REVIEW ARTICLE

SOME CHEMICAL AND MEDICAL ASPECTS OF THE ANTIBIOTICS*

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THERE is no uniformity in the use of the word antibiotic, but we propose here to accept Waksman's definition that an antibiotic is a substance produced by one micro-organism which is capable of interfering with the growth of others[1]. This definition excludes synthetic drugs, such as the sulphonamides, isoniazid, and *p*-aminosalicylic acid, and also the antibacterial substances produced by higher plants.

The use of moulds for the local treatment of infected wounds has a long history in folk medicine, but these early remedies seem to have had little rational basis. Scientific observations on the inhibition of the growth of one micro-organism by the products of another were first made in the second half of the nineteenth century[2], and at that time there began the growth of the idea that such inhibitory products might be used, by injection into the blood stream, to combat systemic infections in man. A substance which is used in this way, as a systemic chemotherapeutic agent, must clearly be much more toxic to the infecting organism than to the cells of the human body, and it must retain its antimicrobial activity in the presence of the blood and body fluids.

During the first thirty years of the present century no chemotherapeutic agent which could safely be injected into the blood stream was obtained from a micro-organism. Ehrlich[3] and his school had striking success in the synthesis of drugs that were effective against diseases caused by protozoa, spirochaetes, or the malaria parasite, but their synthetic approach did not yield any compound which could be used to deal with bacterial infections. The view therefore arose that attempts to find substances with antibacterial activity but no significant toxicity to animals had little hope of success. This view was held when Fleming[4] discovered penicillin in 1929, and it may account for the fact that he did not try to establish whether the antibiotic could be used systemically in medicine. Penicillin thus remained scarcely more than a curiosity for ten years, but, during this time, the advent of the sulphonamides showed that the chemotherapy of systemic bacterial infections was indeed possible.

The investigations of Dubos and others in 1939 on tyrothricin[5], an antibacterial product of *Bacillus brevis*, were probably the first in which both the biological and the chemical properties of an antibiotic were carefully studied[6], but unfortunately tyrothricin was too toxic for systemic use. Soon afterwards, however, the work of Florey and Chain and their

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colleagues at Oxford[7,8] led to the discovery of the remarkable chemotherapeutic properties of penicillin. This discovery stimulated an intensive search by the pharmaceutical industry, especially by firms in America, for other antibiotics of therapeutic value. The American efforts have been rewarded, for most of the antibiotics now used in medicine, other than penicillin, have been isolated and characterised in the United States of America.

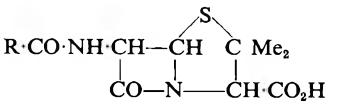
The present article will be mainly concerned with antibiotics of medical importance, but it should not be forgotten that this group of substances represents only a very small proportion of the vast number of antibiotics which have been detected, and that many of the antibiotics which do not have the exacting properties required of a chemotherapeutic agent are nevertheless of interest from a biochemical point of view.

Apart from their antimicrobial activity the antibiotics as a whole have little in common, but they can be classified, to some extent, on the basis of their biological or chemical properties, or the type of organism from which they come. We shall group them here according to their chemical structures, and it will be evident that some of the groups consist of families of closely related substances. In general, the order in which the various groups have been placed is the order in which they were discovered.

Structural Features of some Antibiotics of Medical Importance

The Penicillins and Polypeptide Antibiotics

The penicillins (I) are derivatives of a condensed dipeptide structure and may thus be grouped with other peptide antibiotics. The side-chain (R) of the penicillin molecule is variable, being Δ^2-pentenyl in penicillin F, benzyl in penicillin G, *p*-hydroxybenzyl in penicillin X and *n*-heptyl in penicillin K. A large number of different biosynthetic penicillins, in which R·CO is derived from a substituted acetic acid, have been obtained by adding suitable precursors to the fermentation medium of *Penicillium chrysogenum* or *Penicillium notatum*[9].



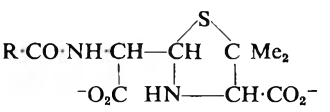
(I)

The collaborative Anglo-American work on the chemistry of penicillin during the last war[10] was followed by a period in which relatively little further progress was made. In recent years, however, there have been some new developments in this field. One of the biosynthetic penicillins (penicillin V) has been found to be much more stable than penicillin G in dilute acid solution and to be useful, in consequence, for oral administration[11]; a rational synthesis of penicillin V and certain other penicillins has now been developed[12]; a penicillin with a new type of side-chain and antibacterial activity, known as cephalosporin N[13] or synnematin B[14], has been discovered among the metabolic products of certain species of

Cephalosporium; and some light has been thrown on the way in which the molecule of penicillin G is formed by *Penicillium chrysogenum*[15].

The Penicillin Structure

Essential features of the structure of the penicillins are illustrated by the chemical behaviour of the penicilloates (II)[10], the products obtained when the penicillins are inactivated, by hydrolysis under mild alkaline conditions or in the presence of the enzyme penicillinase. When a solution of a penicilloate is treated with mercuric chloride a mercaptide is precipitated and one mole of carbon dioxide is liberated. The mercaptide can be decomposed with hydrogen sulphide to give the characteristic amino acid penicillamine. Penicillamine contains the sulphur atom of the penicillin molecule and belongs to the "un-natural" D-series.



(II)

The solution remaining after removal of the mercaptide contains an aldehyde, called penilloaldehyde, which is characteristic of the penicillin being studied. In general, the penilloaldehyde can be precipitated as its 2:4-dinitrophenylhydrazone. Oxidation of the penilloaldehyde with silver oxide yields an acid which can be hydrolysed with strong acid to give glycine, a product obtained from all penicillins, and substituted acetic acid which varies from one penicillin to another.

Consideration of these degradation reactions led to the proposal of a thiazolidine-4-carboxylate structure (II) for sodium penicilloate. Thiazolidine-4-carboxylic acids are readily formed by condensation of an aldehyde group with the nitrogen and sulphur atoms of an α-amino β-thiol acid such as penicillamine. Four stereoisomers of II, derived from D-penicillamine, are possible and by 1945 three of these isomers including one which is identical with the product obtained from penicillin, had been synthesised.

When these facts had been established it remained to formulate the structure of the penicillins by removal of the elements of water from the corresponding penicilloates. A number of physico-chemical arguments could be adduced in favour of the general structure I. The common penicillins have one carboxyl group but no basic group, while the penicilloates have two carboxyl groups and one weak basic group. The simplest way of accounting for the absence of a basic nitrogen atom in the antibiotic is to assume that a carboxyl group of penicilloic acid is condensed with the NH of the thiazolidine ring to give a four membered β-lactam ring. Today this may seem a plausible assumption, but when the β-lactam-thiazolidine structure for penicillin was first proposed in October, 1943[16], it was not generally accepted and other possibilities were given much attention. The question was only finally settled by the crystallographic X-ray studies of Crowfoot and Low[17], which showed

that the relative positions of the atoms in the molecule was consistent only with structure I.

Synthesis of Penicillins

In spite of the relative simplicity of the penicillin molecule, a satisfactory synthesis of a natural penicillin was not announced until the spring of 1957[12]. Workers both at Oxford and in America had undoubtedly synthesized benzylpenicillin in minute yields (0·1 per cent) during 1944[10], but a great deal of effort on both sides of the Atlantic failed to increase the efficiency of the process used. The main cause of the failure of attempts to form a β-lactam thiazolidine ring system by cyclisation of penicilloic acids lay in the fact that the latter readily underwent an alternative type of cyclisation characteristic of *N*-acylated α-amino acids: a carboxyl of penicilloic acid condensed with the C=O group of the side chain to give a five-membered azlactone (oxazolone) ring.

Sheehan and Henery-Logan have now shown that this difficulty can be avoided by use of the penicilloic acid corresponding to phenoxymethylpenicillin (penicillin V)[12]. Phenoxymethylpenicillin, one of a number of biosynthetic penicillins first obtained in the Lilly Research Laboratories[9], was found by Brandl and Margreiter in 1954 to be unexpectedly stable to acid[11]. It would seem that the structural features of the side-chain which are responsible for this acid stability also confer on the corresponding penicilloate a resistance to azlactonisation. By the action of di*cyclo*hexylcarbodi-imide on potassium phenoxymethylpenicilloate (II, R =  —) in aqueous solution at room temperature a β-lactam ring was formed and phenoxymethylpenicillin (penicillin V) (I, R =  —) was obtained in a yield of from 15 to 20 per cent. In contrast, an attempt to synthesise benzylpenicillin by the same method gave yields of only about 0·1 per cent.

During an extensive investigation of the problems of penicillin synthesis Sheehan and his colleagues have discovered methods for making new types of penicillin which have not yet been found in nature. These synthetic penicillins contain the fused β-lactam thiazolidine ring system of the natural products, but their side-chains are not derived from a substituted acetic acid. One of the new types contains a benzylsulphonamido (I, R·CO— is replaced by $R{\cdot}SO_2$) and another a carbamido group (I, R·CO— is replaced by RO·CO—) in place of the phenylacetamido group of benzylpenicillin[18].

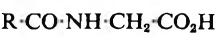
Cephalosporin N

In 1953 a new type of penicillin was discovered which was named cephalosporin N[19]. This antibiotic is produced by a fungus of the genus *Cephalosporium* which had been sent to Oxford from Sardinia[20]. It is distinguished from the natural and biosynthetic penicillins hitherto encountered by its marked hydrophilic character and by its antibacterial activity. It could not be extracted into any organic solvent other than liquid phenol and it is significantly more active than benzylpenicillin

against *Salmonella typhi* but very much less active against *Staphylococcus aureus*[21].

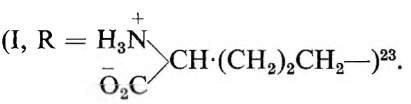
Cephalosporin N is inactivated by penicillinase and it yields the characteristic amino acid, D-penicillamine, on hydrolysis with acid. However, another amino acid, D-α-aminoadipic acid, is also found in the acid hydrolysate. Aminoadipic acid is an uncommon aminodicarboxylic acid and, although the L-isomer was known to occur in nature, the D-isomer had not previously been found.

Cephalosporin N, like other penicillins, could be degraded to D-penicillamine, carbon dioxide, and a penilloaldehyde. The penilloaldehyde, unlike that from the common penicillins, formed a 2:4-dinitrophenylhydrazone which was water-soluble and therefore failed to precipitate from solution. It was evetually identified, however, by oxidation to an acid which was isolated in crystalline form. This acid is a dipeptide of α-aminoadipic acid and glycine and was shown by degradation and synthesis to be D-δ-amino-δ-carboxyvalerylglycine (III)[22]. The aldehyde



(III)

is therefore D-δ-amino-δ-carboxyvalerylaminoacetaldehyde and cephalosporin N is (D-4-amino-4-carboxy-*n*-butyl) penicillin



This structure, with its zwitterionic side-chain, accounted for the hydrophilic character of the antibiotic.

While work on cephalosporin N was in progress at Oxford, an antibiotic produced by *Cephalosporium salmosynnematin*, which was called synnematin B, was being studied in the U.S.A. The chemical nature of this antibiotic had not been determined, but consideration of the published data suggested to us that the substance might well be very similar to cephalosporin N and therefore a hydrophilic penicillin[24]. In 1955 samples of cephalosporin N and synnematin B were exchanged and it was agreed that the two substances were identical[25]. Synnematin B was found to be a useful chemotherapeutic agent for the treatment of typhoid fever in man[26], but economic difficulties have so far hindered its production on a commercial scale.

Cephalosporin C

Examination of partially purified cephalosporin N revealed that it contained a small amount of another hydrophilic antibiotic which was named cephalosporin C. This antibiotic resembles cephalosporin N in some of its chemical and biological properties, but not in others. Cephalosporin C, like cephalosporin N, contains sulphur and a residue of D-α-aminoadipic acid that is linked to the rest of the molecule through its δ-carboxyl group. Unlike cephalosporin N, it is relatively stable in

dilute acid at room temperature and is not inactivated by purified preparations of penicillinase. Its ultra-violet absorption spectrum (ϵ max at 260 mμ) shows that it contains a chromophore which is not present in the normal penicillins. It yields no penicillamine on hydrolysis with acid, although it does yield valine after the sulphur atom has been removed by treatment with Raney nickel, and this indicates that it differs from the penicillins in the thiazolidine-β-lactam portion of the molecule[27].

