to the assay plate in the order determined by any 2 + 2 latin square design. Diffusion of the polymyxin from the centres of application is allowed to proceed for 5 hours at room temperature, during which time the growth of the *Brucella bronchiseptica* is considerably retarded. The plates are then incubated for 10 hours at 37° C., and the zones of inhibition read after magnifying them 15·5-fold. Even with this high magnification the edges of the zones are clearly defined.

The potency of a sample is usually derived as the statistical mean calculated from the data obtained from two assay plates, giving 8 responses at each dose.

Assay of Certain Pharmaceutical Preparations

This method may be employed for the assay of pharmaceutical preparations containing polymyxin.

Polymyxin B Sulphate Otic Solution

The otic solution is a preparation containing 10,000 units of polymyxin B sulphate per ml. in propylene glycol acidified with Acetic Acid B.P. It is highly effective against *Pseudomonas aeruginosa* commonly found in ear infections. The preparation may be assayed simply by dilution with glycine-hydrochloric acid buffer (*p*H 2·0) to the concentrations of polymyxin required for the assay. At these concentrations the other substances present in the solution do not interfere. The results of the assay of 5 recent samples are presented in Table II.

Ointment Containing Polymyxin B Sulphate and Bacitracin

The ointment contains 10,000 units of polymyxin B sulphate and 500 units of bacitracin per g. of a soft paraffin base. Since bacitracin is active against gram-positive micro-organisms the ointment has a very wide bacterial spectrum and may be used topically for many infections with an extremely low risk of obtaining bacterial resistance.

(*a*) *Assay for polymyxin B sulphate.* About 1 g. of the ointment is accurately weighed in a centrifuge tube. 10 ml. of chloroform and 17 ml.

TABLE II

ASSAY OF OTIC SOLUTION OF POLYMYXIN B SULPHATE

(Labelled potency, 10,000 units per ml.)

Sample from batch	Found Potency ÷ Expected Potency (per cent.)	Limits of error ($p = 0.95$) of Found Potency ÷ Expected Potency (per cent.)
1	95·1	82·9–109·0
2	104·8	97·0–113·4
3	94·2	85·6–103·6
4	98·7	88·7–109·9
5	102·5	91·3–115·2

of glycine-hydrochloric acid buffer (*p*H 2·0) for each g. of ointment are added. The tube is stoppered and shaken vigorously for one hour in an automatic shaker and the contents of the tube are then centrifuged. The chloroform layer is discarded and a further 10 ml. of chloroform is added and the extraction is repeated.

The final solution of the polymyxin in glycine-hydrochloric acid buffer is assayed by the method previously described using cavity plates.

(*b*) *Assay for bacitracin.* The extraction procedure given for the extraction of polymyxin B sulphate is employed except that 50 ml. of phosphate buffer (*p*H 6·8) is used in place of the 17 ml. of glycine-hydrochloric acid buffer for each g. of ointment. The solution of bacitracin in phosphate buffer is assayed in cavity plates by a method similar to that given for the assay of polymyxin, using the American National Type Culture No. 7743 of *Micrococcus flavus* as test organism. The organism is maintained on nutrient agar slopes (similar to that described for the assay of polymyxin) which are sub-cultured weekly. 48 hours before the assay one "loopful" is inoculated into 7 ml. of nutrient broth and 3 ml. of this 48-hour culture is added to 140 ml. of nutrient agar and poured into a levelled assay plate.

The standard of bacitracin at present employed by us is a batch from Société Industrielle pour la Fabrication des Antibiotiques, containing 54·5 units per mg.

The results obtained on both polymyxin and bacitracin contents of five samples from recently manufactured batches of the ointment are given in Table III.

TABLE III

ASSAY OF AN OINTMENT CONTAINING POLYMYXIN B SULPHATE AND BACITRACIN

(Labelled potency, 10,000 units of polymyxin B sulphate and 500 units of bacitracin per g. of ointment)

Sample from batch	Polymyxin content		Bacitracin content	
	Found potency ÷ Expected potency (per cent.)	Limits of error ($p = 0.95$) of Found potency ÷ Expected potency (per cent.)	Found potency ÷ Expected potency (per cent.)	Limits of error ($p = 0.95$) of Found potency ÷ Expected potency (per cent.)
1	100·3	95·7–105·2	102·2	97·0–107·8
2	97·5	93·0–102·3	96·0	92·5–99·6
3	97·6	91·2–104·6	102·8	93·6–113·2
4	101·4	97·6–105·4	103·3	96·0–110·9
5	108·1	102·1–114·5	98·9	95·3–102·7

Chemical Estimation of Polymyxin

While numerous methods have been described for the chemical determination of proteins[19], little work of a comparable nature has been carried out on the assay of naturally occurring polypeptides, particularly those of a bacterial origin. Polymyxin B has been estimated by the ninhydrin reaction[20] and fractions of this antibiotic obtained in counter-current distribution experiments[21] have been estimated by measurement of their ultra-violet absorption at $\lambda = 259$ mμ. Other methods which are available for estimation of polypeptides include elementary analysis, particularly determination of nitrogen by the method of Kjeldahl, and colorimetric assay by the biuret reaction. These methods have the disadvantage either of being insufficiently specific or of lacking reproducibility or both.

A search was therefore made for a method in which these disadvantages would be reduced to a minimum and experiments indicated that gravimetric estimation with phosphotungstic acid, taken in conjunction with the results of chromatographic examination of the material would achieve this object. The proposed method is of limited application should the results of chromatographic examination reveal the presence of more than traces of impurities which give insoluble phosphotungstates. In this case the phosphotungstate factor must be determined for the actual batch of polymyxin present in the preparation under examination. None the less, in our experience with polymyxins and bacitracin, the method is capable of giving reproducible results and is applicable not only in the presence of non-nitrogenous substances but also when the polypeptide is accompanied by ammonium salts, nitrates and organic compounds containing one primary amino-group only, such as monoamino-acids. It is also applicable to pharmaceutical preparations of polypeptides and may be regarded as a useful adjunct to the biological method.

As we could find no publication describing the use of phosphotungstic acid for the assay of polypeptides of bacterial origin, an investigation was made to establish the optimum conditions of precipitation, washing and drying of the phosphotungstates. The effects of systematic changes in the following variables were studied in turn, (1) acidity of precipitation medium, (2) quantity of precipitant, (3) volume of liquid to be filtered, (4) volume of wash liquid, (5) acidity of wash liquid and (6) conditions for drying of the phosphotungstates. It was found that the phosphotungstates are virtually insoluble in water (e.g., the solubility of polymyxin B phosphotungstate is 0·0011 per cent. at 20° C.) and are even less soluble in acid medium. They are, however, soluble in organic solvents such as methanol, ethanol and acetone.

A 10 per cent. w/v solution of choline chloride in 2 per cent. w/v sulphuric acid proved a useful reagent for testing washings for the presence of phosphotungstic acid, one part of acid in 50,000 parts of solution being detected. Experiments using several batches of AnalaR phosphotungstic acid proved that the results were consistent when this quality of acid was employed. The precipitates are unaffected by heating for prolonged periods at 100 to 110° C. and are readily obtained in the anhydrous state.

The following general method was adopted for polymyxin B sulphate, polymyxin E sulphate and bacitracin.

Procedure

Transfer about 65 mg. accurately weighed to a 50-ml. beaker and dissolve in 12 ml. of water. Add 5 ml. of dilute sulphuric acid and 6 ml. of a 5 per cent. w/v solution of AnalaR phosphotungstic acid in water, previously filtered through a Whatman No. 5 filter paper. Allow to stand for 10 minutes, stirring intermittently, and filter with the aid of suction through a tared No. 4 sintered glass crucible. Completely transfer the precipitate to the crucible with three portions (20 ml. each) of 2 per cent. w/v sulphuric acid and wash the residue with two portions (20 ml. each) and one portion (10 ml.) of water. Maintain the precipitate in a wet condition during the entire filtering and washing operations and allow it to be dried by suction only after the final washing. Dry the precipitate either at 50° C. for 2 hours or over phosphorus pentoxide *in vacuo* at room temperature for 4 hours; finally heat at 110° C. for 1½ hours, cool and weigh.

Phosphotungstate Factor

For the purpose of assay work a factor equal to the weight of anhydrous polypeptide equivalent to 1 g. of anhydrous phosphotungstate complex, was carefully determined with samples of the purest antibiotics which we had available. Our results are summarised in Table IV.

TABLE IV

Polypeptide	Temperature at which "loss on drying" was determined	Potency of anhydrous material (u./mg.)	Phosphotungstate factor
Polymyxin B sulphate ..	37° C.	8150	0·265
Polymyxin E sulphate ..	37° C.	8430	0·259
Bacitracin	60° C.	76·1	0·322

The Assay of Otic Solution of Polymyxin B Sulphate (10,000 *u./ml.*)

The chemical assay has been applied to the assay of production batches of otic solution, of the composition described under the section on biological assay. For this purpose the following procedure was found to be satisfactory.

Transfer an accurately weighed quantity of otic solution, equivalent to about 360,000 units (approximately 37·5 g.) to a 250-ml. beaker. Add 100 ml. of water and 20 ml. of dilute sulphuric acid with stirring, followed by 5 ml. of a previously filtered 10 per cent. w/v solution of AnalaR phosphotungstic acid in water. Allow to stand for 30 minutes and filter with the aid of suction through a tared No. 4 sintered glass crucible. Complete the assay as described under the assay of polymyxin B sulphate. Calculate the potency of the otic solution using the following formula:—

$$\text{Potency in units per ml.} = \frac{1000\ \text{D.F.P.W.}}{\text{M}}, \text{ where D} = \text{specific gravity}$$

of solution; F = phosphotungstate factor; P = potency of polymyxin, used in preparation of solution, expressed as units per mg. of anhydrous material; W = weight in g. of anhydrous phosphotungstate obtained in assay; M = weight in g. of sample taken for assay.

When the above procedure is carefully followed assay results usually fall within 99 to 101 per cent. of the expected value. For routine checking of strengths of solutions during manufacturing operations it may be more convenient to use the standard phosphotungstate factor of 0·265 without determination of the factor for each batch of polymyxin B sulphate used. In our experience the results obtained by this modification are often close to the theoretical figures but an error of not more than 5 per cent. may occur.

Assay of Tablets of Polymyxin B Sulphate (500,000 *units*)

The procedure may be applied to tablets providing the tablet contains no base which will interfere with the assay. For tablets containing 500,000 units of polymyxin B sulphate per product, we have proceeded as follows.

Take a sample of 10 tablets and determine the average weight. Powder the sample and transfer an accurately weighed quantity, equivalent to about 400,000 units of polymyxin B sulphate, to a small beaker. Add 10 ml. of water and 3 ml. of dilute sulphuric acid and triturate the powder with the liquid. Filter through a Whatman No. 2 paper, washing the residue and filter with two 10-ml. portions of 2 per cent. w/v sulphuric acid and two 5-ml. portions of water. Add to the filtrate 5 ml. of dilute sulphuric acid and 8 ml. of a previously filtered 5 per cent. w/v solution of AnalaR phosphotungstic acid in water. Complete the assay as described under the assay of polymyxin B sulphate.

Discussion

Development of assay procedures for the polymyxins has followed a course similar to that experienced with many other antibiotics. The antibacterial properties of polymyxin and its preparations are of major importance and it will therefore be necessary to depend upon the microbiological assay until such time as physical or chemical techniques of equal specificity are available. A serial dilution method of assay, originally used, was abandoned for the plate diffusion method, now widely accepted, with the advantages of accuracy and simplicity. The results given in Tables II and III indicate that polymyxin B may now be assayed in conformity with the general requirements of the B.P. 1953 for the biological assay of antibiotics; namely that the estimated potency shall not be less than 90 per cent. and not more than 111 per cent. of the stated potency and the limits of error of the estimated potency ($p = 0{\cdot}95$) shall lie within 80 and 125 per cent. of the stated potency.

The chemical assay, employing phosphotungstic acid, was adopted by us after the use of other precipitating agents, such as ammonium reineckate and picric acid, had been explored. Conditions for the precipitation of polymyxin B as the phosphotungstate were carefully investigated

and the method, as described, may have wider applications for water-soluble polypeptides, particularly those of bacterial origin. It is intended that the chemical assay should supplement and not replace the microbiological assay which, at this stage of our knowledge, is fundamental. In order to obtain the most accurate results it is recommended that the phosphotungstate factor for each batch of polymyxin B sulphate should be determined, but the standard factor given may be employed usefully for much work involving the study of manufacturing operations. Polymyxin B phosphotungstate dissolves in some organic solvents and, for this reason, the application of the chemical assay to liquid preparations needs careful study. For example, it is necessary to dilute the otic solution to the extent described in order to prevent low assay figures being obtained owing to the solubility of the precipitate in the presence of propylene glycol, a constituent of the preparation.

In recent years studies on the uptake of polymyxin by bacterial cells have been reported and some of the data provided may form the basis of further assay procedures. Newton[22] and Few and Schulman[23] have shown that bacteria sensitive to polymyxin liberate substances which absorb ultra-violet light at $\lambda = 260$ mμ, when polymyxin is taken up by them. They found that within a suitable range of polymyxin concentrations the amount of light absorbing material released into the surrounding medium was a function of the amount of antibiotic present. Newton[24] has also shown that when washed cells of *Pseudomonas æruginosa* are suspended in a weak solution of *N*-tolyl-α-naphthylamine-8-sulphonic acid the addition of polymyxin produces an immediate fluorescence, which as a percentage of the maximum is linearly related to the dose of polymyxin added to the suspension of cells. These methods would appear to repay further investigation.

The methods of assay described in this paper deal especially with polymyxin B but there is little doubt that they may be extended for use with the other polymyxins. In particular, we have applied both the microbiological and the phosphotungstate assay to samples of polymyxin E sulphate with satisfactory results. No official standard preparation of polymyxin E is yet available and we have found it convenient, under our experimental conditions, to assay this antibiotic against the provisional British Standard polymyxin B sulphate and to express the potency in terms of units of polymyxin B sulphate.

It has been emphasised that these polypeptides should be substantially free from interfering impurities if the phosphotungstate assay is to yield reliable results. We have employed paper chromatography as a test of purity but it must be remembered that "chromatographically pure" is a relative term and workers must be for ever vigilant for the presence of unsuspected impurities. For example, we have been unable to separate polymyxins B and E in the intact state on paper chromatograms in spite of trials with a large number of developing solvents, while the composite nature of polymyxin B has been indicated by the recent work of Hausmann and Craig[21] using their counter-current distribution technique.

SUMMARY

1. The development of the series of antibiotics known as polymyxins is reviewed.

2. Details are given for the identification of the polymyxins by paper partition chromatography.

3. A plate diffusion microbiological assay using *Brucella bronchiseptica* as test organism has been developed and its application to the assay of pharmaceutical preparations is described.

4. Results are recorded to show that the proposed assay method conforms to the general limits of error of the B.P. 1953 for the biological assay of antibiotics.

5. A chemical method is described for the determination of polymyxin B by precipitation as phosphotungstate.

6. The application of the chemical assay to otic solution and tablets of polymyxin B sulphate is described.

7. Reference is made to the extension of the microbiological and chemical assay to other polymyxins, particularly polymyxin E. Some preliminary work with bacitracin is also described.

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DISCUSSION

The paper was presented by MR. G. A. STEWART.

DR. G. BROWNLEE (London) said that as polypeptides of identical chemical composition could have different activities there would be grave danger if the chemical method were ever practised in the absence of the microbiological method.

MR. T. D. WHITTET (London) suggested that it would be helpful if the authors could extend their work to studying the stability of various preparations as there were conflicting reports in the literature.

MR. D. JACK (Harrow) emphasised the importance of the strain of *Brucella bronchiseptica* used in the assay. Using the British strain he had found that he could only estimate concentrations of between 100 and 400 u./ml. With the American strain it was possible to determine concentrations of the order of 25 to 50 u./ml. That was important in the work being undertaken because it was a low potency preparation.

DR. G. E. FOSTER (Dartford) said that there was information available concerning the stability of polymyxin and its preparations.

It was rather unfortunate that the first official standard for polymyxin had to appear in the United States Pharmacopeia XV. Unfortunately, there was no official British standard preparation of polymyxin B sulphate available at the time of the preparation of a monograph for the B.P.C. 1954.

MR. D. STEPHENSON (Dartford) said that polymyxin B sulphate in freeze dried powder retains its potency for many years. Aqueous solutions may be autoclaved with losses of activity not greater than 10 per cent. Compressed tablets of polymyxin E sulphate and polymyxin B sulphate have retained full potency for about three years. The otic solution and the anhydrous ointment may be kept at room temperature for two years without significant loss of activity. It was desirable to prepare ointments at as low a temperature as possible because if soft paraffin were heated during the incorporation of the antibiotics, some loss of activity occurred.

MR. G. A. STEWART, in reply, said that he had not found it necessary to use a more sensitive strain than the Wellcome culture for assaying preparations of polymyxin. However when very low concentrations of antibiotic have to be estimated one would seek for the most sensitive strain of test organism with which to conduct the assay. Results with the American strain of *Brucella bronchiseptica* were in agreement with those obtained by him when assaying similar polymyxin preparations.

THE PREPARATION AND ANTIBACTERIAL ACTIVITY OF 2-PHENACYLPYRIDINE AND RELATED KETONES

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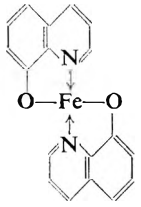
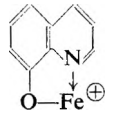
It has been shown[1] that, of the 7 mono-hydroxyquinoline isomers, only 8-hydroxyquinoline (oxine) exhibits antibacterial properties, and this alone of the isomers is capable of binding metals by chelation. Oxine is non-toxic to *Staph. aureus* unless traces of iron (or copper) are present in the medium[2]. Evidence has been presented which indicates that oxine probably exerts its antibacterial effects by penetrating into the cell as a lipoid soluble complex in which 1 atom of metal is bound by 2 molecules of oxine (1 : 2 complex) (I), whereas the toxic effects inside the cell are due to the unsaturated 1 : 1 complex (II)[3]. The introduction of *N* atoms



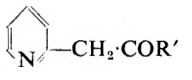
I



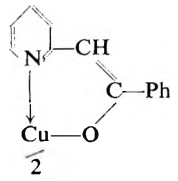
II

into the aromatic rings of the oxine molecule to give aza-oxines results in the reduction of the lipoid solubility of the system and a decrease in the antibacterial properties, whereas both these effects are reversed by increasing the lipoid solubility by further substitution with alkyl groups. The success which has attended the investigations of the co-ordinating properties of the oxine type of molecule, and their implications in the antibacterial activities of these compounds, has stimulated the search for useful antibacterial substances amongst potential complexing agents[1,4,5,6,7,8].

This present study was envisaged as an attempt to provide further information concerning co-ordination phenomena and antibacterial properties. The 2-picolylketone system (III) was chosen because



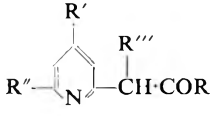
III



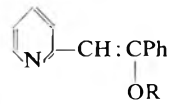
IV

Goldberg *et al.*[9] had already shown that the parent ketone, 2-phenacylpyridine (III, R = H, R′ = Ph) could co-ordinate with cupric ions. Co-ordination presumably occurs via the enolic form of this substance to yield a copper complex (IV), which may be regarded as analogous to the oxine-copper complex. Furthermore, the 2-picolylketone system offers many possibilities for suitable modifications. These include the alteration of the electron density at the nitrogen atom by substitution of the pyridine ring, and the variation of the percentage of the enolic form and the acidity of the enol function by substitution of the methylene and phenyl groups. Such changes could be used to vary the stability and the lipoid solubility of the metal complexes[10,11] and so makes possible an examination of co-ordination phenomena and antibacterial activity.

Preparation of the compounds. The *C*-alkyl derivatives of 2-phenacylpyridine (V, R = R″ = H, R = Ph, R‴ = alkyl groups) were prepared



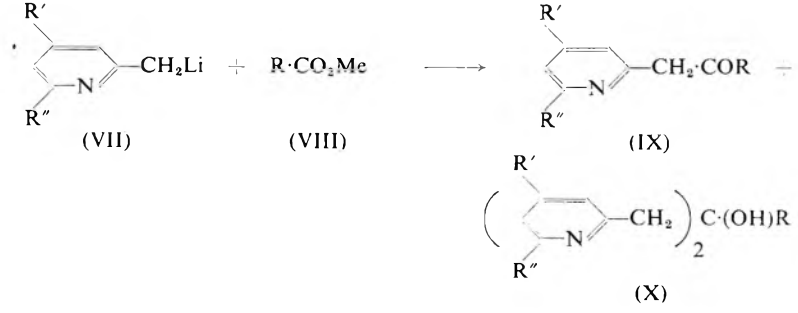
V



VI

by the reaction of the sodio-derivative of 2-phenacylpyridine with the appropriate alkyl halide. Methyl, ethyl, propyl, allyl and benzyl halides gave the *C*-derivatives exclusively (Compounds 2 to 6), but 2-dimethylaminoethyl chloride gave the *O*-derivative (VI; R = $CH_2 \cdot CH_2 \cdot NMe_2$) (Compound 11). The *C*-aroyl compounds (V; R′ = R″ = H, R = Ph, R‴ = CO Aryl) (Compounds 8 to 10) were prepared by the reaction of the appropriate anhydride or acid chloride with the sodio-derivative of 2-phenacylpyridine. Acetic anhydride, on the other hand, gave the *O*-acyl compound (VI; R = Ac) (Compound 12). The details of the methods of preparation and the proof of the structures of the above compounds have been given elsewhere[12].

Those substances possessing substituents in the phenyl ring of 2-phenacylpyridine (Compounds 17 and 19 to 25), or those derived by the replacement of the phenyl by other groups (Compounds 13 to 16), were prepared by the condensation of 2-pyridylmethyl-lithium with the methyl ester of the appropriate acid using a 2 : 1 molar ratio of lithium derivative to ester. As outlined in the following scheme, there is a possibility of the reaction proceeding to give the tertiary alcohols (X) as well as the desired



(VII) (VIII) (IX) (X)

ketones (IX). When the above-mentioned molar ratios were used, however, acylation of 2-pyridylmethyl-lithium with the methyl esters of furoic, naphthoic, benzoic and halogen, methoxy, and methyl substituted benzoic acids yielded the desired ketones in 60 to 80 per cent. yields and tertiary alcohols could not be isolated from the products of the reaction. Likewise, the ketone was the major product when methyl *p*-aminobenzoate was employed as the acylating agent and a 3 molar proportion of 2-pyridylmethyl lithium was used (cf. Scholfield[13] and Schofield and Nunn[14], who used those molar proportions for the condensation of *o*-aminobenzophenone with the lithium derivatives of pyridyl bases). From the reactions in which methyl esters of aliphatic acids were employed, both tertiary alcohols (X; R′ = R″ = H, R = Me or Et) and the desired ketones (IX; R′ = R″ = H, R = Me or Et) could be isolated, e.g., the tertiary alcohol and the ketone were isolated in yields of 30 per cent. and 25 per cent. respectively from the reaction product of methyl acetate and 2-pyridylmethyl-lithium (cf. Levine *et al.*[9]).

The presence of additional methyl-substituents in 2-methylpyridine allows of the possibility of the reaction with lithium yielding a mixture of lithium derivatives which may result in a complex mixture of ketones and tertiary alcohols being formed upon acylation. However, there is evidence that the 4-methyl group is less susceptible to attack by lithium than the 2-methyl group because Erlenmeyer and colleagues[15] were unsuccessful in their attempts to prepare 4-pyridylmethyl-lithium. From the product of the reaction of 2:4-dimethylpyridine (2:4-lutidine) with an equimolecular proportion of phenyllithium followed by a semi-molecular proportion of methyl benzoate, we were able to isolate 4-methyl-2-phenacylpyridine (IX; R″ = H, R′ = Me, R = Ph) in a 65 per cent. yield. Similarly, the use of methyl *m*-chlorobenzoate as the acylating agent resulted in 2-*m*-chlorophenacyl-4-methylpyridine in a 73 per cent. yield. (The fact that the 2-methyl position has been acylated is demonstrated by the green colours given by ethanolic ferric chloride solution with the ketones derived from 2:4-dimethylpyridine, and by the metal co-ordination properties of these compounds.) It is evident that the reaction of equimolecular proportions of phenyllithium and 2:4-dimethylpyridine gives 4-methyl-2-pyridylmethyl-lithium almost exclusively. Under comparable conditions, 2:6-dimethylpyridine gave 6-methyl-2-phenacylpyridine (82 per cent. yield) and 2-*m*-chlorophenacyl-6-methylpyridine (80 per cent. yield) respectively. Carbinols could not be isolated from any of the above reactions involving dimethylpyridines.

The pertinent analytical data and certain properties of those ketones not already reported in an earlier communication, are recorded in Table I and the attached footnotes. Details of the two carbinols (X; R = Me or Et; R′ = R″ = H) isolated are also given in the footnote to this table.

Experimental

Chemical

Microanalyses were made by Mr. G. S. Crouch, School of Pharmacy, University of London. Equivalent weights, except those of picrates, were

TABLE I

KETONES PREPARED FROM 2-METHYLPYRIDINE, 2:4- AND 2:6-DIMETHYLPYRIDINE

	Compound	M.pt. °C.	Physical form	Found			Formula	Required			Yield per cent.	Equivalent	
				C	H	N		C	H	N		Reqd.	Found
13	1:2'-Pyridylpropan-2-one (*a*)	—	yellow oil	—	—	—	C_8H_9NO	—	—	—	25	135	137
14	1:2'-Pyridylbutan-2-one (*b*)	—	,,	—	—	—	$C_9H_{11}NO$	—	—	—	35	149	147
15	α-(2'-Furoyl)-2-methylpyridine (*c*)	50–51	yellow prisms (*i*)	—	—	—	$C_{11}H_9NO_2$	—	—	—	48	187	190
16	α-(1'-Naphthoyl)-2-methylpyridine (*d*)	48	,, ,, (*i*)	82·8	5·4	5·8	$C_{17}H_{13}NO$	82·55	5·3	5·7	68	247	246
17	2-*p*-Aminophenacylpyridine	119·5	,, ,, (*j*)	73·2	5·8	13·2	$C_{13}H_{12}N_2O$	73·6	5·7	13·2	50	106	107
18	2-*p*-Hydroxyphenacylpyridine	148	,, ,, (*j*)	74·0	5·1	6·5	$C_{13}H_{11}NO_2$	73·2	5·2	6·6	—	211	213
19	2-*p*-Methoxyphenacylpyridine	84·5	,, ,, (*j*)	74·2	5·7	6·3	$C_{14}H_{13}NO_2$	74·0	5·7	6·2	75	227	227
20	2-*p*-Methylphenacylpyridine	67	,, ,, (*k*)	79·5	6·15	6·6	$C_{14}H_{13}NO$	79·4	6·0	6·6	80	211	211
21	2-*p*-Fluorophenacylpyridine	92·5	,, ,, (*j*)	72·6	4·8	6·4	$C_{13}H_{10}NOF$	72·55	4·65	6·5	76	215	211
22	2-*o*-Chlorophenacylpyridine (*e*)	—	yellow oil	67·9	4·4	—	$C_{13}H_{10}NOCl$	67·4	4·35	—	74	232	230
23	2-*p*-Chlorophenacylpyridine (*f*)	89	yellow prisms (*j*)	—	—	—	$C_{13}H_{10}NOCl$	—	—	—	80	232	235
24	2-*p*-Bromophenacylpyridine	99	,, ,, (*k*)	56·5	3·7	4·9	$C_{13}H_{10}NOBr$	56·5	3·65	5·1	72	276	274
25	2-*m*-Chlorophenacylpyridine	58	,, ,, (*k*)	67·4	4·45	6·3	$C_{13}H_{10}NOCl$	67·4	4·35	6·05	70	232	229
26	6-Methyl-2-phenacylpyridine (*g*)	78·5	,, ,, (*k*)	—	—	—	$C_{14}H_{13}NO$	—	—	—	82	211	212
27	2-(*m*-Chlorophenacyl)-6-methylpyridine	53	,, ,, (*k*)	68·7	5·0	5·85	$C_{14}H_{12}NOCl$	68·4	4·9	5·7	85	246	244
28	4-Methyl-2-phenacylpyridine (*h*)	53	,, ,, (*k*)	79·8	6·3	6·8	$C_{14}H_{13}NO$	79·6	6·2	6·6	65	211	213
29	2-(*m*-Chlorophenacyl)-4-methylpyridine	84	,, ,, (*j*)	68·4	4·7	5·7	$C_{14}H_{12}NOCl$	68·4	4·9	5·7	78	246	243

(*a*) B.pt. 50–60° C./0·5–1 mm., picrate, m.pt. 141–142° C. In addition 2-methyl-1:3-di-(2'-pyridyl)propan-2-ol (X; R = Me; R′ = R″ = H), b.pt. 130–140° C./0·5–1 mm., dipicrate, m.pt. 214° C. (decomp.), was also isolated in 30 per cent. yield cf. ref. 9.

