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The Journal of
PHARMACY
and
PHARMACOLOGY

VOLUME I. No. 6

JUNE, 1949



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Telegrams : Pharmakon, Westcent, London

Editor: C. H. Hampshire, M.B., B.S., B.Sc., Ph C., F.R.I.C.

Annual Subscription 40s. Single Copies 5s.

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June, 1949

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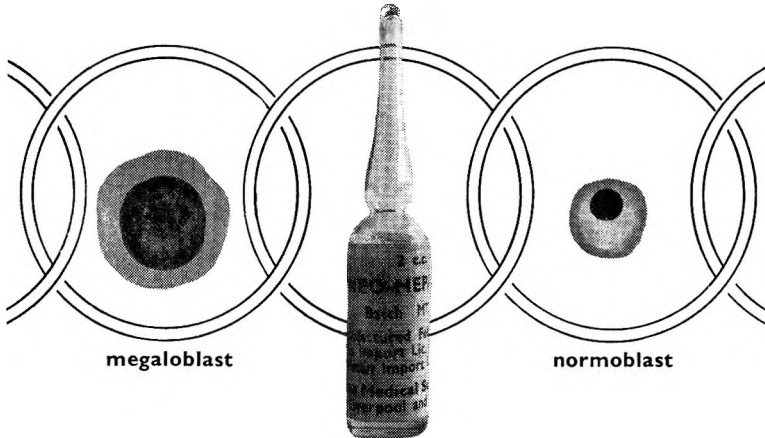
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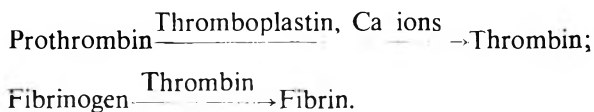
REVIEW ARTICLE

ANTICOAGULANTS

By F. C. MACINTOSH, PH.D.

National Institute for Medical Research, Hampstead, London

THE processes that underly the clotting of blood are still far from adequately understood, in spite of the many facts uncovered by generations of patient investigators. The classical scheme associated with the name of Morawitz¹, although neither complete nor universally accepted, is still useful as a summary of the main events. According to this scheme, the plasma contains three essential ingredients of the complete clotting system: these are ionised calcium and the proteins prothrombin and fibrinogen. The addition of a fourth ingredient, thrombokinase (or better thromboplastin, since its enzymic nature is still in doubt) completes the system, and initiates clotting. Thromboplastin is present in the tissues generally and also in the blood platelets. When blood makes contact with tissue fluid, or the platelets are injured by contact with a hydrophilic surface, prothrombin reacts with thromboplastin and with calcium ions to form an enzyme, thrombin: this is the first stage of clotting. In the second stage of clotting thrombin acts upon fibrinogen, changing it into the insoluble protein fibrin, whose threads constitute the matrix of the clot. Or in summary:



Any substance, or treatment, which removes or inactivates any of the five clotting factors will prevent coagulation. Anticoagulants might therefore be divided into five groups, according to the factor interfered with: such a classification, however, would not be a very useful one, since some important anticoagulants affect more than one component of the system, and since a single component may be attacked by anticoagulants differing completely in their mode of action. In addition, it must be emphasised that the Morawitz scheme is oversimplified. Thus, at least one further plasma protein^{2,3,4} is involved in the conversion of prothrombin to thrombin; and prothrombin itself has been regarded as a complex of two easily separable factors^{5,6,7}. The velocity of both stages of clotting is related to the nature and concentration of the electrolytes present, and that of the second stage is reduced by antithrombin, of which traces are always present in plasma and further amounts are set free during clotting. Finally, the relation of the plasma proteases and their inhibitors to clotting is still poorly defined. No further mention will be made of these factors, and the action of anticoagulant substances will be discussed in terms of the Morawitz scheme alone.

MODES OF ACTION OF ANTICOAGULANTS

A list of the possible mechanisms of anticoagulant action is given below, with a number of the most important substances exhibiting each type of activity.

Prevention of platelet disintegration: hydrophobic surfaces (paraffin, amber, perspex, collodion, silicone, etc.); most anticoagulants.

Removal of calcium ions: oxalates, fluorides, citrates, soaps of alkali metals, ion-exchange resins.

Interference with prothrombin formation: dicoumarol, salicylates.

Inhibition of the conversion of prothrombin to thrombin: heparin, other sulphuric acid esters, salts of the rare earth metals, organic bases, reducing agents, trypsin inhibitors.

Inhibition of the action of thrombin on fibrinogen: heparin, other sulphuric acid esters, reducing agents (cysteine, glutathione, bisulphite, etc.), 'lipid inhibitors', organic bases, trypsin inhibitors.

Inactivation of fibrinogen: fibrinolysin, protamines.

Release of heparin from the tissues: peptone, antigens (in sensitised animals), radioactive substances, nitrogen mustards, diamines, diamidines, etc.

The following discussion will be concerned chiefly with those substances that are now used with the primary aim of inhibiting coagulation *in vitro* or *in vivo*. Only brief mention will be made of substances formerly so used, but now discarded in favour of more active or less toxic materials, and of substances whose anticoagulant action is important only as a side-effect of their therapeutic employment.

SUBSTANCES PREVENTING THE DISINTEGRATION OF THE PLATELETS

It is difficult to withdraw blood from a vein or artery without contaminating it with tissue fluid, but with good technique (clean puncture, avoidance of stasis, discarding of the first portion of effluent blood) the contamination may be kept small. The speed of clotting then depends, other factors being equal, on the rate of platelet disintegration, and this in turn depends on the surface with which the blood is in contact. Clotting is promoted by increasing the area of contact; the large contact area is the main factor in the hæmostatic efficiency of gauze and of its absorbable substitutes such as fibrin foam and gelatin foam. The chemical nature of the surface is equally important. Clotting is slower in a Pyrex glass vessel than in a soda-glass vessel, and still slower in a vessel lined with a water-repellant substance such as paraffin, amber, collodion or any of a variety of plastics. The best of all surfaces for the delay of platelet lysis is provided by the silicone film formed by the hydrolysis of dimethyldichlorosilane⁶. Blood taken with silicone-coated syringes and needles, and kept in silicone-coated vessels, may remain fluid for several hours. The silicone technique should facilitate the study *in vitro*, or in perfusion experiments of phenomena depending on the presence of normally reactive platelets: for example, the liver of a sensitised dog may be perfused with normal whole blood, and can then respond with a maximum anaphylactic reaction when the specific antigen

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is added to the perfusion stream, an effect not obtained when heparinised or defibrinated blood is used for the perfusion⁹.

The mechanism of platelet lysis by contact with foreign surfaces is little understood: there is some evidence¹⁰ that a plasma factor takes an active part. Anticoagulants generally, and notably heparin, delay the lysis, perhaps through an action on the plasma factor rather than directly on the platelets themselves. *In vivo*, platelets agglutinate on a damaged area of endothelium; *i.e.* they stick to one another as well as to the injured blood vessel wall, forming a "white thrombus" or "platelet clot." When the clumped platelets lyse, fibrin is formed locally; in addition, the clumping is itself in some way favoured by the processes giving rise to the fibrin clot. Wright¹¹, applying a simple quantitative test for the measurement of platelet "stickiness," found that this was reduced in animals treated with anticoagulant drugs, including dicoumarol¹², which has no important direct effect on any component of the clotting system, but owes its activity entirely to its ability to prevent prothrombin formation. A platelet thrombus may form within a vessel even when fibrin formation is completely inhibited, as for instance by the administration of heparin; if the dose of heparin is pushed still higher, platelet agglutination is stopped too. The effect of heparin on platelet agglutination begins later and lasts longer than that on the clotting time¹³. These observations are among many which emphasise the dissociability of platelet agglutination and clotting in the ordinary sense; yet the two processes are favoured or prevented by various common influences, and their interrelationships are hard to disentangle, except for the obvious fact that the platelet thrombus is a rich potential source of thromboplastin, and to that extent a likely site of fibrin formation. Further study is needed of the plasma factors affecting platelet adhesiveness and fragility. The observation of Wright¹⁴ that the platelets become more sticky after surgical operations may perhaps be related to the finding by Macfarlane and Biggs¹⁵ of increased fibrinolytic activity at this time, as well as to the greatly increased risk of intravascular clot formation.

DECALCIFYING ANTICOAGULANTS

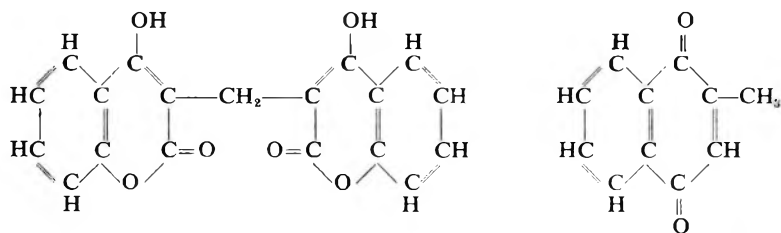
It has been known for over fifty years that calcium precipitants like oxalates, fluorides and the soaps of alkali metals can inhibit the clotting of shed blood, and that the inhibition can be removed by adding an excess of soluble calcium salt. Citrates act similarly, but without precipitating calcium, which becomes bound as part of a complex anion¹⁶. The cheapness and low toxicity of citrate have made it the anticoagulant of choice in blood transfusion. The injected citrate is so greatly diluted by the body fluids that it has no anticoagulant action *in vivo*: the clotting time may even be shortened somewhat¹⁷. When very large volumes of blood or plasma have to be transfused within a short time, the toxicity of citrate may become significant.¹⁸

The prevention of clotting in blood treated with ion-exchange resins, through replacement of the plasma Ca by Na, has recently been described¹⁹.

SUBSTANCES INTERFERING WITH PROTHROMBIN FORMATION

It has long been believed that prothrombin is manufactured by the liver, and this belief has been fully substantiated by recent investigations. When the liver is extirpated or severely damaged (*e.g.* by carbon tetrachloride) the plasma prothrombin falls within a few days to a negligibly low level. Less extensive liver injury produces a smaller, but still readily detectable, reduction in plasma prothrombin. The formation of prothrombin can, however, be depressed without noticeably interfering with the other functions of the liver. The substance which most clearly acts in this way is dicoumarol.

Dicoumarol. The observations of Schofield²⁰ and Roderick²¹ showed that the hæmorrhagic condition of cattle fed on spoiled sweet-clover hay was due to the ingestion of a water-soluble toxic principle, and made it probable that prothrombin was the point of attack. The remarkable work of Link and his colleagues^{22,23,24} led to the identification of the hæmorrhagic agent as 3,3'-methylene-bis-hydroxycoumarin, now known as dicoumarol.



Dicoumarol

2-methyl-1:4-naphthoquinone
(Menaphthone)

There is no doubt that the whole of the anticoagulant action of dicoumarol is due to its interference with prothrombin synthesis: it has practically no effect on clotting *in vitro*. The structural similarity of the dicoumarol half-molecule to the compounds of the vitamin K group (menaphthone is a synthetic vitamin-K analogue: the natural vitamins have branched unsaturated alkyl chains instead of methyl in the 2-position) early suggested that dicoumarol acted as an antagonist to the vitamin. Although the first attempts to counteract the action of dicoumarol by treatment with menaphthone were unsuccessful, it is now clear that the two compounds act antagonistically over a certain range: the vitamin must, however, be given in doses far exceeding the usual therapeutic ones. Ascorbic acid potentiates the anti-dicoumarol effect of menaphthone, and it has been suggested²⁵ that the bleeding tendency characteristic of chronic dicoumarol poisoning may be due in part to a disturbed vitamin C metabolism. Further evidence of the relation of dicoumarol to vitamin K comes from the observations of Meunier and his colleagues^{26,27}, who have described coumarin derivatives with vitamin K activity as well as naphthoquinones acting like dicoumarol. The view that dicoumarol acts by blocking the vitamin is now generally accepted,

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but its mode of action cannot be further analysed, since the role of the vitamin itself in prothrombin formation is unknown.

Dicoumarol is a weak acid, nearly insoluble in water but forming water-soluble salts. Unlike most other anticoagulants, it is active by mouth. The clotting time as ordinarily measured does not provide a satisfactory index of the effectiveness of dicoumarol, and some form of prothrombin-time test is universally used in both experimental and clinical work to gauge the effectiveness of the drug. The action on prothrombin-time is a delayed one, since time must be allowed for the existing prothrombin to disappear: the maximum prolongation is seen in 2 to 4 days after the administration of a single dose. A rather longer time is required for the restoration of prothrombin after the effect has begun to wear off. In the presence of liver injury the effect of the drug is enhanced both in degree and in duration; the same is true when the kidney is damaged. There is no doubt that the response to dicoumarol varies considerably from subject to subject: the effect of an excessive reduction of prothrombin can be counteracted by the administration of a vitamin K preparation, or (since symptoms do not occur unless the plasma prothrombin is depleted to a small fraction of the normal value) by the transfusion of normal blood or plasma.

The toxicity of dicoumarol for both animals and man is related almost entirely to its anticoagulant action, and death when it occurs is due to hæmorrhage. Morphological changes in the liver, when seen at all, are usually secondary to local hæmorrhage, and most liver function tests reveal no impairment of the organ. The plasma fibrinogen level is, however, somewhat raised by moderate doses of dicoumarol, and reduced by large doses²³: similar effects are produced by various agents toxic to the liver, and it may be that the abnormality in this organ is not strictly confined to the prothrombin-forming system. Plasma fibrinogen levels are, however, distinctly labile, and the degree of general hepatic injury produced by dicoumarol is at most an extremely slight one.

Salicylates. Link²⁴ has suggested that the action of dicoumarol may be an indirect one: its breakdown within the body may liberate salicylates, and these may be responsible for inhibiting the formation of prothrombin. While there is not yet enough evidence to prove or disprove this idea, the deleterious action of salicylates on prothrombin formation has been amply confirmed in both human and animal studies.

SUBSTANCES INHIBITING THE CONVERSION OF PROTHROMBIN TO THROMBIN AND/OR THE ACTION OF THROMBIN

It might be thought convenient to consider in separate sections the substances opposing the formation of thrombin, and the substances impeding its action. In practice this is difficult. Heparin, the most important of all, acts on both stages of clotting; and the same is true of some, at least, of the anticoagulants which resemble it in being sulphuric acid esters of high molecular weight. Even in the case of heparin it is very hard to assess the relative contribution made by its antiprothrombin and antithrombin activities to its overall effect on clotting systems containing

whole blood; and the same statement applies with greater force to the related anticoagulants, none of which has been investigated in such detail as heparin. Antiprothrombin activity, in turn, might be due to inactivation of either thromboplastin or prothrombin itself. In practice, the amount of anticoagulant required to prevent thrombin formation goes up if the clotting system is enriched in either thromboplastin or prothrombin. This, however, would be expected on the basis of any theory postulating a reversible combination between either of the clotting factors and the inhibitor. It is likely that both prothrombin and thromboplastin may combine with the anticoagulant and lose activity on so doing; but until the factors concerned in thrombin formation can all be isolated and studied by adequate physio-chemical methods, the relative importance of the various possible reactions can only be guessed at.

Most of the anticoagulants listed as possessing antiprothrombin or anti-thrombin activity have large molecules, or can form more or less stable complexes with protein constituents of the plasma. They thus remain in the blood for some time after injection, and their anticoagulant action, unlike that of the decalcifying anions, is demonstrable *in vivo* as well as *in vitro*. Many of them are compounds containing sulphuric acid in ester linkage. Of these heparin has received by far the most attention, both because it is a natural constituent of the body and may be concerned in maintaining the normal fluidity of the blood, and because its toxicity is low enough to permit its prolonged administration to patients in danger from intravascular thrombosis.

Heparin. The monograph by Jorpes³² is a recent and comprehensive treatise on the chemistry, physiological action and clinical applications of the substance. Earlier summaries by Best^{33,34} and Wilander³⁵, both of whom have made important contributions in this field, are still well worth consulting.

History and chemistry of heparin. Heparin was discovered by McLean³⁶ in 1916 during an investigation, under Howell's direction, of the thromboplastic action of phosphatide preparations from liver and heart. During the next ten years Howell and his colleagues studied it intensively and showed that it was not, as had been thought at first, a lipid; their best preparations contained uronic acids and had a high ash content. In the further purification of heparin and in the elucidation of its chemical nature, the major part was played by Charles and Scott in Toronto and by Jorpes and his co-workers in Stockholm. The Canadian workers³⁷ devised an alkaline extraction technique which gave an improved yield of a purer product, and they showed that many mammalian tissues contained heparin, ox lung being a particularly rich source. The Swedish investigators^{38,39} confirmed the presence of a uronic acid in the purified material, and identified glucosamine and ester sulphate as further constituents: the remarkably high content of sulphate explained the large proportion of ash in Howell's material. Jorpes and Bergstrom³⁹ concluded that heparin is a mucoitin polysulphuric acid, and this view has been sustained by subsequent investigation.

About the same time Charles and Scott⁴⁰, who had been continuing

their purification studies, reported the isolation of a crystalline barium salt of heparin having a constant composition. There has been a good deal of controversy about the significance of this material. Its isolation has been repeated and the formula $C_{26}H_{44}O_{58}N_2S_5$ has been suggested^{41,42} for the corresponding heparin acid, but Jorpes³² regards the barium salt as a mixture of compounds varying in their degree of esterification. There is little doubt that the barium salt of Charles and Scott was really crystalline and that the crystallisation procedure is a valuable method of purification. The heparin molecule is, however, such a large one (the molecular weight of the barium salt is 3462 according to the formula of Charles and Todd and the compound may be a polymer) that crystallinity alone is an insufficient guarantee of purity. There is, indeed, a good deal of evidence that the crystallised preparation does not necessarily represent a chemical individual; samples of it vary appreciably in composition³² and have been separated into fractions of unequal potency⁴³; the potency may go down with repeated recrystallisation⁴⁴; and the barium salts obtained from different mammalian species are of quite divergent potency, although similar in elementary composition, crystalline form, and chemical behaviour⁴⁵. It is hard to say whether heparin in its natural state ought to be regarded as a chemical individual, difficult to obtain in strictly unmodified condition, or whether a family of closely related heparins exists in the tissues: the latter is perhaps the more likely alternative, if only because so complex a substance is probably not synthesised in a single step. Nevertheless, it seems probable that the best preparations obtained by the Canadian and the Swedish workers, and by others, represent a near approach to the most potent heparin obtainable, at any rate from bovine tissue.

The standardisation of heparin. The difficulty of obtaining heparin of uniform quality makes it desirable that each preparation should be standardised for potency. Howell⁴⁶ originally defined a unit of heparin activity as the minimum amount which, when added to 1 c.c. of freshly drawn cat's blood, would keep it fluid for 24 hours. Experience with many drugs has shown that such a unit, defined in terms of a poorly reproducible biological system, cannot be relied on to be constant; and in accordance with the practice now generally accepted, several groups of workers set up stable reference preparations, by comparison with which the potency of other samples was determined. These have now been replaced by an International Standard Heparin⁴⁷, the bulk of which is preserved at the National Institute for Medical Research, London, N.W.3, under the auspices of the Committee on Biological Standardisation of the World Health Organisation. The International Unit represents the strength of 1/130 mg. of this preparation: it is practically identical with the Toronto unit⁴⁸, defined in terms of a sample of barium salt, and, so far as can be ascertained, roughly equal to the Howell unit. It is to be expected that the practice of labelling potency in units will become superfluous in time, because all commercial preparations will be of the same maximum strength; but meanwhile the common practice of reporting doses in mg. introduces an uncertainty of at least 30 to 40 per cent.

and is to be deprecated. It is perhaps unfortunate, as Jorpes³² points out, that the established unit is such a small one: it is inconvenient to have to write out "30,000 units", for instance, when referring to a single human dose.

A great variety of assay methods has been suggested for the standardisation of heparin preparations: the clotting systems which have been used include fresh blood; citrated or oxalated blood or plasma, recalcified with or without the addition of tissue extract; fresh fowl plasma plus tissue extract; and whole blood or plasma plus thrombin. Reproducible results can be obtained with any of these systems, but when two samples of heparin are compared by different methods the values obtained for the relative potency may differ somewhat: this is particularly true when the samples are obtained from different species⁴⁹ or when one of them has been denatured by treatment with acid⁵⁰. The cause of such discrepancies is presumably the varying affinity of the several active principles for different constituents of the clotting mixture. As it appears that most of the heparin now on the market is of bovine origin, and is at least two-thirds as active as the best preparation obtainable from the species, the uncertainties due to the use of varying assay methods are not very serious.

Physical and chemical properties of heparin. Heparin and its salts with the alkali and alkaline earth metals are colourless substances, soluble in water but not in most organic solvents; they do not dialyse appreciably through ordinary collodion membranes. The sodium salt, which is the form usually supplied, is very stable in neutral or alkaline solution and can be sterilised by heating; when heated in acid solution it slowly loses its activity. Heparin is precipitated by many organic bases, including benzidine, protamines, toluidine blue and streptomycin: this property has been employed for inactivating heparin both *in vitro* and *in vivo*. Toluidine blue changes when combined with heparin into its reddish-violet "metachromatic" tautomer⁵¹; a similar sort of colour change is shown by other basic dyes in the presence of heparin. The metachromatic reaction was shown by Lison⁵² to be specific for sulphuric acid esters of high molecular weight: it is given by a large number of substances, both natural and synthetic, which possess anticoagulant activity. The Swedish workers, in a series of particularly elegant experiments^{53,54,35}, have shown that the granular material of the mast cells, which takes an intense metachromatic stain, is rich in heparin.

The most striking property of heparin is the remarkably strong negative charge carried by its molecule in aqueous solution. Indeed, as Jorpes points out, this is almost its only important chemical property, since apart from its acidic (sulphuric and carboxyl) radicals the molecule has no reactive groups. The acidic strength of heparin enables it to react with the basic groups of proteins and other substances and is certainly the basis of its anticoagulant action. That its affinity for proteins is a general one and is not confined to the proteins concerned with clotting was shown by Fischer⁵⁵, who observed that the isoelectric point of casein was shifted to the acid side in the presence of heparin.

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Mode of action of heparin. Although there is no doubt that the negative charge carried by its molecule is responsible for the characteristic activity of heparin, the activity is not simply a function of the proportion of esterified sulphuric acid that is present. The large size of the molecule is also involved in some way. The low potency of the heparin obtained from some mammalian species, and the reduced activity of heparin subjected to mild treatment with acid, which have been referred to, probably indicate a correlation between potency and degree of polymerisation⁴⁴. That potency increases with molecular size is more obvious in the case of the synthetic esters of sulphuric acid, which will be referred to later.

While the key to the action of heparin must be sought in the large size and electronegativity of its molecule, these properties do not explain why heparin should act particularly on the blood-clotting system. Indeed, it is by no means certain that its physiological function has to do with the maintenance of the fluidity of the blood. Heparin does, in fact, act on other enzyme systems: it neutralises complement⁵⁶; antagonises fumarase⁵⁷, trypsin⁵⁸, and fibrinolysin⁵⁹; and it has some inhibitory effect on a variety of allergic reactions. When it is injected or liberated into the blood stream, its low diffusibility will tend to keep it there, and it may be in part for this reason that it has so few extravascular actions.

Heparin can prevent both the conversion of prothrombin to thrombin and the clotting of fibrinogen by thrombin. In either case the inhibitory action disappears when the isolated clotting reagents are used. Thus, heparin does not inhibit the clotting of purified fibrinogen by purified thrombin⁶⁰, or the formation of thrombin from purified prothrombin⁶¹. An additional factor, heparin complement^{62,63,64}, must be present if either reaction is to be prevented: this substance is a constituent of the albumin fraction of the plasma, but is not the crystallisable serum albumin proper. The antithrombin of normal plasma is also found in the albumin fraction, and the attractive suggestion has been made that it may be heparin-complement combined with a small fraction of its possible charge of heparin. The balance of evidence^{64,65} seems at present, however, not to support this hypothesis.

Heparin: fate in the body. Heparin given by vein exhibits its greatest effect on clotting within the first few minutes. It is then rather rapidly inactivated or removed from the circulation. Some is excreted in the urine^{66,35,57}; the greater part apparently escapes slowly into the tissues, where it may be enzymatically inactivated⁶⁸. The duration of the anticoagulant effect is roughly proportional to the dose, but does not exceed 2 to 3 hours unless the dose is so great that its initial effect is to raise the clotting time to infinity.