Structural studies on cephalosporin C are not yet complete, but there can be little doubt that the substance is related, biogenetically, to cephalosporin N. Two of its biochemical properties are in harmony with this interpretation of the chemical evidence. Although not destroyed by penicillinase, it is a competitive inhibitor of the enzyme[28] and it induces the formation of the enzyme by *Bacillus cereus*[29].

Cephalosporin C shows about 0·1 per cent only of the activity of benzylpenicillin against many Gram-positive bacteria, but it is virtually non-toxic to animals and its insensitivity to penicillinase endows it with a higher activity than that of benzylpenicillin against penicillinase-producing strains of staphylococci. In adequate doses it gave complete protection to mice infected with a penicillin-resistant strain of *Staph. aureus*[30]. This antibiotic might thus find a place in medicine if the problem of producing it economically on a large scale could be overcome.

Biogenesis of Penicillin

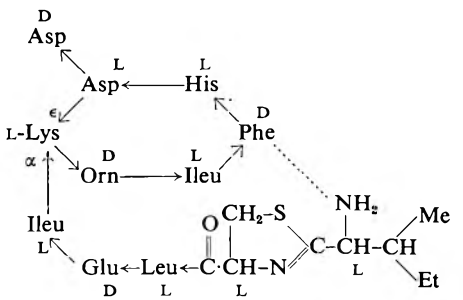
Inspection of the thiazolidine-β-lactam structure of the penicillins suggests that it is built up from two amino acids. Work with isotopically labelled amino acids has shown that the nucleus of benzylpenicillin is formed, in *Penicillium chrysogenum*, from an intact residue of L-cysteine and from the carbon skeleton, at least, of valine[31,32]. Since much of the valine added to the culture fluid undergoes deamination, or transamination, the origin of the nitrogen of the β-lactam ring is still uncertain, but it may be that L-cysteinylvaline is an intermediate. The present evidence is consistent with the view that the penicillin nucleus is synthesised first and is then acylated with phenylacetic acid. It has recently been suggested that the formation of the nucleus involves the $\alpha\beta$-dehydrogenation of the valine fragment of an intermediate β-lactam, and the subsequent addition of a thiol group to the double bond[33].

Cephalosporin N, with its amino acid side-chain, has a more obvious peptide nature than the common penicillins, and it may be regarded, perhaps, as a link between the latter and the larger polypeptide antibiotics. Like the penicillins, these polypeptides do not consist of open chains of amino acid residues but contain one or more ring systems. Most of them are too toxic to the kidneys to be injected into man, but two of them—bacitracin and polymyxin—have had a limited systemic use.

Bacitracin and Polymyxin

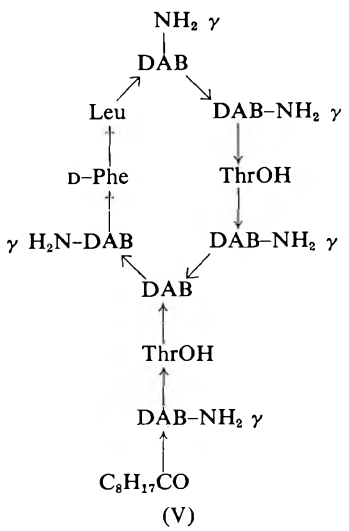
Bacitracin is the name given to a family of sulphur-containing polypeptides formed by *Bacillus licheniformis*[34,35]. The main peptide, bacitracin A, which has two acidic and three basic groups and yields a free

thiol group on mild hydrolysis with acid, has been assigned the provisional structure IV[36,37]. It has at least two ring systems, both of which are of an unusual type. One ring, made up of six amino acid residues, contains lysine linked through the ε-amino group of its aliphatic side-chain, and a branch from this ring arises from the α-amino group of the lysine. The second ring is a thiazoline which appears to have been formed by condensation of both the thiol and the amino group of a cysteine residue with the carboxyl-carbon of an *N*-terminal *iso*leucine residue (IV)[38]. Bacitracin A is the first protein-like substance to be discovered in which a masked thiol group can be attributed to the presence of a thiazoline ring.



(IV)

(The arrows represent C–N bonds and the dotted line symbolises an interaction whose nature is uncertain. An amide group is probably located at D–Asp.)



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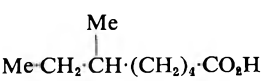


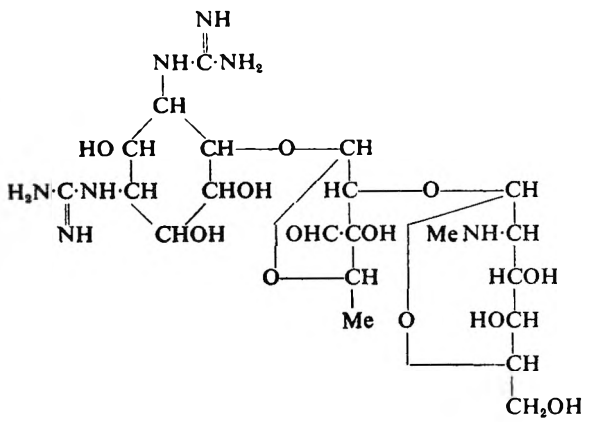
(VI)

Polymyxin B_1, a member of a family of polypeptides produced by *Bacillus polymyxa*, probably has the structure V[39]. It has no free carboxyl group, but contains five basic groups owing to the fact that five of its six residues of α,γ-diaminobutyric acid (DAB) have one of their NH_2 groups free. The sixth residue of DAB has both its α- and its γ-amino group bound, and forms a bridge between a ring and a side chain comparable

to that formed by lysine in bacitracin A. Another unusual feature of the structure of polymyxin B_1 is the *N*-acylation of the terminal DAB residue by the rare C_9 fatty acid, 6-methyloctanoic acid (VI). A residue of an *iso*octanoic acid is found in this position in another member of the polymyxin family known as polymyxin B_2.

The Streptomycins

In 1939 Waksman and his colleagues, who had made an extensive study of the actinomycetes, began to search among these organisms for antibiotic-producing strains[1]. After some initial disappointments they discovered streptomycin[40], and this substance quickly became established in medicine. Streptomycin, which is produced by *Streptomyces griseus*, is complementary to penicillin as a chemotherapeutic agent, since it is highly active against many Gram-negative bacteria and against mycobacteria.



Streptidine Streptobiosamine *N*-Methyl-L-glucosamine

(VII)

Streptomycin has the properties of both a strong base and a sugar, its basicity being accounted for by two guanidino groups and an *N*-methylamino group. It also contains a free aldehyde group, the reduction of which to an alcoholic group by catalytic hydrogenation results in the formation of dihydrostreptomycin.

Work on the structure of streptomycin[41] by several groups of organic chemists showed that the molecule could be dissected into three components which all had uncommon features. On treatment with methanolic hydrochloric acid the antibiotic was readily degraded to a strong base, streptidine, and a disaccharide, streptobiosamine, which was a moderately weak base. Streptidine was shown to be a diguanidinocyclitol, and since it was optically inactive it was a *meso*-form of this compound.

Streptobiosamine (see VII) was isolated as methylstreptobiosaminide dimethyl acetal. On further hydrolysis with acid, followed by acetylation of the products of hydrolysis, the acetal yielded a penta-acetyl hexosamine. Removal of the acetyl groups from the latter gave a compound

which was identified as *N*-methyl-L-glucosamine (see VII). The first hint of the nature of the remaining part of streptobiosamine was furnished by the isolation of the γ-pyrone, maltol, from the products obtained by heating streptomycin with hot alkali for a few minutes. The presence of another six-carbon fragment in the streptomycin molecule was thus clearly established. This fragment, which was named streptose, contained the aldehyde group of streptomycin and its conversion to maltol involved a carbon-carbon rearrangement.

The ease with which streptomycin was cleaved with acid strongly suggested that the streptidine, *N*-methyl-L-glucosamine and streptose fragments were joined by glycosidic linkages. After a great deal of careful work the positions of these linkages were determined and streptomycin was shown to have the structure VII.

A second antibiotic, resembling streptomycin, was found in crude material from *Streptomyces griseus*. This substance was isolated by counter-current distribution and shown to contain a residue of D-mannose in addition to the usual products of hydrolysis of streptomycin itself. It was therefore named mannosidostreptomycin. The D-mannose residue was shown to be glycosidically linked to carbon atom 4 of the *N*-methyl glucosamine residue[42].

It has been suggested that streptomycins are formed which are of the mannosido type but which have polysaccharides, in place of a simple hexose, attached to *N*-methylglucosamine[43]. If this turns out to be so, it may help to throw light on the relationship of the biogenesis of streptomycin to some of the essential metabolic processes of *Streptomyces griseus*.

The Neomycins

Neomycin[44], which is produced by *Streptomyces fradiae*, is active against a wide range of Gram-positive and Gram-negative bacteria and also against *Mycobacterium tuberculosis*. Neomycin appears to resemble streptomycin more closely than do the other antibiotics considered here, but it can easily be distinguished from streptomycin by its chemical and antibacterial properties. Thus, strains of *Mycobacterium tuberculosis* which are sensitive to both antibiotics remain sensitive to neomycin after they have become streptomycin-resistant[45]. Unfortunately, neomycin is too toxic to be used as a systemic chemotherapeutic agent, but it is used extensively for local application, and, in conjunction with other antibiotics, for pre-operative sterilisation of the gut[46].

Crude neomycin is a complex which has been shown to consist of two isomers called neomycins B and C[47]. These substances are bases, but, unlike streptomycin, they do not contain guanidino groups or give a maltol reaction. Neomycins B and C both have the molecular formula $C_{23}H_{46}N_6O_{12}$. When cleaved by methanolysis they yield the same base, neamine ($C_{12}H_{25}N_4O_5$), and the methyl glycoside of different disaccharides called neobiosaminide B and C respectively. The two neobiosamines are diaminohexosides of D-ribose and differ only in the diaminohexose portion of the molecule.

The partial structure VIII has been proposed for the neomycins[48].

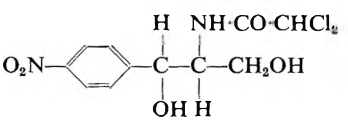
$$C_{12}H_{25}N_4O_5—O—C_5H_7O(OH)_2—O—C_6H_7O(OH)_2(NH_2)_2$$

(VIII)

Chloramphenicol

Chloramphenicol is not only quite different chemically from the antibiotics already described but also has a wider range of action against micro-organisms. Gram-positive bacteria, Gram-negative bacteria, and rickettsiae (which cause diseases such as epidemic typhus) are susceptible to its action. The term "broad spectrum antibiotic" has been coined to describe antibiotics of this type. Chloramphenicol, which is produced by *Streptomyces venezuelae*, was the first member of the type to be discovered[49,50]. Its isolation, characterisation and synthesis were carried out at the Parke Davis Research Laboratories.

Chloramphenicol ($C_{11}H_{12}O_5N_2Cl_2$) is a neutral substance with a characteristic ultra-violet absorption spectrum due to the presence of a *para*-substituted nitrophenyl group[51]. It is hydrolysed by alkali to dichloroacetic acid and an optically active base. The base yields *p*-nitrobenzaldehyde, ammonia and formaldehyde on treatment with periodate, whereas chloramphenicol itself does not react with periodate. Further investigation showed that the side-chain belonged to the *threo* configurational series. It could then be deduced that chloramphenicol was (−)-D-*threo*-2-dichloroacetamido-1-*p*-nitrophenylpropane-1 : 3-diol (IX)[51]. The validity of this structure was confirmed by synthesis[52].



(IX)

The synthetic (−)-D-*threo* isomer of IX was identical with the natural product, but the (+)-L-*threo* isomer had only 0·5 per cent of the antibacterial activity of the D-isomer and the two *erythro* forms were inactive.

The total synthesis of chloramphenicol represented the first efficient synthesis of an antibiotic of medical importance. There followed a period of great activity in the preparation of analogues of chloramphenicol. The antibacterial activity of many of these analogues has not been given, but it appears that changes in the aliphatic side-chain cause a drastic reduction in activity and changes in the aromatic portion of the molecule a smaller reduction. No analogue prepared so far has proved to be superior to the parent compound as a chemotherapeutic agent[53].

