REVIEW ARTICLE

FOLIC ACID, VITAMIN B_{12} AND ANÆMIA

II—MICROBIOLOGICAL ASPECTS
III—FOLIC ACID AND VITAMIN B_{12} IN MEGALOBLASTIC ANÆMIA

II—MICROBIOLOGICAL ASPECTS*

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Studies with microorganisms† have made a considerable contribution to research and development work in this field from three aspects :—(*a*) the quantitative estimation of the two B-group vitamins involved, (*b*) the production of the two substances in quantity sufficient for fundamental research on their chemical properties and possibly even bulk production pending chemical methods, and (*c*) fundamental studies on their function in cell metabolism which may eventually throw light on the basic biochemical lesions in anæmia. All three of these applications spring from the fact that comparative studies of the nutrition of organisms of widely different types (mammals, birds, insects, protozoa, fungi, bacteria) show a common requirement for substances of the vitamin B group ; it is likely therefore that these substances play their part in basic cell reactions which are common to all cells. A given organism may not have an essential nutritional requirement for a certain factor but this normally means that it is able to synthesise it for itself—the requirement in metabolism still exists. In those cases in which it is known, the function in metabolism of members of the vitamin B group has proved to be that of coenzyme or prosthetic group of enzymes concerned in key cell reactions. This has not been demonstrated with folic acid and B_{12} but it may form a useful working hypothesis.

Microbiological Assay‡

Since several microorganisms require the presence of either folic acid or vitamin B_{12} in the growth medium, and since there is a quantitative relationship between dosage and growth-response, it has been possible to estimate these substances in this way ; it must be stated however that these two microbiological assays (especially that of B_{12}) have proved more difficult in practice than those for other substances. Thus the organisms (*Lactobacilli*) most commonly used in the assay of vitamin B_{12} are micro-aerophilic whilst the factor appears not to be required for purely anærobic growth. The quantitative response may therefore depend on fine control of the degree of aeration and also on the quantity of reducing agents present.

The advantages of a microbiological assay method are mainly (*a*) that the criterion is a simple one (amount of growth or some dependent

* Based on a paper read at the British Association for the Advancement of Science Annual Meeting, Newcastle, 1949.

† See references 1, 2, and 3.

‡ See references 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15.

function such as acid production), (*b*) that results may be obtained rapidly, often in less than 24 hours, and (*c*) that cells relatively deficient in factor for use as inoculum may readily be obtained by growth on partially deficient media. There are, of course, also disadvantages. In the case of folic acid, for example, conjugated forms exist in natural materials which are available sources to the animal (which has enzymes for liberating the free factor) but not to the test microorganism. With vitamin B_{12} it is known that other red substances exist in liver concentrates which support the growth of the assay organism but whose possible activity in pernicious anæmia is not yet known. It is necessary with both factors to bear in mind the possibility that the microbe may respond to unknown precursors of the vitamin (*i.e.*, be able to complete the synthesis) whilst the animal cannot; the reverse may also be true. Such difficulties have been encountered with other vitamins.

The fact that rapid microbiological assays were available certainly considerably aided the purification, isolation and synthesis of pteroylglutamic acid (synthetic "folic acid") by the Lederle workers; it also played an important part in the isolation of crystalline B_{12} by one of the two teams (Merck) who were engaged on the problem.

Microorganisms as Sources of Folic Acid and Vitamin B_{12}§

It was mentioned above that when organisms do not require added growth factors they are often found to synthesise them. Sometimes the amount synthesised appears to be in excess of cell requirement and the organism may prove to be a comparatively rich source of the factor. The chemical degradation studies which led to the synthesis of pteroylglutamic acid were carried out with a crystalline folic acid conjugate isolated from the culture fluid of an unspecified *Corynebacterium*. Again, there is a recent report of the isolation of crystalline vitamin B_{12} from a strain of *Streptomyces griseus*; in view of the small amount present in liver and the already undoubted complexity of chemical structure, this might well be an important industrial source.

Function in Metabolism¶

Although immediate practical considerations may be met by the isolation and sufficient purification of factors such as folic acid and vitamin B_{12}, it is clear that for a full understanding of the disorders which follow their absence it is necessary to know their precise function in cell metabolism; the observed clinical symptoms may not be primary. Although this problem is not yet solved for the two substances under consideration, work on microorganisms is providing valuable initial clues. Such work has been of considerable importance in working out the function of several other members of the vitamin B group. Briefly, the present evidence suggests that both folic acid and vitamin B_{12} may be involved in some way in the biosynthesis of the desoxyribonucleic acids which are key constituents of the nuclear material of cells.

Now we know comparatively little about the synthetic mechanisms

§ See references 6, 8 and 16.
¶ See references 2, 3, 5, 7, 17, 18, 19, 20, 21, 22, 23, 24 and 25.

of the cell and only in rather few cases has it been possible to obtain such synthesis in non-viable systems. But even in the more complicated case in which growth occurs it is possible to obtain valuable information from studies of the nutrition of microorganisms. Thus if it is found that a given growth factor may be effectively replaced by larger quantities of another substance of quite different chemical type it suggests that the former is required in some catalytic system involved in the synthesis of the latter. Similarly when growth is specifically inhibited in a competitive manner by a substance which is chemically analogous to the growth factor, growth may be found to be restored by a substance of different chemical type and the same very tentative conclusions may be drawn.

Evidence of this sort indicates a function for folic acid (probably in the form of a higher compound, folinic acid, [26,27]) in the synthesis of pyrimidine, thymine (or perhaps its riboside, thymidine) and the purine bases. This view is supported by the finding that cells of *Lactobacillus casei* grown in medium partially deficient in folic acid contain only half the normal amount of desoxyribonucleic acid.

p-Aminobenzoic acid, an essential growth factor which is a moiety of the folic acid molecule, appears also to be concerned in the synthesis of certain amino-acids but it is not yet certain that this function occurs *via* the formation of folic acid.

In the case of the *Lactobacilli* which need vitamin B_{12} it has been found that the requirement can be met by various desoxyribosides, including thymidine. It is possible therefore that at least one function of vitamin B_{12} is in the synthesis of this type of structure.

It is unlikely however that vitamin B_{12} and folic acid are involved at different stages in the synthesis of some coenzyme-like factor required for desoxyriboside formation : *Lb. leichmannii*, for example, requires for growth both factors ; neither can replace the other. It therefore seems more likely on the present evidence that they are involved at separate stages of the synthetic pathway which eventually leads to desoxyribosides. Thus with *Lb. Leichmannii* the requirement for both factors can be met (though not optimally) by thymidine alone. The latter completely replaces vitamin B_{12}, but gives growth without folic acid or without both only after a lag. Finally there are organisms (*e.g.*, *Lb. bifidus*) whose need for thymidine cannot be replaced by vitamin B_{12}, which would indicate a failure in synthesis at a later stage.

It must be emphasised strongly that such inter-relationships are at present not proved and must be considered only as working hypotheses ; they may serve as a guide to further experiments which will yield further facts.

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III—FOLIC ACID AND VITAMIN B_{12} in MEGALOBLASTIC ANÆMIA*

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This is a brief survey of work done at the Royal Victoria Infirmary, Newcastle upon Tyne, in co-operation with Dr. R. B. Thompson, who performed marrow cultures, Dr. W. Walker, who followed the survival of transfused erythrocytes, and Dr. L. W. Carstairs, who intubated the small intestine. Vitamin B_{12} and vitamin B_{12C} were supplied by Dr. E. Lester Smith, and Dr. W. F. J. Cuthbertson, also of Glaxo Laboratories, was responsible for microbiological assays. A fuller account of the various investigations is being published elsewhere.

Two groups of megaloblastic anæmia will be considered:—(a) Addisonian pernicious anæmia in which there is gastric atrophy and permanent loss of Castle's intrinsic factor; (b) Non-Addisonian megaloblastic anæmias associated with pregnancy or with intestinal disorders such as stenosis or the sprue syndrome.

Parenteral Administration of Vitamin B_{12} in Pernicious Anæmia

Our findings amplify earlier reports on the effect of vitamin B_{12} given parenterally in pernicious anæmia (West[1], Ungley[2,3,4], Hall and Campbell[5], Spies, Stone, Kartus and Aramburu[6], Berk, Denny-Brown, Finland and Castle[7], Bethell, Meyers and Neligh[8], Spies, Suarez, Garcia Lopez, Milanes, Stone, Lopez Toca, Aramburu and Kartus[9], West and Reisner[10]).

Vitamin B_{12} is effective in Addisonian pernicious anæmia but in only

* Based on a paper read at the British Association for the Advancement of Science, Annual Meeting, Newcastle, 1949.

some types of non-Addisonian megaloblastic anæmia. The response of some 50 patients with pernicious anæmia to the injection of vitamin B_{12} have been analysed. The earliest change was a maturation of megaloblasts in the bone marrow. Clinical improvement followed and the erythrocytes, platelets and leucocytes increased to normal levels. For constructing a dosage response curve reticulocyte responses proved unreliable and we used the increase of erythrocytes in 15 days. Doses were spaced logarithmically from 1·25 to 160 μg. Ten μg. was enough, on average, to give a response satisfactory according to the "average" standard of Della Vida and Dyke[11], but larger doses gave greater responses which were roughly proportional to the logarithm of the dose in the range 5 to 80 μg. (Ungley[12]). Further tests at high and low levels of dosage are necessary to complete the dose response curve which will probably be sigmoid in form, rising gradually then steeply from the threshold dose and flattening out to a horizontal level when supramaximal doses are reached.

In 21 patients followed for 6 to 18 months neurological manifestations if present were improved or unchanged, and fresh symptoms did not develop. More direct evidence of the efficacy of vitamin B_{12} against the neurological manifestations of pernicious anæmia was obtained by treating 8 established cases of subacute combined degeneration. The usual dosage was 40 μg. a week. Using a quantitative method of neurological assessment, significant improvement was demonstrated in each case. The improvement continued for about 6 months, after which a state of arrest was maintained. The degree of residual neurological defect was proportional to the duration of difficulty in walking. Comparison with 44 cases treated before the war showed that vitamin B_{12} was as effective as parenteral liver extract, crude or refined, in the treatment of subacute combined degeneration of the cord (Ungley[13]).

These findings are in agreement with other published work which has already been reviewed (Ungley[12,13]).

Vitamin B_{12} in Non-Addisonian Megaloblastic Anæmias

In 5 patients with megaloblastic anæmia of pregnancy or the puerperium single injections of 63 to 80 μg. of vitamin B_{12} had no effect (apart from a slight reticulocytosis in one case), whereas subsequently all patients responded to folic acid.

In megaloblastic anæmia with intestinal disorders the results were variable. A patient with intestinal stenosis responded quite well to 80 μg. In a patient with non-tropical sprue vitamin B_{12}, even in repeated large doses, was only partially effective; in this patient folic acid, too, was only partially effective. In a patient suffering from thyrotoxicosis with steatorrhœa, vitamin B_{12} failed completely, although subsequently there was an excellent response to small doses of folic acid.

Factors Related to Vitamin B_{12}

By a combination of microbiological assay and partition chromatography on paper Cuthbertson and Lester Smith[14] have demonstrated that

liver extracts contain at least 4 substances related to vitamin B_{12}:—(a) A fast-moving red component—vitamin B_{12} itself. (b) A slow-moving red component. (c) An unidentified substance ("Band 3"). (d) Thymidine. The slow-moving component, which consists of several compounds, has proved effective in a dose colorimetrically equivalent to 10 μg. of vitamin B_{12}. Thymidine, on the other hand, appears to be without effect, at least in a dose of 48 mg. (Ungley[4]). Since vitamin B_{12} proved just as effective as crude liver extract there is no necessity to postulate a need for multiple factors in pernicious anæmia and subacute combined degeneration.

Absorption of Vitamin B_{12} from the Alimentary Tract

Oral administration of 5 μg. daily was ineffective, whereas the same quantity with 50 ml. of normal gastric juice daily produced a satisfactory response. In another patient an excellent response was obtained by administering 50 μg. with 500 ml. of normal gastric juice in a single dose. In a third patient 40 μg. with only 150 ml. of gastric juice proved inadequate. Filtration of the gastric juice through a Seitz filter led to loss of intrinsic factor activity.

The mechanism whereby normal gastric juice facilitates absorption of vitamin B_{12} or prevents its destruction in the gastrointestinal tract remains obscure. No response followed the application of 5 μg. of vitamin B_{12} daily to the buccal mucosa, although the same quantity given by mouth with 50 ml. of gastric juice gave a good response.

To test the possibility that, even without gastric juice, vitamin B_{12} might be absorbed if protected from contact with the intestinal contents, we isolated a segment of the small intestine between two balloons on a Miller-Abbott tube. After washing to remove intestinal contents 40 μg. of vitamin B_{12} was instilled into this segment. There was no response. Subsequently there was a submaximal response to the same dose of vitamin B_{12} given orally with 150 ml. of normal gastric juice (an inadequate amount), and thereafter maximally to a single injection of 40 μg. The problem of the mechanism of absorption of vitamin B_{12} remains unsolved. A full account of this work will appear shortly[15].

Toxic and Hæmolytic Aspects

Not all the facts can be explained on a simple nutritional basis. Both in true pernicious anæmia and in non-Addisonian megaloblastic anæmias toxic and hæmolytic factors may play a part. Since methæmalbumin may be present in the plasma, some of the hæmolysis must be intravascular. Destruction of poorly-formed red cells is not a sufficient explanation. In collaboration with Dr. W. Walker we have followed the survival of transfused cells from normal donors. In most patients with pernicious anæmia such cells are eliminated at a normal rate, surviving about 120 days. In three cases, however, the transfused cells were rapidly destroyed. A change to a normal rate of elimination occurred after vitamin B_{12} in 2 cases and spontaneously in 1 case. In 3 patients with non-Addisonian megaloblastic anæmia associated with pregnancy or

intestinal disorders excessive hæmoylsis changed to a normal rate of elimination about 2 weeks after giving folic acid. The dramatic change in the rate of destruction after treatment suggests that vitamin B_{12} and folic acid may be concerned in detoxicating or preventing the production of a hæmolytic agent.

My colleague, Dr. R. B. Thompson, confirms the finding of Rusznyák, Löwinger and Lajtha[16] that the maturation of megaloblasts in marrow culture is accelerated by the addition of normal plasma, but inhibited by pernicious anæmia plasma. The greater the concentration of pernicious anæmia plasma the less the megaloblasts mature. This suggests active inhibition rather than mere absence of a maturation factor. Low concentrations of folic acid (1 μg./ml.) added to an inert medium cause rapid maturation of megaloblasts, but pernicious anæmia plasma antagonises this effect. The maturing effect of small amounts of normal plasma is also antagonised by the addition of pernicious anæmia plasma. Larger amounts of folic acid or of normal plasma overcome this antagonism. Cerebrospinal fluid from patients with pernicious anæmia has an effect similar to their plasma, so that the inhibiting factor is probably ultrafiltrable. The action of vitamin B_{12} on maturation of megaloblasts *in vivo* is presumably indirect, for unlike folic acid it fails to accelerate maturation *in vitro*.

Other relevant facts follow. Early lesions in the spinal cord in pernicious anæmia are spotty in distribution and are often related to vessels. They suggest the action of a substance destructive to myelin rather than a simple nutritional deficiency. The urinary excretion of certain phenolic compounds is excessive in relapse and becomes normal in remission. These phenolic substances possibly arise from the incomplete metabolism of tyrosine or from bacterial action on tyrosine in the small intestine.

The metabolism of liver slices from rats deficient in folic acid is deficient unless folic acid is added (Rodney, Swendseid and Swanson[17]) Another potentially toxic substance is indol, a product of the metabolism of tryptophane. Indol given to pigs receiving a diet deficient in vitamin B-complex produces hæmolysis and macrocytic anæmia, a result not observed in normal pigs (Rhoads, Barker and Miller[18]).

For the production of macrocytic anæmia following intestinal stenosis, loops or blind sacs, stagnation of intestinal contents and bacterial infection seem to be essential. In the rats of Watson, Cameron and Witts[19] many weeks elapsed before the animals suddenly became ill and anæmic. My tentative interpretation is that a toxic and hæmolytic factor was produced in the infected contents of the blind sac. During the latent period detoxication occurred through enzymes using folic acid and possibly vitamin B_{12}, stores of which were gradually depleted in the process. When these stores were exhausted detoxication failed, resulting in sudden illness and anæmia. Folic acid restored powers of detoxication and relieved the anæmia.

Something of the same kind may occur in the small intestine of patients with pernicious anæmia as a result of bacterial infection and alteration in food residues due to lack of gastric enzymes.

A tentative hypothesis based on these findings, some of which require confirmation, is that in megaloblastic anæmias, toxic as well as nutritional factors play a part. These are responsible for megaloblastic erythropoiesis, for some of the hæmolysis and possibly for the lesions in the spinal cord. Potentially toxic material, for example indol or a phenolic compound, arises either from bacterial action on protein metabolites in the small intestine or from a defect in intermediary metabolism of some substance such as tyrosine or tryptophane. Detoxication or a return to normal metabolism in which production of toxic material ceases, occurs through the action of enzymes using folic acid and vitamin B_{12}.

Wills Factor

Is there a hæmatopoietic factor other than vitamin B_{12} or folic acid present in whole liver and in yeast? Why should yeast extracts which appear to contain no vitamin B_{12} when tested microbiologically or in animals be effective as a source of Castle's intrinsic factor? Can the effect of yeast extract in non-Addisonian megaloblastic anæmias be explained by their content of folic acid or folic acid conjugates? In a patient with pernicious anæmia of pregnancy an alcoholic extract of yeast was effective in doses which contained less than 40 μg. of folic acid tested both microbiologically and in rats for conjugates. Moreover, the daily excretion of folic acid in the urine during the period of administration of yeast was extremely low—only 1 to 5 μg. per day. There was no secondary reticulocyte response after the subsequent administration of 2·5 mg. of folic acid daily, although the average daily excretion of folic acid in the urine rose to 700 μg. per day. The evidence is against folic acid being the cause of the hæmatopoietic response observed with yeast. Further work is necessary.

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RESEARCH PAPERS

THE PHOTOMETRIC DETERMINATION OF 2:3:5:6-TETRACHLORONITROBENZENE

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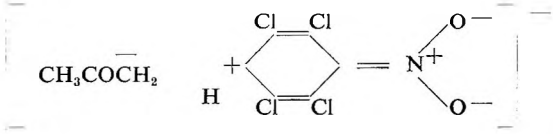
The current interest in 2:3:5:6-tetrachloronitrobenzene as a prominent inhibitor of sprouting and rotting of potatoes during storage has made a rapid method for the determination of small amounts of this substance a topical problem. The toxicity of the compound has recently been discussed in this journal by Buttle and Dyer[1].

This substance was first prepared by Jungfleisch[2] and later by Beilstein and Kurbatow[3] by nitration of 1:2:4:5-tetrachlorobenzene. Page[4] prepared the compound by chlorination of nitrobenzene in presence of iron chloride. It was studied later by Holleman[5], Berckmans and Holleman[6], Dyson, George and Hunter[7], and by Hüffer[8]. Berckmans and Holleman[6] were able to demonstrate that when treated with sodium methylate it was hydrolysed to 2:3:5:6-tetrachloroanisol and nitrite ions.

The benzene ring is heavily loaded with negative substituents. In this case the single nitro group is enough to stabilise a *p*-quinoid structure of the addition product (see below) when tetrachloronitrobenzene is allowed to react with active methylene groups in alkaline solution. This reaction may be formulated in the same way as the general reaction between aromatic nitro compounds and active methylene groups, see Canbäck[9]. Thus when tetrachloronitrobenzene is dissolved in acetone and alcoholic potassium hydroxide is added a brilliant red-bluish colour is produced. When the colour has faded and the solution is acidified with nitric acid, chloride ions are not present in the solution while nitrite ions are easily shown to be present. The structure of the coloured anion might thus be formulated in the following way: —



We have tried to use this reaction in a quantitative way. If the amount of the reagents, the reaction time, the water content of the acetone, etc., are rigidly controlled it is possible to use the reaction for the determination of small amounts. However, the shape of the standard curve indicates that the destruction of the compound is rather rapid and not always uniform. We tried, therefore, to find a better method. If tetrachloronitrobenzene is hydrolysed quantitatively to 2:3:5:6-tetrachloroanisol and nitrite ion the latter may be easily determined by any of the classical methods for the determination of nitrite, see Allport[10] and Snell and Snell[11]. We have preferred to use the reagents recom-

mended by Jendrassik and Falcsik-Szabó[12], who used procain and α-naphthylamine. After some trials the following method was adopted:—

50 to 300 μg. of tetrachloronitrobenzene is dissolved in 5 ml. of acetone and 5·0 ml. of 0·5N alcoholic potassium hydroxide is added. The solution is refluxed on a boiling water bath for 15 minutes. After cooling to room-temperature 5·0 ml. of diazo reagent I (see below) and 5·0 ml. of diazo reagent II are added. After standing for 30 minutes the coloured solution is transferred to a 25 ml. volumetric flask by the aid of ethyl alcohol (95 per cent.) and made up to the volume with ethyl alcohol (95 per cent.). The extinction is measured in a photometer with a filter with maximum transmission at about 500 mμ. A blank is made on 5 ml. of acetone.

Diazo reagent I: 3·0 g. of procain hydrochloride is dissolved in a mixture of 15 ml. of glacial acetic acid and 85 ml. of distilled water.

Diazo reagent II: 0·20 g. of α-naphthylamine is dissolved in 30 ml. of boiling distilled water and filtered through a warm funnel. The filter is washed with 2 × 30 ml. of hot water. To the filtered solution 30 ml. of glacial acetic acid and water to 150 ml. are added.

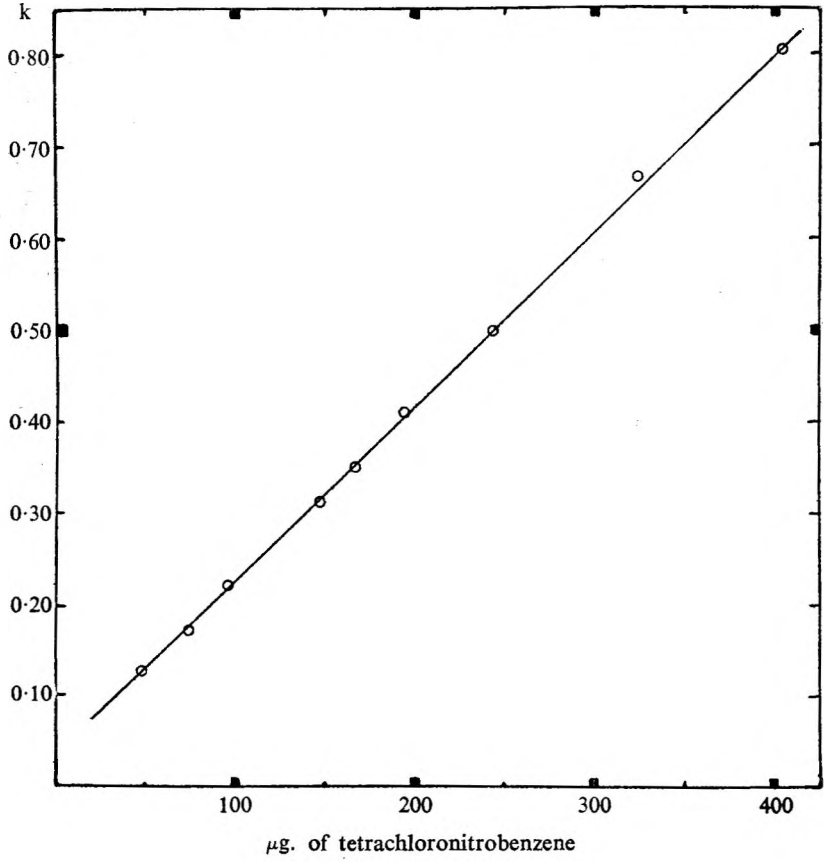


FIG. 1. Standard curve.

The reagents are stable for at least 1 week.

In Figure 1 the standard curve is shown (Lumetron photo-electric Colorimeter, filter M 515). The absorption curve of the azo dye is given in Figure 2. Figures 3 and 4 give respectively the rates of hydrolysis (expressed as extinction coefficient *versus* time) when treated (as described in the method) with 0·5N alcoholic potassium hydroxide at room-temperature and when refluxed on a boiling water-bath. In Figure 5 the rate of development of the colour after the addition of the diazo reagents is shown.