Organic esters of sulphuric acid. The possibility of obtaining a cheap, synthetic anticoagulant suitable for use *in vivo* began to receive attention about 20 years ago. The anticoagulant action of the trypanocidal drug suramin (sodium *m*-benzoyl-*m*-amino-*p*-methylbenzoyl-*l*-aminonaphthalene-4:6:8-trisulphonate, germanin, Bayer 205) had been known for some time⁶⁹, and in 1930 Stuber and Lang⁷⁰ reported its successful use

in the therapy of thrombosis. In the same year Rous, Gilding and Smith⁷¹ noted the anticoagulant effect of the dye chiorazol sky-blue FF, the sodium salt of tetrazotised dianisidine coupled with 1-amino-8-naphthol-2:4-disulphonic acid); and Demole and Reinert⁷² prepared a variety of substances of high molecular weight and found some of them active against clotting, the most potent being sulphonated aromatic polymers. One of these, the sodium salt of polyanetholesulphonic acid, was later marketed as "liquid," and has had some popularity as an anticoagulant for *in vitro* and animal experiments: it is a rather toxic substance causing delayed death in doses not far above the effective ones. Chlorazol sky-blue, however, and some related azo-sulphonic dyes, especially Chlorazol fast-pink BKS (sodium 3:5-disulpho-diphenylurea-4:4-diazo-*bis*-2-amino-8-naphthol-6-sulphonate) were found by Huggett and his colleagues^{73,74} to be relatively non-toxic, and have been used extensively in physiological experiments. They act, apparently, mainly in the first stage of clotting. In this group of dyes there is no simple relationship between chemical structure and anticoagulant activity: all have heavy molecules containing a number of $-SO_3$ groups and azo linkages; and it is of interest that they resemble heparin, and the other synthetic anticoagulants containing ester sulphate, in having a strong affinity for toluidine blue and other metachromatic dyes.

The next series of synthetic anticoagulants to be investigated represented, chemically at least a nearer approach to heparin. Chargaff and his colleagues⁷⁵ and Jorpes' collaborator Bergstrom⁷⁶ independently reported the activity of carbohydrates esterified with sulphuric acid by treatment with chlorosulphonic acid. The esters derived from mono- and di-saccharides were inactive, but all the polysaccharides tested gave rise to active compounds. The treatment with chlorosulphonic acid, if unduly prolonged, yielded less active products, presumably because the materials were being depolymerized⁷⁶. These compounds probably act on both stages of clotting, and the best of them^{77,78,79} approach heparin in potency: the ratio of their activity to that of heparin cannot be exactly stated, since it varies greatly with the conditions of assay. All are too toxic for clinical use. It is of interest that their toxicity is due, at least in part, to their effect on clotting factors: they either cause the platelets to agglutinate, or precipitate fibrinogen, or both. According to Karrer and his colleagues⁸⁰ the toxicity is reduced by the introduction of other acid groups into the polysaccharide molecule before the treatment with chlorosulphonic acid.

Many high-molecular-weight esters of sulphuric acid occur naturally in both animals and plants; some of these are anticoagulants and some are not: activity is correlated at least roughly with ester sulphate content. Mucoitin and chondroitin sulphuric acids are not active, but become so when further esterified⁶. The mucus of the mollusc *Charonia lampus* contains a potent anticoagulant⁸¹ with many chemical similarities to heparin. Two anticoagulants formerly popular with physiologists may possibly belong to this group, but little is known of their chemistry except that they seem to be acidic in nature: these are hirudin^{82,83}, from

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the buccal glands of the medicinal leech, and novirudin⁸⁴, a melanin-like substance of vegetable origin. A number of marine algae contain polysaccharides esterified with sulphuric acid. Agar-agar, the best known of these, is not an anticoagulant; but related substances from *Chondrus crispus*⁸⁵ and *Iridaea laminarioides*⁷⁵ are moderately active.

Basic dyes and other basic substances. All the substances just discussed presumably act in virtue of their strongly acid SO_3H groups and the size of their molecules. The possibility that basic substances of high molecular weight are also anticoagulants has been investigated. This too is the case: protamine and histones^{86,87} have well-marked activity, as have a number of basic dyes⁸⁷, including methylene blue, crystal violet and Janus green. Even so simple a base as diethylamine has some anticoagulant activity⁸⁸. The mode of action of these substances is supposed to be analogous to that of the synthetic heparin analogues, but in a reverse direction: *i.e.* they displace the isoelectric point of protein clotting-factors toward the alkaline side, and so reduce their reactivity. The antagonism between compounds of this group and the ester-sulphate anticoagulants has already been mentioned; it is due, however, not so much to a cancelling-out of opposed actions on the electric charge of protein clotting factors, as to a simple co-precipitation of the acidic and basic anticoagulants. None of the bases so far investigated appears likely to have any practical value in delaying coagulation.

Reducing substances. A number of reducing agents, both inorganic and organic, have anticoagulant activity; they appear to act on both stages of clotting. Sodium bisulphite⁸⁹ and thiosulphate⁹⁰, cysteine⁹¹ and glutathione⁹² may be mentioned.

Salts of rare earth metals. The trivalent cations of neodymium^{93,94} praseodymium, lanthanum and other elements of this group⁹⁵ are extremely active anticoagulants both *in vitro* and *in vivo*. They appear to act mainly on the first stage of clotting^{94,96}, but the mechanism of their action is not understood. The compounds are too toxic to be of practical value⁹⁷.

“*Lipid inhibitors*” of coagulation have been detected in phosphatide fractions of tissue extracts by Chargaff⁹⁸ and de Sütö-Nagy⁹⁹. Their mode of action has not been studied in detail, and whether they have any physiological significance for the prevention of clotting is unknown.

Trypsin inhibitors. The purified trypsin inhibitors obtainable from pancreas and from soya beans are fairly active in delaying coagulation^{100,101,102,103}. Their mode of action is obscure.

SUBSTANCES INACTIVATING FIBRINOGEN

The fibrinolytic enzyme of normal plasma, discovered forty years ago by Nolf¹⁰⁴, should be listed for the sake of completeness, since it can digest fibrinogen as well as fibrin¹⁰⁵. It is usually present in an inactive form, but can be activated *in vitro* or *in vivo* by a number of different ways. Whatever the relation of this enzyme to the clotting system, it is doubtful whether it ever, *in vivo*, destroys fibrinogen so rapidly as to make the blood incoagulable.

Fibrinogen is precipitated more or less selectively by both protamines and certain anticoagulants of the sulphonic ester group, but this is certainly not the main reason why such substances inhibit clotting.

SUBSTANCES CAUSING THE RELEASE OF HEPARIN

The blood of dogs thrown into shock by the injection of large amounts of Witte's peptone, or of an antigen (in previously sensitised animals), becomes incoagulable; and it has been conclusively demonstrated that the inhibition of clotting seen in such animals is due to circulating heparin^{107,108}. The liver is the principal source of the released heparin. A similar effect is produced by large doses of ionising radiation¹⁰⁹, by the radiomimetic drugs of the nitrogen mustard series¹¹⁰, and by certain simple basic drugs, particularly diamines and diamidines¹¹¹. Evidence that these stimuli release heparin in the same way from human tissues is lacking, except in the case of ionising radiations.

THE CLINICAL USE OF ANTICOAGULANTS

The utility of a non-toxic anticoagulant in the therapy and prophylaxis of thrombosis in man has long appeared probable, and was confirmed as soon as purified heparin became available in quantity. The first favourable clinical reports from Stockholm¹¹² and Toronto¹¹³ have been followed by some hundreds of papers describing the successful use of both heparin and dicoumarol in a variety of thrombo-embolic conditions. It is impossible to give a brief adequate summary of this work; an excellent account will be found in the monograph by Jorpes³², who seems, however, to emphasise unduly the toxic action of dicoumarol on the liver. Both drugs appear to have an established place in therapy. As compared with heparin, dicoumarol has the advantages of cheapness and of effectiveness on oral administration: its drawbacks are its slow onset of action, which makes it useless in emergencies unless supplemented by heparin, and the considerable variability in the response of individual patients. All anticoagulant therapy involves the risk of hæmorrhage, and this risk can only be minimised by close supervision of the patient and frequent checks of clotting time (in the case of heparin) or prothrombin time (in the case of dicoumarol).

The numerically most important field of usefulness for these drugs has been the prevention and treatment of post-operative thrombosis, particularly after pelvic operations. The incidence of this complication is notoriously variable, and the availability of an effective therapy should not distract attention from the importance of simpler measures, especially active and passive movement of the limbs. Treatment is usually begun on the second day, when the risk of bleeding at the site of operation is small, and continued till the patient is ambulant. Opinions vary as to whether anticoagulant therapy should be used routinely after pelvic and abdominal surgery or reserved until signs of clot formation appear. Early diagnosis of latent thrombosis is naturally of the greatest importance, and

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phlebography of the lower extremities¹¹⁴ and tests revealing hypercoagulability of the blood, such as the heparin tolerance test of de Takats¹¹⁵, have been found useful for this purpose.

Other forms of active venous thrombosis respond equally well to anti-coagulant therapy, which undoubtedly reduces the incidence of embolic complications. Thrombosis of the mesenteric veins, the retinal veins and the cavernous sinus, have all been treated successfully, in addition to the more common condition in which the initial site of clot formation is one of the deep veins of the lower leg. The status of the anticoagulant drugs in the treatment of occlusive coronary artery disease is still uncertain. They are quite useless in subacute bacterial endocarditis. Overdosage with heparin is treated by withdrawal of the drug, when the blood regains its normal clotting power within a few hours, or in emergency by the intravenous injection of protamine, which has an instantaneous effect. Dicoumarol overdosage can be corrected by the administration of massive doses of vitamin K preparations, or more rapidly by the transfusion of fresh blood or plasma.

Finally it should be mentioned that heparin is a valuable adjunct to vascular surgery, and has some advantages over citrate as an anticoagulant in blood transfusion.

The expense and inconvenience of heparin therapy have undoubtedly restricted its field of usefulness. While intravenous administration, either several times a day or by continuous drip, is still the method most commonly used, a number of menstrua for the incorporation of heparin have been devised^{116,117}, which permit a prolonged effect to be obtained with a smaller number of intramuscular or subcutaneous injections.

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RESEARCH PAPERS

THE IDENTIFICATION OF THE CLINICALLY-IMPORTANT SULPHONAMIDES

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Received March 23, 1949

SINCE the discovery in 1935 of the chemotherapeutic value of the red azo-dye prontosil rubrum, and the subsequent proof that its activity *in vivo* is due to its conversion into sulphanilamide, an immense number of sulphonamide derivatives has been synthesised. The vast majority of these derivatives has found no place in medicine, and in this country some dozen only are in current clinical use.

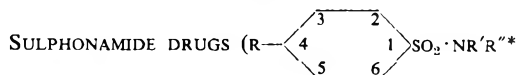
Owing to their relatively low toxicities, the sulphonamide drugs are unlikely suicidal agents, but during their therapeutic use toxic symptoms of varying severity not infrequently develop, necessitating clinical and chemical supervision. Excretion of the drugs occurs essentially *via* the urine, in which they are found either unchanged, in acetylated form, or to a lesser degree as the sulphates or glycuronates of derived phenols, the relative proportions of each form varying with the nature of the original substituent radical (Williams¹). Thus urine, or blood, is the usual material examined in the biochemical control of sulphonamide therapy, but the toxicologist may be further concerned with the isolation and identification of these drugs when occurring in viscera and medicinal preparations. From an analytical standpoint, a serious difficulty arises from the fact that no reactions specific for the typical sulphonamide linkage have been described. Quantitative determinations of the sulphonamide content of urine and blood are usually based upon such reactions of the free primary amino group as diazotisation (Fuller² Bratton, Marshall, Babbitt and Hendrickson³), production of the yellow Schiff's bases with *p*-dimethylaminobenzaldehyde (Werner⁴), or the indophenol reaction (Lapière⁵⁻¹²). Whilst such reactions have some merit of simplicity, it is evident that interference would result from the presence of other aryl primary amino compounds (Pons and Abel¹³), and clearly the method is ineffective with sulphonamides in which the primary amino group is substituted.



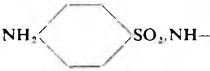
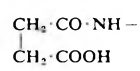

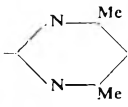

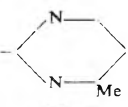
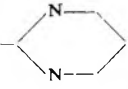
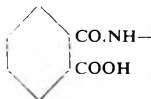

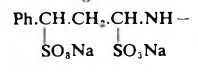
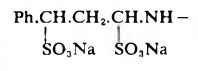

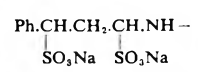

Methods for the qualitative identification of individual sulphonamides figure widely in the literature, and range from the synoptic schemes of Burkham¹⁴, Deniges¹⁵, Pesez¹⁶, and Hoffmann and Wilkens^{17,18}, to the colour reactions of Chavez¹⁹, Sample²⁰, and other workers and the crystal reactions suggested by Beck²¹, Dodson and Todd²², and Lapière⁵⁻¹². The present authors have repeatedly investigated the various methods hitherto described, but nevertheless have felt the need for a simple technique for identification of the drugs in the micro-quantities in which they may be encountered in toxicological analyses.

The number of sulphonamides is so large, whilst those of clinical importance are so relatively few, that any attempt at systematic identifi-

IDENTIFICATION OF SULPHONAMIDES

TABLE I



	R	R'	R''	M pt. (°C)
Sulphanilamide B.P. ...	NH ₂ —	—H	—H	165
Proseptasine ...	C ₆ H ₅ ·CH ₂ ·NH—	—H	—H	175
Sulphacetamide B.P. ...	NH ₂ —	—H	—CC·CH ₃	183
Sulphaguanidine B.P....	NH ₂ —	—H		191
Sulphapyridine B.P.C. ...	NH ₂ —	—H		191
Uleron ...		—CH ₃	—CH ₃	194
Succinylsulphathiazole B.P.		—H		195 (185)
Sulphadimidine ...	NH ₂ —	—H		199 (176)
Sulphathiazole B.P. ...	NH ₂ —	—H		201
Sulphamerazine ...	NH ₂ —	—H		236
Sulphadiazine B.P. ...	NH ₂ —	—H		255 d.
Phthalylsulphathiazole ...		—H		260 d.
Soluseptasine ...		—H	—H	—
Solupyridine ...		—H		—
Soluthiazole ...		—H		—

* The drugs are listed under the name given in the British Pharmacopœia or British Pharmaceutical Codex, or if non-official, under a common trade name. The obsolescent azo-compounds prontosil rubrum and prontosil soluble are not included in this survey.

cation in the field as a whole would be unnecessarily cumbersome and would have little practical value. The investigations described in this publication, therefore, have been confined to those sulphonamide drugs commercially available at the present time in this country (Table I).

Residues isolated from medicinal preparations, or from viscera or other biological sources, are examined in a series of separate stages: (1) purification of crude residues, (2) provisional identification as a sulphonamide compound, (3) demonstration of the presence or absence of a free primary amino group, (4) simple crystal tests, (5) colour test indicative of the substituted sulphapyrimidines, (6) final conclusive identification by micromixed melting-point determination. The complete scheme provides a simple and rapid method for the identification of the listed sulphonamides, but in many instances characterisation may be achieved without inclusion of all the stages. It is emphasised that the object of the preliminary stages is essentially the indication of the likely compound, and whilst by the use of control experiments it is frequently possible to obtain a clear identification of an unknown sulphonamide by means of the preliminary tests alone, the ultimate proof should be by micromixed melting-point determination.

EXPERIMENTAL

Isolation of the sulphonamides. Sulphonamide therapy is of so recent introduction that few of the standard toxicological works have any mention of this group of drugs. Bamford²³, in a brief treatment of the sulphonamides, deals largely with methods of determination, and observes "Identification must depend on their isolation—often a difficult process—and examination of physical properties. Generally, however, the history of the case, combined with the results of the non-specific diazotisation and condensation reactions suffice to establish (or, more usually to confirm) the nature of the poison." The difficulties of isolation which this author so rightly mentions are largely attributable to the low solubilities of the sulphonamides in the water-immiscible organic solvents. Literature figures for the solubilities are by no means consistent, but the comparative record of available data (Table II) is useful, particularly when dealing with mixtures of sulphonamides.

In the Stas-Otto process, and also in the tungstic acid method described by Valov²⁴, sulphonamides are found in the ether extract of the aqueous acidic solution. The solubilities in ether, however, are so low that a small percentage only of the sulphonamide is recovered in this way, but in dealing with mixtures of sulphonamides with other compounds this is a definite advantage, as it frequently affords a ready means of separation. Complete removal from the aqueous acid medium may be effected by addition of half the volume of acetone, followed by thorough extraction with ether. For quantitative determination of total sulphonamides in material from biological sources, hydrolysis of the various derivatives is necessary. Sulphonamides generally, are present partly in the form of N₄-acetyl derivatives, whilst the three disodium cinnamylidene bisul-

IDENTIFICATION OF SULPHONAMIDES

phite compounds listed occur (Wien and Hampton²⁵) as the parent sulphonamides, their acetyl derivatives, and possibly also as the unchanged bisulphite compounds. Hydrolysis is readily accomplished by refluxing with 2N hydrochloric acid for 20 to 30 minutes.

Purification of crude residues. The low solubilities of the drugs are an undoubted advantage in connection with their purification. As a general method, heating with activated charcoal in acetone solution is found satisfactory; losses due to absorption are negligible, and a clean product, suitable for a preliminary melting point determination, is usually obtained.

Wide differences are found in the reported melting points of several sulphonamides. In the case of sulphadimidine, this variation is attributed

TABLE II
SOLUBILITIES OF SULPHONAMIDE DRUGS.*

	Water	2N sulphuric acid	2N sodium hydroxide	Alcohol	Ether	Chloroform	Acetone	Light Petroleum b.pt. 60° to 80° C.
Sulphanilamide ...	1-125	Soluble	Soluble	1-37	1-600	1-4000	1-5	Insoluble
Sulphaguanidine	1-1000	Soluble	Insoluble	1-200	Insoluble	Insoluble	1-300	Insoluble
Sulphapyridine	1-3500	Soluble	Soluble	1-340	1-2200	1-1500	1-65	Insoluble
Sulphadiazine ...	1-13,000	Soluble	Soluble	1-1100	Insoluble	Insoluble	1-170	Insoluble
Sulphamerazine	1-6250	Soluble	Soluble	1-400	Insoluble	1-2000	1-60	Insoluble
Sulphadimidine ...	1-5000	Soluble	Soluble	1-200	1-5000	1-600	1-20	Insoluble
Sulphacetamide	1-150	Soluble	Soluble	1-15	1-600	1-1200	1-7	Insoluble
Sulphathiazole ...	1-2000	Soluble	Soluble	1-200	1-2400	1-375	1-23	Insoluble
Succinylsulphathiazole ...	1-4800	Slightly soluble	Soluble	1-100	Insoluble	Insoluble	1-110	Insoluble
Phthalylsulphathiazole	1-7500	Slightly soluble	Soluble	1-500	Insoluble	Insoluble	1-250	Insoluble
Proseptasine ...	1-32,000	Slightly soluble	Slightly soluble	1-145	1-320	1-1500	1-10	Insoluble
Uleron ...	1-50,000	Slightly soluble	Soluble	1-250	Insoluble	1-1000	1-20	Insoluble

* Solubilities in water are given numerically throughout. Organic solvent solubilities lower than 1 in 5000 are indicated as Insoluble.

(Northey²⁶) to the occurrence of unstable hydrates. A similar explanation may hold for other sulphonamides, and the figures in parenthesis in Table I may represent the melting points of these hydrates. It is further likely that this may also be the explanation of the wide differences in solubilities recorded in the literature.

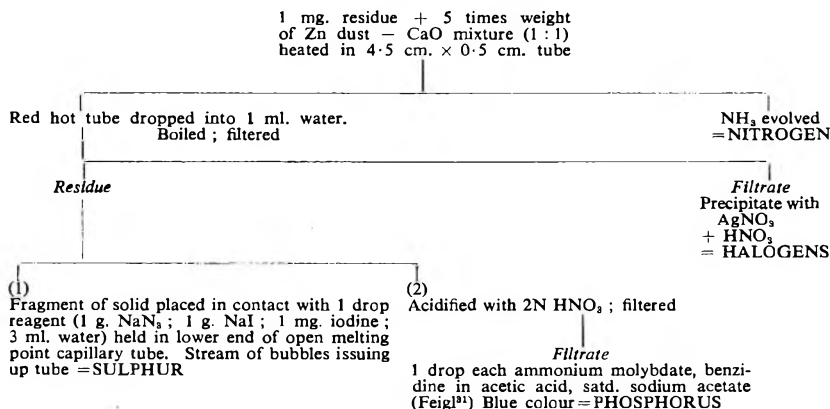
Provisional identification as a sulphonamide. Using macro-quantities, identification by standard analytical methods presents no particular problem, but on a micro-scale the lack of a specific reaction for the sulphonamide grouping is a serious disadvantage. Lapière²⁷ has investigated the cobalt reaction described by Parri²⁸ in connection with the identification of barbiturates, and has found that certain sulphonamides, notably sulphathiazole, sulphapyridine and sulphadiazine, also give the characteristic purple-violet colour. Unfortunately, the reaction cannot be regarded as a general test for the presence of sulphonamides.

Provisional characterisation of isolated and purified residues as

sulphonamide compounds has been accomplished by the present authors in the following manner:

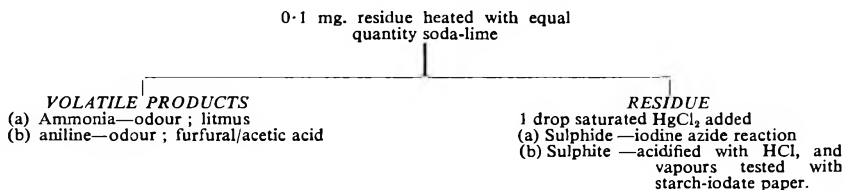
(i) Detection of elements by an adaptation of the micro-method described by Bennett, Gould, Swift and Niemann²⁹. The procedure is given in detail in Table III.

TABLE III
MICRO-IDENTIFICATION OF ELEMENTS



(ii) If nitrogen and sulphur only are detected sulphonamides may be present, and the effect of heating in admixture with soda-lime is then investigated according to the scheme in Table IV.

TABLE IV
DECOMPOSITION ON HEATING WITH SODA-LIME



The clinically-important sulphonamides listed are all primary amino derivatives, and on fusion with soda-lime are decomposed with the production of ammonia or readily volatile amines, aniline bases and sodium sulphite; sulphathiazoles and the cinnamylidene bisulphite compounds yield, in addition, sodium sulphide. Thus, by the method in Table IV, positive tests for ammonia, aniline and sulphite provide presumptive evidence of the presence of a sulphonamide; sulphide in addition suggests the sulphathiazoles or cinnamylidene bisulphite compounds.

Individual sulphonamides differ considerably in the rate at which the various products are formed, but by using a standard technique the reactions with quantities of the order of 0.1 mg. are equally as satisfactory as those utilising larger amounts of material.

0.1 mg. of the test material intimately mixed with an equal weight

IDENTIFICATION OF SULPHONAMIDES

of soda-lime is gently warmed in a small test tube (4.5 cm. x 0.5 cm.). The issuing vapours are tested with a pointed strip of moist red litmus paper introduced just inside the tube. Stronger heat is now applied until an oily distillate accompanied by white fumes approaches to within approximately 0.5 cm. from the open end of the tube. The tube is placed on a white tile, and a pointed strip of filter paper moistened with a 2 per cent. solution of furfural in glacial acetic acid carefully introduced. In the presence of aniline (which may also frequently be detected by odour) a distinct reddish-pink band appears around the tube in the region of the oily distillate. At this stage one drop of saturated aqueous mercuric chloride solution is added to the solid residue in the tube, in order to obviate interference from hydrogen sulphide on subsequent acidification; a fragment of the treated residue is tested for sulphide by the iodine-azide reaction. The contents of the tube are finally heated with dilute hydrochloric acid, and the issuing vapours tested with a pointed strip of moistened filter paper impregnated with starch and potassium iodate, when a blue colour develops in the presence of sulphur dioxide. The results of this test with the listed sulphonamide drugs are summarised in Table V. It should be emphasised that this procedure does

TABLE V
REACTIONS OF SULPHONAMIDES

	Decomposition by soda-lime fusion				Furfural reaction for free NH ₂
	Ammonia	Aniline	Sulphite	Sulphide	
Sulphanilamide	+	+	+	-	+
Proseptasine	+	+	+	-	-
Sulphacetamide	+	+	+	-	+
Sulphaguanidine	+	+	+	-	+
Sulphapyridine	+	+	+	-	+
Uleron	+	+	+	-	+
Succinylsulphathiazole	+	+	+	+	-
Sulphadimidine	+	+	+	-	+
Sulphathiazole	+	+	+	+	+
Sulphamerazine	+	+	+	-	+
Sulphadiazine	+	+	+	-	+
Phthalylsulphathiazole	+	+	+	+	-
Soluseptazine	+	+	+	+	-
Solupyridine	+	+	+	+	-
Soluthiazole	+	+	+	+	-

not provide unequivocal proof that the material is a sulphonamide, but positive reactions here coupled with indications of unusual insolubility is strong presumptive evidence.