The Tetracyclines

Soon after the clinical value of chloramphenicol had been established two more broad spectrum antibiotics of great importance appeared in medicine. These substances were aureomycin, discovered in 1948 by a research team of the Lederle Laboratories[54], and terramycin, discovered

about a year later by workers at Chas. Pfizer Inc.[55] They were found to be active not only against bacteria and rickettsiae but also against some of the larger viruses, such as those responsible for psittacosis and lymphogranuloma venereum. Aureomycin ($C_{22}H_{23}O_8N_2Cl$) is produced by *Streptomyces aureofaciens* and terramycin ($C_{22}H_{24}O_9N_2$) by *Streptomyces rimosus*. It soon became evident, from a consideration of their chemical and physical properties, that these two antibiotics were closely related[56]. Both are amphoteric substances which contain two acidic groups (forming part of two conjugated enolic systems) and one basic group. The two nitrogen atoms can be accounted for as $-N(CH_3)_2$ and $-CONH_2$ groups respectively and one carbon is present as C-methyl. Other data suggest that both antibiotics contain a core, $C_{18}H_9O_4$, to which similar functional groups are attached, but that aureomycin contains a chlorine atom which appears as an OH group in terramycin.

Terramycin

Early attempts to degrade terramycin showed that, unlike streptomycin, it could not be split easily into a number of well defined fragments. It was found later, however, that a relatively simple procedure could be used to throw light on the nature of the C_{18} nucleus. When terramycin was exposed to the prolonged action of zinc and acetic acid, the dimethylamino group and one oxygen atom were removed to give desdimethylaminodesoxyterramycin. Treatment of the latter with acid under anhydrous conditions removed two molecules of water to give a crystalline red substance, called desdimethylaminoterrarubein, which was fully aromatic, and distillation of the terrarubein from zinc dust yielded the parent hydrocarbon, naphthacene[57]. The formation of this hydrocarbon was clearly consistent with the assumption that terramycin itself had a similar tetracyclic structure. The complete structure of terramycin[58] (X, R = H, R′ = OH) was eventually arrived at by skilful deductions based on the properties of a large number of degradation products, most of which had undergone molecular rearrangement during the degradation process. Much use was made of careful analyses of the absorption spectra of the two chromophoric centres in the molecule, and of the spectra of suitable model compounds. Subsequently, the results of degradations of aureomycin[57] were interpreted in the light of the structure suggested for terramycin[59].

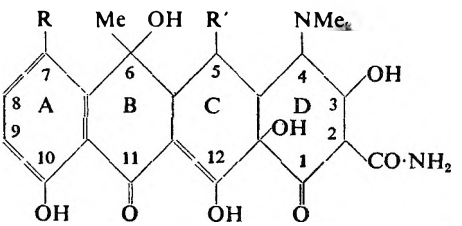
Aureomycin

The structural investigations on aureomycin confirmed the suggestion that this antibiotic only differed from terramycin in containing a chlorine atom instead of a hydroxyl group. It was realised at an early stage, however, that the chlorine atom in aureomycin and the corresponding hydroxyl in terramycin were located at different positions on the carbon skeleton[60]. A clue to the placing of the chlorine atom was the formation of 5-chlorosalicylic acid when aureomycin was fused with alkali. Salicylic acid, derived from the aromatic ring A of X, was obtained under similar conditions from terramycin. On the basis of this and other evidence the

chlorine atom of aureomycin was placed at C(7) and the antibiotic was assigned the structure X, R = Cl, R′ = H[57,59].

Tetracycline

Catalytic hydrogenation of aureomycin removed the chlorine atom and led to a product (X, R = H, R′ = H) which is now known by the generic name tetracycline[59–62]. Subsequently tetracycline was found to occur naturally in the culture fluid of a *Streptomyces*[63]. This substance may be regarded as the parent from which both aureomycin and terramycin are derived: aureomycin is a chlorotetracycline and terramycin an oxytetracycline. Tetracycline is similar to aureomycin and terramycin in biological activity and is itself used in medicine[64].



(X)

Demethyltetracyclines

A mutant of *Streptomyces aureofaciens* has recently been found which produces a tetracycline without a methyl group in the C(6) position (6-demethyltetracycline). The same mutant can also produce 7-chloro-6-demethyltetracycline when grown in the presence of chloride.

The demethyltetracyclines are very much more stable under acidic and alkaline conditions than the parent compounds, yet they retain most of the antibacterial activity of the latter[65]. If the demethyltetracyclines can be produced economically it is conceivable that they may replace the tetracyclines that are now in use.

The Macrolides

By 1950 most bacterial infections could be controlled either by the well established antibiotics, such as penicillin and streptomycin, or by the newer broad spectrum antibiotics. However, as time went on, the need was felt for substances which would be effective against strains of staphylococci that were resistant to penicillin or the tetracyclines. There followed an intensive search for chemotherapeutic agents active against Gram-positive bacteria and this revealed, among others, a large group of interesting antibiotics called the macrolides. These antibiotics, of which erythromycin is perhaps the best known, are found in the culture fluids of *Streptomyces*. Their range of activity is similar to that of penicillin, but they are active against penicillin or tetracycline-resistant strains of micro-organisms[66].

The term macrolide was coined to express the fact that each member of this group of substances has a large lactone ring. The macrocyclic lactone

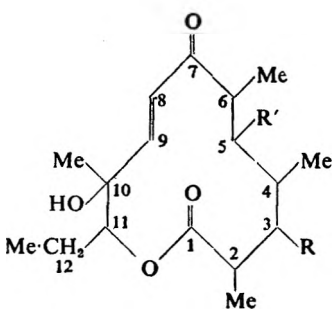
which contains from 12 to 17 carbon atoms, is linked glycosidically to one or more dimethylamino sugars. In some cases other pyranose sugars are linked to the lactone ring or to the dimethylamino sugar.

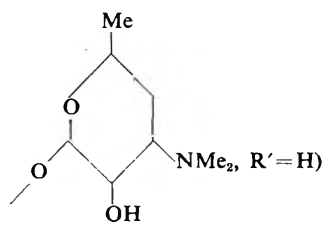
Table I shows the molecular formulae and the types of sugar that the various macrolides contain. Methymycin[67] and pikromycin[68,69] are isomers having very similar structures, and the foromacidines[76] and the spiramycins[77] have been shown to be identical. Several of the known macrolides contain the same dimethylamino sugar, desosamine, and at least two others contain a closely related dimethylamino sugar, mycaminose.

TABLE I

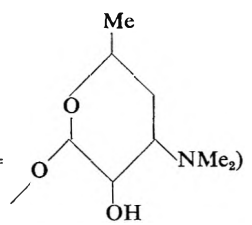
	Streptomyces Sp.	Molecular formula	Dimethylamino sugar	Other sugar	Macrolide ring	
					Name	No. of atoms
Methymycin[67]	M-2104	$C_{28}H_{49}O_7N$	Desosamine	None	Methynolide	12
Pikromycin[68,69]		$C_{28}H_{47}O_7N$	,,	,,	Kromycin	12
Narbomycin[70]	*narbonensis*	$C_{28}H_{47}O_7N$	,,			
Griseomycin[71,78]	*griseolus*	$C_{28}H_{45}O_8N$	,,			
Oleandomycin[72]	*antibioticus*	$C_{35}H_{61}O_{12}N$	,,	Oleandrose		
Erythromycin[73]	*erythreus*	$C_{37}H_{67}O_{13}N$	,,	Cladinose	Erythronolide	14
Magnamycin[74,75]	*halstedii*	$C_{42}H_{67}O_{16}N$	Mycaminose	Mycarose		17
Foromacidines[76] / Spiramycins[77]	*ambofaciens*	$C_{48}H_{82}O_{18}N_2$	Mycaminose and another[81]	Mycarose		
Angolamycin[79]	*eurythermus*	$C_{49-51}H_{87-91}O_{18}N$				
Miamycin[80]	*ambofaciens* (?)					

The foromacidines (or spiramycins) contain a second dimethylamino sugar, 5-dimethylamino-6-methyl-2-hydroxytetrahydropyran[81]. There is more variation in the nature of the non-nitrogeneous sugar component than in the dimethylamino sugar. Cladinose, found in erythromycin[73], and mycarose, in magnamycin[74] and foromacidine[76], are new sugars. But oleandrose, obtained from oleandomycin[72], had previously been found in the cardiac glycoside oleandrin[82].



(XI) (R = 

, R′=H)

(XII) (R=H, R′= 

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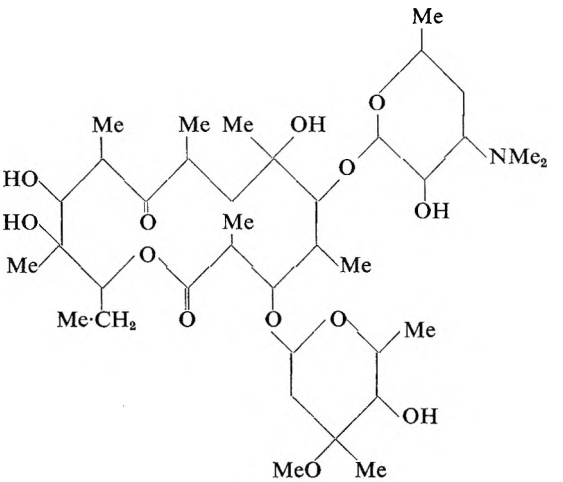
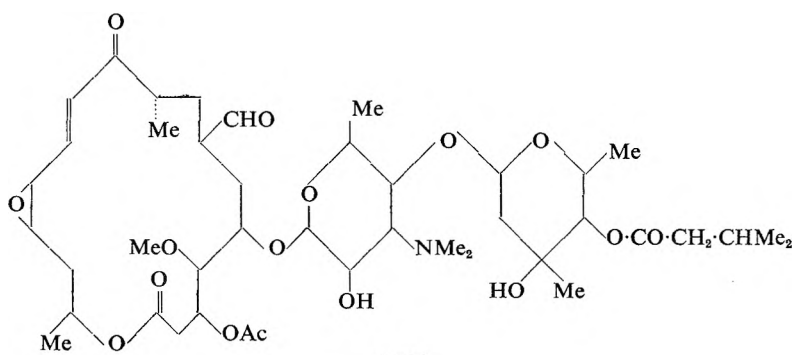
Complete structures have now been worked out for methymycin[67,83,84] (XI), pikromycin[68,69] (XII), erythromycin[85,86] (XIII) and magnamycin[87–89]

(XIV). The first two substances contain twelve membered lactone rings while the other two contain a fourteen and a seventeen membered ring respectively. Methymycin has a desosamine residue glycosidically linked to the lactone ring (called methynolide) at C(3). Neomethymycin[90], a



(XIII)

minor component of crude methymycin, has a hydroxyl group at C(12) instead of that at C(10) in methymycin. Pikromycin appears to differ from methymycin in that the desosamine is linked to C(5) of the methynolide. Erythromycin contains desosamine and also cladinose, each sugar being linked separately to the erythronolide core. Erythromycin B[91], found in



(XIV)

the mother liquor from the crystallisation of erythromycin, differs from the latter in having no hydroxyl substituent at C(12). Magnamycin, on the other hand, contains the dimethylamino sugar, mycaminose, linked directly to the macrolide ring and another sugar, mycarose, which is linked glycosidically to the mycaminose. The mycarose is acylated with an *iso*valeryl residue. Magnamycin B[92], which is found as a minor component in the mother liquors from the crystallisation of magnamycin,

differs from the major component in having a double bond between C(14) and C(15) in place of an epoxide ring.

The non-nitrogenous sugar which is found (in addition to the dimethylaminosugar) in erythromycin and magnamycin can be removed by mild methanolysis, leaving compounds which have been named erythrolosamine and carimbose respectively. Much more drastic conditions are required to remove the desosamine or mycaminose fragment from erythrolosamine or carimbose, since the dimethylamino group, which is positively charged in acid media, has a protective effect on the glycosidic linkage. Indeed, the amino sugar can be removed hydrolytically from erythromycin and magnamycin only under conditions which result in the break up of the acid-labile nucleus. Reduction of the C(9) carbonyl group of erythromycin led to the isolation of dihydroerythronolide, but no well defined product consisting only of the intact macrolide nucleus of magnamycin has been obtained. For these reasons the determination of the structures of the large lactone rings has been a difficult task. Nevertheless, the structure of methynolide was elucidated by Djerassi and his colleagues at Wayne State University[67,83,84] and later the structure of erythronolide was discovered in the Lilly Research Laboratories[85,86] and that of the lactone from magnamycin was determined by the combined efforts of chemists at the Pfizer Research Laboratories and Harvard University[87–89]. Somewhat later, the structure of kromycin (the macrolide from pikromycin) was determined by Brockmann and Oster[68] and by Anliker and Gubler[69].