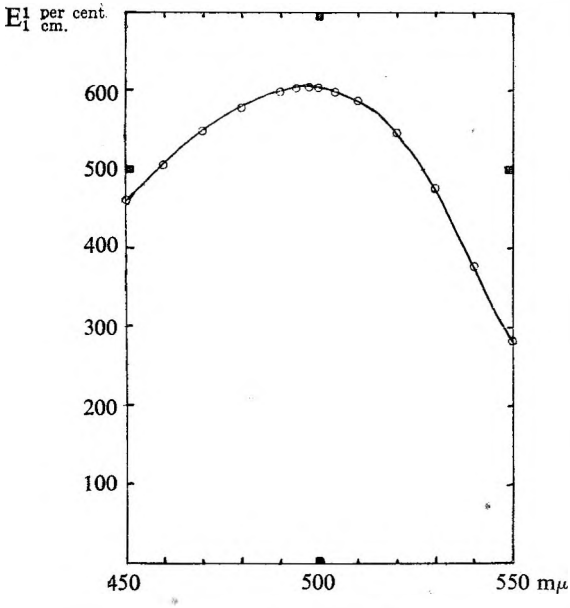


FIG. 2. Absorption curve (Beckman Quartz spectrophotometer model DU).

To extract the compound from potatoes, etc., acetone, light petroleum (e.g., Skellysolve F) or other organic solvents which can be removed by distillation may be used. This extraction increases the specificity of the method as the number of organic compounds giving nitrite ions when treated with alkali is relatively small Aliphatic nitrates and some nitro benzene derivatives may interfere.

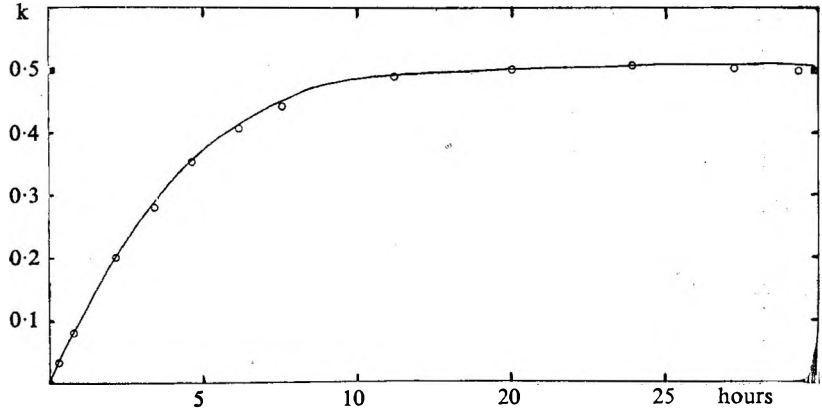
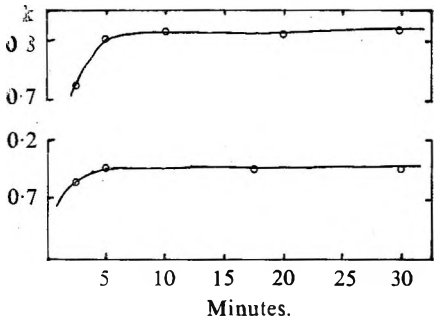


FIG. 3. Hydrolysis at 20°C.



If large quantities of fat are present in the acetone or light petroleum extract (e.g., when testing rat fæces) the final coloured solution may be turbid. However, the coloured solution can be filtered clear, but this operation must be included in the operations leading to the standard curve and in the blank.

FIG. 4. Hydrolysis on the water-bath. Lower curve about 60 μg. Upper curve about 400 μg.

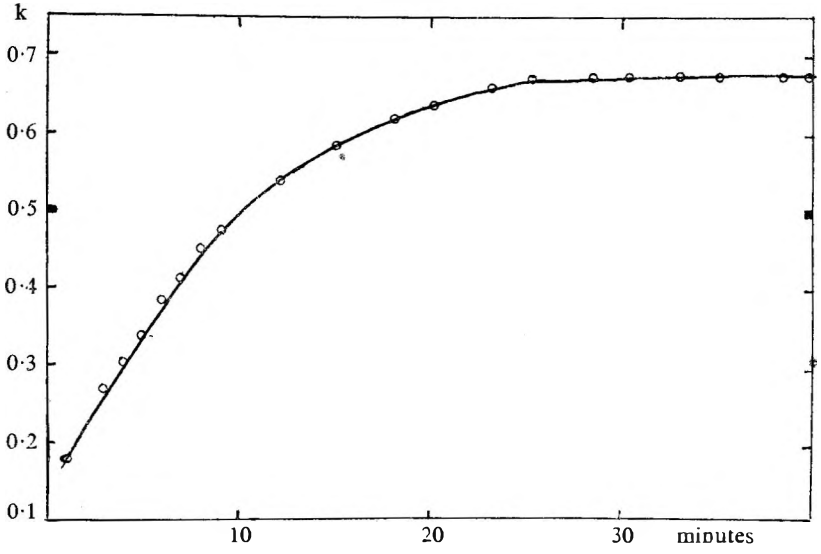


FIG. 5. The development of the colour.

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THE ANALYSIS AND DETERMINATION OF PHENYL GLYCEROL ETHERS WITH PARTICULAR REFERENCE TO PHARMACEUTICAL PREPARATIONS

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Aromatic ethers of glycerol have for some time been known to possess interesting pharmacological properties[1,2]. The group of compounds is also interesting from a physicochemical standpoint in that the attachment of the glycerol molecule to the benzene nucleus produces a derivative soluble to some extent both in polar and in non-polar solvents. A general investigation of the group of compounds by Berger and Bradley[3,4,5] showed that αβ-dihydroxy-γ(2-methylphenoxy)-propane (myanesin, mephenesin) produced muscular relaxation and paralysis without causing respiratory arrest or influencing the blood pressure; the injection of the compound in anæsthesia was described by Mallinson[6], who advocated its use as a synthetic muscle-relaxing agent. Subsequently its use was reported in tetanus[7] and by mouth in the form of tablets and elixir for the treatment of spastic and hyperkinetic states by Berger and Schwartz[8].

Another compound in the same chemical group which has become available is α-*p*-chlorophenyl glyceryl ether (αβ-dihydroxy-γ-(*p*-chlorophenoxy)-propane, (gecophen, chlorphenesin), introduced by Hartley[9] as an antifungal agent. This is used in an ointment and in a dusting powder, while pessaries for the treatment of mycotic infection of the vagina have been used by Mackinlay[10]. It is clear that methods of analysis of such compounds, particularly with reference to their pharmaceutical preparations are required.

αβ-*Dihydroxy-γ-(2-methylphenoxy)-propane* (myanesin, mephenesin) forms colourless crystals m.pt. 70° to 71°C., soluble to the extent of approximately 1 per cent. in water at 22°C., very soluble in alcohol and propylene glycol; solutions have a neutral reaction and are stable to heat, light, acids and alkalis. A proposed D.A.K. specification[11] for the substance under the name glykresin has been published by the Control Laboratory of the Danish Pharmaceutical Society; this specification gives limit tests for acidity and alkalinity, for loss on drying, for ionised halogen, for sulphates, for heavy metals, for glycerol monochlorhydrin and in addition an assay depending on bromine absorption.

Published methods for the preparation of αβ-dihydroxy-γ(2-methylphenoxy) propane involve the condensation of glycerol and *o*-cresol in the presence of sodium acetate[12], or the action of glycerol monochlorhydrin on *o*-cresol[13]; if the compound is prepared by either of these routes *o*-cresol is a likely impurity and tests for its limitation should be included in any specification. The following test has proved to be satisfactory in the author's hands. To 0·1 g. dissolved in 5·5 ml. of water, add 3 ml. of a 4 per cent. solution of sodium hexametaphosphate, 1·5 ml. of Folin and Ciocalteau's reagent and 0·4 g. of anhydrous sodium carbonate. The mixture is heated in a water-bath for 5 minutes, cooled, and

the blue colour is compared with standards prepared from known amounts of *o*-cresol after allowance for the colour produced in a blank determination on the reagents used.

Among the assay processes available the following two have been used; the first, found to be the most useful, is based on a determination of the hydroxy-groups, while the second employs a periodic acid oxidation.

For hydroxy-groups.—Heat approximately 2 g. accurately weighed, with 20 ml. of a 15 per cent. solution of acetic anhydride in pyridine in a flask fitted with a reflux condenser and a ground glass joint on a water-bath for 2 hours; cool, add 40 ml. of water and titrate the free acid with N sodium hydroxide using phenolphthalein as indicator. A blank determination is performed at the same time omitting the substance under test; each ml. difference between the titrations is equivalent to 0·0911 g. of $C_{10}H_{12}O.(OH)_2$. Not less than 99·5 per cent. and not more than 100·5 per cent. should be indicated.

Assay using periodic acid. This is carried out according to the conditions given for the assay of propylene glycol in the National Formulary VIII; it has been found to be necessary to allow the period of contact with the periodic acid solution to be extended from the 15 minutes specified. In a few experiments a 24 hours' reaction time gave satisfactory results although this was not pursued in view of the reliability of the assay for hydroxyl groups.

p-*Chlorophenyl-α-glycerol ether, αβ-dihydroxy-γ*(p-*chlorophenoxy*) *propane* (gecophen, chlorphenesin) is a colourless crystalline solid, m.pt. 80°C. soluble in water to the extent of approximately 0·6 per cent. at 25°C., more readily soluble in organic solvents; aqueous solutions are neutral and are unaffected by dilute acids or alkalis or by exposure to light. The substance can be prepared[15] by the action of glycerol monochlorhydrin on *p*-chlorophenol and analytical determinations of purity should include limit tests for acidity and alkalinity, for loss on drying, for ionised halogen, for melting-point and for free phenols. The test for free phenols can be performed using Folin and Ciocalteau's procedure as outlined above for αβ-dihydroxy-γ-(2-methylphenoxy) propane. Three methods for the assay of *p*-chlorophenyl glycerol ether have proved to be satisfactory—an assay based on the determination of hydroxyl groups in the molecule, one based on the chlorine content, and a periodate oxidation process.

For hydroxyl groups. This can be carried out using the method described above for hydroxyl groups in mephenesin. Each ml. difference between the titrations is equivalent to 0·1013 g. of $C_9H_{11}O_3Cl$.

For halogen. Removal of the chlorine from the benzene nucleus can be effected quantitatively with sodium and amyl alcohol. Weigh accurately about 0·5 g. of sample into a flask of 250 ml. capacity, containing 50 ml. of amyl alcohol (AnalaR) and fitted with a wide bore air condenser. Pellets of sodium metal (about 2 g.) are added and the whole is warmed until a steady evolution of hydrogen takes place. When all the sodium has dissolved the liquid is refluxed gently for 2 hours, cooled, 80 ml. of water is added, followed by 40 ml. of nitric acid and 50 ml. of

0·1 N silver nitrate; the resulting mixture is cooled and back titrated with 0·1 N ammonium thiocyanate following the usual Volhard technique. Each ml. of 0·1 N of silver nitrate required is equivalent to 0·02026 g. $C_9H_{11}O_3Cl$.

Assay by oxidation. This can be carried out as in the periodic acid assay of mephenesin, using the conditions given for the assay of propylene glycol[14]. Each ml. of sodium arsenite solution is equivalent to 0·01013 g. $C_9H_{11}O_3Cl$.

The Determination of Phenyl Glycerol Ethers in Pharmaceutical Preparations

The method of Titus, Ulick and Richardson[15] for the determination of αβ-dihydroxy-γ-(2-methylphenoxy) propane in body fluids and tissues involves coupling with diazotised 2:4-dinitroaniline, while an alternative procedure involves the colorimetric estimation of the formaldehyde

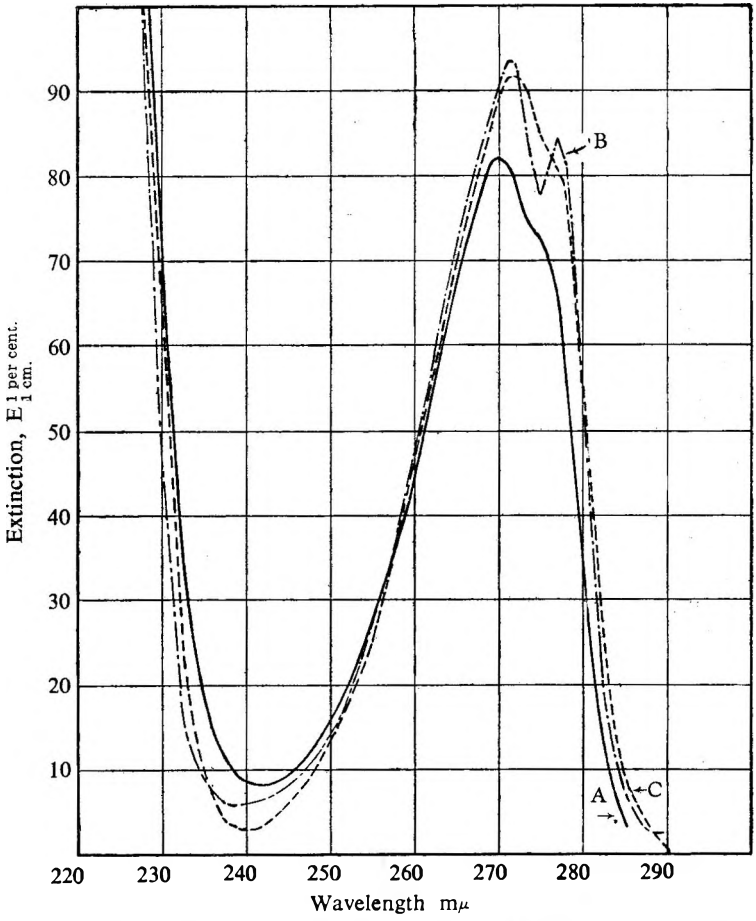

Fig. 1. Absorption spectra of mephenein. A, in water; B, in cyclohexane; C, in isopropyl alcohol.

resulting from periodate oxidation. Neither of these methods as given has been found to be reliable when applied to pharmaceutical preparations and considerable adaptation would be necessary to make them applicable.

The value of the determination of the ultra-violet absorption spectrum in the analysis of pharmaceutical preparations has become established in recent years. In preparations where there is an inert vehicle this method has much to recommend it as regards simplicity, accuracy, and speed of operation; with this in view an examination of the absorption spectrum of the two phenyl glycerol ethers was undertaken.

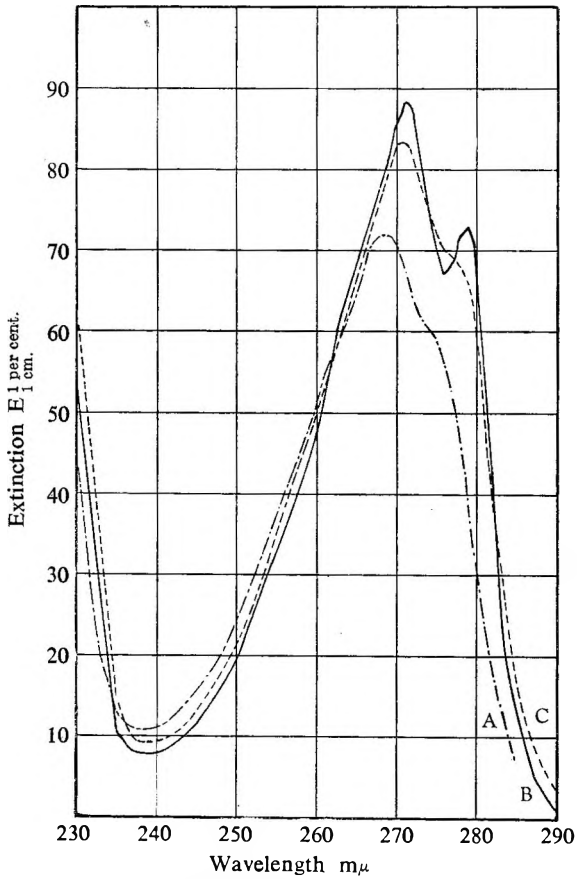

FIG. 2. Absorption spectra of chlorphenesin. A, in water; B, in cyclohexane; C, in isopropyl alcohol.

The spectrum of mephenesin in various solvents is shown in Fig. 1. A change of *p*H in aqueous medium did not produce a change in spectrum, an interesting point in view of the fact that the glycerol ethers generally are slightly more soluble in solutions of alkali hydroxides than in water; greater resolution of the peak is obtained in cyclohexane solution, two distinct maxima being visible.

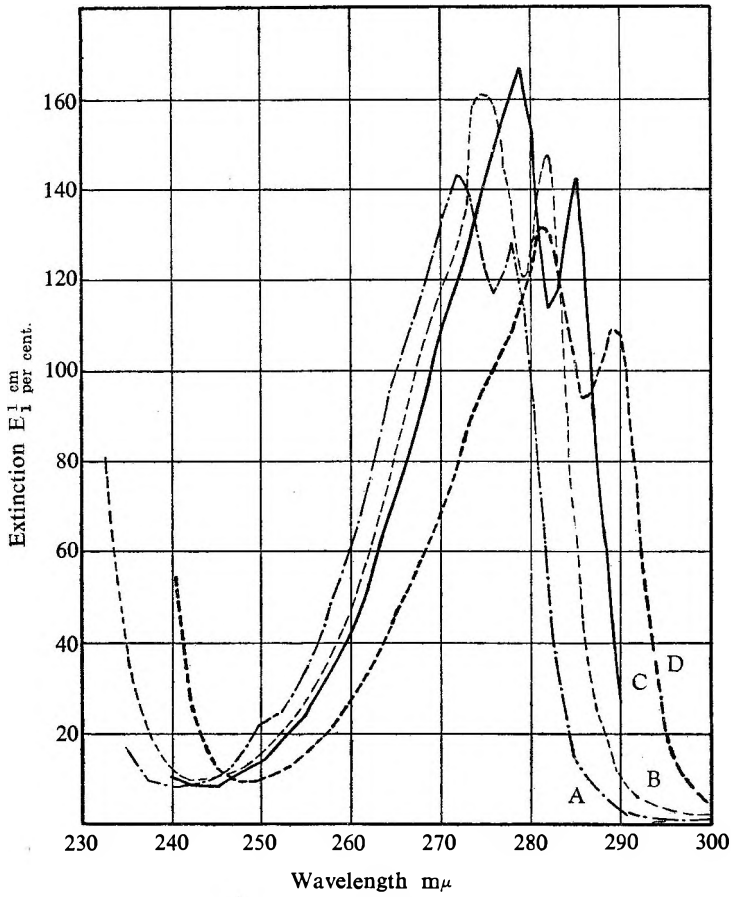

FIG. 3. Absorption spectra of phenyl ethers in cyclohexane. A, *o*-cresyl ethyl ether; B, *o*-chloroanisole; C, *p*-cresyl methyl ether; D, *p*-chloroanisole.

Chlorphenesin (Fig. 2) shows a similar absorption curve, although the combined effect of the halogen substituent coupled with a para-orientation has produced a shift of *ca.* 10 mμ in the peak wavelength. There is a complete analogy between chlorphenesin and mephenesin in the small relative peak differences between the various solvents; again cyclohexane produces greater resolution.

Generally the spectra of the above two compounds are those of substituted benzene derivatives where it has been shown that there is a constant ratio between the two peaks; in the spectra above, the shorter wavelength peak has not been realised. The actual maxima would, however, be below 220 mμ and would be of little value in pharmaceutical work owing to the strong end-absorption of most substances in this region. The glycerol moiety of the molecule plays little or no part in determining either the intensity of the absorption or the peak wavelength. To illustrate this point the spectra of a number of analogous benzene derivatives have been determined in which $-OCH_2-CHOH-CH_2OH$ has been

substituted by an alkyl group; these, in cyclohexane, are shown in Figure 3. It will be seen that *o*-cresyl-ethyl ether has a spectrum almost identical with that of mephenesin, the analogy being more apparent if the absorption maxima are calculated on a molecular basis. Para-substitution causes a shift to longer wavelength of *ca.* 7 mμ over the ortho-compound, a difference which is remarkably constant for both peaks.

TABLE I

	Solvent	λ max	$E^{1\%}_{1cm}$
Mephenesin	isopropyl alcohol	272·0 mμ	91·5
	cyclohexane	277·0 mμ	84·4
		271·5 mμ	93·5
	water	277·0 mμ	66·0
		270·0 mμ	82·1
Chlorphenesin	isopropyl alcohol	281·0 mμ	83·6
	cyclohexane	281·0 mμ	89·0
		289·0 mμ	73·2
p-Cresyl methyl ether	cyclohexane	285·0 mμ	142·2
		279·0 mμ	166·0
o-Cresyl ethyl ether	cyclohexane	278·0 mμ	128·0
		272·0 mμ	143·5
p-Chloranisole	cyclohexane	289·0 mμ	109·2
		281·5 mμ	131·3
o-Chloranisole	cyclohexane	282·0 mμ	148·0
		275·0 mμ	161·4

Elixirs of Mephenesin. A number of preparations of this compound have been formulated containing in the main propylene glycol or alcohol as solvents. In many cases where there are few absorbing impurities a direct determination of $E^{1\%}_{1cm}$ 270 mμ will yield a reliable result for the mephenesin content; in some formulations a correction for the solvent and flavouring may be necessary.

An alternative method of analysis is available in dilution with water, extraction with an immiscible solvent, and weighing the residue. Ether was found to give impure oily residues after extraction and was rejected; chloroform, however, gave surprisingly clean residues and although the pure compound is low in melting-point the extracted product was nearly always crystalline on cooling. The following method is suggested. A quantity of elixir (containing about 0·5 g. of mephenesin) is diluted to 50 ml. with water and extracted 5 times with 25 ml. quantities of chloroform. The resulting chloroform extracts after washing with 10 ml. water are bulked, evaporated to dryness, and dried at 100°C. to constant weight.

Injections of mephenesin are usually formulated with alcohol and/or propylene glycol; they can be treated similarly for analytical purposes.

Mephenesin Tablets. These can be extracted with chloroform, the chloroform washed to remove absorbing impurities and the absorption determined as before.

The estimation of *p*-chlorophenyl glycerol ether can be performed as indicated for mephenesin by the determination of $E^{1\%}_{1cm}$ 281 mμ;

this method is valid for simple aqueous solutions and for preparations which yield little irrelevant absorption on treatment. For more complex formulations, e.g., ointments and dusting powders, a different method must be used. Analysis will then depend on the constituents of the ointment or preparation; solvent extraction with a variety of solvents is usually most successful, followed by purification of the extract. The extracted chlorphenesin is then determined by weighing or by a determination of $E_{1\,cm.}^{1\ per\ cent.}$ on the final solution if this is sufficiently pure; the determination of total halogen content using sodium and amyl alcohol can also be used.

Summary

1. The examination of αβ-dihydroxy-γ-(2-methylphenoxy) propane (myanesin, mephenesin) and of αβ-dihydroxy-γ-(*p*-chlorophenoxy) propane (gecophen, chlorphenesin) is described, a method based on the use of Folin and Ciocalteau's reagent being proposed for the detection of phenolic impurities.

2. Methods of assay based on acetylation or oxidation of the hydroxy groups are described and discussed.

3. The absorption spectra of phenyl glycerol ethers and related substances are described and their suitability for the determination of phenyl glycerol ethers in pharmaceutical preparations, is discussed.

4. Methods are proposed for the determination of mephenesin in injections, elixirs, and tablets and of chlorphenesin in ointments, dusting powders, and pessaries.

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A COMPARISON OF THE ACTIVITY OF SOME DRUG PRODUCTS INJECTED IN HYDROLYSED CALCIUM GELATINATE AND IN DISTILLED WATER

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During the past few years a number of pharmaceutical companies have incorporated into some of their formulations substances which are known to prolong the action of the active ingredient following parenteral injection. This type of formulation has resulted in some cases in a reduction in the amount of active ingredient necessary to attain a desired blood level for adequate medication. Where the active ingredients are rapidly eliminated or detoxified in the body these substances have been most useful in maintaining adequate blood levels. Special diluents have also been advocated for the same purpose.

The purpose of this paper is to report on an investigation on Hydrolysed Calcium Gelatinate, a diluent which has been reported to prolong the action of a number of drugs, such as morphine, penicillin, certain vitamins and sex hormones[1]. The drugs which were chosen for this study were morphine sulphate, *d*-tubocurarine chloride and œstrone.

A comparison of the analgesic activity of morphine sulphate in distilled water and hydrolysed calcium gelatinate in the rat, and also determinations of the free morphine content of rabbit blood at various intervals after intramuscular injection were made. The activity of the *d*-tubocurarine chloride in distilled water and hydrolysed calcium gelatinate was compared and a similar comparison on œstrone was also made.