Detection of free NH₂ groups. Aryl primary amino groups may be detected by a variety of reactions, of which diazotisation followed by coupling is perhaps the most usual. The information can however be obtained equally satisfactorily, and much more rapidly, by means of the furfural condensation. 0.1 mg. to 1 mg. of material is placed in a white porcelain dish and treated with 1 drop of 2 per cent. solution of furfural in glacial acetic acid. The liquid is allowed to evaporate spontaneously, during which process a free primary amino group is indicated by the production of an intense red colour, rapidly turning reddish-violet.

The reactions of the sulphonamides are listed for convenience in Table V. It is noteworthy that in the case of the pyrimidine derivatives, the colour is appreciably slower in developing, and may not be apparent until evaporation is complete. Of the listed sulphonamides, positive tests for both sulphide and free $-NH_2$ are given by one compound only, sulphathiazole. Similarly, one compound only, proseptasine, gives negative tests for both sulphide and free $-NH_2$.

Crystal tests. Since the sulphonamides are, in general, soluble both in acid and alkali, acidification of an ammoniacal solution as recommended for the identification of barbiturates (Turfit³⁰) is valueless. It has been found, however, that by a variation in the technique, crystals of the sulphonamides may in most cases be readily obtained. The

TABLE VI
CRYSTAL FORMATION

	Strong solution of ammonia 0.880	Acetic acid vapour
Sulphanilamide ...	Soluble with difficulty	* No peripheral precipitation. Long fine needles radiate from undissolved particles; also occasional hexagonal forms.
Proseptasine ...	Insoluble	—
Sulphacetamide ...	Very readily soluble	—
Sulphaguanidine ...	Insoluble. Slightly soluble hot; long needles on cooling.	—
Sulphapyridine ...	Readily soluble	* Rapid peripheral precipitation. Characteristic 'banded' crystals, with some hexagonal and arborescent needle formations.
Uleron ...	Readily soluble	* Rapid peripheral precipitation. Minute globules with characteristic 'dumb-bell' shaped crystals. Wrinkled surface skin develops over drop.
Succinylsulphathiazole	Very readily soluble	—
Sulphadimidine ...	Readily soluble	Rapid peripheral precipitation. Minute globules giving large rosettes of brownish needles. Wrinkled surface skin develops over drop.
Sulphathiazole ...	Very readily soluble	* Slow peripheral precipitation. Minute drops coalescing rapidly into large globules, and giving arborescent needle-shaped growths.
Sulphamerazine ...	Readily soluble. On evaporation 'curved' crystals usually obtained	Rapid peripheral precipitation. Three crystalline forms usually obtained: (a) rods, often with bifurcated ends, followed by (b) 'twinned' crystals, and finally (c) aggregates of 'curved' crystals. Any one of these forms is characteristic of the compound.
Sulphadiazine ...	Readily soluble	Fairly rapid peripheral precipitation. Needles individually or in clusters.
Phthalylsulphathiazole	Readily soluble	* Slow peripheral precipitation. Thin massed needle rosettes. Wrinkled surface skin forms over drop.
Soluseptasine ...	Very readily soluble	—
Solupyridine ...	Very readily soluble	—
Soluthiazole ...	Very readily soluble	—

* The same crystalline forms are obtained on spontaneous evaporation of the cold ammoniacal solution.

IDENTIFICATION OF SULPHONAMIDES

crystalline forms are generally highly distinctive, and it is frequently possible to identify a sulphonamide by means of this test alone.

0.1 mg. of material is finely powdered on a microscope slide and 1 drop of 0.880 ammonia added. The mixture is stirred thoroughly with a fine glass rod and observation made of the ease or difficulty of solution. A drop of glacial acetic acid on the end of a glass rod is then held just above the surface of liquid until a white turbidity appears at the margin of the drop, or, as in the case of sulphanilamide, crystals appear within the drop itself, when the slide is examined microscopically at a magnification of approximately X 50.

A general description of the crystals obtained with the various sulphonamides is given in Table VI, whilst for reference purposes the characteristic forms are illustrated in Figures 1 to 11.

It is regarded as essential that control crystal tests should be made with authentic material, and it is further recommended that after examination of the crystals these should be redissolved by treatment with ammonia vapour or solution, reprecipitated with glacial acetic vapour, and again examined. No loss of material is incurred during this repeated test, but confirmation of the typical crystalline form is obtained.

Vanillin reaction for substituted sulphapyrimidines. When warmed with vanillin and concentrated sulphuric acid the majority of the sulphonamides give a yellowish-green colour; the methylpyrimidine compounds sulphamerazine and sulphadimidine however, give an intense bright red colour. This property has been found useful as a confirmatory test, and has been adapted for micro-quantities.

A quantity of vanillin of the order of 0.01 mg. is mixed on a microscope slide with 1 small drop of concentrated sulphuric acid. Into the liquid is dropped approximately 0.01 mg. of the sulphonamide, and the mixture warmed over a micro-flame until fumes are just observable. The slide is placed upon a white tile, and the presence or absence of a red colour arising from the sulphonamide particles is noted.

Final mixed melting-point. The information derived from the foregoing tests is invariably conclusive for any one of the listed sulphonamides, but the conclusion should be checked by a mixed melting-point with a specimen of the indicated compound.

MIXTURES OF SULPHONAMIDES

With the introduction of proprietary mixtures, e.g. sulphatriad, containing sulphathiazole, sulphadiazine and sulphamerazine, the toxicologist may be required to identify the individual components of such mixtures, either in biological material or during the analysis of actual tablets.

It is clearly essential in such cases to effect a preliminary separation of the components. This may be achieved satisfactorily on the basis of solubility differences (Table II), subsequent identification of each fraction being accomplished by the described methods.

The authors have encountered no insuperable difficulties in the qualitative analysis of mixtures treated in this manner.

SUMMARY

1. A scheme is described for the identification of all the sulphonamide drugs at present available in this country.
2. The six stages of the process are simple and rapid operations, involving no unusual reagents or apparatus:
 - (i) purification of the crude material by charcoal treatment in acetone solution, followed by melting-point determination.
 - (ii) preliminary identification as a sulphonamide compound by decomposition with soda-lime.
 - (iii) detection of free primary amino group by condensation with furfural.
 - (iv) simple crystal tests based on precipitation from ammonia solution by acetic acid vapour.
 - (v) vanillin confirmatory test for sulphamerazine and sulphadimidine.
 - (vi) mixed melting-point determination.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to Messrs. May and Baker Ltd. for samples of their proprietary products and for much helpful information throughout the course of this work; to Mr. A. Hay for his valuable assistance with the photomicrographs; and to Dr. H. S. Holden, Director of the Metropolitan Police Laboratory, for his kind permission to publish the results of this investigation.

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IDENTIFICATION OF SULPHONAMIDES

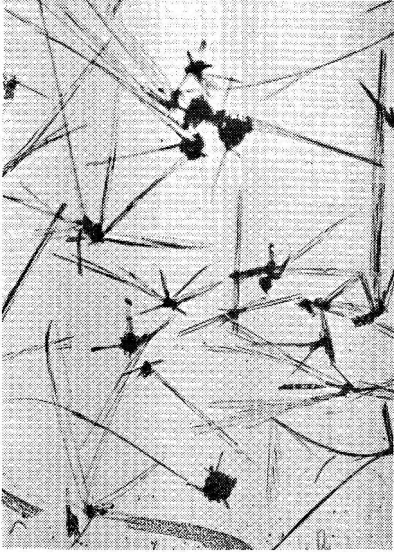


FIG. 1. Sulphanilamide.

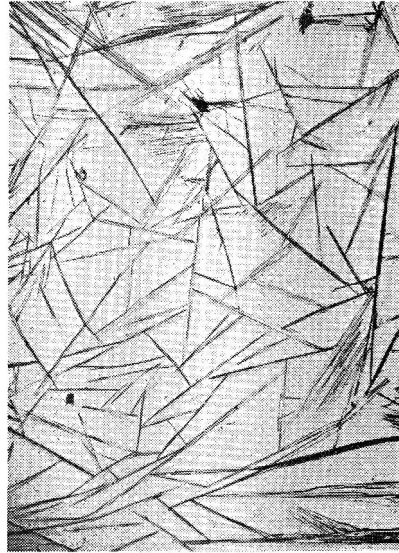


FIG. 2. Sulphaguanidine.

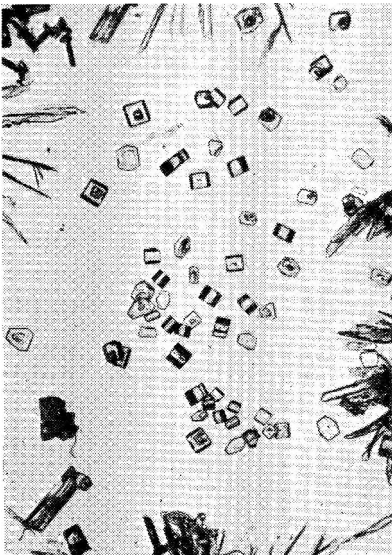


FIG. 3. Sulphapyridine.

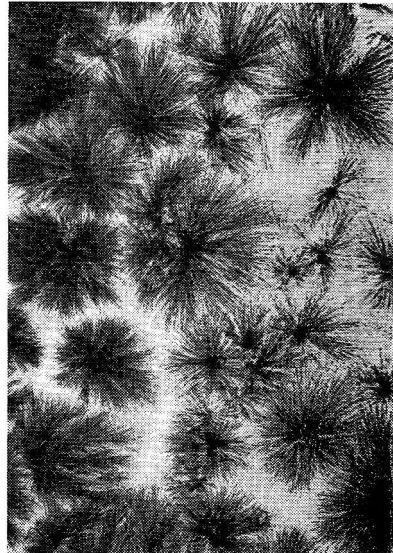


FIG. 4. Sulphadimidine.

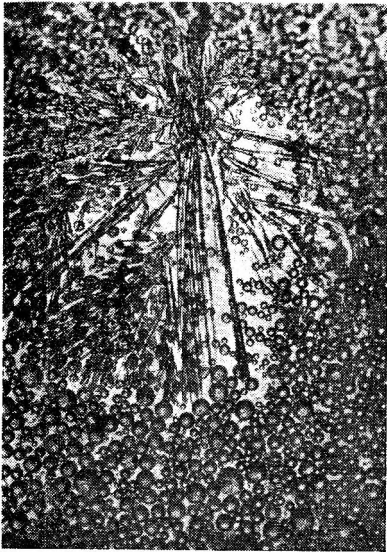


FIG. 5. Sulphathiazole.

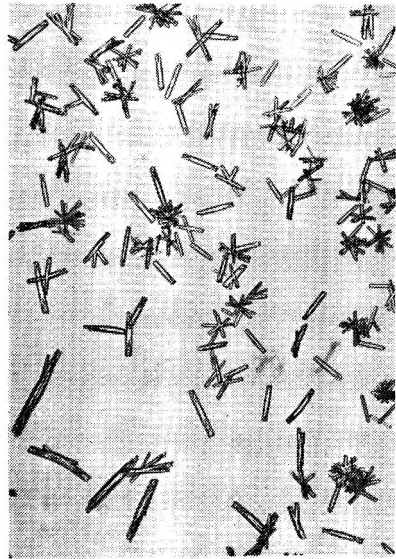


FIG. 6. Sulphamerazine (1).

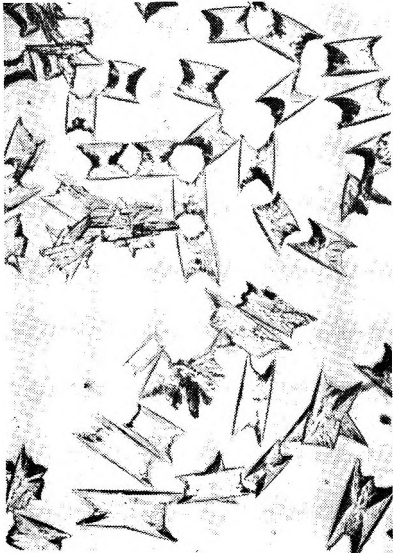


FIG. 7. Sulphamerazine (2).



FIG. 8. Sulphamerazine (3).

IDENTIFICATION OF SULPHONAMIDES

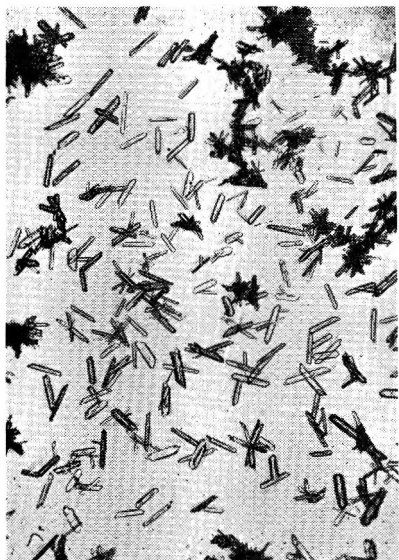


FIG. 9. Sulphadiazine.

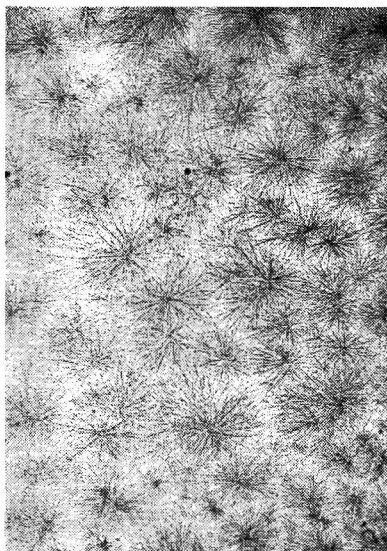


FIG. 10. Phthalysulphathiazole.

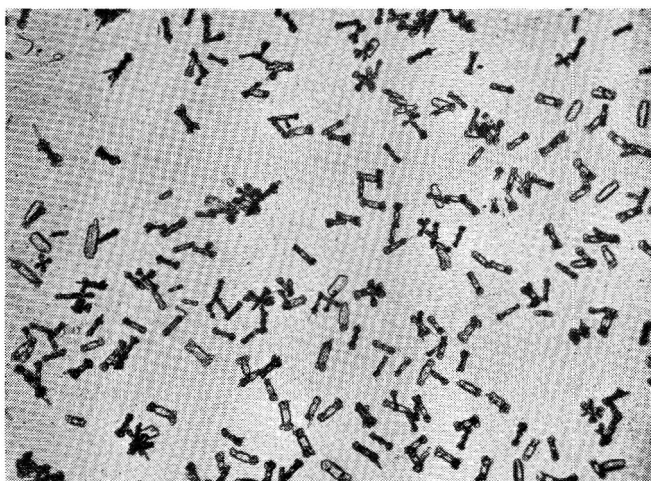


FIG. 11. Uleron.

The magnification of Figures 1-10 is approximately $\times 40$, and of Figure 11, $\times 50$.

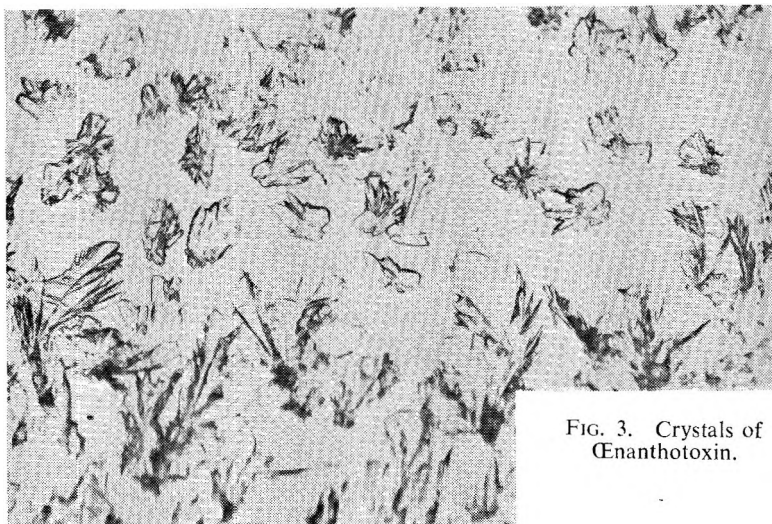


FIG. 3. Crystals of Enanthotoxin.

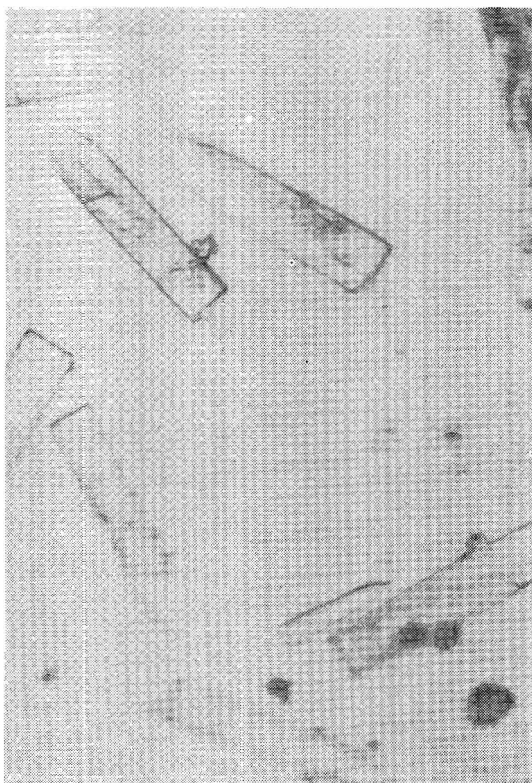


FIG. 4. Enanthotoxin after recrystallisation from ethyl alcohol.

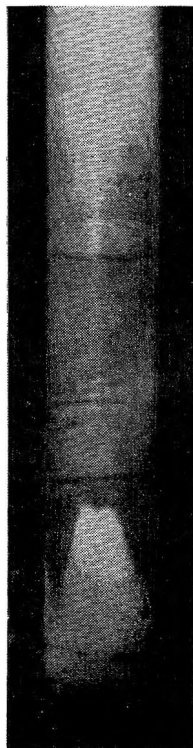


FIG. 1. Chromatogram of toxic principle of *Enanthe crocata*.

THE ISOLATION OF THE TOXIC PRINCIPLE OF *ÆNANTHE CROCATA*

BY E. G. C. CLARKE, D. E. KIDDER AND W. D. ROBERTSON

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Received March 29, 1949

INTRODUCTION

ÆNANTHE CROCATA Hemlock Water Dropwort, belonging to the family Umbelliferae, is common in wet places all over Western Europe, including the British Isles. Its toxicity has been known for a great many years, Linnaeus having noted it in Sweden in 1746 (Skarman¹), while Orfila² quotes a number of instances of poisoning in the 17th and 18th centuries. Witthaus³ cites 159 cases, 42 of them (i.e., 26 per cent.) being fatal, while there are numerous references to the subject in more recent medical literature (Thomas⁴; McGarthy⁵). Holmes⁶ refers to *Ænanthe crocata* as the most poisonous plant in England, while Fenton and Robertson⁷ state that it is responsible for more stock poisoning than any other. The frequency with which cases of poisoning occur is probably because it has a pleasant taste, and an attractive smell, rather like celery.

In spite of its well-established toxicity, very little work has been done on the chemistry of the plant. Cormerais and Pihan-Dufeillay⁸ found the active ingredient to be contained in a resinoid material in the root, while Gerding⁹ discovered a similar material in *Æ. fistulosa*. By purifying an ethyl alcoholic extract of the root by dissolving in ether, washing with sodium hydroxide and precipitating with light petroleum, Pohl¹⁰ obtained a neutral resinous substance, which he named ænanthotoxin, and to which he assigned the formula $C_{17}H_{22}O_5$. Tutin¹¹ isolated several non-toxic substances (triacontane, hentriacontane and ipuranol) from an ethyl alcoholic extract of the root, but he also found that the toxicity was associated with the ether-soluble neutral resin. He considered that "Pohl's ænanthotoxin" was not a pure substance. We are not aware of any other chemical investigation of value.

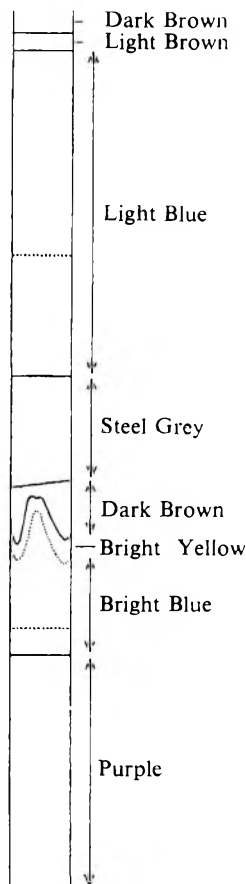


FIG 2. Diagram showing sections of Chromatogram

EXPERIMENTAL

Extraction. As a preliminary experiment, ethyl alcoholic extracts of roots, seeds and green

stems were made, and tested for toxicity by intra-peritoneal injection in mice. Only the extract from the root proved lethal, so investigation was confined to this part of the plant. The roots were obtained from the barks of the Thames near Kew, and passed, while still fresh, through an ordinary kitchen mincer. An extract made with 0.9 per cent. sodium chloride solution showed no activity, while there was little to choose in toxicity between extracts made with ethyl alcohol, chloroform or ether. The latter solvent was chosen owing to the ease of removal. Air-dried roots extracted with either ethyl alcohol or ether gave less toxic preparations.

6 kg. of freshly minced roots were gently refluxed in a 20-l. bolt-head flask for a total time of 12 hours with 11 l. of ether. The residue was washed with 6 l. of ether, the washings added to the extract, and the whole dried over anhydrous sodium sulphate. The ether was then distilled off, leaving 25 g. of a toxic brown oily residue, with an A.L.D. in mice of approx. 25 mg./kg. This oil was dissolved in 30 ml. of ether and 600 ml. of light petroleum was added. This threw down 17.5 g. of a toxic resinous material. The fraction remaining in the light petroleum was not toxic. The resinous precipitate was dissolved in 300 ml. of ether and shaken with 300 ml. of 40 per cent. sodium hydroxide, then with 300 ml. of 8 per cent. sodium hydroxide solution, and finally washed with saturated sodium sulphate solution (it having been found that water gave troublesome emulsions) until the washings were neutral. The sodium hydroxide extract, neutralised with hydrochloric acid, threw down a dark resinous material that was not toxic. The ethereal solution was dried over anhydrous sodium sulphate and evaporated to dryness, yielding 8 g. of light brown viscous oil, which was highly toxic. This oil deposited a few crystals on standing, but insufficient for investigation.

CHROMATOGRAPHY

Chromatography in daylight was unsatisfactory, but on elution on alumina columns under ultra-violet light a series of coloured bands was given by most organic solvents. The best separation was obtained with a mixture of dry benzene and ethyl alcohol (99:1). Therefore a solution of the light brown oil in benzene/ethyl alcohol (99:1) was placed on an alumina column and developed with the same mixed solvent. The bands seen under ultra-violet light were removed, eluted separately with ethyl alcohol and the extracts tested on mice. The toxicity was found to be associated with a band which gave a steel-grey fluorescence (Figs. 1 and 2).

The 5 g. of pale yellow oily material obtained on evaporating the extract from this band deposited oily crystals on standing. These were recrystallised by dissolving in the minimum of chloroform and cooling to -15°C . 400 mg. of colourless crystals was obtained, which were highly toxic (*v. infra.*). They can be purified, either by recrystallisation from chloroform, methyl alcohol or benzene, dissolving at room temperature

TOXIC PRINCIPLE OF *ÆNANTHE CROCATÀ*

and cooling as above, or by chromatography on an alumina column, eluting with benzene/ethyl alcohol (99:1), when a single steel-grey zone is obtained. These purifications do not alter the melting point or crystalline habit.

PROPERTIES

Crystalline "ænanthotoxin" prepared by this method forms small colourless irregular crystals, (Fig. 3) m.pt. 80° to 81°C., insoluble in water, light petroleum, alkalis and dilute mineral acids, but freely soluble in ether, ethyl alcohol and chloroform. If dissolved in ethyl alcohol, and the solvent allowed to evaporate, the substance crystallises in flat plates, many of them with a characteristic "bullet-shaped" appearance (Fig. 4). Found C, 71.75; H, 7.29; O, 20.96 per cent. Molt. wt. (cryoscopic in benzene) 292 $C_{18}H_{22}O_4$ requires C, 71.50; H, 7.43; O, 21.16, Mol. wt. 302.