(*b*) B.pt. 80–90° C./2 mm., picrate m.pt. 146–147° C. In addition 2-propyl-1:3-di-(2'-pyridyl)propan-2-ol (X; R = Et; R′ = R = H), b.pt. 160–170° C./0·5–1·5 mm., dipicrate, m.pt. 196–198° C. (decomp.), was also isolated in 20 per cent. yield, cf. ref. 9.

(*c*) Picrate, m.pt. 149–150° C.; see ref. 16.

(*d*) Picrate, m.pt. 201–202° C. (decomp.); found: N, 11·6 per cent.; equiv., 476. $C_{23}H_{16}N_4O_8$ requires; N, 11·8 per cent.; equiv., 476.

(*e*) n_D^{20} 1·5990. Picrate, m.pt. 173° C.; found: N, 12·4 per cent.; equiv., 462. $C_{13}H_{13}N_4O_8Cl$ requires; N, 12·15 per cent.; equiv., 461.

(*f*) See ref. 17.

(*g*) See ref. 18.

(*h*) Picrate, m.pt. 153° C.; found: N, 12·95 per cent.; equiv., 441. $C_{20}H_{16}N_4O_8$ requires; N, 12·7 per cent.; equiv., 440.

(*i*) Solvent, ether.

(*j*) Solvent, 95 per cent. ethanol.

(*k*) Solvent, light petroleum. B.pt. 40–60.

determined by titration with 0·2N-perchloric acid in acetic acid; those of the picrates were determined by titration with 0·02N-sodium hydroxide in 1:1 ethanol-acetone with ethyl bis-2:4-dinitrophenylacetate as indicator.

Preparation of the compounds. The preparation of compounds 2 to 12 has been described in an earlier communication (Beckett and Kerridge[12]).

The general procedure adopted for the preparation of compounds 13 to 17 and 19 to 29 is described below.

Preparation of the lithium derivatives of the methylpyridine bases. The appropriate pyridine base (0·05 mole), freshly distilled over barium oxide, was added with vigorous stirring to a solution of phenyllithium (0·05 mole; from 0·69 g. of lithium and 7·9 g. of bromobenzene) in ether (60 ml.). Addition was carried out under nitrogen and at such a rate that the ether did not reflux. The dark red-brown solution of the lithium derivative was stirred at room temperature for a further period of 15 minutes.

General procedure for the preparation of compounds nos. 13 *to* 16, 19 *to* 29. Since all the compounds were prepared under essentially the same conditions, only the procedure for 2-phenacylpyridine is described.

Methyl benzoate (3·66 g., 0·025 mole), in ether (15 ml.), was added over a period of 10 minutes to a rapidly stirred solution of 2-pyridylmethyl-lithium (0·05 mole) in a nitrogen atmosphere. After the addition of the ester, the mixture was poured onto a slurry of ammonium chloride (4 g.) and crushed ice (30 g.). The ethereal layer was separated and the aqueous phase extracted with ether (3 × 20 ml.) or until the extracts no longer gave a colour with alcoholic ferric chloride solution. The total organic extract was dried ($MgSO_4$), the ether distilled under reduced pressure, and the residue fractionally distilled in vacuum. In this way 2-phenacylpyridine (4·0 g., 75 per cent.), b.pt. 140 to 150° C./2 mm., yellow needles (from ethanol), m.pt. 56° C., was obtained.

Isolation of 2-pyridylmethyl ketones. Picrates, of those compounds which were oils or did not crystallise, were formed by mixing equivalent quantities of picric acid and pyridyl base in ethanol. The pyridyl base was regenerated by dissolving the picrate in a mixture of equal parts of water, ethanol, and acetone and warming with an equivalent quantity of lithium hydroxide. The solution was diluted with water, the free pyridyl base extracted with ether, and the residue obtained by evaporating the ethereal extract chromatographed on alumina. Compounds which had failed to crystallise on standing or after chromatography, usually crystallised on being purified via the picrate.

2-*p*-*Aminophenacylpyridine* (*Cpd. no.* 17). Methyl *p*-aminobenzoate (3·8 g.; 0·025 mole), in ether (15 ml.) was added, under an atmosphere of nitrogen, to a solution of 2-pyridylmethyl-lithium (0·075 mole; from 1·03 g. of lithium in 90 ml. of ether). The mixture was stirred at room temperature for 4 hours and then decomposed and worked up as described under "General procedure."

2-*p*-*Hydroxyphenacylpyridine* (*Cpd. no.* 18). 2-*p*-Methoxyphenacylpyridine (2 g.) was heated with hydriodic acid (15 ml.), sp.gr. 1·7, and red phosphorus (0·2 g.) for 1 hr. at 130 to 140° C. The cooled solution was neutralised with ammonia, and the precipitated base extracted with ether (3 × 15 ml.). After distilling off the ether, under reduced pressure, the residue was recrystallised from 70 per cent. aqueous ethanol to give 2-*p*-hydroxyphenacylpyridine (1·3 g., 70 per cent., m.pt. 148° C.)

The pertinent analytical details for the above compounds and others are given in Table I.

Metal sensitivity tests. The sensitivities of the compounds towards biologically important metal ions were determined by the method of Butler, Irving and Ring[19] as modified by Hollingshead[20]. Sensitivities are expressed in Table III in terms of pL, which is defined as $-\log_{10}$ limiting concentration of metal in gram equivalents per litre which gives a colour or precipitate under conditions of the test.

The molar ratio of glycine necessary to prevent precipitation at *p*H 7·3 of selected compounds was determined using the same concentration of the reagent (0·0032M) as in the above tests and a 0·0016M concentration of cupric ions. The results are summarised in Table IV.

Bacteriology

Determinations of minimum inhibitory concentration. Nine species of bacteria (National Collection of Type Cultures) were revived on recovery media (Table II), and then transferred to maintenance media. After incubating for 24 hours on recovery media, a washed suspension in glass-distilled water was prepared containing 10,000 organisms per ml.

TABLE II

Strain of bacteria	Culture media		
	Recovery	Maintenance	Test
Staph. aureus 6571 ..	Nutrient broth (Oxoid C.M.1)	Nutrient agar (Oxoid C.M. 55)	Peptone water (Oxoid C.M.9 + 0·5 per cent. dextrose)
B. subtilis 3610	,,	,,	,,
Corynebact. hofmannii 231	,,	,,	,,
Bact. coli 86	,,	,,	,,
Proteus vulgaris 4175 ..	,,	,,	,,
Sh. sonnei 8220	,,	,,	,,
Salm. typhimurium 5170 ..	,,	Dorset egg (Oxoic P.M.5)	,,
Myco. phlei 8151	Glycerol broth (Oxoid C.M.1 + 10 per cent. glycerol)	,,	,,
Str. pyogenes 8198.. ..	Serum broth (Oxoid C.M.1 + 10 per cent. normal horse serum [B.W. and Co.])	Blood agar (Oxoid C.M.55 + 10 per cent. rat blood Heparinised])	Serum broth

The compounds to be tested were prepared in M/40 concentration in 70 per cent. ethanol and then serially diluted with glass distilled water so that each dilution was twice as great as the preceding one in the series. To 1·8 ml. of the test media (see Table II) was added 0·1 ml. of these dilutions and 0·1 ml. of bacterial suspension, determinations being carried out in quadruplicate. The dilutions of the substances under test covered the range M/1600 to M/800,000. Incubation was at 37° C. for 48 hours except for tests involving *Mycobacterium phlei* in which the period was extended to 5 days. The minimum inhibitory concentration was taken as the greatest dilution showing no growth at the end of the specified period. Oxine was used as a control substance in all the tests. Concentrations of M/800 and M/400 were employed for some of the compounds tested.

The results are summarised in Tables V and VI.

Tests showing the role of metal ions. For each compound being studied, 5 test systems were prepared in sterile glass distilled water. One

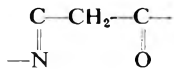
contained only the compound in minimum inhibitory concentration. A second and third contained the compound in minimum inhibitory concentration with equivalent amounts of ferrous sulphate and cupric sulphate respectively. The remaining 2 tubes contained the equivalent amount of these two metal salts alone. All test systems were inoculated and the final volume (2 ml.) contained 2000 washed organisms. They were incubated at 37° C. and subcultures of 0·1 ml. made at fixed times after inoculation. The subculture tubes were incubated for 24 hours and the presence or absence of visible growth noted.

The organisms used were *Proteus vulgaris* and *Bacillus subtilis*. The results are summarised in Tables VII and VIII.

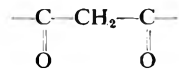
Results and discussion

Metal Co-ordination Properties

It has been shown[21] that substitution by alkyl substituents of the methylene group of aceto-acetic ester results in a reduction in the percentage of the enol form; the effectiveness of the substituents in accomplishing this effect is ethyl>methyl>*n*-propyl. Likewise, the order of the groups in reducing the percentage of the enol form in acetyl acetone is ethyl> methyl>benzyl[21]. Since 2-methylpyridine is regarded as an ammono ketone ether[22], the tautomeric system of 2-phenacylpyridine (XI) may be



(XI)

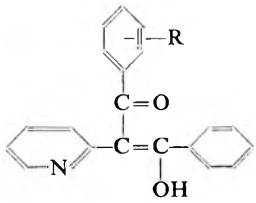


(XII)

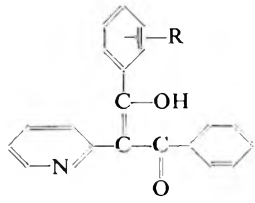
considered as analogous to a β-diketone (XII). Substitution of the active methylene group in 2-phenacylpyridine with methyl, ethyl, *n*-propyl, allyl, and benzyl groups led to a complete absence of the enol form. This was demonstrated by failure of the compounds to give colours with ethanolic ferric chloride solution (contrast with the parent compound) and the presence of only one absorption peak at 247 mμ in the ultra-violet absorption spectra of ethanolic solutions of the compounds (see Beckett and Kerridge[12]), the absorption peak at 337 mμ characteristic of the enol form of 2-phenacylpyridine being absent. Furthermore, unlike the ultra-violet absorption spectra of the parent molecule, the spectra of these alkyl substituted derivatives were unaffected by the presence of cupric ions.

Since the substitution of the active methylene group of a β-diketone system by an electron attracting mesomeric group has been shown to increase the percentage of the enolic component[23], aroyl substitution of 2-phenacylpyridine was expected to have a similar effect. In fact the aroyl derivatives (Bz, $-CO\cdot C_6H_4Cl$-*p*, $-CO\cdot C_6H_4\cdot NO_2$-*p*, $-CO\cdot C_6H_3\cdot(NO_2)_2$–1:3:5) of 2-phenacylpyridine all gave colours with ethanolic ferric chloride solution indicating that the keto-enol tautomerism had not been supressed. All these compounds in ethanolic solution exhibited an ultra-violet absorption peak at 370 mμ (ε values 9000 to 14,000)[12] which indicates the existence of these compounds in the enol form (XIII), rather

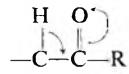
than in the alternative form (XIV) which would be expected to absorb at varying wavelengths dependent upon the group R. These compounds, unlike the *C*-alkyl derivatives, precipitated metal ions from aqueous solutions (see Table III).



(XIII)



(XIV)



(XV)

It is known that prototropy is facilitated in the system (XV), as shown, if group R is electron attracting[24]. Consequently, the substitution of the phenyl ring of 2-phenacylpyridine by electron attracting groups would be expected to increase the enol/keto ratio and in consequence the apparent acidity of the system. The results of the metal sensitivity tests (Table III) indicate that, of the compounds tested, the *m*-chloro-derivative shows the greatest sensitivity towards metal ions, whereas those compounds in which enolisation is repressed, by the substitution of an electron donating group such as amino or hydroxyl in the phenyl ring, fails to precipitate metal ions under the conditions of the test. Although the sensitivity towards metal ions is dependent upon both the stability and the solubility of the chelate, the observed differences in sensitivity are not due solely to difference in the solubility (e.g., the replacement of a hydrogen atom of the ring by methyl or chlorine would be expected to diminish the solubility of the reagent similarly[11], whereas these derivatives show different sensitivities to metal ions). Moreover, differences in sensitivity were observed in the isomeric mono-chlorophenacylpyridines.

The substitution of a methyl group in the 6-position of 2-phenacylpyridine (Cpd. 26) completely repressed metal precipitation under the conditions of the test. This result is probably due to the steric interference of the group with the co-ordination by the adjacent nitrogen atom, and is comparable with the observed reduction of chelating properties upon the introduction of a methyl group into the 2-position of 8-hydroxyquinoline[25]. On the other hand, 4-methyl-2-phenacylpyridine (Cpd. 28) is more sensitive to metal ions than 2-phenacylpyridine, probably due to the increased electron density at the nitrogen atom in the former compound.

The replacement of the phenyl group of 2-phenacylpyridine by alkyl groups (Cpds. 13 and 14) caused a complete loss of metal-precipitating properties and lowered sensitivity towards metal ions resulted from the replacement of the phenyl by the α-furyl group (Cpd. 15).

It is known that amino-acids and proteins can bind metal ions, e.g., glycine solubilises zinc serum albumin and zinc insulin complexes by successfully competing for the complexed metal ions[26]. Consequently, before a molecule can be considered as a potential complexing agent under

TABLE III

METAL SENSITIVITY TESTS

Metal	Cu^{++} (as $CuSO_4 \cdot 5H_2O$)			Fe^{++} (as ferrous alum)			Fe^{+++} (as ferric alum)			Co^{++} (as $Co(NO_3)_2 6H_2O$)		
*p*H	8·4	7·3	5·3	8·4	7·3	5·3	8·4	7·3	5·3	8·4	7·3	5·3
Cpd. 1†	3·8	3·5	N.P.	2·2*	2·2*	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.	N.P.
" 7†	3·8	3·5	"	2·2*	2·2*	"	"	"	"	"	"	"
" 8†	4·3	3·8	"	2·3	2·2*	"	"	"	"	"	"	"
" 9†	3·8	3·5	"	2·2*	N.P.*	"	"	"	"	"	"	"
" 10†	4·0	3·8	"	N.P.	"	"	"	"	"	"	"	"
" 1	4·5	4·2	"	3·2*	3·2*	"	"	"	"	"	"	"
" 13	N.P.	N.P.	"	N.P.	N.P.	"	"	"	"	"	"	"
" 14	N.P.	N.P.	"	"	"	"	"	"	"	"	"	"
" 15	3·5	3·5	"	"	"	"	"	"	"	"	"	"
" 16	4·5	4·3	3·5	3·8	4·2	"	"	3·8	"	"	"	"
" 17	N.P.	N.P.	N.P.	N.P.	N.P.	"	"	N.P.	"	"	"	"
" 18	"	"	"	"	"	"	"	2·5*	"	"	"	"
" 19	4·2	4·2	3·8	"	3·2	"	"	3·2	"	"	"	"
" 20	4·8	4·5	N.P.	3·5	4·2	2·2	"	N.P.	"	3·5	"	"
" 21	4·5	4·2	3·8	N.P.	4·3	N.P.	"	3·8	"	N.P.	"	"
" 22	3·8	3·8	N.P.	N.P.	N.P.	"	"	N.P.	"	"	"	"
" 23	4·3	4·3	3·8	2·5	4·5	"	"	4·5	"	"	"	"
" 24	4·8	4·2	3·8	2·2	4·2	"	"	3·5	"	"	"	"
" 25	4·8	4·5	4·2	2·8	4·3	"	"	3·8	"	4·2	3·2	"
" 26	N.P.	N.P.	N.P.	N.P.	N.P.	"	"	N.P.	"	N.P.	N.P.	"
" 27	"	"	"	"	"	"	"	"	"	"	"	"
" 28	4·2	4·2	3·5	"	2·2	"	"	2·2	"	"	"	"
" 29	4·8	4·5	3·8	3·2	4·2	"	2·5	4·2	2·5	3·8	2·5	"

N.P. = no precipitate. * Colour only. † Reagent concentration 0·0008 molar.

biological conditions, it must be shown that it can compete successfully with amino-acids or proteins for metal ions. The results obtained in the metal sensitivity test are dependent upon the solubility as well as upon the stability of the complexes and although both these factors are important in biological action, a measure of the ability of the reagent to bind metals under biological conditions is not obtained. A simple test of this latter effect has been devised for the present investigation because this preliminary exploration of a series of compounds did not warrant the more detailed study of stability constants of the complexes. The test involved the determination of the sensitivities of the compounds towards cupric ions under standard conditions in the presence of varying amounts of glycine; the molar ratio of glycine necessary to prevent precipitation was observed. It seems reasonable to assume that if a molecule can precipitate copper in the presence of a large molar excess of glycine, then it is likely to form a complex with these ions under biological conditions. The results of the tests upon certain of the compounds of the series are shown in Table IV.

TABLE IV

MOLAR RATIOS OF GLYCINE NECESSARY TO PREVENT PRECIPITATION OF COPPER IONS BY DERIVATIVES OF 2-PHENACYLPYRIDINE

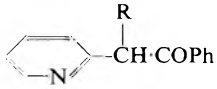
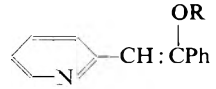
Compound No.:	19	1	23	24	25	28	29
Molar ratio of glycine	4	5	55	45	50	7·5	90

Antibacterial Properties

The bacteriological results (Tables V and VI) indicate that although many of the compounds are capable of co-ordinating with metals such

TABLE V

ANTIBACTERIAL ACTIVITIES OF 2-PHENACYLPYRIDINE AND ITS DERIVATIVES

(I)  (II) 

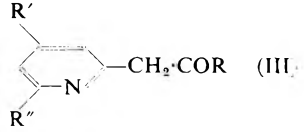
Compound No.	Derivative (I)	M.I.C. in reciprocal molar concentration against*							
		Staph. aureus.	*Str. pyogenes*	*B. subtilis*	*Corynebact. hofmannii*	*Bact. coli*	*Proteus vulgaris*	*Sh. sonnei*	*Myco. phei.*
1	R = H	—	1600	—	—	—	1600	—	—
2	R = Me	400	1600	—	—	—	3200	—	800
3	R = Et	1600	800	—	—	—	1600	—	1600
4	R = Pr^n	6400	1600	1600	1600	—	1600	—	3200
5	R = CH_2·CH : CH_2	3200	1600	1600	1600	—	1600	—	1600
6	R = CH_2Ph	800	—	—	—	—	1600	—	—
7	R = Bz	800	1600	1600	1600	—	3200	—	1600
8	R = CO·C_6H_4·NO_2-*p*	1600	3200	—	—	1600	1600	—	1600
9	R = CO·C_6H_4Cl-*p*	1600	1600	—	800	—	800	—	1600
10	R = CO·$C_6H_3(NO_2)_2$ −1 : 3 : 5 (II)	1600	3200	1600	3200	1600	1600	1600	3200
11	R = CH_2·CH_2·NMe_2	800	—	—	—	—	1600	—	—
12	R = Ac	800	1600	—	—	800	1600	—	—
	Oxine	100,000	100,000	50,000	100,000	1600	800	1600	25,000

— Signifies growth at M/1600.
* All these compounds listed in Table V are inactive at M/1600 concentration against *Salm. typhi.*

as iron or copper (Table III), and many of them exhibit antibacterial effects, the two actions do not appear to be directly related. For example, compounds 2 to 6 which exist in the ketonic form and do not co-ordinate

TABLE VI

ANTIBACTERIAL ACTIVITIES OF KETONES PREPARED FROM 2-METHYLPYRIDINE, 2 : 4- AND 2 : 6-DIMETHYLPYRIDINE

 (III)

Cpd. No.	Derivative (III)	M.I.C. in molar concentration against*			
		Staph. aureus	*Str. pyogenes*	*B. subtilis*	*Proteus vulgaris*
13	R′ = R″ = H; R = Me	—	—		—
14	R′ = R″ = H; R = Et	—	—		—
15	R′ = R″ = H; R = 2-furyl	—	—		800
16	R′ = R″ = H; R = 1-naphthyl	800	3200		3200
17	R′ = R″ = H; R = C_6H_4·NH_2 -*p*	—	—	—	1600
18	R′ = R″ = H; R = C_6H_4·OH -*p*	—	—	—	1600
19	R′ = R″ = H; R = C_6H_4·OMe -*p*	1600	1600	—	1600
20	R′ = R″ = H; R = C_6H_4Me -*p*	—	3200	—	3200
21	R′ = R″ = H; R = C_6H_4F -*p*	—	—	6400	3200
22	R′ = R″ = H; R = C_6H_4Cl -*o*	—	—	3200	3200
23	R′ = R″ = H; R = C_6H_4Cl -*p*	—	1600	3200	1600
24	R′ = R″ = H; R = C_6H_4Br -*p*	—	3200	—	6400
25	R′ = R″ = H; R = C_6H_4Cl -*m*	—	—	—	6400
26	R′ = H; R″ = Me; R = Ph	—	—	1600	3200
27	R′ = H; R″ = Me; R = C_6H_4Cl -*m*	—	—	25,000	3200
28	R′ = Me; R″ = H; R = Ph	—	—	25,000	3200
29	R′ = Me; R″ = H; R = C_6H_4Cl -*m*		—	50,000	3200
	Oxine	100,000	100,000	50,000	3200

— Signifies growth at M/1600.
* Compounds 17 to 29 are inactive at M/1600 concentration against *Corynebact. hofmannii, Bact. coli, Salm. typhi, Sh. sonnei,* and *Myco. phei.*

with metals, exhibit antibacterial activities of a higher order and against more organisms than do the metal co-ordinating compounds 1 and 19 to 25. Furthermore, even the enol ether (Cpd. 11) and enol ester (Cpd. 12) are antibacterials.

Although the correlation (see Compounds 13 to 29) between the antibacterial effect recorded as minimum inhibitory concentration and the chelating power of the molecules as determined by the precipitation tests is slight, it was decided to investigate a few of the metal chelating compounds to see whether metal ions played any part in their possible bactericidal action. Two similar non-chelating compounds were used as controls. Oxine was used as reference compound because it is known that it fails to exert its bactericidal action upon certain organisms in distilled water if certain concentrations of ferrous or cupric ions are absent.

The role of metal ions in the antibacterial action. The results reported in Table VII show the effect of ferrous and cupric ions on the bactericidal action of compounds 2, 24, 25, and 26 and of oxine on *Proteus vulgaris* in glass-distilled water. Compounds 24 and 25 coordinate with metals both in the absence and in the presence of 45 and 50 molar proportions of glycine, respectively, but compounds 2 and 26 fail to precipitate metal ions under the conditions of the test used. All four of these compounds, in contrast to oxine, are bactericidal to *Proteus vulgaris* in the absence of metals, and both ferrous and cupric ions potentiate this activity, although the effect is slight. However, since these metal ions have a similar effect on compounds 2 and 26 as on compounds 24 and 25, this observed potentiation of activity is probably independent of chelation phenomena; it could be attributed to the additive effect of two independent bactericidal actions because the concentrations of metal ions used are themselves bactericidal.

Compounds 28 and 29 are highly active against *B. subtilis* (Table VI), and are bactericidal in the presence of cupric but not ferrous ions (Table VIII). In contrast to these results, oxine is bactericidal in the presence of both cupric and ferrous ions (Table VIII). This difference in effect of cupric and ferrous ions on the bactericidal activity of compounds 28 and 29 may be due to the lower order of metal chelation of the latter ions (Table III).

Although complete success has not attended this attempted design of potential antibacterial agents in which biological activities can be correlated with metal chelating properties, this mechanistic chemotherapeutic approach has, at least, led to compounds exhibiting antibacterial properties. Two interesting facts which may have practical applications also arise from the investigation.

(1) Nearly all the compounds of the series possess activity, albeit of a low order, against *Proteus vulgaris*. This fact, coupled with the difficulty of erradicating *Proteus vulgaris* in certain conditions of infection and the low toxicity of the compounds, e.g., compounds 28 and 29 can be tolerated in mice at doses as high as 620 mg./kg. by subcutaneous injection, makes certain compounds worthy of a more detailed investigation.

TABLE VII

THE EFFECT OF FERROUS AND CUPRIC IONS ON THE ACTIVITY OF SELECTED COMPOUNDS AGAINST *Proteus vulgaris* IN GLASS DISTILLED WATER

Compound No.	Concn.	Metal	Concn.	Time of subculture in hours 0	1	2	3	4	5	6	8
		Fe	M/3200	+	+	+	−	+	−	−	−
		Cu	M/3200	+	+	−	−	−	+	−	−
2	M/3200	—	—	+	+	−	−	−	+	−	−
2	M/3200	Fe	M/3200	+	+	−	+	−	−	−	−
2	M/3200	Cu	M/3200	+	+	−	−	−	−	−	−
26	M/3200	—	—	+	+	−	−	−	+	−	−
26	M/3200	Fe	M/3200	+	−	−	−	−	−	−	−
26	M/3200	Cu	M/3200	+	−	−	−	−	−	−	−
		Fe	M/6400	+	+	−	−	+	+	+	−
		Cu	M/6400	+	+	+	−	+	−	−	−
24	M/6400	—	—	+	+	−	−	−	−	−	−
24	M/6400	Fe	M/6400	+	+	−	−	+	−	−	−
24	M/6400	Cu	M/6400	+	+	+	−	−	−	−	−
25	M/6400	—	—	+	+	+	−	+	+	−	−
25	M/6400	Fe	M/6400	+	+	−	−	−	−	−	−
25	M/6400	Cu	M/6400	+	+	−	−	−	−	−	−
Oxine	M/6400	—	—	+	+	+	−	+	+	+	+
Oxine	M/6400	Fe	M/6400	+	+	−	−	+	−	−	−
Oxine	M/6400	Cu	M/6400	+	+	+	−	+	−	−	−
—	—	—	—	+	+	+	−	+	+	+	+

(2) Compounds 27, 28 and 29 are almost specific in their action against *B. subtilis*, e.g., compound 29 is inactive against 7 of the organisms used in the present work at concentrations of M/1600 but is active in concentrations as low as M/50,000 against *B. subtilis*.