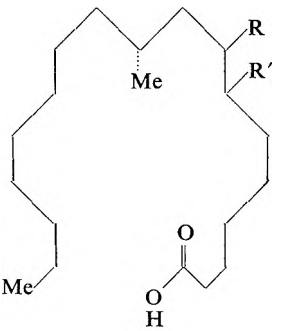
Although these macrocylic lactones are complex, it is likely that they are built up in nature by the condensation of units of acetic acid (and possibly propionic acid) to give polyketonic acids of the type XV, in which R is H or Me.

(XV)

This hypothesis is based on the way in which oxygen atoms (or functions such as double bonds which can be formed by the elimination of oxygen) and methyl groups are distributed in the rings. In methynolide we find oxygen on each odd numbered carbon atom except C(5) and in erythronolide on each odd numbered carbon except C(7). Moreover, in methynolide we find methyl groups on four of the even numbered carbon atoms and in erythronolide on all of them. The fact that it is possible to dissect the structure of erythronolide into seven consecutive three-carbon units, each responsible for a branched methyl group, has led to the suggestion that it may be possible to apply to some compounds a "propionate rule",[85] analogous to the "isoprene rules" which have facilitated the prediction of structures among the terpenes.

A lactone built from acetate or propionate units should have a carbon chain with an even number of atoms. The lactone ring of magnamycin, however, contains an odd number of carbon atoms. To overcome this difficulty Woodward has suggested that the aldehyde branch at C(7) is

formed by a pinacol-pinacolone rearrangement[88,89]. Thus, the hypothetical precursor of magnamycin, which has been called protomagnamycin[88,89], would have a C_{18} carbon skeleton (XVI, R = R′ = OH). This skeleton is very similar to that of the fatty acid, tuberculostearic acid (XVI, R = R′ = H), which has been obtained from the tubercle bacillus, the analogy even extending to the absolute configuration of the carbon atoms from C(10).



(XVI)

The common fatty acids are now known to be built up by the stepwise addition of acetic acid residues (in the form of acetyl coenzyme A) to give β-keto acids[93]. In some instances, at least, it seems that oxygen is eliminated from the β-carbon atom of the chain before each new acetate unit is added. However, the structures of the macrolides, and of a number of other natural products, indicate that the chain can also be lengthened while oxygen on odd numbered carbons is retained. The enzymic mechanisms which govern the varying degrees of oxygenation of these fatty acid-like substances are a subject for future biochemical investigation.

Novobiocin and Vancomycin

Recently, two new antibiotics which are highly active against the staphylococcus and against Gram-positive bacteria in general have been introduced into medicine. They have been named novobiocin and vancomycin. Although they probably have few chemical properties in common, they are placed together here as very little information about the chemistry of vancomycin has yet been published.

Novobiocin

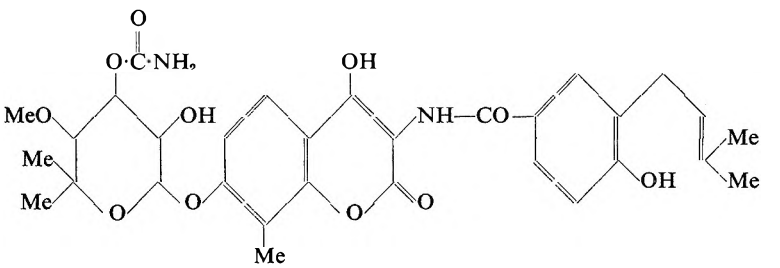
Novobiocin was discovered independently at the research laboratories of both the Upjohn Co., and Merck Sharp and Dohme. The Upjohn workers obtained the antibiotic from the culture fluid of *Streptomyces niveus* and originally called it streptonivicin[94]. The Merck group obtained the same substance from *Streptomyces spheroides* and named it cathomycin[95]. It is now apparently called novobiocin[96]. Novobiocin has been shown to have the structure[97–100] XVII. It is readily cleaved into three fragments by treatment with hot ethanolic hydrochloric acid. These fragments are a substituted benzoic acid, a substituted coumarin, and a

new sugar, noviose, respectively. The enolic group on C(4) of the coumarin moiety imparts an acidic character to the molecule.

At pH 10 novobiocin is converted in about 30 per cent yield to an isomeric product called *iso*novobiocin. Analysis of the equilibrium mixture shows that the carbamyl group in the noviose fragment has migrated from C(3) to C(2)[101]. This change is accompanied by the loss of about one third of the original activity.

Vancomycin

Vancomycin is produced by *Streptomyces orientalis* and has been obtained in a highly purified form at the Lilly Research Laboratories. It is an amphoteric substance with an isoelectric point of 5·0, and has a molecular weight of 3200–3500. Its hydrochloride contains 7 per cent of nitrogen and from 16 to 17 per cent of carbohydrate[102].



(XVII)

Polyene Antibiotics

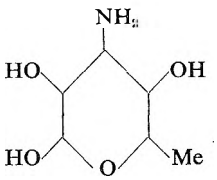
Although a number of human diseases, some trivial, some disabling, and others fatal, are caused by fungi, the progress of chemotherapy has been less rapid in this field than in others. The introduction of the broad-spectrum antibiotics into medicine raised the problem of fungal infections in a new form, because these antibiotics may eventually eliminate much of the normal bacterial flora of the gut, leaving the way open for pathogenic fungi to become established. The need to cope with this situation undoubtedly stimulated the search for antifungal antibiotics.

Since 1950 we have seen the discovery of a group of antifungal substances which are produced by streptomyces and have certain chemical features in common. The substances in this group contain a chromophoric centre characteristic of a conjugated polyene and are therefore referred to as polyene antibiotics[103]. Tetraenes, a pentaene, hexaenes and heptaenes with antifungal properties have been isolated. Nystatin[104] (fungicidin), produced by *Streptomyces noursei*, and amphotericin B[105], produced by another *Streptomyces* sp., are probably the best known members of the group.

Little information about the structure of nystatin and amphotericin B, apart from that provided by their ultra-violet absorption spectra, is yet available. Both substances are amphoteric and are practically insoluble in water. Nystatin, which contains a conjugated tetraene, has the molecular formula $C_{46}H_{77}NO_{19}$, and amphotericin, which contains a

heptaene, has the formula $C_{46}H_{73}NO_{20}$[76]. On prolonged acetolysis both antibiotics yield the same tetra-acetate of an aminodesoxyhexose, mycosamine[107] (XX).

In spite of their low solubility in water, both nystatin and amphotericin B are active against systemic infections when administered by mouth, and their toxicity, when they are given in this way, is low[108]. They appear to have been used successfully, by the oral route, to eliminate pathogenic fungi from the stools of patients in whom fungal infections of the gut had followed treatment with broad spectrum antibiotics[109].



(XX)

Chemical Structure and Antibacterial Activity

It will now be clear that the antibiotics form an extremely heterogeneous collection of substances and that no single type of chemical structure can be regarded as the seat of their antibacterial activity. Chloramphenicol and the tetracyclines provide an illustration of this point. These substances show a similar pattern of activity against a variety of bacteria and are classed in every clinician's mind as "broad spectrum antibiotics"; but it would be hard to find two types of compound having fewer structural features in common.

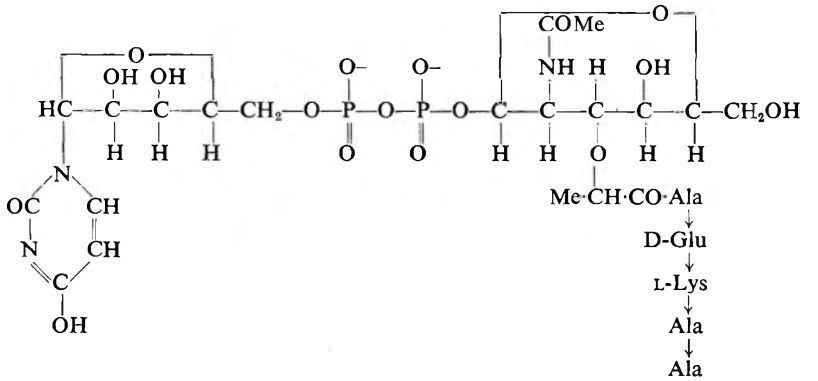
Nevertheless, the antibiotics appear to have a common property in the possession of structural features which have not so far been found in animal products. The penicillins contain the unique β-lactam-thiazolidine ring system and amino acids belonging to the D-configurational series. The polypeptide antibiotics also contain new ring systems and D-amino acids. Chloramphenicol is almost unique in possessing an aromatic nitro group and a dichloroacetyl group, and the tetracyclines have a naphthacene ring system which is not known to be formed by animal cells. Streptomycin and the macrolides contain new sugars. Large lactone rings, which are present in the macrolides, have also been found in the peptide antibiotics enniatin[110], etamycin[111], amidomycin[112] and valinomycin[113], but apparently not elsewhere in nature.

These peculiarities of the antibiotics are clearly reflections of structural and enzymic patterns peculiar to the micro-organisms themselves. The presence of such patterns makes chemotherapy possible, for a chemotherapeutic agent must interfere with some process which is vital in a pathogenic organism but is unimportant, or inaccessible, in animal cells, and it must also be resistant to destruction by the enzymes of animal tissues.

In general, we still know little about the specific biochemical reactions with which antibiotics interfere, but we are beginning to obtain some idea,

at least, of the kind of disorganisation that can be brought about by penicillin. Recent work has indicated that penicillin prevents the synthesis of bacterial cell walls[114], and that these walls are quite different, in chemical structure, from the outer membranes of animal cells.

The cytoplasm of a Gram-positive bacterium lies within a fragile membrane which is surrounded by a rigid wall. The contents of the cytoplasm appear to exert a high osmotic pressure, but the cell is prevented from bursting by the strength of its wall[115]. The cell walls of some organisms can be dissolved by the action of the enzyme lysozyme[116]. In ordinary media, removal of the wall is followed by the rupture of the cytoplasmic membrane and escape of the cytoplasm, but in media containing a high concentration of sucrose the membrane and its contents may survive as a spherical body called a protoplast[117].



(XXI)

Methods are now available for separating the bacterial walls from the remainder of the cell[118], and analysis of the walls of staphylococci has shown that they contain residues of D- and L-alanine, D-glutamic acid, lysine, glycine, glucosamine, and the *N*-acetyl derivative of a new amino sugar which is thought to be 3-*O*-carboxyethyl hexosamine[119] and has been given the trivial name "muramic acid". This new sugar is widely distributed in the walls of certain micro-organisms, but has not so far been found in other forms of life.

It has long been known that penicillin causes no damage to resting bacteria, but that sensitive organisms are killed, and often lysed, when they begin to grow in the presence of the drug[120]. More recently it has been found that certain growing bacteria are not lysed by penicillin in a medium of high osmotic pressure, but are converted to protoplasts, and that the latter undergo lysis when the medium is diluted with water[121]. These facts have led to the suggestion that bacteria growing in the presence of penicillin may die because they fail to maintain their rigid cell walls[114].

Evidence in support of this hypothesis has come from investigations of a different kind. Several years ago, Park[122] found that three uridine nucleotides accumulated in staphylococci which had grown for a short time in the presence of penicillin, while very little of these substances

were present in the normal cells. Later, Park and Strominger[114] showed that the principle nucleotide consisted of uridine diphosphate linked glycosidically to *N*-acetylmuramic acid and a peptide composed of D-glutamic acid, D- and L-alanine and L-lysine, and they proposed the provisional structure XXI for this compound. Further work established that the amino sugar and amino acids were present in the same molecular ratio in the nucleotide as they were in the staphylococcal cell walls[123]. Park and Strominger have therefore suggested that the uridine diphosphate *N*-acetylaminosugar-peptide is a precursor of the cell wall and that it accumulates in penicillin-treated staphylococci because the drug prevents the incorporation of the *N*-acetylaminosugar peptide fragment of the nucleotide into new cell wall material[114]. This leads to a picture of penicillin treated cells growing normally except that they soon fail to have enough cell wall to go round. In consequence, the wall can no longer protect the cytoplasmic membrane and cellular organisation is obliterated by lysis.