Nitrogen is absent. The substance is extremely unstable, changing comparatively rapidly into a brown insoluble resinous material, decomposing without melting above 200°C., and possessing no pharmacological activity. This change is accelerated by a high temperature and by oxygen. At 4°C. under oxygen-free nitrogen ænanthotoxin is much more stable, only a slight yellow colour developing in a period of weeks. The specific rotation in chloroform is + 14.7°. Ænanthotoxin gives an immediate black colour with concentrated sulphuric acid.

PHARMACOLOGY

Ænanthotoxin, in the form of an emulsion, was injected intraperitoneally into white mice. In the earlier preparations the emulsion had been prepared by dissolving the substance in 0.5 ml. of ethyl alcohol

TABLE I

Results of injecting 0.5 ml. of lecithin-saline suspension of crystalline ænanthotoxin containing the dose shown, intraperitoneally into white mice, c.18 g. weight. C—Interval before onset of convulsions. (In minutes from time of injection). D—Time elapsing before death. S—Survived.

Mouse No.	...	1	2	3	4	5	Average
.25 mg.	{C ...	3	3	3	2	3	2.8
	{D ...	22	10	27	21	7	17.4
.12 mg.	{C ...	3	4	4	3	4	3.4
	{D ...	23	51	28	51	7	32.0
.06 mg.	{C ...	13	8	4	4	5	6.8
	{D ...	26	45	44	42	59	43.2
.03 mg.	{C ...	11	19	13	20	16	15.8
	{D ...	83	58	57	88	27	62.6
.015 mg.	{C ...	28	20	32	42	23	29.4
	{D ...	S	38	S	S	32	
.007 mg.	{C ...	—	—	—	—	—	—
	{D ...	S	S	S	S	S	
0	{C ...	—	—	—	—	—	—
	{D ...	S	S	S	S	S	

Controls received 0.5 ml. lecithin saline only.

This gives an A.L.D. of approximately 0.83 mg./kg. Pohl's ænanthotoxin killed one rabbit in 105 minutes at a dose of 24 mg./kg.

and pouring this into 9.5 ml. of 0.9 per cent. sodium chloride. In the case of the crystalline product, the emulsion produced by this method was unstable, owing to the removal of some emulsifying agent by the purification. The emulsion of crystalline *œnanthotoxin* for injection was made by pouring an ethyl alcoholic solution into a 0.1 per cent. solution of lecithin in 0.9 per cent. sodium chloride solution.

The results of the experiment on mice with the crystalline substance are given in Table I.

SIGNS

Shortly after injection the animal becomes perceptibly less active, respiration is accelerated but the animal shows no sign of distress. 1 to 20 minutes after injection the animal becomes restless and adopts the characteristic feeding posture with excessive movement of the fore limbs.

Soon tremors of varying intensity are observed which may be confined to only local regions, but more frequently are general. The onset of convulsions may be sudden, but it is usually preceded by a generalised tremor of the whole body. Depending on the dose the convulsive movements vary from those involving tonic contractions and rolling, to wild jumping movements. The convulsive stage varies in its duration: usually several convulsions follow in rapid succession, but occasionally an interval of some minutes may intervene between any two.

The terminal phase is invariably heralded by pedalling movements of the hind limbs and irregular vigorous movements of the fore limbs. Abduction of the digits is marked, and always accompanies the pedalling movements. At this stage hæmorrhages from the buccal cavity may be observed accompanied by trismus of the jaw muscles. Prior to death the animal assumes a characteristic posture, lying on one side with the fore limbs acutely flexed and the hind limbs rigidly extended to the full. Death follows.

These symptoms correspond closely with those recorded in cases of poisoning in man and domestic animals following the ingestion of the roots of *œnanthe crocata*.

SUMMARY

1. A method is described for the preparation of highly toxic crystals from water dropwort root. Previous workers have only reported oils or resins of far less toxicity.

2. These can be purified by recrystallisation, or by chromatography on an alumina column, when a single steel-grey zone is obtained. These purifications do not alter the melting point or the crystalline habit.

3. The crystalline material, m.pt. 80° to 81° C., is insoluble in water, light petroleum, alkalis, and diluted mineral acids, but readily soluble in chloroform, ethyl alcohol and ether.

4. The crystals are extremely unstable, yielding an insoluble infusible resinous material, with no pharmacological activity.

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5. If kept under nitrogen at 4°C., the crystals can be preserved for weeks with little loss of activity.

6. Death after characteristic convulsions follows the intraperitoneal injection of an emulsion of the crystalline material into mice.

7. The A.L.D. is 0.83 mg./kg. of body weight.

We wish to express our thanks to Mr. G. Knight, of the Beaumont Hospital, for the loan of apparatus and to Mrs. King for her drawing of Figure 2. We are also indebted to Mr. Burgess for the photo-micrographs, and to our technician, Mr. Waterman, for his help and for the collection of material.

The photograph of the chromatographic column was taken at the Optical Department of the Medical Research Council, Hampstead, and to Dr. Smiles, of this unit, we wish to accord our thanks. Thanks are also due to Dr. Klyne, of the Postgraduate Medical School, for the use of the micropolarimeter.

Our thanks are also due to Professor E. C. Amoroso, Head of the Department of Physiology, for his sympathetic interest and helpful suggestions during the course of this investigation.

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THE ABSORPTION SPECTRA AND IONIC DISSOCIATION OF THIOURACIL DERIVATIVES WITH REFERENCE TO THEIR ANTI-THYROID ACTIVITY

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From The British Drug Houses, Limited, London

Received March 23, 1949

A NUMBER of derivatives of thiourea and especially the substituted thiouracils inhibit thyroid activity in animals^{1,2}; in particular thiouracil and methylthiouracil have been used in the treatment of hyperthyroidism in man. Miller, Roblin and Astwood³ studied the reaction between iodine and 2-thiouracil and found that at *pH* 7.4 in the presence of sodium bicarbonate a disulphide from two molecules of thiouracil was formed. The absorption of iodine was such that tyrosine and casein were protected from iodination under these conditions in the presence of 2-thiouracil. This was held to support the hypothesis that thio-derivatives may prevent hormone synthesis in the thyroid gland by blocking the iodination of hormone precursors. Doubts, however, have been expressed by Rimington and Lawson⁴ concerning the validity of this reaction ($2 \text{ RSH} + \text{I}_2 \rightleftharpoons \text{RS.SR} + 2 \text{ HI}$) as a mechanism owing to the activity of sulphonamide derivatives which have no free -SH group.

Williams and Kay⁵ found that the activity of thiouracil was distinctly decreased or in some instances lost by the addition of methyl or ethyl substituents on the nitrogen atoms or by the addition of substituents on the sulphur atom. Since both of these alterations to the molecule are connected directly or indirectly with the ionisable hydrogen atom it seems probable that ionisation is an important factor in thiouracil activity. It was therefore decided to investigate the dissociation of 2-thiouracil and 2-thio-4-methyluracil and to try and determine the probable structure of the resulting ion. With the latter object in view the methylated thiol structures corresponding to 2-thio-4-methyluracil, namely, 2-methylmercapto-4-methyl-6-oxypyrimidine and 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine, were prepared and their ultra-violet absorption spectra determined under varying *pH* conditions. At the same time the dissociation of the compounds prepared was investigated by means of an electro-metric titration.

EXPERIMENTAL

Absorption Spectra. The absorption curves were determined at varying *pH* values using a Beckman photoelectric spectrophotometer. Samples of 2-thiouracil and 2-thio-4-methyluracil of appropriate strengths were dissolved in potassium dihydrogen phosphate-sodium hydroxide buffer solutions and examined in a 1 cm. cell. Solutions of high and low *pH* were obtained by dissolving in carbon dioxide-free water and adding sodium hydroxide or hydrochloric acid until the required *pH* was obtained as indicated by *pH* meter; solutions obtained in this manner were used immediately.

Electrometric Titrations. These were carried out in general by running N/10 aqueous sodium hydroxide into solutions of thiouracil

ABSORPTION SPECTRA OF THIOURACIL DERIVATIVES

derivatives in 50 per cent. aqueous ethyl alcohol, the pH of the solution being determined at intervals by a standard Cambridge pH meter. A typical result is shown for 2-thio-4-methyluracil:

Per cent. neutralised	10	20	30	40	50	60	70	80			
pK_a	8.6	8.5	8.5	8.6	8.5	8.5	8.4	8.4

The temperature throughout the pH measurements was $18^\circ \pm 2^\circ C$. The results are not corrected for the effects of alcohol.

Preparation of the methylated derivatives.

2-Methylmercapto-4-methyl-6-oxypyrimidine was prepared by methylation of 2-thio-4-methyluracil in sodium ethylate using methyl iodide according to List⁶. After crystallisation from alcohol it had m.pt. $220^\circ C$. Di-methylation of 2-thio-4-methyluracil, by the method of Wheeler and MacFarland⁷ produced 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine which after crystallisation from alcohol had m.pt. $94^\circ C$. The constitution of the methylated compounds was proved by Wheeler and MacFarland (*loc. cit.*) since on heating with hydrochloric acid 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine gave 1:4-dimethyluracil.

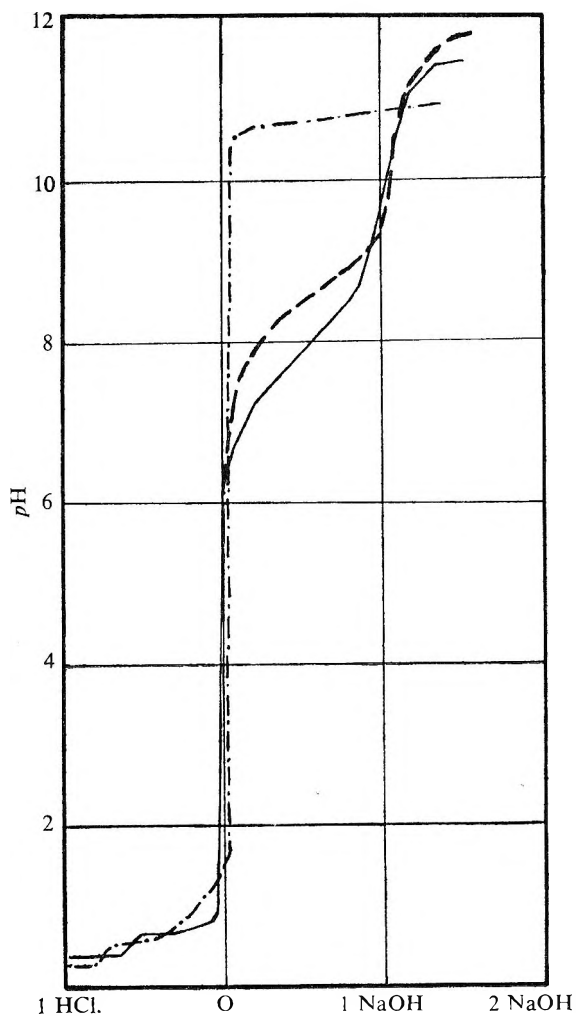


FIG. 1. Electrometric titration curves :

- 2-thio-4-methyluracil;
- 2-methylmercapto-4-methyl-6-oxypyrimidine;
- · - · - 1 : 4 - dimethyl - 2 - methylmercapto-6-oxypyrimidine.

RESULTS

The results in general are of interest in showing the close correlation between dissociation as shown by the pH curves and changes in absorption

spectrum, i.e. structural changes are due primarily to ionisation. Thus 2-thio-4-methyluracil possesses a potentiometric titration curve (Fig. 1) in aqueous ethyl alcohol with a break corresponding to an acidic dissociation

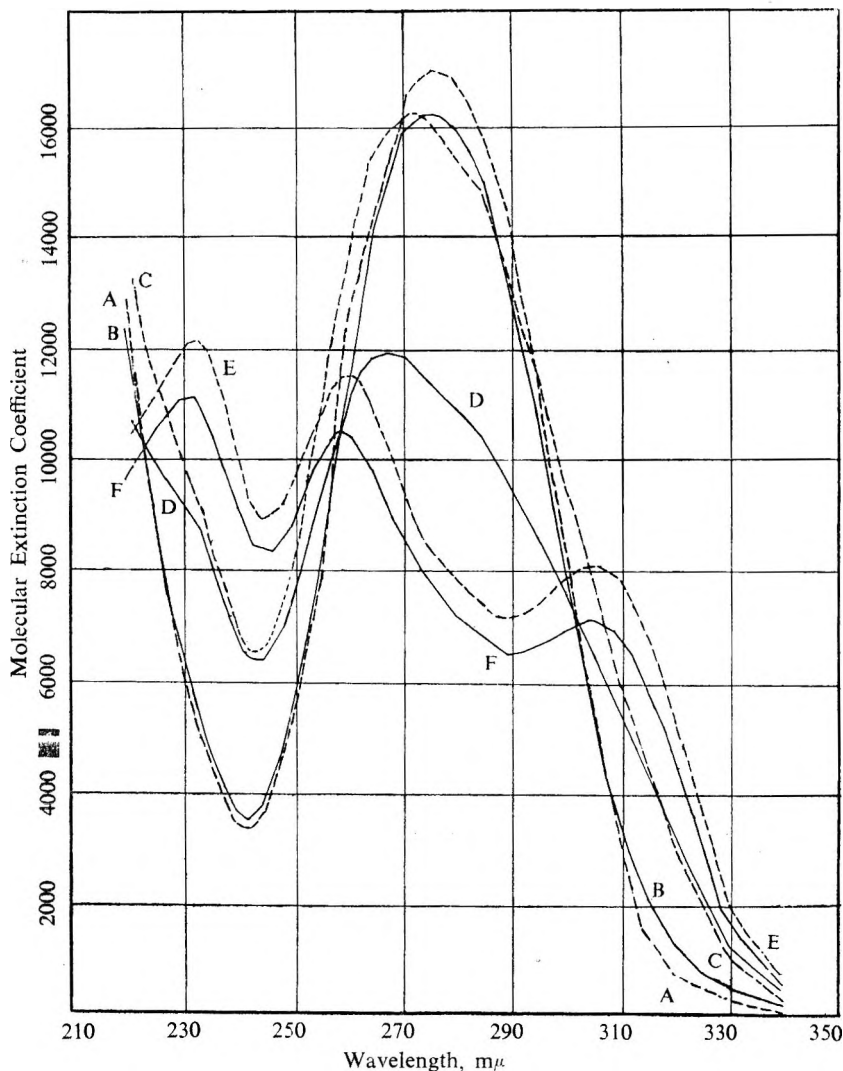


FIG. 2. Absorption spectra of 2-thio-4-methyluracil. A, at pH 2; B, at pH 7; C, at pH 8; D, at pH 9; E, at pH 10; F, at pH 12.

constant of pH 8.5. The spectra of this compound (Fig. 2) show minor differences only at pH values up to 7, and at pH 8 the main peak λ_{\max} ca. 270 m μ ϵ_{\max} ca. 16,000 is still not appreciably changed. At higher pH values, corresponding to increasing ionisation three peaks become evident, a transition curve occurring at pH 9.

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2-Methylmercapto-4-methyl-6-oxypyrimidine does not show any main peak ϵ_{\max} *ca.* 16,000 as for 2-thio-4-methyluracil, and the changes are not so fundamental (Fig. 3). A break in the *pH* curve (Fig. 1) between *pH* 0 and *pH* 2 corresponds to a change in absorption spectra at these

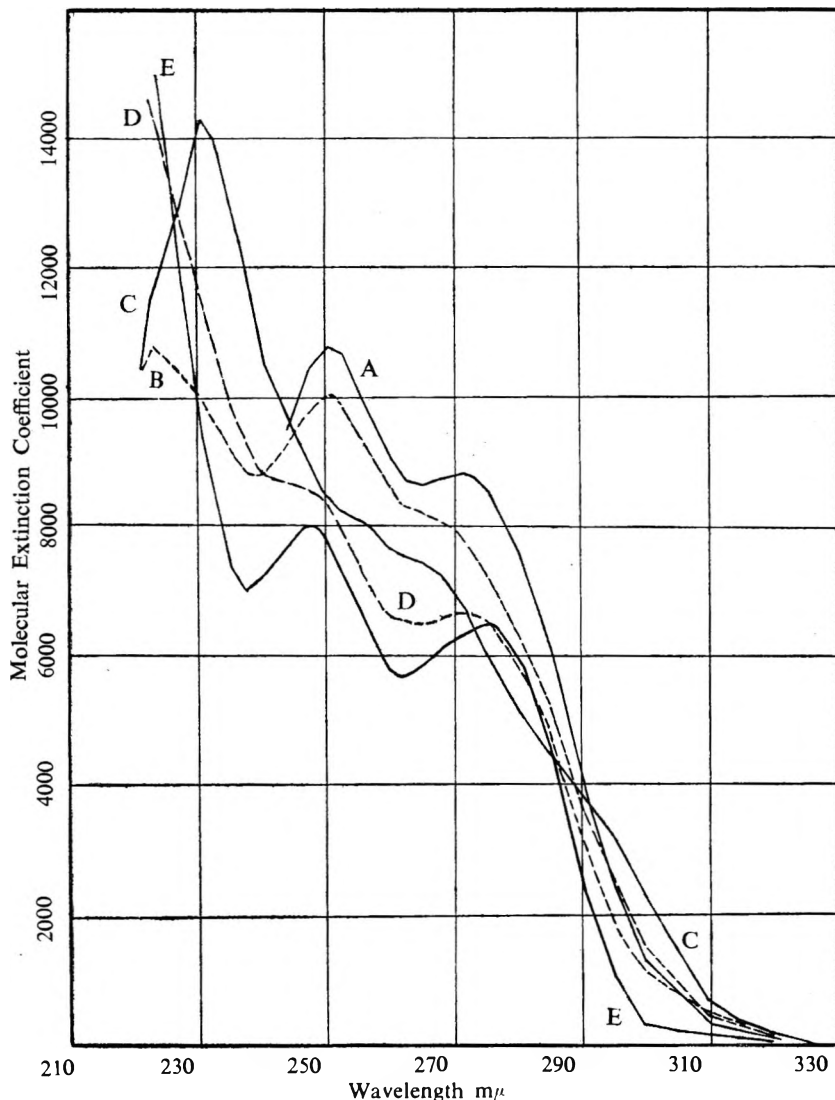


FIG. 3. Absorption spectra of 2-methylmercapto-4-methyl-6-oxypyrimidine. A, at *pH* 0; B, at *pH* 1; C, at *pH* 2; D, at *pH* 8; E, at *pH* 9.

values. The electrometric titration results for this compound show it to be a weak acid, having a pK_a value of 7.9.

1:4-Dimethyl-2-methylmercapto-6-oxypyrimidine shows absorption changes only at *pH* values less than 2 (Fig. 4), again corresponding to a

break in the electrometric titration curve (Fig. 1). The spectrum of 2-thiouracil (Fig. 4) is in general agreement with the graphical results of Elion, Ide and Hitchings⁵ and of Miller, Roblin and Astwood³.

It was not possible directly to calculate the dissociation constants from absorption measurements (see e.g. Morton and Tipping⁹ for violuric acid, Stuckey¹⁰ for barbituric acid), as the relationship between absorption maxima and ionisation was not linear. It is obvious from this, and from

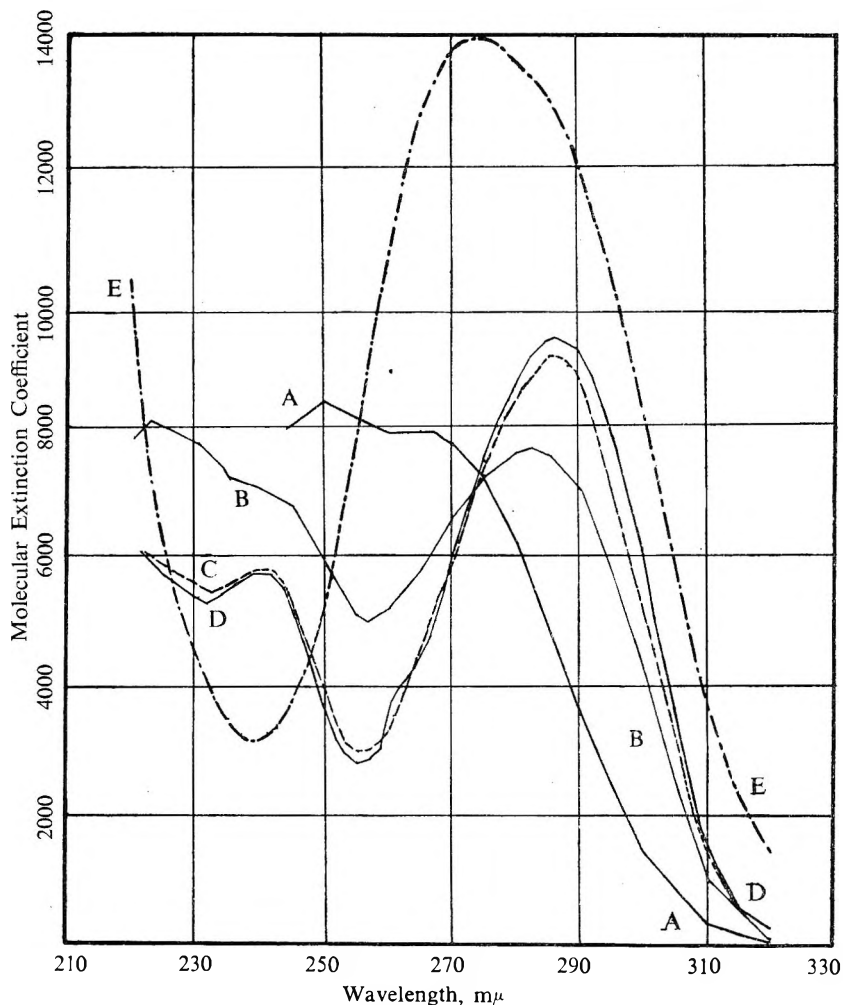


FIG. 4. Absorption spectra of 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine. A, at pH 0; B, at pH 1; C, at pH 2; D, at pH 12. E, 2-Thiouracil, at pH 7.

the fundamental spectrum changes, that variations in pH must be causing spectrum changes associated with resonance effects or with more than one group in the molecule.

ABSORPTION SPECTRA OF THIOURACIL DERIVATIVES

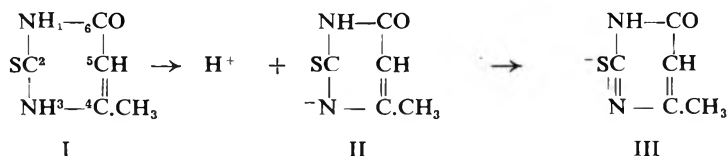
DISCUSSION

Tautomerism in 2-thio-4-methyluracil (I) can theoretically occur in more than one part of the molecule, notably involving the hydrogen attached to the nitrogen atom in the 3-position producing a $\Delta^{2,3}$ structure, or involving the hydrogen attached to the nitrogen atom in the 1-position producing either a $\Delta^{1,2}$ or a $\Delta^{1,6}$ structure. The potentiometric titration curve for this compound (Fig. 1) shows a single dissociating group with a pK_a value in aqueous ethyl alcohol of 8.5, but the fact that the absorption

TABLE I

	pH	λ_{\max} m μ	ϵ_{\max}
2-Thiouracil	7	274	13,940
2-Thio-4-methyluracil	2	275	16,980
	7	275	16,160
	8	272	16,100
	9	267	11,800
	10	232.5	12,180
		260	11,500
		305	8,050
	12	232.5	11,070
		260	10,430
		305	7,080
2-Methylmercapto-4-methyl-6-oxypyrimidine ...	0	250	10,800
		270	8,810
	1	222.5	10,730
		250	10,000
	2	230	14,260
	8	272.5	6,600
1 : 4-Dimethyl-2-methyl-mercapto-6-oxypyrimidine	9	247.5	7,980
		275	5,960
	0	250	8,320
	1	222.5	8,040
	2	282.5	7,620
		241	5,750
		285	9,050
	12	241	5,740
		286	9,420

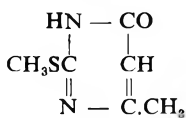
changes do not show a linear relationship between peak values and ionisation indicates that spectrum changes are not directly associated with a single ionic structure.