TABLE VIII

THE EFFECT OF FERROUS AND CUPRIC IONS ON THE ACTIVITY OF SELECTED COMPOUNDS AGAINST *B. subtilis* IN GLASS DISTILLED WATER

Compound No.	Concn. 1/M	Metal	Concn. 1/M	Time of subculture in hours 0	1	2	3	4	5	6	8
		Fe	25,600	+	+	+	−	+	+	+	+
		Cu	25,600	+	+	−	−	−	+	+	+
28	25,600	—	—	+	+	+	−	+	+	+	+
28	25,600	Fe	25,600	+	+	−	−	+	+	+	+
28	25,600	Cu	25,600	+	+	−	−	−	−	−	−
		Fe	51,200	+	+	+	−	+	+	+	+
		Cu	51,200	+	+	+	−	+	+	+	+
29	51,200	—	—	+	+	−	−	+	+	+	+
29	51,200	Fe	51,200	+	+	+	−	+	+	+	+
29	51,200	Cu	51,200	+	+	−	−	−	−	−	−
Oxine	51,200	—	—	+	+	+	+	+	+	+	+
Oxine	51,200	Fe	51,200	+	−	−	−	−	−	−	−
Oxine	51,200	Cu	51,200	+	−	−	−	−	−	−	−
—	—	—	—	+	+	+	−	+	+	+	+

SUMMARY

1. Substituted acyl and aroyl derivatives of 2-methylpyridine and 2:4- and 2:6-dimethylpyridine have been prepared as potential antibacterial and chelating agents, and their sensitivities towards metal ions determined. The effect of glycine upon the precipitation of cupric ions by certain of the compounds is recorded.

2. The bacteriostatic values of these compounds against Gram-positive and Gram-negative organisms are reported. The effect of metal ions on the bactericidal action of selected compounds has been investigated and compared with their effect upon the action of oxine.

3. Some of the substances possess a specific action against *B. subtilis*.

One of us (K. A. K.) thanks the Pharmaceutical Society for the award of a scholarship.

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DISCUSSION

The paper was presented by DR. A. H. BECKETT.

DR. F. HARTLEY (London) said that although in the particular series chosen it had not been possible to draw the rather interesting conclusions which Albert was able to obtain, nevertheless it was only from the examination of potentially useful series that fundamental correlation between action and structure could be determined. Another potentially useful aspect of the paper was whether it could add anything useful to the detection and evaluation of metals by the reagents which had been studied. From Table III it appeared that there might be one or two interesting possibilities. By analogy with 8-hydroxyquinoline it might well be the case that if other metallic ions were studied some specific colour or precipitation would be obtained.

DR. G. E. FOSTER (Dartford) asked why iron and copper had been chosen for the chelating test. Bacteria were sensitive to other metals. Had the authors any data with regard to the chelating properties of their compounds with calcium and magnesium?

MR. H. D. C. RAPSON (Dorking) said that at least three mechanisms for the bactericidal action of chelating agents are possible, firstly a lipoid soluble form of the compound diffuses into the bacterium and forms a metal complex inside; secondly, a lipoid soluble complex with a metallic ion diffuses into the bacterium and then dissociates with the liberation of metallic ions or unsaturated complexes; and, thirdly, more complicated processes. Hence it is important to investigate more directly and precisely the chelating ability of the compounds.

DR. G. BROWNLEE (London) referred to the statement in the paper "Oxine is non-toxic to *Staph. aureus* unless traces of iron (or copper) are present in the medium" and said that that was not what Albert *et al.* had shown. They showed that a substantial part of the activity of oxine could be reversed under very special conditions of choice of medium and testing. At least two other authors have shown that this simplification of the possible mechanism of action would have to be critically investigated before it could be accepted that chelation played the only part in the mechanism. That copper and iron—and, incidentally, magnesium—played a part seemed to be implicit in the discussion, but in his view one ought, in all humility, to say "Is it true?" Of course nothing of this detracted from the value of the investigation.

DR. A. H. BECKETT, in reply, agreed with Dr. Hartley that some of the compounds might have analytical uses and infra-red studies would be of interest. Iron and copper were chosen for the test since those metals are implicated in the activity of oxine against *Staph. aureus*. Since the work was only a preliminary study, a quickly performed test seemed desirable when the substances were not of a high order of antibacterial activity. On the points raised by Dr. Brownlee, he agreed that the conditions of Albert's tests were rather different from practical conditions, but nevertheless such work was essential to limit the variables. The paper was an attempt to provide further information on compounds which could compare with metals based on the premise that chelation could be involved in certain types of antibacterial activity.

THE SPECTROPHOTOMETRIC DETERMINATION OF VITAMIN D IN PHARMACEUTICAL PREPARATIONS

PART II. TABLETS OF CALCIFEROL B.P.

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INTRODUCTION

TABLETS of calciferol are the subject of monographs in the National Formulary 1949 and the British Pharmaceutical Codex 1949 under the title, Strong Tablets of Calciferol, and in the British Pharmacopœia 1953 as Tablets of Calciferol. The various monographs relate to substantially the same preparation, namely coated tablets which, unless otherwise stated, each contain 50,000 units of antirachitic activity. The preparation which appeared in the British Pharmaceutical Codex 1949 and 1954 under the title Tablets of Calcium with Vitamin D will not be discussed in this paper.

Very little has been published about the assay of tablets containing vitamin D. The British Pharmacopœia 1953 describes a procedure involving shaking the powdered tablets with light petroleum followed by evaporation of the solvent and subsequent biological assay. The extraction of vitamin A from tablets is a similar problem, and several methods are described in the literature[1–6], including continuous extraction in a Soxhlet apparatus. Another possibility is elution of the powder with a suitable solvent using the technique of adsorption or partition chromatography[7].

The vitamin D, extracted from the tablets and brought into solution by one or other of these methods, may then be assayed biologically[8], microbiologically[9], by direct ultra-violet spectrophotometry (using the absorption maximum at ca. 265 mμ), or colorimetrically[10]. Infra-red methods are probably unsuitable because of the large amounts of vitamin D that would be needed[11].

APPARATUS, MATERIAL AND REAGENTS

Spectrophotometry. The work in this paper was carried out in part on a Beckman Model DU spectrophotometer and in part on a Unicam SP 500 spectrophotometer. Matched 1 cm. glass stoppered cells with a tungsten lamp as light source were used for colorimetry in the visible region of the spectrum and matched 1 cm. fused silica cells with a hydrogen lamp for the ultra-violet measurements.

Extraction apparatus. For the elution method, a chromatography-type tube of internal dimensions 30 cm. × 1 cm. fitted at the bottom with a cotton-wool plug on which the powder to be extracted was supported. The rate of flow of the eluant was of the order of 3 ml. per minute.

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A small Soxhlet apparatus attached by ground-glass joints to a 150 ml. flat-bottomed flask and an efficient double-surface condenser. Heat was supplied by an electric hot-plate. The size of extraction thimble used was 2·2 cm. × 8·0 cm.

Antimony trichloride. Antimony trichloride, reagent grade. *Calcium phosphate.* Calcium phosphate, B.P. *Calciferol.* Crystalline calciferol, B.P. *Chocolate basis.* A mixture of non-alkalised cocoa powder 15 parts, sucrose B.P. 15 parts and lactose B.P. 70 parts. *Lactose.* Lactose, B.P. *Starch.* Maize starch, B.P. *Acetyl chloride.* Acetyl chloride, reagent grade. *Carbon tetrachloride.* Carbon tetrachloride dried over anhydrous calcium chloride, and distilled, the first and last 10 per cent. portions being rejected. *Chloroform.* Chloroform B.P., containing 1 to 2 per cent. of ethanol, shaken successively with three equal volumes of water, dried over anhydrous calcium chloride, and distilled, the first and last 10 per cent. portions being rejected. Used within 24 hours of preparation. cyclo*Hexane.* *cyclo*Hexane dried over anhydrous potassium carbonate, passed through a column of activated silica gel[12,13], and distilled, the first and last 10 per cent. portions being rejected. The transmission, compared with water in 1 cm. cells, exceeded 90 per cent. at all wavelengths down to 245 mμ, and exceeded 95 per cent. at 265 mμ. *Ethanol.* Dehydrated ethanol refluxed for two hours with 2 per cent. of potassium hydroxide pellets and 2 per cent. of zinc dust, and distilled, the first and last 10 per cent. portions being rejected. The transmission, compared with water in 1 cm. cells, exceeded 90 per cent. at all wavelengths down to 235 mμ, and exceeded 95 per cent. at 265 mμ. *Ethylene dichloride.* 1:2-dichloroethane dried over anhydrous calcium chloride, and fractionally distilled, the portion of boiling range 82° to 84° C. being collected. *Light petroleum.* "Aromatic-free" light petroleum fractionated, the portion of boiling range 40° to 50° C. being collected. This was preferred to the 50°/60° material used in the pharmacopœial assay, since it is more suitable as a spectroscopic solvent in the ultra-violet region. The transmission, compared with water in 1 cm. cells, exceeded 90 per cent. at all wavelengths down to 225 mμ, and exceeded 95 per cent. at 265 mμ.

Antimony trichloride reagent. For the B.P. identification test, a solution of 20 per cent. of antimony trichloride and 1 per cent. of acetyl chloride in chloroform. For the assay, a solution of 20 per cent. of antimony trichloride in ethylene dichloride with the addition of 2 per cent. of acetyl chloride, prepared according to the method of de Witt and Sullivan[11].

Methods of Extraction

Shaking. Weigh and powder 20 tablets. To an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol, add 50 ml. of solvent, shake for 5 minutes, adjust the volume to 100 ml. with solvent, shake for 2 minutes, and centrifuge or allow to stand until the insoluble matter has settled. Determine the calciferol in the supernatant liquid by the colorimetric method.

Elution. Weigh and powder 20 tablets. Elute an accurately weighed

quantity of the powder, equivalent to about 1·5 mg. of calciferol, with solvent, collecting 100 ml. of eluate. Mix by shaking, and determine the calciferol in the solution spectrophotometrically or colorimetrically.

Soxhlet extraction. Weigh and powder 20 tablets. Extract an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol with 90 ml. of solvent for 60 minutes. Cool the solution, and adjust the volume to 100 ml. with solvent. Mix by shaking, and determine the calciferol in the solution colorimetrically.

Methods of Determination

Spectrophotometric. Compare the extinction of the solution with that of pure solvent at the wavelength of maximum absorption, 265 mμ (or 267 mμ in the case of ethylene dichloride). The value of $E_{1\,cm.}^{1\%}$ (265 mμ) for calciferol in *cyclo*hexane, ethanol or light petroleum is 470; the value of $E_{1\,cm.}^{1\%}$ (267 mμ) for calciferol in ethylene dichloride is 420. Calculate the weight of calciferol in each tablet of average weight.

Colorimetric. To 0·4 ml. of solution add 2·2 ml. of antimony trichloride reagent, and compare the extinctions at 500 mμ at ½-minute intervals for 3 minutes with those of 0·4 ml. of solvent + 2·2 ml. of reagent in the blank cell. The colour reaches a maximum in about one minute, then slowly fades; record the maximum extinction. The value of $E_{1\,cm.}^{1\%}$ (500 mμ) for the colour given by pure calciferol in these conditions is about 2000; it varies slightly from batch to batch of reagent, and should be determined afresh for each batch which is prepared. Calculate the weight of calciferol in each tablet of average weight.

This is essentially the method described in a previous paper[10]. It has been found with a few batches that the reagent blank was rather high, and the use of a mixture of reagent and solvent in the blank cell instead of solvent only has been adopted to correct this source of error. Also, recent batches of reagent indicate a value of $E_{1\,cm.}^{1\%}$ (500 mμ) for calciferol nearer to 2000 than 1900 which was the figure previously reported.

Other Tests

B.P. test of identification. Powder one tablet, shake with 5 ml. of chloroform, filter, and to 1 ml. of filtrate add 9 ml. of antimony trichloride reagent. A brownish-red colour should be produced.

B.P. test of disintegration. Carry out the official test in the 1955 addendum.

Commercial Tablets

Samples of calciferol tablets, labelled as Tab. Calciferol. B.P., or Tab. Calciferol.Fort. B.P.C. 1949, or Tab. Calciferol.Fort. N.F. 1949, or otherwise stated to contain 50,000 units of antirachitic activity (vitamin D) per tablet, were purchased from fourteen different manufacturers, A to N. All samples were analysed within 2 months of purchase. Sample E_2 was obtained from the manufacturers of sample E_1 when the results of the analysis of E_1 were known.

The calciferol in the tablets was extracted by three methods with a

number of solvents and determined spectrophotometrically or colorimetrically, and the B.P. tests of identity and of disintegration were applied. The results are recorded in Tables I to V. Although physico-chemical and not biological methods were used, the estimates of calciferol are stated in units of vitamin D, on the assumption that calciferol contains in 1 mg. 40,000 units of antirachitic activity.

Discussion

Ethylene dichloride appears to extract the most calciferol from the tablets (see Table II), although chloroform and carbon tetrachloride are nearly as good. The use of ethylene dichloride simplifies the experimental work since it is the solvent used in the preparation of the antimony trichloride reagent.

The Soxhlet technique appears to be the least satisfactory of the three methods of extraction studied. This may be due to partial destruction of the calciferol by heat; extension of the length of time of extraction to three hours gave lower results. The elution technique has the advantage of presenting fresh solvent to the partially exhausted tablet powder, but extraction may be incomplete because of channelling effects, and Table I shows that the shaking technique gives slightly higher results in most instances.

The higher results obtained spectrophotometrically compared with colorimetrically (Tables II and IV) are attributed to the ultra-violet light absorption of material extracted from the tablet excipients and coating material. The recovery experiments (Table VI) show that significant blanks are obtained by the spectrophotometric method on commonly used tablet excipients. While the spectrophotometric method of determination may be regarded as satisfactory for the routine control of production batches of tablets by the manufacturer, when the precise composition of the ingredients of the tablets is known and allowance can be made for interference, it cannot be recommended for adoption as an official method of assay.

Those samples which contained more than 50,000 units of vitamin D per tablet all gave an orange-brown colour in the B.P. identity test; this presumably corresponds to the official description "brownish-red." Those which assayed 10,000 to 40,000 units per tablet gave a light brown or light orange-brown colour; the samples with less than 1000 units per tablet gave none or at the most a pale straw-yellow colour.

The samples from ten manufacturers complied with the official disintegration test. Of the four which did not comply, one sample made no labelled claim to be of B.P. quality.

Proposed Methods of Assay

As a result of the work described above, the following alternative methods are recommended for the assay of tablets of calciferol.

Method 1. Weigh and powder 20 tablets. To an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol, add 50 ml. of ethylene dichloride, shake for 5 minutes, adjust the volume to

TABLE I

COLORIMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL BY VARIOUS METHODS. CALCIFEROL CONVERTED TO VITAMIN D UNITS

Manufacturer	Calciferol "units" per tablet			
	Shaking with ethylene dichloride	Elution with ethylene dichloride	Soxhlet extraction with ethylene dichloride	Shaking with light petroleum
A	—	56,900	56,600	49,000
B	27,600	26,500	26,500	26,200
C	28,400	27,300	23,100	23,600
D	50,600	52,200	51,600	47,300
E_1	0	0	0	0
E_2	1200	900	900	0
F	27,400	26,800	28,500	24,400
G	31,100	29,900	30,000	29,400
H	62,400	63,400	62,500	59,500
I	12,300	11,300	10,900	10,800
J	50,500	51,000	52,000	50,000
K	51,300	51,400	51,000	49,200
L	38,500	38,500	37,800	36,800
M	21,300	20,100	20,900	19,600
N	33,300	33,000	33,200	34,400

TABLE II

COLORIMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL BY ELUTION WITH VARIOUS SOLVENTS. CALCIFEROL CONVERTED TO VITAMIN D UNITS

Manufacturer	Calciferol "units" per tablet				
	Carbon tetrachloride	Chloroform	*cyclo*Hexane	Ethylene dichloride	Light petroleum
A	56,100	54,200	51,100	56,900	51,100
C	25,300	28,300	25,000	27,300	24,300
D	48,100	—	45,800	52,200	45,000
G	30,200	30,000	29,300	29,900	27,400
I	11,400	7600	10,900	11,300	10,500
J	51,800	48,500	49,600	51,000	51,000

TABLE III

COLORIMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL USING SOXHLET APPARATUS WITH VARIOUS SOLVENTS

Manufacturer	Calciferol "units" per tablet		
	*cyclo*Hexane	Ethylene dichloride	Light petroleum
A	56,300	56,600	52,000
C	28,100	23,100	23,300
D	57,600	51,600	52,600
I	11,100	10,900	10,600
J	43,900	52,000	43,600

TABLE IV

SPECTROPHOTOMETRIC DETERMINATION OF CALCIFEROL EXTRACTED FROM COMMERCIAL TABLETS OF CALCIFEROL BY ELUTION WITH VARIOUS SOLVENTS

Manufacturer	Calciferol "units" per tablet			
	*cyclo*Hexane	Ethanol	Ethylene dichloride	Light petroleum
A	67,200	—	79,400	69,800
C	28,300	34,400	31,200	25,500
D	47,600	57,200	57,500	52,200
E_2	5200	10,800	9100	3700
G	30,700	45,000	36,700	30,400
I	13,700	19,900	19,100	14,800
J	55,200	100,000	90,900	53,400

TABLE V

B.P. TESTS OF IDENTITY AND DISINTEGRATION APPLIED TO COMMERCIAL TABLETS OF CALCIFEROL

Manufacturer	Test of identity	Disintegration time (minutes)
A	Orange-brown	31
B	Light orange-brown	17
C	Light brown	More than 150
D	Orange-brown	19
E_1	Colourless	79
E_2	Straw-yellow	—
F	Light brown	More than 150
G	Light brown	11
H	Orange-brown	More than 150
I	Light brown	21
J	Orange-brown	32
K	Orange-brown	31
L	Light orange-brown	27
M	Light orange-brown	10
N	Light orange-brown	29

TABLE VI

RECOVERY OF CALCIFEROL FROM VARIOUS TABLET EXCIPIENTS USING THE COLORIMETRIC METHOD OF DETERMINATION

	Calciferol "units" per g.						
	Blank				Calciferol found		
Diluent	Method 1	Method 2	Method 2a*	Calciferol added	Method 1	Method 2	Method 2a*
Calcium phosphate	1000	2000	20,000	206,000	203,000	207,000	234,000
Chocolate basis ..	1000	1000	83,000	193,000	192,000	193,000	266,000
Lactose	0	0	15,000	197,000	195,000	196,000	217,000
Starch	0	0	29,000	201,000	202,000	201,000	233,000

* Method 2a is extraction by elution with ethylene dichloride followed by spectrophotometric determination.

100 ml. with ethylene dichloride, shake for 2 minutes, and centrifuge or allow to stand until the insoluble matter has settled. To 0·4 ml. of the supernatant liquid add 2·2 ml. of antimony trichloride reagent, and compare the extinctions at 500 mμ at ½-minute intervals for 3 minutes with those of 0·4 ml. of ethylene dichloride + 2·2 ml. of reagent. Record the maximum extinction. Calculate the weight of calciferol in each tablet of average weight.

Method 2. Weigh and powder 20 tablets. Transfer an accurately weighed quantity of the powder, equivalent to about 1·5 mg. of calciferol, into a chromatography-type tube of internal dimension 30 cm. × 1 cm., and fitted at the bottom with a cotton-wool plug. Elute with ethylene dichloride, collecting 100 ml. of eluate. Mix by shaking. To 0·4 ml. of solution add 2·2 ml. of antimony trichloride reagent, and complete the assay as in method 1.

VALIDITY OF METHODS

The colorimetric and spectrophotometric methods of determination were applied to the solutions obtained by the elution and the shaking methods of extraction from calcium phosphate, chocolate basis, lactose and starch, and to mixtures of each of these excipients with calciferol.

The results are recorded in Table VI. The colorimetric method gave the correct concentration of calciferol in all cases, within ± 2 per cent. By the spectrophotometric method, however, there were positive "blanks" in the absence of calciferol, and the estimates of calciferol concentration were high by as much as 35 per cent.

Table I shows that the methods of extraction recommended in this paper gave results as high as those obtained by a method, involving shaking with light petroleum, almost identical with that specified in the official assay. Also, for method 2, preliminary work had shown that the powder was completely extracted by the first 80 ml. of ethylene dichloride.

Evers and Smith[15] state that neither colour tests nor the absorption band at 265 mμ can be used as evidence for the presence of vitamin D in pharmaceutical products such as tablets, since they may be given by the decomposition products of vitamin D. On the other hand, Mariani[16] has isolated a product of decomposition from calciferol which gave a negative reaction with antimony trichloride, and the data of Brockmann and Chen[17] and of Mueller[18] also indicate that other steroids are unlikely to produce a colour with antimony trichloride comparable in intensity with that given by calciferol in 2 to 3 minutes at 500 mμ.

Samples obtained from manufacturers A and B in May 1952 were assayed in 1955 when the tablets were at least three years old; the results were 33,100 and 13,300 units per tablet respectively. This is in accordance with expectation.

Biological assays made at the same time as the chemical determinations are of course necessary to establish complete validity, but the indications are that the methods proposed in this paper correctly assay fresh tablets, and give good estimates of the amount of calciferol in partly decomposed tablets.

Summary

1. Two methods are recommended for the colorimetric determination of calciferol in Tablets of Calciferol B.P.

2. The tablets of fourteen manufacturers have been examined by these and other methods, and the results are analysed.

The author thanks Mr. Wilfred Smith and Mr. R. V. Swann for helpful suggestions and criticisms.

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THE CHEMICAL ESTIMATION OF CALCIFEROL IN PHARMACOPŒIAL PREPARATIONS

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The chemical estimation of calciferol (ergocalciferol) presents many problems, but a considerable number of methods including colorimetric[1–7] and spectrophotometric[8,9], as well as methods based on volumetric estimations have been published. Among the latter are the iodine trichloride method of Green[10] and the *p*-benzoquinone method of Tamayo[11], but both suffer from the major disadvantage that very large quantities of calciferol are required, and their usefulness is therefore chiefly confined to the determination of calciferol in high concentrations such as in irradiation products. Among the colorimetric methods, reagents based on antimony trichloride-acetyl chloride have found the widest acceptance[9,12–19], and although the reagent is corrosive and unpleasant to handle the sensitivity is high. Results obtained with such reagents on natural oils have been found to be in good agreement with those obtained by biological assays[9,16,19,20], but so far there has been no proof of the validity of the results obtained in the assay of pharmaceutical preparations which might contain decomposition products.

The biological assay method is a long and expensive procedure and it is desirable that it should be replaced by chemical assay where possible. The problems are considerably simplified if vitamin A is absent, the general composition of the preparations is known, and comparatively large quantities of calciferol are present. The aim of this communication is to recommend simple and rapid methods which give results in good agreement with biological assays and which could therefore be acceptable as official methods.

It was hoped that by suitable extraction procedures followed by chromatography where necessary, the antimony trichloride–acetyl chloride reagent of Nield, Russell and Zimmerli[21], would give satisfactory results with Tablets of Calciferol B.P. and Solution of Calciferol B.P. This reagent, which has been in use in our laboratories for a number of years, was chosen because solutions of antimony trichloride in alcohol-free chloroform are commercially available as Carr-Price reagent, and chloroform is more easily available in a pure state than ethylene dichloride, the alternative solvent recommended by De Witt and Sullivan[16] for the reagent. The reagent chosen, when correctly prepared, has the same sensitivity and stability as that of De Witt and Sullivan and the latter therefore offers no advantage.

The following considerations were borne in mind during the investigation. (1) To confirm the optimum conditions for colour formation and to make a thorough investigation of the technique. (2) To show that only calciferol, and not decomposition products, were measured. (3) To

show that materials likely to be present in tablets do not interfere with the estimations. (4) To investigate interference by substances present in oils likely to be used in the preparation of Solution of Calciferol B.P. and to develop a suitable method of purification. (5) To obtain confirmation of the results by biological assay and by other available methods.

General Procedures Used in the Investigation

Colorimetric Estimation Using Antimony Trichloride-Acetyl Chloride Reagent

The method finally adopted was substantially that described by Nield, Russell and Zimmerli[21]. When one volume of a solution, containing about 25 μg. per ml. of calciferol, is mixed with nine volumes of a 21 to 23 per cent. w/v solution of antimony trichloride containing approximately 2·5 per cent. of acetyl chloride, an orange colour develops, which is maximal 1·5 to 2 minutes after addition of the reagent. The absorption spectrum of the brown-orange colour has a maximum extinction at 500 mμ and an almost zero extinction at 550 mμ. In the absence of interference the extinction at 500 mμ is proportional to the quantity of calciferol present. The quantity of acetyl chloride is not critical and from 1·5 to 5 per cent. may be used. Satisfactory colour development can also be obtained if each volume of calciferol solution is mixed with as little as 5 volumes of reagent.

As it is convenient to use undiluted reagent as the blank, and as a partial correction for any general absorption in the sample, the extinction at 550 mμ is always subtracted from the extinction at 500 mμ. The extinction at 550 mμ should be low and certainly not greater than 0·015. The blank solution may become cloudy after a time, and also the cuvette may become misty. If readings at 550 mμ are always taken immediately after readings at 500 mμ, these possible errors are always detected.

The reagent is prepared by dissolving 22 g. of antimony trichloride, preferably vacuum distilled[23], in dry alcohol-free chloroform (sp.gr. not less than 1·499 at 15·5° C.), diluting to 100 ml. with the chloroform and adding 2·5 ml. acetyl chloride A.R. The reagent must be allowed to stand for at least 30 minutes before use and is stable for several weeks if kept in the dark.

Method

To 1 ml. of the chloroform solution under test (containing about 25 μg. of calciferol) in a stoppered tube, add 9 ml. of the antimony trichloride-acetyl chloride reagent from a rapid delivery burette or pipette, stopper the tube and shake. Transfer to a stoppered 1 cm. cuvette and measure the extinction at 500 mμ and 550 mμ in a suitable spectrophotometer from one and a half to two minutes after addition of the reagent. If no spectrophotometer is available a filter instrument can be used. An interference filter, Wratten filter No. 65 A, or less satisfactorily, a Chance OB2, is suitable. A typical calibration curve obtained by using the Wratten filter is shown in Figure 1.

Determine $E_{1\text{ cm.}}$ (500 mμ minus 550 mμ) and by comparing the figure so obtained with that obtained at the same time with a sample of calciferol B.P. calculate the amount of calciferol present in the sample. $E^{1\text{ per cent.}}_{1\text{ cm.}}$ (500 mμ minus 550 mμ) for calciferol will be found to vary slightly from one batch of reagent to another and from day to day, but generally lies between 1810 and 1900.

Precautions: The reagent is volatile and corrosive and should be kept away from the skin and from metal parts of the instrument. Apparatus must be thoroughly dry as even traces of moisture cause cloudiness. The glass apparatus can usually be cleaned by washing with acetone, but concentrated hydrochloric acid may be necessary if moisture has been allowed to come in contact with the reagent. After completion of the measurements the cell compartment should always be freed from corrosive fumes by blowing in clean compressed air or by vacuum. If a filter instrument is used it is advisable to prepare a fresh calibration curve daily.

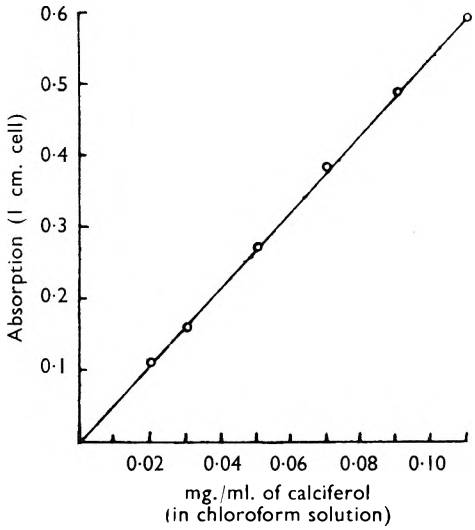

FIG. 1. Typical calibration curve (Wratten filter No. 65A).