The polypeptide bacitracin also appears to interfere with the synthesis of bacterial cell walls. This antibiotic, like penicillin, is bacteriolytic to growing staphylococci[124] and can induce the formation of protoplasts in hypertonic media[125]. We have recently shown that the uridine nucleotides which accumulate in staphylococci treated with penicillin also accumulate in staphylococci treated with bacitracin, and that they do so in similar amounts when the two antibiotics are used in dilutions proportional to their antibacterial activities[126]. This finding does not allow us to conclude that the first effects of bacitracin and penicillin on the staphylococcus are identical, but it does suggest that the modes of action of the two antibiotics are very closely related. It is relevant to recall, therefore, that both bacitracin and penicillin contain highly-reactive, sulphur-containing ring systems, for the reactivity of these rings may well be responsible for the primary lesions which the substances produce in bacterial cells.

Therapeutic Achievements and Problems

The introduction of the antibiotics into medicine has revolutionised the treatment of infections caused by bacteria, spirochaetes, rickettsiae, and some of the larger viruses. Diseases which, twenty years ago, were always dangerous and often fatal can now be treated with every hope of success; the disability which resulted from many less serious diseases has been greatly diminished; and surgical procedures which would once have been attended by grave risk of bacterial infection can now be undertaken with confidence. It would be wrong to assume, however, that the antibiotics are ideal chemotherapeutic agents, even in circumstances in which they are normally used with success.

Although the chemotherapeutic antibiotics are much more toxic to pathogenic micro-organisms than to man, most of them are not entirely harmless to the body tissues. Penicillin is exceptional in being almost devoid of toxicity, in the normal sense of the word, to the patient. Nevertheless, this antibiotic can occasionally cause trouble by inducing sensitivity, for serious discomfort, or even death, may follow the injection of

the drug into sensitised people. Sensitivity to penicillin is not often encountered in this country, but its incidence appears to be as high as 5 per cent among hospital patients in some countries where there is no control over the sale of antibiotics to the public. However, allergic reactions to penicillin can often be controlled by antihistamine drugs and people who are sensitive to benzylpenicillin are frequently able to tolerate the biosynthetic penicillin O[127] (allylmercaptomethylpenicillin). Sensitivity is also encountered among persons treated with novobiocin, but it does not seem to be a problem with the other antibiotics in current use.

Streptomycin has a relatively low toxicity to man and can be used with some margin of safety, but if given in too large a dose over a prolonged period it may cause permanent damage to the eighth nerve with a resulting loss of the sense of balance[128]. Chloramphenicol has been given to many millions of people without serious ill effects, but in a few cases its administration has been followed by disorders of the blood, including fatal aplastic anaemia[129,130], and it has been suggested that the nitro group in the molecule has been responsible for these changes. The tetracyclines and the macrolides do not appear to show any substantial direct toxicity, but some of them are unpleasant to take, being liable to cause vomiting and diarrhoea. Moreover, prolonged administration of the broad-spectrum antibiotics is attended by the risk that pathogenic fungi and yeasts will replace the depleted bacterial flora of the gut[109].

In contrast to these substances, the polypeptide antibiotics bacitracin and polymyxin only just qualify for inclusion among the systemic chemotherapeutic agents, for they are nephrotoxic in doses no larger than those required to combat infections by sensitive bacteria[131]. These antibiotics are seldom used systemically unless other forms of therapy are unavailing and damage to the kidney can be justified by the severity of the disease.

Unfortunate though they are, the toxic effects of the antibiotics now used in medicine seem trivial when viewed against the saving of life and relief of suffering for which these substances have been responsible. A more serious matter has been the emergence of resistant strains among certain species of bacteria where sensitivity to an antibiotic was at first the rule.

When a micro-organism is subcultured in the laboratory in the presence of an antibiotic, its resistance to the antibiotic tends to increase. With some substances, such as streptomycin, this acquired resistance may rapidly attain a high value; with others, such as penicillin, it often rises gradually. In some cases, the change is brought about by the natural selection, in the presence of the drug, of a resistant mutant, and in others it may represent an adaptation which the drug itself induces[132].

It might have been imagined that bacteria would acquire resistance in patients under treatment with antibiotics, and that strains resistant to a new antibiotic would begin to replace sensitive strains soon after the introduction of the substance into medicine. Fortunately, the development of resistance in this way has not, with most organisms, been an important clinical problem; but with two species of bacteria, *Myco.*

tuberculosis and *Staph aureus*, it has been sufficiently serious to endanger the success of chemotherapy.

In the treatment of pulmonary tuberculosis with streptomycin the emergence of streptomycin-resistant strains of *Myco. tuberculosis* has been favoured by the need for prolonged therapy and the ability of bacteria to undergo large and sudden increases in resistance to the drug. During 1949–1950 the problem of resistance, together with the limitation imposed on the use of streptomycin by its toxicity, made it seem possible that this substance would fail to retain its position as an effective chemotherapeutic agent against chronic tuberculosis. However, streptomycin is still firmly established today. This is partly due to the finding that simultaneous administration of streptomycin and *p*-aminosalicylic acid (PAS)[133] or isoniazid[134] delays the emergence of resistant strains and enables smaller quantities of streptomycin to be used.

The problem of the resistant staphylococcus is more serious[30]. By 1949 a high proportion of the strains of staphylococci encountered in some hospitals were penicillin-resistant[135]. This resistance was of a special type, being due to penicillinase-producing strains of staphylococci; the penicillinase-producers survived in the presence of the drug, were carried on the skin and in the nasal passages of nurses and doctors, and passed from patient to patient. However, it was soon found that the staphylococcus became resistant to other antibiotics when the latter were used extensively in medicine. The discovery of the broad spectrum antibiotics greatly improved the position for a time, but today many strains of staphylococci isolated in hospitals are resistant to both penicillin and the broad spectrum antibiotics[136]. Moreover, a strain that has become resistant to one of the tetracyclines is commonly resistant to others. The macrolides, vancomycin, and novobiocin are now available to deal with such strains, but staphylococci readily acquire resistance to the erythromycin (macrolide) group of antibiotics and cross-resistance is found, in some degree, to all the members of the group[66]. A marked increase in the resistance of staphylococci to erythromycin was encountered in some American hospitals two years after the introduction of the drug[137] and it has therefore been recommended, in this country, that erythromycin should be held in reserve for the treatment of staphylococal infections which do not respond to other antibiotics[66].

Fortunately, other aspects of the staphylococcal problem are more hopeful. Preliminary reports on vancomycin[138,139] and novobiocin[140] are encouraging. These substances are active against staphylococci which are not sensitive to the well-established antibiotics; vancomycin, in particular, is bactericidal in high dilution and staphylococci do not readily acquire resistance to it *in vitro*[141]. Quite a different kind of substance, cephalosporin C, has been shown to protect mice infected with penicillin-resistant staphylococci[30]; although a penicillin-like compound, it is not destroyed by penicillinase[28], and the elucidation of its structure might lead to the production of more powerful substances of a similar type. Furthermore, the proportion of penicillin-resistant strains of staphylococci encountered in a hospital falls considerably when the administration of

penicillin is temporarily restricted[136,137]. Something may therefore be done by the careful use of the different antibiotics available to keep resistant strains in check.

A good deal of effort is now being expended on a search for antibiotics which will extend the range of infections that can be treated, and even for microbial products which will selectively inhibit the growth of human tumour cells[142]. Whether these efforts will be rewarding it is scarcely possible to predict. It is clear, however, that sustained success in some fields where chemotherapy is already well established has been dependent on the slow but steady introduction of new antibiotics into medicine. If this position is to be maintained new drugs will have to be forthcoming in the future. Of the many hundreds of thousands of strains of micro-organisms that have already been examined only a very small proportion have yielded antibiotics which have proved to be clinically useful. The chance of finding further antibiotics able to cope with infections caused by an organism such as the staphylococcus must now decrease as each new substance is discovered. But in the long run, this law of diminishing returns may be offset, to some extent, by an increase in our knowledge of the factors which govern the biological activity of the antibiotics that have already been isolated and characterised. Although little progress has so far been made by the organic chemist in his efforts to improve the antimicrobial substances produced by micro-organisms, we may have seen, in the production of certain new synthetic penicillins[20], the beginning of one successful chapter in this field. Certainly, further studies of the mode of action of antibiotics already in use and of the mechanisms by which micro-organisms become resistant to them will contribute to our understanding of some of the fundamental aspects of microbiology. In doing so they may eventually help to make the rational design of new chemotherapeutic substances possible.

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RESEARCH PAPERS

THE QUANTITATIVE SEPARATION OF PAPAVERINE FROM NARCOTINE IN MIXTURES

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A method for the quantitative separation of papaverine from narcotine in mixtures by using ammonium reinckate is described, and also its application to the determination of these alkaloids in opium. The identity and purity of these alkaloids isolated from opium is shown by means of ultra-violet, visible and infra-red spectrophotometry. The complete extraction of these alkaloids from the triturate of opium by chloroform was demonstrated by paper chromatography. The regeneration of pure papaverine from the reineckate complex is also described.

VARIOUS methods have been proposed for the separation and determination of papaverine and narcotine which are usually isolated from opium in admixture. Plugge[1–3] separated papaverine from narcotine by precipitation of the former with potassium ferricyanide and decomposition of the resulting hydroferricyanide with dilute sodium hydroxide solution. The method gave gummy precipitates when applied to opium[4]. Isolation of papaverine as the acid oxalate was described by Hesse[5] but this procedure is useful only as a means of purification of papaverine.

Annelar reported a method based upon the opening of the lactone ring of narcotine with alcoholic potash to form the soluble potassium narcotinate followed by the extraction of papaverine[6]. A modification of this method was adopted as the official method for the analysis of these alkaloids in papaveretum by the British Pharmaceutical Codex 1954. We found that this method did not give quantitative recoveries even when applied to the determination of mixtures of pure drugs. A brief description of the use of ammonium reineckate for the quantitative separation of papaverine from narcotine has recently been reported by us[8] and it is the purpose of this paper to describe the method in detail.

EXPERIMENTAL

The Separation and Determination of Papaverine and Narcotine in Mixtures of the Drugs

Reagents. (i) Papaverine m.p. 145 to 146°; (ii) Narcotine m.p. 175 to 176°; (iii) Chloroform (Analar); (iv) Acetone (Analar); (v) 0·1N hydrochloric acid; (vi) Ammonium reineckate—approximately 2 per cent solution prepared by dissolving 2 g. of ammonium reineckate in 100 ml. cold water and filtering through a Whatman No. 42 paper. This solution is stable in a refrigerator for about a week and it should be filtered before use if precipitation has occurred; (vii) Crystal violet in 0·5

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per cent solution prepared by dissolving 0·5 g. in 100 ml. glacial acetic acid; (viii) Standard perchloric acid in 0·05N solution is prepared by dissolving about 7·15 g. of 70 per cent perchloric acid in 200 ml. glacial acetic acid. Thirty ml. of acetic anhydride are added and the solution diluted with glacial acetic acid to 1/1. After a day this solution is standardised by the method of the B.P. 1958, page 809; (ix) Silver nitrate—1 per cent aqueous solution.

Procedure. Mixtures of varying amounts of papaverine 6 to 32 mg., and narcotine 30 to 150 mg., are dissolved in 70 ml. of chloroform in 250 ml. flasks fitted with ground glass stoppers. Thirty ml. 0·1N hydrochloric acid and 10 ml. ammonium reineckate solution is then added and the resulting solution is shaken mechanically for 30 minutes. The solution is cooled in the refrigerator for a further 30 minutes and filtered through sintered glass with suction. The papaverine reineckate in the filter is washed with three 5 ml. portions of cold water and the residue dried by suction. The filtrate is set aside for the estimation of narcotine.