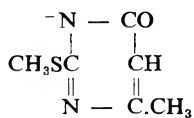
The absorption spectra of 2-thio-4-methyluracil show a main broad peak *ca.* 270 m μ with relatively little change at pH values up to 8. This peak corresponds to the undissociated form I, by analogy with other pseudo-acids, e.g., barbituric acid derivatives, the thio-ketone form being present in aqueous acid solution. It is noteworthy that Schneider and Halverstadt¹¹ found from a study of dipole moments in dioxan that 2-thiouracil possessed the structure corresponding to I. The change in

spectrum accompanying dissociation (from pH 7 to pH 10) is fundamental, with the splitting of the main band into three subsidiary bands; at pH 10 the change is virtually complete, the absorption maxima at higher pH values showing a slight decrease only, common to the spectra of many pseudo-acids in alkaline solution (cf. Stuckey¹⁰), the main characteristics being retained. The reason for the main spectrum change is not easily interpreted, but it is probable that this follows ionic rearrangement, predominantly with the production of structures II and III with associated resonance effects. This is supported by the fact that pH measurements show only one dissociating group. Any second dissociation with the addition of a further double link (from subsequent tautomerism) would produce the basic pyrimidine structure with three double links; this would have characteristically lower absorption values, which are not actually found. Methylation, with the production in the first instance of 2-methylmercapto-4-methyl-6-oxypyrimidine (IV), gives support to the supposition that the nitrogen in the 3-position is the dissociating group. The spectra of IV in alkaline solution show, broadly, a similar pattern to 2-thio-4-methyluracil in alkaline solution.

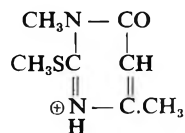
The spectrum of 2-thiouracil (Fig. 4) agreed with the graphical results of Elion, Ide and Hitchings⁸ and of Miller, Roblin and Astwood³. The peak values in acid solution were considerably lower than for the 4-methyl compound and lower than for 2-thiothymine. Although the introduction of a methyl group in the 4-position has no direct effect on the tautomerism it must obviously have some effect other than the normal weighting although exactly what is causing the increase in ϵ_{max} is rather obscure. The parent uracil has ϵ_{max} 11,000 at 258 $m\mu$. (Loofbourow, Stimson and Hart¹²) so that the replacement of oxygen by sulphur has caused only a slight increase in ϵ_{max} .



IV



V



VI

2-Methylmercapto-4-methyl-6-oxypyrimidine (IV, Fig. 3) shows an absorption curve at pH 2 with a maximum at 230 $m\mu$, but lacking any peak values at longer wavelengths; at this pH , ionisation accompanied by the splitting off of a hydrogen atom does not take place to any extent and the spectrum will be that of the undissociated structure IV. There is a change in spectrum with the development of two subsidiary peaks in alkaline solution (pH 9) corresponding to dissociation as shown by the electrometric titration curve. The spectrum at pH 9 is due to the ion (V) with the possibility of resonance with a fully unsaturated pyrimidine ion having a 1:6 double link. The pH curve for 2-methylmercapto-4-methyl-6-oxypyrimidine shows a slight break with an inflexion corresponding to a radical change in the spectra *ca.* pH 1 and

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pH 0, undoubtedly due to the partial conversion of the tertiary nitrogen to a $=\overset{\oplus}{N}H-$ structure.

1:4-Dimethyl-2-methylmercapto-6-oxypyrimidine shows no change from pH 2 to pH 12, which is to be expected in the absence of an ionisable hydrogen atom. The break in the pH curve between pH 0 to pH 1 corresponds, as with the monomethylated compound IV, to the addition of a proton to the tertiary nitrogen in the 3-position (VI).

The iodine absorption theory of anti-thyroid activity is supported by the work of Albert, Rawson, Merrill, Lennon and Riddell¹³, who found that the loss of thyrotropic activity occurring during exposure of a pituitary extract to iodine, was restored by treatment of the iodinated hormonal material with 2-thiouracil. The production of a disulphide compound incorporating the thiol form of the thiouracil molecule (III) in Astwood's iodination experiments, together with the findings of Williams and Kay⁵, suggest that any *mechanism* of iodine absorption depends on ionisation and subsequent ion tautomerism. In view of this it appears that ionisation is necessary for antithyroid activity in thiouracil derivatives. The present work has shown that 2-thio-4-methyluracil has a pK_a value in 50 per cent. aqueous alcohol of 8.5; in order to make this figure applicable to an aqueous solution it is necessary to apply a correction. Mizutani¹⁴ found values for ΔpK_a between water and 50 per cent. aqueous ethyl alcohol to be of the order of 0.9 to 1.2, with an approximate average of 1.1, for the numerous acids studied. It is unfortunate that a more precise correction cannot be applied since a small difference in pK_a in the region pK_a 6.6—8.0 corresponds to a big difference in percentage dissociation at pH 7.3; the very limited solubility of 2-thio-4-methyluracil, however, makes an accurate determination in aqueous solution a matter of difficulty. Assuming ΔpK_a to be 1.1 units, the corrected figure for 2-thio-4-methyluracil is, therefore, 7.4. This corresponds to a dissociation of 44 per cent. at a blood pH of 7.3. On the limited evidence available an appreciable dissociation of thiouracil derivatives at pH 7.3 would seem to be necessary for iodine absorption in anti-thyroid activity.

The peak absorption values in acid solution shown by 2-thio- and 2-thio-4-methyluracil provide a useful property for the analytical estimation of these compounds. This, together with the dissociation properties of thiouracil derivatives, is the subject of further investigation.

SUMMARY

1. The ultra-violet absorption spectra of 2-thiouracil, 2-thio-4-methyluracil, 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine and 2-methylmercapto-4-methyl-6-oxypyrimidine have been determined at varying pH values.

2. Electrometric titration curves have been plotted for 2-thio-4-methyluracil, 2-methylmercapto-4-methyl-6-oxypyrimidine and 1:4-dimethyl-2-methylmercapto-6-oxypyrimidine, and pK_a values for these compounds in aqueous ethyl alcohol have been recorded.

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3. The close connection between change in pH and change in ultra-violet light absorption for the compounds studied shows that absorption changes are due primarily to ionisation and subsequent ionic rearrangement.

4. On the basis of the iodine oxidation theory, the probable ionic nature of the iodine absorption mechanism suggests that thiouracil derivatives which are appreciably ionised at pH 7.3 are most likely to exhibit anti-thyroid activity.

The writer would like to thank Miss H. M. Oster for technical assistance and the Directors of The British Drug Houses, Ltd., for permission to publish these results.

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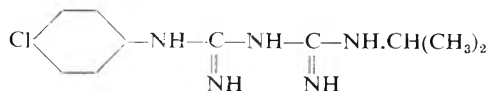
THE DETERMINATION OF PROGUANIL

BY H. E. STAGG

From the Analytical Laboratories, Imperial Chemical Industries, Ltd., Blackley, Manchester

Received March 31, 1949

PROGUANIL (Paludrine), introduced as an antimalarial drug in 1946, has the constitution N_1 -*p*-chlorophenyl- N_5 -isopropylbiguanide.



It is a strongly basic substance which is administered orally as tablets containing the monohydrochloride and by injection as a 5 per cent. solution of the lactate.

Methods for the determination of traces in biological fluids have been described by Spinks and Tottey¹ and Gage and Rose². Spinks and Tottey employ hydrolysis with N/4 hydrochloric acid in a sealed tube at 100°C. followed by diazotisation of the *p*-chloroaniline, coupling with N- β sulphatoethyl-*m*-toluidine and colorimetric determination of the azo dye which is formed. Gage and Rose's method depends on formation and extraction of the copper-complex $(C_{11}H_{15}N_5Cl)_2Cu$ (see page 392) with benzene and determination of the copper in the extracted complex by the well-known dithiocarbamate method.

Neither method, however, is of sufficient accuracy to be satisfactory for the determination of major amounts, either in the bulk product or in pharmaceutical preparations, and the methods which have been developed for these purposes are described herein.

METHODS OF ANALYSIS

1. VOLUMETRIC DETERMINATION IN THE HYDROCHLORIDE BY TITRATION WITH ACID. The drug is in reality a diacidic base, and although the form in which it is mainly encountered (the monohydrochloride) is neutral in aqueous solution, this salt can be dissolved in glacial acetic acid and the second basic group can be titrated with a standard solution of perchloric acid in glacial acetic acid in the manner described by Bandel and Blumrich for the titration of weak bases with strong acids³. The indicator is α -naphthol benzein, which changes from yellow to green at the end-point.

This method has the advantage of rapidity and the results are not affected by normal tablet excipients (e.g. starches, gums, sugars, etc.); it can, therefore, be used for the direct determination of the content of tablets.

Reagents required: 1. Acetic acid, glacial, analytical reagent quality.

2. N/10 solution of perchloric acid in glacial acetic acid: dissolve the equivalent of 10.05 g. of perchloric acid (calculated from the acid content

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of the 60 per cent. aqueous solution) in 50 ml. of acetic acid; add this mixture, a drop at a time, to an amount of freshly distilled acetic anhydride (boiling point 137° to 139°C.) which is exactly sufficient to combine with the water present in the perchloric acid, cooling the mixing vessel in ice during the addition. Dilute with acetic acid to 1 litre.

3. Standard solution of sodium acetate in acetic acid: cautiously dissolve 1.325 g. of sodium carbonate, previously dried for 5 hours at 300°C., in 50 ml. of acetic acid, transfer to a 250-ml. measuring flask and make up to volume with acetic acid.

4. α -Naphthol benzein indicator: dissolve 0.2 g. of α -naphthol benzein in 100 ml. of acetic acid.

Determination. Standardise the perchloric acid (reagent 2) by titrating 50.0 ml. of the standard solution of sodium acetate (reagent 3) and 1 ml. of the indicator (reagent 4) until the colour changes from brown to green. The temperature of the perchloric and sodium acetate solutions should be kept as near to 20°C. as possible for all measurements of volume in order to minimise errors due to the large coefficient of expansion of acetic acid.

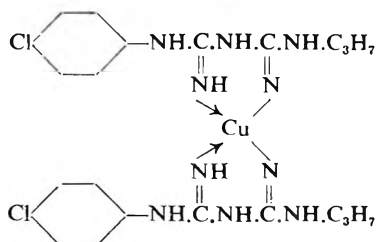
Experience has shown that the normality of this solution remains constant, under ordinary conditions of storage in a stoppered bottle, for at least 3 months.

Weigh about 0.8 g. of the sample, or its equivalent in tablet form, into a dry conical flask and dissolve in 30 ml. of acetic acid, warming to a temperature not exceeding 80°C. Cool to room temperature, add 0.5 ml. of indicator solution and titrate with the perchloric acid solution until the colour of the titration matches that obtained in the standardisation.

If B = vol. of N/10 perchloric acid required

$$\frac{2.90B}{\text{wt. of sample}} = \text{per cent. calculated as hydrochloride, mol. wt. 290}$$

2. GRAVIMETRIC DETERMINATION IN THE HYDROCHLORIDE OR LACTATE BY PRECIPITATION OF THE COPPER COMPLEX. When an excess of ammoniacal cupric chloride solution is added to a cold aqueous solution of the hydrochloride or lactate, the base is precipitated as a copper complex containing ten atoms of nitrogen for each atom of copper. There is little doubt that this precipitate is an inner complex having the formula:



and it has been found to be suitable for gravimetric determination, being easily washed free from the excess of reagent and dried at 130°C.

DETERMINATION OF PROGUANIL

Reagent required. 5. Ammoniacal cupric chloride solution: dissolve 22.5 g. of cupric chloride, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, in 200 ml. of water and add 100 ml. of ammonia solution (880).

Determination. Weigh 0.5 to 0.7 g. of sample into a 250-ml. beaker, and dissolve in 50 ml. of water by gentle warming. Cool in an ice bath to 5° to 10°C . and add ammoniacal cupric chloride solution (reagent 5), with stirring, until the solution remains a deep blue colour. Allow to stand at room temperature for at least 1 hour and then filter on a sintered glass crucible, porosity 3—preferably one having a large filtering surface—fitted with an asbestos pad and previously dried at 130°C . to constant weight. Wash the precipitate with 100 ml. of dilute ammonia and then with cold water until the washings are quite colourless. Dry at 130°C . ($\pm 5^\circ$) to constant weight.

$$\frac{\text{Wt. of precipitate} \times 1.020}{\text{Wt. of sample}} = \text{per cent. calculated as hydrochloride, mol. wt. 290}$$

DISCUSSION

The colour change which occurs at the end-point of the volumetric method extends over a range of about 0.5 ml. of titrant in a titre of 25 ml., and the averages of duplicate tests by two operators upon the same sample often differ by 1.5 per cent. The method has, however, the advantage of rapidity and is, therefore, of special value where large numbers of determinations have to be carried out, as, for example, in the routine examination of tablets.

The precision of the gravimetric method is considerably higher than that of the volumetric method, and the averages of duplicate tests by two operators upon the same sample usually agree within 0.5 per cent. Recovery from specially purified hydrochloride has not been determined; this was not considered to be necessary, since the technical hydrochloride (i.e., the material which is normally manufactured for use in pharmaceutical preparations) is itself a very pure product, and the sum of the determined constituents—active agent, *p*-chloroaniline, moisture and ash in technical samples is normally over 99.0 per cent.

Analysis of a typical sample of technical proguanil hydrochloride is given below:

content, by gravimetric method, calc. as hydrochloride, mol. wt. 290	99.5 per cent.
<i>p</i> -Chloroaniline	Less than 0.01 per cent.
Moisture (by loss of weight on heating at 100°C .)	0.1 per cent.
Sulphated ash	0.05 per cent.
Total	99.6 per cent.

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SUMMARY

1. Two methods are described for the determination of proguanil hydrochloride: one method depends upon titration with perchloric acid in glacial acetic acid solution, and the other upon gravimetric determination of the copper-complex precipitated from cold aqueous solution by ammoniacal cupric chloride.

2. The volumetric method is rapid and is applicable to the determination in tablets, but is less precise than the gravimetric method.

3. The gravimetric method is applicable also to the lactate.

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A NOTE ON THE CONVERSION OF ω -TRIBROMOQUINALDINE TO ω -DIBROMOQUINALDINE AND THE PRODUCTION OF QUINALDIC ALDEHYDE

By L. K. SHARP.

Received March 3, 1949.

ω -DIBROMOQUINALDINE cannot be prepared by selective bromination of quinaldine, the only product isolated being the ω -tribromo derivative, but the former may be prepared by reduction from the latter. Hammick¹ reduced tribromoquinaldine by means of stannous chloride in acetone solution, obtaining a yield of 60 per cent. of theory. In order to separate the product from tin compounds, steam distillation was employed. As dibromoquinaldine is only slightly volatile in steam the purification process is tedious. The reduction may however be performed by refluxing ω -tribromoquinaldine with a 20 per cent. v/v solution of sulphuric acid in alcohol and pouring the mixture into water. The product separates almost pure in excellent yield, and a single crystallisation from alcohol is all that is necessary. The identity of the product was confirmed by its conversion, by means of alcoholic silver nitrate, to quinaldic aldehyde by the method of Hammick².

EXPERIMENTAL.

ω -Tribromoquinaldine (10 g.) prepared by Hammick's³ method was refluxed for 4 hours with a mixture of alcohol (97 per cent.) (80 ml.) and concentrated sulphuric acid (20 ml.), and the mixture then poured into water. The white precipitate was washed and dried. Yield 7 g. (88 per cent. of theory). M.pt. 119°C. After recrystallisation from alcohol the pure product melted at 120°C. Found: C, 39.95; H, 2.67; N, 4.9; Br, 52.9 per cent.: $C_{10}H_7NBr_2$. requires C, 40.0; H, 2.3; N, 4.7; Br, 53.3 per cent.

PROOF OF CONSTITUTION.

2.5 g. in 15 ml. of boiling alcohol was treated with silver nitrate solution and the product worked up in the manner described by Hammick². After steam distillation 0.9 g. (70 per cent. of theory) of quinaldic aldehyde was obtained: M.pt. 69°C.; mixed melting-point with quinaldic aldehyde, 70°C.

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**A PRELIMINARY INVESTIGATION ON THE GROWTH OF
CEPHAËLIS IPECACUANHA (BROT) A. RICH, UNDER
TROPICAL CONDITIONS AT CALCUTTA**

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Received October 5, 1948

INTRODUCTORY

THE first plant of *Cephaëlis Ipecacuanha* was taken to Calcutta by Dr. King in 1866, and in about 1868 experiments with a view to the introduction of the plant into India were begun by Dr. Anderson in the Royal Botanic Gardens, Calcutta. The original stock of plants came from Kew and Edinburgh. Gammie observed that the varieties from Kew and Edinburgh differed greatly, the leaves of the former being more rough and hence more hardy than the latter. Balfour^{1,2} had previously described the difference in the character of the leaves between the plants received from Kew and those sent by Gunning from Rio de Janeiro. The leaves of the Kew plants are firmer in texture, somewhat coriaceous, elliptical or oval, with apex rather blunt, and margin wavy. The leaves of the Rio de Janeiro plants are thinner and more delicate in texture, the shape is rather elliptico-lanceolate, the apex pointed, and the margin less wavy; in the young state the leaves are fringed with hairs; the plants grow more freely and are less shrubby. The Kew plants were also characterised by flowers possessing long stamens and short style as described by Hooker³, while the plants produced from Rio de Janeiro specimens showed two forms of flowers, viz., one with a short style and long stamens as in the Kew plant and the other with a long style and short stamens. Under cultivation by Gammie the Edinburgh plants did not survive for more than a year or two, but plants of the Kew variety, in the shade, lived. The Kew variety thus seemed to be more hardy and more suitable for cultivation in India. Later ipecacuanha was successfully cultivated in the Sikkim Himalayas. The seeds and plants for the present investigation were obtained from a nursery at Labdah. Evidently the original stock of these plants is the Kew variety, and they resemble the description and drawings given by Bentley and Trimen⁴ and by Balfour². But to the authors it seems that the characters of the two forms as mentioned by Balfour have become less clearly defined during natural fertilisations at the early period of their acclimatisation in India. The present production of the drug in India is insufficient to meet the demand, and more attention and systematic research is needed to improve the quality and yield.

EXPERIMENTAL

All the preliminary experiments on the cultivation of *Cephaëlis Ipecacuanha* in India show that for favourable growth and development the plant requires:—a forest area with sandy loam soil rich in humus,

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potash, magnesia and lime; a minimum temperature of 50°F. and a maximum of 100°F., with smaller variations in temperature during summer and winter and during day and night; and a minimum rainfall of about 90 in. distributed during monsoon, winter and spring.

The experiments reported here were chiefly concerned with germination of seeds, growth of cuttings and of young plants of *Cephaelis Ipecacuanha* (Kew variety) obtained from Mungpoo under the changed tropical conditions of the plain. The experiments were made under thatched sheds and on prepared soil beds, as is usual in Mungpoo.

Germination from seeds. The seeds, which usually take 4 to 6 months to germinate, have a very hard coat, are plano-convex and 3 to 5 mm. in length. They were sown on prepared beds in December, 1946, and January, 1947. They were watered morning and evening. By the end of April, the December and January sowings started germination almost at the same time. The curved seedlings were very weak and took 15 to 20 days to become erect with the hard seed coat still enclosing the apices of the first pair of leaves. The seedlings were so delicate and the hard seed coats were so tough that it was not possible to remove the seed coats without damaging the seedlings. The seedlings died under these conditions. Later on monthly sowings of seeds and the treatment of the seeds with acids to remove the hard seed coat were tried. The seeds were also subjected to low temperatures for different periods and to alternate high and low temperatures before sowing. But in no case did the seeds sprout even after 6 or 7 months. The causes of dormancy are being studied in detail.

Growth from Cuttings. By the end of February, 1947, some young plants were obtained from Mungpoo. A few cuttings of roots, stems and leaves from two such young plants were made and planted on prepared beds. The cuttings were kept under humid conditions by a cover of wet moss. They were watered every 3 hours during the day with a suitable arrangement to avoid waterlogging. During April some of the root cuttings began to sprout, but the stem cuttings did not and the leaf cutting began to rot. With the approach of the monsoon (i.e., during the months of May, June, July, August and September) more of the root cuttings sprouted and showed progressive activity by the production of minute leaves and 3 to 4 nodes. The internode varied in length from 1 to 2 cm. The activity of the cuttings was best in August. By the beginning of November the root cuttings were affected for the time with browning of the apices of their leaves. Browning progressed with the advance of the month and resulted in gradual drying up of the cuttings.

Growth of young plants. A few of the young plants obtained from Mungpoo by the end of February, 1947, were planted on prepared beds in earthen pots. They were watered every three hours during the day. The leaves of young plants within a few days showed browning of their apices and periphery. Browning progressed from these areas to the middle region of each lamina. As a result, the leaves soon crumpled

and were shed. Microscopical examination of the brown patches of the leaf lamina showed that they were not due to fungal attack, and the anatomical structure of the leaf showed the characteristic tissue development and differentiation of a shade and moisture-loving plant. Frequent spraying with water failed to check the browning of the leaves, and by the end of April all the leaves of the young plants were shed. Though they were completely defoliated, they were alive, as shown by the freshness of their terminal buds, and the gradual appearance of axillary buds gradually increased during the months of May, June, July, August and September. The buds only slightly expanded into one or two pairs of minute leaves. The growth was best in August. By the beginning of November the young plants were again affected with browning of the apices of the small leaves of terminal and axillary buds. Browning of the apices of leaves of the buds similarly progressed very quickly, and by the end of the third week of the month resulted in their drying up.

Experiments under scientifically increased humid conditions. Towards the end of the third week of November, when the root cuttings and the young plants were both showing definite signs of drying up, they were subjected to increased humidity for the purpose of studying its effect upon their growth. Accordingly, on November 21, 1947, the root cuttings and young plants, which were already affected with browning of their leaves, were covered with bell-jars leaving a little space for the access of air; inside each bell-jar a dish containing water was kept. Each morning and evening, fresh air was admitted so as to avoid the accumulation of carbon dioxide. Both root cuttings and young plants responded quickly to this treatment. It was observed that browning of apices of leaves of the root cuttings, which had already started, could not be checked, but, later, new leaves were formed. The effect was more marked in young plants. One of the axillary buds of a young plant near the base of its stem steadily increased in length and in the surface area of its leaves. At the beginning of the experiments the length of the axillary shoot was 1 cm., and 8 weeks later it had increased to 5 cm., while in the same period the length of one of the first pair of leaves of the axillary shoot which was 1.2 cm. and the other one which was 0.6 cm. had increased to 5.3 cm. and 3.1 cm. respectively. Two more pairs of leaves of the axillary shoot were developing by this time.

DISCUSSION

The preliminary experiments in the acclimatisation of plants in India show that the plants can easily be propagated from root cuttings and seeds, and that they prefer moist shady spots where there is much vegetable mould in the soil and an equable steamy atmosphere. The plants and cuttings, especially the roots, showed the best growth in August in nursery conditions but, in artificially increased humidity, during the winter months, the growth was much more than in August. The mean minimum temperature curve of Labdah is always far below that

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of Calcutta throughout the year, and in some months the difference is as great as 20°F., whilst the mean maximum temperature curve at Labdah is slightly below that of Calcutta, excepting in the months of July and August. So the mean maximum temperature of the plain may not affect the growth of the plants, but the duration of the maximum temperature in the hills and in the plains is to be taken into consideration. In the hills the mean maximum temperature lasts for a few hours, while in the plains it persists for a longer time. As regards the duration of the mean minimum temperature it is just the reverse. The rainfalls of the two places show wide differences. The total rainfall during a year at Labdah is 137 in., while at Calcutta it is 63 in.

It was seen from these experiments that the high temperature of the summer months, April, May and June, did not prevent the natural growth of the cuttings and young plants and the germination of seeds. Further, under natural conditions, the growth of the cuttings and of young plants improved with the onset of rains, and was found to be the best in August. In November, however, the cuttings and young plants showed the signs of decay as seen by the browning of their leaves. Again, it was found that on increasing the humidity, which was 64, by growing the cuttings and young plants under bell-jars their growth was revived to a great extent as seen by the development of the branches and leaves. Thus from November onwards, through the winter months, the condition of the growth of the cuttings and young plants, if kept in increased humid condition, was much better than what was found in the natural condition during the month of August. The humidity inside the bell-jar may be assumed to approximate to that prevailing in the monsoon, say, in the month of August. The observations indicate the favourable influence of the lower temperature of the winter months as compared with August, provided the humidity is not allowed to decrease, and the mean maximum temperature which persists for a longer duration in the plain appears to be above the optimum temperature, otherwise we would have expected the best growth in August.

The anatomical structure of the leaves also indicates that the plants are sensitive to desiccation, which was supported by the revival of growth of the cuttings and young plants in November when they were grown under the bell-jars in increased humid conditions. The browning of leaves in November can be ascribed to the desiccatory effect of lowering of humidity in that month.

SUMMARY

1. Under the tropical conditions of the suburbs of Calcutta the seeds of *Cephaëlis Ipecacuanha* (Kew variety) sprouted in April, 4 and 5 months after their sowing, but quickly died.

2. The young plants and cuttings, especially the roots, showed the greatest activity in August when the rainfall was 15.4 inches and the mean minimum temperature 79.5°F., whilst in November, when there

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was no rain and the mean minimum temperature was 64.6°F., they showed signs of drying up.