Ultra-violet Absorption

The ultra-violet absorption spectrum of calciferol has a characteristic peak at 265 mμ, and this was used as the basis of another assay method. Decomposition products no longer show this selective absorption because of the disruption of the conjugated triene structure, and their absorption at 265 mμ is small compared with that of calciferol and is approximately linear over the range 254 mμ to 272 mμ. The specificity of the method can therefore be improved by the application of a geometric three point correction similar to that described by Morton and Stubbs[24] for vitamin A. It is undesirable to lay down a general formula for this purpose based on data obtained from a limited number of instruments and it is advisable that each laboratory should work out its own formula using pure calciferol for calibration. The formula in use in our laboratories is:—

$$E\ 264\ \text{m}\mu\ \text{(corrected)} = 10{\cdot}50\ \text{B} - 4{\cdot}666\ \text{A} - 5{\cdot}834\ \text{C}$$

(where A, B, and C are the readings at 254 mμ, 264 mμ and 272 mμ respectively using *n*-hexane as solvent) and is based on the ratio:

$$\frac{E\ 254\ \text{m}\mu}{E\ 264\ \text{m}\mu} = \frac{E\ 272\ \text{m}\mu}{E\ 264\ \text{m}\mu} = 0{\cdot}905$$

Since the absorption peak of calciferol is rather flat the correction is of limited value, but the procedure can be used for the assay of high potency tablets and oils, particularly in control laboratories where blank readings are available.

Method

Prepare a solution in spectroscopically pure *n*-hexane containing approximately 10 μg./ml. of the calciferol under test. Measure the extinction in a 1 cm. cuvette over the range 245 mμ to 280 mμ, paying particular attention to 254, 264 and 272 mμ. Calculate the amount of calciferol present using the formula given above or a similar formula. $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ 264 mμ for calciferol = 470.

Chromatography

Calciferol can be separated from its decomposition products by chromatography on an alumina column using mixtures of *n*-hexane as basic solvent, with ether and ethanol to increase its polarity for elution purposes. Separation is also effected from phytosterols but is not always complete. It is difficult to lay down standard procedures owing to variation in the alumina and to such factors as rate of flow, but a typical method which has been found effective in our hands is as follows.

Reagents. Alumina: standardised according to the method of Brockman[25] and deactivated by water if necessary to give activity intermediate between grades 1 and 2. *n*-Hexane: This must be of spectroscopic purity, and such that when 100 ml. are evaporated to dryness and redissolved in 10 ml., the extinction (using the original *n*-hexane as the blank) is not greater than 0·03 in the range 255 to 275 mμ. Ether: anæsthetic B.P. washed thoroughly with water, dried with anhydrous sodium sulphate and redistilled from anhydrous sodium sulphate and anhydrous ferrous sulphate. Ethanol: absolute, A.R.

Method. Prepare a chromatographic column of alumina in amber glass 15 to 20 cm. long by 1 cm. diameter by pouring in a slurry of alumina in *n*-hexane. Tamp the alumina down to give a flow rate of about 1 to 2 ml. per minute under slight nitrogen pressure. Wash the column with spectroscopic *n*-hexane until the washings have an absorption at 255 mμ no higher than the original hexane. Transfer the sample containing about 1 mg. (as little as 50 μg. can be used) to the top of the column using the minimum quantity of *n*-hexane, wash with 50 ml. of 10 per cent. ether in *n*-hexane calling the eluate fraction I. Wash further with 50 ml. 20 per cent. ether in *n*-hexane—fraction II, elute the calciferol with 100 ml. 1 per cent. ethanol in a mixture of 1 : 1 ether in *n*-hexane—fraction III, further wash with 10 per cent. ethanol in 1 : 1 ether, *n*-hexane—fraction IV. Evaporate each fraction to dryness, in a stream of nitrogen at a temperature not exceeding 30° C., dissolve in a suitable amount of *n*-hexane and examine for calciferol using the spectrophotometric method given above (it may be found more convenient to dilute fraction III to 100 ml. in a volumetric flask and measure the absorption directly), evaporate an aliquot to dryness, redissolve in chloroform and examine by the colorimetric

method. 95 to 100 per cent. of the calciferol will normally be found in fraction III, but fraction IV should always be examined.

Using this technique recoveries of pure calciferol of 98 to 102 per cent. have regularly been obtained by either method of determination.

To confirm that fraction III really contains calciferol and not an artefact or decomposition product, an experiment was run on a larger scale. 100 mg. of a naturally decomposed sample of calciferol, shown to contain 70 per cent. calciferol by the above colorimetric and spectrophotometric methods, was subjected to the above chromatographic procedure. Fraction III evaporated to dryness weighed 70·4 mg. Table I gives the physical characteristics of the solid material obtained, compared with those of calciferol B.P.

TABLE I

PHYSICAL CONSTANTS OF CALCIFEROL OBTAINED FROM CHROMATOGRAPHIC FRACTION III

	Sample	Calciferol B.P.
m.pt.*	117° C.	114° to 117° C.
Mixed m.pt.	117° C.	,,
$[\alpha]_D^{20}$	+102·9°	+102·5° to 107·5°
m.pt. dinitrobenzoate ..	144° C. rext. 147° C.	About 148° C.
$E_{1\text{ cm.}}^{1\text{ per cent.}}$ 264 mμ	466	460
$E_{1\text{ cm.}}^{1\text{ per cent.}}$ 500 mμ minus 550 mμ ..	1830	1850
Infra-red	Normal	—

* m.pt. of original material = 101° to 107° C.

Infra-red Absorption

The infra-red absorption curves of calciferol have been published[26,27] and these have been confirmed. For diagnostic purposes three peaks with wave numbers between 1600 and 1650 are important and are thought to be due to the conjugated triene structure. In a Nujol mull an examination of both pure calciferol and of partially decomposed materials showed that this absorption band disappears as decomposition proceeds and a very broad band due to C=O bonding appears centred at about 1700 wave numbers. At this wavelength calciferol shows no absorption; see Figure 2.

For quantitative purposes solution spectra are required. Two per cent. solutions of the same pure and decomposed samples of calciferol in carbon disulphide were run over the range 700 to 1400 wave numbers and a peak at about 1050 wave numbers found to be suitable for assay purposes, since it is produced only by calciferol, and suffers no interference from decomposition products, Figure 3.

Method

Prepare a two per cent. solution of the sample in carbon disulphide and fill into a 1 mm. cuvette. Record its absorption between 950 and 1150 wave numbers, calculate the extinction of the peak at about 1050 wave numbers using a base line technique, and relate this to a standard curve obtained by using pure calciferol.

Interfering Materials

In the assay of pharmaceutical preparations containing calciferol, interference may be due to decomposition products of calciferol or the vehicle used in the preparation.

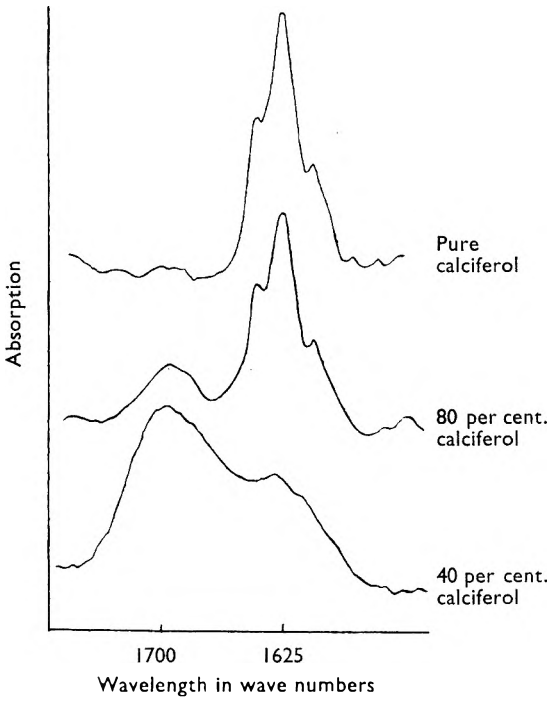

FIG. 2. Nujol mull spectra of calciferols.

Decomposition Products

Four samples of calciferol which had been allowed to decompose naturally to varying degrees were examined by all the techniques outlined above (see Table II). The figures quoted in this table for spectrophotometric and colorimetric determination after chromatography, were obtained from fraction III in the chromatographic method outlined above.

As a further check in an experiment on sample 2 the column was stripped as free as possible by increasing the solvent polarity stepwise up to two per cent. acetic acid in 2 : 1 : 1 ethanol : ether : *n*-hexane mixture.

TABLE II

EXAMINATION OF DECOMPOSED CALCIFEROL SAMPLES

Sample	Per cent. calciferol				
	Method				
	Colorimetric		Spectrophotometric		Infra-red
	Direct	After chromatography	Direct	After chromatography	
1	83·0	81·0	85·0	82·0	88·5
	84·0	—	82·0	83·0	
2	40·0	39·0	no results possible	39·5	40·0
	36·0	—		39·0	
3	6·0	4·2	no results possible	4·5	2·85
	7·0	5·5			
4	1·0	0·9	no results possible	1·0	0·80
	0·9	1·1		1·2	

All fractions were examined by both spectrophotometric and colorimetric procedures. The results are summarised in Table III, which emphasises that the direct determination using the colorimetric method will give the correct result on a decomposed sample.

Vegetable Oils

The B.P. directs that in the preparation of tablets crystalline calciferol (Calciferol B.P.) shall be dissolved in a suitable vegetable oil and tablets prepared using the normal excipients. Solution of Calciferol B.P. is a solution of crystalline calciferol in a suitable vegetable oil. It is well known that phytosterols present in vegetable oils interfere with the estimation of calciferol by both colorimetric and spectrophotometric methods[15,19]. Table IV gives E (500 mμ minus 550 mμ) figures given by some vegetable oils (without saponification) after colour development with antimony trichloride-acetyl chloride reagent.

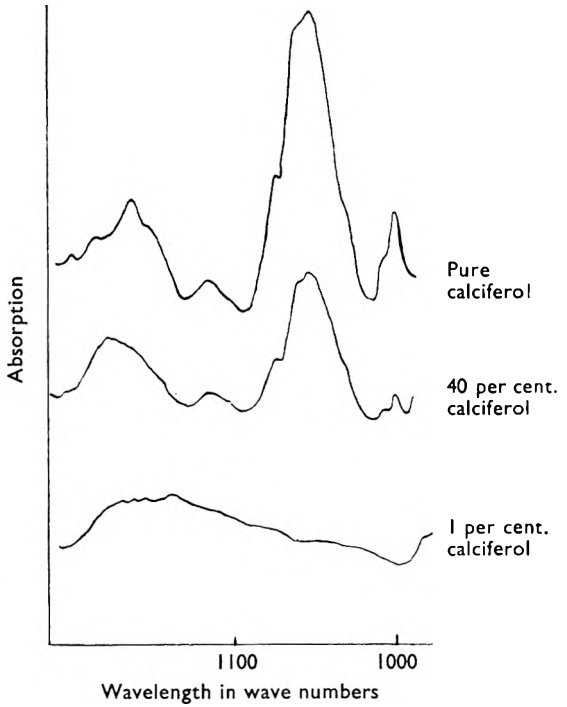

FIG. 3. Infra-red spectra of 2 per cent. solutions of pure and decomposed calciferols in carbon disulphide solution.

A number of tablets made by different manufacturers were found to contain between 2·5 and 5 per cent. vegetable oil. Assuming a 50,000 I.U. tablet to weigh 5 grains uncoated and to contain say 10 per cent. of oil the interference due to a hypothetical oil with an $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$ (500 mμ minus 550 mμ) value of 0·2 will be only 0·2 per cent.

In Solution of Calciferol B.P. the interference of the vegetable oil is a major problem[22]. The chromatographic method outlined above, applied to the unsaponifiable fraction of the oil followed by the colorimetric procedure, has given satisfactory results on oil solutions made in olive oil, arachis oil, cotton seed oil and tea seed oil. One batch of arachis oil gave a chromatographic fraction III which contained interfering material amounting to 5 per cent. of the calciferol. This interference was reduced to less than 2 per cent. by modifying the chromatographic procedure. The column was eluted with 15 to 20 per cent. ether in *n*-hexane, 25 ml.

TABLE III

SUMMATION OF THE ABSORPTION OF CHROMATOGRAPHIC FRACTIONS OF SAMPLE 2

Fraction	Volume (ml.)	Solvent	$E^{1\%}_{1cm}$ 265 mμ	$E^{1\%}_{1cm}$ (500 minus 550 mμ)
1	90	10 per cent. ether in *n*-hexane	1·04	3·1
2	20	20 per cent. ether in *n*-hexane	zero	0·2
3	50	0·25 per cent. ethanol in 1 : 1 ether : *n*-hexane	184·9	725
4	20	1 per cent. ethanol in 1 : 1 ether : *n*-hexane	4·5	5·1
5	25	5 per cent. ethanol in 1 : 1 ether : *n*-hexane	4·5	3·6
6	25	10 per cent. ethanol in 1 : 1 ether : *n*-hexane	6·1	1·5
7	25	20 per cent. ethanol in 1 : 1 ether : *n*-hexane	zero	zero
8	30	50 per cent. ethanol in 1 : 1 ether : *n*-hexane	zero	zero
9	50	50 per cent. ethanol in 1 : 1 ether : *n*-hexane + 2 per cent. acetic acid	19·5	0·6
		Total	220·5	739·1
		Original before chromatography	244·9	743
		Per cent. calciferol from fraction 3	39·5	39·0
		Per cent. calciferol on total unchromatographed material	—	40·0

fractions were collected and examined separately. One interesting feature arising from this work was that although the interfering materials, present in the calciferol fraction, when examined by the extrapolation method of Rogers[22], gave zero colour at zero time, those present in the other fractions did not

TABLE IV

COLOUR DEVELOPMENT GIVEN BY VEGETABLE OILS WITH ANTIMONY TRICHLORIDE-ACETYL CHLORIDE REAGENT

Oil	$E^{1\%}_{1cm}$ 500 minus 550 mμ*
Arachis	0·1
Olive	0·1
Cotton Seed	0·08
Theobroma	0·07

* Readings taken after 2 minutes.

Antioxidants

It seems likely that vegetable oils used in the future may contain small quantities of antioxidants, the addition of 0·01 per cent. propyl or octyl gallate and 0·02 per cent. of butylated hydroxy anisole to vegetable oils being permitted in the United States. These and similar compounds even in considerably greater quantities do not interfere with the determination of calciferol either in tablets or in oily solutions.

Tablet Excipients

Excipients commonly used in calciferol tablets include lactose, dextrose, starch, cocoa powder, calcium phosphate, sucrose, and acacia. The coating consists of sugar and varnish. None of these materials have been found to interfere with the direct colorimetric estimation.

Conclusions

From the results obtained by the various methods and a detailed study of the ultra-violet and infra-red absorption curves, it was concluded that although the absorption curves of the calciferol-containing fraction

obtained after chromatography are generally identical to those of calciferol, a three point correction is sometimes necessary. This is particularly so when samples are being analysed in which a very high proportion (60 per cent. or more) of the calciferol originally present has undergone decomposition. Such a three point correction is always a source of error, and only very rarely corrects for all the irrelevant absorption. With tablets made by one manufacturer, the spectrophotometric method, even after chromatography, always gave very much too high results. In the analysis of comparatively low potency oils, saponification, followed by chromatography of the unsaponifiable matter does not remove everything which interferes with the spectrophotometric assay, and such an assay, even if a correction procedure is used, will give very high results. On the other hand, decomposition products of calciferol appear to give practically no colour with the antimony trichloride-acetyl chloride reagent, and the colorimetric method applied direct to a chloroformic extract of a decomposed sample of calciferol, or of ground tablet material, gives the same result before and after chromatography. It was therefore concluded that a chromatographic step is unnecessary in the colorimetric analysis of Tablets of Calciferol. In the case of the comparatively low potency oily solutions of calciferol where the phytosterols present in the oil give colours which interfere, chromatography is necessary. This then separates the calciferol from the bulk of the phytosterols and from any decomposition products. The colorimetric procedure is then applied, as before, to the purified calciferol.

Recommended Methods

Tablets of Calciferol B.P.

Method. Weigh 20 tablets, crush and grind to a very fine powder. Weigh accurately the equivalent of about 5 tablets and shake mechanically during one hour in the dark with 20 ml. of *n*-hexane. The shaking must be vigorous and should be continuous for one hour because decomposition products of calciferol, which may be present, are rather insoluble in *n*-hexane and prevent the calciferol from dissolving.

Allow to settle, centrifuge if necessary, and evaporate 2 ml. to dryness under nitrogen keeping the temperature below 30° C. Dissolve the residue in chloroform and make up to 20 ml. Transfer 1 ml. to a stoppered glass tube and add 9 ml. of antimony trichloride-acetyl chloride reagent from a fast flowing pipette or burette. Shake, transfer a portion to a stoppered 1 cm. cuvette and measure the absorption at 500 mμ and 550 mμ from $1\frac{1}{2}$ minutes to 2 minutes after the addition of the reagent.

Prepare a chloroform solution containing a known quantity (about 30 μg./ml.) of a standard sample of calciferol, and treat 1 ml. exactly as above, using the same reagents.

From the $E_{1\,\mathrm{cm.}}$ (500 mμ minus 550 mμ) figures obtained calculate the calciferol content in a tablet of average weight.

Solution of Calciferol B.P. To 1·5 g. sample add 0·1 g. hydroquinone and 25 ml. 0·5N ethanolic potassium hydroxide solution. Boil under reflux during 20 minutes, cool, add 50 ml. of water and transfer to a

TABLE V

SUMMARISED RESULTS ON TABLETS

No.	Sample	Labelled potency	Age	Method 1†	2†	3†	4†	5†	6†	7‡	Limits of error p = 0·95
1	Tablets non-chocolate basis ..	50,000	5 years	58,600	36,600	37,800	36,900			34,000	(29,000–39,000)
2	"	50,000	5 years		43,200		41,700				
3	"	50,000	4½ years	65,000	36,700	37,800	36,500			43,000 43,000	(35,000–53,000) (37,000–51,000)
4	"	50,000	3 years	47,700	37,700	38,400	37,300			39,000	(30,000–51,000)
5	"	50,000	2 years		48,500		46,600				
6		50,000	2 years		44,600						
7		50,000	2 years		44,600						
8	"	50,000	2 years		50,800						
9	"	50,000	2 years		52,800						
10	"	50,000	1 year	85,500	54,600 54,400	53,800	53,800				
11	Tablets chocolate basis	50,000	1 year	58,800	55,600	54,100	54,900				
12	Tablets non-chocolate basis ..	50,000	less than ½ year	72,400	51,500	53,200	50,500			60,000	(35,000–72,000)
13	Tablets chocolate basis (dragées)	50,000	unknown	36,650	39,400 33,800*	36,250 31,500*	36,200 33,600*	35,800	38,800	25,000*	(21,000–30,000)
14	Non-chocolate basis	50,000	"	95,250 100,700	47,500 47,500	90,000 62,000	46,000 44,400	47,000	46,750	44,000	(37,000–53,000)
15	"	50,000	"	28,250 36,200	26,250 27,300	28,250 27,700	25,500 27,000			31,000	(26,000–38,000)
Tablets Calc. & Vit. D. B.P.C. 1949		500 500 500 500 500 500 500					400 250 290 360 370 480 280			400 280 330 420 350 350 290	(300–530) (140–590) (220–500) (330–550) (240–520) (250–470) (210–420)

* 6 months after first assay. † Calciferol converted to units. ‡ vitamin D units.

KEY TO TABLE V

Methods:—
(1) Spectrophotometric—direct, correction applied.
(2) Colorimetric—direct (recommended method for tablets).
(3) Spectrophotometric—on chromatographic fraction III, correction applied.
(4) Colorimetric—on chromatographic fraction III.
(5) Spectrophotometric—after saponification, correction applied.
(6) Colorimetric—after saponification.
(7) Biological.

separator. Extract with 3 × 30 ml. portions of anæsthetic ether. Wash the combined ethereal layers with 20 ml. of water followed by 10 ml. 0·5N aqueous potassium hydroxide and then with 20 ml. portions of water until the washings are no longer alkaline to phenolphthalein.

Dry the ethereal solution with anhydrous sodium sulphate, wash the latter with two 10 ml. portions of ether and evaporate the combined solution and washings to dryness under nitrogen at a temperature not exceeding 30° C.

Dissolve the residue in about 10 ml. of *n*-hexane and transfer to an alumina column 20 cm. long and 1 cm. diameter; using a flow rate of 1 to 2 ml. per minute with nitrogen pressure if necessary, elute continuously with 15 to 20 per cent. ether in *n*-hexane collecting that fraction which contains the calciferol Its position is determined previously for the alumina and solvents actually used.

TABLE VI

SUMMARISED RESULTS ON OILY SOLUTIONS OF CALCIFEROL

No.	Sample	Comments	Theory	Found (recommended method) (Calciferol converted to vitamin D units)
1	Soln. B.P. in arachis oil	Freshly prepared	3100	3050 3130
2	,, ...	Freshly prepared	3450	3450 3280
3	,, ...	Freshly prepared. Production batch	3200	2920 3230
4	Soln. in arachis oil		1000	1090
5	,, ...		1000	900
6	Soln. B.P. in olive oil	Freshly prepared	2800	2980
7	Soln. B.P. in cotton seed oil	Freshly prepared	3270	3060
8	Soln. in arachis oil	Production batch	51,500	51,700
9	,, ...	Production batch	51,500	50,800
10	Soln. B.P. in arachis oil	2½ years old	3070	3070
11	,, ...	1½ years old	3070	3100

Evaporate the solvent under nitrogen at a temperature not exceeding 30° C., and dissolve the residue in 5 ml. chloroform. Transfer 1 ml. to a glass stoppered tube and add quickly 9 ml. of antimony trichloride-acetyl chloride reagent. Measure the absorption at 500 mμ and 550 mμ. A high 550 mμ value is an indication that separation from interfering materials is not complete, because the alumina is too inactive or the concentration of ether in the *n*-hexane used for elution too high, or both.

Determine *E* (500 mμ minus 550 mμ) for the sample and for calciferol, and calculate the amount of calciferol in the sample. All work in both of the above methods must be performed in non-actinic glassware.

Biological assays have been carried out on a sufficient number of samples of tablets to confirm the recommended methods. In addition some results obtained by biological and chemical assays on tablets of Calcium

and Vitamin D B.P.C., are given as further evidence of the good agreement obtained by the two methods. The various results on tablets are summarised in Table V. The results obtained by the recommended method on various solutions of calciferol in oil are summarised in Table VI.

SUMMARY

1. The antimony trichloride-acetyl chloride reagent of Nield, Russell, and Zimmerli has been applied to the assay of calciferol in a number of preparations.

2. Results obtained by this method have been compared with results obtained by spectrophotometric and infra-red methods.

3. A chromatographic procedure of purification of decomposed calciferol has been described.

4. Assay procedures have been recommended for calciferol in Tablets of Calciferol B.P. and Solution of Calciferol B.P.

5. The results obtained by chemical assays have been found to be in agreement with results obtained by biological assays.

We should like to express our thanks to Mr. A. V. May and Mr. C. B. Baines for assistance with the experimental work, Dr. S. W. F. Underhill and Mr. K. L. Smith for biological results, and Dr. R. E. Stuckey and Dr. D. C. Garratt for helpful comment and criticisms.

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DISCUSSION

The papers were presented by MR. A. R. ROGERS and MR. P. S. STROSS respectively.

DR. F. WOKES (Kings Langley) said that there were other colour tests, including that of Schaltegger (*Helv. chim. Acta*, 1946, **29**, 285) which had shown reasonable agreement with biological tests but was not mentioned in the papers. Because of the fading of the colour there was only time in the antimony trichloride test to take readings at one extinction. That did not characterise the absorption band, and as the test was not specific it would be helpful if methods could be devised for taking readings on either side of 500 mμ. Nearly thirty years ago he had shown that the antimony trichloride reagent could give colours with many sterol derivatives but paper chromatography might overcome this difficulty. The spectrophotometric method should be more specific than any colour test, provided the peak could be adequately characterised by taking readings around the maximum. That had been done by the authors of the second paper and he agreed with their suggestion to use the Morton-Stubbs correction. Their extinction readings had been taken rather close to maximum so that at 254 to 264 there was a ratio of 0·905 which, in his view, was not as accurate as if the readings had been taken lower down from the peak. The nearer the ratio came to 1 the less accurate it was. He questioned the statement by Stross and Brealey that "Decomposition products no longer show this selective absorption because of the disruption of the conjugated triene structure, and their absorption at 265 mμ is small compared with that of calciferol and is approximately linear over the range 254 mμ to 272 mμ." His own experience had been that when calciferol was exposed to light an initial decomposition product had a peak at 275 to 280 mμ. There was another decomposition product which appeared later with a peak round about 250, and it was only when calciferol continued to be exposed for long periods that the peaks disappeared.

MR. W. H. C. SHAW (London) observed that Mr. Rogers used ethylene dichloride as solvent whilst Messrs. Stross and Brealey preferred chloroform. Some time ago he reached the conclusion that ethylene dichloride offered no advantages over chloroform, particularly as it was difficult to obtain in a satisfactorily pure condition. In Table IV Mr. Rogers included some direct spectrophotometric results with ethylene dichloride. It should be remembered that a solution of calciferol could readily be polarized, particularly in a chlorinated solvent, with the formation of the compound known as *iso*tachysterol. This had a much higher extinction value than calciferol. In a brief investigation carried out some time ago on the atmospheric oxidation of calciferol at 37° C. it was found that when there was no detectable biological activity left, a small reaction with antimony trichloride was still obtained. He agreed that simple chromatography was usually effective in eliminating oxidation products which were undoubtedly a very complex mixture. It should be remembered that the Morton-Stubbs correction was only an approximation. In his view

the antimony trichloride method was preferable to that employing the Morton-Stubbs correction.

DR. G. E. FOSTER (Dartford) said that in his experience the chemical and spectrophotometric methods were usually applicable to high potency preparations of calciferol. Difficulties were encountered with low potency products. That was illustrated to some extent by the present work, particularly in the Tables showing the assays on the B.P. tablets of calcium with vitamin D which only contained 500 units. The authors of the second paper stated that their results were supported by the results of biological tests, but in Table V there were no limits of error stated for the biological figures. In his experience the biological estimation for vitamin D was subject to a very large experimental error, and for this reason there would be some difficulty in stating categorically that the colorimetric method agreed with the biological activity.

DR. F. HARTLEY (London) said that the errors of the biological assay were quite considerable even in one laboratory with one worker and if an attempt were made to obtain correlation in a combined test in several laboratories the range of error would be widened. The colorimetric method was recommended, and he suggested that in fact the validity had indeed been established by taking the potencies as set out in Table V of the second paper. If necessary a further column could be added setting out in detail the limits of error. He wished to avoid the situation where calciferol and its preparations continued to be determined only by biological assay. Since the second paper was prepared for publication biological results were completed on some of the solutions, and it was to be hoped that Mr. Stross would outline those results in his reply. The object of the work could be said to be a move from the biological assay for vitamin D to a chemical or physical method.

DR. D. C. GARRATT (Nottingham) said that in considering the determination of biologically active material which was liable to decomposition it was necessary to correlate the method used with the biological assay. Mr. Roger's paper was incomplete. It was no use getting correlation between chemical and physical assays unless they could be correlated with the potency of the material being examined. The potencies of various manufacturers' products had been estimated, but that did not necessarily mean that they were correct.

MR. H. E. BROOKES (Nottingham) said he understood that the term "error" was very different in bioassay work from that used in analytical chemistry. When one talked of error in analytical chemistry it was rather due to some fault of the person. The limits of error of the bioassayist were rather the limits of probability than limits of error, and it was surprising in the case of calciferol that a series of chemical tests and a series of biological results correlated so well.

MR. K. L. SMITH (Nottingham), speaking as one of those who supplied some of the biological figures, said the estimates in the column referred to were made to $\pm$ 25 per cent., and if the authors had stated this, precise limits of error would not be necessary. The only biological assay carried out on a chocolate-base substance was the assay of tablet 13. The result,

for which he was responsible, was significantly different from the chemical assay result.