Methods

The analgesic tests in rats were conducted according to the method described by D'Amour and Smith[2] and further modified by Miller[3]. The apparatus used for this study was one designed and made to the specification of Wolff, Hardy and Goodell[4]. The free morphine determinations in rabbit blood were done by the silicomolybdic acid method as proposed by Shideman and Kelley[5]. The activity of the *d*-tubocurarine chloride solutions was determined by a sloping screen method which has been used in this laboratory for testing the strength of *d*-tubocurarine solutions[6]. The details of the method for the assay of œstrone has been previously described[7].

For all these assays, with the exception of the free morphine determinations in blood, the object of the experiments was to determine the activity of the product in both diluents and to ascertain if possible if the diluent delayed the onset and prolonged the effect.

For the analgesic tests the observations were made at various intervals after the injections were made in order to find out the approximate time of the onset and the duration of analgesia.

The rats used in the *d*-tubocurarine chloride assays were observed continuously for several hours. It has been found that rats injected

with *d*-tubocurarine chloride in distilled water respond before 20 minutes has elapsed or do not respond at all[6]. In these tests the period of observation was extended to several hours in order to find out if the onset of effect might be delayed. The computations for all the assays reported in Tables I, III and IV were done by the methods of Bliss[8,9].

TABLE I

COMPARISON OF THE ANALGESIC EFFECT OF MORPHINE SULPHATE INJECTED INTRAMUSCULARLY IN DISTILLED WATER AND HYDROLYSED CALCIUM GELATINATE RADIANT HEAT-TAIL METHOD

Morphine sulphate in hydrolysed calcium gelatinate diluent in terms of morphine sulphate in distilled water Minutes after Injection							
30		60		90		120	
[a]Per cent and Confidence Limits, P = ·05							
133	112–159	107	94–122	90	75–107	68	67–110

NOTE.—Instrument setting, 350–400 millicalories. Cut off time, 3·0–5·4 secs. [a]Weighted mean of 2 or more assays.

RESULTS AND DISCUSSION

In Tables I, III and IV comparative results are shown of the analgesic

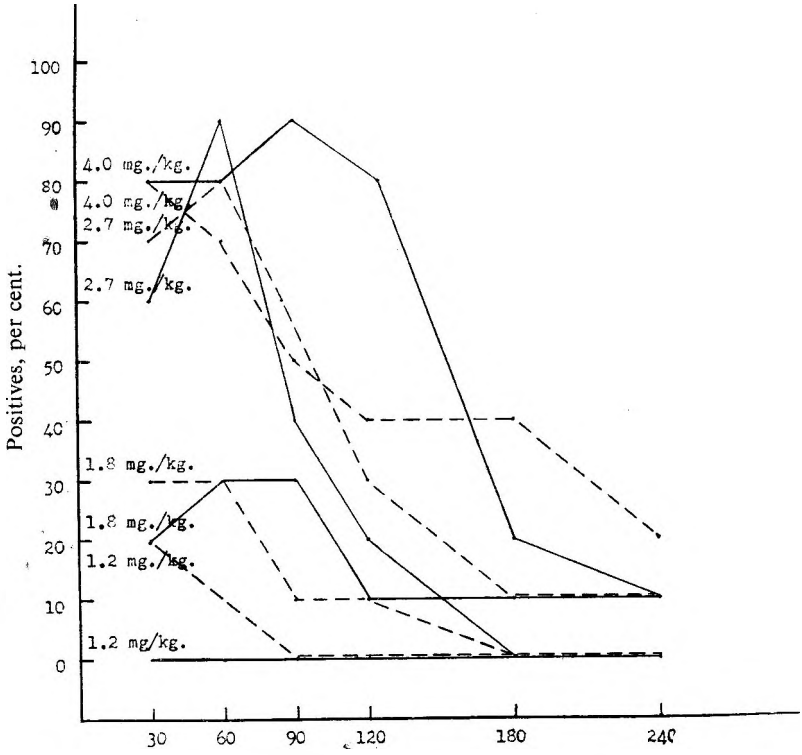

FIG. 1. Minutes after injection—tail method.

——— Morphine sulphate in aqueous solution
– – – Morphine sulphate in hydrolysed calcium gelatinate diluent

potency of morphine in distilled water and hydrolysed calcium gelatinate and the activity of *d*-tubocurarine and œstrone in both diluents.

For the analgesic tests there was no evidence that those rats injected with hydrolysed calcium gelatinate showed greater analgesic effect at any time during the observation period, which in some cases lasted for several hours. The number of rats responding positively at the end of the observation period were about the same for groups which received the same dose of the drug. This may be seen from an examination of the results of a typical experiment (Fig. 1). If the drug had been slowly absorbed one would have expected that a greater proportion of the rats injected with morphine in distilled water would have shown an analgesic effect on the first readings, and on the later readings a greater proportion of the rats injected with morphine in hydrolysed calcium gelatinate would have shown an analgesic effect. The results do not show this trend as may be seen from an examination of the comparative estimates of potency shown in Table I.

As may be seen in Table II the determinations of free morphine in rabbit blood at various intervals after injection shows a somewhat

TABLE II

COMPARISON OF THE FREE MORPHINE CONTENT OF RABBIT BLOOD FOLLOWING INTRAMUSCULAR INJECTION OF MORPHINE SULPHATE IN DISTILLED WATER AND HYDROLYSED CALCIUM GELATINATE

[a] Free morphine concentration in blood, (mg. per cent., ±S.E.) after intramuscular injection of 50 mg./kg. of morphine sulphate in hydrolysed calcium gelatinate diluent and distilled water

Minutes after Injection					
5		15		45	
2·62±0·33[b]	4·23±0·53[c]	3·15±0·47[b]	3·47±0·38[c]	2·04±0·22[b]	1·50±0·22[c]

NOTE :—*a* 14 rabbits were used on each diluent. The results at each time interval are the means of 14 determinations.

b refers to determination of morphine sulphate in distilled water.

c refers to determination of morphine sulphate in hydrolysed calcium gelatinate.

similar agreement in results. At 5 minutes after the injections were made the free morphine recovered from blood was significantly greater in those rabbits injected with morphine in distilled water, but at 15 and 45 minutes after injection there was no significant difference in the amount of free morphine recovered in the respective groups. If the morphine had been released slowly a greater amount of free morphine should have been recovered at the 45-minute interval after injection. Determinations were made at later times but there was not sufficient present in the blood to be detectable.

In Tables II and IV are shown the results of comparative assays on *d*-tubocurarine chloride and œstrone in distilled water and hydrolysed calcium gelatinate diluent. As was mentioned previously the rats on the *d*-tubocurarine chloride test were observed continuously for several hours from the time the injections were made, but in no case was any effect observed after an elapsed time of 20 minutes. The rats that

responded did so within 20 minutes and the effect did not last longer on the rats injected with *d*-tubocurarine chloride in hydrolysed calcium gelatinate diluent. As may be seen from Table III there was no difference in the activity of *d*-tubocurarine chloride in the two diluents. If the action had been delayed and prolonged in those rats injected with *d*-tubocurarine chloride in hydrolysed calcium gelatinate diluent the relative activity for *d*-tubocurarine chloride in the two diluents would have been different.

TABLE III

COMPARISON OF THE ACTIVITY OF *d*-TUBOCURARINE CHLORIDE FOLLOWING INTRAMUSCULAR INJECTION IN DISTILLED WATER AND HYDROLYSED CALCIUM GELATINATE

[a]Potency of *d*-tubocurarine chloride in hydrolysed calcium gelatinate diluent in terms of *d*-tubocurarine chloride in distilled water, per cent.	Confidence Limits P = ·05
102·5	96·7—108·8

NOTE :—[a]Weighted mean of 2 assays.

A similar result was obtained on œstrone. The proportion of rats showing œstrus after injection of œstrone in both diluents was about the same for those groups receiving the same doses and this was evident from the computed activity of œstrone in distilled water in terms of œstrone in hydrolysed calcium gelatinate diluent, as may be seen from the results in Table IV. There was no evidence of a prolonged action

TABLE IV

COMPARISON OF THE ACTIVITY OF ŒSTRONE INJECTED SUBCUTANEOUSLY IN DISTILLED WATER AND HYDROLYSED CALCIUM GELATINATE

[a]Potency of œstrone in hydrolysed calcium gelatinate diluent in terms of œstrone in distilled water, per cent.	Confidence Limits P = ·05
110·4	87·8—137·5

NOTE :—[a]Weighted mean of 2 assays.

as the rats injected with œstrone in both diluents returned to their preinjection state at the same time.

SUMMARY

1. A comparison of the analgesic effect of morphine sulphate and the activity of *d*-tubocurarine chloride and œstrone in distilled water and hydrolysed calcium gelatinate diluent is presented.

2. No appreciable prolongation of analgesic effect of morphine nor activity of *d*-tubocurarine chloride and œstrone in hydrolysed calcium gelatinate was found.

3. Free morphine blood levels except at 5 minutes after injection were about the same following intramuscular injection of morphine in distilled water and hydrolysed calcium gelatinate diluent.

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THE ASSAY OF AMMI VISNAGA FRUITS

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In previous communications[1,2] a photoelectric colorimetric method is described for the assay of khellin in injections and tablets based on the intensity of the yellow colour which the chromone imparts to sulphuric acid of a definite concentration. In this work, the same method is applied for the assay of ammi visnaga fruits.

Abdel Rahman[3] has proposed a gravimetric method for the assay of ammi visnaga fruits based on exhausting the powdered drug by percolation with alcohol (95 per cent. v/v), removing the alcohol by distillation, diluting the concentrated extract with water, purifying it by treatment with lead acetate solution, removing the excess of lead from the filtrate with sodium dihydrogen phosphate, extracting the filtrate with chloroform, evaporating the chloroform, and weighing the dried residue. This residue was found by the authors to be impure and the results obtained by this method are higher than the actual quantity of non-glycosidal chromones present in the drug.

On replacing the alcohol (95 per cent. v/v), in the above method, by alcohol (70 per cent. v/v), or, on exhausting the powdered drug by boiling under a reflux condenser with alcohol (50 per cent. v/v), or water; no improvement in the purity of the residues could be obtained. On extracting these residues with boiling water, filtering, cooling the filtrate, extracting it with chloroform and evaporating the chloroform, a purer residue could be obtained. But on applying the photoelectric colorimetric method to such residues, it can be proved that they are still impure.

Experimental

Four quantities, each of about 5 g. of genuine ammi visnaga fruits, in moderately fine powder, are accurately weighed and separately exhausted by percolation with alcohol (95 per cent. v/v), alcohol (70 per cent. v/v), and by boiling under reflux with alcohol (50 per cent. v/v) and water, until the marc in each case fails to give a rose-red colour with a solution of sodium hydroxide (1 in 1).

From the alcoholic extracts, the alcohol is removed by distillation and each extract is diluted to about 100 ml. with distilled water; while the aqueous extract is concentrated to about 100 ml. Each extract is then separately treated with 10 ml. of a 10 per cent. solution of lead acetate, boiled and filtered while hot and the precipitate is washed on the filter with 3 quantities, each of 20 ml., of boiling water. To the combined filtrate and washings, 5 g. of sodium dihydrogen phosphate is added; after boiling, the precipitated lead phosphate is removed by filtration, and the precipitate on the filter washed with 3 quantities each of 10 ml. of boiling water. The filtrate and washings are concentrated in a porcelain

dish on the water-bath to about 50 ml. and filtered while hot into a separator. The dish and filter are washed with 3 quantities, each of 20 ml., of boiling water. The filtrate is cooled to room temperature, then extracted with 4 successive quantities, each of 25 ml., of chloroform. The combined chloroform extracts are washed with about 5 ml. of water, the water rejected and the chloroform extract is dehydrated with about 2 g. of anhydrous sodium sulphate and filtered through a dry filter paper into a tared flask. The sodium sulphate and the filter are washed with 3 quantities, each of 5 ml., of chloroform, adding the washings to the chloroform extract in the flask. The chloroform is then evaporated, the residue dried to constant weight at 100°C. and weighed.

The residues obtained are purified by extracting them separately with boiling water, filtering the aqueous solution while hot, cooling the filtrate to room temperature extracting with chloroform evaporation of the chloroform extract, drying the purified residues at 100°C. to constant weight and weighing. The amount of non-glycosidal chromones is determined in the residues thus obtained by the photoelectric colorimetric method[1,2], and calculated as khellin.

The mean results of three experiments for each method of extraction are given in Table I.

TABLE I

Method of Extraction	Percentage of residue	Percentage of purified residue	Percentage of chromones
Percolation with alcohol (95 per cent. v/v)	2·71	2·31	1·348
Percolation with alcohol (70 per cent. v/v)	2·57	2·16	1·538
Refluxing with alcohol (50 per cent. v/v)	2·71	2·23	1·558
Refluxing with water	2·64	2·04	1·629

From the above table it may be concluded that:—

(1) The residue obtained in the gravimetric method of assay of ammi visnaga fruits is impure.

(2) The different methods of extraction used did not help to obtain a pure residue.

(3) The method used for the purification of the residue did not succeed.

Therefore, the gravimetric method is unsuitable for the assay of ammi visnaga fruits as shown by the estimation of the chromones in the residues by the photoelectric colorimetric method.

Moreover, extraction of the fruits with boiling water gives better results than extraction with alcohol.

A Recommended Method for the Assay of Ammi Visnaga Fruits

From the foregoing investigation, it has been shown that the chloroform extract of an exhaustive decoction of ammi visnaga fruits is the most suitable for carrying out an accurate assay of the fruits, as the residue obtained by this method gives the highest colorimetric value and repre-

sents the true content of the non-glycosidal chromones (viz. khellin and visnagin) in the fruits.

Working with quantities of the drug not exceeding 0·5 g., the volume of the decoction resulting from the exhaustion of the fruits with boiling water is considerably reduced, making possible an easy application of the photoelectric colorimetric method for the assay of the fruits.

By this method the time of the assay is reduced to a maximum of 3 hours.

Procedure. Introduce about 0·25 g. of ammi visnaga fruits in moderately fine powder, accurately weighed, into a flask of about 150 ml. capacity; add 50 ml. of distilled water and boil the mixture under reflux for 30 minutes. Add to the boiling mixture 2 ml. of a 10 per cent. solution of lead acetate and continue the boiling for 3 minutes more. Filter the hot mixture by suction. Wash the flask and filter with 3 quantities, each of about 20 ml., of boiling water. Transfer the filtrate and washings to a beaker of about 250 ml. capacity; add 1 g. of sodium acid phosphate and boil for 3 minutes. Filter the hot solution directly into a separating funnel without suction. Wash the beaker and filter with 3 quantities, each of about 20 ml., of boiling water. Cool to room temperature. Extract the aqueous solution with 4 quantities, each of 25 ml., of chloroform. Wash the combined chloroform extracts with 5 ml. of water, reject the water, dehydrate the chloroform extract with about 2 g. of anhydrous sodium sulphate. Filter through a dry filter paper into a flask of about 200 ml. capacity; wash the sodium sulphate and the filter with 3 quantities, each of about 10 ml., of chloroform adding the washings to the chloroform extract in the flask.

Evaporate completely the chloroform on the water-bath. Add to the residue in the flask 80 ml. of 10 N sulphuric acid. Dissolve by the aid of gentle heat. Cool. Transfer the cooled acid solution to a volumetric flask of 100 ml. capacity. Make up to volume with distilled water. Mix well and leave to stand for about 5 minutes. Filter about 15 ml. of the solution into a dry colorimeter tube and read the percentage transmission of the solution at room temperature (25°C.) in a Lumetron Photoelectric colorimeter using blue filter 420 against water as the blank set at 100 per cent. transmission.

Find the concentration of khellin corresponding to the percentage transmission from a calibration table prepared at room temperature (25°C.) using standard concentrations of khellin. The figure obtained is the amount in mg. of the total non-glucosidal chromones contained in the weighed sample calculated as khellin. The percentage of these chromones in the drug can then be obtained by calculation.

Accuracy of the results. It has been found experimentally that the most accurate results are obtained on applying this method to weighed samples of the crude drug assaying about 4 mg. of khellin to get a reading of the per cent. transmission of the solution lying between 40 and 60. In this case, the results of the assay did not differ by more than $\pm$ 5 per cent.

Applying this method to different varieties of ammi visnaga fruits,

Table II shows the mean percentage of the non-glucosidal chromones calculated as khellin, resulting from 6 assays for each variety.

TABLE II

Variety	Percentage of chromones per cent.
Egyptian (Lower Egypt)	1·676
Egyptian (Upper Egypt)	1·530
Lebanon	1·120
Morocco	1·200

The above table shows that the fruits of the Egyptian variety of ammi visnaga are the richest in chromones.

Summary

1. A recommended method for the assay of ammi visnaga fruits is described.

2. The results of the gravimetric method of assay of ammi visnaga fruits are high by not less than 50 per cent.

3. The fruit of the Egyptian variety of ammi visnaga contains the highest percentage of chromones.

The authors wish to express their thanks to the Memphis Chemical Co. for the help extended to them in carrying out this work.

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STUDIES OF RICINOLEIC ACID AND A TURBIDIMETRIC METHOD OF EVALUATING THE BACTERICIDAL ACTION OF SOLUTIONS OF PHENOLS IN POTASSIUM RICINOLEATES

PART III—REPRODUCIBILITY OF NEPHELOMETER RESULTS

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INTRODUCTION

IN Part II of this series[1] it was shown that further investigations were necessary before the nephelometer results could be used for evaluating the bactericidal action of a bactericide. The large daily variation noted in the earlier investigations was attributed to the treatment accorded the suspension of *Bacterium coli* after the initial standardisation of the suspension. This initial suspension was mixed with a solution of phenol and then sampled at predetermined time intervals, the samples were incubated for 5 hours and their turbidities were measured on the nephelometer. The assumption that equal nephelometer readings, in two different experiments, indicated an equal number of survivors in the samples appeared incorrect and so a technique was evolved in this part of the work to make this assumption unnecessary.

REFERENCE TURBIDITY

As a direct relationship between potentiometer reading and percentage survivors was not possible it was necessary to introduce a reference turbidity which, whilst subject to the same variation as the test material, remained a reference in that it was grown from a known or standard inoculum. The principle being that if test and reference cultures were incubated under identical conditions then the number of survivors in the test could be found by direct comparison of its turbidity with an equal turbidity produced in the reference culture.

It was decided that the standard suspension prepared daily for each experiment would serve to provide this reference turbidity. However the initial suspension itself could not be used undiluted since its turbidity after 5 hours incubation would be too great to be measured on the nephelometer ; nor could a shorter period of incubation of the reference culture be used since reference and test would not then be receiving identical incubation treatments and so not allow of any turbidity comparisons. A system of diluting the initial suspension was evolved which enabled it to be used as a reference turbidity.

APPARATUS

The apparatus used was that described in Part II of this work[1] with the exception of the water-bath used for incubation in the final part of this work.

Incubation. In the initial experiments when a batch of 10 bottles of

medium had been inoculated it was transferred from the water-bath at 37°C. to an electrically heated incubator at 37° ± 0·5°C. for the 5 hour incubation period. During the experiment the door of the incubator had to be opened and closed three or four times during the first hour or 90 minutes ; because of this a careful check was carried out on the incubator temperatures on each of several days and a temperature drop of 0·8° to 1·0°C. for periods as long as 20 minutes was observed. An experiment was performed to ascertain the effects if any of these small temperature changes upon the turbidities of the cultures. The results of this experiment, summarised in Table I, clearly indicated that the temperature of incubation was very important, an observation which was supported by the consideration of the fact that the incubation period was during the initial stages of the logarithmic growth phase of the bacteria.

TABLE I

SHOWING THE EFFECT OF A SMALL CHANGE OF INCUBATION TEMPERATURE ON THE TURBIDITY AFTER 5 HOURS INCUBATION OF A SUSPENSION OF *BACTERIUM COLI*

Inoculum (measured as drops of initial suspension)	1	2	3	5	5	10	15	25
Turbidity (nephelometer reading) after incubating at 36·5°C. ±0·3°C.	12 11	14 12	17 17	28 24	20 19	30 32	44 43	58 64
Turbidity (nephelometer reading) after incubating at 37·2°C. ±0·2°C.	14 14	18 20	24 22	37 44	38 37	62 64	67 75	75 85

Joslyn[2] encountered a similar difficulty in a 4-hour determination of penicillin activity, and recommended the use of a constant temperature water-bath for short incubation periods. A constant temperature water-bath was designed to hold 80 culture bottles, which, when in place, were immersed up to their necks. The temperature was kept constant by means of an 11-inch bulb type toluene thermo-regulator and a hot-wire vacuum switch, which controlled the current to a 750 watt immersion heater ; efficient stirring was maintained throughout the incubation period by two 1/60th H.P. high-speed stirrers. Trials indicated that a temperature of 37° ± 0·2°C. could be maintained for 8 hours, although it was noticed that removal of the bottles individually at the end of the 5-hour incubation period caused a drop in water level in the bath and the range was increased during this final period to ± 0·5°C.

EXPERIMENTAL

The experimental details for this work were essentially the same as those outlined in Part II[2]. A further detail was the inoculation of 10 culture bottles at the start of the experiment and a further 10 bottles at the end of the experiment to give the reference turbidity. The method was as follows :—

1.—The initial suspension was prepared as before, labelled S.100 per cent. and placed in the water-bath at 20°C.

2.—10ml. of this suspension was pipetted into a bottle containing 90ml. of sterile water. This bottle was labelled R.10 per cent. shaken and placed in the water-bath at 20°C.

3.—2ml. of the suspension S.100 per cent. was pipetted into a bottle

containing 98ml. of sterile water. This bottle was labelled R.2 per cent. and also placed in the bath at 20°C.

4.—10ml. of sterile distilled water was pipetted into each of 4 sterile medication tubes and the tubes placed in the bath at 20°C.

5.—Into each of 2 tubes containing 10ml. of sterile water, 5ml. of R.10 per cent. was pipetted and the tubes returned to the water-bath, after labelling R.10 per cent.

6.—Similarly 5ml. of R.2 per cent. were pipetted into each of the two remaining tubes containing 10ml. of sterile water, the tubes labelled R.2 per cent. and returned to the bath.

7.—At a predetermined time one of the R.10 per cent. tubes was taken from the water-bath and shaken. A sterile dropping pipette was rinsed with the suspension in the tube by drawing the mixture up and down the pipette twice, the pipette was filled with the suspension and the tube discarded.

8.—A culture bottle containing 50ml. of sterile peptone broth was taken from the 37°C. water-bath and inoculated with 5 drops of the R.10 per cent. suspension of (7), after shaking to disperse, the bottle was returned to the 37°C. bath.

9.—This was repeated with 4 other culture bottles adding respectively 4, 3, 2, and 1 drops of the R.10 per cent. suspension.

10.—Repeated (7), (8), (9) with 1 of the medication tubes labelled R.2 per cent., a fresh sterile dropping pipette and 5 further culture bottles each containing 50ml. of sterile broth.

This established the reference turbidity at the beginning of the experiment, a similar procedure at the end of the experiment gave a similar

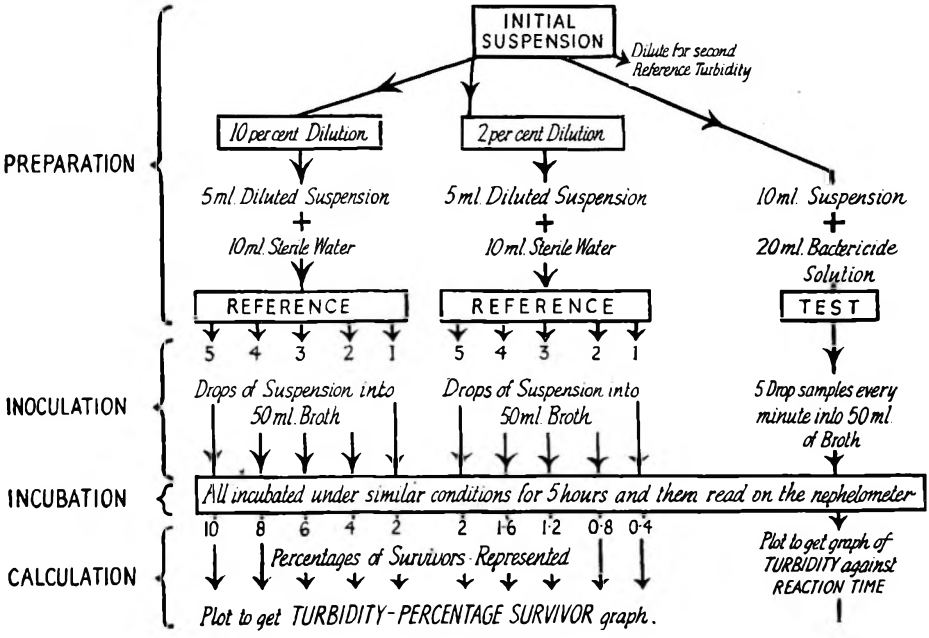

FIG. 1 Schematic diagram of a single experiment.

reference turbidity finally. Any difference in these reference turbidities indicated a variation in the culture during the inoculation stage of the experiment.