Determination of Papaverine

The stem of the funnel containing the papaverine reineckate is rinsed with a little acetone to remove water and is then placed in a second dry suction flask. About 5 ml. of acetone is poured on to the papaverine reineckate. After the reineckate salt has dissolved, the solution is collected under gentle suction. The process is repeated with fresh 1 ml. portions of acetone until the effluent is colourless. The red coloured acetone solution is quantitatively transferred to a volumetric flask and diluted with acetone to exactly 10 ml. or 25 ml. volume, depending on the amount of papaverine reineckate present which is judged by the intensity of the colour of the original solution. The solution is shaken and the optical density determined at 525 mμ in a spectrophotometer using acetone as the blank. The amount of papaverine present can be calculated by means of a calibration curve obtained under similar conditions or by using the following equation:

$$w = \frac{A}{110{\cdot}0} \times M \times \frac{v}{1000}$$

where w = weight of papaverine in mg.
A = observed optical density using one cm. cell.
M = molecular weight of papaverine in g. (339·4).
v = volume in ml. in which reineckate complex is dissolved.
110 = ϵ (gram-molecular extinction coefficient for papaverine reineckate).

Determination of Narcotine

The chloroform : water filtrate obtained after the filtration of papaverine reineckate is transferred to a separating funnel, and the chloroform layer is separated. The suction flask is rinsed with 25 ml. of chloroform which is used to re-extract the alkaloids from the aqueous layer in the separatory funnel. The aqueous solution is then rejected.

The red-coloured chloroform which contains the narcotine is placed in a separatory funnel and to it is added 30 ml. of water and 10 ml. of 1 per cent silver nitrate solution. This solution is shaken until the chloroform layer becomes colourless. The chloroform is separated from the aqueous layer which is further extracted with two fresh 10 ml. portions of chloroform. At this stage vigorous shaking should be avoided to prevent the formation of a suspension of silver reineckate. The combined chloroform fractions are filtered through a funnel plugged with cotton wool. The filtrate is collected in a 250 ml. volumetric flask and made up to volume with chloroform. The narcotine can then be determined by two methods.

Method A—spectrophotometric determination. The optical density at 310 mμ of the chloroform solution or a diluted solution is determined by means of the spectrophotometer. The molecular extinction coefficient of narcotine in chloroform at 310 mμ is 4750.

Method B—determination by non-aqueous titration. An aliquot of the chloroform solution containing at least 20 mg. narcotine is evaporated to dryness in an evaporating dish. The residue is dissolved in 25 ml. of glacial acetic acid and 3 ml. of acetic anhydride and this solution is titrated with standard perchloric acid using crystal violet as the indicator.

Extraction of Papaverine and Narcotine from Opium

Opium 4·5 g. is triturated with 25 ml. of glacial acetic acid for 15 minutes followed by 20 ml. of water, and the resulting mixture is filtered through a Whatman No. 42 paper. This amount of opium is used to provide sufficient volume of filtrate for replicate determinations. A 10 ml. aliquot of the filtrate is extracted successively with 10 ml. portions of chloroform, each of which is passed through a series of separating funnels containing water, sodium hydroxide, sulphuric acid and aqueous sodium bicarbonate solution shown below.

Separatory funnel	Containing
No. 1	Opium filtrate (10 ml. opium solution in acetic acid)
No. 2	15 ml. water
No. 3	15 ml. water and 15 ml. 1 : 1 sodium hydroxide and a few grains sodium bisulphite
No. 4	15 ml. water
No. 5	15 ml. 0·1N sulphuric acid
No. 6	10 ml. 0·1N sulphuric acid
No. 7	10 ml. water and 0·5 g. sodium bicarbonate.

The process is considered completed when two drops of chloroform taken from separatory funnel No. 4 gives no yellow colour when tested for thebaine with syrupy phosphoric acid.

The chloroform extracts are combined (about 70 to 80 ml.) and evaporated on a hot water bath. Unnecessary heating of the dry residue is avoided to prevent decomposition of the alkaloids. The residue is then dissolved in about 50 ml. of carbon tetrachloride and passed through a funnel plugged with cotton wool to remove insoluble impurities. The resulting filtrate is passed through a column of calcium hydroxide using suction which removes further impurities from the carbon tetrachloride

solution leaving the papaverine and narcotine in solution. The calcium hydroxide column is then washed with two 10 ml. portions of carbon tetrachloride. The combined carbon tetrachloride fractions are then shaken with two 20 ml. portions of 0·1N hydrochloric acid in a separatory funnel. A small portion of the carbon tetrachloride solution is then tested for the complete removal of papaverine and narcotine with Frohde's reagent and the persulphate reagent, respectively. When negative tests are obtained this carbon tetrachloride solution is reserved for redistillation.

Determination of Papaverine and Narcotine

The hydrochloric acid fractions from above are combined in a 250 ml. stoppered flask and to it is added 70 ml. of chloroform. This mixture is shaken for 10 minutes and then 10 ml. of ammonium reineckate solution added and the resulting mixture shaken mechanically for 30 minutes. The solution is cooled in the refrigerator for a further 30 minutes and then filtered through sintered glass with suction. The papaverine reineckate which is collected on the filter is dissolved in acetone and determined as described previously.

The narcotine present in the chloroform-water filtrate is also determined in the manner described for the determination of pure drugs.

Discusion of Results

Principles of the Method

The reaction between a base and ammonium reineckate, in acid solution, can be represented by the following equation:

$$BHX + NH_4R \longrightarrow NH_4X + BHR$$

However, narcotine in the presence of excess chloroform does not form an insoluble reineckate, whereas papaverine does, and this difference in chloroform solubility forms the basis of a method of separation of papaverine and narcotine. Table I shows the recoveries obtained using various proportions of papaverine and narcotine.

Under the conditions described earlier, the red coloured chloroform solution obtained after papaverine reineckate had been removed could not be used for direct narcotine determination, since no linear relation was found between concentration and absorbance. Of sixty reineckates which have been studied only narcotine reineckate showed this chloroform solubility behaviour. It was further observed that the red colour attributed to the reineckate can be removed by shaking the chloroform with water. However, the complete removal of the colour could be effected only by shaking the chloroform with silver nitrate solution. The silver reineckate, insoluble in both phases, can be removed by filtration.

The Separation of Papaverine and Naroctine from Opium

The extraction procedure described separates the major opium alkaloids into three groups, (A) morphine and codeine which remain in the acid-containing separatory funnels (1) and (2); (B) thebaine which is retained in the sulphuric-acid-containing funnels (5) and (6); (C) narcotine and

papaverine which are present in the chloroform. They are then determined by the procedure previously described. For ultra-violet absorbance measurements, it was found necessary to remove other ultra-violet absorbing materials present in the solution of the papaverine and narcotine by redissolving them in carbon tetrachloride after evaporation of the

TABLE I

RECOVERIES OF PAPAVERINE AND NARCOTINE FROM MIXTURES

Papaverine and narcotine used		Papaverine (recovered as the reineckate complex)			Narcotine recovered as the free base	
Amount of papaverine used (mg.)	Amount of narcotine used (mg.)	Amount of acetone used in dissolving the reineckate complex (ml.)	Observed optical density "A"	Amount of papaverine recovered (mg.)	Amount of 0·502N aceous perchloric acid used (ml.)	Amount of narcotine recovered (mg.)
6·7	42·4	10	0·218	6·7	2·06	42·5
9·0	65·4	10	0·295	9·1	3·15	65·2
10·8	79·9	10	0·344	10·6	3·86	80·0
11·65	174·4	25	0·152	11·7	8·36	173·2
14·5	32·4	10	0·470	14·5	1·54	31·8
17·0	74·0	10	0·550	17·0	3·52	72·9
20·95	146·4	25	0·269	20·8	7·0	145·0
25·6	101·6	25	0·339	26·2	4·88	101·0
28·6	107·0	25	0·368	28·4	5·18	107·1
32·35	81·1	25	0·423	32·6	3·92	81·1

TABLE II

DETERMINATION OF NARCOTINE AND PAPAVERINE IN OPIUM*

	Narcotine		Papaverine
	Non-aqueous titration	Spectrophotometric determination	
	mg.	mg.	mg.
Indian export sample ..	63·4	62·1	6·2
	63·6	61·7	6·3
Yugoslavian sample ..	63·7	62·8	29·1
	63·9	64·3	29·4

* 10 ml. acetic acid-opium filtrate used equivalent to about 1 g. opium.

chloroform. Further purification was achieved by passing the carbon tetrachloride through a column of calcium hydroxide; using a final back extraction by means of dilute (0·1N) hydrochloric acid to ensure spectral purity of the drugs. At this stage the separation of the papaverine and narcotine was made in the same way as mixtures of the pure drugs. The results shown in Table II illustrate the amounts of papaverine and narcotine recovered from replicate volumes (10 ml.) of the "aliquots" obtained from 4·5 g. of each of two authenticated United Nations opium samples. The agreement between replicates is good. However, the agreement between duplicates has been found to be less satisfactory. This is attributed to irregularities in the initial extraction of opium by acetic acid.

In order to ascertain that complete papaverine and narcotine extraction from the aqueous acetic acid solution has been achieved, paper chromatographic experiments were made on the contents of the various separatory funnels previously listed. The results of this experiment showed the absence of papaverine and narcotine in all the funnels.

TABLE III

RECOVERIES OF NARCOTINE AND PAPAVERINE FROM OPIUM*

Alkaloids	Anneler's method	Proposed method
	mg.	mg.
Narcotine ..	58·3	63·5
Papaverine ..	28·7	29·8

* 10 ml. acetic-acid-opium filtrate used equivalent to about 1 g. opium.

Papaverine and narcotine are not separated by the *iso*butanol-acetic acid-water mobile solvent used for chromatography. They travel close to the solvent front and comprise the leading spot in the mixture. For a comparison of results of papaverine and narcotine recovered by the Anneler method, given in the United Nations document[7], and the proposed method, Table III should be consulted.

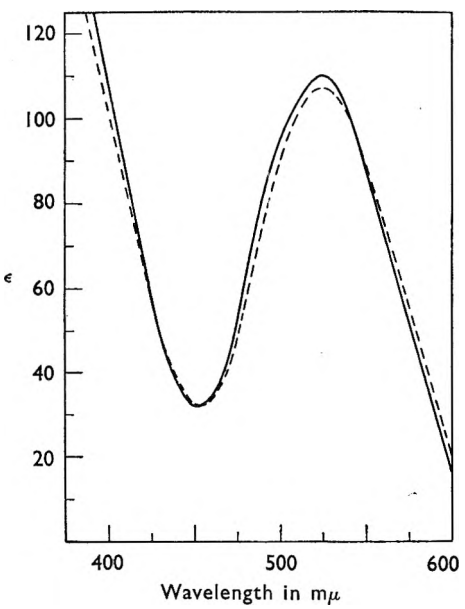

FIG. 1. Ultra-violet curves of papaverine reineckate. — Papaverine reineckate (standard), - - - papaverine reineckate (from opium). Solvent: acetone.

The proposed method gives significantly higher results than Anneler's method which may be explained by loss of narcotine through decomposition. Both alkaloids recovered from the Anneler procedure are found to be spectrally impure. The acetone solution of papaverine reineckate produced by the Anneler method very often has an orange tinge, and the narcotine solution cannot be determined by spectrophotometric means.

Purity of the Extracted Papaverine and Narcotine Fractions

To establish the purity of the papaverine and narcotine separated by this method the physical properties of each drug isolated from opium was established. The spectra of the drugs and their reineckates in the visible, ultra-violet and infra-red regions were measured.

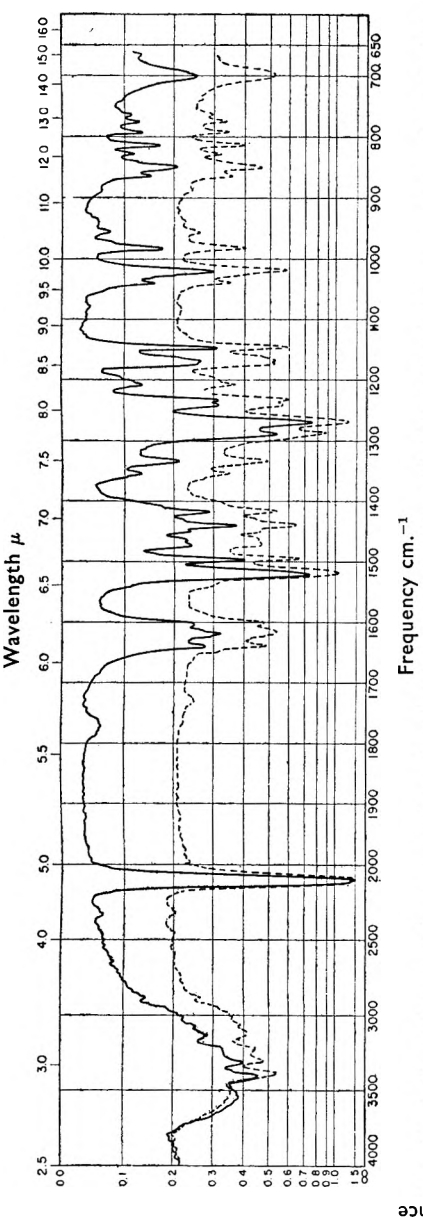

FIG. 2. Infra-red curves for papaverine reineckate. — papaverine reinekate (standard), - - - papaverine reinekate (opium).