MR. A. R. ROGERS, in reply, said that no adsorbent was used in his elution method of extraction, the eluent being added directly to the powdered tablets in the column. The batches of ethylene dichloride used had always been found to be free from interfering impurities, including hydrochloric acid. The main objection to the use of chloroform was the formation of carbonyl chloride on storage. He would like to ask the other authors if it had been established that carbonyl chloride did not interfere with the colour reaction. The limitations of the Morton-Stubbs correction formula must be stressed. It was unusual to find linear irrelevant absorption, and errors were large. He also asked Mr. Stoss if he and his co-author had established by recovery experiments that no decomposition of calciferol occurred during evaporation. Interfering materials which did not show zero colour at zero time with the reagent were reported to be present in some samples of vegetable oils, and he enquired whether they were present in amounts sufficient to invalidate the extrapolation procedure. He also asked for information regarding the chief factors causing deterioration of calciferol preparations on storage. MR. P. S. STROSS, in reply, said that recovery experiments had shown that there was no loss of calciferol during evaporation when below 30° C. if the solution were protected from light and oxygen-free nitrogen was used. The solution used in the B.P. identification test is too strong and the specificity could be increased by measuring the extinction at 450, 500 and 550 mμ. Alcohol-free chloroform need be used only for the preparation of the reagent and not as solvent for the sample, and no trouble has been encountered with the formation of phosgene. The absence of air and light and the use of peroxide-free oils is essential for the stability of calciferol preparations, which should be stored in a cool place. *n*-Hexane was chosen for the chromatographic and spectrophotometric work as chlorinated hydrocarbons should be avoided as pointed out by Mr. Shaw. The low results obtained by Mr. Rogers using hexane (or light petroleum) for this extractions were probably due to decomposition products and to insufficient time being allowed for the extractions.

Results which were more than 25 per cent. high were sometimes obtained using Mr. Roger's extrapolation technique for the assay of Solution of Calciferol B.P., and he considered that unless blanks are available chromatography is necessary for low potency oils. In reply to Dr. Wokes, he said that they had realised that the antimony trichloride-acetyl chloride reagent was not specific for calciferol. He considered that when analysing calciferol preparations whose general composition is known, high specificity though very desirable is not absolutely essential. One must, however, be certain that the concentrations of decomposition products and other constituents are not sufficient to interfere. The selection of wavelengths for the three point correction was a compromise. Whilst decomposition products of calciferol formed by the action of light, particularly ultra-violet light are well known not to have linear absorption

over the range 254 to 272 mμ, the absorption of oxidation products and decomposition products formed in the dark seems to be linear over this range. In reply to Mr. Shaw, he said that they had avoided using chlorinated hydrocarbons for extraction, and only allowed the calciferol to be in contact with chloroform for the minimum possible time. He agreed that some colour is given by decomposition products as seen from Table III, but is so small as to be almost negligible. On the points raised by Dr. Foster, Mr. Stross said that the limits of error of the various biological assays were now available and that it may be possible to include these in the paper. It was realised when this work was started that the limits of error would be rather wide, and every effort was made to confirm the results by other methods as well as by the biological tests. In answer to other points raised, biological figures of 3100 units per g. and 3700 units per g. had since been found for oils Nos. 10 and 11 (Table VI). As could be seen from Table V, they had attempted to obtain a large number of biological results rather than a single result with a narrow limit of error. The interference of chocolate base and other excipients was assessed by blank and recovery experiments, but for brevity the description of these had been omitted.

LIGNOCAINE HYDROCHLORIDE AND ITS SOLUTIONS: PURITY, TESTS, STABILITY AND ASSAY

By Kenneth Bullock and James Grundy

From the Department of Pharmacy of the University of Manchester

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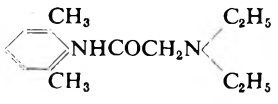
Introduction

Lignocaine (I), like cinchocaine (II), is an amide and thus differs from the large group of local anæsthetics which are aromatic esters of tertiary amino-alcohols and to which procaine and cocaine belong. While cinchocaine resembles this latter group in that it is the amide of an aromatic acid and possesses a tertiary nitrogen atom in the non-acidic portion of the molecule, lignocaine, on the other hand, has its tertiary nitrogen atom in the acidic portion of the molecule and is the substituted anilide of an aliphatic acid; in this way it resembles the analgesic acetanilide.



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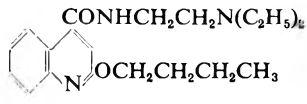

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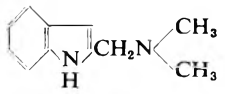

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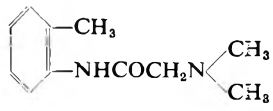

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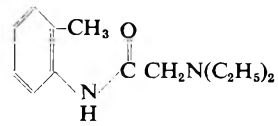


(V)

The first synthesis of lignocaine, in 1943 by Löfgren[1], followed from an earlier observation made by Erdtman and Löfgren[2] that 2-dimethylaminomethyl indole (III) possessed considerable local anæsthetic activity as did 2-dimethylaminoacetamido-1-methylbenzene (IV) in which the nitrogen containing ring of the indole group has been opened. The structural similarity of these earlier compounds to lignocaine can easily be seen if the latter is written as in (V).

Ehrenberg[3] using conduction measurements on frog muscle-nerve preparations showed that lignocaine was 3·3 times as active as procaine at *p*H 7·39 and has a shorter latency time. Compared with procaine it has been claimed that lignocaine is more toxic[4] but may be used in the same concentrations, is less irritating when injected into the tissues[5,6,7] and gives a deeper anæsthesia with more rapid onset and more extensive spread[8,9]. Lignocaine can be used for surface as well as infiltration anæsthesia without, but preferably with, adrenaline. As a local anæsthetic for use in dental and oral surgery it may be said to rival procaine.

Lignocaine hydrochloride is used clinically in aqueous solution as follows. (1) 1 per cent. usually containing 1 in 200,000 adrenaline for nerve block. (2) 2 per cent. usually containing 1 in 80,000 to 1 in 50,000

adrenaline for dental surgery and infiltration anæsthesia. (3) 4 per cent. for surface anæsthesia.

Probably the above mentioned slight advantages of lignocaine over procaine would not have led to its wide-spread use had it not been for one other important chemical characteristic. While almost all other local anæsthetics are relatively easily hydrolysed in solution, particularly at neutral or higher *p*H values, the lignocaine molecule is extremely resistant to hydrolysis by both acid and alkali[10]. This is the more surprising since acetanilide and most other amides readily undergo hydrolysis. These interesting considerations, coupled with the fact that requests were received from the Dental Department of this University for information about the stability, methods for analysis and the detection of decomposition in neutral or alkaline solutions of lignocaine, caused us to undertake an investigation of such solutions similar to the investigations carried out previously in this department relating to other local anæsthetics[11,12,13]. The results of this work, which was commenced several years ago, are reported in this paper.

Experimental

Purity of Materials

All melting points recorded in this paper are uncorrected.

Lignocaine and its Hydrochlorides

At the commencement of this work neither lignocaine nor its salts were available commercially, but only solutions of the hydrochloride. A sample of lignocaine was therefore synthesised by the method described by Löfgren[1]. After six recrystallisations from light petroleum (b.pt. 40° to 60°) the melting point was 67° C. (Löfgren[1] 67° C.). Found: C, 71·9; H, 9·6; N (Dumas), 11·9 per cent.; calculated for $C_{14}H_{22}ON_2$, C, 71·8; H, 9·46; N, 11·96 per cent.

A portion of this material was retained as an analytical reference sample of the free base while the remainder was converted, as described by Löfgren[1], into the anhydrous hydrochloride. After three recrystallisations from anhydrous ethyl methyl ketone and drying at 65° C. under reduced pressure over phosphorus pentoxide the melting point was found to be 128° C. (Löfgren[1] 128 to 129° C.). Found: C, 61·9; H, 8·85; N, 10·23 per cent.; calculated for $C_{14}H_{22}ON_2$,HCl, C, 62·1; H, 8·56; N, 10·34 per cent.

After reserving a portion as analytical standard the rest of the hydrochloride was converted into the monohydrate by recrystallisation from moist ethyl methyl ketone and drying under reduced pressure at room temperature. The melting point was found to be 77° C. (with decomposition). Found: C, 58·45; H, 8·75; N, 9·40 per cent.; calculated for $C_{14}H_{22}ON_2HCl{\cdot}H_2O$, C, 58·2; H, 8·72; N, 9·70 per cent. Later, during the course of the work, lignocaine base, the anhydrous hydrochloride and the monohydrate of the hydrochloride, all of good purity (Table I), became available commercially.

2-*Amino*-1 : 3-*dimethylbenzene* (*ADB*). A sample of ADB of commercial quality was purified by fractionating 3 times under reduced pressure. The fraction selected distilled at 56° C./2 mm. The hydrochloride, obtained by passing dry hydrogen chloride into a solution of the amine in sodium-dried ether, was purified by recrystallisation from

TABLE I

PURITY OF COMMERCIAL LIGNOCAINE AND ITS HYDROCHLORIDE

Commercial subs.	m.pt. ° C.	Mixed m.pt. ° C. with pure salts	Per cent. lignocaine HCl from ionisable Cl per cent.
Lignocaine base	67·0	67·0	—
Anhydrous hydrochloride ..	128·0	128·0	99·8
Hydrochloride monohydrate ..	77·0	77·0	100·2

butan-1-ol. The pure ADB hydrochloride melted at 234° C. (with decomposition). Found: C, 60·95; H, 7·66; N, 8·88; Cl, 22·49 percent.; calculated for $C_8H_{12}NCl$, C, 60·80; H, 7·80; N, 9·35; Cl, 22·50 per cent.

Diethylaminoacetic acid (*DEAA*) was prepared according to the method of Heintz[14] with the following modifications. The reaction between monochloroacetic acid and diethylamine was carried out in absolute ethanol which, after completion of the reaction, was distilled together with any excess of diethylamine. After drying under reduced pressure over phosphorus pentoxide the DEAA was separated from diethyl-ammonium chloride by extraction with ether in a continuous extractor of the type described in the British Pharmacopœia, 1953. It was purified by recrystallisation from either benzene or ethyl methyl ketone and melted at 130° C. (Bowman and Stroud[15] 131° C.).

ASSAY PROCESSES FOR LIGNOCAINE, ITS SALTS AND SOLUTIONS

As with other basic substances and their salts the assay processes for lignocaine may be divided into two parts, (*a*) isolation of the base, usually by making a solution of a salt alkaline and shaking with a suitable organic solvent, and (*b*) estimation of the isolated base. The latter will be discussed first.

(i) *The Kjeldahl Process*

A sample of pure lignocaine base was assayed for total nitrogen by the Kjeldahl process as modified by Middleton and Stuckey[16] (British Pharmaceutical Codex, 1954, method I). Multiplying the percentage total nitrogen found by the factor 0·02343 gave in 4 determinations 99·95, 99·43, 99·87 and 100·03 (relative mean deviation 0·19) as the percentage purity of the sample.

(ii) *The Reineckate Method*

The reineckate method of assay adapted for lignocaine and described by Örtenblad and Jonsson[17] was found to be satisfactory within the usual

limitations of a colorimetric process. Recoveries of 98·95 per cent. and 100·7 per cent. were obtained.

(iii) *Weighing the Recovered Base*

Attempts to estimate lignocaine base in solution in organic solvents by distilling the latter, drying at 100° C. and weighing the residue led to low and variable results. This was shown to be due to the volatility of lignocaine base both alone and in steam. A sample of pure lignocaine base heated at 100° C. gradually lost weight, the loss amounting to 13 per cent. in 5 hours. Another sample of lignocaine base was subjected to steam distillation. The distillate gave a positive reaction with potassio-mercuric iodide solution, but no reaction for ADB when tested by the sensitive colour reaction described below.

(iv) *Titrating the Base*

The curve shown in Figure 1 was obtained by dissolving 0·2110 g. of pure lignocaine base in 20 ml. (an excess) of 0·1084N hydrochloric acid and titrating with 0·1089N sodium hydroxide. The *p*H at half neutralisation, i.e., the pKa value of lignocaine, was found to be 7·90 at room temperature (Lofgren[10] pKa 7·85). Possible indicators for the back titration of excess acid in a solution of lignocaine base in hydrochloric acid are therefore bromocresol green and methyl red.

Results of quintuplicate determinations obtained by using both these indicators and also a mixture containing 3 parts of 0·1 per cent. bromocresol green and 1 part of 0·2 per cent. methyl red are shown in Table II.

TABLE II

ASSAY OF A SAMPLE OF PURE LIGNOCAINE BY TITRATION USING DIFFERENT INDICATORS

Indicator	Percentage lignocaine indicated	Mean	Relative mean deviation
Methyl red	99·34 99·29 99·30 99·40 99·70	99·4	0·11
Bromocresol green	100·00 100·00 100·50 99·86 100·23	100·12	0·20
Methyl red and bromocresol green ..	100·45 100·30 100·30 100·50 100·30	100·37	0·084

Extraction of Lignocaine from Alkaline Solution by Means of Volatile Solvents

In all experiments 10 ml. of a 2 per cent. solution of lignocaine hydrochloride was made alkaline with 0·5 g. sodium carbonate (anhydrous) and the precipitated base extracted with several quantities of volatile solvent. After washing the combined extracts with 5 ml. of water and washing the

latter with 5 ml. of volatile solvent the combined extracts were shaken with 10 ml. of 0·1N hydrochloric acid. After separation, the excess acid was titrated with 0·1N sodium hydroxide using bromocresol green as indicator. Using 3 quantities of 20 ml. each of a mixture of 3 parts of chloroform and 1 part of *iso*propanol as volatile solvent, low results showing between 94·4 per cent. and 96·6 per cent. recovery of the lignocaine were obtained. The following modifications were tried without any improvement in the results obtained.

(1) 5 extractions with the same volatile solvent, (2) the use of 0·5 g. sodium hydroxide in place of sodium carbonate to render the solution alkaline, (3) the use of chloroform only in place of chloroform-*iso*propanol mixture.

Washing the volatile solvent with two 5 ml. quantities of water after the extraction with hydrochloric acid and adding the washings to the acidic solution before back titrating, as was to be expected, slightly, but only slightly, lowered the percentage recovery[12]. It was found that approximately 100 per cent. recovery could be obtained by any of the following modifications, (*a*) using 3 quantities of 20 ml. each of ether as the volatile solvent, (*b*) extracting the chloroform or chloroform-*iso*propanol solutions of the base with one 20 ml. or two 10 ml. quantities of 0·1N hydrochloric acid, with careful separation of the acidic layer and omission of the washing of the volatile solvent with water before back-titrating the excess hydrochloric acid, or (*c*) evaporation of the chloroform or chloroform-*iso*propanol extracts followed by solution of the residue in 10 ml. 0·1N hydrochloric acid and back-titration of the excess acid with 0·1N sodium hydroxide using bromocresol green as indicator. The results obtained by these modifications are shown in Table III.

TABLE III

SATISFACTORY METHODS FOR THE ESTIMATION OF LIGNOCAINE DISSOLVED IN A VOLATILE SOLVENT BY TITRATION TO BROMOCRESOL GREEN

Method	Percentage recovery lignocaine	Mean
Ether as volatile solvent + 10 ml. 0·1N HCl	99·6 99·7 100·1	99·8
$CHCl_3$-*iso*propanol + 20 ml. 0·1N HCl	99·9 99·7	99·8
$CHCl_3$ but evaporation before addition of 10 ml. 0·1N HCl	99·9 99·7 99·6	99·7

It may be mentioned that if, in method (*b*) after separation of the hydrochloric acid, the volatile solvents were washed with water and the washings added to the acidic extract before back titration low results were again obtained. Thus the low results at first obtained must be due to pecularities in the acid-base partition between chloroform and water. Solid anhydrous lignocaine hydrochloride was found to be insoluble in ether but soluble to the extent of 1·95 per cent. in dry chloroform at room

temperature. It was also shown that when lignocaine hydrochloride solutions are shaken with chloroform both lignocaine and chloride ions could be recovered from the chloroform layer. This presumably contributes to the cause of the low results using chloroform. The difficulty may be overcome by providing a considerable excess of hydrochloric acid in the aqueous phase. This was confirmed by the results shown in Table IV. In these experiments 10 ml. quantities of a 2 per cent. solution of lignocaine hydrochloride were treated with various quantities of 0·1N hydrochloric acid solution and shaken with 30 ml. of chloroform and *iso*propanol mixture. 4 ml. or more of 0·1N excess acid has to be present in the aqueous solution to prevent an increase of acidity on shaking with the mixed organic solvents. On repeating these experiments but using ether as organic solvent in no case did shaking with the ether increase the titratable acidity.

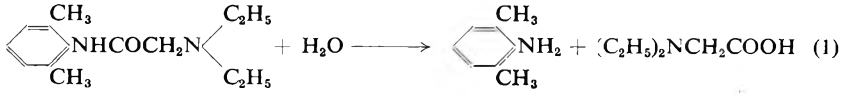
TABLE IV

INCREASE IN ACIDITY ON SHAKING LIGNOCAINE HYDROCHLORIDE SOLUTIONS WITH CHLOROFORM/*iso*PROPANOL MIXTURE AND VARIOUS QUANTITIES OF STANDARD ACID

Amount of 0·1N hydrochloric acid (in ml.) added to a 2 per cent. solution of lignocaine hydrochloride (10 ml.)	Titre of 0·1N sodium hydroxide (in ml.)		Difference
	After shaking with solvent chloroform/*iso*propanol mixture	Omitting shaking with solvent chloroform/*iso*propanol mixture	
1	1·14	1·03	0·11
2	2·12	2·06	0·06
3	3·12	3·09	0·03
4	4·12	4·12	0·00
5	5·14	5·14	0·00
10	10·28	10·28	0·00

THE STABILITY OF LIGNOCAINE IN SOLUTION

To establish the stability of lignocaine in solution it was first necessary to obtain a process which could be shown to be capable of detecting a slight degree of decomposition. In solution lignocaine would be expected to decompose by hydrolysis as follows:



It was to be expected that on such hydrolysis the *p*H of the solution would fall since ADB is a very weak amine while DEAA is a disubstituted amino-acid of which a 2 per cent. solution in water shows a *p*H of 6·5. In fact, two solutions containing DEAA, and lignocaine and ADB hydrochlorides in the proportions corresponding to 1 per cent. and 50 per cent. decomposition showed *p*H values of 3·7 and 2·8 respectively, whereas the original solution had a *p*H of 4·8. This small fall in *p*H cannot be used as an indicator of slight decomposition. It was considered possible that the two *ortho*-methyl groups might well suppress the usual diazo reaction of the aromatic amino group and so interfere with the detection of ADB. In the first place therefore some commercial ADB was diazotised and coupled with 2-naphthol when a vivid red colour developed.

The reaction would detect 100 μg., but not 10 μg., of ADB in 1 ml. of solution. If a 2 per cent. solution of anhydrous lignocaine hydrochloride undergoes hydrolysis to the extent of 1 per cent., calculation shows that the resultant solution will contain 89·5 μg. of ADB per ml. Thus 1 per cent. but not 0·1 per cent. decomposition would be detected by the simple diazotisation test.

A 2 per cent. solution of lignocaine hydrochloride made alkaline to *p*H 7·3 with phosphate buffer (solution F, Table VI) was heated in an autoclave at 116° C. for 30 minutes. Subsequent test by diazotisation showed no colouration and therefore less than 1 per cent. decomposition. Further, 100 per cent. of the lignocaine originally present was recovered by the assay process described above. Other similar but more drastic attempts failed to bring about hydrolysis detectable in this way.

Finally some hydrolysis was obtained by two methods. Two 0·1 g. quantities of lignocaine base, known to be pure, were dissolved, one in 25 ml. of 50 per cent. sulphuric acid and the other in 20 per cent. ethanolic potash. Both solutions were heated in an autoclave at 116° C. for 5 hours. The acidic solution was treated with excess sodium hydroxide and the bases extracted with ether and transferred to 0·1N hydrochloric acid. The alkaline solution was diluted with water and most of the ethanol removed by evaporation. The bases were extracted with ether and transferred to 0·1N hydrochloric acid. The simple diazo colour test described above indicated that in the 20 per cent. ethanolic potash lignocaine was hydrolysed to the extent of approximately 1 per cent. while in 50 per cent. sulphuric acid between 1 per cent. and 5 per cent. had been hydrolysed. All attempts to isolate a sample of ADB from the residual liquids failed but a sample of pure lignocaine m.pt. 67° C. was readily obtained.

Later the two solutions were examined using the more sensitive colour test described below. The material heated in an autoclave in 50 per cent. sulphuric acid showed 3 per cent. decomposition, and that similarly heated with ethanolic potash showed 0·5 per cent. decomposition. These figures can only be considered to be approximate owing to the difficulty of isolating small quantities of ADB from solutions containing large amounts of acid or alkali.

These preliminary experiments appeared to justify the conclusions that the lignocaine molecule in aqueous solution is resistant to heat, acid and alkali, but when decomposition does occur it is by the hydrolysis shown in equation (1). Later quantitative experiments described below confirmed these conclusions. To estimate the degree of decomposition it is necessary to know (*a*) either the lignocaine originally added or (*b*) one of the hydrolysis products. (*a*) The amount of lignocaine originally present in the solution may be obtained by estimation of the total bases, i.e., lignocaine plus ADB, or the undecomposed lignocaine, i.e., the lignocaine but not the ADB. Since one is usually concerned with solutions showing less than 50 per cent. decomposition titration methods are sufficiently accurate for this section of the work. (*b*) DEAA in solutions of relevant concentrations has a *p*H value approximating to 7 so that

titration methods are not to be recommended; it does not give the ninhydrin reaction and no other colour reactions could be traced in the literature. ADB is too weak a base to be titrated accurately but it can be estimated colorimetrically.

Estimation of Undecomposed Lignocaine

Various pK values have been reported for ADB. Thomson[18] found the pKa 25° C. for the free base in 75 per cent. ethanol to be 3·42, and in 50 per cent. ethanol 3·19. Spryskov[19] using an aqueous solution of the hydrochloride found pKb 10·39.

ADB in mixtures of lignocaine and ADB should not therefore interfere with the estimation of lignocaine by addition of excess hydrochloric acid and back titration with alkali provided that a suitable indicator is chosen. That this is true is shown in Figure 2, where the titration of a mixture of

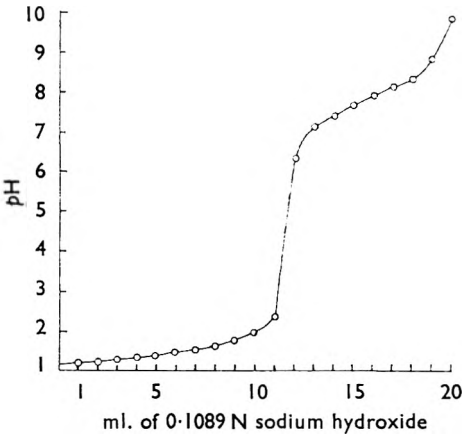


FIG. 1. Titration of lignocaine base (0·2110 g.) dissolved in 20 ml. of 0·1084N hydrochloric acid with 0·1089N sodium hydroxide.

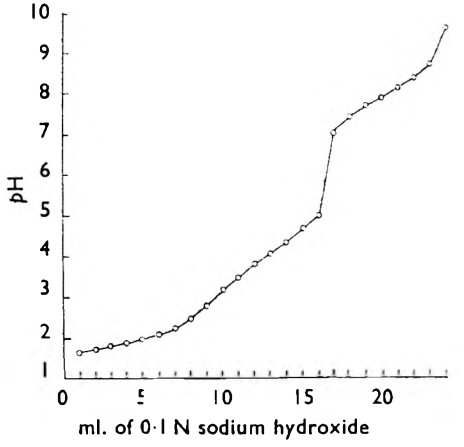


FIG. 2. Titration of 2-amino-1 : 3-dimethylbenzene hydrochloride (0·1 g.) and lignocaine base (0·1 g.) dissolved in 20 ml. of 0·1N hydrochloric acid with 0·1N sodium hydroxide.

0·1 g. of ADB hydrochloride and 0·1 g. lignocaine in 20 ml. of 0·1N hydrochloric acid with 0·1N sodium hydroxide is shown. Comparison of Figures 1 and 2 show that while the end-point for lignocaine alone is *p*H 4 to 6, for lignocaine in presence of ADB it is over the range 5 to 7. It was in fact found that chlorophenol red (*p*H range 4·8 to 6·4) gave accurate results for the lignocaine only in the mixture, while methyl red or bromocresol green gave results approximately 2 per cent. high. No attempt was made to estimate the ADB as well as the lignocaine by titration; examination of Figure 2 shows that there would be no satisfactory end-point even if a suitable indicator in the *p*H range 2 to 3 could be found.

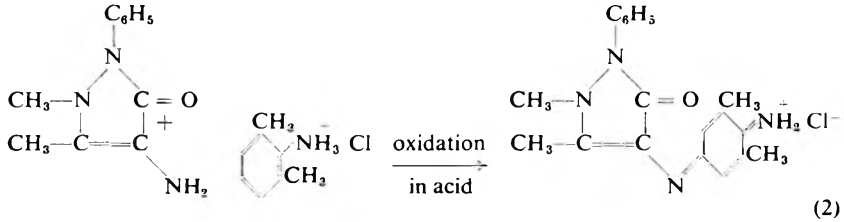
(*b*) *Colorimetric Assay of ADB*

Estimation of ADB by diazotisation and coupling in alkaline solution would be undesirable on account of the precipitated lignocaine which

would render the solution turbid. To avoid turbidity would necessitate a difficult separation of the two bases.

Following the method suggested by Bratton and Marshall[20] for sulphonamides and modified by Brodie and Axelrod[21] for aniline, ADB was estimated by diazotisation, removal of excess nitrous acid with ammonium sulphamate and coupling with *N*-(1-naphthyl)-ethylenediamine dihydrochloride in acidic solution. This process gave only a faint colour with 100 μg. ADB per ml. and so was unsuitable for the estimation of less than 1 per cent. decomposition of lignocaine solutions. With larger quantities of ADB, owing to insolubility, the coupling reagent had to be dissolved in ethanol and it was found that the colour developed varied with the proportion of ethanol present. Diazotisation methods were therefore abandoned.

In 1938 Eisenstædt[22] showed that aromatic amines combine with 4-amino-2:3-dimethyl-1-phenyl-5-pyrazolone(4-amino-phenazone) with loss of 4 hydrogen atoms in the presence of potassium ferricyanide as an oxidizing agent to give blue-red dyes of the indamine type. This reaction was found to occur with ADB and may be represented as follows:



(2)

Neither lignocaine nor acetylated ADB gave any colour under the conditions used. The absorption spectrum of the colour produced showed maximum absorption in the region of 540 mμ. Estimations were subsequently carried out on the Spekker absorptiometer using a Chance OGI filter.

The effect of *p*H is shown in Figure 3. As the *p*H increased from 1·5 to 5·5 the colour and therefore the optical density at 540 mμ of both test and blank increased. Up to *p*H 2·9 the colour of the test increased more rapidly than that of the blank. Over *p*H 3·2 the colour of the blank began to increase more rapidly than that of the test. Thus the greatest difference between test and blank occurred between those two *p*H values and *p*H 3·1 was chosen as the optimum.