As in Part II the turbidities were read on the nephelometer after exactly 5 hours incubation.

Figure I shows a schematic diagram of a single experiment and how the data for the two graphs for each experiment were obtained.

Calculation of Results. Tables II and IV are the results obtained in a typical experiment.

TABLE II

RESULTS OF THE NEPHELOMETER READINGS OBTAINED IN A TYPICAL EXPERIMENT FROM WHICH A REFERENCE TURBIDITY CURVE WAS PLOTTED

Suspension used	R. 10 per cent.					R. 2 per cent.				
No. of drops ...	5	4	3	2	1	5	4	3	2	1
	NEPHELOMETER READINGS									
Before test ...	84	81	77	67	48	46	40	32	30	19
After test	78	77	70	56	49	40	41	31	—	20
Average	81	79	73½	61½	46		40½	31½	30	19½
Percentage of survivors	10	8	6	4	2		1·6	1·2	0·8	0·4

From the results in Table II the graph in Figure 2 was drawn using a logarithmic scale for the percentage survivors.

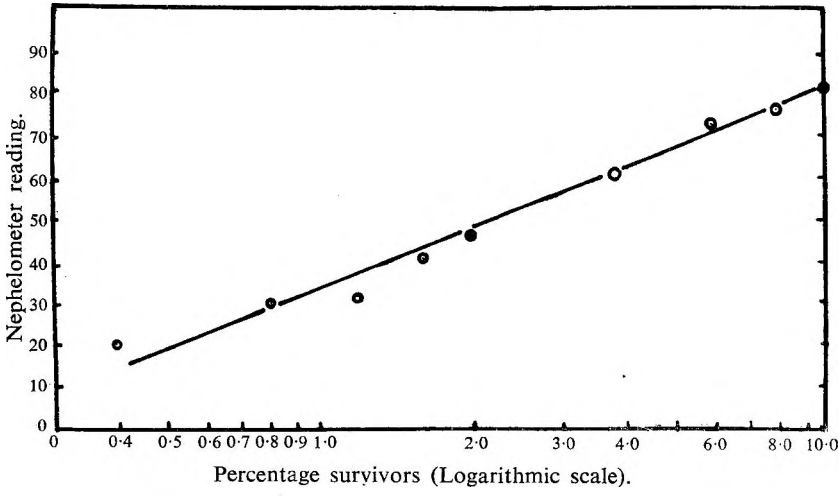

FIG. 2. Reference turbidity. Nephelometer reading plotted against the logarithm of the percentage of survivors.

From this graph another table (Table III) was compiled giving the nephelometer readings corresponding to various percentages of survivors.

In this way the reference turbidity was used to convert the nephelometer readings into percentage survivors.

TABLE III

NEPHELOMETER READINGS CORRESPONDING TO VARIOUS PERCENTAGES OF SURVIVORS, COMPILED FROM FIG. 2

Survivor Percentage ...	0·5	0·6	0·7	0·8	0·9	1·0	2·0	3·0	4·0	5·0	6·0	7·0	8·0	9·0	10·0
Nephelometer Reading ...	19	23	26	29	31	33	48	56	62	67	71	74	77	80	82

From the results of test I in Table IV a graph of nephelometer reading against reaction time was drawn (Fig. 3).

TABLE IV

RESULTS, FROM A TYPICAL EXPERIMENT, OF THE NEPHELOMETER READINGS OBTAINED USING THREE SAMPLES OF A 1 PER CENT. PHENOL SOLUTION AGAINST BACT COLI.

Reaction Time	Nephelometer Readings I	II	III
1 minute	100	95	B.S.
2 ,, ...	97	99	B.S.
3 ,, ...	90	89	91
4 ,, ...	89	79	80
5 ,, ...	73	75	77
6 ,, ...	64	60	62
7 ,, ...	54	41	58
8 ,, ...	41	37	49
9 ,, ...	41	24	41
10 ,, ...	30	25	28
11 ,, ...	22	19	31
12 ,, ...	26	16	22
13 ,, ...	17	17	18
14 ,, ...	13	18	31
15 ,, ...	16	15	16

B.S.—Beyond the scale of the instrument.

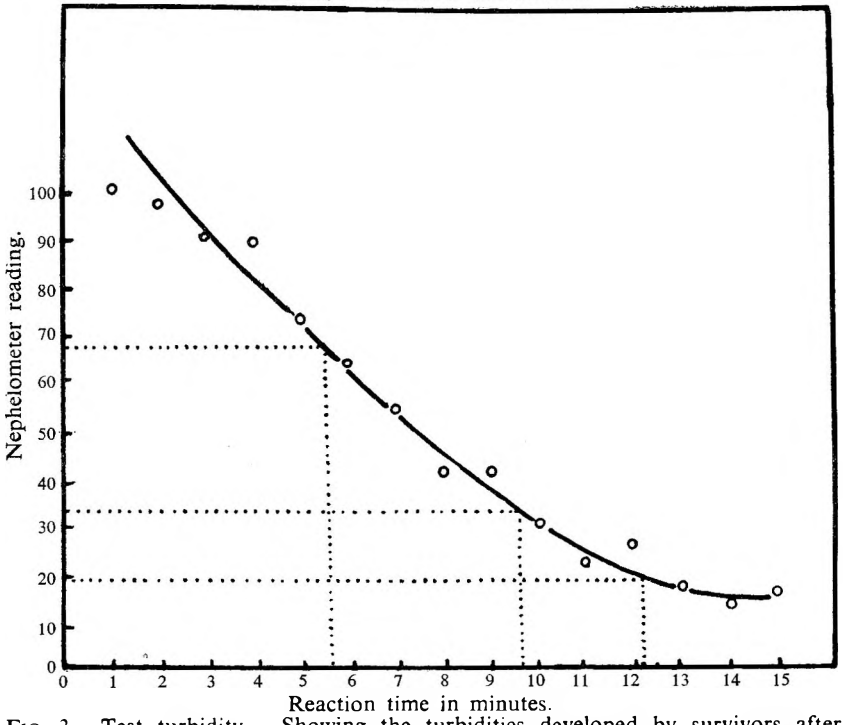

FIG. 3. Test turbidity. Showing the turbidities developed by survivors after various times of exposure to 1 per cent. phenol.

Using the nephelometer readings in Table III on the graph in Figure 3 the reaction times corresponding to various survivor levels were obtained —the dotted lines in Figure 3 illustrate the method with reference to the 0·5 per cent., 1·0 per cent. and 5·0 per cent. survivor levels. In this way it is possible to draw up, for each experiment, a table (Table V) showing the reaction times corresponding to various survivor levels.

TABLE V

REACTION TIMES CORRESPONDING TO VARIOUS SURVIVOR LEVELS

Survivors	0·5	0·6	0·7	0·8	0·9	1·0	2·0	3·0	4·0	5·0	6·0	7·0	8·0	9·0	10·0
Reaction Times	12·1	11·2	10·7	10·2	9·8	9·5	7·7	6·8	6·2	5·7	5·2	4·9	4·6	4·3	4·1

The principles of this technique were applied to tests on 1·0 per cent. and 0·9 per cent. phenol using 20 drops, 10 drops, and 5 drops samples, and 0·8 per cent. phenol using 5 drops and 10 drops samples ; by analysis of the results it was expected that the effects of the varying size of sample and the difference in strength of the phenol would be evident. The arithmetical averages of the experiments were plotted on Figure 4.

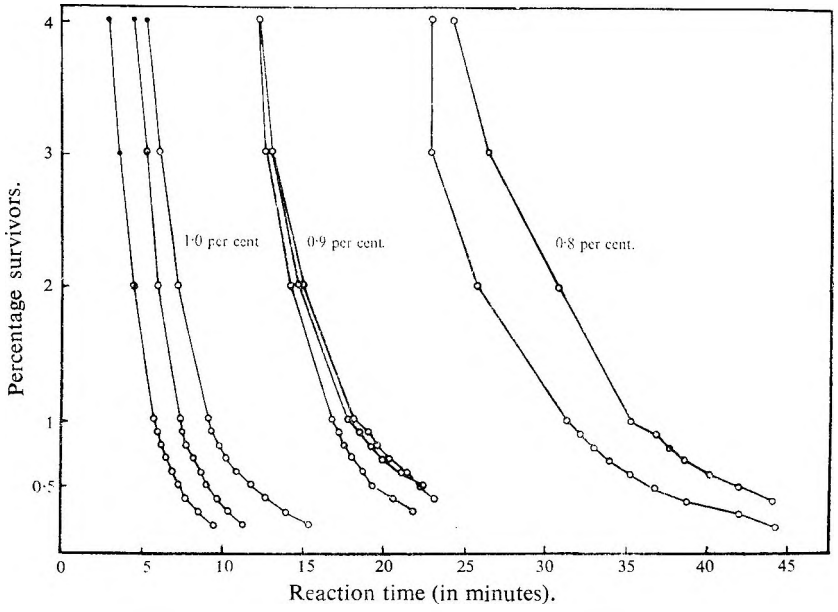

FIG 4. Reaction time—percentage survivor graph for *Bact. coli* against various percentages of phenol.

ANALYSIS OF RESULTS

Linear Regressions. The curves plotted in Figure 4 were of such a shape, as to warrant further investigation.

Curvilinear relationships, such as those of Figure 4 would be extremely cumbersome mathematically and it was desirable if possible to convert the results into linear regressions. Gaddum[3] and Hemmingson[4] suggested that individual sensitivities of experimental animals to many drugs

would exhibit a normal distribution if logarithms of the doses were used to plot the dose effect curve. Withell[5] showed that the resistances of bacteria to bactericides were normally distributed if a logarithmic scale was used to plot the reaction time on a survivor-reaction time regression. Applying a logarithmic scale to the curves of Figure 4 gave a fresh series of curves, the shapes of which more nearly approximated the lower portion of a typical sigmoid curve for an ideal normal distribution. By making use of the normal equivalent deviation (N.E.D.) proposed by Gaddum[3] or the Probit proposed by Bliss[6] such a sigmoid curve is transposed into a straight line. The averages which formed the basis of Figure 4 were then plotted on a probit percentage mortality-log. reaction time graph, and the results showed an approximately linear relationship and thus justified the decision that further analysis of the individual results was desirable.

Taking the results of each experiment the percentages of survivors were converted to percentages of mortality measured in probits and the reaction times to a logarithmic scale. The equation to a linear regression of log. reaction time on probit per cent. mortality was calculated for the results of each experiment. The figures and calculations for one experiment involving 0·8 per cent. phenol with a 5 drops sampling are quoted below and the collected results summarised.

Probit X	X^2	Log. time Y	Y^2	XY
7·6521	58·554634	1·6395	2·687960	12·545618
7·5758	57·392746	1·6233	2·635103	12·297796
7·5121	56·431646	1·6149	2·607902	12·131290
7·4573	55·611323	1·6107	2·594355	12·011473
7·4089	54·891799	1·6064	2·580521	11·901336
7·3656	54·252063	1·6021	2·566436	11·800428
7·3263	53·674672	1·5888	2·524286	11·640025
7·0537	49·754684	1·5211	2·313745	10·729383
6·8808	47·345408	1·4564	2·121101	10·021197
6·7507	45·571950	1·4082	1·983027	9·506336
6·6449	44·154696	1·3655	1·864590	9·073611
6·5548	42·965403	1·3346	1·780890	8·747381
86·1830	620·601024	18·3714	28·259916	132·405674

$\bar{X}=7·1820$ $\bar{Y}=1·5309$

$$\Sigma(X-\bar{X})^2=1·641900$$
$$\Sigma(Y-\bar{Y})^2=0·134221$$
$$\Sigma(X-\bar{X})\,(Y-\bar{Y})=0·463811$$

	Sum of Squares	Variance
Total	0·134221	
Due to regression $\dfrac{[\Sigma(X-\bar{X})\,(Y-\bar{Y})]^2}{\Sigma(X-\bar{X})^2}$	0·13039	0·13039
Residual	0·00383	0·00038

As the residual variance was small compared with the variance due to

regression it indicated that the data could be expressed in the form of a linear regression.

$$\text{Slope of regression line} = \frac{\Sigma(X-\overline{X})\,(Y-\overline{Y})}{\Sigma(X-\overline{X})^2} = 0{\cdot}2825$$

Hence equation to regression line is :—

from $(y-y^1)=m(x-x^1)$

$$Y=1{\cdot}5309+0{\cdot}2825\ (X-7{\cdot}1820)$$

The summarised results of the equation to the regression lines for other results are given in Table VI.

TABLE VI

SHOWING THE REGRESSION EQUATIONS CALCULATED FROM THE RESULTS OF 40 EXPERIMENTS ON THE BACTERICIDAL VALUE OF PHENOL AGAINST *BACTERIUM COLI*

Percentage Phenol	Number of Drops	Experiment Number	Regression Equation $y=\overline{y}+b(x-\overline{x})$		
			$\overline{y}$	b	$\overline{x}$
0·8	5	105	1·5309	0·3209	7·1820
		106	1·5295	0·3234	7·1820
		107	1·5309	0·2825	7·1820
		108	1·4105	0·4240	7·1820
	10	117	1·4829	0·2786	7·2254
		118	1·5109	0·3165	7·4417
		119	1·4634	0·3297	7·2254
		120	1·3610	0·4637	7·4417
		121	1·5113	0·3124	7·2721
		122	1·5306	0·2485	7·4417
0·9	5	127	1·2550	0·4082	7·1820
		128	1·2172	0·3416	7·1820
		129	1·2109	0·2907	7·2721
		130	1·1971	0·3489	7·2841
		131	1·2515	0·3040	7·1820
		132	1·1871	0·3406	7·3980
	10	123	1·1684	0·2501	7·3272
		124	1·1489	0·1774	7·4417
		125	1·3003	0·3784	7·2813
		126	1·2201	0·2987	7·4417
	20	133	1·2029	0·3867	7·1329
		134	1·1656	0·3952	7·4417
		137	1·2794	0·2879	7·2721
		138	1·2778	0·3007	7·4417
1·0	5	89	1·0043	0·4117	7·3272
		90	0·9727	0·3946	7·3272
		91	0·9884	0·4176	7·3272
		92	0·7887	0·3481	7·3272
	10	111	0·9515	0·3484	7·2721
		112	0·9131	0·4175	7·4417
		113	0·9258	0·3078	7·3272
		114	0·9226	0·2859	7·4417
		115	0·7329	0·3524	7·2721
		116	0·5854	0·2510	7·3272
	20	74	0·7081	0·3766	7·3272
		75	0·4430	0·3570	7·3272
		76	0·9162	0·4314	7·4978
		77	0·7137	0·2339	7·4978
		78	0·8138	0·5619	7·3272
		79	0·8042	0·4141	7·3272
		80	0·7779	0·4126	7·3841

Further analysis of the 0·8 *per cent. Phenol* 5*-Drop Sample Results.* In this instance there were four regression lines :—

(i) $Y=1\cdot5309+0\cdot3209\ (X-7\cdot1820)$
(ii) $Y=1\cdot5295+0\cdot3234\ (X-7\cdot1820)$
(iii) $Y=1\cdot5309+0\cdot2825\ (X-7\cdot1820)$
(iv) $Y=1\cdot4105+0\cdot4242\ (X-7\cdot1820)$

It was desired to find if :—

(a) all the lines could be represented as going through their respective means with the same slope ;

(b) all the lines could be represented by one common line going through the grand mean of Y's (Tippet[7])

(a) *To test if the four lines could be represented as going through their respective means with the same slope.* The residual sums of squares and their corresponding degrees of freedom were :—

	Residual Sum of Squares	Degrees of Freedom
(i)	0·002749	10
(ii)	0·020037	10
(iii)	0·00383	10
(iv)	0·00189	10
	0·028506	40

The sum of these residuals divided by the sum of the degrees of freedom gave an estimate of the residual variance of Y (which was assumed to be constant for all samples).

i.e. First estimate of residual variance of Y ... $=\frac{0\cdot0285}{40}$
$=0\cdot00071$

Then, if it was assumed that the samples have one regression coefficient but different means of Y for a given value of X the residual sum of the squares from the regression lines with a common slope and their degrees of freedom were calculated from :—

	$\Sigma(X-\overline{X})$	$\Sigma(Y-\overline{Y})$	$\Sigma(X-\overline{X})(Y-\overline{Y})$	Degrees of Freedom
(i)	1·642	0·171815	0·526969	11
(ii)	1·642	0·191757	0·530987	11
(iii)	1·642	0·134221	0·463811	11
(iv)	1·642	0·297502	0·697110	11
	6·568	0·795595	2·218777	44

from which

$$\text{Residual Sum of Squares}=0\cdot795595-\frac{(2\cdot218777)^2}{6\cdot568}$$
$$=0\cdot046046$$

the number of degrees of freedom is 43 that is 44 less 1 as one is absorbed in fitting the regression line. Thus :—

$$\text{Second Estimate of Residual Variance of Y}=\frac{0\cdot046046}{43}$$
$$=0\cdot00107$$

These two estimates of the residual variance in Y (0·00071 and 0·00107) could not be compared as they were not independent. When however the sum of the squares used to get the first estimate (0·028506) was subtracted from the sum of the squares used to get the second estimate (0·046046) and the remainder divided by the difference in the number of degrees of freedom then a third estimate of the variance was obtained which was independent of the first and could be compared with it. This is best summarised :—

Sum of Squares	Degrees of Freedom	Variance
0·046046	43	
0·028506	40	0·00071
0·01754	3	0·00584

from which Variance Ratio $(F) = \frac{0{\cdot}00584}{0{\cdot}00071}$

$= 8{\cdot}22$

(From Tables F at 3 to 40 degrees of freedom is 2·84 at 5 per cent. level.)

Thus, the variances are significantly different and so the four lines (i), (ii), (iii) and (iv) above cannot be regarded as having the same slope.

(b) *To test if the four lines could be represented by one common line going through the grand mean of Y's.*

For this the total regression was calculated as if all the points were scattered along one regression line :—

ΣX	ΣX^2	ΣY	ΣY^2	ΣXY
86·183	620·601024	18·3538	28·263588	132·346449
86·183	620·601024	16·9259	24·171343	122·257513
86·183	620·601024	18·3714	28·259916	132·405674
86·183	620·601024	18·3711	28·296591	132·466578
344·732	2482·404096	72·0222	108·991438	519·476214

From which

$\Sigma(X-\bar{X})^2 = 6{\cdot}5676$ $\Sigma(Y-\bar{Y})^2 = 0{\cdot}9248$ $\Sigma(X-\bar{X})(Y-\bar{Y}) = 2{\cdot}2188$

and

	Sum of Squares	Degrees of Freedom	Variance
Total	0·9248	47	0·0197
Due to regression	0·7496	1	0·7496
Residual	0·1752	46	0·0038

As in case (a) above an independent estimate of variance was calculated and gave :—

	Sum of Squares	Degrees of Freedom	Variance
Residual total	0·1752	46	
Residuals from individual regressions	0·0285	40	0·00071
	0·1467	6	0·02445

from which $$F=\frac{0\cdot02445}{0\cdot00071}=34$$

Hence the four lines cannot be regarded as having the same slope and also they lie at different levels.

Later Results. As previously stated above the bottles in the earlier experiments were incubated in an electric incubator whereas for later experiments a constant temperature water-bath was used. Typical results for two experiments using the water-bath are given in Table VII.

TABLE VII

RESULTS OF TWO TYPICAL EXPERIMENTS GIVING THE REACTION TIMES CORRESPONDING TO VARIOUS SURVIVOR LEVELS IN THE ACTION OF 1·0 PER CENT. PHENOL ON A SUSPENSION OF *BACTERIUM COLI*

Percentage Survivors	Reaction Times					
	Experiment A			Experiment B		
0·5	12·1	11·0	12·5	9·7	10·0	11·5
0·6	11·2	9·9	11·4	8·9	9·3	10·8
0·7	10·7	9·3	10·9	8·3	8·8	10·0
0·8	10·2	8·8	10·3	7·9	8·5	9·4
0·9	9·8	8·5	10·0	7·3	8·0	9·0
1·0	9·5	8·2	9·7	7·1	7·7	8·7
2·0	7·7	6·7	8·1	5·1	6·1	7·0
3·0	6·8	6·1	7·2	4·6	5·3	6·0
4·0	6·2	5·7	6·4	4·2	4·8	5·4
5·0	5·7	5·3	5·8	3·9	4·3	5·0
6·0	5·2	5·0	5·4	3·6	4·0	4·6
7·0	4·9	4·8	5·1	3·4	3·7	4·3
8·0	4·6	4·5	4·8	3·3	3·5	4·0
9·0	4·3	4·2	4·4	3·2	3·4	3·8
10·0	4·1	4·0	4·0	3·1	3·2	3·7

The probit mortality-log. reaction time regressions for these were :—

$$Y=0\cdot7534+0\cdot3865\ (X-7\cdot3272)$$

and

$$Y=0\cdot8201+0\cdot3784\ (X-7\cdot3272)$$

The sums of the squares and their corresponding degrees of freedom were

	Sum of Squares	Degrees of Freedom
Total (Expt A)	1·433037	44
Due to regression (Expt A)	1·402917	1
Residual (Expt A)	0·030120	43
Total (Expt B)	1·423083	44
Due to regression (Expt B)	1·345134	1
Residual (Expt B)	0·077949	43
Total (A and B)	2·957224	89
Due to regression (A and B)	2·746908	1
Residual (A and B)	0·210316	88

From these results using the analysis outlined above, it can be shown that the two lines could be represented as going through their respective means with the same slope, that is the lines are parallel, but they could not be combined to give a single line representative of 1 per cent. phenol with a 5 drops sampling.

Discussion

The two main criticisms of the second part of this work[1] are partially answered in this third part, the use of a reference turbidity increases the accuracy of measurement, and the results show that more stringent control of incubation temperature considerably reduced the variation in results.

Alper and Sterne[8] and Huntingdon and Winslow[9] have shown that the size of individual bacteria in a *Bact. coli* culture varied considerably during the first 7 hours of growth. It was thus reasonable to assume that two cultures of *Bact. coli* grown from the same size inoculum of the same parent culture would contain the same number of organisms *only* when the conditions of incubation of the two cultures were identical. Such an assumption also precludes the use of a reference turbidity of the barium sulphate type or the calibration of the nephelometer scale in number of organisms. In this part of the work cultures of inocula from definite dilutions of the original suspension of *Bact. coli* and from a test mixture (of original suspension and bactericide) were incubated under exactly similar conditions and the turbidities were compared : on the assumption that ' n ' bacteria in the test mixture would produce the same turbidity as ' n ' bacteria in the original suspension, then an estimate of the percentage of survivors in the test mixture was made. One criticism of this is that although the conditions of time and temperature of incubation were identical for test and reference cultures the bacteria in the test sample had been subject to the action of the bactericide and they may have been so injured that ' n+a ' bacteria had been taken as an inoculum from the test mixture to produce a turbidity equal to that produced by ' n ' bacteria from the original culture unaffected by the bactericide. This does not however detract from the principle of the reference turbidity since in every case the reference turbidity graph (Figure 2) shows the turbidities produced by 0·5 per cent. to 10·0 per cent. of the original suspension under the conditions of the test. It was realised that in calculations what is referred to as 99·5 per cent. mortality may in fact be very slightly less than this ; until some reliable counting method with extremely small limits of error is available for checking these figures it has been assumed that this small error, inherent in this method, can be neglected.

Withell[5], Jordan and Jacobs[10], and Berry and Michaels[11] in similar work have all calculated Probit-Log. Time regressions since in their work the time has been the independent variable, although contrary to normal procedure all the above workers have used the regressions to calculate a value for the independent variable for a known value of the dependent variable, that is the time for a given mortality. In this work however although in the experiments the time was the independent variable in the results obtained for plotting the final graphs the percentage of survivors became the independent variable and thus allowed a regression of log. time on probit per cent. mortality to be calculated. The equation to such a regression line is :

$$Y=\bar{Y}+b(X-\bar{X})$$

where Y = expected value of log. reaction time
X = value of probit per cent. mortality for which Y is desired
$\bar{X}$ & $\bar{Y}$ = mean values of X and Y obtained in test
b = regression coefficient (slope of line).