3. Artificial increase of humidity at this stage revived the growth of young plants and cuttings. Onwards through the winter months the growth of the cuttings and young plants was maintained.

4. The observations indicate the favourable influence of the lower temperature of the winter months provided that the humidity is not allowed to decrease. The monthly mean maximum temperature of the plains appears to be above the optimum for growth of the plant.

This work has been carried out under the direction of Mr. S. N. Bal, Director, Pharmacognosy Laboratory, Government of India. The authors are grateful to Mr. S. C. Sen for providing plant material.

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THE PREPARATION OF INDONES

By D. DALEV

Received September 25, 1948

THERE are many methods of preparing indones. One of the commonest is that which was given more or less at the same time by Löwenbein and Ulich¹ on the one hand, and by Weiss and Sauermann² on the other. This method is not very convenient because most of the indones produced by it contain much impurity in the form of 3-benzalphthalide and some by-products, and the mixture of the latter with the indones is a sticky, resinous substance which prevents the indones from crystallisation. Weiss and his fellow worker removed the benzalphthalide by saponification with concentrated alcoholic ammonia. Under such conditions the purification of the greater proportion of the indone is difficult. Weiss does not mention the reasons which made him choose ammonia for the saponification of benzalphthalide.

My experiments have shown that dilute aqueous solutions of alkaline hydroxides or carbonates do not change the indones to any marked extent when boiled. The latter do not change even when they are mixed with benzalphthalide. The crude indones which are produced, after they are distilled from the ether-benzene mixture at a temperature below 100° C., form a thick substance resembling molten resin. The by-products of the reaction, such as hydrocarbons and unchanged halogenides, are separated from this mass by steam distillation.

The use of steam has this advantage, that it continuously stirs the resinous substance and almost completely separates the volatile compounds. From this it appeared that the benzalphthalide in the crude indone would be easily soluble under these conditions if the medium was alkaline. Experiment proved that a 1 to 2 per cent. solution of sodium hydroxide is suitable for saponification. An aqueous solution of sodium or potassium carbonate can be used equally successfully. What quantity of unchanged benzalphthalide there is with the different indones is, of course, not known. It can safely be assumed that the hydroxide or the carbonate can be calculated for at least 50 per cent. of unchanged benzalphthalide. The saponification is completed in most cases in about half an hour.

EXPERIMENTAL

General method. The organomagnesium compound is produced from 0.1 g. mol. of halogenide with a corresponding excess according to Gilman's table³ in order to produce a 100 per cent. yield. The ether solution of the organomagnesium compound is transferred to a separating funnel in such a way as to prevent oxidation from the air. The flask is closed with a rubber stopper through which passes a short glass tube. The flask is turned over quickly, the tube is put in the separating funnel and almost all the ether solution is poured out of the flask in which there always remains a little of the ether solution of the organomagnesium com-

pound and a little magnesium. A solution of 22.2 g. of benzaldehyde in 100 ml. of dry benzene is quickly poured in, followed by the solution, first drop by drop, and then in a thin stream, at the same time continually shaking the flask. Very often the inside of the flask becomes covered with a thick yellow-brown precipitate which fills the whole flask before all the ether solution is added. The temperature rises and the solution in the flask begins to boil. Boiling is prevented by cooling with water from time to time. After adding all the ether solution, the contents are stirred well with a metal rod and boiled over steam for 1 hour. At the bottom of the flask there almost always gathers a dark red sticky substance which is scraped off and stirred with a rod, two or three times while the heating lasts. It is left overnight and decomposed with ice and dilute sulphuric acid. In some cases water followed by sulphuric acid may be used—the temperature does not rise very much. Decomposition is completed in a short time, and the ether-benzene layer, of a colour something between orange and red, is separated. It is washed several times with a dilute alkali and with water and distilled directly in steam. At first the ether and the benzene distil quickly, and afterwards the unchanged halogenide and the other by-products of the reaction which are volatile in steam. The distillation in steam is continued until a clear liquid passes, sometimes over 10 or 12 hours. Distillation is then stopped and, without cooling, a sufficient quantity of dilute sodium hydroxide solution to produce about a 2 per cent. solution is poured in and distillation in steam is continued for another hour. When the resinous substance has deposited, the deep-red aqueous layer is poured off, and the resinous substance is again distilled in steam for another hour. When treated with alkali the indone often turns into a solid mass, and its removal from the flask becomes impossible. The solid mass contains a good deal of water, and for this reason its solubility in ether is very small, but it dissolves easily in chloroform. The chloroform solution is washed with water, and dried with dehydrated sodium sulphate and the chloroform is distilled off.

The following 12 indones have been prepared by this method and identified:—

	m.pt.
2:3-diphenylindone ⁴	153°C.
2-phenyl-3-tolyindone ⁵	123° to 125°C.
2-phenyl-3- <i>m</i> -tolyindone	113°C.
2-phenyl-3- <i>p</i> -tolyindone ⁷	133 to 134°C.
2-phenyl-3- <i>p</i> -bromo-phenylindone ⁷	172 to 174°C.
2-phenyl-3- α -naphthylindone	182 to 183°C.
2-phenyl-3-benzylindone	135°C.
2-phenyl-3- β -naphthylindone	132°C.
2-phenyl-3-cyclohexylindone	162 to 163°C.
2-phenyl-3-isopropylindone	110°C.
2-phenyl-3-isoamyindone	74°C.
2-phenyl-3-ethylindone	98°C.

PREPARATION OF INDONES

This method can be applied for the preparation of many different indones, and the work is being continued with a view to the producing triketones from which to prepare anthracene derivatives similar to those used as carcinogenics in cancer research.

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THE SPECTROPHOTOMETRIC DETERMINATION OF RUTIN AND QUERCETIN IN MIXTURES

BY R. V. SWANN

This Journal, 1949, **1**, 323-329

Corrections

Page 324, figure 1, horizontal scale, *for 520 read 250.*

Page 328, figure 2, horizontal scale, *for 367 read 347.*

vertical scale, *for 0, 10, 20 per cent., etc., read 0.0,
1.0, 2.0, etc.*

Bottom line, *for Porter et al.² read Porter et al.¹.*

ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Boric Acid, Estimation of Minute Amounts of. N. Trinder. (*Analyst*, 1948, **73**, 494.) Conditions have been worked out for the determination of small amounts of boric acid using the dyes alizarin blue S and the unsulphonated dye base of Solway purple (Colour Index No. 1073). Alizarin blue S in strong sulphuric acid changed in colour from purple to brown to green. The final concentration of sulphuric acid for this dye is important, a high acidity being needed. Alizarin blue S is only half as sensitive as the base of Solway purple but it has the advantage that it is stable in sulphuric acid solution and, except for fluorides and nitrates, is unaffected by the presence of large amounts of impurities. The base of Solway purple gives a colour change from pale yellowish-green to deep blue. It has the disadvantage that it sulphonates slowly to a blue compound at high sulphuric acid concentrations and gives a slight colour change if comparatively large amounts of manganese are present. It has the advantage that it is twice as sensitive as alizarin blue S and the dye colour deepens progressively over a wide range of boric acid concentrations. The most suitable dye for use in any particular determination will depend on the impurities present and the concentration of borate. Details are given of the methods using both dyes, the colour estimations being made using a Spekker absorptiometer with Ilford violet filters (No. 601) for alizarin blue S and Ilford Yellow filters (No. 606) for the unsulphonated dye base of Solway purple.

R. E. S.

Drying Conditions. A Study of U.S.P. XIII and N.F. VIII. N. L. Deahl, J. L. Powers and M. W. Green. (*Bull. Nat. Form. Comm.*, 1948, **16**, 153.) The results of investigations on the drying conditions of over 1,000 substances contained in the U.S.P. XIII and N.F. VIII are given. Attempts were made in the work to standardise on a few different temperatures and conditions which were consistent with accurate and reproducible results. Another object was to establish the temperature and the time required for the drying of a large class of substances which are at present dried to "constant weight." Where substances gave identical results when dried under a wide range of conditions, the easiest and most acceptable laboratory procedure was recommended; weighings were made to within ± 0.1 mg., weighing bottles after removal from the ovens were cooled in desiccators over calcium chloride until they reached room temperature (kept constant at 25°C.); drying over sulphuric acid was done at 25°C. and vacuum drying was carried out at a pressure of less than 1 mm. Hg.; the temperature of drying was controlled to within $\pm 1^\circ\text{C.}$ of the desired temperature; other precautions taken followed the directions of the U.S.P. XIII; dryings were carried out on two or more samples of each substance. In general drying was carried out wherever possible under "standard conditions" as follows: (1) at 60°C.; (2) at 80°C.; (3) at 105°C.; (4) at 120°C.; (5) at 150°C.; (6) over sulphuric acid at room temperature; (7) in vacuum. The following general results are recommended for drying over sulphuric acid: (1) for the removal

of "surface moisture" only, when the amount present is small and the material is non-hygroscopic in character, 2 to 4 hours drying time was usually sufficient; (2) for the removal of "surface moisture" from materials of a hygroscopic nature, overnight drying (16 to 24 hours) may be required; (3) drying over sulphuric acid for removal of water of hydration was generally unsatisfactory, either because of incomplete dehydration or because of the time involved. It is recommended that substances dried at normal pressure and high temperatures should, in many cases, be dried at 105°C. rather than at 100°C. With drugs such as acacia and sterculia gum, the particle size was found to influence the loss on drying to a considerable extent, indicating that definite specifications for particle size as well as for temperature and time of drying were necessary; thus losses ranged from 10.2 to 14.4 per cent. on the same sample when reduced to different particle sizes. In vacuum drying, the working pressure is important, since greater losses are obtained with a low vacuum. It is recommended that vacuum drying should be carried out at a pressure of less than 5 mm. Hg. Tables are given covering over 1,000 recommended drying conditions: for U.S.P. XIII drugs; for the methods of drying applicable in general tests and analytical processes; and for the methods of drying applicable to U.S.P. XIII reagents. Methods of drying are similarly given for drugs, processes, and reagents of the N.F. VIII, following the principles outlined above. Graphs are included, showing the loss on drying with time of acetophenetidin, cotarnine chloride, emetine hydrochloride, sulphathiazole, digitalis, stibophen, ovary, and liquid glucose, under varying conditions.

R. E. S.

Morphine, Ammoniacal Zinc Ferrocyanide Solution as Reagent for. G. Dénigès. (*Bull. Soc. Pharm. Bordeaux*, 1947, 85, 29.) The reduction of ferricyanide by morphine, with subsequent formation of Prussian Blue, forms a familiar test for the alkaloid. Owing to the strong colour of ferric ferrocyanide, the reaction is not very sensitive and cannot be employed quantitatively. These objections are removed by employing zinc in place of iron to detect the ferrocyanide formed. The reagent is prepared by mixing immediately before use, equal volumes of a 6 per cent. solution of potassium ferricyanide (free from ferrocyanide) and of a solution of 2.5 g. of zinc sulphate in 50 ml. of water to which is added 20 ml. of ammonia. A few drops of this reagent is added to about 1 ml. of the solution to be tested, when in presence of morphine a turbidity or precipitate appears in a short time, and may be estimated nephelometrically. The method may be applied to any clear preparation of morphine, even if coloured.

G. M.

Oxydimorphine in presence of Morphine, Determination of. N. Thörn and A. Ågren. (*Svensk. farm. Tidskr.*, 1949, 53, 33, 49.) Both morphine and oxydimorphine give colours with aromatic aldehydes in presence of sulphuric acid, but in the former case the extinction is very small at 600 μ . The method described below may be used for the determination of oxydimorphine formed in solutions of morphine after storage or sterilisation. For the test, 0.50 ml. of the solution to be tested is transferred to a dry test tube and treated with 10.0 ml. of a solution of 1.0 g. of vanillin in 100 ml. of sulphuric acid (95 \pm 1 per cent.) The reagent must be added slowly from a pipette, with continuous shaking and cooling. The mixture is stirred with a glass rod, immersed in a water-bath for 20 minutes, and then cooled in running water. A blank test is carried out at the same time using a solution of pure morphine of the same concentration. The difference in the extinctions is measured at 600 m μ . The official solvent of

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the Swedish Pharmacopœia for morphine injection contains 5 per cent. of glycerol and 15 per cent. of alcohol, and the method may be applied directly to such solutions, but the extinction is somewhat different, as shown by the table below.

Oxydimorphine in solution per cent.	Extinction	
	in water	in dilute glycerol-alcohol
0.2	0.190	
0.4	0.372	0.315, 0.325
0.6	0.530, 0.549	0.456, 0.463
1.0	0.894, 0.907	0.814, 0.844

G. M.

Procaine Penicillin G, Spectrophotometric Determination of Procaine in. C. V. St. John. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, **37**, 343.) Weigh accurately approximately 50 mg. of procaine penicillin G and transfer to a 100 ml. volumetric flask. Dilute to volume with distilled water and shake until completely dissolved. Transfer 5 ml. of this dilution to a 250 ml. volumetric flask, dilute to volume, and measure the optical density of the resulting solution at 290 μ against water in the reference cell. Obtain the concentration of the solution by calculation from an extinction coefficient determined on a sample of pure procaine hydrochloride or a sample of pure procaine penicillin G analysed by the chloroform shake-out titrimetric procedure. The accuracy of the method compares very favourably with that of the conventional method. Care must be taken, however, to clean the cells frequently with dichromate-sulphuric acid cleaning solution and to make careful adjustment of the instrument. Cell calibration should also be checked. The arithmetical average of the readings for several adjustments of the instrument on each of two fillings of the cell should be taken.

S. L. W.

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Anti-Pernicious Anæmia Factor, Presence of Cobalt in. E. Lester Smith. (*Nature*, 1948, **162**, 144.) The use of the borax bead test and the specific red colour reaction with nitroso R salt reveals the presence of cobalt in the ash of the anti-pernicious anæmia factor recently isolated as red needle-shaped crystals. The crystals, dried in vacuo at 56°C. contain 4.0 per cent. of cobalt, estimated colorimetrically with α -nitroso- β -naphthol. Assuming 8 per cent. loss on drying and one atom of cobalt per molecule, this corresponds to a molecular weight of 1,600, in agreement with the result (1,500 to 1,750) obtained by X-ray crystallography. The different molecular weight (3,000) found by diffusion may be due to errors in the method, impure material, or possibly association in solution. The molecule also contains three atoms of phosphorus. American workers have confirmed the presence of cobalt and phosphorus in vitamin B₁₂ isolated by them. G. B.

Bacitracin, Stability of. G. C. Bond, R. E. Himelick and L. H. Macdonald. (*J. Amer. pharm. Ass., Sci. Ed.*, 1949, **38**, 30.)

Bacitracin, the antibiotic produced by the growth of the "Tracy I" strain of *Bacillus subtilis* was studied to ascertain its stability in various pharmaceutical preparations. It was found to parallel penicillin quite closely in its stability, or lack of it, with various substances. The dry substance was quite stable at 37°C., but showed definite decomposition at 56°C. At 80°C. decomposition occurred within 48 hours. Within a pH range of 5 to 7, aqueous solutions of bacitracin, with or without buffers, were stable for several months at refrigerator temperature, but lost about 50 per cent. of their activity in a week at room temperatures. Ointments prepared with anhydrous fatty bases showed good stability at room temperature, but attempts to prepare a stable water-miscible ointment were unsuccessful. A lozenge and a powder containing ephedrine for the preparation of nasal solutions were shown to be stable. Assay was by the cylinder-plate method using *Staphylococcus aureus* or *Micrococcus flavus*.

G. R. K.

***Lactobacillus casei*, Growth Factors for.** F. W. Chatterway, D. E. Dolby and F. C. Happold. (*Biochem. J.*, 1948, **43**, 567.) The concentration and separation from liver of further factors promoting acid production by *Lactobacillus casei* are described. Importance is attached to the preparation of the casein digest used in the growth medium as some processes do not remove appreciable *L. casei* activity. Growth factors insoluble in saturated baryta were separated from the crude liver extract together with at least three factors soluble in saturated baryta and in silver nitrate at pH 1.0; these consist of a filtrate fraction which is not adsorbed on alumina at pH 3, and two fractions which are adsorbed, one of which is eluted with a 20 per cent. v/v solution of methyl alcohol or water and the other with 0.5 per cent. w/v solution of ammonia. The three latter components have properties dissimilar to both pteroyl glutamic acid and the folic acid of Mitchell *et al.* (1941). The greatest concentration of folic acid-like material was found in the silver salts insoluble at pH 1 (from the baryta-soluble material) which contained none of the components discussed above. The differential action of ninhydrin and nitrous acid, and of esterification and acetylation, upon the activity of the material eluted by a 2 per cent. w/v solution of ammonia for *Streptococcus faecalis* R. and *L. casei*, confirms that there are two components present in this material. The fact that the above chemical treatment affects the filtrate fraction and the baryta-insoluble fraction dissimilarly to one another, and to the above ammonia-eluate material, is evidence that four separate and distinct factors are present.

R. E. S.

Penicillin Activity *in vitro*, Enhancement by Vitamin K₅. Robertson Pratt, J. Dufrenoy and P. P. T. Sah. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, **38**, 435.) The addition of vitamin K₅ (2-methyl-4-amino-1-naphthol hydrochloride) in concentrations of 0.1 to 10 mg./l. enhances the effect of penicillin against *Staphylococcus aureus*, *Proteus vulgaris* and *Escherichia coli* as measured by the cup plate method to a greater extent than would be expected from the sum of the activities of the two components acting separately. The magnitude of the enhancement depended on the concentration of K₅ and of penicillin and on the test organism. The enhancement is particularly marked with *E. coli*.

A. L.

Penicillin, New Absorption Delaying Vehicle. F. H. Buckwalter and H. L. Dickison. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, **37**, 472.)

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In this preliminary report the authors describe experiments which show that peanut oil-aluminium stearate gels are superior to peanut oil or peanut oil-beeswax combinations as repository forms for penicillin. J. W. F.

Penicillin G Sodium in Aqueous Solution, Stability of. T. J. Macek, E. J. Hanus and B. A. Feller. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, **37**, 322.) Solutions of crystalline sodium benzylpenicillinate (penicillin G sodium) in water lost 50 per cent. of their activity in about 2 days at 25°C. and in about 16 days at 10°C. The rate of decomposition was unchanged when normal saline solution and 5 per cent. dextrose solution were the solvents, and was independent of the initial concentration. It was also observed that the pH of the solutions fell from pH 6 to pH 4 in about the time required for 50 per cent. decomposition, but thereafter rose to about pH 4.5, the rise being attributed to the decarboxylation of benzylpenicilloic acid. The greater stability of solutions of partly purified penicillin sodium suggested that the process of purification removed substances capable of acting as buffers and preventing the rapid fall in pH, which accelerates the rate of decomposition. By adding various phosphate and citrate buffers to maintain the pH at about 6.0, the stability of solutions of sodium benzylpenicillinate was increased so that at 25°C. the time for 50 per cent. decomposition was 15 days and at 10°C. solutions still had 75 per cent. of their original activity after 56 days. The stabilising effect was shown to be due to the buffering action of the phosphate or citrate ion and not to a specific ion effect; it was also shown to increase with increase in the concentration of the buffer mixture. G. R. K.

Streptomycin, Degradative Studies on. M. L. Wolfrom and W. J. Polglase. (*J. Amer. chem. Soc.*, 1948, **70**, 2835.) An inactive product was obtained by degradation of dihydrostreptomycin; this, when acetylated with pyridine and acetic anhydride gave crystalline $C_{16}H_{24}O_{12}N_3 \cdot (CH_3C)(COCH_3)_{10} \cdot (I)$ m.pt. 261.5° to 262.5°C. $[\alpha]_D^{23} - 84$ (c, 1; water). Methanolysis of (I), designated deca-acetyldideguanyldihydrostreptomycin with subsequent reacylation yielded hexa-acetylstreptamine, transition point 250°C. m.pt. 341 to 345°C., and methyl penta-acetyldihydro- α -L-streptobiosamide, m.pt. 194 to 195°C., unchanged on admixture with a specimen prepared from dihydrostreptomycin trihydrochloride, $[\alpha]_D^{23} - 120$ (c, 0.5; chloroform). (I) was found to be readily soluble in methyl alcohol, water and hot ethyl alcohol, sparingly so in chloroform, ethyl acetate and ethyl alcohol, and insoluble in benzene and ethyl ether. Aqueous solutions of N,N,N-tetra-acetyldideguanyldihydrostreptomycin (II), N¹,N³, diacetyl streptamine (III) and N-acetyldihydro- α -L-streptobiosaminide were prepared by partial deacetylation of the aforementioned acetyl derivatives with 0.05N sodium hydroxide in water-dioxane. Treatment with periodate showed the presence in II of an α -glycol which is not in III, and this α -glycol is present in the streptamine moiety of II. The presence of such a glycol group indicates that streptobiosamine is attached at C4 of streptidine, thus confirming the result of Folkers. R. E. S.

Streptomycin and Mannosidostreptomycin, Crystalline Trihydrochlorides of. L. J. Heuser, M. A. Dolliver and E. T. Stiller. (*J. Amer. chem. Soc.*, 1948, **70**, 2833.) Crystalline streptomycin trichloride dihydrate has been prepared as monoclinic prisms showing birefringence. On heating, gradual decomposition without melting took place. Drying at 55°C. *in vacuo* gave $C_{21}H_{39}N_7O_{12} \cdot 3HCl \cdot 2H_2O$; after drying at 100°C. *in vacuo* a substance was

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obtained which gave $[\alpha]_D^{26-6^\circ} - 86.1^\circ$ (1 per cent. in water) and $C_{21}H_{39}N_7O_{12} \cdot 3HCl$. When assayed with *Klebsiella pneumoniae* in a broth-dilution test, the trihydrochloride dihydrate had a potency of 820 units/mg. and on this basis the anhydrous material would have an activity of 891 units/mg. The trihydrochloride of mannosidostreptomycin crystallised in the form of isotropic hexagonal plates. By means of the counter-current distribution method, this material was also shown to be a single entity and to be free of streptomycin. Drying at $55^\circ C$. *in vacuo* gave $C_{27}H_{49}N_7O_{17} \cdot 3HCl \cdot 2H_2O$; at $100^\circ C$. *in vacuo* analysis showed $C_{27}H_{49}N_7O_{17} \cdot 3HCl$, $[\alpha]_D^{26-6^\circ} - 54.1^\circ$ (1 per cent. in water). When assayed with *K. pneumoniae* in a broth-dilution test, the anhydrous mannosidostreptomycin had a potency of ca.210 units/mg.

R. E. S.

Vitamin A in Whale-Liver Oil, Chromatographic Estimation of. N. T. G r i d g e m a n, G. P. G i b s o n and J. P. S a v a g e. (*Analyst*, 1948, **73**, 662.) The method given is based on the fact that the main components of the unsaponifiable fraction of whale-liver oil are selectively adsorbed on weakly active alumina in the order: anhydro-vitamin A < oxidised vitamin A < vitamin A (alcohol) < kitol < sterols < selachyl alcohol. The technique consisted of depositing the material on the column from a non-polar solvent and then developing and eluting the chromatogram with solvents of progressively increasing polarity; the eluate was collected fractionally and Carr-Price spot tests were used to identify the vitamin A portions, from which aliquots were bulked for spectrophotometric estimations. A quantity (approximately 0.35 to 0.40 g.) of the unsaponifiable fraction in light petroleum was used for chromatography and 25 ml. quantities of light petroleum-ether mixture were used to elute the fractions, the proportion of ether being gradually increased in each succeeding fraction. The eluate was collected in 5 ml. fractions and a few drops of solution from individual tubes were tested with Carr-Price reagent to establish the range of tubes containing the vitamin. The tubes corresponding to the zone below the vitamin A usually give a reddish-purple colour with the reagent; while those corresponding to the zone above the vitamin gave a bluish-purple or greenish-purple colour. Both these colours are readily distinguished from the bright blue of the vitamin A solution. Moreover, in a good chromatogram, the set of vitamin A tubes will be separated at either end from the sets containing the adjacent zones by one of two tubes whose eluate content is almost nil; these correspond to the inter-zone regions in the chromatogram and will give only a faint coloration with the Carr-Price reagent. Aliquots drawn from those tubes showing vitamin A reaction were pooled, diluted with cyclohexanol and $E_{1\text{ cm.}}^{1\text{ per cent.}}$ 326 to 328μ was measured; if the fraction is pure, the maximum of the absorption curve will lie between 326μ and 328μ , $E_{300\mu}/E_{\lambda_{\text{max}}}$ will be not more than 0.63, and $E_{360\mu}/\lambda_{\text{max}}$ not more than 0.35. Details are given of the full analysis by this method of a sample of whale-liver oil. The method could be extended to more normal oils. Chromatography of the unsaponifiable matter of a shark liver oil, a mixed fish-liver oil diluted in vegetable oil, a distilled vitamin A ester concentrate, and a cod-liver oil, showed that of the total absorptions at 326μ (on the whole oil for the first three samples and "via unsap." for the cod-liver oil) the following fractions were due to vitamin A: 85, 92, 91 and 90 per cent., respectively. Recovery experiments on vitamin A acetate dissolved in vegetable oil and in dolphin oil gave results of 97 to 99 per cent. on the unsaponifiable fractions.