At all *p*H values the colour reached a maximum and then faded. At low *p*H values the maximum colour was reached in 5 to 15 minutes and remained fairly constant at the maximum for a period of 5 to 10 minutes. At *p*H 5·5 the maximum was reached in 4 minutes and rapid fading set in at once. At *p*H 3·05 the maximum was reached in 5 to 10 minutes and remained fairly constant over that period of time as shown in Figure 4. For a concentration of 20 μg. per ml. of ADB, which is suitable for the colorimetric assay as described below, 0·05 ml. of 3 per cent. 4-amino-phenazone solution and 0·7 ml. of 2 per cent. potassium ferricyanide

solution were required for each 1 ml. of solution examined. More 4-amino-phenazone resulted in increased optical density in the blank without increasing the excess of optical density in the test. Increase in the quantity of potassium ferricyanide solution added resulted in a slight increase in

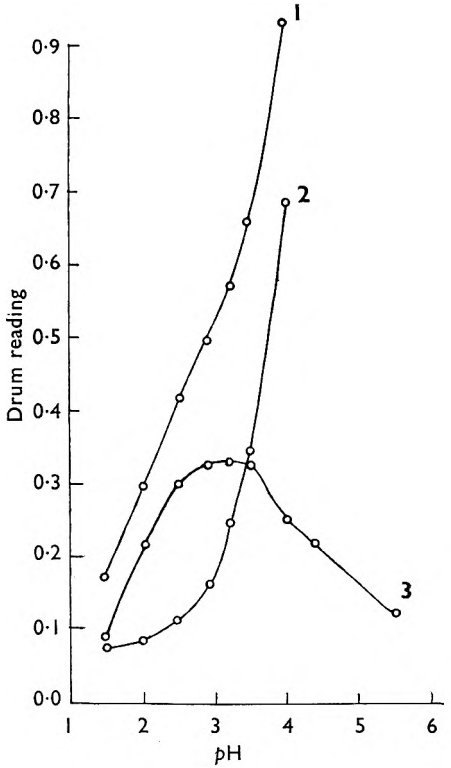


FIG. 3. Effect of *p*H on the development of the colour in the colorimetric assay of 2-amino-1 : 3-dimethylbenzene.

1. Variation in extinction of the test solution when compared with water.
2. Variation in extinction of blank when compared with water.
3. Variation in extinction of test solution when compared with blank.

Quantity of ADB in test solution, 20 μg.
Quantity of 3 per cent. solution of 4-amino-phenazone, 0·3 ml.
Quantity of 2 per cent. solution of potassium ferricyanide, 0·7 ml.

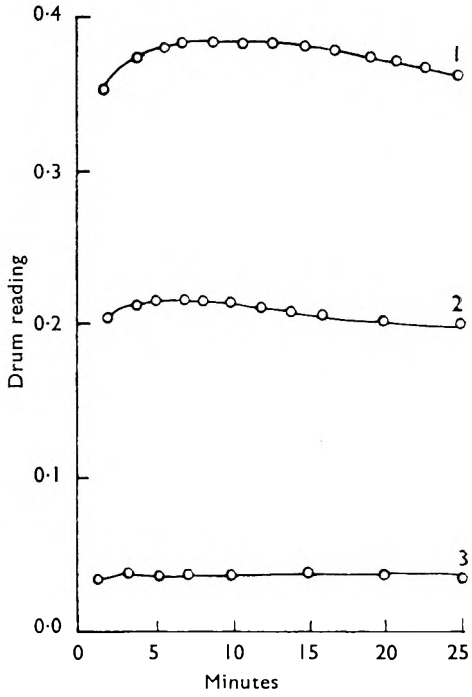


FIG. 4. Stability of colour in the colorimetric assay of 2-amino-1 : 3-dimethylbenzene.

1. Extinction of test solution when compared with water. Concentration of ADB is 20 μg.
2. Extinction of test solution when compared with water. Concentration of ADB is 10 μg.
3. Extinction of blank when compared with water.

colour in the blanks with only a slight corresponding increase in colour in the test. Extraction of the colour with chloroform, as recommended by Ettinger, Ruchhoft and Lishka[23] for the estimation of phenol by a similar process, was found to be unsatisfactory because the colour in the chloroformic extract faded rapidly.

The Process Recommended

(*a*) *Assay for undecomposed lignocaine.* Make alkaline 10 ml. of a solution containing the equivalent of 0·2 g. of lignocaine, by addition of 0·5 g. of sodium carbonate (anhydrous) and extract the precipitated bases by shaking with three quantities of 20 ml. each of ether. Wash the mixed ethereal extracts with 5 ml. of water and wash this in turn with 5 ml. of ether before discarding it. Shake the mixed ethereal solutions successively with 10 ml. 0·1N hydrochloric acid and two 5 ml. quantities of water. Titrate the excess acid in the mixed aqueous extracts with 0·1N sodium hydroxide using chlorophenol red as indicator. 1 ml. 0·1N hydrochloric acid = 0·02888 g. $C_{14}H_{22}ON_2HCl{\cdot}H_2O$, = 0·02708 g. $C_{14}H_{22}ON_2HCl$, = 0·02343 g. $C_{14}H_{22}ON_2$.

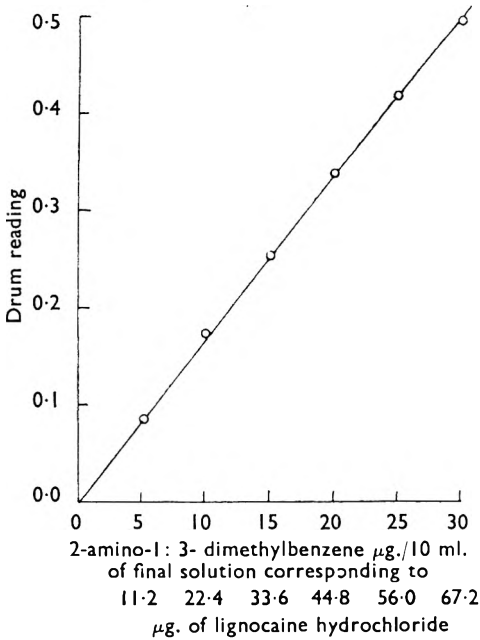


FIG. 5. Calibration curve of 2-amino-1 : 3-dimethylbenzene.

(*b*) *Assay for ADB resulting from decomposition of lignocaine.* Take 1 ml. of solution previously diluted if necessary so as to contain approximately 20 μg. of ADB per ml. Add 8·25 ml. B.P. phthalate buffer *p*H 3·1, 0·05 ml. 3 per cent. solution of 4-amino-phenazone and 0·7 ml. 2 per cent. solution of potassium ferricyanide, mixing thoroughly after each addition. At the same time carry out a blank determination using 1 ml. of water. Between 5 to 10 minutes later, i.e., during the period of maximum colour intensity, measure the optical density in a Spekker absorptiometer using a 1 cm. cell and a Chance OGI green filter. Any other suitable instrument may be used.

From a calibration graph (see Fig. 5) read off the mg. of decomposed lignocaine, corresponding to the resultant ADB, per ml. of the solution tested. From (*a*) and (*b*) the concentration of lignocaine or its hydrochloride in the original fresh solution and the percentage decomposition which it has undergone may be calculated.

The accuracy of the above process was assessed by analysing mixtures of lignocaine hydrochloride, ADB hydrochloride and DEAA of known composition and mixed in the proportions which would result from a 2 per cent. solution of lignocaine hydrochloride undergoing decomposition to the extent of 10 and 50 per cent. respectively. The results are shown in Table V.

TABLE V

ASSAY OF SOLUTIONS CORRESPONDING TO 2 PER CENT. SOLUTION OF LIGNOCAINE HYDROCHLORIDE AFTER 10 PER CENT. AND 50 PER CENT. DECOMPOSITION

	Per cent. composition corresponding to					
	10 per cent. decomposition			50 per cent. decomposition		
	Made up	Found by analysis	Per cent. recovery	Made up	Found by analysis	Per cent. recovery
Lignocaine hydrochloride ..	1·8000 g.	1·7950 g.	99·7	1·300 g.	0·9980 g.	99·8
ADB hydrochloride	0·1164 g.	0·1192 g.	102·4	0·5822 g.	0·5995 g.	103·0
DEAA	0·0969 g.	—	—	0·4844 g.	—	—

SOLUTIONS OF LIGNOCAINE HYDROCHLORIDE SUITABLE FOR USE IN DENTAL SURGERY

The preparation and stability of solutions for dental work will be described here. The pharmacological experiments and clinical trials are proceeding and will be reported later.

All the quantities of materials to be used in preparation of the solutions described refer to the dried anhydrous substances, sodium chloride, sodium phosphate, sodium acid phosphate and anhydrous lignocaine hydrochloride. This has been done so that dry ampoules may easily be made if required. All freezing points quoted are uncorrected for disengaged ice[24]. Lignocaine base was found to be soluble in water at room temperature to the extent of 0·38 per cent., the *p*H of the saturated solution being 9·85. On addition of 0·1N sodium hydroxide to 10 ml. of a 2 per cent. solution of lignocaine hydrochloride at room temperature the solution became turbid due to separation of free base at *p*H 7·8 after the addition of 3·8 ml. of the alkali solution.

The Effect of Temperature on Alkaline Buffered Solutions of Lignocaine Hydrochloride

All the alkaline solutions described below became turbid on heating but became clear again on cooling. That it was lignocaine base which separated was shown by removing some of the separated material and, after dissolving in dilute hydrochloric acid, obtaining a precipitate with potassio-mercuric iodide solution. This same phenomenon occurred previously with amylocaine[12] and was attributed to alteration of the dissociation constant of water with temperature. Probably changes, with

increase of temperature, of the hydrolysis and dissociation constants of the local anæsthetic salt are also involved.

Isotonicity. Figures for the depression of the freezing point by lignocaine hydrochloride could not be found in the published literature. Experiments using the Beckmann apparatus showed that a 2 per cent. solution of the anhydrous salt possessed an average depression of 0·278° C. This corresponds to the figure, subsequently adopted by the British Pharmaceutical Codex, 1954, of 0·130° C. for the depression produced by 1 per cent. of the monohydrate. It had previously been ascertained[11] that isotonic solutions of anhydrous sodium phosphate and anhydrous sodium acid phosphate contain respectively 2·2 per cent. and 2·0 per cent. of the salts.

Formulation of solutions of lignocaine hydrochloride for use in surgery. The composition of the six solutions formulated is shown in Table VI.

TABLE VI

PERCENTAGE COMPOSITION OF LIGNOCAINE SOLUTIONS FORMULATED

Solution	A	B	C	D	E	F
Lignocaine HCl (anhydrous) ..	2·0	2·0	2·0	2·0	2·0	2·0
NaCl	0·478	0·366	—	—	—	—
Na_2HPO_4	—	0·262	0·800	1·10	0·889	0·977
NaH_2PO_4	—	—	0·289	—	0·200	0·112
*p*H	4·8	7·1	7·1	7·5	7·2	7·3

Availability of anæsthetic base in the solutions. 10 ml. quantities of the various solutions were each shaken with 3 successive quantities of 10 ml. each of ether. The free base in the first and in the mixed second and third ethereal extracts was estimated as described earlier. The *p*H of the aqueous solution was determined electrometrically before and after extraction with ether. The results obtained are recorded in Table VII.

TABLE VII

PERCENTAGE OF THE TOTAL BASE REMOVED BY ETHER FROM SOLUTIONS OF LIGNOCAINE HYDROCHLORIDE

Solution	A	B	C	D	E	F
1st extraction	1·5	20·0	44·9	55·5	48·0	50·6
2nd and 3rd extractions ..	1·2	2·8	15·2	17·3	18·9	17·6
Total 1st, 2nd and 3rd extractions	2·7	22·8	60·1	72·8	66·9	68·2
Initial *p*H	4·8	7·1	7·1	7·5	7·2	7·3
*p*H after extraction	2·95	3·2	5·3	6·1	5·55	6·05

TABLE VIII

PERCENTAGE DECOMPOSITION OF LIGNOCAINE SOLUTIONS ON HEATING IN AN AUTOCLAVE

	Approximate per cent. decomposition		*p*H		
Solution	30 min.	3 hours	Initial	30 min.	3 hours
C	0·03	0·05	7·1	7·1	7·1
F	0·03	0·05	7·3	7·3	7·3

Stability on sterilisation and storage. The most alkaline and heavily buffered solutions C and F were autoclaved for 30 minutes and for 3 hours at 115° C. The resultant decomposition and *p*H changes are shown in Table VIII.

The result obtained by analysis of the solutions after storage at room temperature for 33 and 84 weeks respectively are shown in Table IX.

TABLE IX

PERCENTAGE DECOMPOSITION OF LIGNOCAINE SOLUTIONS AT ROOM TEMPERATURE

Solution	Approx. per cent. decomposition after		*p*H		
				After	
	33 weeks	84 weeks	Initial	33 weeks	84 weeks
A	0·005	0·005	4·8	5·3	5·5
B	0·005	0·005	7·1	7·05	7·15
C	0·015	0·15	7·1	7·0	7·2
D	0·015	0·15	7·5	7·3	7·5
E	0·02	0·02	7·2	7·2	7·3
F	0·02	0·02	7·3	7·2	7·4

DISCUSSION

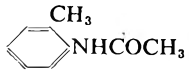
Commercially available samples of lignocaine and its hydrochloride were found to be as pure as samples prepared in the laboratory by synthesis and purified by repeated recrystallisations.

In the assay of lignocaine salts and solutions two facts should be borne in mind:—(*a*) the free base is volatile at 100° C. alone and in steam so that methods involving recovery from volatile solvents by evaporation of the latter and drying the residue at 100° C. or over should be avoided. (*b*) The hydrochloride is soluble in chloroform. There are three satisfactory methods available for the estimation of recovered lignocaine base. The Kjeldahl process is accurate but tedious. The colorimetric reineckate method is accurate but unless the estimation of very small quantities is involved it has the disadvantages common to colorimetric processes. The most convenient method is titration of the excess acid added to the base with standard alkali using bromocresol green as indicator. If this method is used it is unnecessary to evaporate the organic solvent in which the base is usually, at this stage, dissolved provided that ether is used. If chloroform is employed either it must be removed by evaporation or considerable excess acid must be added to extract the lignocaine base and the chloroformic solution must not subsequently be washed with water (Table IV). The physico-chemical behaviour of the hydrochloride of a base soluble in a volatile solvent has been discussed by Davis[25] and Moede and Curran[26]. These workers showed that the hydrochloride of a base soluble in chloroform could undergo dissociation into free base and hydrogen chloride. Such behaviour almost certainly accounts for the difficulties introduced by using chloroform in the extraction of lignocaine solutions. The assay process found to be most satisfactory was thus similar to that now official in the British Pharmaceutical Codex, 1954.

Solutions of lignocaines have been reported to be exceptionally stable. Löfgren[10] claimed that such solutions "endured eight hours' boiling with

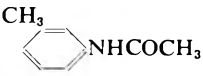
30 per cent. aqueous hydrochloric acid or lengthy boiling with strong alcoholic caustic potash."

The stability of lignocaine to hydrolysis by acid or alkali is undoubtedly due to the presence of the two methyl groups in the *ortho*- position to the diethylaminoacetamido group. Thus 2-acetamido-1 : 3-dimethyl benzene, a compound structurally similar to lignocaine, was found by Nölting and Pick[27] to undergo no decomposition when heated in strong hydrochloric acid solution although at 200° C. in 70 to 75 per cent. sulphuric acid it was hydrolysed, after prolonged heating, into ADB and acetic acid. Davis[28] calculated from experimental data that the corresponding monomethyl derivative VI would require 17·16 hours refluxing in alkaline solution for 50 per cent. hydrolysis. On the other hand, compounds VII, VIII, and IX, under similar conditions required only 3·79, 3·89 and 3·90 hours respectively for 50 per cent. hydrolysis. The unreactivity of groups which have other groups in the *ortho*-position is a general property known as the *ortho*-effect[29a].



(VI)

(VII)



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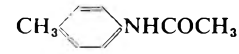

(IX)

Originally attributed to simple steric hindrance[29b] the *ortho*-effect is now thought [30,31], to be due to reduced mesomerism between the benzene nucleus and substituent resulting from a loss of planarity between the nucleus and substituents caused by the *ortho*-groups—in the case of lignocaine, the *ortho*-methyl groups. This steric inhibition of mesomerism has recently been renamed the secondary steric effect[32] and has been demonstrated in lignocaine by Löfgren[10].

Preliminary experiments soon confirmed the stability of the lignocaine molecule, but it was also established that when lignocaine in solution does decompose it is hydrolysed as shown in equation (1).

Local anæsthetics of the procaine type are easily hydrolysed, particularly in alkaline solutions, and the object of the previous research work carried out with them in this department was to obtain a quantitative estimate of the degree of hydrolysis under various conditions and then to formulate solutions and devise methods of sterilisation and storage which would result in a maximum of activity combined with a minimum of decomposition. In the case of lignocaine the object was different. The molecule is stable. A colorimetric test was elaborated which would detect even less than 0·1 per cent. of decomposition in a 2 per cent. solution of lignocaine hydrochloride. This test was then applied to show that under any of the usual conditions of preparing, sterilising and storing such solutions there was not more than 0·1 per cent. of decomposition.

Reactions based upon diazotisation and coupling, which, owing to the presence of undecomposed lignocaine, have to be carried out under acid conditions, were not sufficiently sensitive to detect the concentration of ADB corresponding to 0·1 per cent. decomposition of a 2 per cent. solution of lignocaine. A colour reaction represented by equation (2) was found to be of the required sensitivity as can be seen from Figure 5.

The accuracy of the method devised for the estimation of the degree of decomposition in lignocaine solutions was tested on solutions containing lignocaine hydrochloride, ADB hydrochloride and DEAA in proportions corresponding to 10 and 50 per cent. decomposition of a 2 per cent. solution of lignocaine hydrochloride. The results, shown in Table V, indicate the success of the method.

Although not applicable without considerable modification and consequent loss of accuracy, the method was used to show that heating lignocaine in an autoclave with 50 per cent. sulphuric acid for 5 hours at 116° C. caused approximately 3 per cent. decomposition, while similar treatment with 20 per cent. ethanolic potash caused approximately 0·5 per cent. decomposition.

Usually amides are more readily hydrolysed by alkalis than acids[33]. The reverse phenomenon in the case of lignocaine may probably be attributed to the inhibited mesomerism in that substance (*ortho*-effect) giving rise to higher electron densities at the amide nitrogen and acyl carbon atoms. Such conditions would facilitate the electrophilic attack by a proton and water molecule at the two points required for the hydrolysis.

The advantages of using in dental surgery local anæsthetics in buffered alkaline solutions have been stated previously[11,34]. More recent work[3] has confirmed the enhanced activity of solutions of local anæsthetics at higher *p*H values although there has been a suggestion[35], based perhaps on a not very critical examination of the data, that the local anæsthetic cation as well as the free base may have anæsthetic activity. Previously the relatively easy hydrolysis of the local anæsthetics as well as the instability of adrenaline at *p*H values over 7 has prevented the general use of alkaline buffered solutions which had to be freshly prepared involving the employment of two solution cartridges or dry ampoules. It was obviously of interest to investigate the pharmacological activity and clinical use of alkaline buffered solutions of the very stable lignocaine hydrochloride.

Since lignocaine resembles procaine in potency it was decided to prepare solutions for surgical use similar to those previously described for procaine[11], i.e., 2 per cent. solutions made alkaline and buffered with sodium phosphate alone or in admixture with sodium acid phosphate and, if necessary, rendered isotonic by addition of sodium chloride. Adrenaline was omitted for two reasons, (*a*) lignocaine has been recommended for use without adrenaline and (*b*) the dispensing and storage of alkaline solutions of adrenaline constitute a separate problem which has been previously investigated and discussed[11,36]. It was decided that adrenaline would be added if required immediately before use, at any rate in the preliminary experiments, in the Dental Hospital.

Six different solutions, A to F, having the percentage compositions shown in Table VI were formulated and examined. All contained 2 per cent. of lignocaine hydrochloride. A was made isotonic by the addition of sodium chloride. B was a similar solution brought to *p*H 7·1 by addition of sodium phosphate before being made isotonic by addition of sodium chloride. Thus the buffering capacity was the minimum consistent with the *p*H. On heating it became turbid at 65° C. Solution C

like B had a *p*H of 7·1 but it had the maximum buffering capacity consistent with isotonicity. On heating it became turbid at 43° C. D had maximum buffering capacity and maximum *p*H consistent with the use of sodium phosphate as the alkalising agent. It had a *p*H of 7·5 and on heating became turbid at 34° C., i.e. below body temperature. E and F were similar to C and D in having maximum buffering capacity consistent with isotonicity but, by varying the proportions of sodium phosphate and sodium acid phosphate, the *p*H values were respectively 7·2 and 7·3. On heating solution E became turbid at 37° C. and solution F at 34° C.

If the current belief that on reaching the tissues a local anæsthetic base must dissolve in fatty tissue before anæsthesia is attained is true, then clearly when the base is removed the acid with which it was associated will be liberated in the tissues. Unless this acid is neutralised by the alkalinity and buffering capacity of the blood supply the *p*H of the tissues will fall and the further supply of anæsthetic base will be restricted until the *p*H is raised again. The effects of rendering alkaline and buffering solutions of lignocaine hydrochloride are clearly shown in Table VII. The base available to the tissues, estimated in these experiments by shaking with ether, is greatly increased and the fall in *p*H after removal of the base is lessened.

All six solutions proved to be remarkably stable. Heating in an autoclave for three hours at 115° C. or storage for over 18 months at room temperature in all instances brought about less than 0·1 per cent. of decomposition (Table VIII and IX). On autoclaving the more alkaline buffered solutions for 30 minutes almost the whole of the lignocaine came out of solution. Solutions C and F on removal from the autoclave both showed an oily layer consisting of free base. With occasional shaking during cooling this redissolved to yield a perfectly clear solution.

The pharmacological activity and clinical suitability of the different solutions are at present being examined. It is intended that the results of these investigations shall be published later.

Summary

1. Samples of lignocaine and its anhydrous and monohydrated hydrochloride have been synthesised, purified and used as analytical standards.

2. A number of assay processes for lignocaine, its salts and solutions have been examined and are reported upon.

3. A method for determining the percentage decomposition of solutions of lignocaine has been described.

4. Formulæ for alkaline buffered solutions of lignocaine hydrochloride suitable for clinical trial have been suggested. Their stability and properties have been examined.

This work was carried out at the request of the British Pharmacopœia Commission.

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DISCUSSION

The paper was presented by MR. J. GRUNDY.

DR. W. MITCHELL (London) asked whether the authors had tried extracting the lignocaine base from chloroform with sulphuric acid. He would be surprised if the sulphate were soluble in chloroform.

MR. T. D. WHITTET (London) said that he had examined the effect of prolonged sterilisation of lignocaine solutions and his results were largely in agreement with those of the authors. After six hours autoclaving at 115° C. there was slight discoloration. The solutions were estimated by extraction of the base with chloroform, dissolving in sulphuric acid, back titration with alkali, using as an indicator a solution of bromocresol blue and cresol red. The *p*H of the solutions showed no change. The low figure for *p*H given by the authors was rather surprising as he had found it to be about 6·2 both in commercial samples and in his own. As a further check the melting point of the base was determined and no significant difference was observed. Some pharmacological tests were also carried out, and a slight lowering of analgesic potency was found by the corneal reflex method, and there was a slight increase in toxicity in rabbits.

DR. G. E. FOSTER (Dartford) said that as lignocaine was often used with adrenaline could the colour reaction be used in the presence of adrenaline.

DR. L. SAUNDERS (London) asked whether the authors had considered using ion exchange resins to simplify some of the extraction procedures in the analysis.

MR. J. GRUNDY, in reply, said that he had carried out experiments using sulphuric acid to extract the lignocaine base from a chloroform solution. Low results were obtained similar to those when using hydrochloric acid for the extraction. That was presumably due to lignocaine sulphate being soluble in chloroform and subsequently dissociating in that solvent. He found that there was no difference in toxicity between an ordinary aqueous solution of the hydrochloride and alkaline buffered solutions. Why Mr. Whittet should obtain a rather high value for the *p*H of an aqueous solution of the hydrochloride was difficulty to understand. It was quite possible that the presence of adrenaline would interfere in the estimation of lignocaine in a solution containing both. He had not considered the use of ion exchange resins to separate the decomposition products of lignocaine.

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Acriflavine, Polarographic Determination of. A. J. Zimmer and K. Mansur. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 204.) Solutions of acriflavine were examined polarographically. It was established that although acriflavine is a mixture it behaves as a single compound the half-wave potential in acid solution *p*H 1·1, after the removal of oxygen, being given by the equation $E_{\frac{1}{2}} = 0{\cdot}39\ V + 4{\cdot}8\ c$ for the first wave and $E_{\frac{1}{2}} = 0{\cdot}59\ V + 4{\cdot}8\ c$ for the second wave, where c is the percentage concentration. The waves were well defined and independent of the supporting electrolyte. They were affected by changes in the hydrogen ion concentration, and in neutral or alkaline solutions the electrode reaction was irreversible for the first wave. The polarographic method of analysis was applied to preparations, including tablets and ointments and was more accurate and convenient than that of the U.S. National Formulary (9th edition), which also suffers from the disadvantage of determining only the chloride ion, and not the active acridine compounds. G. B.

Amanita Toxin, Chemical Identification of. S. S. Block, R. L. Stephens, A. Barreto and W. A. Murrill. (*Science*, 1955, **121**, 505.) The following procedure was used. Mince a sample of 0·1 g. or more of fresh fungus and extract by boiling in methanol. Filter or centrifuge the extract and evaporate the liquid to dryness on a water bath. Redissolve the residue in a little methanol and submit the solution to paper chromatography using methyl ethyl ketone, acetone, water and butanol (20:6:5:1) as the solvent. Spray the dried chromatogram with a 1 per cent. solution of cinnamaldehyde in methanol. Violet spots indicate the presence of amanitines and bright blue spots are due to phalloidine. This method was applied to 46 species, and only the poisonous ones gave a positive reaction for amanitines. An exception was observed in the case of *Lepiota cretacea* which gave a weak violet colour although it was not toxic. *Amanita muscaria*, which contains the quicker-acting poison muscarine, was not submitted to the test. G. B.

Barbiturates, Potentiometric Non-aqueous Assay for. C. J. Swartz and N. E. Foss. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 217.) When pure chloroform is used as a non-aqueous medium for the titration of barbiturates, the end-point cannot be determined accurately by potentiometry. This disadvantage may be overcome by the addition of polyethylene glycol 400, the sharpest end-point being obtained when chloroform containing 10 per cent. of polyethylene glycol 400 is used. The following is an accurate and convenient method of assay. Dissolve 0·1 to 0·2 g. of the dried barbituric acid under test in 5 ml. of polyethylene glycol 400 and 45 ml. of chloroform, and titrate with 0·1 N sodium methoxide in dehydrated methanol, the end-point being determined electrometrically. Sodium derivatives of barbituric acids should first be dissolved in water, the solution acidified, extracted with chloroform and the extract concentrated to 45 ml. After the addition of polyethylene glycol 400, the solution is titrated as above. G. B.

Magnesium Citrate Solution, Assay for. D. D. Abbott and L. A. Reber. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 287.) To a 10-ml. sample, freed from excessive carbon dioxide by repeated pouring, add sufficient water to produce 100 ml. To 25 ml. add 50 ml. of standard solution of sodium ethylenediaminetetra-acetate, allow to stand for 10 minutes, add 20 ml. of ammonium chloride/ammonium hydroxide buffer solution and titrate the excess of ethylenediaminetetra-acetate with standard solution of magnesium sulphate (1 mg. of Mg/ml.,) using "inhibited versenate" indicator solution, and titrating until the colour changes from dark blue to wine red. The standard solution of magnesium sulphate may be standardised by the hydroxyquinoline gravimetric method. The above method is rapid and comparable in accuracy with the U.S.P. XIV gravimetric method. For the determination of total citric acid, heat almost to boiling, 25 ml. of the diluted sample (see above) and pour it through a column of ion exchange resin (amberlite IR-120 (H)). Wash the sample through the column with 2 quantities each of 25 ml. of hot water, and titrate the effluent, containing the free acid, with 0·1N sodium hydroxide using phenolphthalein as indicator. This method, while not specific for citric acid, appears to give results comparable to those of the U.S.P. method of precipitation as calcium citrate, and the precision of the method is greater. G. B.