The fact that each experiment consisted of 10 measurements made over the comparatively short range of mortality, namely 90 per cent. to 99·5 per cent., and also the fact that the correlation coefficients were all very close to unity, justified the conclusion that the calculated regression was linear. The correlation coefficients being close to unity (0·972 and 0·989) means also that the regression coefficients calculated (0·387 and 0·378) would approximate the reciprocals for those calculated for probit-log. time regressions ; in this respect it is interesting to note that the reciprocals of the coefficients calculated by Withell[5] for 0·5 per cent. phenol against *Bact. coli* at 20°C. were 0·5, 0·32 and 0·30, and two of these are of the same order as the above coefficients. Withell's coefficients were calculated from data spread over a much wider mortality range, but similarity of the above coefficients and a consideration of the values of the coefficients obtained for 1·0 per cent., 0·9 per cent. and 0·8 per cent. as listed in Table VI are an indication that the probit-log. time regressions for all strengths of phenol against *Bact. coli* will be parallel.

The slopes of the regression lines for 0·8 per cent. phenol in the earlier experiments were shown to be significantly different but, in later experiments in which the temperature of incubation was more constant this difference in slope was reduced and the lines obtained may be reasonably taken as parallel and this permits of a comparison of these two later lines. For a complete comparison adjustment to the common regression of 0·382 would be necessary but, since in both experiments $\bar{X}$ had the same value then the two values of $\bar{Y}$ afford a means of estimating the difference in level of the two lines and this shows a difference of only 0·94 minute in the reaction time at a mortality level of approximately 97·5 per cent. This difference is a measure of the day to day variation and is considerably smaller than the difference in levels obtained by using different strengths of phenol, as expressed by the averages in Table VIII.

TABLE VIII

SHOWING THE DIFFERENCE IN AVERAGE LEVELS OF THE REGRESSION LINES OBTAINED USING DIFFERENT STRENGTHS OF PHENOL AGAINST *BACTERIUM COLI*

Phenol	$\bar{Y}$ at $\bar{X}$ = 7·3272	Standard Deviation
per cent.		
0·8	1·50	0·07
0·9	1·22	0·06
1·0	0·81	0·12

Thus the daily variation which completely masked the results in Part II[1] has been reduced to a level that permits of closer examination of results.

Berry and Michaels[12] have shown that the concentration of the initial inoculum affects the velocity of the bactericidal action. In this respect

a more rigid control of the initial suspension may help in reducing even further the daily variation.

Summary

1. To reduce the daily variation noted earlier a new form of reference turbidity for use with nephelometers has been introduced. This consists of diluting the initial suspension of *Bact. coli* from 10 per cent. to 0·5 per cent. with sterile water and incubating inocula from these dilutions under conditions identical with those for similar inocula from the bactericidal test on the same initial suspension; comparison of turbidities after 5 hours incubation enables a time survivor relationship to be calculated.

2. The analysis of results obtained by this new method still showed an unduly large variation which was later reduced by more efficient control of incubation temperature and the final results showed that parallel time-survivor regression lines were obtained, although complete reproducibility of results without a small daily variation appears to be more difficult. There are however indications that the technique will allow of comparisons between different strengths of the same bactericide.

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THE CHEMISTRY OF ANTI-PERNICIOUS ANÆMIA FACTORS

PART V.

THE INTER RELATIONSHIP AND STRUCTURE OF THE α-, β-, AND γ-COMPONENTS

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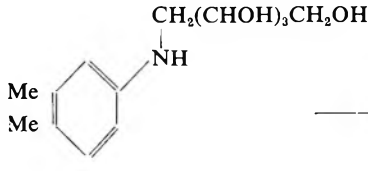
PREVIOUS work by Beaven, Holiday, Johnson, Ellis, Mamalis, Petrow and Sturgeon[1] had shown that: (i) hydrolysis of vitamin B_{12} leads to the formation of three closely related substances designated *components* α, β and γ, (ii) *component* γ is spectroscopically and chromatographically indistinguishable from 5:6-dimethylbenziminazole (VII)[2], (iii) *components* α and β are spectroscopically identical with certain 1-substituted 5:6-dimethylbenziminazoles (*vide infra*), (iv) the effect produced by the 1-substituent in the α- and β-*components* on the absorption spectrum of the parent 5:6-dimethylbenziminazole is similar to that exerted by a methyl grouping.

Consideration of these facts led to the following conclusions: 1. The substituent attached to N^1 in the α- and β-*components* is capable of stepwise degradation by acid. 2. The spectroscopic similarity of the α- and β-*components* with 1:5:6-trimethylbenziminazole excludes the identity of the 1-substituent present in these two compounds with any polar grouping capable of exerting an auxochromic effect. 3. The α- and β-*components* bear a structural similarity to the *N*-D-ribityl-4:5-dimethyl-*o*-phenylenediamine portion of the riboflavine molecule.

In so far as the facts allowed, Beaven *et al.*[1] interpreted the evidence as indicating that the α- and β-*components* might well prove to be sugar derivatives of 5:6-dimethylbenziminazole or their acid transformation products. Experiments on the synthesis of benziminazole glycosides were accordingly initiated in these laboratories.

From this point our studies on vitamin B_{12} developed simultaneously in two main directions. In the first place, procedures for the synthesis of benziminazole glycosides were elaborated[3]. Secondly, chromatographic studies were initiated with the object of determining the sequence in which phosphate[4], the "ninhydrin-reacting" fragment[5] (1-aminopropan-2-ol[6,7]) and the α-, β-, and γ-components were released from vitamin B_{12} during hydrolysis.

The quantity of vitamin B_{12} at our disposal at this stage was, unfortunately, extremely limited owing to heavy commercial demands upon available supplies. It was thus not possible to attempt the direct isolation and identification of the α- and β-*components* by the methods of classical organic chemistry. Nevertheless, we hoped to obtain the desired evidence regarding their structure by chromatographic comparison with synthetic glycosides of known structure.



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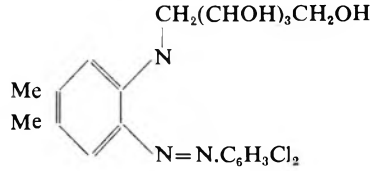

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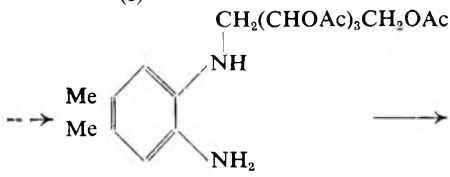

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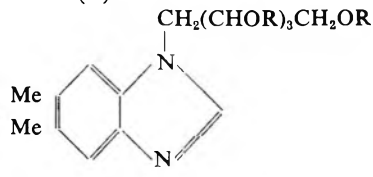

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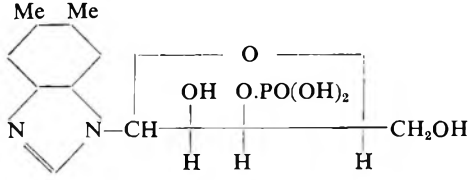
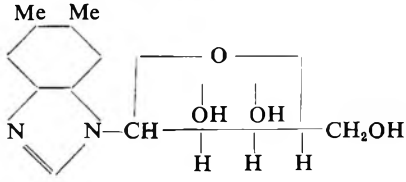


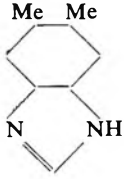
(V)

α—*Component*



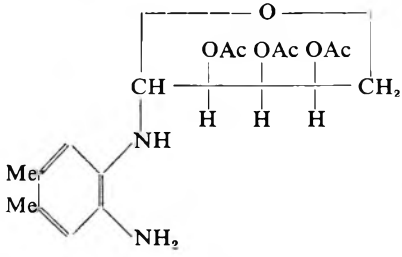
(VI)

β—*Component*

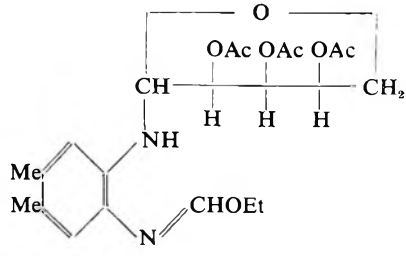


(VII)

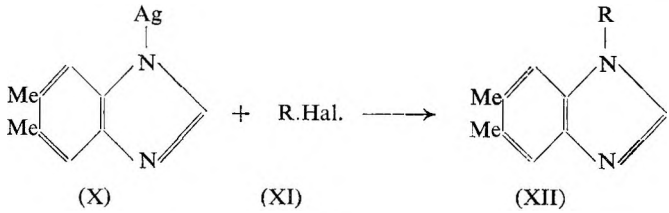
γ—*Component*



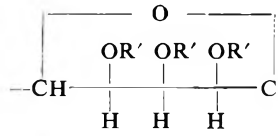
(VIII)

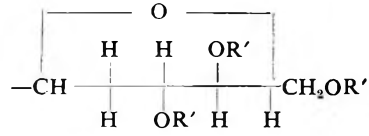


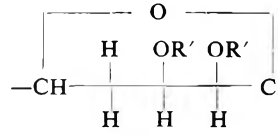
(IX)



(X) (XI) (XII)







Treatment of vitamin B_{12} with 6N hydrochloric acid for very short periods at room temperature led to the rapid liberation of the α-*component*. Thus the latter fragment could be detected on chromatograms prepared from an aliquot of hydrolysate withdrawn and dispensed on to the paper only 5 minutes after solution of the vitamin in mineral acid. Spectrophotometric estimation of the α-*component* eluted from a chromatogram of a 5-hour hydrolysate indicated that not less than 0·7 mol. had been split off from the B_{12} molecule by this treatment. These results were obtained in collaboration with Dr. E. R. Holiday (M.R.C. Spectrographic Unit, The London Hospital, E.1), whose help we gratefully acknowledge.

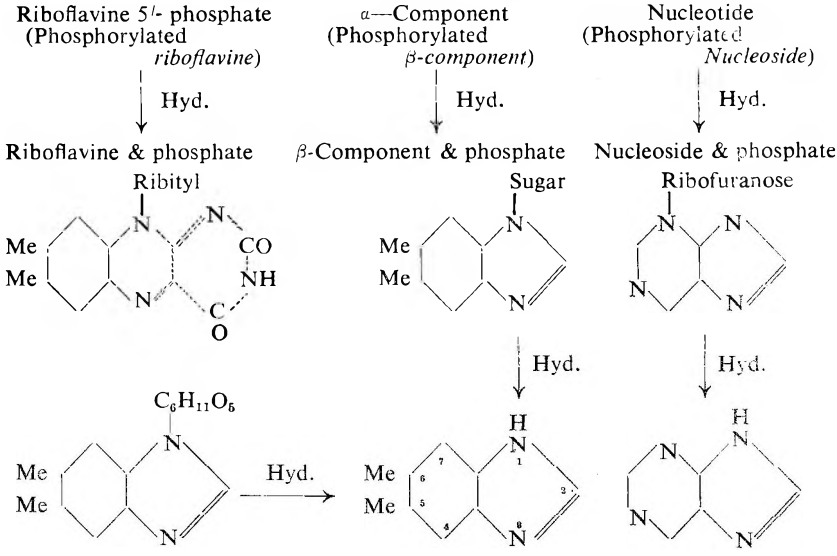
Further contact with 6N hydrochloric acid led, after not less than 18 hours, to the gradual release of 1-aminopropan-2-ol, which formed the second moiety liberated from vitamin B_{12} under these experimental conditions. Neither phosphate nor the β- or γ-*components* made their appearance on chromatograms at this stage.

Accordingly we isolated the α-*component* on the micro-scale and submitted it to further degradation with 6N hydrochloric acid at 100°C. Slow hydrolysis occurred to give, after 6 hours, unchanged α-*component*, some β-*component*, and phosphate, an observation which led us to conclude that the α-*component* was a phosphorylated derivative of the β-*component*. Finally, hydrolysis of the β-*component* with 6N hydrochloric acid at 150°C. in a sealed tube gave the γ-*component* 5:6-dimethylbenziminazole. The fate of the erstwhile 1-substituent which had been eliminated during the last stage could not be determined.

This sequence of changes brought to mind the behaviour of the nucleotides on acidolysis. These compounds are known to be converted, with

liberation of phosphate, into nucleosides, which then undergo further hydrolysis into the heterocyclic base and the sugar (see below). On this basis the α-*component* would correspond to the nucleotide, and the β-*component* to the nucleoside.

Pursuing this line of reasoning further, we were led to compare the behaviour, on acidolysis, of the β-*component* with that of 5:6-dimethylbenziminazole-1-β-D-glucopyranoside, the synthesis of which had already been accomplished at this stage. A remarkable degree of similarity was observed. Thus the two compounds were recovered unchanged after 12 hours' heating with 6N hydrochloric acid at 100°C. whilst at 150°C. profound degradation occurred to give the γ-*component*.



This result furnished us with the first experimental evidence, although admittedly of an indirect character, that the β-*component* could rationally be formulated as a benziminazole glycoside. It left unanswered, however, the nature of the sugar residue involved in glycosidic union with the benziminazole residue.

The structural analogy between riboflavine and the β-*component* (see above) appeared to indicate that the latter compound might be a ribityl-benziminazole. The resemblance to a nucleoside, on the other hand, pointed to a ribosido-benziminazole formulation. Unfortunately we had no direct means of determining the accuracy of these speculations and were thus forced to adopt a purely empirical approach and to attempt the synthesis of the ribosido- and ribityl-derivatives of 5:6-dimethylbenziminazole.

1-D-Ribityl-5:6-dimethylbenziminazole (IV; R = H), prepared as described in the Synthetical Section proved completely stable to 6N hydrochloric acid at 150°C., an observation which eliminated it from further consideration. 5:6-Dimethylbenziminazole-1-β-D-ribopyranoside (XIIa;

R′=H), in contrast, closely resembled the β-*component* both in its stability to 6N hydrochloric acid at 100°C. and in its degradation to 5:6-dimethylbenziminazole at 150°C. In addition, the R_F values of the two compounds on paper chromatography proved to be identical in three different solvent systems.

The results are collected in the Experimental Section together with parallel data obtained with some related benziminazole glycosides synthesised during the course of this work.

Cooley, Ellis and Petrow[8] have previously shown that chromatographic methods, *per se*, cannot be used to identify an unknown compound in the absence of supporting chemical data. We therefore attempted to obtain evidence of this character by periodate oxidation of (XIIa; R′=H) and of the β-*component*, followed by chromatographic examination of the products. The results proved disappointing. Both compounds underwent facile oxidation, as was indeed expected, but the chromatograms obtained from the oxidation products were not satisfactory.

Nevertheless, in spite of these results, we felt convinced that, on biogenetic grounds alone, the β-*component* could not be formulated as (XIIa; R′=H) but, if our speculations were correct, should be assigned the constitution of a 5:6-dimethylbenziminazole-1-ribofuranoside. This conviction was based upon the universal occurrence of D-ribose in the furanose form in such naturally occurring substances as the nucleosides. We therefore turned our attention to the reaction between 5:6-dimethylbenziminazole silver and acetobromribofuranose, hoping thereby to obtain the desired product. Before this work could be completed, Brink, Holly, Shunk, Peel, Cahill and Folkers[9] reported the isolation of 5:6-dimethylbenziminazole-1-α-D-ribofuranoside (VI) from acid hydrolysates of vitamin B_{12} and its synthesis by an unambiguous route. This compound (VI) is undoubtedly identical with the β-*component* of Beaven *et al.* (Part III)[1], the complete structure of which may now be considered as finally elucidated.

Our observation that conversion of the α-*component* into the β-*component* is accompanied by release of phosphate (*vide supra*) has recently been confirmed by Buchanan, Johnson, Mills and Todd[10], who have successfully isolated the former fragment of the B_{12} molecule as its barium salt. Like ourselves, they find the α-*component* stable to periodic acid, and accordingly assign it the constitution of a 2′- or 3′-phosphoryl-5:6-dimethylbenziminazole-1-α-D-ribofuranoside (V). We accept these conclusions, but are unable to adopt their view that the D-1-aminopropan-2-ol present in vitamin B_{12} is likewise attached to the phosphoryl grouping. Our own results (*vide supra*) show that release of the α-*component* is not necessarily accompanied by concomitant liberation of 1-aminopropan-2-ol, for which an alternative location must obviously be found. On general grounds of symmetry, too, the existence of two molecules[11] of 1-aminopropan-2-ol in the B_{12} molecule would appear to render the attachment of only one of them to the phosphoryl grouping somewhat unlikely.

Synthetical Section

Ribityl-and-2′-Deoxyribosido-benziminazoles

The preparation of 1-D-ribityl-5:6-dimethylbenziminazole (IV; R=H) was effected in the following way.

N-D-Ribityl-*o*-4-xylidine (I) was condensed with 2:4-dichlorophenyldiazonium chloride when 5(2′:4′-dichlorophenylazo)-N-D-ribityl-*o*-4-xylidine (II) was readily obtained in excellent yield. Acetylation of this product, followed by reduction with zinc dust and acetic acid in ethyl acetate solution, furnished tetraacetyl-D-ribityl-4:5-dimethyl-*o*-phenylene diamine (III). The latter compound was not isolated but was condensed *in situ* with ethyl orthoformate to give 1-tetraacetyl-D-ribityl-5:6-dimethylbenziminazole (IV; R=Ac), isolated as the picrate. Hydrolysis in the usual way furnished 1-D-ribityl-5:6-dimethylbenziminazole (IV; R=H).

In addition to the foregoing compound, the preparation of the related 2′-deoxyriboside was undertaken, as 2-deoxy-D-ribose is known to occur in certain nucleosides. Before embarking on its synthesis, however, we explored the preparation of the corresponding deoxy-D-glucoside.

Reaction of 1-bromo-3:4:6-triacetyl-2-deoxy-D-glucose (XIb, R′=Ac) with 5:6-dimethylbenziminazole silver (X) gave a non-crystalline mixture of αβ-isomers which could not be resolved by fractionation procedures. Deacetylation, followed by treatment with picric acid, however, readily gave a crystalline 5:6-dimethylbenziminazole-1-(2′-deoxy-D-glucopyranoside) picrate from which, by acetylation and regeneration of the base, 5:6-dimethylbenziminazole-1-(3′:4′:6′-triacetyl-2′-deoxy-D-glucopyranoside) (XIIb; R′=Ac) was obtained in crystalline form. Hydrolysis of the latter now furnished the pure deoxy-D-glucoside (XIIb; R′=H). The second isomer, which was no doubt present in the initial reaction mixture, was not isolated.

In contrast to these results, reaction between 5:6-dimethylbenziminazole silver (X) and 1-chloro-3:4-diacetyl-2-deoxy-D-ribose (XIc; R′=Ac) in xylene solution at 100°C. readily gave a homogeneous 5:6-dimethylbenziminazole-1-(3′:4′-diacetyl-2′-deoxy-D-ribopyranoside) (XIIc; R′=Ac). Hydrolysis furnished 5:6-dimethylbenziminazole-1-(2′-deoxy-D-ribopyranoside) (XIIc; R′=H) characterised by conversion to the picrate.

Similar results were obtained by reaction between benziminazole silver and (XIc; R′=Ac). The product readily gave crystalline benziminazole-1-(3′:4′-diacetyl-2′-deoxy-D-ribopyranoside) picrate, decomposed to the glassy base (XIIc; R′=Ac). Hydrolysis of the latter compound, followed by purification *via* the picrate, gave benziminazole-1-(2′-deoxy-D-ribopyranoside) (XIIc; R′=H), isolated as the hydrochloride.

These observations contrast somewhat with those recorded by Davoll and Lythgoe[12], who obtained two isomeric deoxyribosides and only one deoxyglucoside by condensing (XIb; R′=Ac) and (XIc; R′=Ac) with theophylline silver.

5:6-Dimethylbenziminazole-1-β-D-*ribopyranoside*

The ribopyranoside (XIIa; R′=H) required for comparison with the

β-*component* was readily prepared by an extension of the synthetic methods elaborated in Part IV[3].

By condensing 4:5-dimethyl-*o*-phenylenediaminetriacetyl-D-ribopyranoside (VIII) with ethyl orthoformate, the *iso*formanilide (IX) was obtained in excellent yield. Treatment with dilute hydrochloric acid furnished 5:6-dimethylbenziminazole-1-β-triacetyl-D-ribopyranoside (XIIa; R′=Ac). The β-configuration assigned to the anomeric centre in this compound followed from its alternative synthesis from 5:6-dimethylbenziminazole silver (X) and α-acetobromribopyranose (XIa; R′=Ac). Hydrolysis with hydrochloric acid gave the desired 5:6-dimethylbenziminazole-1-β-D-ribopyranoside (XIIa; R′=H) which was isolated as the hydrochloride hemihydrate. The pyranoside character of the lactol ring in the latter compound was confirmed by periodate titration.

Experimental

Melting points are corrected. Microanalyses are by Drs. Weiler and Strauss, Oxford.

Section A

*The preparation of 1-*D*-Ribityl-5:6-dimethylbenziminazole. 5-(2′:4′-Dichlorophenylazo)-N-*D*-ribityl-o-4-xylidine.*—2:4-Dichloroaniline (1·65 g.) was diazotised by treating an ice-cold solution in water (75 ml.) and concentrated hydrochloric acid (5 ml.) with sodium nitrite (0·75 g.) in water. A paste of *N*-D-ribityl-*o*-4-xylidine (2·5 g.) in a little water was added with stirring, followed at once by sufficient sodium bicarbonate to make the mixture just alkaline. The *azo*-compound (2·0 g.) was collected after 24 hours, and was recrystallised from aqueous alcohol, forming red needles, m.pt. 158° to 160°C. (decomp.) Found: C, 52·8; H, 5·2; N, 9·7. $C_{19}H_{23}O_4N_3Cl_2$ requires C, 53·3; H, 5·4; N, 9·8 per cent. The compound gave a syrupy tetraacetate which could not be crystallised.

*1-Tetraacetyl-*D*-ribityl-5:6-dimethylbenziminazole.*—The acetyl-derivative of the foregoing compound (1·6 g.) was dissolved in ethyl acetate (240 ml.) and zinc dust (24 g.) added. The suspension was boiled and mechanically stirred whilst acetic acid (12 g.) in ethyl acetate (110 ml.) was slowly added over 1 hour. The colourless filtrate and washings were evaporated to dryness and the 2:4-dichloroaniline removed by washing with light petroleum. The residue was heated at 100°C. for 3 hours with ethyl orthoformate (5 ml.) and then concentrated to a syrup. 1-Tetraacetyl-D-ribityl-5:6-dimethylbenziminazole was isolated as the *picrate*, which crystallised from alcohol in yellow needles. M.pt. 153° to 154°C. Found: C, 49·7; H, 4·8; N, 10·8. $C_{22}H_{28}O_8N_2$, $C_6H_3O_7N_3$ requires C, 49·6; H, 4·6; N, 10·9 per cent.

The foregoing picrate (60 mg.) was decomposed by passing a chloroform solution through a column of alumina, and the tetraacetate hydrolysed by treatment with methyl alcoholic sodium methoxide. 1-D-Ribityl-5:6-dimethylbenziminazole was obtained as a syrup which was used without further purification for stability and chromatographic studies.

*5:6-Dimethylbenziminazole-1-(3′:4′:6′-triacetyl-2′-deoxy-*D*-glucopyranoside).* 5:6-Dimethylbenziminazole silver (9·6 g.), 1-bromo-3:4:6-

triacetyl-2-deoxy-D-glucose [prepared from 7·7 g. of triacetyl-D-glucal by the method of Davoll and Lythgoe (loc. cit.)] and xylene (80 ml.) were heated at 100°C. for 30 minutes with frequent shaking. The product, isolated in the usual way, was dissolved in alcohol (100 ml.) and treated with picric acid (5 g.). An insoluble, gummy picrate separated, which was washed several times by decantation and then decomposed by percolation through alumina in chloroform solution. The resulting syrupy triacetate (3 g.) was allowed to stand for 3 days with methyl alcohol containing a trace of sodium methoxide, whereafter the deacetylated deoxy-glucoside was isolated as the picrate (1·5 g.). 5:6-*Dimethylbenziminazole*-1-2′-*deoxy*-D-*glucopyranoside picrate* crystallised from alcohol in glistening yellow needles. M.pt. 189° (decomp.), $[\alpha]$ $-4{\cdot}9°$ (c = 1, in pyridine). Found: C, 47·7; H, 4·7; N, 13·5. $C_{15}H_{20}O_4N_2$, $C_6H_3O_7N_3$ requires C, 48·4; H, 4·5; N, 13·4 per cent. The foregoing picrate was acetylated with acetic anhydride/pyridine and after removal of the solvents, the crude triacetate picrate was decomposed on alumina in chloroform solution. 5:6-*Dimethylbenziminazole*-1-(3′:4′:6′-*triacetyl*-2′-*deoxy*-D-*glucopyranoside*) crystallised from chloroform and light petroleum in sparkling needles, m.pt. 125° to 127°C. $[\alpha]_D^{25°C.}$ $-39{\cdot}4°$ (c = 1, in chloroform). Found: C, 60·8; H, 6·6; N, 6·0. $C_{21}H_{26}O_7N_2$ requires C, 60·3; H, 6·3; N, 6·1 per cent.