R. E. S.

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Folic Acid, Polarographic Determination of. W. J. Mader and H. A. Frediani. (*Anal. Chem.*, 1948, **20**, 1199.) The determination of folic acid can be made quantitatively and rapidly. The sample is dissolved in 1 per cent. tetramethylammonium hydroxide solution containing cadmium chloride as internal standard with sufficient ammonium chloride to prevent precipitation of the cadmium from alkaline solution. A polarogram of this solution yields two clearly defined waves, one at 0.74 volt (against the saturated calomel electrode) for the cadmium, and one at 0.98 for the folic acid. With known folic acid concentrations (and fixed cadmium concentration) a straight line results on plotting the step-height ratios of cadmium-folic acid against folic acid concentration using log log coordinates. Replicate runs on a fixed sample indicated a reproducibility of ± 2 per cent. if the drop rate was controlled within 0.7 second, and the temperature within 10°C ., variations which exceed the conditions ordinarily encountered in an analytical laboratory. The diffusion current constant as defined by Lingane was calculated to be 1.72. It was found more convenient to utilise the ratio-concentration curve and thus to read off sample concentrations directly in mg./ml. than to calculate sample concentrations. The method could be applied to folic acid tablets and to tablets with vitamin B₆ added, but could not be used in the presence of iron. R. E. S.

Penicillin, Spectroscopic Estimation of. G. H. Twiggs. (*Analyst*, 1948, **73**, 211.) The author has reviewed various applications of ultra-violet and infra-red spectroscopy for the estimation of total and individual penicillins, and has discussed the limitations of these methods. The aim of spectroscopy is to discover in the absorption spectrum a band which is characteristic of each penicillin molecule as a whole. This is not attained in practice and the absorptions arising from separate parts of the molecule have to be used. Such a procedure may provide an estimate of total penicillin, but it leads to fundamental difficulties in assaying individual penicillins; impurities and deactivated penicillin products may contain similar molecular groupings and thus have bands almost identical with those of the penicillins. It is, therefore, likely that spectroscopic estimation of individual penicillins can only be applied to the pure material. The ultra-violet analysis of total penicillin depends on the development of an absorption band at 3220 \AA when an aqueous solution of penicillin is treated with acid under standard conditions. The band, which is due to an intermediate product, disappears after a time and the reading must be taken at its maximum intensity. The method can be used with impure material and gives an accuracy to within 5 per cent.; chemical methods of estimation are probably more speedy and accurate. The ultra-violet analysis of penicillin G depends on the development of absorption bands in the ultra-violet spectrum due to the phenyl group. Two methods have been developed. One compares the ultra-violet spectrum of the unknown sample with that of a known standard by photography and for pure samples of penicillin G gives an accuracy of ± 2 per cent. The second method is based on the relative absorption at 2630 \AA and 2800 \AA . Penicillin G has no absorption band at the higher wave-length but impurities and decomposition products have. All measurements of optical density are made with solutions of constant penicillin content (1.8 mg. per ml.) as both penicillin K and F show some absorption at 2630 \AA . The optical density difference $E_{2630} - E_{2800}$ is plotted against percentage of penicillin G content and gives a straight line, the analyses being evaluated from this calibration curve. Both these methods suffer from similar defects, phenyl-

containing inactivated materials interfere and concentrations of penicillin X in excess of 1 per cent. will invalidate the results. Infra-red analysis of the individual penicillins is theoretically the ideal method, as the infra-red spectrum of any substance is unique. Unfortunately serious practical difficulties arise; the penicillin may contain impurities or inactivated materials which have absorption bands in the same position as the bands used for analysis, the penicillin may also be in the form of a salt which is insoluble in all the solvents that are of use in infra-red measurements, and degradation may occur when converting it to the free acid and transferring to a suitable dry solvent. The effect of crystal structure is not known and a separate calibration may be necessary with amorphous material, and it is not easy to measure I_0 , the intensity of incident radiation. A suitable internal standard, generally *dl*-alanine, in known concentration has to be mixed with the penicillin. The ratio $R = \log(I_0/I)_{703} / \log(I_0/I)_{851}$ is determined for penicillin G, where I_0 and I are the intensities of incident and transmitted radiations respectively at both 703 cm^{-1} and 851 cm^{-1} . A calibration curve R against percentage of penicillin G is plotted by diluting the pure penicillin G with magnesium oxide; results using this curve have an accuracy of ± 2 per cent. For other penicillins different bands are used.

L. H. P.

Salicylate in Blood, Fluorophotometric Method for the Estimation of.

A. Saltzman. (*J. biol. Chem.*, 1948, 174, 399.) One ml. of oxalated or citrated plasma is shaken in a test-tube with 9 ml. of a freshly prepared mixture of 1 volume of a 10 per cent. solution of sodium tungstate and 8 volumes of N/12 sulphuric acid, and the precipitated proteins are filtered off after 10 minutes. To 5 ml. of the filtrate 7 ml. of sodium hydroxide solution (40 per cent.) is added and the mixture is placed in a fluorophotometer. The fluorescence is directly measured within 30 minutes using the same filters as in the vitamin B₁₂ determination. The values are read off a standard reference curve, plotted by adding varying amounts of a standard salicylate solution, containing 1.16 mg. of sodium salicylate in 12 ml. of water to a reagent blank mixture, consisting of 5 ml. of tungstic acid and 7 ml. of sodium hydroxide solution (40 per cent.). These values must be multiplied by 2 to correct for the dilution. Salicylate concentrations of 1 to 2 mg. in 100 ml. are detectable, and both free and combined salicylate is determined. A modification of the ethylene dichloride method for determining salicylate concentration in the blood is also described.

L. H. P.

Streptomycin B, Chemical Assay of. W. B. Emery and A. D. Walker. (*Nature*, 1948, 162, 525.) The use of 0.2 per cent. anthrone, a reduction product of anthraquinone, in 95 per cent. sulphuric acid is described for distinguishing streptomycin B (a mannoside) from streptomycin A; it can also be used for estimating the former in a mixture. The results obtained are in accord with those calculated from biological and chemical assays, making the accepted assumption about the relative biological activities of the two streptomycins. The glucosamine moiety, present in both molecules, does not react with the reagent.

R. E. S.

Urea in Blood, An Improved Diacetyl Reaction for the Estimation of.

V. R. Wheatley. (*Biochem. J.*, 1948, 43, 420.) The enhancing effect of a number of substances on the diacetyl-urea reaction has been studied. Phenols were unsatisfactory and produced precipitates; with aromatic amines the colour produced was orange or red, and not yellow, whilst diphenylamine and its derivatives produced an intense magenta colour. The reaction with

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N-phenyl anthranilic acid was studied in detail and adopted for the estimation of urea in blood. Investigations of the reaction conditions were made involving a study of the quantities of reagent used, the time of heating, the development of the colour and the specificity of the reaction. Calibration and absorption curves are given for the reaction colour, which obeyed Beer's law up to a urea concentration of 20 $\mu\text{g./ml.}$ Under the conditions prescribed ammonia, histidine, tyrosine, cystine, caffeine, uric acid, barbiturates, acetamide, asparagine, creatine, sulphonamides and thiouracil all give negative reactions. Proteins and monosubstituted ureas give red colorations; semi-carbazide gives a magenta colour similar to that obtained with urea, while biuret gives a brown colour. Creatinine gives a positive reaction, but fortunately the sensitivity in this case is only one hundredth of that with urea, so that creatinine will not interfere with the estimation of urea in biological fluids. The method showed fairly good agreement with the manometric hypobromite method except that in 10 per cent. of cases errors of 8 to 10 mg./100 ml. were obtained. The estimation can be performed with as little as 0.2 ml. of blood and compares favourably with other colorimetric urea determinations, although it is not sufficiently accurate for urea clearance tests.

R. E. S.

CHEMOTHERAPY

Fungistatic Activity and Structure in a Series of Simple Aromatic Compounds. G. W. K. Cavill, J. N. Phillips and J. M. Vincent. (*J. Soc. chem. Ind., Lond.*, 1949, **68**, 12.) Derivatives of benzene are assessed for activity against *Aspergillus niger*. For comparison, the logarithm of the reciprocal of the millimolar concentration giving 50 per cent. inhibition is used to express fungistatic activity. In the case of ionised substances, this is calculated relative to the un-ionised form as there is some evidence that the ionised forms are not so active fungistatically. Benzene and toluene have a small activity, aniline, benzoic acid, phenol and nitrobenzene are less active, but chlorobenzene is more active. Saturated compounds are considerably less active than the corresponding aromatic substances. In general, halogen substitution increases activity. Substitution of aniline, benzoic acid or phenol with hydroxyl or amino groups nearly always reduces activity, except that $-\text{OH}$ substituted *ortho* to carboxyl groups increases activity slightly. *Meta* or *para* substitution of a carboxyl group in phenol or in aniline decreases activity, but *ortho* carboxyl groups cause an increase, or little change, in activity. Nitration of phenol or aniline increases activity (except for picric acid and trinitroresorcinol which are ionised), but nitrobenzoic acid is less active than benzoic acid. The introduction of $-\text{CH}_2-$ groups between ring and carboxyl group does not enhance the activity of benzoic acid, and decreases that of 4-aminobenzoic acid; the same applies to the methyl esters. To conform to the conclusions above, 4-hydroxybenzoic acids have to be regarded as substituted benzoic acids, not as substituted phenols. Alkylation, alkyl esterification or alkyl etherification generally increases activity. There is some correlation between reciprocal water solubility and activity for a homologous series, but this breaks down if a wider range of compounds is taken. Slightly better agreement is obtained when the logarithm of relative solubility in alcohol and water is compared with activity, but there are considerable deviations.

G. B.

Polymethylene bis-Quaternary Ammonium Salts, Curare-like action of. R. B. Barlow and H. R. Ing. (*Brit. J. Pharmacol.*, 1948, **3**, 298.) The following series of polymethylene bis-quaternary ammonium dibromides

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were prepared and tested for curare-like activity on the phrenic-nerve diaphragm preparation of the rat (n = number of carbon atoms in the polymethylene chain):—*bis*-trimethylammonium series, n = 2, 3, 4, 5, 7, 8, 9, 10, 11, 12 and 13; *bis*-triethylammonium series, n = 2, 3, 4, 5, 7, 8, 9, 10 and 13; *bis*-strychninium series, n = 2, 3 and 5; *bis*-quinolinium series, n = 3, 5 and 10; *bis*-(phenyldimethylammonium) series, n = 3 and 5. In the *bis*-trimethyl series, the salt with n = 2 is about twice as active as tetramethylammonium iodide; salts with n = 3, 4 or 5 are only feebly active; activity increases from n = 7 to n = 9; salts with n = 9, 10, 11 and 12 are about 5 to 6 times as active as tetramethylammonium. In the *bis*-triethyl series, salts with n = 2 or 3 are relatively inactive; activity increases from n = 4 to n = 13. None of the members of the other three series was so active as the most active members of the *bis*-trimethyl series. In the rabbit head-drop test the *bis*-trimethyl member with n = 9 was nearly as active as tubocurarine chloride; the member with n = 10 was about 3 times as active. The *bis*-triethyl member with n = 13 was about two-fifths as active as tubocurarine. Some *bis*-onium salts augment the response of the rat diaphragm to maximum stimuli and inhibit the cholinesterase of caudate nucleus (dog). The sensitivity of the rat diaphragm to *bis*-onium salts differs greatly from that of the rabbit, and the rat diaphragm is less sensitive to methylstrychninium and more sensitive to tetramethylammonium iodide than the frog's sartorius, suggesting that synthetic curare-like drugs ought to be tested on a variety of species.

S. L. W.

PHARMACY

DISPENSING

Fatty Oils, Neutralisation of, for Injection. C. G. van Arkel and J. J. M. van Sonsbeek. (*Pharm. Weekbl.*, 1949, 84, 70.) Neutralisation of fatty oils is sometimes carried out by shaking the oil with excess of calcium or magnesium oxide, possibly with the addition of a trace of alcohol. The method was found effective in reducing the natural acid value of an oil from 0.32 to 0.16 (with magnesia) or 0.09 (with lime). Traces of these metals could be detected in the neutralised oils. When using an oil to which fatty acid had been added to give an acid value of 9.63, it was found necessary to add water in order to obtain a satisfactory result, but too much water causes formation of emulsions and difficulty in filtration. The dissolved metal amounted to, for magnesium 0.2 mg./100 ml., and for calcium, 6.5 mg./100 ml. If, however, the filtration is carried out with the aid of heat, larger quantities are dissolved. It is concluded that the method of neutralisation with soda is to be preferred on account of greater reliability and the possibility of filtration at a raised temperature.

G. M.

Penicillin Ointment, Stability of. S. H. Culter. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 370.) A number of penicillin ointments, using various types of bases, with sodium, potassium, and calcium salts of varying degrees of potency were prepared. These ointments were stored in collapsible tin tubes at room and refrigerator temperatures and assayed from time to time to determine the stability of the penicillin. It was found that impure sodium penicillin (400 I.U./mg.) is very unstable in an aqueous or a non-aqueous water-miscible ointment base and has only limited stability in an anhydrous petrolatum base. Calcium penicillin (500 to 650 I.U./mg.) is much more stable than the impure sodium salt in the same bases, while the high potency (1583 to 1620 I.U./mg.) crystalline sodium or potassium salts are equal, if

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not superior, to the impure calcium salt for ointment purposes in respect of stability. Penicillin ointments are somewhat more stable at refrigerator temperatures than at higher temperatures. The inclusion of sulphadiazine, sulphathiazole, adrenaline, or benzocaine in a penicillin ointment does not materially influence the stability of the penicillin, but water, zinc stearate, aluminium hydroxide gel hasten its deterioration to a marked degree.

S. L. W.

Penicillin Powders, Preparation of. J. B ü c h i and F. O. G u n d e r s e n. (*Pharm. Acta Helvet.*, 1949, **24**, 31.) A penicillin powder, prepared by dilution of penicillin with lactose, had a limited stability, since 15 per cent. of the activity was found to be lost after keeping for 2 months at 4°C. Preparations made according to the following formula were more satisfactory and showed no loss after 4 months at 4°C., provided that the materials were dried thoroughly before use, and the powder was kept over calcium chloride. Crystalline sodium penicillin, 1,000,000 units; hydrogenated arachis oil (m.pt. 37°C.), 0.25 g.; anæsthetic ether, 5 ml.; sodium laurylsulphate, 0.50 g.; de-enzymated gum acacia, 1.50 g.; diluent, to 50.00 g. In the preparation the penicillin is rubbed down with a solution of the hardened arachis oil in the ether, in order to coat the particles of penicillin, and the other substances are then added. The mixture is finally passed through sieve VI (approx. 100 mesh/inch; wire 0.08 mm. diam.). Anhydrous lactose, sulphanilamide or dried milk may be used as diluent.

G. M.

Sterility of Chemicals, Employment of Filtration in Testing. O. B a n g, G. B o w i t z and A. T. D a l s g a a r d. (*Arch. Pharm. Chemi.*, 1949, **56**, 643.) The authors have examined the method of testing for sterility proposed by Davies and Fishburn (*Quart. J. Pharm. Pharmacol.*, 1946, **19**, 36). Their results show that the risk of infection arising during manipulation cannot be ignored, since out of 113 tests (30 with a dry filter, the others with sterile solutions) 15 gave positive results. Tests were carried out with a number of pure chemicals, and positive results were obtained with a proportion in the cases of ascorbic acid, hexobarbitone, morphine hydrochloride, dextrose, allylisopropyl barbitone, phenobarbitone, oxedrine tartrate, benzocaine, and boric acid. "Sterilised" boric acid powder gave positive results in 9 tests out of 10. Generally the contaminating organism was a Gram-positive rod. The authors consider that the method is worthy of further study and possible official adoption.

G. M.

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Antimalarial Plants, Chinese. S. T. Y a n g. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, **37**, 458.) A brief description of five Chinese medicinal plants which may have antimalarial activity is given. 1. *Fraxinus malacophylla* Hensl. This tree occurs in S.E. Yunnan and is known as *Pei Chiang Kan*; the root-bark is used. Recent tests indicate the absence of antimalarial activity. 2. *Fraxinus chinensis* Roxb. Grows in Szechuan and Yunnan; the bark yields fraxetin which earlier workers claimed to possess antimalarial activity; however recent work indicates it is ineffective. 3. *Clerodendron yunansis* Hu. Grows near Kunming where thin slices of the twigs of this tree are sold as *Tien Ch'ang Shan*. No investigations have been conducted on this plant. 4. *Alstonia yunansis* Diel. Also grows near Kunming; the bark, twigs and pods are sold as *Chih Ku Ch'ang Shan*. Several alkaloids and a resinous substance are present, but they show no antimalarial activity. 5. *Dichroa febrifuga* Lour. This is the only plant in the group whose anti-

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malarial activity has been established. It is an evergreen shrub growing in Szechuan and Yunnan; the dried roots are known as *Ch'ang Shan*. In Yunnan the leaves and twigs are used and these have been shown to be much more active than the roots.

J. W. F.

Belladonna, Indian, Pharmacognosy of. H. W. Youngken and W. E. Hassan, Jr. (*J. Amer. pharm. Ass., Sci. Ed.*, 1948, 37, 450.) A complete description and comparison of this plant with *Atropa Belladonna* Linn. is given. The materials used were grown by the workers from authenticated seeds and surplus material has been deposited in the Herbarium of the Massachusetts College of Pharmacy. *Leaves*: the following data are reported; *Vein Islet Number*, species average = 10; *Palisade Ratio*, 5.0 - 8.3 - 12.4 (*A. Belladonna*, 4.5 - 6.9 - 9.2), *Stomatal Index*, Upper Surface, 3.4; Lower surface, 17.6; (*A. Belladonna*, 2.9 and 17.6 respectively). *Floral Members*; the Indian variety has larger flowers than European, and the corolla is bright yellow. *Roots and stems*: similar basic structure except that the cells of the Indian variety are larger than those of the European. *Alkaloidal content*: the Indian variety has a high alkaloidal content. Drying under infra-red lamps at 145° C. destroys the alkaloidal content. No significant increase in alkaloidal content resulted from the injection of amino-acids into the growing plant. The authors suggest that Indian belladonna is a variety of the European and should be named *Airopa Belladonna* variety *acuminata*.

J. W. F.

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Adrenaline and norAdrenaline, Action on Human Heart-rate. H. Barcroft and H. Konzett. (*Lancet*, 1949, 256, 147.) The actions of intravenous infusions of noradrenaline and of adrenaline on the heart-rate and arterial blood pressure of normal men and women have been studied. In doses of 10 to 20 μ g./minute noradrenaline causes bradycardia, whereas adrenaline causes tachycardia. Subjective effects during the adrenaline infusions included mild palpitation, hyperventilation, tightness in the chest, and muscular fatigue; there were usually no subjective symptoms during infusions of noradrenaline. The explanation of the different actions of adrenaline and noradrenaline on the heart-rate is not known, but the authors suggest that two factors are probably concerned—the direct excitatory action of the drug on the pacemaker, and its reflex inhibitory action due to its pressor action on the vascular system.

S. L. W.

Courbonia virgata. Identification of Toxic Principle as a Tetramethylammonium Salt. A. J. Henry. (*Brit. J. Pharmacol.*, 1948, 3, 187.) The tuberous root of *Courbonia virgata* A. Brongn. (Fam. Cappariaceae), a plant occurring in the Southern Sudan, Northern Uganda, Kenya, and French Equatorial Africa, has been found to contain a toxic principle, tetramethylammonium iodide. This was also found in the scaly shoots and the superstructure, and in the leaves of the subsidiary shoots. It has been named tetramine and the fresh root contains about 0.2 per cent. About 0.25 g. of the base taken orally (in the form of the root) has proved lethal to adult human beings within an hour. Lethal dose of the iodide subcutaneously was 0.5 to 1 mg./25 mg. of mouse, the symptoms being convulsive spasms, collapse, and death within 30 minutes. Intravenous injection of 8 mg. of the iodide into a rabbit caused death within 2 minutes. The

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toxic principle is not extracted from alkaline solution by organic solvents and unless its presence is suspected and its properties known, it might easily be overlooked. The toxic properties of the plant are well known to the natives. As a qualitative test for tetramine, the crystalline precipitate which it produces with Wagner's reagent can be used. From warm dilute solutions, either acid or neutral, the periodide rapidly separates as well-shaped rhombic crystals which are readily recognised under the microscope. S. L. W.

Dextran as a Plasma Substitute. J. J. Bull, C. Ricketts, J. R. Squire, W. d'A. Maycock, S. J. L. Spooner, P. L. Mollison and J. S. C. Paterson. (*Lancet*, 1949, **256**, 134.) Dextran is produced by the growth in culture of certain micro-organisms, in particular of *Leuconostoc mesenteroides*, in a substrate of glucose and phosphate. After removal of protein and inorganic salts from the culture fluid dextran is precipitated as a syrupy gum by organic solvents such as acetone; so obtained, it is a polysaccharide composed entirely of glucose units. The molecules of this crude dextran are too large for infusion purposes and preparations of smaller molecular size are produced by partial hydrolysis with acid. In defining a specification of dextran for intravenous use the proportion of dextran of low molecular weight should be kept to a minimum; it is probably also important to define the upper limit of molecular size. Physico-chemical methods for controlling molecular size are described. The solution for infusion is colourless or pale straw colour and of about the same specific gravity and saline concentration as plasma, with a colloid osmotic pressure 1.5 to 2.0 times that of normal plasma. It is well tolerated as an infusion by man and is not pyrogenic, toxic, or antigenic. Immediately after infusion the erythrocyte-sedimentation rate is increased and rouleaux can be observed in smears of blood. No increase in the osmotic fragility of the red cells has been observed. Dextran has proved efficacious as a plasma substitute in cases of burns and has produced a sustained increase in the venous return in patients with surgical shock or hæmorrhage, but as there is still doubt as to the ultimate fate of dextran in the body it cannot yet be recommended unreservedly for intravenous infusion. S. L. W.

Histamine Antagonists, Comparison of. J. J. Reuse. (*Brit. J. Pharmacol.*, 1948, **3**, 174.) Comparison of a number of histamine antagonists on isolated organs placed them in the following order of descending activity: neoantergan, 3277 RP, benadryl, antistin, nupercaine. Besides being the most active of these drugs against histamine, neoantergan is also the most specific; it had the least action against acetylcholine, and its action against nicotine, potassium and adrenaline was much smaller than its action against histamine. It is thus clearly the best of the drugs studied to use in specific tests for histamine in unknown solutions, but it is useless if high concentrations are used. A satisfactory method for carrying out tests of this kind is first to find doses of the tissue extract and of histamine which cause equal effects on a piece of guinea-pig's ileum, and then to continue giving these doses alternately and to study the effect, in a series of responses, of a brief addition (1 minute) of a small dose of neoantergan to the bath. The dose of neoantergan is chosen so as to produce 50 to 70 per cent. inhibition of the subsequent response to histamine. The concentration of neoantergan for this effect is usually about 1/10 the concentration of histamine. A method for the rough biological assay of neoantergan is described which involves the use of only about 0.002 μ g. of the drug per dose. The antihistamine drugs

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were found to depress the isolated heart and dilate the coronary vessels. The activity of these drugs as local anaesthetics on the frog's lumbar plexus appeared to be more nearly related to their activity against acetylcholine than to their activity against histamine; benadryl and 3277 RP were, in fact, much more potent than antistin and neoantegan both as local anaesthetics and as acetylcholine antagonists.

S. L. W.

Isomeric Heptylamines, Comparative Pharmacology of. D. F. Marsh, (*J. Pharmacol.*, 1948, **94**, 225). In this study the vasopressor activity of the isomeric heptylamines in anaesthetised and unanaesthetised dogs was determined and compared with adrenaline. By limiting the investigation primarily to compounds with a total of seven carbon atoms it was possible to determine the relationship between spatial configuration and pharmacological activity without having to consider differences in molecular weight. The most potent of the compounds was 4-methyl-2-heptylamine which is about 1/200 as active as adrenaline and has a long duration of action; orally, the heptylamines have but little pressor action in man. With the exception of the 4-heptylamine, they increased the tone of isolated rabbit jejunum with a concentration of 4 mg./100 ml., caused contraction of the rat uterus and antagonised the relaxant action of adrenaline. In the perfused heart they produced a decrease in rate, force of contraction and outflow of perfusate. They do not antagonise histamine constriction in the perfused guinea-pig lung.