Opium, Determination of Morphine in. R. Fischer and K. Folberth. (*Arzneimitt.-Forsch.*, 1955, **5**, 66.) 1 g. of opium is rubbed down with 3 ml. of water, and the mixture is passed through a column of 10 g. of acid alumina (Woelm), being washed through with 5 ml. portions of water, using in all 35 ml. of water. The eluate is treated with 4 ml. of ammonia (23 per cent.), and made up to 40 g. with water. A solution of 0·25 g. of dinitrochlorbenzene in 30 ml. of acetone is then added, and the mixture is allowed to stand overnight in a cold place. The morphine ether is filtered off, washed with two 2 ml. portions of cold acetone, then with two 2 ml. portions of water, and dried for 2 hours at 70 to 80° C. The method may also be applied to tincture of opium, 10 ml. being evaporated to remove the ethanol and the residue taken up in 5 ml. of water. G. M.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Blood Cholinesterase Values in Excessive Exposure to Phosphorus Insecticides. J. C. Gage. (*Brit. med. J.*, 1955, **1**, 1370.) The red cell and plasma cholinesterase values of 19 normal subjects were measured at monthly intervals for 1 year. The coefficient of variation was 12·8 for red cell and 21·3 for plasma values, composed of a basic individual variation, increased by the experimental error of measurement and temporal variations of unknown origin. The following are suggested as criteria for the detection of excessive exposure to phosphorus insecticides. If an individual shows a red cell or plasma cholinesterase value less than ½ of the population average, or less than ½ of his pre-exposure value, whichever is the higher, he should not be allowed to continue his employment and measures to prevent excessive exposure of workers should be re-examined. A value between 50 and 60 per cent. of the population average indicates the need of special supervision with frequent determinations of the cholinesterase values of the workers involved. It is advisable to determine the normal cholinesterase value for each worker before he has been exposed to the insecticides, so that a more accurate assessment of the amount of anticholinesterase he absorbs can be made. G. B.

Kynurenine and 3-Hydroxykynurenine in Urine. L. Musajo, C. A. Benassi and A. Parpajola. (*Nature, Lond.*, 1955, **175**, 855.) Results are reported on the excretion of kynurenine and 3-hydroxykynurenine in pathological human urines. The urine of normal humans, used as control, gave negative results and the urine of several patients was also negative; in many others a slight excretion of metabolites was noted, mainly kynurenic and xanthurenic acids, anthranilic and 3-hydroxy-anthranilic acids and, sometimes, kynurenine and 3-hydroxy-kynurenine. A positive result was found in hæmoblastotic forms, 76 cases being examined. In 59, kynurenine and 3-hydroxykynurenine (both free and conjugated) were found in quantity with small quantities of kynurenic and xanthurenic acids; in 11 cases kynurenine was present, but 3-hydroxykynurenine was absent. In 6 subjects, some of uncertain diagnosis, the kynurenines were absent including 4 patients treated with a colchicin derivative which was considered to inhibit the excretion of the tryptophan metabolites. Excretion took place during both normal and fever periods; the recession of the fever did not stop the excretion. In 4 cases, quantities up to 40 mg. kynurenine sulphate and 16·4 mg. of 3-hydroxykynurenine were recovered from urine. R. E. S.

BIOCHEMICAL ANALYSIS

Adrenaline and Noradrenaline in a Mixture, Colorimetric Estimation of. T. Ozaki. (*Tohoku J. exp. Med.*, 1954, **61**, 83.) A method is described for the differential estimation of adrenaline and noradrenaline in a mixture. At *p*H 3·6 adrenaline is oxidised completely in 2 minutes with potassium permanganate while only 10 per cent. of noradrenaline is oxidised. At *p*H 5·6 both are completely oxidised within 3 minutes. For the estimation 0·1 to 1·0 ml. of the sample to be tested or a standard adrenaline solution is measured. The *p*H is adjusted to 3·6 or 5·6 by the addition of acetate-acetic acid buffer solutions, and 0·1 ml. of permanganate reagent added. (The permanganate reagent consists of 3 g. of potassium permanganate dissolved in 24 ml. of distilled water and 8 ml. of 75 per cent. lactic acid solution is added.) After 2 minutes at *p*H 3·6 or 3 minutes at *p*H 5·6, 0·05 per cent. hydrogen peroxide solution, in an amount equivalent to the permanganate, is added and the mixture is diluted with cold water to a volume of 6 to 10 ml. The oxidation with permanganate is always made at 20° C. The colour density of the test and standard are compared within 5 minutes in a colorimeter. The amount of adrenaline and noradrenaline is calculated from the formulas $A = X + n_{3\cdot6}.Y$ and $B = X + n_{5\cdot6}.Y$ where X and Y are the amount of adrenaline and noradrenaline respectively, A and B are μg. equivalents of adrenaline estimated at *p*H 3·6 and 5·6 respectively and $n_{3\cdot6}$ and $n_{5\cdot6}$ the colorimetric activity ratio of noradrenaline to adrenaline estimated at *p*H 3·6 and 5·6 respectively. The method has been used to assay the noradrenaline and adrenaline contents of the adrenals of cattle and horses. Noradrenaline was found in the medullary tissue in an amount of 29 per cent. of the total in cattle and 18·5 per cent. in the horse, which is in agreement with the findings of Holtz and Schuman and Shepherd and West. G. F. S.

Human Whole-blood Cholinesterase, Assay of. D. R. Davies and J. D. Nicholls. (*Brit. med. J.*, 1955, **1**, 1373.) Collect 20 cu. mm. of blood in a hæmoglobin pipette and wash it into 1 ml. of dilute solution of bromothymol blue in a test-tube. Add 0·5 ml. of a 0·6 per cent. solution of acetylcholine chloride in water, and observe the time required for the solution to become deep orange. The blood cholinesterase value may be read from a chart relating cholinesterase value to rate of change of *p*H during the test. A result 50 per cent. of the mean normal cholinesterase value (ΔpH/hour = 67) indicates that the level of

hypersensitivity to anticholinesterase has been reached, while 20 per cent. of the normal value (ΔpH/hour = 26) may be regarded as the clinical danger level. The lower fiducial limit of normal activity is 109 (ΔpH/hour). The above figures relate to determinations at 20° C. A chart is given showing the times corresponding to these limits, for determinations at 10 to 30° C. G. B.

Noradrenaline and Adrenaline in Urine, Chemical Determination of. A. Pekkarinen and M-E. Pitkänen. (*Scand. J. clin. Lab. Invest.*, 1955, **7**, 1.) The adrenaline and noradrenaline contained in the urine sample are adsorbed on to aluminium oxide at *p*H 8·5 and subsequently eluted with oxalic acid. Care is taken to eliminate all interfering substances. The solution is then treated with manganese dioxide and the fluorescence of the resulting adrenalutine or noradrenalutine is measured. The two substances are distinguishable by the difference in the time that they take to reach maximal intensity. About 70–80 per cent. recovery of either amine from the urine is obtained. M. M.

Steroids, Extraction of, from Blood. M. E. Lombardo, P. H. Mann, T. A. Viscelli and P. B. Hudson. (*J. biol. Chem.*, 1955, **212**, 345.) A simple method is described for the estimation of steroids in blood, combining dialysis and extraction in one operation. Equal volumes of blood, water and methanol are placed in Cellophane dialyzing tubing and extracted with 60 per cent. aqueous methylene chloride in a special extractor. The extracts are evaporated to dryness on a water bath at 40° C. *in vacuo* and then chromatographed in an appropriate solvent system. All steroids possessing a Δ^4-3-ketone group are located on the paper as ultra-violet-absorbing areas. Steroids such as androsterone and dehydroepiandrosterone are detected with the Zimmerman reagent. The steroid containing zones on the paper are cut into tiny squares and eluted quantitatively with a methanol-chloroform mixture. Steroids possessing the Δ^4-3-ketone group are estimated by their absorption at 238 to 242 mμ in a Beckman spectrophotometer. Compounds such as deoxycorticosterone, Compound E, Compound F can also be determined by the blue tetrazolium method, and except Compound E by the Porter-Silber reaction. The results of recovery experiments with various steroids at different concentrations are reported. The method is useful for the analysis of adrenal venous blood. G. F. S.

Xanthines and Organic Mercurials, Bioassay and Diuretic Potency of, in Humans. T. Greiner, H. Gold, F. Palumbo, L. Warshaw, T. McGowan, J. Weaver and H. Otto. (*J. Pharmacol.*, 1955, **113**, 140.) A method for the bioassay of diuretic substances, using ambulant patients with congestive heart failure, is described and the results analysed statistically. The diuretic effect is measured by the loss of body weight 24 hours after the dose. This method is used to compare the potency of aminophylline with meralluride (Mercuhydrin), to determine the degree to which the diuretic action of meralluride may be augmented by aminophylline, to compare the diuretic action of aminophylline by intramuscular and oral administration and to compare the therapeutic value of oral theophylline in the form of the ethylenediamine with that of the calcium salt. It was found that 0·5 g. of aminophylline causes a diuretic effect equal to that of 0·6 ml. of meralluride, both being given intramuscularly, but that aminophylline is less effective orally. In terms of molecular weight, aminophylline and calcium theophyllinate have equal diuretic potency. Aminophylline may enhance the diuretic activity of the organic mercurials by approximately 50 per cent. This is due to a simple summation of effects and not to a potentiation. There is, however, insufficient evidence that the xanthines will restore a diuretic response in patients refractory to the organic mercurials.

M. M.

CHEMOTHERAPY

Antibiotic of Bacterial Origin, A New. A. T. Fuller. (*Nature, Lond.*, 1955, **175**, 722.) This antibiotic was isolated from a spore-bearing organism of the *Bacillus pumilis* group, obtained from an East African soil. It was prepared in good yield by growing the organism in aerated culture in a medium containing inorganic salts, ammonium citrate, glucose and meat extract. The antibiotic was isolated by acidifying to *p*H 2·5, precipitating with ammonium sulphate, extracting the precipitate with ethanol, evaporating to dryness, washing the residue with ether to remove impurities, dissolving the remainder in ethanol and passing the solution through an alumina column. The eluate was recrystallised from a mixture of acetic acid and ethyl acetate, in the form of a white solid, melting point 252° C., empirical formula $C_8H_9O_2N_2S$. It was effective *in vitro* against *Streptococcus aureus*, *Pasteurella muriseptica*, *Streptococcus hæmolyticus* group A and *Mycobacterium tuberculosis*. It was not active against *Bacterium coli*, *Shigella flexnerii* or *Candida albicans*. Administered intraperitoneally to mice it was non-toxic and effective against hæmolytic streptococcal infections. It was less effective when given by mouth. G. B.

Ethylene Bis-dithiocarbamate Esters as Fungicides. A. P. Collins and G. A. Wiese. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 310.) Disodium ethylene bis-thiocarbamate was prepared by the reaction of a solution of ethylenediamine and sodium hydroxide with carbon disulphide. The resulting solution was used directly for the preparation of the bis-2-hydroxyethyl, bis-3-hydroxypropyl and di-*n*-propyl esters. The isolated salt, prepared by a modified procedure, was used for the preparation of the diallyl and dibenzyl esters. All these esters were tested for activity against pathogenic fungi (*M. audouini*, *A. schoenleinii*, *M. lanosum*, *M. gypseum* and *T. rubrum*) using paper discs impregnated with acetone solutions of the fungicides, which were placed on plates of medium streaked with the organism. Zones of inhibition were measured after incubation for 7 days at 37° C. The diallyl ester was more active than undecylenic acid against all the test organisms. Bis-2-hydroxyethyl, bis-3-hydroxypropyl and di-*n*-propyl esters were of about the same order of activity as undecylenic acid. A certain amount of specificity of action was observed, the bis-2-hydroxyethyl ester being much more active against *M. lanosum*, *M. gypseum* and *T. rubrum* than against the other organisms. G. B.

PHARMACY

NOTES AND FORMULÆ

Aspirin, Decomposition of, in Aspirin, Phenacetin and Caffeine Tablets. D. Ribeiro, D. Stevenson, J. Samyn, G. Milsovich and A. M. Mattocks. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 226.) Experimental batches of aspirin, phenacetin and caffeine tablets were prepared to determine the effect of varying the form of aspirin, lubricant, moisture content and pressure in the tablet machine. The tablets were stored at 45° C. for 4 weeks and the decomposition assessed in terms of the content of salicylic acid, determined by measuring the colour intensity after reaction with ferric ammonium sulphate. Stearic acid and stearates caused significantly greater decomposition than talc, mineral oil or Aldo 33, especially when a large proportion of lubricant was included. Crystalline aspirin was preferable to aspirin-starch granulation made by precompression, especially when Aldo 33, stearic acid, talc or mineral oil

were employed. A moderate water content of the phenacetin-caffeine granules did not increase the rate of decomposition, and variations in the pressure applied to the machine punches had no effect on the keeping properties of the tablets. G. B.

Neomycin in Pharmaceutical Preparations, Stability of. R. M. Simone and R. P. Popino. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 275.) Preparations were assayed before and after storage, using a cup-plate method, with *Bacillus subtilis* as the test organism. Aqueous solutions, tablets and soluble and water-miscible bases were diluted and assayed directly. Oily preparations were dissolved in ether/light petroleum mixture and extracted several times with phosphate buffer, *p*H 8, the mixed extracts being used for the test. Aqueous solutions appeared to be stable for up to 2 years at room temperature, the presence of 0·1 to 0·3 per cent. of antioxidant and buffering to *p*H 6 to 7 preventing the discoloration which otherwise occurs, especially in concentrated solutions (50 to 400 mg./ml). A series of nasal preparations, mouthwashes and tinctures were stable for 1 year at room temperature. Tablets, lozenges and ointments in water-miscible and water-immiscible bases were stable for 2 years, but a 4 per cent. ointment in hydrous lanolin lost 90 per cent. of its activity in 1 year at 23° C. Autoclaving of a 0·325 per cent. w/v solution of neomycin sulphate in water at 120° C. for 40 minutes caused no loss in potency. Preparations containing neomycin with penicillin, bacitracin or dihydrostreptomycin were equally stable. Sterilisation of dry powdered neomycin sulphate at 110° C. for 10 hours caused no loss in potency, but under similar conditions a 0·325 per cent. ointment in white petroleum jelly showed a loss of 13 per cent. with darkening of the preparation. G. B.

PHARMACOLOGY AND THERAPEUTICS

Acetazoleamide in Acute Respiratory Acidosis. M. Wishart and B. Isaacs. (*Lancet*, 1955, **218**, 995.) Acetazoleamide (Diamox) inhibits the enzyme carbonic anhydrase, its main pharmacological effect being exerted on the renal tubule cells, leading to increased urinary excretion of sodium, potassium, and bicarbonate; reduced excretion of ammonium ion; a rise in *p*H of the urine; and a fall in the serum bicarbonate level and in *p*H of the blood. Acetazoleamide was given to 3 patients with acute exacerbation of chronic respiratory acidosis in the hope of preventing or relieving carbon-dioxide narcosis. Increased urinary excretion of bicarbonate and a fall in the serum-bicarbonate resulted. In all 3 cases there was clinical deterioration—2 of the patients died and the third recovered when the acetazoleamide was withheld. It was concluded that in these 3 cases acetazoleamide was of no value, and that its property of promoting renal excretion of bicarbonate may have been harmful to the patients. S. L. W.

Adrenaline, Noradrenaline and Dihydroergotamine, Effects of, on Excised Human Myometrium. W. J. Garrett. (*Brit. J. Pharmacol.*, 1955, **10**, 39.) The action of (−)-adrenaline, (−)-noradrenaline and dihydroergotamine on isolated strips of human myometrium were investigated at various stages of the menstrual cycle, after the menopause, during pregnancy and at parturition. The myometrial segments from non-pregnant uteri were cut as vertical strips from the body of the uterus; those of pregnant uteri were transverse strips from the lower segment. Both adrenaline and noradrenaline stimulated the muscle under all the above conditions, noradrenaline being considerably the more potent on the non-pregnant uteri. The stimulant actions were blocked by dihydroergotamine and potentiated by cocaine. The *in vitro* results obtained

for adrenaline with the late-pregnancy strips differed from those obtained for this drug *in vivo*, where inhibition was the usual response. Noradrenaline stimulated the myometrium both *in vivo* and *in vitro*. G. P.

Anticholinesterase Agents, Effect of, on the Rats' Blood Pressure. P. Dirnhuber and H. Cullumbine. (*Brit. J. Pharmacol.*, 1955, **10**, 12.) Sarin, dyflos, eserine, TEPP and E600 all produced hypertension when administered to rats in near-lethal doses. Spinalisation of the rat, hexamethonium, tolazoline, ergotamine and large doses of atropine reduced the response to a small short-lasting rise in pressure. In the skinned rat the prolonged rise was also absent. It was concluded that the anticholinesterases increase, by a central action, the sympathetic tone in the blood vessels of the skin. G. P.

Antithyroid Drugs: Mode of Action, *In Vitro* Inhibition of Oxidative Protein Iodination. R. Fraser, M. G. W. Hanns and W. R. Pitney. (*Brit. J. Pharmacol.*, 1955, **10**, 1.) The mechanisms of action of antithyroid drugs were investigated by two *in vitro* tests. The first and more important test measured the inhibition by the drug of a protein-iodinating enzyme system, consisting of milk enzyme powder, xanthine, casein and radio-iodide. The other determined the interference with a non-enzymic casein iodination with peroxide and radio-iodine. In a series of known antithyroid drugs, differentiation of mode of action was possible from the results of the two tests. The first group (carbimazole, methimazole, 2-thiouracil and iodothiouracil) acted mainly by enzyme inhibition, the second group (resorcinol, sulphathiazole and isoniazid) had little enzyme-inhibiting activity and apparently acted mainly by iodine removal or by substrate competition, while the third group (thiocyanate, L-thyroxine and *p*-aminosalicylate) showed intermediate characteristics, but resembled more closely the second group. The enzyme test was suggested as a useful screening test for new antithyroid compounds since in the series tested the potency measured by this method correlated well with *in vivo* antithyroid activity. G. P.

Baytenal, A Short-acting Thiobarbiturate. P. Nobes. (*Lancet*, 1955, **268**, 797.) Baytenal is sodium 5:5-allyl-(2-methylpropyl)-thiobarbiturate, which is administered intravenously as a 10 per cent. aqueous solution as a very short-acting anæsthetic. Elimination is so rapid that there is virtually no post-operative somnolence. The drug was tried in 11 patients ranging in age from 17 to 62, selected so as to avoid the need for other intravenous medication. Some patients received Omnopon 1/3 grain and scopolamine 1/150 grain. Dosage of Baytenal was based on the age, weight and general condition of the patient and on the duration of the operation, and varied from 0·45 to 1 g. The drug was also given experimentally to a volunteer (the author). The most striking feature was the rapidity with which consciousness was regained and the freedom from post-anæsthetic confusion or drowsiness. The volunteer regained consciousness in 6 minutes and could write an account of his subjective sensations in 22 minutes. Slight euphoria persisted for 2 hours but did not interfere with work or ability to drive a car. In all cases respiratory depression was slight and apnœa was of short duration. Retching, lasting for ½ to 1 minute, occurred in only 3 cases, two of whom had been given Omnopon and scopolamine premedication. It is suggested that the drug may be especially useful for anæsthesia in out-patients or casualty cases. H. T. B.

B.C.G. Vaccination, Standardisation and Efficacy of, against Tuberculosis. S. R. Rosenthal. (*J. Amer. med. Ass.*, 1955, **157**, 801.) This is a critical evaluation of B.C.G. vaccination based on a 20-year study. With regard to

alleged loss in viability of B.C.G. vaccine, it has been shown that when standard vaccine is stored at ice-box temperatures it loses little of its viability for periods up to one month, and at these temperatures freeze-dried vaccine remains of constant viability for periods up to 3 years. With the freeze-dried vaccine it is now possible to completely standardise the vaccine before use; viability, potency and safety can all be ascertained before distribution. B.C.G. vaccine increases resistance against virulent tuberculosis in man in all age groups bringing about a reduction in the morbidity and mortality from tuberculosis of from 54 to 100 per cent. B.C.G. vaccination should be used only as a part of a tuberculosis control programme in which housing, nutrition and education are also taken into account. The multiple puncture method of vaccination is simple in application and devoid of complication, and leaves no scar. In this method a thin stainless steel disc with 36 sharp protrusions is used. A drop of vaccine is placed on the outer aspect of the arm over the deltoid region and is spread with the wide margin of the disc which is held by a stainless steel magnet. The skin is tensed from beneath with one hand and downward pressure is exerted on the magnet and disc with the other so that the needles puncture the skin through the vaccine. The vaccine is then redistributed with the wide margin of the disc into the apertures made by the disc. Small maculopapules appear after 10 to 14 days and disappear after a month or two. The rate of conversion is high (90 to 100 per cent.) and compares favourably with the results of other parenteral methods. S. L. W.

Cardiac Glycosides in the Treatment of Cardiogenic Shock. R. Gorlin and E. D. Robin. (*Brit. med. J.*, 1955, **1**, 937.) Hesitation about using cardiac glycosides in myocardial infarction is based on the facts that the experimentally infarcted heart shows lowered tolerance of digitalis and that the effects of digitalis and infarction, both of which increase the likelihood of ventricular irritability and arrhythmia, may be additive. The authors have used cardiac glycosides in 4 patients with myocardial infarction who were in both coma and shock. 1 patient in clinical shock was given lanatoside C and 3 with shock and pulmonary œdema were treated with ouabain, both drugs being given intravenously. All showed remarkable clinical response with a rise in blood pressure, decrease in pulmonary œdema and dramatic clearing of the coma. 3 out of the 4 ultimately survived. The first patient was given 50 mg. of procaine amide hydrochloride intravenously, with conversion to a normal sinus mechanism. Noradrenaline produced a rise in blood pressure but the clinical shock remained. 0·4 mg. of lanatoside C intravenously produced a marked response within 30 minutes; a further dose of 0·4 mg. was then given. Death occurred suddenly 1 hour later and post-mortem examination showed a fresh myocardial infarction and pulmonary congestion. Death may have been related to a digitalis induced arrhythmia. The ouabain treated patients were given respectively 0·15 mg. in 3 doses each of 0·05 mg., and single doses of 0·2 mg. and 0·125 mg. Supplementary treatment with morphine, oxygen, procaine amide hydrochloride and venesection was given when necessary. All came out of coma within 20 to 45 minutes and eventually made a complete recovery. The authors suggest the following treatment for myocardial infarction with shock—morphine for pain, adjustment of the patient's position in bed for its effect on blood pressure and venous return, phlebotomy for its effect on venous pressure, vasoconstrictor agents for increasing systemic resistance and arterial pressure, and cardiac glycosides for increasing cardiac output and arterial pressure. The importance of the dose of the cardiac glycosides in avoiding toxicity is emphasised. In this series 25 to 50 per cent. of the generally accepted dose of ouabain was found adequate. H. T. B.

Chlorpromazine, Action of, on the Autonomic Nervous System. F. Jourdan, P. Duchêne-Marullaz and P. Boissier. (*Arch. int. Pharmacodyn.*, 1955, **101**, 253.) The actions of chlorpromazine on autonomic pathways in the dog and the rabbit were studied, using in all cases the same dose, 5 mg./kg. intravenously, a dose which corresponds to that employed clinically. In the dog, blockade of adrenaline vasopressor effects was always obtained and reversal sometimes occurred. In the rabbit, however, only a moderate adrenergic blockade resulted, reversal never appearing. In the dog, chlorpromazine had no action on the cardioaccelerator action of adrenaline; on the contrary, the drug had an accelerator action of its own, which persisted after denervation of the heart. There was also no action on the mydriasis, exophthalmos, retraction of the nictitating membrane or conjunctival vasoconstriction resulting from stimulation of the cervical sympathetic trunk. Similarly, the cardioaccelerator effects of cervical sympathetic stimulation were not affected. Renal vasoconstriction following splanchnic nerve stimulation in the adrenalectomised dog was only moderately depressed. Release of adrenaline from the adrenal medulla on stimulation of the splanchnic nerve was not affected by chlorpromazine. Stimulation of the peripheral vagus was partly blocked, but the duration of action was prolonged, especially in the rabbit. Salivary secretory response to chorda stimulation was slightly decreased. The hypotensive and cardiac slowing effects of injected acetylcholine were not modified. In the dog, the hypotension induced by chlorpromazine was mainly central, no hypotension occurring in the spinal dog. Also, small doses, inactive when injected peripherally, led to a rapid fall in pressure when injected into the vertebral artery.

G. P.

Chlorpromazine, Psychiatric Use of. W. H. Trethowan and P. A. L. Scott. (*Lancet*, 1955, **268**, 781.) A clinical trial was carried out on 30 men and 29 women patients with psychoneuroses or personality disorders who showed some symptoms of an obsessive or compulsive kind. The patients were observed for 7 to 18 weeks, during which all received chlorpromazine and an inert placebo, and more than half received no treatment for a week during the trial. In 31 patients treatment began with chlorpromazine while in the remainder the placebo was given first. The initial dosage of the drug was 50 to 75 mg. daily, increased after a week to 100 mg., and after a further week to 150 or 200 mg. at which level it was maintained for the remainder of the course. A significant response to chlorpromazine occurred in 27 of the patients. All had a return of symptoms on replacing the drug by the placebo, usually within 3 to 5 days. 6 patients became considerably worse after the drug was withdrawn than they had been before treatment began; of these, 3 developed a state of agitated depression which necessitated admission to hospital. Of the 32 patients who did not respond to chlorpromazine, 10 showed some improvement but as it continued whether the drug or the placebo was given it was assumed to be spontaneous. After withdrawal of chlorpromazine there was a significantly smaller response to a subsequent course of treatment. In addition to the patients completing the course of treatment, 16 commenced treatment but were not included in the results. Of these, three developed jaundice. Pyrexia sometimes occurred, and 2 patients developed hypnagogic hallucinations. The best results were obtained in patients in whom tension and anxiety were prominent. Little relief of obsessive-compulsive symptoms apart from aggressive urges and hypochondriacal ideas was obtained. It is emphasised that sudden withdrawal of the drug is inadvisable.

H. T. B.

Chlorpromazine, Inhibitory Actions of, on Motor Activity. S. R. Dasgupta and G. Werner. (*Arch. int. Pharmacodyn.*, 1955, **100**, 409.) Chlorpromazine causes a reduction of muscle tone in cats, which is not due to a neuromuscular blocking action but to central depressant action on tone and reflex regulating centres. Experiments in anæsthetised cats show that the effects of cortical stimulation (pericruciate area) are depressed by 0·5 to 1·0 mg./kg. of chlorpromazine and similar results are obtained with 20 to 30 mg./kg. of mephenesin. Stimulation of the cerebellar cortex in decerebrate cats show that 0·5 mg./kg. of chlorpromazine suppresses the postural responses and decreases decerebrate rigidity. Stimulation of suitable areas of the lateral reticular formation (area of the restiform body) show the drug to reduce turning movements. It also reduces the height of the contraction elicited by stimulation of descending medullary tracts, like mephenesin. The crossed extensor reflex of decerebrate cats is completely suppressed by 0·5 mg./kg. of chlorpromazine, and is antagonised by strychnine (20–30 μg.). The crossed extensor reflex of spinal cats is comparatively resistant to the blocking action of chlorpromazine. The results show the close similarity of the inhibitory actions of the drug on motor activity with the effect of a typical "interneuron-blocking" drug mephenesin.

G. F. S.

Chlorpromazine in Mental Hospital Patients. J. Lomas. (*Brit. med. J.*, 1955, **1**, 879.) This is a report on the treatment of 205 patients with chlorpromazine over a period of 4 months. The generally adopted scheme of dosage was to give 50 mg. three times daily orally in the first week, 75 mg. three times daily in the second week, and 100 mg. three times daily in the third and subsequent weeks. Very few patients failed to tolerate this dosage. Some patients received higher dosages than 300 mg. daily, but most patients who improved on the drug did so on 150 mg. daily. The more disturbed and chronic psychotic patients both tolerated higher dosages and required more drug to produce improvement. Treatment was not abandoned as useless in under 2 months and was not usually prolonged beyond 3 months. It was terminated abruptly without any withdrawal effects being noticed in any case. Almost all cases treated were ambulant. Although a few patients complained of slight giddiness, no marked hypotensive effects were observed except in those with cardiovascular disease. The risk of potentiation of sedatives was negligible in normal doses and was noticeable only in those in whom continual narcosis was produced when it was possible to manage with much smaller doses of barbiturates given in association with chlorpromazine than with barbiturates alone. The results in this series suggest that chlorpromazine may prove useful in the treatment of acute states of excitement of all sorts, in neurotics in whom tension or anxiety is a prominent symptom, and in some depressive patients in whom E.C.T. has proved ineffective. The most striking results were obtained in chronic psychotic patients in whom the prognosis would normally be very bad; it enabled a few of these patients to be discharged and many more to make a good hospital adjustment. In chronic patients the chief part played by chlorpromazine was to make possible the rehabilitation of those in whom this was formerly impossible. It would appear that the results of chlorpromazine treatment are almost identical in both inhibited and overactive patients; this suggests that some revision is needed in the conception of the drug as being mainly a symptomatic treatment for excited states. As good results were found after leucotomy as in other chronic patients.