5:6-*Dimethylbenziminazole*-1-2′-*deoxy*-D-*glucopyranoside* slowly crystallised from a solution of the triacetate (300 mg.) in methyl alcohol (20 ml.) containing sodium (5 mg.) and was recrystallised from methyl alcohol/light petroleum forming colourless prismatic needles, m.pt. 250°C. Found: C, 62·0; H, 7·0; N, 8·4. $C_{15}H_{20}O_4N_2$ requires C, 61·6; H, 6·9; N, 8·3 per cent. 5:6-*Dimethylbenziminazole*-1-(3′:4′-*diacetyl*-2′-*deoxy*-D-*ribopyranoside*). Diacetyl-D-arabinal (2 g.) was converted into 1-chloro-3:4-diacetyl-2-deoxy-D-ribose by the method of Davoll and Lythgoe[12]. The crude compound was reacted with 5:6-dimethylbenziminazole-silver (3 g.) in xylene (60 ml.) at 100°C. for 4 hours. Isolation in the usual way gave 5:6-*dimethylbenziminazole*-1-(3′:4′-*diacetyl*-2′-*deoxy*-D-*ribopyranoside*) *picrate* (1 g.), yellow needles from alcohol and chloroform, m.pt. 203°C. Found: C, 49·9; H, 4·3; N, 11·8. $C_{18}H_{22}O_5N_2$, $C_6H_3O_7N_3$ requires C, 50·1; H, 4·4; N, 12·2 per cent. The *diacetyl*-2′-*deoxy*-D-*riboside* formed tablets from ethyl acetate/light petroleum, m.pt. 118°C. $[\alpha]_D$ $+2{\cdot}6°$ (c = 1, in chloroform). Found: C, 62·7; H, 6·2; N, 8·2. $C_{18}H_{22}O_5N_2$ requires C, 62·4; H, 6·4; N, 8·1 per cent.

5:6-*Dimethylbenziminazole*-1-2′-*deoxy*-D-*ribopyranoside*. A solution of the foregoing diacetate (500 mg.) in dry methyl alcohol (20 ml.) containing a trace of sodium was allowed to stand at room temperature for 3 days. Carbon dioxide was bubbled in and the solution then evaporated to dryness. Crystallisation of the residue from alcohol-light petroleum gave 5:6-dimethylbenziminazole-1-2′-deoxy-D-ribopyranoside as the *hydrate* in silky needles, m.pt. 160°C. $[\alpha]_D^{25°C.}$ $+30{\cdot}9°$ (c = 0·5, in pyridine). Found: C, 60·1; H, 7·2; N, 9·8. $C_{14}H_{18}O_3N_2$. H_2O requires C, 60·0; H, 7·2; N, 10·0 per cent. The *picrate* formed yellow needles from

alcohol, m.p. 203°C. Found: C, 48·7; H, 4·4. $C_{14}H_{18}O_3N_2,C_6H_3O_7N_3$ requires C, 48·9; H, 4·3 per cent.

Benziminazole-1-2′-deoxy-D-ribopyranoside. 1-Chloro-3:4-diacetyl-2-deoxy-D-ribose, from D-arabinal (4·9 g.) was condensed with benziminazole silver (7·4 g.) in xylene solution at 100°C. The filtrate and washings were taken to dryness and treated with alcoholic picric acid (5 g.). The resulting picrate was extracted with chloroform and the soluble fraction (2·8 g.) recrystallised from alcohol to give *benziminazole-1-(3′:4′-diacetyl-2′-deoxy-D-ribopyranoside picrate* in yellow needes, m.p. 167° to 168°C. $[\alpha]_D^{26°C.}$ −8·6° (c = 1, in pyridine). Found: C, 48·3; H, 3·9; N, 12·7. $C_{16}H_{18}O_5N_2,C_6H_3O_7N_3$ requires C, 48·3; H, 3·9; N, 12·8 per cent. Decomposition of this compound by passing a chloroform solution through alumina gave only a resinous triacetate which was therefore directly deacetylated with sodium methoxide in methanol. The deoxy-D-riboside so obtained was isolated as the *picrate,* yellow needles from alcohol, m.p. 170°C.$[\alpha]_D^{22°C.}$ −14·8° (c = 1, in pyridine). Found: C, 46·5; H, 3·7; N, 14·7. $C_{12}H_{14}O_3N_2$, $C_6H_3O_7N_2$ requires C, 46·6; H, 3·7; N, 15·1 per cent., which was decomposed by shaking with dilute hydrochloric acid and nitrobenzene. Evaporation of the aqueous layer gave *benziminazole-1-2′-deoxy-D-ribopyranoside hydrochloride hydrate,* long needles from alcohol, m.p. 150°C.$[\alpha]_D^{24°C.}$ −34·5° (c = 0·5, in water). Found: C, 50·0; H, 6·0; N, 9·7. $C_{12}H_{14}O_3N_2HCl,H_{20}$ requires C, 49·9; H, 5·9; N, 9·7 per cent.

5:6-*Dimethylbenziminazole-1-β-triacetyl-D-ribopyranoside.* (i) 5-Nitro-*o*-4-xylidine-triacetyl-D-riboside (3 g. Kuhn and Strobele[13]) in ethyl acetate (50 ml.) was shaken with hydrogen in the presence of a palladised charcoal catalyst until hydrogen uptake was complete. After removal of the catalyst, ethyl orthoformate (10 ml.) was added and the solution heated on the steam bath in an open flask for 3 hours. The resin remaining after removal of the solvent was heated with 0·1N hydrochloric acid (15 ml.) for 10 minutes, whereafter the solution was basified with potassium carbonate and the product extracted with chloroform and converted to the picrate. 5:6-*Dimethylbenziminazole-1-β-triacetyl-D-ribopyranoside picrate* (500 mg.) formed yellow needles from alcohol, m.p. 186° to 187°C. Found: C, 48·9; H, 4·7; N,10·9. $C_{20}H_{24}O_7N_2$, $C_6H_3O_7N_3$ requires C, 49·3; H, 4·3; N, 11·0 per cent. The *base* crystallised in colourless plates from chloroform and light petroleum, m.p. 155°C.$[\alpha]_D^{24°C.}$ −40·4° (c = 1, in chloroform). Found: C, 59·6; H, 6·2; N, 7·0. $C_{20}H_{24}O_7N_2$ requires C, 59·4; H, 6·0; N, 6·9 per cent. (ii) 5:6-Dimethylbenziminazole silver (1·3 g.) and acetobrom-D-ribose (1·65 g.) were condensed together in xylene (40 ml.) at 140°C. in the usual way. The product was purified by passing a chloroform solution through a short column of alumina and then converted into the *picrate* (600 mg.), which crystallised from alcohol in yellow needles identical in m.p. and mixed m.p. with the compound prepared by method (i).

5:6-*Dimethylbenziminazole-1-β-D-ribopyranoside hydrochloride hemihydrate* was prepared by hydroylsis of the foregoing triacetate (500 mg.) with 2N hydrochloric acid (50 ml.) at 100°C. followed by evaporation to

dryness. It separated from alcohol/ether in small needles, m.pt. 229° to 230°C. (decomp.). Found: C, 51·5; H, 6·3. $C_{14}H_{13}O_4N_2,HCl,\frac{1}{2}H_2O$ requires C, 51·9; H, 6·2 per cent. The *riboside* crystallised from alcohol-light petroleum in flat needles, m.pt. 250° to 251°C. (decomp.). Found: S, 60·2; H, 6·2; N, 10·0. $C_{14}H_{18}O_4N_2$ requires C, 60·4; H, 6·5; N, 10·1 per cent. This compound consumed 2·05 moles. of periodic acid.

Section B. Whatman No. 1 filter paper was employed for the chromatograms. The benziminazole derivatives examined on chromatograms were detected by inspection of the papers under the ultra-violet light transmitted by a low-pressure mercury resonance lamp fitted with a Chance OX7 filter, when they appeared as blue-violet fluorescent spots. The latter filter was found to be just as suitable as the Corning 9863 filter previously employed for this purpose[1]. The α- and β-*components* present in hydrolysates of vitamin B_{12} and required for degradation studies, were obtained from chromatogram segments which were eluted by the method of Dent[14].

The stabilities to hydrochloric acid of the following compounds were investigated.

(a) The β-*component.*
(b) 5:6-Dimethylbenziminazole-1-β-D-ribopyranoside (XIIa; R′=H).
(c) 1-D-Ribityl-5:6-dimethylbenziminazole (IV; R=H).
(d) 5:6-Dimethylbenziminazole-1-(2′-deoxy-D-ribopyranoside) (XIIc: R=H).

Solutions of each of the above substances in 6N hydrochloric acid were heated in sealed tubes for 12 hours at (i) 100°C. and (ii) 150°C. and the products submitted to paper chromatography employing *n*-butyl alcohol-acetic acid[15] as the irrigation solvent. The results are recorded in Table I.

TABLE I

Compound	R_F value of compound	R_F values of products obtained after 12 hours at		R_F value of 5:6-dimethyl-benziminazole
		(i) 100°C.	(ii) 150°C.	
(a)	0·76	0·76	0·84	0·84
(b)	0·76	0·76	0·84	0·84
(c)	0·76	0·76	0·76	0·84
(d)	0·79	0·84 (0·79)	0·84	0·84

Compounds (a) and (b) behaved identically in every respect. Thus both were characterised by identical R_F values, were stable under conditions (i), and were completely degraded into the parent benziminazole under conditions (ii). Compound (c) proved to be completely stable even under the latter drastic conditions of acidolysis. In contrast to these results, compound (d) under conditions (i) gave rise on the chromatogram to two spots of differing intensity, the R_F value of the less intense spot being shown in brackets in the above Table. The degradation of this substance into the parent benziminazole was therefore largely complete under conditions (i).

Compounds (a) and (b) were also chromatographically indistinguishable when *n*-butyl-alcohol-acetic acid-water (4 : 1 : 1) or aqueous saturated secondary butyl alcohol were employed as the irrigation solvents. With these systems, R_F values of 0·72 and 0.90 respectively were obtained.

The chromatographic behaviour and stabilities to 6N hydrochloric acid of a number of synthetic benziminazole glycosides[3] were determined in a like manner. The compounds examined were : —

1. Benziminazole-1-β-D-glucopyranoside.
2. 4 : 5-Dimethylbenziminazole-1-β-D-glucopyranoside hydrochloride.
3. 5 : 6-Dimethylbenziminazole-1-β-D-glucopyranoside.
4. Benziminazole-1-α-L-arabopyranoside.
5. Benziminazole-1-β-D-xylopyranoside.
6. 5-Methylbenziminazole-1-β-D-xylopyranoside.
7. 5 : 6-Dimethylbenziminazole-1-L-rhamnopyranoside.

The R_F values obtained employing *n*-butyl-alcohol-acetic acid[15] as the irrigation solvent are shown in Table II.

TABLE II

Compound No.	R_F value of compound	R_F values of products obtained after 12 hours at		R_F value of parent benziminazole unsubstituted in 1-position
		(i) 100°C.	(ii) 150°C.	
1	0·46	0·46	0·76 (0·47)	0·75
2	0·61	0·61	0·84 (0·61)	0·84
3	0·59	0·60	0·83 (0·61)	0·84
4	0·50	0·51	0·76	0·75
5	0·58	0·58	0·75	0·75
6	0·62	0·61	0·75	0·74
7	0·71	0·71	0·83 (0·72)	0·84

Glycosides nos. 1, 2, 3 and 7 each gave rise to two benziminazole spots of differing intensity under conditions (ii). In each of these cases the R_F value of the less intense of the two spots is given in brackets.

The close correspondence between the R_F values of the compounds themselves with those obtained under (i) indicates that all the glycosides examined were unaffected by 6N hydrochloric acid for 12 hours at 100°C. On the other hand, comparison of the figures under (ii) with those given in the last column of Table II indicates that under the more drastic conditions of acidolysis employed, four of the glycosides were mostly, and the other three completely, degraded into the parent benziminazoles unsubstituted in the 1-position.

Summary and Conclusions

1. The order in which phosphate, the "ninhydrin-reacting" fragment (1-aminopropan-2-ol), and the *α-, β- and γ-components* are released from vitamin B_{12} on acid hydrolysis has been determined.

2. The presence of phosphorus in the *α-component* is reported.

3. The bearing of the results on the inter-relationship and structure of *α-, β-* and *γ-components* is discussed.

4. 1-D-Ribityl-5:6-dimethylbenziminazole and 5:6-dimethylbenziminazole-1-β-D-ribopyranoside have been synthesised and each compared with the *β-component* in respect to both stability towards hydrochloric acid and behaviour on paper chromatography.

5. 5:6-Dimethylbenziminazole-1-β-D-ribopyranoside and the *β-component* behaved identically on acidolysis and also on paper chromatograms irrigated with three different solvent systems.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Charcoal, Determination of Adsorptive Power of. R. Charonnat and J. Leclerc. (*Ann. pharm. franc.*, 1949, **7**, 625.) Differences between the adsorptive power of different samples of charcoal are least marked when the concentration of the substance to be adsorbed is either very high or very low. The most favourable character for differentiation is the concentration of solution from which the adsorbent takes up one half of the dissolved substance. For the determination, 100mg. of the charcoal is shaken for 30 minutes with 10 ml. of a solution of iodine, the mixture is centrifuged, the residue is washed with a little water, and the iodine in the solution is determined by titration. From the results of a series of determinations, the concentration of 50 per cent. adsorption is determined by interpolation. Some results obtained are as follows: graphite 0·004N; vegetable charcoal 0·020N; animal charcoal 0·082N; activated charcoal A 0·13N; activated charcoal B 0·235N. G. M.

Glucose, Microdetermination of. M. Herbain. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1104.) A solution of glucose containing 5 to 25μg. in 2ml. is treated with 2ml. of ferric-ferrocyanide reagent and 2ml. of water and is heated for 15 minutes in a boiling water-bath followed by 10 minutes at 20°C. Phospho-ferric reagent is added to a volume of 10ml. and, after mixing, the solution is kept at 20°C. for 1 hour before the colour is measured in a suitable spectrophotometer; a blank determination is taken through the whole procedure. The ferric-ferrocyanide reagent consisted of potassium ferricyanide (0·03g.), potassium ferricyanide (0·165g.) and anhydrous sodium carbonate (2·0g.) dissolved in water to 250ml.; the phospho-ferric reagent consisted of potassium ferricyanide (0·2g.) and phosphoric acid (85 per cent., 23·5g.) dissolved in water to 500ml. Detailed results are given of the development work carried out in the selection of the conditions of the reaction and on the composition of the reagent in order that quantitative results could be obtained. R. E. S.

Oxalate, Colorimetric Determination of. S. Burrows. (*Analyst*, 1950, **75**, 80.) A number of colorimetric methods for the routine determination of calcium oxalate in unknown composts were found to be unsatisfactory and an absorptiometric method based on the fading effect of oxalates on the green complex of tervalent iron and 7-iodo-8-hydroxyquinoline-5-sulphonic acid (ferron) was shown to be suitable. The ground compost is extracted with citric acid solution containing calcium chloride and saturated with calcium oxalate to remove the iron and most of the phosphate and colouring matter. The residue is extracted with hydrochloric acid (0·4N), filtered, and a buffered ferric chloride-ferron solution is added, the light absorption of the solution being measured spectrophotometrically using Ilford filters No. 607; the amount of oxalate is calculated by using a standard curve prepared using solutions containing between 0 and 4mg. of calcium oxalate. The reproducibility obtained (± 4 per cent. in samples containing about 1 per cent. of calcium oxalate) was considered satisfactory in view of the rapidity of the method, although the recovery of added calcium oxalate amounted to only 90

per cent. The test solutions for absorption measurements were stable in the dark for a number of hours, but fading occurred on exposure to sunlight; this fading was related quantitatively to the amount of oxalate added. R. E. S.

Phenylbarbiturates, Detection of. E. Rathenasinkam. (*Analyst*, 1950, **75**, 108.) Three phenyl barbiturates, phenobarbitone, methylphenobarbitone, and 5-methyl-5-phenylbarbituric acid were examined. The barbiturate (about 1·0mg.) was heated with potassium nitrate (200mg.) and sulphuric acid (20 drops) in boiling water for about 20 minutes. After cooling and diluting with water to about 30ml., the mixture was transferred to a separating funnel and extracted first with 30ml. of chloroform, and then with 30 ml. of ethyl ether. Each extract was washed and evaporated separately, the residue being dissolved in acetone (2ml.), and 1 drop of sodium hydroxide solution (50 per cent.) added; after shaking and allowing to stand, the three compounds examined showed colours ranging from blue to purple in the chloroform or ether extracts. R. E. S.

Phenylephrine (Neo-synephrine), in Pharmaceutical Products Colorimetric Determination of. M. E. Auerbach. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 50.) 5 ml. of a solution containing about 25 μg. of phenylephrine in water or alcohol is mixed with 1 ml. of a 5 per cent. solution of borax and 0·5 ml. of diazo reagent. After 10 minutes 1 ml. of 10 per cent. sodium hydroxide is added, the mixture diluted to 10 ml. with water, and the percentage transmittancy measured at 495 mμ. The content of phenylephrine is read from the straight line obtained by plotting percentage transmittancy against concentration for a number of standard solutions similarly treated. The diazo reagent is prepared by adding 0·5 ml. of a 7 per cent. solution of sodium nitrate to an ice-cold solution of 30 mg. of *p*-nitroaniline in 2 ml. of 6N hydrochloric acid, diluting after 2 minutes with 100 ml. of cold water and adding 1 ml. of sulphamic acid ; it should be freshly prepared. Penicillin, amethocaine, chlorbutol, procaine, quaternary ammonium germicides and dyes in the proportions normally used in elixirs do not interfere, whereas sulphathiazole and marfanil do. G. R. K.

Procaine, Sulphanilamide and Related Compounds, Determination of. K. R. Srinivasan. (*Analyst*, 1950, **75**, 76.) Satisfactory results are obtained in such determinations by direct titration with bromate and bromide in acid solution, since the reaction stops at the stage of dibromo-substitution. The difficulty in the determination of the end-point is obviated by the use of a pair of polarised platinum electrodes; a sharp end-point is obtained, the slightest excess of bromine in the solution at once depolarising the cathode and causing a large deflection of the galvanometer. An aliquot containing about 5mg. of a sulphonamide is taken, potassium bromide solution and diluted hydrochloric acid are added and the electrodes are dipped into the solution and connected up in circuit; the solution is then titrated with the potassium bromate solution (0·01N) the end-point being indicated by a permanent deflection of the galvanometer from the zero position. Details are given of the preparation of the electrodes and of obtaining the polarising voltage of 10 millivolts. Satisfactory recovery results were obtained for procaine hydrochloride, benzocaine, sulphanilamide, sulphapyridine, sulphaguanidine, sulphathiazole and sulphadiazine. In the determination of procaine penicillin the procaine is freed from the penicillin by extraction with chloroform after liberating the free base with ammonia; the procaine is taken up in

hydrochloric acid from the chloroform solution and the acid solution is then titrated with standard bromate. R. E. S.

Reducing Sugars, Colorimetric Determination with Triphenyltetrazolium Chloride. A. M. Mattson and C. O. Jensen. (*Anal. Chem.*, 1950, **22,** 182.) A method has been worked out for the determination of reducing sugars based on the reduction of triphenyltetrazolium chloride to triphenylformazan which is red and water-soluble. The solution under test in a dry flask is maintained at 25°C., in a water-bath for 10 minutes together with a blank consisting of distilled water. N sodium hydroxide is added to each flask, the solutions are mixed by swirling and returned to the water-bath for 6 minutes; triphenyltetrazolium chloride solution (0·5 per cent., in water) is added to each of the flasks which are then heated for a further 30 minutes in the water-bath. Acidified pyridine (15ml. of hydrochloric acid per 100ml. of pyridine) is added and the light absorption of the clear red solution is taken at 490mμ. on a photoelectric colorimeter; the sugar content can be obtained from a standard curve previously prepared. The graph of optical density and sugar concentration was a straight line, the average slope of the lines being determined for each sugar; values are given for lactose, glucose, fructose and invert sugar. Milk samples, after clarification with lead acetate and ammonium oxalate, were analysed by the above method and by that of Quisumbing and Thomas; the results obtained showed good agreement. The determination of glucose and fructose in mixtures could be accomplished by estimating the total sugars as glucose by the tetrazolium method (S) and by the Quisumbing and Thomas method (S′) and then applying the equation $(S - S') = AF$ where A is a constant (found experimentally to be 6·48) and F is the percentage of fructose. The method was applied to the analysis of honey. R. E. S.

Reducing Sugars, Estimation of by Ferricyanide. M. Plumel. (*Bull. Soc. Chim. biol.*, 1949, **31,** 1163.) A study has been made of the principal factors affecting the oxidation of glucose in an alkaline medium and a method was devised suitable for the determination of glucose in blood. The blood sample (0·1ml.) in a centrifuge tube is diluted with a little water (3·4ml.) and 1ml. of ferric sulphate reagent (2g. of anhydrous ferric sulphate and 25ml. of N sulphuric acid diluted to 100ml. with water) is added followed by N sodium hydroxide (0·5ml.). The supernatant liquid after centrifuging is colourless and slightly acid (*p*H 6·0); 2ml. aliquots are taken for the actual determination, 2ml. of water is added and 2ml. of alkaline potassium ferricyanide solution. The mixture is heated in a boiling water-bath for 8 minutes, cooled, and acid ferric sulphate solution is added followed by centrifuging and the addition of acid sodium fluoride. The precipitate redissolves and the resulting colour is measured spectrophotometrically, the amount of glucose present being determined from a calibration graph prepared previously. R. E. S.

Saponins, Hæmolytic Assay of. W. Awe and H. Häussermann. (*Arch. Pharm., Berl.*, 1950, **283,** 7.) Comparison of the saponin content of vegetable extracts by means of the hæmolytic index is often unsatisfactory on account of the difficulty in determining the end point owing to turbidity in the extracts. This difficulty may be avoided by determining the "Index hæmolyticus initialis" (HI_1), which is defined as the minimum concentration at which the hæmolytic tubes show a reddish-yellow coloured liquid zone above the red blood corpuscles; it can be detected even in slightly turbid liquids.

A comparison was made of the results of this test with determinations of the surface tension, for extracts of senega root and of horse chestnut. Within certain limits the two sets of figures were found to run parallel for each extract. The determination of surface tension is subject to a smaller range of error than that of hæmolytic index, and it would therefore appear that a determination of the former (i.e. measurement of drop size) would be the simplest method of comparison of saponin extracts derived from a single drug. The method would be invalidated by adulteration with surface-active (wetting) agents, but this objection also applies to the hæmolytic method, more especially as such agents, like saponins, are precipitated by cholesterol.

G. M.

INORGANIC CHEMISTRY

Sodium Hydroxide, Carbonate-free, Preparation of. C. W. Davies and J. H. Nancollas. (*Nature*, 1950, **165**, 237.) Rather more than the required amount of the alkali is made up from washed sticks, and a column of the resin Amberlite IRA-400, which may be initially in the form of its chloride, is prepared in the usual way. The capacity of the air-dry resin is 1·4 milli-equivalents/g., and a 50-ml. tube $\frac{2}{3}$ full of the resin would be suitable for preparing 1 l. of 0·1N sodium hydroxide. The alkali is passed through the resin until the effluent is chloride-free (if necessary), and the tube is then transferred to the neck of the stock bottle. When the rest of the alkali has passed through, the resin column is replaced by a soda lime guard tube, and the carbonate-free sodium hydroxide is ready for standardisation. The column can be easily regenerated by the passage of hydrochloric acid, which destroys the carbonate, and the subsequent replacement of the chloride by hydroxide.