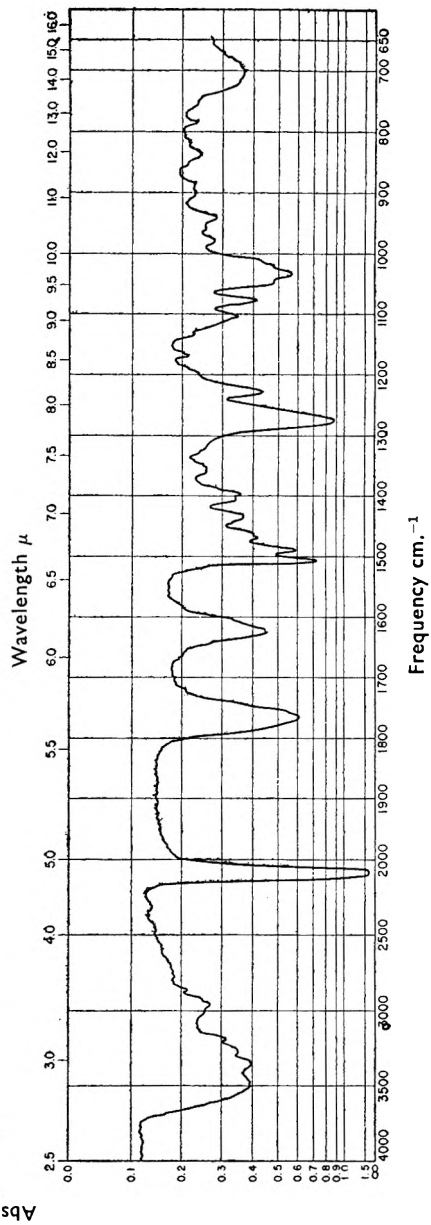

FIG. 3. Infra-red curves of narcotine reineckate.

Spectral Curves of Papaverine Reineckate (Fig. 1)

The ammonium reineckate spectrum (acetone solution) in the wavelength region 350 to 600 mμ is the same when all opium alkaloid reineckates are measured including narcotine reineckate. Indeed, this curve appears to be generally the same for reineckate derivatives of most bases. Papaverine reineckate is exceptional in its spectral behaviour since it shows no maximum in the spectral curve at 395 mμ for papaverine reineckate (Fig. 1).

The infra-red spectra of papaverine reineckate and narcotine reineckate were obtained using the pressed potassium bromide pellets technique. In Figure 2 the infra-red spectra of papaverine reineckates obtained from opium and the pure reference drugs are compared. The two spectra are identical in all features. Figure 3 shows a narcotine reineckate infra-red spectrum obtained from an authenticated sample of the drug. This shows a strong absorption band at 1760 cm.$^{-1}$ which is due to the carbonyl group of the lactone ring. This band is absent in the infra-red spectrum of papaverine reineckate. The possibility of using infra-red spectrophotometry for the quantitative estimation of mixtures of papaverine and narcotine is also shown.

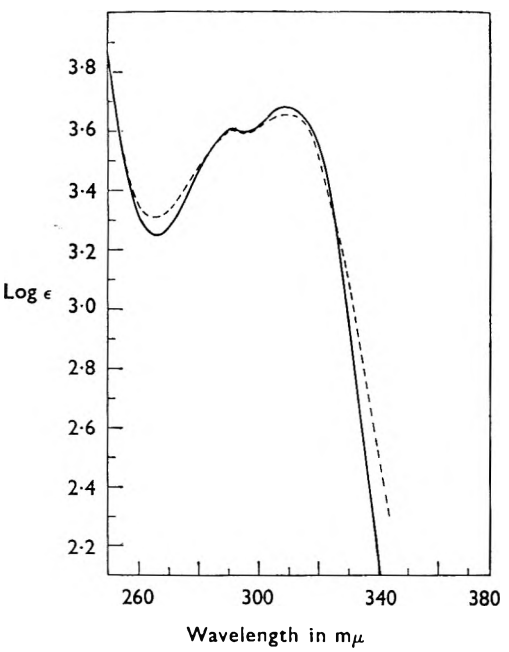

FIG. 4. Ultra-violet curves for narcotine. — Narcotine (standard), - - - narcotine (from opium). Solvent: chloroform.

The Ultra-violet Spectrum of Narcotine from Opium

In Figure 4 the purity of narcotine in the chloroform solution obtained after the separation of papaverine from narcotine by this method is shown. The spectral curves were obtained by means of a Cary (Model 11 N) recording spectrophotometer. To obtain the concentration of narcotine in the chloroform, a non-aqueous titration of a portion of the chloroform for narcotine was made. This concentration was used for calculation of the logarithm of the molecular extinction coefficient (log ε) employed in Figure 4. The standard narcotine log wavelength curve in chloroform is also shown. The spectra are practically identical. The absence of additional maxima or minima in the spectral curve of the opium isolate compared with the standard is a clear indication of its purity.

Isolation of Pure Papaverine and Narcotine from Opium

Pure narcotine and papaverine were extracted from opium by the method described. To obtain pure narcotine the chloroform solution containing the alkaloid after the separation of the papaverine was evaporated and the resulting crystals were dissolved in ethanol (95 per cent) and recrystallised twice. The melting point (175 to 176°) was determined by means of the Fisher John's melting point apparatus. A mixed melting point with standard narcotine showed no depression.

Pure papaverine was obtained from the reineckate by treating an acetone solution of the latter with silver nitrate and filtering the resulting mixture. The aqueous-acetone filtrate containing papaverine was extracted with chloroform. Evaporation of the chloroform gave a residue which on recrystallisation with ethanol yielded pure papaverine base m.p. 145 to 146° alone and mixed with an authenticated sample. The papaverine thus obtained gave no colour with concentrated sulphuric acid which indicates the absence of cryptopine.

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VEGETABLE PURGATIVES CONTAINING ANTHRACENE DERIVATIVES

PART VIII.—THE PAPER CHROMATOGRAPHY OF CERTAIN ANTHRAQUINONES AND THEIR GLYCOSIDES

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A simple paper chromatographic technique, using toluene alone as a running solvent, is described for identifying free anthraquinone compounds in crude drugs. A second system is described for anthraquinone glycosides: the running solvent is the lower aqueous phase of a water:acetone:benzene mixture. This system was found particularly successful for cascara bark *Rhamnus purshiana* D.C. as it readily revealed the presence of at least three anthraquinone glycosides.

METHODS of estimating the amount of glycosidal and free anthraquinones in several drugs have been published in this series[1–3]. They were designed to evaluate crude drugs and their extracts but did not enable individual glycosides to be estimated separately. As we wish to obtain more information on the distribution and quantities of individual anthraquinone compounds in these drugs we have investigated the possible application of paper chromatography. As a result suitable methods of separating and identifying some of the individual substances have been developed.

About seven papers have been published on the paper chromatography of the anthraquinones, mainly by the Japanese workers Shibata, Tsukida and Takido[4–8]. In all instances the glycosides are first hydrolysed with acid and then the liberated aglycones (free "anthraquinones) are examined by paper chromatography. The published methods therefore deal in practice with the paper chromatography of the free anthraquinones only. We decided not only to investigate these methods but also to attempt to devise suitable methods for separating the individual glycosides without previous hydrolysis. By this means they could be eluted from the paper in virtually the same form in which they occur in the living plant.

EXPERIMENTAL METHODS AND RESULTS

FREE ANTHRAQUINONES

Samples of aloe-emodin, aloe-emodin anthranol, chrysophanol, emodin, and rhein were prepared, authenticated and used to test the published methods. Fair agreement was found between our R_F values and those of the Japanese workers although the latter used "Toyo" papers while we used "Whatman" papers and sometimes our solvents were slightly different. But a number of irregular results were obtained and the methods were found to suffer from some defects. (*a*) A confusing number of light petroleums of boiling ranges from 45° to 110° is quoted. Each

fraction results in a different R_F value for a given anthraquinone compound. (*b*) Some of the solvent systems are very volatile and quick-running and therefore susceptible to slight variations in temperature. (*c*) It was almost impossible to avoid entrainment of one phase in another when preparing certain solvent systems; this entrainment also caused results to vary. (*d*) The spots frequently showed bad tailing.

Method Recommended for Free Anthraquinones

We found the most satisfactory of the solvent systems previously used to be toluene saturated with water. Further work on this system showed that toluene alone was equally effective; at first we assumed that the paper contained enough moisture to saturate the toluene during use, but when paper which had been dried in an oven for a few hours was used equally satisfactory results were obtained. Results can be obtained

TABLE I

R_F VALUES OF PURE ANTHRAQUINONES, USING TOLUENE AS THE MOBILE PHASE

Anthraquinone compound	R_F values*			
	Whatman paper No. 1	Whatman paper No. 20	Whatman paper 3 MM.	Japanese workers Toyo No. 131
Chrysophanol	0·98 (0·99)	0·91 (0·96)	0·98 (0·99)	(0·99)
Aloe-emodin	0·65 (0·80)	0·58 (0·65)	0·65 (0·80)	(0·87)
Aloe-emodin anthranol ..	0·66 (0·84)	0·56 (0·65)	0·67 (0·88)	—
Emodin	0·40 (0·50)	0·32 (0·40)	0·40 (0·50)	(0·63)
Rhein	0	0	0	(0·03)

* R_F values in brackets were measured from the front of the spot.

after one hour with a simple ascending technique. The paper after removal from the tank and drying is sprayed with a 0·5 per cent solution of magnesium acetate in methanol and heated at about 90° for a few minutes; anthraquinone compounds become pink to red in daylight and ultra-violet light. Table I shows the R_F values obtained with pure anthraquinones using toluene as the mobile phase, three varieties of Whatman paper and a range of temperatures between 18° and 22°. Whatman No. 20 paper gives a better separation as it slows down this quick-running solvent more than the other papers.

"Tailing" of the spots still occurred which makes this method unsuitable for quantitative work but if care is taken not to overload the paper the "tailing" can be sufficiently reduced to measure the R_F values in the usual manner. Where "tailing" was pronounced the R_F value was measured from the front of the spot as was done by the Japanese workers. In Table I we record both R_F values for each anthraquinone. The figures quoted are the average of several determinations which showed some variation, but the relative position of the spots was always the same. The ratio of the R_F values was also fairly constant, for example that for aloe-emodin and emodin was about 1·7 to 1. The R_F values for chrysophanol and rhein are inconvenient, values greater than 0·9 or less

than 0·1 being unreliable; fortunately rhein has a more suitable value in the next solvent system we discuss and Hillis[9] quotes an R_F value of 0·71 for chrysophanol. He used Whatman 3 MM paper at 22° and his running solvent was methanol saturated with *n*-heptane at 10°. We have confirmed this observation but found that aloe-emodin and emodin have similar R_F values to each other and to chrysophanol. However, the system can be used to confirm the presence of chrysophanol by eluting the spot from the toluene chromatogram and running it in this second system.