S. L. W.

Desacetylmethylcolchicine in Myeloid Leukæmia. B. J. Leonard and J. F. Wilkinson. (*Brit. med. J.*, 1955, **1**, 874.) 8 patients with chronic myeloid leukæmia were treated with desacetylmethylcolchicine (Colcemid). The initial oral dose of 3 mg. daily was increased after 3 or 4 days to a total of 10 mg. daily according to the white-cell count. This dosage was continued until the leucocytes dropped to approximately 25,000 per cu.mm. and was then stopped for 3 to 4 days. A daily maintenance dose of 3 to 5 mg. was then instituted. Daily white-cell counts, platelet counts and hæmoglobin estimations were performed until the maximal effect on the white cells had been attained; thereafter counts were done twice weekly and finally every 1 to 2 weeks. In 6 of the 8 patients there was distinct clinical improvement; their appetites improved, they gained weight and had more energy; their spleens rapidly decreased in size; there was a dramatic reduction in the white-cell count and an increase in the hæmoglobin content and the red-cell count; the platelet counts were unaffected. These 6 patients have been maintained in satisfactory clinical and hæmatological states for periods of from 4 to 9 months and have carried on with their normal occupations. It appears that the compound is a more selective granulocyte depressant than other chemotherapeutic agents. The disadvantages of myleran in producing thrombocytopenia and aplastic anæmia, and in some cases in precipitating an acute myeloblastic leukæmia, were not seen in this group of cases. In 2 patients with myelofibrosis the white cells were extremely sensitive to Colcemid and dropped to low levels in 3 and 5 days respectively. The results of the treatment in 6 patients with acute myeloid leukæmia were disappointing; in spite of some reduction in the number of myeloblasts clinical deterioration continued and in no case was a remission obtained. The use of Colcemid would appear to be contraindicated in chronic lymphatic leukæmia, and evidence (in one case) suggests that it may even aggravate the condition. S. L. W.

Hydrocortisone, Intravenous, Clinical Uses of. F. Dudley Hart. (*Brit. med. J.*, 1955, **1**, 454.) Intravenous hydrocortisone is available for use in patients suffering from acute adrenal insufficiency when cortisone or hydrocortisone acetate by mouth or by intramuscular or intra-articular injection do not act with sufficient promptness. In Addisonian crisis an intravenous drip of 10 to 20 mg. hydrocortisone for 6 to 8 hours usually effects sufficient improvement to allow of oral or intramuscular therapy. In adrenalectomised patients affected by some additional stress such as infection, intravenous hydrocortisone may be life-saving. Partial adrenal insufficiency may occur as a result of additional stress, such as a surgical operation, in patients being treated with cortisone. Other conditions in which the use of hydrocortisone should be considered include the Waterhouse-Friderichsen syndrome, acute renal insufficiency due to stress in Simmonds's disease, and severe status asthmaticus, acute disseminated lupus erythrematosus and overwhelming allergic reactions. Sodium retention and œdema are not a serious danger during the short periods of treatment needed in adrenal crisis and the hazard is diminished by using 5 per cent. dextrose. The drug is rapidly eliminated and after its use full doses of oral or intramuscular cortisone must be given, or intramuscular cortisone can be given simultaneously. The patients must be closely watched, especially those with metastatic malignant disease who have been adrenalectomised; if signs of post-operative infection are observed antibiotic therapy must be instituted. H. T. B.

Isoniazid in the Treatment of Pulmonary Tuberculosis in Children. R. McL. Todd. (*Lancet*, 1955, **269**, 794.) Isoniazid was administered to alternate

children in a group of 50 with primary pulmonary tuberculosis. Children not given isoniazid received no specific chemotherapy. All were kept in bed for 4 to 8 weeks and were then allowed to return gradually to full activity. Dosage of isoniazid was 3 mg./lb. body weight per day, in 3 doses, for 3 months. Progress was assessed by (1) vitality, appetite and clinical examination; (2) change in body weight; and (3) radiological evidence. Improvement in appetite was seen in almost every treated case. Except in infants under 3 years of age, the increase in body weight was greater in the treated children than in the controls and a statistically significant difference was found in the 3 to 6 year age group. X-ray shadows tended to clear earlier in the treated group but after 1 to 3 years there was little difference in the clinical and radiological condition of the treated and the untreated groups. An impression was formed that the size of the primary lesion decreased more often during the first 3 months in the treated patients. No side effects of isoniazid were observed and the progress made by the patients confirmed previous reports that drug resistance is less likely to occur in children. H. T. B.

Lysergic Acid Diethylamide, Action of, on Mammalian Cholinesterases. R. H. S. Thompson, A. Tickner and G. R. Webster. (*Brit. J. Pharmacol.*, 1955, **10**, 61.) Lysergic acid diethylamide (LSD) caused an increase in acetylcholine levels in the guinea-pig brain (Poloni and Maffezzoni, *Sistema nerv.*, 1952, **4**, 578). In view of this report the authors studied the actions of LSD and other ergot alkaloids on cholinesterases. The enzyme-inhibitory activity was determined manometrically. LSD had relatively powerful inhibitory activity on pseudocholinesterase of human serum and brain, the true cholinesterase and tributyrinase of the brain being only slightly affected by concentrations of LSD which almost completely inhibited the pseudocholinesterase. The inhibition was reversible and competitive in nature. In the rat, guinea-pig, rabbit, chicken and monkey the brain pseudocholinesterase was much less sensitive than was the corresponding enzyme in human brain. 5-Hydroxytryptamine had no effect on the anticholinesterase action of LSD. Ergometrine, methylergometrine, ergotamine, ergotoxine and dihydroergotamine, in decreasing order of activity, all had considerably less action than LSD on human serum cholinesterase.

G. P.

Morphine and Diaminophenylthiazole, Treatment of Intractable Pain with Large Doses of. F. H. Shaw and A. Shulman. (*Brit. med. J.*, 1955, **1**, 1367.) This paper gives a full account of the earlier short report to *Nature* by the authors (*see J. Pharm. Pharmacol.*, 1955, **7**, 431) on the use of diaminophenylthiazole in doses which prevent the respiratory depression and soporific action of morphine, diamorphine and synthetic narcotics without diminishing their analgesic effect. Large, graded doses, up to 2 grains of morphine are given, with 15 mg. of diaminophenylthiazole by intramuscular injection. It is possible in this way to provide 6 to 8 hours of analgesia per dose, and 4 treatments a day may be given so as to obtain continuous analgesia if required. Diaminophenylthiazole has a high therapeutic index, whether used as a morphine antagonist, as a β:β-methylethylglutarimide synergist or in the treatment of barbiturate intoxication. The dose may be increased to as much as 100 mg. if necessary to prevent respiratory depression. Slow and irregular breathing are not signs of respiratory danger, provided that the respirations are deep and cyanosis is absent. Hyoscine, atropine, barbiturates, chlorpromazine and meclozine may be given at the same time. G. B.

Piperazine, *In Vitro* Activity against *Ascaris lumbricoides*. O. D. Standen. (*Brit. med. J.*, 1955, **2**, 20.) Piperazine citrate, adipate and phosphate were tested *in vitro* against *A. lumbricoides* from the pig. All three salts were equally effective in inducing a state of narcosis in the worms. The drug effect was gradual and did not irritate or kill the worms, and when placed in drug-free medium all affected worms recovered in half to two hours. The appearance of worms in the stools of patients treated less than 24 hours previously and the recovery of such worms when placed in warm Ringer solution suggest that the action of piperazine against *A. lumbricoides* in man is very similar to that against *A. lumbricoides* from the pig *in vitro*. It seems almost certain that the worms in the small bowel are likely to be affected 5 to 6 hours after the drug has been given and will remain fully narcotised while a piperazine concentration of 1 : 560 or more persists. Obviously, to achieve successful treatment drug-affected and narcotised worms must be voided in the fæces before the effects of the drug have worn off. In persons with normal bowel movement this can be achieved by a single large dose in the morning, but it is likely that worm clearance would be more certain if the drug was given before the evening meal. In costive patients it would appear desirable that the normal rate of bowel movement should be assisted by a purge timed to take effect within the limits of duration of worm narcosis. S. L. W.

Poliomyelitis Vaccine: American Trials in 1954. (*Brit. med. J.*, 1955, **1**, 1083.) Two distinct but concurrent trials were carried out in areas with a consistently high incidence of poliomyelitis. In one, uninoculated children were used as controls, while in the other the controls were children who were given an injection of something having no influence on immunity to poliomyelitis. In the first study, covering 127 areas in 33 states, 221,998 children received a complete course of vaccinations while the controls numbered 725,173; in the second study, covering 84 areas in 11 states, 200,745 received a complete course while 201,229 were given the placebo injections. Dosage consisted of 3 intramuscular injections each of 1 ml., the intervals between the first and second injections and between the second and third injections being 1 week and 4 weeks respectively. 129 cases of presumed poliomyelitis were reported up to 4 weeks after completion of the course but it is considered that there was no evidence of disproportionate frequency of the disease in vaccinated children. Within a period of 6 to 7 months after vaccination, 1013 children were reported as having poliomyelitis but of these 14·8 per cent. were doubtful or definitely not suffering from that disease, 67·6 had the paralytic form of poliomyelitis and 17·6 per cent. had the non-paralytic form. Of those definitely suffering from poliomyelitis, 79 per cent. had the paralytic form. In the placebo control areas, the ratio of poliomyelitis cases among patients treated with the placebo to those among the vaccinated patients was 2 to 1, and for the paralytic cases 3·5 to 1; on the other hand there was no difference between the two groups in the attack rates of non-paralytic poliomyelitis. Differences between the treated and untreated groups increased with the severity of the disease; there were no fatal cases in the treated groups. Analysis of the results by reference to age showed a significant protective effect against paralytic poliomyelitis for every age except for 6-year-olds, where, based on 16 and 23 cases respectively, the attack rates were 37 and 53 per 100,000 of population in vaccinated and placebo controls respectively. Effectiveness increased with age. The general conclusions were that the vaccine was 80 to 90 per cent. effective against paralytic poliomyelitis; against disease due to Type I virus the effectiveness was 60 to 70 per cent., but

against disease due to Types II and III virus the effectiveness was 90 per cent. or more. H. T. B.

Radioactive Phosphorus in Treatment of Polycythæmia. J. B. Harman, P. L. de V. Hart and E. M. Ledlie. (*Brit. med. J.*, 1955, **1**, 930.) Radioactive phosphorus (^{32}P) is obtained as a solution of orthophosphoric acid and is prepared by the irradiation of sulphur. Its half-life is 14·3 days, but excretion in the urine reduces the effective half-life to 10 days. Phosphorus is largely incorporated into cells in which proliferation is most active, so that ^{32}P provides a method of irradiating the bone marrow in polycythæmia more selectively than can be done by external irradiation. The ^{32}P was given intravenously as sodium phosphate and the patient was discharged from hospital when excretion reached a safe level (100 mC. in 24 hours), usually after 2 to 3 days. At first, in 28 patients, two injections were given, usually of 5 mC. and 4 mC., at intervals of 6 to 12 weeks; subsequently, in 16 patients, a single dose of 4 to 7 mC. was given. There was no obvious difference in the results. The effect on circulating red cells becomes apparent gradually over 2 to 3 months, and the period before improvement occurs can be shortened by massive venesection with simultaneous infusion of a plasma substitute. Observations over a period of up to 6 years show that in most cases the blood concentration can be kept at or near normal and symptoms are relieved, particularly ulceration and sepsis of toes, hæmorrhages, "congested" feelings and enlargement of the spleen. Pruritus may be resistant. Dyspepsia seems to improve except when ulcers are present. The relief lasted for from nearly 1 to over 4 years, with an average of about 2 years; further treatment can then be given. The largest total amount of ^{32}P given to a patient so far is 36 mC. There was no evidence that radiation treatment produced leukæmia or myelosclerosis, both of which may supervene on polycythæmia. ^{32}P may be harmful if leukæmia does develop. H. T. B.

Reserpine and Chlorpromazine, Potentiating Action of. B. B. Brodie, P. A. Shore, S. L. Silver and R. Pulver. (*Nature, Lond.*, 1955, **175**, 1133.) Reserpine and chlorpromazine have both been shown to potentiate the action of hexobarbitone and ethanol on the central nervous system. Mice injected with 100 mg./kg. of hexobarbitone and 5 mg./kg. of chlorpromazine sleep for considerably longer than those given the barbiturate only. Chlorpromazine does not influence the metabolism of the barbiturate, as barbiturate assays of suitably treated homogenates from animals killed at definite times revealed. Furthermore chlorpromazine has no hypnotic action of its own, even in large doses. Finally, animals killed at various times after injections of hypnotics showed no significant difference in the concentration of barbiturate or ethanol in the brain, regardless of whether or not they had also received chlorpromazine. Experiments with reserpine gave similar results and its potentiation of ethanol was striking. The mode of potentiation of hypnotics by SKF-525A is different from that of chlorpromazine and reserpine, since animals recovering from a hexobarbitone hypnosis revert to a deep sleep when treated with chlorpromazine but are not visibly affected when SKF-525A is given intravenously. G. P.

Reserpine Parenterally in Acute Hypertension. F. A. Finnerty, Jr. and J. G. Sites. (*J. Amer. med. Sci.*, 1955, **229**, 379.) Parenteral reserpine was given to 192 hypertensive and toxæmic patients in hospital. The drug was given intravenously or intramuscularly in a dose of 2·5 mg., repeated at 8 to 12 hour intervals if necessary. In addition 47 of the patients received a preparation of veratrum viride intramuscularly and 24 received hydrallazine intravenously. 91 patients showed an average reduction of 23 mm. Hg in systolic and 19 mm.

Hg in diastolic pressure, an effect which lasted an average of 6·5 hours. More striking than the hypotensive effect was the calming influence of reserpine with frequent complete relief of anxiety. As with oral reserpine the greatest use of the drug given parenterally was as background therapy to the more potent hypotensive agents. Though veratrum or hydrallazine was needed to reduce the arterial pressure and control the toxæmic state in 71 patients reserpine enhanced and prolonged the action of these more potent drugs and reduced the dosage requirement, thus greatly reducing the toxicity and making these agents easier to administer. Since reserpine takes 1½ to 2½ hours to exert its effect when given intravenously (3 to 4 hours when given intramuscularly) it should not be relied on as the sole form of therapy in severe toxæmia, particularly in eclampsia. It should not be administered concomitantly with barbiturates since it potentiates their sedative action in some patients. Experience suggests that reserpine is as effective intramuscularly as intravenously and a rational plan of therapy for acute hypertension in the moderately sick patient should consist of reserpine 2·5 mg. intramuscularly every 12 hours, and, if no hypotensive effect is noted after 2 hours, or if the condition becomes worse, give 0·5 mg. of purified veratrum intramuscularly. S. L. W.

Synnematin B in the Treatment of Typhoid. L. Benavides, B. H. Olson, G. Varela and S. H. Holt. (*J. Amer. med. Ass.*, 1955, **157**, 989.) 16 children ranging from 2½ to 11 years of age, suffering from typhoid (12 severe cases and 4 moderate) were treated with the antibiotic synnematin B; all the patients were poorly nourished. There were 6 controls; 3 were untreated, one was treated with tetracycline, one with tetracycline and chloramphenicol, and one with synnematin B on the 26th day of the disease. The dosage of synnematin B was increased gradually as the study progressed as there was no previous dosage experience in human beings with typhoid. Group 1 consisted of 7 patients receiving 80 mg./kg./day body weight (20 mg. of pure synnematin B per kg./day). Group 2 consisted of 4 patients receiving 160 mg./kg./day (40 mg. of the pure drug per kg./day). Group 3 consisted of 5 patients receiving 350 mg./kg./day (87·5 mg. of the pure drug per kg./day). Patients in Group 1 received the drug for 14 days, and patients in Group 2 and 3 for 12 days. Synnematin B was reconstituted with sterile distilled water and given 4-hourly intramuscularly into the buttocks. In all but one case the response to the treatment was similar. Regardless of the day of the disease on which treatment began, the temperature returned to normal, the toxæmia was relieved, the appetite increased, and the mental alertness improved in 2 to 6 days. There was a pronounced improvement in general well-being. In some patients the fever fell by crisis and in others by lysis during the period of treatment, irrespective of the dosage schedule used. The clinical response in Groups 2 and 3 was closely comparable and was far superior to that in Group 1. There were 3 relapses and 1 persistent case, in all of which recovery occurred on further treatment; 2 of the relapses and the persistent case were in Group 1. *Salm. typhi* disappeared from the blood and fæces of every patient during treatment, the organism was recovered from the blood of 3 of the 16 patients after therapy but was never recovered from the fæces of any patient after therapy. This is contrary to the usual experience with chloramphenicol. On the basis of observation in this series it would appear that synnematin B is bactericidal rather than bacteriostatic. Data on blood levels and excretion indicate that the highest levels are attained in 1 hour after injection and that the drug is excreted rapidly by the 4th hour. Urinary concentrations were very high,

(ABSTRACTS *continued on page 792.*)

PHARMACOPŒIAS AND FORMULARIES

THE PHARMACOPEIA OF THE UNITED STATES OF AMERICA

FIFTEENTH REVISION*

REVIEWED BY FRANK HARTLEY

The publication in July, 1955, by the Board of Trustees of the United States Pharmacopeial Convention of the U.S.P. XV, to become official from 15th December, 1955, inevitably arouses much interest among those concerned with substances and preparations of therapeutic importance. Such interest may lie in the choice of substances and preparations added or deleted by the revision, in the range of information now given, in the techniques adopted for the various standardisations, or in the method of presentation.

In the Fourteenth Revision, 119 substances and preparations were deleted and 202 new substances and preparations were added. Of these 202 items no fewer than 44 have failed to secure inclusion in the U.S.P. XV. In all, 163 articles included in 1950 in the U.S.P. XIV have not been admitted to the U.S.P. XV. There are 242 "new admissions" to the U.S.P. XV.

The selection of substances and preparations for addition and deletion is perhaps the most difficult task of all in the revision of a pharmacopœia. In the words of the preface, "the Pharmacopeia must reflect with fidelity the best practices of medicine and pharmacy in providing standards of purity and potency for drugs of established merit and indispensability. These drugs must constitute, in the words of the first Pharmacopeia, therapeutic agents 'the utility of which is most fully established and best understood'. To this extent, the U.S.P. is a therapeutic guide, the soundness of which is tempered only by that of the judgment of those who select the articles to be included. Yet, by its nature, the process of selection can scarcely be perfect, for no means has been found to ensure, at least by the time of publication, that all drugs included are of equal merit and that no others equally meritorious are omitted. In view of to-day's rapid progress in medical sciences, a varying degree of lag is inevitable".

Many of the additions were clearly to be expected, for example, cortisone acetate, hydrocortisone and its acetate, bacitracin, erythromycin, neomycin sulphate, oxytetracycline and tetracycline, polymixin B sulphate, aminosalicylic acid and its sodium and calcium salts, and isoniazid, diethylcarbamazine citrate, sulphacetamide, sulphamezathine and sulfisoxazole (sulphafurazole). But there are also some surprises. It is surprising to find that in 1955 the following qualified to become "new additions"—calcium pantothenate, dehydrocholic acid, dienoestrol, juniper

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tar, sulphurated potash and white lotion (prepared from zinc sulphate and sulphurated potash) but, perhaps most of all, sulphapyridine, especially as sulphaguanidine, sulphanilamide and sulphathiazole are deleted. Specially interesting additions appear to be protein hydrolysate injection, sterile suspensions of progesterone and testosterone, water-miscible vitamin A, sodium radio-iodide (^{131}I) solution and sodium fluoride (given the category "Dental prophylactic" and Dose—usual—0·7 to 1 part per million in drinking water). Among the deletions are amphetamine and its inhalant, aloin, barbital (barbitone) and its sodium salt and tablets, boric acid ointment, calcium lactate, cascara sagrada tablets, digitalis tincture, ephedrine and its hydrochloride (though the sulphate is retained), ferric ammonium citrate, gentian, neocinchophen, pancreatin and scopolamine hydrobromide tablets.

Interesting innovations are the addition to the monographs of statements of category to "indicate the therapeutic basis of admission of the drug and . . . the best known pharmacologic action of the article or of its active ingredient". As the definition of category is often limited to one word or at most a sentence, the resolution of many definitions of category must have involved much discussion. The reviewer believes, however, that with the increasing number of new substances whose names tax the memory of practitioner and pharmacist alike, the addition of a definition of action, however brief and approximate, cannot fail to be helpful and will diminish the risk of accidental confusion between the names of some drugs used for entirely different purposes. As an example, bethanechol chloride is given as "Category: parasympathomimetic", and benzethonium chloride as "Category: local antibacterial".

Cautionary italicised notes are added to monographs about poisonous substances, as for example, "Caution—Atropine sulfate is very poisonous" and to monographs of substances whose solutions deteriorate, as for example, "Caution—Prepare solutions of Calcium Acetylsalicylate within 24 hours of administration. Under no circumstances use a solution if its color is darker than that of a freshly prepared solution".

Especially interesting also are pages 800–945, prefaced by a separate contents page on "General Tests, Processes and Apparatus". In the section on General Information and Procedures are descriptions of chromatography (on columns and on paper), pharmaceutical preparations (capsules, elixirs, emulsions, extracts, gels, inhalants, lotions, magmas, ointments and ointment bases, pastes, solutions, spirits, sprays, suppositories, suspensions, syrups, tablets, tinctures and aromatic waters), radioactivity and its determination, U.S.P. reference standards, including a list of twenty-four U.S.P. steroid substances now available, spectrophotometry, sterilisation, titrimetry, including titration in non-aqueous solvents, and analysis of vegetable drugs. There follow sections on apparatus for tests and assays, bacteriological tests, biological reagents for clinical tests, including blood grouping and typing serums and thromboplastin, biological tests and assays (including an excellent 15-page section on the design and analysis of biological assays), chemical tests and assays, and physical tests and determinations.

The pyrogen test is an improvement on the previously official test in using summation of rectal temperature rises in groups of rabbits as well as maxima in individual rabbits, but still leaves potential ambiguity in the borderline case, which could be removed without necessarily employing more rabbits, by applying sequential sampling technique. The vitamin B_{12} activity assay continues to be based on the response of *Lactobacillus leichmanii* and the medium still includes centrifugal canned tomato juice, whereas, in this country, we now regard *Ochromonas malhamensis* as more specific in its response and in consequence obtain lower but probably truer potencies.

The Kjeldahl nitrogen determination still adheres to fixed proportions of potassium or sodium sulphate and sulphuric acid for digestion with copper sulphate as catalyst, whereas the B.P. 1953 recognises that greater precision is achieved by adapting the proportions to the nature of the nitrogenous substance being examined.

The weight variation of tablets and of contents of containers of sterile drugs continues to depend on the use of 20 unbroken tablets and 20 containers respectively. A weight variation test for contents of capsules is now added using initially 20 capsules, but if the prescribed limits are exceeded, may be repeated on a further 40 capsules.

For implantation, pellets of desoxycortone acetate and testosterone may be prepared only by compression of the steroid, unlike the B.P. 1953 which recognises fusion as the preferred method. Further, the B.P. 1953 requires pellets to be distributed singly in sterile containers sealed so as to exclude micro-organisms, whereas the U.S.P. XV directs the pellets to be preserved singly in *tight containers*. The general notices define a tight container as capable of tight re-closure, which hardly seems likely to ensure the retention of the sterility of the pellets.

In striking contrast to the British Pharmacopœia, the general notices of the U.S.P. XV state that capsules and tablets may be manufactured with suitable diluents, bulking agents, *colors*, lubricants and adhesives, that "tablets may be coated with harmless ingredients and that capsules, and tablet coatings, may be colored with a pharmacopeial article or a color certified as suitable for coloring drugs under the terms of the Federal Food, Drug and Cosmetic Act". Enteric coatings are permitted and hypodermic tablets continue to be recognised. Buccal and sublingual tablets are permitted to soften but not disintegrate in the disintegration test, which continues to employ the basket-rack assembly with the tablets raised and lowered on a wire mesh through aqueous liquids at about 37° C. Disintegration times of 30 minutes for antihistaminic or barbiturate tablets, of 1 hour for tablets such as carbasone, nicotinamide or penicillin and 2 hours for tablets such as those of ethisterone and ferrous gluconate are permitted.

Substances other than those specified in the monographs are permitted to be added to enhance the permanency or usefulness of the pharmacopœial article or preparation, provided they are non-toxic and harmless in the amounts administered and do not interfere with the therapeutic efficacy or with the tests and assays prescribed. Alcohol denatured by

not more than 10 per cent. by volume of methyl alcohol or acetone may be used in the manufacture of preparations in which alcohol is used as a solvent, provided the finished preparations are identical with those in the monographs and comply with the prescribed standards.

Latin sub-titles which were retained in the U.S.P. XIV have now been omitted and are retained as synonyms only where the Latin stem differs markedly from the English. Apothecary equivalents have been omitted from the statements of dosage and sizes available. It is stated that an intention to retain the equivalents for those drugs still customarily prescribed in apothecary units proved to be a greater source of confusion than outright abandonment. It is also stated that "For those who still use the apothecary measures a table given on the back cover will prove useful". These equivalents are as near to being out of the U.S.P. as is physically possible!

Non-proprietary names recommended for international use by the World Health Organisation are given as synonyms where these differ from those common in the medical literature of the United States. As to identity of the standards of the U.S.P. and of the International Pharmacopœia, it is recognised that small differences may exist, but that there are few instances in which an article conforming to the U.S.P. standards will fail to conform also to those of the Ph.I.

The U.S.P. XV, providing as it does the most up-to-date compilation of modern medicaments and their standards and tests, cannot fail to merit close study. There is much to be learnt from the vast mass of new information which it contains. Not all British readers will agree with all the decisions which have been taken in its compilation. All will, however, appreciate and admire the skill and attention to detail with which Dr. Lloyd C. Miller, Director of Revision, and his team of collaborators have completed their huge task.

(ABSTRACTS *continued from page 788.*)

and concentrations in blood and urine increased with increasing dosages. No toxic effects were observed except for a chill reaction with one lot of the drug. The carrier state was not observed in any patient up to 3 months after treatment.

S. L. W.

Tricyclamol, a New Anticholinergic Agent. A. G. Zupko and L. D. Prokop. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 160.) The degree to which sweating was suppressed in human subjects to whom tricyclamol had been administered was determined by measuring the sweat excreted from several areas of the body. Suppression was greatest on the forehead and dorsum of the hand and least on the upper arm and thigh. Hyperhidrosis was effectively controlled in 76 per cent. of the subjects. The peak effectiveness was reached 60 to 90 minutes after administration of the drug and the duration of the effect was 4½ to 5 hours. The maximum suppression of the parotid salivary output was 26·8 per cent., 60 minutes after the administration of 0·1 g. of tricyclamol. The substance compared favourably with other anticholinergics in regard to anhidrotic and antisialogogic side effects, but some subjective manifestations of parasympathetic inhibition were observed. Low doses were found to inhibit locomotor activity in the rat, but cerebral stimulation was indicated by convulsions when the toxic dose was reached.

G. B.

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