S. L. W.

ORGANIC CHEMISTRY

Adrenochrome and Ephedrine, "Biuret reaction" of. P. Bouvet. (*Ann. pharm. franc.*, 1949, **7**, 640.) Both adrenochrome and ephedrine give, apparently, a "biuret reaction" with copper salts in presence of strong alkali. The coloured compounds produced in these two cases were isolated and examined. In the case of adrenochrome, the compound was an organic substance of green colour, containing no copper, and soluble in alkalis to give a violet solution. The compound from ephedrine, separated by extraction with light petroleum from alkaline solution, formed violet crystals of m.pt. 180°C. (with decomposition). It differs from the biuret compounds by its lack of acid properties. A suggested graphic formula is given. G. M.

Glycerophosphates, Stability of. E. Bamann, E. Nowotny and E. Heumüller. (*Arch. Pharm., Berl.*, 1950, **283**, 4.) In testing for free phosphate in glycerophosphates, using nitro-molybdate, a slight positive reaction is permitted in the case of the ferric salt. This is because this salt is much more easily hydrolysed by acid than are those of sodium or calcium. The salts of aluminium and of zinc are also comparatively readily hydrolysed, and there is also a difference between the α and β salts. The β isomer of the cerium salt was found to be hydrolysed twice as quickly as the α isomer.

G. M.

Steroids, Nomenclature of. W. Klyne. (*Nature*, 1950, **165**, 313.) The nomenclature of the cardiac aglycones, toad poisons and steroid sapogenins is unsatisfactory. New names should be introduced for a few fundamental structures and the naturally occurring substances, and their derivatives named as substitution products of these. Thus, most cardiac aglycones are derivatives of the 14-*iso*-norcholenic acid lactone I (lactone structures I to IX are set out in the text), which might be called digitenolide—indicating the source of the compounds and their unsaturated lactone character. Digitoxigenin would then be named 3β : 14β-dihydroxydigitenolide, and strophanthidin 3β : 5β : 14β-trihydroxy-19-aldo-digitenolide. Toad poisons are derivatives of the 14-*iso*-choladienic acid lactone structure V, which might be called bufadienolide. Thus bufalin would be 3β : 14β-dihydroxybufadienolide. The names of the steroid sapogenins might be based on the name steroketal VI, so that sapogenin would be steroketal, and sarsasapogenin would be 3β-hydroxysteroketal. Compounds in which both oxide rings have been opened might be best named as derivatives of coprostane or cholestane, for example, tetrahydrosarsasapogenin =coprostane—3β : 16β : 26-triol. S. L. W.

TOXICOLOGY

Arsenic in the Body, Distribution of. V. Brustier, P. Bourbon and R. Vignes. (*Ann. pharm. franc.*, 1949, **7**, 729.) Samples of blood and hair from patients suffering from arsenical polyneuritis, were examined at intervals, with the following results:—

Case	Blood mg. per cent.	Hair mg. per cent.	Date of Sampling
C	0·108	7·1	18.6.48
	0·04	18·2	5.7.48
M	—	2·77	22.10.48
	0·08	9·5	5.11.48
	0·02	0·38	11.2.49
N	0·13	0·12	11.2.49
	0·08	0·04	19.3.49
	0·08	0·04	26.4.49
L	0·025	0·7	30.11.48
	0·024	0·6	23.4.49

These figures suggest that arsenic only passes from the blood into the hair when the arsenæmia has reached a certain value. In toxicological investigation, the blood level should be a better guide to the probable date of ingestion of the arsenic than the hair, since the latter may have been cut in the interval. The localisation of arsenic in the hair is not solely a function of the time, but depends on the degree of arsenæmia. Consequently an estimate of the probable date of commencement of poisoning, based on the assumption of continuous and regular passage into the hair, and on the determination in different parts of the latter, is very liable to error. G. M.

Bismuth, Toxicological Determination of. R. Castagnou, P. Cazaux and P. David. (*Bull. Trav. Soc. Pharm. Bordeaux*, 1949, **87**, 106.) In solutions obtained by wet combustion, with nitric and sulphuric acids, of organic matter, bismuth may be determined directly by means of hypophosphorous acid, although concentrations of sulphuric acid greater

than 25 per cent. produce a reduction in the sensitivity of the reaction. The determination is carried out nephelometrically, after heating 10 ml. of the solution under examination with 2ml. of hyphosphorous reagent on the water-bath for 30 minutes.

BIOCHEMISTRY

GENERAL BIOCHEMISTRY

Adrenaline, Oxidation of, by Halogens and Iodic Acid. P. Bouvet. (*Ann. pharm. franc.*, 1949, **7**, 721.) The oxidation of adrenaline by halogens is incomplete, as the formation of acid prevents the continuation of the reaction. In the presence of calcium carbonate, iodine reacts quantitatively, producing adrenochrome. The same result is obtained with bromine in theoretical quantity, but in this case an excess reacts further with production of brominated derivatives. In weak acid solutions, iodic acid also gives adrenochrome, but in presence of mineral acid there is an immediate and quantitative production of iodo-adrenochrome. For the latter reaction it is necessary to have an excess of 6 molecules of iodic acid for 1 of adrenaline.

G. M.

Heparin Sodium, Ultraviolet Absorption of. F. K. Bell and J. C. Krantz, Jr. (*J. Amer. pharm. Ass., Sci Ed.*, 1950, **39**, 95.) The ultraviolet absorption of 15 preparations of heparin sodium of varying potency was measured in a Beckmann ultraviolet spectrophotometer equipped with a hydrogen tube as the source of radiation. The absorption spectra of the low and medium potency preparations showed no consistent relation to the anti-coagulant activity. The high potency preparations gave curves of a recumbent S-shape when optical density was plotted against wavelength, consisting of a region of transmission followed by one of absorption at the higher wavelengths. The lack of any commonly recognised chromophore group in proposed heparin structures suggests that this absorption pattern in the high potency preparations is due to an impurity or to an unidentified part of the heparin molecule.

G. R. K.

Polyhydric Alcohols, Chromatographic Separation of. L. Hough. (*Nature*, 1950, **165**, 400.) The behaviour of a number of polyhydric alcohols in paper partition chromatography has been examined using ammoniacal silver nitrite to develop the spots. The R_F values were determined on Whatman No. 1 filter paper in various solvents using the normal procedure for sugar analysis. Small circular spots of aqueous solutions of the alcohols (approximately 2·5 per cent w/v) were put on the paper strip; much larger amounts of glycerol and ethylene glycol could be used, however, small spots of the syrup giving excellent results. A solution of silver nitrate (5 per cent.) to which ammonia (sp.gr. 0·88) had been added in excess was used for developing the paper chromatogram. Ethylene glycol moves rapidly on the paper chromatogram and is readily separated from glycerol, which in turn, is easily distinguished from the hexitols, which travel relatively slowly. The R_F values of trimethylene glycol, ethylene glycol, glycerol, α-methyl galactoside, α-methyl mannoside, β-methyl maltoside, sorbitol, dulcitol, mannitol, inositol and sucrose are given for *n*-butyl alcohol-water; for *n*-butyl alcohol 4·0, ethyl alcohol 1·1, water 1·9; for *n*-butyl alcohol 4, ethyl alcohol 1, water 5; for benzene 1, *n*-butyl alcohol 5, pyridine 3, water 3; and for *n*-butyl alcohol 5, acetic acid 1, water 2. Ammoniacal silver nitrate

will detect as little as 1μg. of a polyhydric alcohol, 10μg. of ethylene glycol, and 10μg. of sugar glycosides such as α-methyl galactoside, α-methyl mannoside and β-methyl maltoside; sucrose also reduces the reagent under these conditions. R. E. S.

BIOCHEMICAL ANALYSIS

***p*-Aminosalicylic Acid, Determination of.** A. L. Tarnoky and V. A. L. Brews. (*Biochem. J.*, 1949, **45,** 508.) A method for the estimation of "total *p*-aminosalicylate" in capillary blood is described in which *p*-aminosalicylic acid and its *N*-acetyl derivatives are quantitatively converted to *m*-aminophenol; this compound gave deeper colours than *p*-aminosalicylic acid when diazotised and coupled with *N*-1-naphthylethylenediamine. In the details given oxalated or freshly drawn capillary blood (0·2 ml.) is added to water (3·2 ml.) and allowed to stand for 30 minutes, trichloroacetic acid (20 per cent., 0·6 ml.) is added, the mixture is shaken, centrifuged and the supernatant liquid (2·0 ml.), transferred to a centrifuge tube graduated at 4 ml., is heated with sulphuric acid (21·5 ± 0·5 N., 0·5 ml.) in a boiling water bath for 1 hour. The solution is cooled in tap water partially neutralised with sodium hydroxide (8·1N, 1·0 ml.) and again cooled, water being added to adjust the volume to 4·0 ml. Sodium nitrite solution (1 per cent., 0·2 ml.) is added, the solution is shaken and after 5 minutes ammonium sulphamate solution (5 per cent.) is added, followed 20 ± 1 sec. later by naphthylethylenediamine hydrochloride solution (1 per cent. in water, 1·0 ml.) the tube being shaken after each addition. The pink colour is allowed to develop for at least 2·5 hours and is then compared with previous colour standards. In a modification of this method ethyl alcohol (95 per cent., 1·0 ml.) is added 20 ± 1 sec. after coupling with the naphthylethylenediamine solution, the tubes are shaken and the colours compared after 1 hour. The colours given in both cases follow Beer's Law at concentrations from 2·5 to 20 mg./100 ml. using an Ilford 625 filter. Graphs of the absorption spectra of the dyes obtained from equimolar solutions of *p*-aminosalicylate after heating and of *m*-aminophenol were identical; unheated solutions of *p*-aminosalicylate develop colour more rapidly, producing a dye with lower extinction values and an absorption maximum at a shorter wavelength. In estimations carried out on fresh oxalated blood to which a known amount of *p*-aminosalicylate had been added mean recoveries of 96 per cent. (range 89 to 102) on 17 experiments, and 98 per cent. (range 91 to 102) on 8 experiments were obtained. R. E. S.

Citric Acid, Micro-estimation of. P. Cartier and P. Pin. (*Bull. Soc. Chim. biol.*, 1949, **31,** 1176.) A study has been made of the pentabromacetone method of determination of citric acid; the conditions of the oxidation, of the bromination, and of the colour estimation, were examined and varied until the best results were obtained. Preliminary treatment is performed with trichloracetic acid solution (10 per cent. for powdered bones, 20 per cent. for other tissues) so that the filtrate contains between 100 and 1000μg. of citric acid. The extract is taken in a test tube, sulphuric acid and bromine water are added, the mixture is heated in a boiling water-bath for 10 minutes, and then cooled and diluted. Bromide-bromate solution is added followed by potassium permanganate (1·5N) drop by drop; after vigorous shaking the tube is stoppered and allowed to stand for 30 minutes

at room temperature when hydrazine sulphate solution (10N) is added gradually until decolorised. The pentabromacetone is extracted by shaking twice for 3 minutes with light petroleum, washing each extract with water; an aliquot of the light petroleum layer is taken in a tube protected from light and 3ml. of a solution of sodium iodide, 10 per cent. in alcohol (96 per cent.), is added. After shaking for 30 seconds and allowing to stand in the dark for 70 minutes, the light petroleum is removed and the intensity of the yellow colour in the aqueous phase is measured with a suitable spectrophotometer, the amount of citric acid being deduced from a standard curve previously prepared. Using the technique described it is possible to estimate 100 to 1200μg. of citric acid in tissues and biological fluids with an accuracy of the order of ±2 per cent. R. E. S.

Glucose in Blood, Microdetermination of. P. Castaigne. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1184.) A method has been investigated for the determination of small quantities of glucose in blood using the reducing action of glucose on potassium ferricyanide followed by spectrophotometric estimation of the ferrocyanide formed by means of the prussian blue reaction under specified conditions. The sample of blood (0·02ml.) is added to one of two tubes each containing 2ml. of copper sulphate solution (7 per cent.). After shaking and allowing to stand for 5 minutes, 0·4ml. of sodium tungstate solution (1·4 per cent.) is added to each tube with shaking, followed by 0·1ml. of barium chloride solution (1 per cent.). The tubes are shaken for 5 minutes and centrifuged and 1ml. of the clear supernatant liquid in each case is pipetted into a 10ml. graduated tube and 2ml. of Herbain's ferric-ferrocyanide reagent is added with 3ml. of distilled water. After shaking, the tubes are left for 15 minutes in a boiling water bath and the determination is completed as described previously (*Bull. Soc. Chim. Biol.*, 1949, **31**, 1104); the colour is measured in a suitable spectrophotometer, the amount of glucose present being calculated from a standard curve constructed previously. The results obtained on normal subjects generally averaged from 0·7 to 0·9 g./l. The concentrations of glucose were studied for 2 days at varying times using the above method as well as that of Baudouin and Lewin as modified by Fleury and Marque; good agreement between the two methods was shown.

R. E. S.

Iodine in Blood, Micro-estimation of. F. Lachiver and J. Leloup. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1128.) A method is described for the determination of iodine present in organic material, in which oxidation is first performed with chromic and sulphuric acids. The iodic acid formed is reduced by phosphorous acid to elemental iodine which is steam distilled in a special apparatus, being absorbed in an alkaline solution on which the determination is made. Where proteins are present, they are precipitated by heating with acetic acid or by the addition of zinc sulphate solution and after 15 minutes in a thermostat at 35°C., ceric sulphate is added. The optical density of the solution is then measured at time intervals of 10, 20, 30 and 40 minutes. After plotting a curve with known amounts of iodine the data can be used to determine the iodine in an unknown sample using an algebraically deduced expression. Detailed results are given showing the variation in colour obtained with temperature and time, with *p*H, on the effect of the addition of sodium chloride, and on the effect of differing concentrations of ceric sulphate. Using this method the recoveries of iodine from potassium iodide, di-iodotyrosine and thyroxin in different sera, varied from

93 to 100 per cent. The method enables quantities of iodine between 0·005 and 0·2μg. to be determined with an accuracy of 3 to 7 per cent. R. E. S.

Mercury, Bismuth, Antimony and Arsenic, in Biological Material, Detection of. A. O. Gettler and S. Kaye. (*J. Lab. clin. Med.*, 1950, **35,** 146.) To a 20 g. sample of finely macerated tissue, stomach contents, or urine, in a 50-ml. Erlenmeyer flask is added 4 ml. of concentrated hydrochloric acid and 10 ml. of water. A spiral is prepared by winding a length of 20 gauge copper wire tightly and closely over a piece of glass rod 10 times. The copper spiral, after washing with alcohol and with ether, is introduced into the material contained in the flask. The contents of the flask are gently boiled for 1 hour and the original volume maintained constant by the addition of 10 per cent. hydrochloric acid (by volume) from time to time. The spiral is then removed and washed with water. A large amount of mercury or bismuth may require a longer heating period. A silvery coating on the spiral may indicate mercury. A dark discoloration may indicate antimony, arsenic, bismuth, selenium, sulphur or tellurium, or any combination of these substances. To the deposit on the copper spiral a series of tests are then applied. First a contact agent is employed to detect and estimate the mercury ; then appropriate solvents are used completely to remove a particular metal or group of metals from the spiral. To the solutions thus obtained appropriate reagents are added to identify each metal. The procedure is so arranged that an estimation may be made simultaneously. Several of the tests employed were found to be sensitive and specific for quantities as small as 0·01mg. S. L. W.

Penicillinase Activity, Determination of. W. S. Wise and G. H. Twigg. (*Analyst,* 1950, **75,** 106.) The rate of production of the extra acidic grouping in penicillin by penicillinase is followed by electrometric *p*H titration using sodium hydroxide. A penicillin solution containing 200 to 300 units /ml. is placed in a beaker in a thermostat at 25° ±0·2°C., and the electrodes of a *p*H meter are inserted. The contents of the beaker are stirred with a stream of carbon dioxide-free air, the solution is adjusted to *p*H 7·8, a known volume of the penicillinase solution is added and, to prevent a lowering of *p*H, sodium hydroxide (0·01N) is added continuously from a burette at such a rate that the *p*H is constant at 7·8. The slope of a graph of burette readings plotted against time, in ml. of 0·01N sodium hydroxide/minute, is a measure of penicillinase activity. Results, showed graphically, indicated that the rate of reaction was constant with respect to time, i.e., independent of the penicillin concentration, but dependent on the enzyme concentration over a wide range. The rate of reaction was dependent on the purity of the penicillin used, very impure samples giving lower rates; commercial penicillins of potency greater than about 1200 units/mg. were generally satisfactory. The purity of the penicillinase used did not affect the determination unless it was so highly buffered that the change in *p*H on formation of the acid group from penicillin was too small to be measured easily; the penicillinase preparation was then dialysed before examination. R. E. S.

Phenadoxone in Urine, Determination of. J. E. Page and H. King. (*Analyst*, 1950, **175,** 71.) A colorimetric method has been devised for determining phenadoxone (DL-6-morpholino-4:4-diphenylheptan-3-one) in urine. The method depends on the reaction of equimolecular quantities of phenadoxone and bromophenol blue to form a toluene-soluble compound. The free base of the drug is extracted into toluene and the toluene extract is shaken with an aqueous solution of the dye buffered at *p*H 4·0. An amount

of dye, equivalent to the weight of drug in the toluene layer, is carried over into the toluene as a yellow compound, which is decomposed by shaking the toluene with aqueous alkali; the sodium salt of the dye enters the aqueous layer and is then determined colorimetrically. Phenadoxone was examined polarographically; in 0·1N potassium chloride it gave a polarographic step with a characteristic peak at $-1{\cdot}75$v., the step height being approximately proportional to the concentration over the range 0·0075 to 0·075 per cent.; the step height was however sensitive to small amounts of surface-active material and appeared at a relatively high potential, so that the method was unsuitable for biological fluids. Interference with the determination of phenadoxone in urine is caused by basic substances that react with bromophenol blue to form a toluene-soluble compound e.g. alkaloids, such as codeine, and synthetic analgesics, such a pethidine and amidone; bacterial growth also leads to the production of basic substances and a small amount of an antiseptic (e.g., mercuric chloride or toluene) must therefore be added to the urine. R. E. S.

Potassium in Biological Materials, Estimation of. N. I. Joukovsky and A. Lowenthal. (*Bull. Soc. Chim. biol.*, 1949, **31**, 1190.) The method adopted consists of ashing the organic material at 350°C., the precipitation of the potassium by silver cobaltinitrite reagent and the colorimetric estimation of the cobalt. A quantity of biological material containing 100 to 400 μg. of potassium in a silica dish is evaporated on a water-bath with 4 volumes of nitric acid followed by heating at 350°C., until a white ash is obtained. The residue is dissolved in 3ml. of water, 2ml. of this solution is mixed with 2ml. of silver cobaltinitrite reagent in a graduated centrifuge tube and after thoroughly mixing, is allowed to stand for half an hour. The reagent is prepared by powdering 22·6g. of cobalt acetate with 16·9g. of silver nitrate, adding to the mixture 30ml. of acetic acid (8ml. of water and 22 ml. glacial acetic acid), followed by sodium nitrite solution (44g. in 80ml. of water); after standing for 14 to 16 hours the mixture is filtered and a current of air is passed through the filtrate for 4 hours. 4ml. of acetone (50 per cent.) is added to the tube which is centrifuged for 10 minutes; the supernatant liquid is decanted, the precipitate is washed twice with 2ml. of acetone (50 per cent.) and dried on a boiling water-bath. After dissolving in 1ml. of nitric acid (7 per cent.), 14 ml. of alcoholic ammonium thiocyanate is added and the colour is measured after 10 minutes in a photoelectric colorimeter using a standard curve prepared previously and making allowance for the reduced volume taken. R. E. S.

Vitamin A in Whale Liver Oils, Determination of. O. R. Braekkan. (*Anal. Chem.*, 1949, **21**, 1530.) Ultra-violet absorption studies were carried out on purified kitol. It gave an absorption curve in the ultraviolet with absorption maximum at approximately 285 mμ with a corresponding $E_{1\text{ cm.}}^{1\text{ per cent.}}$ value of 465 in absolute ethyl alcohol and 469 in isopropyl alcohol; in cyclohexane the absorption maximum was found at 287·5 mμ with $E_{1\text{ cm.}}^{1\text{ per cent.}}$ equal to 461, indicating an approximate purity of 60 per cent. (assuming pure kitol to have $E_{1\text{ cm.}}^{1\text{ per cent.}}$, 290 mμ = 707). Only in strong concentrations could a colour be obtained with activated glycerol dichlorhydrin. giving an $E_{1\text{ cm.}}^{1\text{ per cent.}}$ (555 mμ) value approximately 3. The absence of a consistent curve between 400 and 700 mμ suggested that the colour was caused by small amounts of impurities, rather than by kitol itself. Examination of mixture of vitamin A, kitol and cholesterol

by the glycerol dichlorhydrin reaction (*Ind. Engng. Chem. Anal. Ed.*, 1946, **18,** 570) indicated that neither kitol nor cholesterol interfered. The values obtained in the determination of vitamin A in whale liver oils by using activated glycerol dichlorhydrin were 10 to 30 per cent. lower than those obtained by the spectrophotometric method based on $E^{1\ per\ cent.}_{1\ cm.}$ (325 mμ). The absorption maxima are given for a number of samples of whale oil; the displacement of the peak wavelength ranged from 10 to 17 mμ below 325 mμ. The results obtained indicated that there is considerable irrelevant absorption in addition to that caused by the presence of kitol, but there did not seem to be any interference with the colour reaction: the conclusion is drawn that determinations by the use of activated glycerol dichlorhydrin give the true values of the vitamin A content of whale liver oils.

R. E. S.

CHEMOTHERAPY

Œstrogenic Action and Chemical Constitution in Azomethine Derivatives. H. H. Keasling and F. W. Schueler. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39,** 87.) The assumption that compounds with a large, rigid, relatively inert, fat-soluble molecular structure containing two active hydrogen-bond-forming groups at an optimum distance of 14·5Å apart possess œstrogenic activity was tested by preparing 16 4 : 4′-substituted benzylideneanilines and examining for œstrogenic activity. The compounds comprised all the substitution combinations of the four groups H, CH_3, OCH_3 and OH, and were prepared by reaction between the appropriate benzaldehyde and aniline in ethyl alcohol. Œstrogenic activity, tested by the vaginal smear test, was shown by 4 : 4′-dihydroxy-benzylideneaniline when injected subcutaneously in a total dose of 12·5 mg. and when applied to the vagina in a total dose of 25 μg. A total subcutaneous dose of 25 mg. of each of the other 15 compounds produced no effect. The distance between the hydroxyl groups in the active compound was 14·5Å, in agreement with the general assumption. Other compounds showing the same characteristic distance were the 2 hydroxy-methyl compounds and the dimethyl compound ; these were inactive because the methyl group does not form hydrogen bonds. The methoxy-hydroxy and dimethoxy compounds, which might show activity following hydrolysis in the body to the dihydroxy compound, were apparently precluded from doing so by the more rapid detoxification of the azomethine link. The big difference between the effective subcutaneous and intravaginal doses of the dihydroxy compound is also attributed to this detoxification process. G. R. K.

Neostigmine-like Compounds, Curarising and Anti-curarising Action of. H. F. Chase, B. K. Bhattacharya and E. M. Glassco. (*J. Pharmacol.*, 1950, **97,** 409.) A series of carbonyl congeners of neostigmine were studied by the authors by means of the rabbit head-drop assay for curare. Most of the compounds studied were shown to have two characteristic and opposing actions, a curare-like and an anti-curare action. Two of the compounds, however, were shown to be purely curare-like ; these were the diethylcarbamate of (2-hydroxy-3-cyclohexylbenzyl) trimethyl-ammonium bromide (NU 906) and the diethylcarbamate of (2-hydroxy-3-cyclohexylbenzyl) methyl-piperidinium bromide (NU 911). The curare-like action of these compounds differs from the true curare action in that it is summated with, not antagonised by, neostigmine. This synergistic action raises the possibility that the curare-

like action of neostigmine here is exaggerated by the presence of molecules of a compound of similar structure and that the anti-curare and the curare-like actions may be due to different chemical properties. Even though relatively large amounts of NU 906 and NU 911 were necessary to produce curare-like effects it is possible that compounds of similar structure might be more potent paralysants and yet be as free of objectionable cholinergic side actions. S. L. W.