S. L. W.

Isuprel in Spontaneous and Induced Asthma. F. C. Lowell, J. J. Currey and I. W. Schiller. (*New Engl. J. Med.*, 1949, **240**, 45.) Isuprel, *l*-(3: 4-Dihydroxyphenyl)-2-isopropylaminoethanol, (*isopropyladrenaline*) has been studied for a number of years in Europe under the name of aleudrin, and has been advocated for use in the form of an aerosol for the relief of attacks of bronchial asthma. The authors report their observations of the administration of isuprel to asthmatic subjects in the out-patient clinic, in the wards, and in the laboratory (induced asthma). In most cases the drug was given as an aerosol, in a concentration of 1.0 and 0.5 per cent., but it was subsequently given in tablets sublingually containing 10 mg., or subcutaneously or intramuscularly in a concentration of 0.02 or 0.01 per cent. Given by aerosol it was very effective in relieving mild or moderately severe asthma and appears the most effective agent available for self-medication; in severe and prolonged attacks it was far less satisfactory. In certain cases other medication, particularly aminophylline intravenously, was required; on recovery, isuprel was again effective in the control of milder attacks. Sublingual and parenteral administration of the drug was not very effective. Side-effects were uncommon in the doses used in this study.

S. L. W.

Procaine, Influence of Potassium and Calcium Ions on. H. J. Bein. (*Brit. J. Pharmacol.*, 1948, **3**, 251.) The action of procaine on the refractory period of the isolated rabbit auricle was determined in the presence of varying amounts of potassium and calcium. The influence of potassium and calcium was shown to be antagonistic; increasing the amount of potassium or decreasing the amount of calcium both potentiated the action of procaine, about the same potential change being obtained by raising the K by 50 per cent. or by lowering the Ca by 50 per cent. A reduction of the potassium or an increase of the calcium content produced the same effect qualitatively but not quantitatively. The action of procaine was depressed, but to obtain

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the same degree of depression as that obtained by reducing the K concentration by 50 per cent. it was necessary to increase the Ca concentration by 600 per cent. The determining factor is therefore not the ratio K/Ca but the absolute amount of potassium present, though the presence of at least the normal amount of Ca is necessary.

S. L. W.

Proguanil (Paludrine), Intravenous. R. N. Chaudhuri and H. Chakravarti. (*Brit. med. J.*, 1949, **1**, 91.) Proguanil acetate was administered by intravenous injection to 11 patients ranging in age from 9 to 60 years, in 8 of whom *Plasmodium falciparum* infection was predominant, while 2 had *P. vivax* infection and 1, mixed infection. Four patients were gravely ill with pernicious symptoms, 4 had heavy parasitic infection with frequent vomiting, and 3 had "ordinary" malaria. Doses varied from 25 to 400 mg., and were repeated in a few cases, the total amount injected ranging from 200 to 600 mg. Although the series was too small to determine the best dosage, in the majority of cases 200 to 400 mg. produced a striking effect, controlling the temperature and clearing the peripheral blood of asexual parasites in 2 or 3 days. One patient admitted in a moribund state, died; another, with typical cerebral malaria, remained unconscious for several days and later developed signs and symptoms of encephalitis, from which, however, he recovered completely. The injections were well tolerated, although 2 patients complained of pain along the injected vein, possibly due to some local phlebitis. A footnote to this paper reports that proguanil lactate is more soluble and less irritant than the acetate, and is being used by intramuscular injection.

G. R. K.

Proguanil (Paludrine) in Prophylaxis and Treatment of Malarial Infections caused by a West African Strain of *Plasmodium falciparum*. G. Covell, W. D. Nicol, P. G. Shute and M. Maryon. (*Brit. med. J.*, 1949, **1**, 88.) Proguanil was found to act as a true causal prophylactic of infections of the strain of *Plasmodium falciparum* used, and the prophylactic dose recommended for non-immune adults exposed to malarial infection in West Africa is 100 mg. daily. Although it controlled the clinical attack caused by infections with the same strain, its action in this respect and in clearing asexual parasites from the peripheral blood was somewhat less rapid than that of mepacrine or quinine, and by itself, it did not effect a radical cure. Nevertheless, a course of 300 mg. twice daily for 10 days effectively sterilised the gametocytes and rendered them non-infective to mosquitos for as long as they continued to be present in the peripheral blood in sufficient numbers for infection to occur. Proguanil also has the lowest toxicity of any known antimalarial drug. In the treatment of *P. falciparum* malaria infections, rapid termination of the clinical attack, a high radical cure rate, sterilisation of the gametocytes and minimum risk of injurious side-effects is achieved by a course of 300 mg. of proguanil twice daily for 10 days with 900 mg. of mepacrine given in 3 doses on the first day, followed by a maintenance dose of 100 mg. of proguanil for the ensuing 6 weeks.

G. R. K.

Pyrogens and Fever of Acute Infection. J. L. Bennett. (*J. exp. Med.*, 1948, **88**, 267, 279.) The possibility that the fever accompanying acute infections is a response to a pyrogen produced by the infecting organism was investigated in rabbits. Animals surviving dermal pneumococcal infections, or peritonitis due to *Escherichia coli*, were given intravenous injections of typhoid or *E. coli* vaccine. They showed no tolerance to the fever-promoting

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effect of these pyrogenic materials, indicating that the pyrogen produced by the organisms is not a significant factor in the production of fever. It was, however, found that tolerance developed on repeated injection of pyrogenic material during fever, showing again that the pyrogen produced by *E. coli* is not the main factor in the causation of a raised temperature. It is suggested that perhaps a product of cell injury is the cause of the fever. Similar experiments were carried out with the injection of sterile exudates of acute inflammation, the exudates being produced by the intrapleural injection of turpentine in dogs. Daily injection of exudate produced no tolerance to its fever-producing effect. Animals tolerant to pyrogens remained fully responsive to exudate. The fever-producing property of exudates is not therefore due to the presence of bacterial pyrogen.

H. T. B.

β -Pyrrolidine-ethyl-phenothiazine (Pyrrolazote), Pharmacology of. M. J. Vander Brook, K. J. Olson, M. T. Richmond and M. H. Kuizenga. (*J. Pharmacol.*, 1948, **94**, 197). This compound was compared with pyribenzamine and was shown to be highly specific as a histamine antagonist. It appears to be effective for a longer period of time than pyribenzamine as judged by both the activity it exhibits against histamine spasms of smooth muscle *in vitro* and the protection it affords against fatal histamine intoxication by aerosol *in vivo*. It possesses anti-anaphylactic properties similar to those of pyribenzamine. Pyrrolazote has no effect on the pressor responses to adrenaline; in this respect it differs from pyribenzamine, benadryl and neoantergan, all of which enhance the pressor response. Acute toxicity experiments show that pyrrolazote is considerably less toxic than pyribenzamine in mice, rats and rabbits, and chronic toxicity studies in rats showed that a dose of 10 mg./kg. orally 5 days each week for 10 weeks produced no gross pathology, and growth was not impaired. Histopathology limited to degenerative fatty infiltration of the liver occurred at doses of 25 mg./kg. and greater.

S. L. W.

Quinine Methiodide, Pharmacology of. F. H. Shaw, P. Keogh and M. MacCallum. (*Austral. J. exp. Biol.*, 1948, **26**, 147). The authors show that while quinine methiodide retains many of the properties of quinine it has also a curare action on the neuromuscular junction and sympathetic ganglia. It weakens the depressor action of adrenaline and in this respect is the complement of yohimbine and ergotoxine. Because of its extreme toxicity towards the respiratory centre it would be an unsuitable clinical substitute for curare. Intravenous doses of as low as 10 mg./kg. in the cat or dog nearly always resulted in immediate cessation of respiration. It is suggested that it may provide another useful pharmacological test for adrenaline.

S. L. W.

Thyroid Activity, Biological Assay of. D. E. Hutcheon (*J. Pharmacol.*, 1948, **94**, 308). The results of two relatively simple methods for estimating the physiological potency of thyroxine are presented. One is a quantal response type of assay depending on the decreased resistance to anoxia of mice treated with thyroxine. For this, adult mice, weighing from 20 to 25 g., were divided into 4 groups of 10 animals, one group serving as a control while the other 3 were given thyroxine 2.5, 5.0 and 10.0 μ g. subcutaneously daily for 7 days. 48 hours after the last injection the mice were all placed in an air-tight chamber of 32 litres capacity containing soda-lime. When approximately half the mice had died the survivors were removed and the mortality-rate of each group noted. The method of calculating the

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BOOK REVIEWS

THE PLANT ALKALOIDS, by T. A. Henry. 4th edition, 1949, p.p. xxii + 783 and Index J. and A. Churchill, Ltd., London.

The international reputation of Henry's "Plant Alkaloids" is such that workers in many branches of science throughout the world will be grateful that Dr. Henry has been able to keep track of, digest, and present in his usual lucid and authoritative manner, the wealth of information that has been published on topics relating to the plant alkaloids and their analogues in the past decade since the third edition appeared. A large part of the volume has had to be rewritten and its bulk continues to increase. Not only have the years since 1939 brought to light a considerable volume of new knowledge of the occurrence and distribution of alkaloids in plants, of their biogenesis, isolation, purification and properties, chemical structure and pharmacological action, but they have also led to very extensive work on the preparation and study of synthetic analogues. Perusal of the edition under review has failed to reveal any important or less important publication having a bearing on plant alkaloids that has not received Dr. Henry's attention. As in the previous edition the material is classified primarily on the basis of nuclear structure although it is admitted that the basis adopted must necessarily be arbitrary in many cases where chemical complexity is such that the structures could have been accommodated under more than one nuclear heading. Two new groups have been required to accommodate new types encountered, namely the pyrrolizidine group comprising so far principally the necine derivatives found in *Senecio* species and the steroidal alkaloid group which includes the alkaloids of *Aconitum*, *Delphinium* and *Veratrum* species and the glucosidal alkaloids of *Solanum* species. The difficulty of keeping pace with additions to knowledge in this field is shown *inter alia* by the fact that while the text was going through the press evidence was published of the steroidal nature of the alkaloid conessine, derived from Kurchi bark which has recently attracted much attention from pharmacologists. A reference to the recent assignment of an *allopregnane* structure to conessine has been inserted in the introduction, though the *Holarrhena* alkaloids had necessarily to be dealt with in the body of the work under "Alkaloids of Undetermined Constitution." Alkaloids derived from an acridine nucleus have recently been isolated from certain members of the *Rutaceae* in Australia and, as Dr. Henry points out, a new group will require to be added to his classification to accommodate these. The literature on synthetic analogues of alkaloids is duly referred to and the repercussions of the extensive war-time work on synthetic potential antimalarials on the correlation of structure with action are taken into account. Due attention is paid to the biogenesis of alkaloids, including Robinson's recent discussions on strychnine and emetine.

In addition to the physical, physicochemical and chemical properties of something like two thousand alkaloids and their derivatives, references are included to the newer techniques applied to alkaloidal analyses, including micro-methods of detection and estimation and the application of procedures involving chromatographic and polarographic methods. All who are concerned, however remotely, with the plant alkaloids and related chemistry will require to ensure that this new edition, which is as carefully written and produced as its predecessors, is among their available works of reference.

F. HARTLEY.

LETTERS TO THE EDITOR

The Assay of Mersalyl

SIR,—Methods of assay of mersalyl [sodium salt of salicyl-(γ -hydroxymercuri- β -methoxypropyl)amide-O-acetic acid], phenylmercuric nitrate, and unguentum hydrargyri nitratis dilutum as proposed by Waterhouse¹ and modified by Pierce² have become official in the British Pharmacopœia, 1948. All involve reduction to metallic mercury under reflux, solution of the precipitated mercury in nitric acid, and titration with ammonium thiocyanate. It was found that erratic results were sometimes obtained in the assay of mersalyl, and also when this assay process was applied to injection of mersalyl. An investigation was therefore undertaken into the cause. Under the conditions of assay, mercury being to some extent volatile, it was found that vigorous boiling caused condensation of the metal on the cold part of the reflux condenser, mercury in such a form often being difficult to wash off. It is suggested, therefore, that glass jointed apparatus be used, and that after the reduction, the condenser should be washed with water and the precipitate transferred to the filter paper as directed. Nitric acid (20 ml.) and water (10 ml.) are then placed in the flask and refluxed in the apparatus for 10 minutes. The condenser is finally washed with 10 ml. of water, and the acid, after cooling, used to dissolve the zinc amalgam in the usual way, the assay then being completed in accordance with the directions given.

P. S. STROSS.
R. E. STUCKEY

The British Drug Houses, Ltd.,
City Road, London, N.1.
March 30, 1949.

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1. Waterhouse, *Quart. J. Pharm. Pharmacol.*, 1938, **11**, 458.
2. Pierce, *ibid.*, 1942, **15**, 367.

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slope of the dose-mortality curve was that described by Finney, allowances being made for the mortality rate of the control groups. The weighted mean slope was found to be 3.53 with a standard error of ± 0.76 . The other method is a graded response method based on the acute weight loss of rats following thyroxine injections. For this, adult male albino rats, weighing 220 to 280 g., were given drinking water containing 0.1 per cent. of thiouracil for 10 days prior to injection. They were then placed in individual cages and assigned at random to the various treatments; food and water were not restricted. Two subcutaneous injections of 0.25 to 2.0 mg. of thyroxine were given on successive days. The body weights were measured for several days before and after the injections. The data obtained from two experiments are presented and were subjected to an analysis of variance according to the method of Bliss and Marks (*Quart. J. Pharm. Pharmacol.*, 1939, **12**, 82, 182). The mean deviation of this method was 4.4 and the slope of the log dose response curve was 9.1; the value for λ was therefore 0.478. Though this method is easily performed in a very short time, a very large number of animals is necessary (400). The asphyxiation test is approximately as accurate and its precision is of the same order as that of other assays based on the all-or-none response.

S. L. W.

SCIENTIFIC MEETINGS

SOME ASPECTS OF PHARMACOLOGICAL CHEMISTRY

By F. BERGEL, D.Sc., F.R.I.C.

A Summary of Three University Lectures given in the Department of Pharmacology, University College, London. January-February, 1949.

ONLY the symptomatic drugs were considered in this series. The first lecture dealt with the significance of total chemical constitution and the theories of modes of drug action¹. Examples indicating constitution-action-parallelism were quoted, but it was emphasised that much more data were required to produce a more secure basis for a rational approach. In the next lecture the work on the analgesic substances, particularly on morphine, was discussed². On the third occasion the chemistry of the parasympathomimetics and parasympatholytics was considered. These drugs are based on acetylcholine, which is not strange to the organism. Acetylcholine has three pharmacological actions, muscarinic, nicotinic and under certain conditions, curare-like. As an ester, it is hydrolysed by the enzymes, cholinesterases of blood cells and plasma and transformed into the weakly active choline. Its re-synthesis may be due to the action of an acetylase, the co-enzyme of which may consist partly of pantothenic acid. Its existence was demonstrated by Feldberg and Mann in brain and by Bülbring and Burn in rabbit heart auricles. There exists also an inactive acetylcholine complex, the formation of which may partly explain the disappearance of the neurohormone. That it also occurs in plants was shown by Feldberg's discovery of acetylcholine in the stinging nettle. The instability and amphotropic properties of acetylcholine stimulated the pharmacological chemist to synthesise substances with more sustained and clear-cut action. When considering substances which, like acetylcholine, possess parasympathomimetic action, Pfeiffer's theory of prosthetic³ distances comes to mind, postulating an optimum distance between the N-methyl group and the two oxygen atoms. Thus it can be understood why three natural drugs—muscarine, pilocarpine, and arecoline—show, on the whole, lower activities and have never gained clinical importance. Virtual changes of the acetylcholine molecule itself have produced evidence that the free aminoalkanols are very much weaker and that, for the existence of full muscarinic action, the alcohol group must be esterified and the nitrogen carry at least two methyl groups. When the chain of the alcohol or the acid is elongated, activity falls considerably. There is one exception, and that is when the aminoalkanol chain is branched, as in mecholyl which shows strong muscarinic effect. Acetic acid has been exchanged against other acidic residues, such as carbamic acid in carbachol, thioacetic acid, etc. Transformation of the choline ester into choline ethers produces more stable but less active compounds. An ether-like product was made by Fourneau (2268 F) and found to be very potent indeed. Another ether-like compound is Esmodil which contains a double bond like arecoline and croton betaine methyl ester. The latter, carrying the nitrogen on the acidic side, is very much like acetylcholine though weaker. While choline itself shows very little activity, 3-hydroxymethylpyridine, the alcohol corresponding to nicotinic acid, possesses interesting parasympathomimetic properties. Other aromatic compounds with phenolic groups in place of alcoholic hydroxy groups, show remarkable properties, especially when esterified with carbamic acid. Such substances, like physostigmine, Prostigmine (neostigmine) are

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not only stable towards cholinesterase, but inhibit the action of cholinesterase on acetylcholine in a reversible manner. A similar action had been described for *bis-p*-dimethylaminobenzylacetone dimethiodide and for various alkyl phosphates, although the latter combine irreversibly with cholinesterase. The "stigmines" act against the effect of curare, but are seemingly not able to counteract the curariform action of *bis*-trimethylammonium decane.

While the nicotinic action of acetylcholine is antagonised either by ganglia-blocking agents, such as tetraethylammonium salts or *bis*-trimethylammonium pentane, or on the skeletal muscle side by curariform compounds, the muscarinic action of the cholinergic neuro-hormone and parasympathomimetics is antagonised by atropine. This drug, together with similarly acting compounds, named parasympatholytics, and the papaverine and anti-histamine group, form what one may call the larger group of anti-spasmodics or spasmolytics. There is hardly any representative of this group which does not possess a multiplicity of action but usually one predominates over the others, either the atropine-like, the musculotropic papaverine-like, or the histaminolytic. The parasympatholytics or anti-acetylcholine drugs represent mostly nitrogen-containing esters which, when Pfeiffer's theory of prosthetic distances is applied, show similar distances between the nitrogen-methyl group and the two oxygens to those of the protagonist group, the parasympathomimetics. This represents a very neat illustration of the receptor theory.

On looking through the list of drugs of the parasympatholytic group, we find that the acid part of the ester is "heavier" than acetic acid, Pfeiffer's umbrella effect being obtained by additional phenyl or hydroxy groups. The alkanolamine part need not be so complicated as the tropine of atropine, but can be represented by dialkylamino alkanols. The original tropic acid has been changed in time to mandelic, benzilic, diphenylacetic and fluorene carboxylic and dihydroanthracene carboxylic acids. Compounds prepared for different purposes, but showing, superficially, similarities with the atropine group, except for the fact that they are not esters, show varied amounts of parasympatholytic activities; but even compounds like Benadryl or diphenylpropylamines, without oxygen, still possess measurable anti-acetylcholine action in addition to anti-histamine and anti-barium activities.

REFERENCES

1. Bergel, *J. Soc. chem. Ind., Lond.*, 1949, in the press.
2. Bergel and Morrison, *Quart. Rev.*, 1948, **2**, 349.
3. Ing, *Science*, 1949, **109**, 264.

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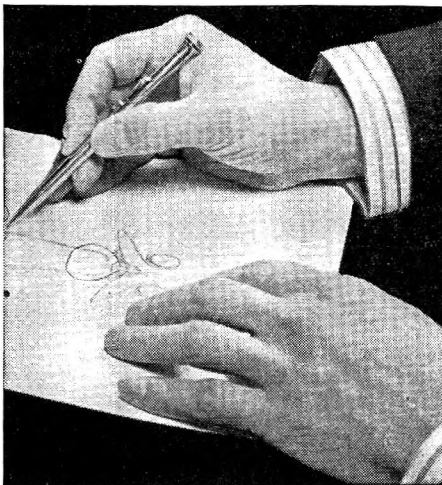


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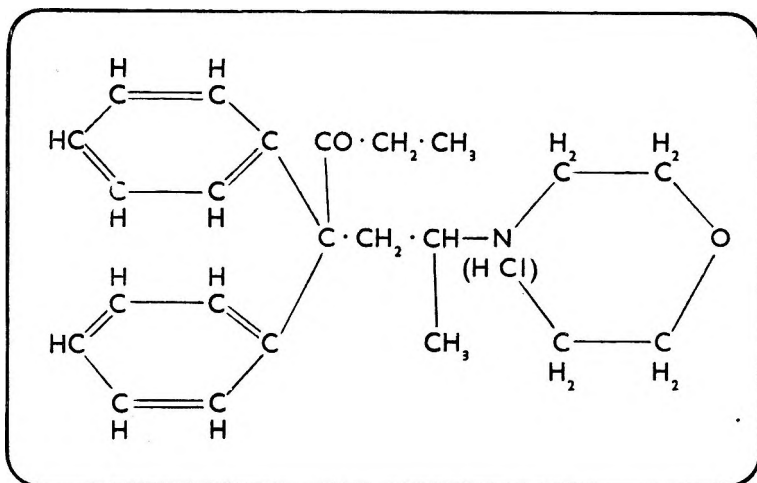
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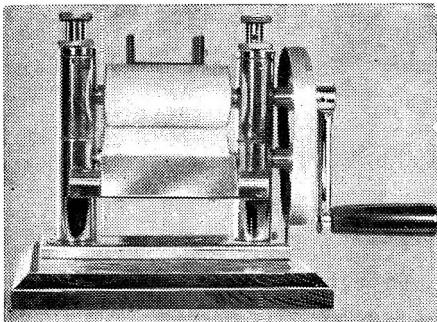
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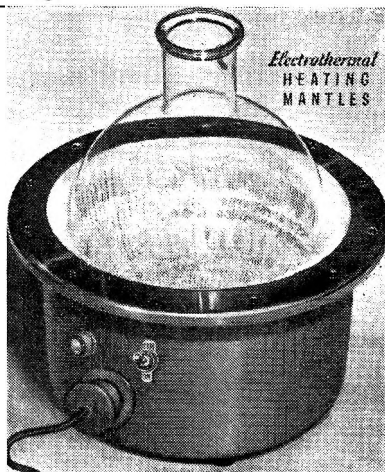
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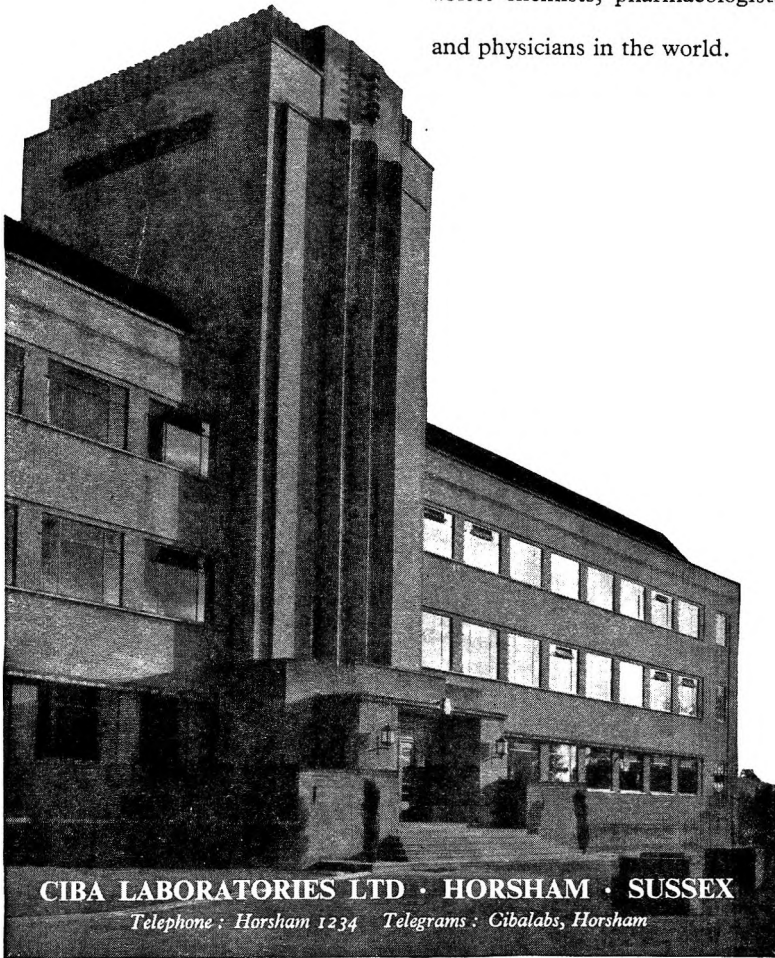


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