PHARMACY

GALENICAL PHARMACY

Suppository Excipients, Physiological Evaluation of. R. Charonnat, L. Chevillard and H. Giono. (*Ann. pharm. franc.*, 1949, **7**, 627.) Suppositories, containing 5·5mg. of methyl nicotinate, are administered to guinea pigs and the rise of temperature is measured in the ear of the animal by means of a thermocouple. Measurements are made every 2 or 3 minutes, the results being recorded on a graph. The length of the base of the curve represents the duration of the action; the height corresponds to the intensity, and the area of the curve to the total amount of effect. Mean results, obtained with 5 to 6 animals, showed that glyco-gelatin caused a maximum rise in temperature of 5·65°C. lasting 30 minutes and gave a curve with an area of 65·3 sq. cm. Corresponding figures for cocoa butter were 5·1°C., 20 minutes and 35·7 sq. cm., and for polymerised ethylene oxide 3·4°C., 20 minutes and 26·3 sq. cm. G. M.

NOTES AND FORMULÆ

Methenamine Mandelate (Mandelamine). (*New and Nonofficial Remedies ; J. Amer. med. Ass.*, 1950, **142**, 487.) Methenamine mandelate is obtained by reaction between equimolecular amounts of hexamine and mandelic acid. It occurs as a white, almost odourless, crystalline powder with a sour taste, m.pt. 127° to 130°C., very soluble in water and soluble in alcohol (1 in 10), chloroform (1 in 20) and ether (1 in 30) ; a 1 per cent. aqueous solution has *p*H 4·2 to 4·4. A 0·1 per cent. solution exhibits an ultraviolet absorption maximum at 2576 to 2577Å. It complies with limit tests for halides, sulphate and heavy metals ; the loss in weight when dried over sulphuric acid for 18 hours is not more than 1·5 per cent. Methenamine mandelate contains 50 to 54 per cent. of mandelic acid when titrated with sodium hydroxide using phenolphthalein as indicator, and 46 to 50 per cent. of hexamine calculated with reference to the dry substance. The content of hexamine is determined by refluxing for 15 minutes with diluted hydrochloric acid, adding the resulting solution to a modified Nessler's reagent cooled in ice and allowing to stand for 1 minute, when acetic acid and iodine are added and the excess iodine titrated with sodium thiosulphate. Methenamine mandelate combines the actions of hexamine and mandelic acid and is used as a urinary antiseptic. It is given by mouth in a dose of 0·75 to 1 g. 3 times daily. G. R. K.

PHARMACOGNOSY

***Hyoscyamus niger*, Cultivation of.** R. Laruelle. (*J. Pharm. Belg.*, 1949, **4**, 281.) Experiments with both the annual and biennial varieties were carried out at the experimental station at Lessines. Considerable difficulty in germinating the seeds was encountered; about 30 per cent.

germinated after 3 weeks, 10 per cent. after 6 weeks, 10 per cent. after 2 months and the remainder did not germinate at all. Lvov and Jakovleva state that preliminary freezing favours germination and it is hoped to apply this technique next winter. Freshly collected seeds, sown in August, did not germinate, thus indicating that the power to germinate is not acquired till some time after collection. Annual plants are not a good source of the leafy drug, but produced good yields of seed; probably 10 kg./100 sq.m. could be obtained for commercial purposes. J. W. F.

Quassia and Quassin, Characterisation of. P. Duquénois and O. Colbe. (*Ann pharm. franc.*, 1949, **7**, 660.) The method of the German Pharmacopœia for the characterisation of quassia wood is useless, since all lignified parts give up a proportion of lignin to alcohol, causing a positive reaction. The reaction of the French Codex for quassin is also unsatisfactory, and should be replaced by the following: to 1 mg. of quassin add a crystal of phloroglucinol and 1 micro-drop of concentrated hydrochloric acid; a rose colour appears immediately. The limit of sensitivity appears to vary with different samples of quassin. G. M.

PHARMACOLOGY AND THERAPEUTICS

Analgesics, Pharmacodynamic Effects of. F. P. Luduena and E. Ananenko. (*Arch. int. Pharmacodyn.*, 1950, **81**, 259.) The pharmacodynamic effects of some new synthetic analgesic drugs have been studied on dogs, using morphine for comparison. In the unanæsthetised dog the progressive intravenous or intramuscular administration of *l*-methadone, *l-iso*-methadone, 1-methyl-4-(3-hydroxyphenyl)-4-piperidyl ethyl ketone (WIN 1539), *l*-3-dimethylamino-1 : 1-diphenylbutyl ethyl sulphone (WIN 1161) and morphine first produced sedation, respiratory depression, analgesia, cardiac slowing, miosis and less frequently salivation and defæcation. With high total doses there was hyperexcitability and even convulsions except with morphine. In most of the dogs at this stage tachypnœa, tachycardia and mydriasis were present. With *d*-methadone the same symptoms were observed, except that the respiratory rate was increased. With doses of 1 to 3mg/kg. intravenously the analgesia produced by *l*-methadone, *l-iso*methadone, WIN 1161, or less frequently WIN 1539, was sufficient to permit surgical operations without any other medication. Larger doses were required for *d*-methadone. This degree of analgesia was not obtained with morphine with doses up to 100 mg./kg. In dogs anæsthetised with soluble thiopentone the first dose of 1 to 1·5 mg./kg. of *l*-methadone, *l-iso*methadone or WIN 1539, by intravenous injection, produced a moderate fall of blood pressure, subsequent doses producing a brief fall followed by a rise. In contrast, *d*-methadone in the same doses produced only a fall of pressure both initially and subsequently. S. L. W.

Dibromoprocaine Hydrochloride ; a Radioactive Spinal Anæsthetic. F. Howarth. (*Brit. J. Pharmacol.*, 1949, **4**, 333.) This substance was prepared, by a method described by the author, for the purpose of studying the fate of a spinal anæsthetic with special reference to its ultimate distribution among the tissues of the body and the routes by which it leaves the spinal theca. It was shown that the concentration in the spinal subarachnoid space rapidly declines associated with a rapid rise in urine concentration, the blood

level remaining persistently low. A study of the tissue distribution showed that only the spinal roots show any capacity to concentrate the drug above the level existing in the cerebrospinal fluid at the site of injection. Of the tissues examined only kidney (and urine), and liver (and bile) appear able to concentrate the anæsthetic above the circulating blood concentration. Large variations in time and dose did not produce large changes in the tissue/blood ratios. It was shown that the anæsthetic enters the spinal cord during spinal anæsthesia, though it is improbable that it is able to produce a functional cord transection. Of the various routes of departure of the anæsthetic from the spinal theca, the venous system appeared to be the most important. S. L. W.

Digitalis, Some Extraction Studies on. R. E. King and O. Gisvold. (*J. Amer. pharm. Ass., Sci Ed.*, 1950, **39**, 109.) Aqueous extracts of fresh leaves of *Digitalis purpurea* were prepared by disintegrating the broken leaves in water, heating to 70°C. to coagulate the chlorophyll and proteins and inactivate the enzymes, and filtering. A 2 per cent. extract thus prepared was inactive when assayed by the frog method, but conversion to an alcoholic tincture by evaporation to low bulk, diluting with alcohol and filtering indicated that the original aqueous extract gave 87 per cent. extraction of the activity as compared with a control tincture prepared by the U.S.P. method ; 5 and 10 per cent. aqueous extracts similarly treated represented 78 and 57 per cent. extraction respectively. The active material from fresh leaves may be stored by saturating the aqueous extracts with anhydrous sodium sulphate, filtering and drying the precipitate. When required, the active material can be extracted from the precipitate with butyl alcohol, methyl ethyl ketone or tetrahydrofuran. The active principles may be obtained directly from the aqueous extract by adding tetrahydrofuran, saturating with anhydrous sodium sulphate and preparing a tincture from the aqueous layer which separates. Fresh digitalis leaves appear to retain their potency when stored frozen for 4 months. Attempts to remove the pigments from butyl alcohol extracts by washing with alkalis failed because the active principles were destroyed. The active principles were separated into two fractions from a purified extract by using the selective solvent action of acetone and benzene. One fraction gave a positive Keller-Kiliani test for digitoxin and purpurea glycoside A and the other a positive test for gitoxin, purpurea glycoside B and gitalin. G. R. K.

Digitoxins, Pharmacology of. D. I. Macht. (*Arch. int. Pharmacodyn.*, 1950, **81**, 345.) In view of reports which have appeared in the literature describing difficulties experienced with digitoxin U.S.P. in the management of congestive heart failure, the author conducted an experimental investigation on Digitaline Nativelle and digitoxin U.S.P. Comparisons were made by three types of experiment : (1) phytopharmacological tests on root growth of *Lupinus albus* seedlings, (2) pharmacological measurements after irradiation with X-Rays, (3) colorimetric assays. All three sets of experiments showed a consistent and marked quantitative difference in the pharmacological properties of the two digitoxins, Digitaline Nativelle revealing a greater activity than digitoxin U.S.P. The reason for this difference remains unexplained but it can be stated that it is not due to the presence of digitonin. It was noted that combinations of digitoxins with blood serum give synergistic effects ; here also Digitaline Nativelle was found to be more potent than digitoxin U.S.P. S. L. W.

Dihydrostreptomycin in Procaine-Pectin Solution. F. Zini. (*Acta med. scand.*, 1950, **146**, 209.) The intramuscular injection in man of 0·5 g. of dihydrostreptomycin or streptomycin in a 2 per cent. pectin solution, with 1 per cent. of procaine (buffered at *p*H 7) produces a concentration of the antibiotic

in the serum corresponding, in 50 per cent. of cases, to 17 to 96 S.U. ml. at the 24th hour. When the antibiotic is diluted in pectin solution the sharp fall in the serum concentration in the 2nd and 3rd hour described by many investigators does not occur. The concentration of antibiotic in the serum at the 24th hour was much greater than that in the whole blood. In the urine, inhibition values corresponding to 6 to 8 S.U. were observed 32 hours after a first intramuscular injection of 0·25 g. of the antibiotic in pectin-procaine solution, and the inhibition values in urine specimens examined in hourly fractions of the 24 hours were consistently higher than those found in serum or blood. S. L. W.

Heparin, Intramuscular Administration of. G. Bauer. (*Acta med. scand.*, 1950, **136**, 188.) A study of the clinical course in 16 cases of acute thrombosis indicates that heparin administered intramuscularly, in a dosage of 150mg. 3 times a day does not have the same immediate and satisfactory influence on the thrombotic process as the same dosage given by intermittent intravenous injection. This is due mainly to the fact that after intravenous injection the coagulation time is raised to a much higher level for 1 to 2 hours than after intragluteal injection. Even if intramuscular injection of heparin is easier than intravenous this is offset by the fact that it is more painful and frequently gives rise to large local hæmatomata, and also that it requires larger total amounts of heparin for each patient. Moreover, assessment of the stage of healing of the thrombotic process is more difficult than in cases treated by intravenous injection. Thus, after intramusuclar injections, in several cases patients found it difficult to state clearly whether they felt pain when their deep veins were palpated. Because of this heparin treatment was stopped and the patients allowed to get up without satisfactory evidence that the pathological process was terminated. This may account for the much greater number of recurrences observed in comparison with those observed in cases treated intravenously ; it was also responsible for one fatal case of pulmonary embolism due to insufficient dosage. Intermittent intravenous injections, 4 times daily, constitute the best method of administration of heparin, though the intramuscular route may be employed as an alternative where intravenous injections are impracticable. S. L. W.

Neoarsphenamine, Toxicity Determination by a Method based on Survival Time. M. G. Allmark and D. Lavallee. (*J. Amer. pharm. Ass. Sci. Ed.*, 1950, **39**, 81.) Neoarsphenamine in 15 per cent. aqueous solution was injected into the saphenous veins in the hind legs of rats of either sex and the time of death recorded ; 3 doses of standard and 3 of sample were used in each test, and each dose was given to 5 rats weighing 125 to 150 g. The doses were chosen so that all the rats would die within 10 hours. The method was quicker and more economical than the usual quantal response method but in 3 of the 15 samples tested it gave the toxicity as equal to that of the standard preparation whereas the quantal response method showed it to be much greater. G. R. K.

Œstrone, Effect of Crystal Size on the Activity of. A. Simond, K. M. Lindquist, F. H. Tendrick and L. W. Rowe. (*J. Amer. pharm. Ass., Sci. Ed.*, 1950, **39**, 52.) Using adult female, ovariectomised rats and the principles of the Allen-Doisy test, the duration of œstrus produced by single massive subcutaneous injections of aqueous suspensions of œstrone crystals of varying size was determined for individual animals in small groups and then

(*Continued on page* 608)

PHARMACOPŒIAS AND FORMULARIES

THE PHARMACOPŒIA OF THE UNITED STATES

Fourteenth Revision

IT is little more than three years since the U.S.P.XIII came into force, and the Fourteenth Revision, now published, becomes official from November 1, 1950. This short interval is a measure of the rapid changes in medical, chemical and pharmaceutical requirements, and must indicate a heavy and intense programme of work accomplished by those responsible for revision. The new issue shows, by its deletions and additions, those drugs and preparations now held in repute in the United States as well as the treatment of problems of drug standardisation.

The deletions include a number of substances and preparations still in extensive use in this country. Some are well known as domestic remedies, such as Seidlitz powder, mustard plaster, cod-liver oil emulsion, saponated cresol solution, alum and sublimed sulphur. Other articles commonly prescribed, although viewed critically by some medical authorities, include caffeine citrate, prepared chalk, methylene blue, potassium citrate, tannic acid, formaldehyde solution, thymol, activated charcoal, β-naphthol and strychnine sulphate (no other salt of strychnine is mentioned). The deletion of arsenic trioxide, neoarsphenamine and sulpharsphenamine is, no doubt, due to the general adoption of antibiotics and oxophenarsine hydrochloride. The therapeutic decline of mercurials is reflected in the deletion of mercury, mild mercury chloride (calomel), mercury oleate and the mercurial ointments. A decrease in the number of vegetable drugs is in accordance with present pharmacopœial trends. Myrrh, nutmeg, red saunders wood, clove, spearmint, juniper tar and pine tar are, not surprisingly, deleted, but the omission of rhubarb, senna, stramonium, hyocyamus and ginger and their galenical preparations is less expected. Seven serological preparations are deleted, namely cholera vaccine, plague vaccine, scarlet fever streptococcus antitoxin and toxin, and bivalent, trivalent and pentavalent gas gangrene antitoxins.

Of the 203 new monographs, most are concerned with synthetic drugs, substances used in diagnosis, antibiotics and preparations, particularly tablets, capsules and injections. The synthetic compounds include the antihistamine drugs diphenhydramine hydrochloride (benadryl) and tripelennamine hydrochloride (pyribenzamine), the anti-malarial drugs chlorguanide hydrochloride (known in this country as proguanil hydrochloride), chloroquine phosphate (aralen diphosphate) and pentaquine phosphate, and the mercurial diuretics meralluride (mercuhydrin) and mercurophylline (mercuzanthin). The only new sulphonamide is phthalylsulphathiazole, with a monograph on tablets of this substance, and there are new injections for three other sulphonamides. Other monographs appearing in the U.S.P. for the first time are amphetamine, amphetamine sulphate, dicoumarol (under the title bishydroxycoumarin), dimercaprol, meperidine hydrochloride (pethidine hydrochloride), methamphetamine hydrochloride (methylamphetamine hydrochloride), naphazoline hydrochloride (privine) and tubocurarine chloride. Substances used in diagnosis include iodoalphionic acid (priodax) employed in cholecytography, methiodal sodium (skiodan), employed in pyelography, and congo red, which is used for the detection of amyloidosis and for the estimation of blood volume.

Those concerned with the preparation, standardisation and use of antibiotics will be interested in the monographs, totalling 22, on these substances,

but may be disappointed by the treatment. In the monograph on penicillin G potassium (benzylpenicillin potassium), there are details of the method of assay and tests including stability, safety and clarity of solution. On the other hand, more than half of these monographs contain little beyond a brief definition and the statement: "It complies with the requirements of the Federal Food and Drug Administration." This restricted treatment is applied to the monographs on the sodium and potassium salts of penicillin, penicillin G procaine and preparations such as ointment, tablets, troches, inhalation and aqueous and oily injections. Tyrothricin is dealt with in detail, but for aureomycin, dihydrostreptomycin and streptomycin the information is limited to Description, Solubility and Identification, and a reference to the Food and Drug Administration. In a similar way, the monographs on some biological products, including diphtheria and tetanus antitoxins and toxoids, smallpox vaccine, blood-grouping sera and Rh-typing sera, refer important requirements to the National Institutes of Health of the United States Public Health Service.

The tradition of the United States Pharmacopœia of providing a range of flavouring agents is maintained. Some of the older preparations, such as anise water, fennel water and orange flower syrup are deleted, but there are new monographs on cacao syrup, cherry juice and syrup, raspberry juice and syrup and vanilla tincture which might well be examined by pharmacists in this country. Among the pharmaceutical preparations it is interesting to note that calamine lotion is no longer prepared with bentonite magma as the suspending agent, but now contains the carbowaxes, polyethylene glycol 400 and polyethylene glycol 400 monstearate, and no glycerin. In addition to a simple benzyl benzoate lotion, a formula is given for benzyl benzoate chlorophenothane lotion containing 11·5 per cent. v/v of benzyl benzoate, 1 per cent. w/v of chlorophenothane (dicophane or D.D.T.) and 2 per cent. of w/v benzocaine emulsified with polysorbate 80 ("Tween 80"). The title amphetamine inhalant is applied to "amphetamine, usually aromatized and contained in a suitable inhaler." An identification test, assay process are given, but there is no requirement for the actual amount of amphetamine present.

The 80 injections of the U.S.P.XIV include new monographs on injections of dimercaprol, globin zinc insulin, sodium iodomethamate (iodoxyl), sodium salicylate, tubocurarine chloride and vitamin B_{12}. The general style in the injection monographs is to define the preparation as a sterile solution, provide identification tests, assay process and tolerances and refer to the "other requirements" given in an appendix. This appendix describes the solvents and vehicles which may be used, permits the addition, with certain limitations, of substances to "increase stability and usefulness," and states the excess volume to be included in single-dose and multiple-dose containers for both mobile and viscous liquids. There is a detailed statement of the testing procedures to be applied to the containers, covering a powdered glass test and whole container tests using water and acid at 121°C. Four grades of container are specified and a table shows the grade to be used for each injection. The difficult problem of controlling particulate matter in injection solutions is referred to only in a statement that "Good pharmaceutical practice also requires that each Injection, in its final container, be subjected individually to visual inspection." Details are given of the sterility tests for injections, but it is surprising to find that there is no reference to methods of sterilisation, even for thermostable substances. Consequently, the pharmacist is given no guidance or directions on the method to be used, and this seems likely to place him at a disadvantage compared with the

large-scale producer. There is the further danger that, in the absence of directions, a process involving the use of heat may be applied to a thermolabile substance.

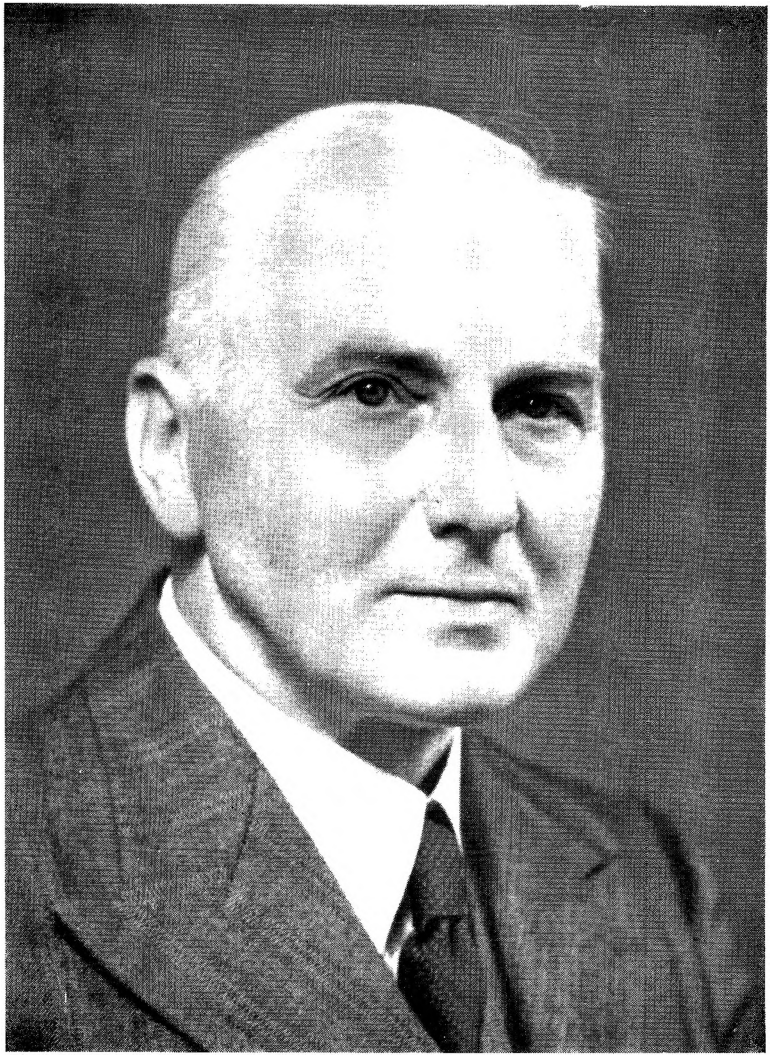
The appendices run to 350 pages descriptive of general tests, processes, apparatus and reagents, and a series of tables. There are First and Second Sheet Supplements. The volume is an important work of refence for all concerned with the study of standards for a wide range of drugs and materials used in modern medicine. T. C. DENSTON.

ABSTRACTS (*continued from page* 605)

averaged. Injections were made in the dorsal area and the total volume injected varied from 0·2 to 1 ml., with doses ranging from 5,000 to 20,000 I.U. When the crystals of œstrone were 10 μ or less in length the average duration of œstrogenic stimulation was 10 days. When at least 50 per cent. of the crystals were 50 to 150 μ in length, the average duration from an equal dose was 24 days. A few tests were also made on oily solutions of œstrone and œstradiol monobenzoate and on aqueous suspensions of œstradiol crystals. The average duration of effect produced by these preparations was similar to or slightly less than that produced by an aqueous suspension of crystals of œstrone 10 μ or less in length. G. R. K.

Procaine Penicillin Preparations. E. Griffiths, A. J. Walker and R. A. Shooter. (*Brit. med. J.*, 1950, **1**, 761.) 113 patients with acute staphylococcal infections were treated with daily doses of a preparation consisting of 300,000 units of procaine penicillin in oil with 1 per cent. aluminium stearate and 100,000 units of sodium penicillin. The results were found to be as satisfactory as those in a similar series of patients treated with 300,000 units of procaine penicillin daily or 300,000 units of sodium penicillin in saline solution twice daily. The preparation provides detectable amounts of penicillin in the blood throughout most of the 24 hours in the majority of patients. Other preparations tried were 300,000 units /ml. of procaine penicillin in oil with 2 per cent. of aluminium stearate, and 200,000 /ml. units of procaine penicillin in oil with 2 per cent. of aluminium stearate and 100,000 units /ml. of sodium penicillin. With these latter two, the clinical results compared unfavourably with those obtained with 300,000 units of procaine penicillin. S. L. W.

***d*-Tubocurarine, Liberation of Heparin and Histamine by.** G. Reid. (*Nature*, 1950, **165**, 320.) When tubocurarine (0·25 to 15 mg.) is injected into the portal vein of the dog under chloralose there is a sharp rise of portal pressure, with a fall in systemic arterial and venous pressures. Systemic intravenous injection causes a fall of arterial and venous pressures, and the portal pressure also falls. This indicates that the rise of portal pressure is mainly due to an action of the drug within the liver itself. That this action is one of histamine liberation is shown by the antagonism of benadryl to small doses of tubocurarine given intra-portally and by the detectable loss of liver histamine with larger doses. A dose of 0·25 mg. caused a rise of portal pressure greater than that caused by the intra-portal injection of 50 μg. of histamine acid phosphate, and with large doses the effect was probably equivalent to that of several mg. of histamine. The loss of histamine, estimated by comparing the content of two pieces of liver removed before and after injection, varied from no detectable loss to a loss of 44 per cent. It was similarly shown that heparin is also liberated by *d*-tubocurarine, since, following the intra-portal injection of 4 to 15 mg., arterial blood withdrawn within 20 minutes after injection had not coagulated 24 hours later in three experiments, and in a fourth experiment the clotting-time had increased from 5 to 60 minutes. S. L. W.



A. D. POWELL

Chairman, 1950