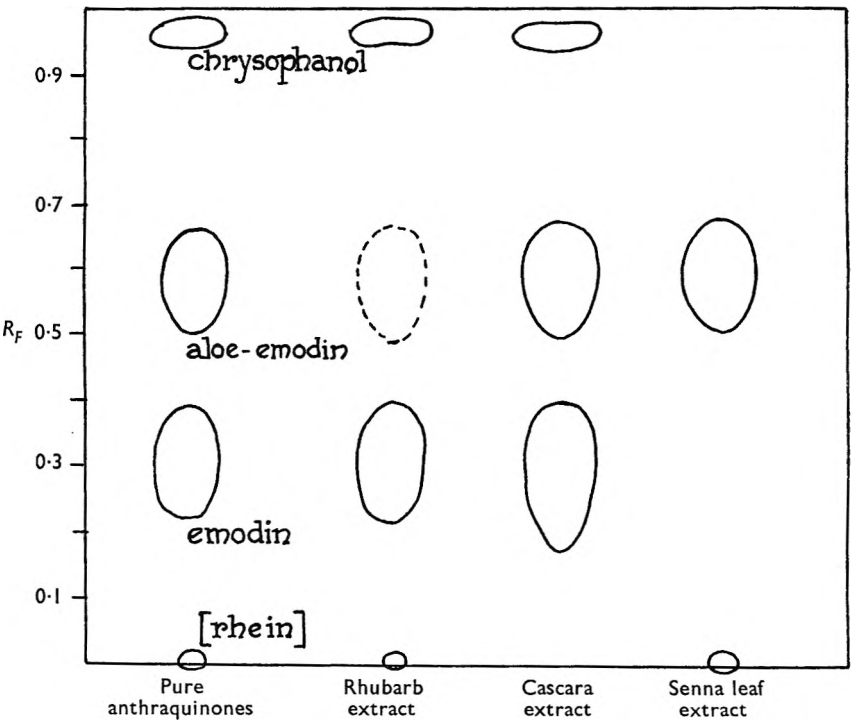

FIG. 1. Paper chromatogram of free anthraquinones and of suitable extracts of certain crude drugs. Running solvent, toluene. Whatman No. 20 paper. Temperature about 20°.

Application to Crude Drugs

The modified Bornträger test[10] was applied to powdered samples of cascara bark, rhubarb and senna leaf and the resulting benzene solutions were treated by the method described above. The results are shown in Figure 1 and indicate the free compounds present in these crude drugs.

COMBINED ANTHRAQUINONES

Danilovic[11] has published a paper chromatographic method for dealing with the glycosides as such, and he claims to be able to separate and identify the glycoside frangulin. He used the system *iso*amyl alcohol : *iso*-butyric acid : acetic acid : water. This system was found by us to be

unsatisfactory as unless the proportions were carefully controlled the four liquids formed one phase; even when two phases formed there was marked entrainment of one phase in another. The resulting chromatograms showed tailing of the spots and when the method was applied to cascara extracts no glycosides were visible on the chromatograms. Krogerous and others[12] also have published an account of the chromatography of frangula glycosides using a single phase solvent mixture of butanol: acetic acid: water. This system achieved a separation of glycosides from cascara extracts but did not give such good separation as the one we describe below. Furthermore, some of the glycosides are decomposed by the acid solvent system.

Method Recommended for Combined Anthraquinones

Our observation that rhein frequently had an R_F value similar to some of our glycosidal material led to a re-investigation of Takido's water: acetone: benzene system[6], which had proved satisfactory for rhein, and we found that, after suitable modification, it was successful for the combined anthraquinones present in cascara bark, aloes and to a lesser extent for senna pod, senna leaf and pure sennosides. The R_F values for some free anthraquinones in this system were also determined. The details are as follows. Water, acetone and benzene (2:1:4) are shaken vigorously and allowed to separate. The lower aqueous phase (the running solvent) is placed on the bottom of a chromatographic tank and a beaker containing the upper phase is also placed in the tank, which is then sealed, and allowed to come to equilibrium overnight. Meanwhile a small quantity of the substance to be investigated is transferred to the starting line of a chromatographic paper which is then placed in the tank, preferably as a cylinder, with its lower end resting in the aqueous phase. The paper is allowed to remain in the re-sealed tank for 2–3 hours or until the solvent front has reached a suitable height (ascending technique). The paper is removed, dried and sprayed with 0·5 per cent magnesium acetate in methanol and heated at about 90° for a few minutes. Combined anthraquinones became yellow to orange when sprayed, and appear in colours ranging from yellow to red in ultra-violet light. Whatman No. 1 and 3 MM papers were suitable but with Whatman No. 20 paper the solvent ran slowly. The R_F values quoted are therefore those obtained using Whatman No. 1 or 3 MM paper.

Application to Cascara Bark

Applied to cascara bark (*Rhamnus purshiana* DC.) the method revealed the presence of at least three anthraquinone glycosides or similar compounds.

A quantity of powdered bark was exhausted with chloroform to remove free anthraquinones, chlorophyll, etc., and dried. When the dried marc was extracted with water and this solution chromatographed by the method described two glycosidal spots were observed and were named Compound A ($R_F = 0{\cdot}87$) and Compound B ($R_F = 0{\cdot}74$). When a methanolic extract was treated in a similar manner spots corresponding to

Compound A and a third glycoside, Compound C ($R_F = 0{\cdot}45$) were revealed. These three substances were shown to contain anthraquinone compounds by preparing adequate quantities of each, using band chromatography and cutting out the appropriate bands, which were then eluted with methanol. A portion of each methanolic solution was heated in 3N HCl at 98° for 15 minutes and a second portion was heated in 3N HCl, containing 25 per cent of ferric chloride at 98° for 15 minutes. Each aqueous portion was extracted with carbon tetrachloride and the latter solutions treated by the method of paper chromatography for the free anthraquinones. Both Compounds B and C produced free anthraquinones by direct hydrolysis in acid, but Compound A produced free anthraquinones only after ferric chloride oxidation[13].

TABLE II

APPLICATION OF THE PROPOSED PAPER CHROMATOGRAPHIC METHOD TO ANTHRAQUINONE GLYCOSIDES

Compound	R_F value
Sennoside A, "free"	0·96
Sennoside A, Na salt	0·86
Sennoside B, "free"	0·96
Sennoside B, Na salt	0·90
Rhein	0·75
Rhein, Na salt ..	0·69
Aloin (m.pt. 142–5°)	0·66
Emodin	0·26*
Aloe-emodin ..	0·18*
Chrysophanol ..	0·0

* Values measured from front of spot.

Application to other Anthraquinone Glycosides

The method was applied to certain pure glycosides and a few free anthraquinones and the results obtained are shown in Table II.

When applied to senna pod and leaf, spots corresponding to the sennosides were observed. However as the R_F values are rather high it was concluded that the method is not as suitable for investigating the glycosides of senna as it is for those of cascara.

DISCUSSION

This work was undertaken in order to obtain a more detailed picture of the anthraquinone compounds present in certain drugs, than could be obtained by our earlier methods of investigation. The two methods have enabled this to be done on a qualitative basis and we have found them particularly useful in the investigation of cascara bark constituents. The methods are simple and results can be obtained fairly quickly; this is particularly true of the method for free anthraquinone using toluene alone as the running solvent. Another important advantage is the fact that the methods require only small quantities of material which makes it possible to use small samples from the living plant for biochemical investigations. No attempt has been made to make the method quantitative but we believe that, by carefully controlling the conditions, the

methods described could be used for quantitative estimations. An example of this is the recent work of Paris and Durand[14], who have devised a quantitative paper chromatographic method for aloin; they controlled the conditions carefully so that the spots were sufficiently well defined to be measured by a suitable densitometer.

The range of compounds we have examined is small but the results indicate that the R_F values are related to chemical structure. In both systems (Table I and II) the order is rhein, emodin, aloe-emodin and chrysophanol, rhein having the highest R_F value when a polar running solvent is used and chrysophanol having the highest value when a non-polar solvent is used. These properties run parallel with the reactivity of the substituent in position 3 of the molecule (β-position). All four compounds have two α-hydroxyl groups but rhein has a carboxylic group in the β-position; emodin an hydroxyl, aloe-emodin a primary alcohol and chrysophanol a methyl group. It is known that a β-hydroxyl group is more acidic than an α-hydroxyl[15] so that the order rhein, emodin, aloe-emodin, chrysophanol appears to represent an order of decreasing acidity, or reactivity of the β-hydroxyl group. This would be expected to affect the solubility in polar and non-polar solvents in the same order.

Acknowledgements. We would like to express our sincere thanks to the Westminster Laboratories Ltd. for generously providing a maintenance grant to one of us (V. K. M.).

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THE DETERMINATION OF MERCURY OXYCYANIDE

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The reaction between mercuric oxycyanide and hydrochloric acid, in the presence of certain salts, has been examined. The reaction between potassium thiocyanate and mercuric oxide in aqueous solution appears to go to completion. The reaction between mercuric cyanide and potassium iodide (or sodium thiosulphate) is complete in the presence of formaldehyde. These reactions form the basis of a method for the determination of mercuric oxycyanide which is considered more satisfactory and accurate than that in the British Pharmacopoeia, 1953.

The method of determination of mercuric oxide in mercuric oxycyanide is due to Holdermann[1], who showed that, in the presence of chloride ions, direct titration of the oxide with decinormal hydrochloric acid (methyl orange indicator) was possible.

$$HgO + 4Cl^- + 2H^+ \rightleftharpoons HgCl_4^{--} + H_2O \quad (1)$$

Tagliavini[2] extended the titration to include a determination of mercuric cyanide, by adding potassium iodide after completion of the titration of mercuric oxide.

$$Hg(CN)_2 + 4I^- \rightleftharpoons HgI_4^{--} + 2CN^- \quad (2)$$

Continued titration with decinormal hydrochloric acid enables this reaction to proceed more or less completely to the right, since the cyanide ions are removed as the slightly ionised hydrogen cyanide. Methyl orange indicator is again used. This is the method described in the British Pharmacopoeia, 1953.

Rupp[3] described a modification in which sodium thiosulphate replaced the potassium iodide.

$$Hg(CN)_2 + 2S_2O_3^{--} \rightleftharpoons Hg(S_2O_3)_2^{--} + 2CN^- \quad (3)$$

He recommended the use of methyl orange (pH 2·8–4·0) or methyl yellow (pH 2·8–4·0).

TABLE I

Titration of cyanide in mercuric oxycyanide with 0·1N hydrochloric acid, in the presence of potassium iodide, using methyl orange indicator

Volume of acid added (ml.)	pH (glass electrode)	Indicator colour
40·3	4·57	Yellow
40·4	4·23	Orange-yellow
40·5	3·88	Orange
40·6	3·58	Orange
40·7	3·35	Orange-red
40·8	3·29	Red

Methyl red indicator (pH 4·2–6·3) was used by Vieböck[4], who adapted the reaction between mercury oxycyanide and halides to determine chloride and bromide resulting in the acid digestion of halogen-containing organic compounds.

Using the procedure of the British Pharmacopoeia, 1953, we have experienced difficulty in detecting the end point, especially in the cyanide determination, as a result of slow change of pH with added acid. Consequently the methyl orange indicator slowly changes colour during the addition of about 0·5 ml. of 0·1N hydrochloric acid (Table I). This is presumably due to buffering caused by the presence of hydrogen cyanide and a change of indicator fails to provide a remedy. The present paper suggests a method to avoid the difficulty.

Experimental

Determination of Mercuric Oxide

The reaction between the oxide portion of mercuric oxycyanide and sodium chloride was investigated by titrating with 0·1N hydrochloric acid (i) 50 ml. of solution of mercuric oxycyanide and (ii) 50 ml. of a solution of mercuric oxycyanide with the addition of 1 g. of sodium chloride. The pH changes were recorded potentiometrically using a glass electrode. The results in Figure 1 show that the first sign of a colour change with methyl red should give a suitable indication of the end point.

The pH of the solution, after addition of sodium chloride, was not higher than pH 9·5, suggesting that the reaction of equation (1) does not go to completion (Fig. 1). The substitution of potassium thiocyanate[5] for sodium chloride was considered, since mercuric thiocyanate ionises only to a slight extent (Equation 4). The titration with 0·1N hydrochloric acid of a solution of mercuric oxycyanide containing 1 g. of potassium thiocyanate is also recorded in Figure 1. The curve approximates closely to that obtained by titrating with 0·1N hydrochloric acid 50 ml. of a carbonate free N/70 (approx.) potassium hydroxide solution containing 1 g. of potassium thiocyanate, thus the reaction

$$HgO + 2SCN^- + H_2O \rightleftharpoons Hg(SCN)_2 + 2OH^- \quad .. \quad .. \quad .. \quad (4)$$

proceeds almost completely to the right.

The end point is well marked by the change to the full yellow colour of phenol red. Any precipitation of mercuric thiocyanate can be disregarded. It is desirable to remove dissolved carbon dioxide from the solution before the addition of potassium thiocyanate.

Determination of Mercuric Cyanide

The reaction of the mercuric cyanide of mercuric oxycyanide with (i) potassium iodide and (ii) sodium thiosulphate was examined. To the neutral solution obtained above was added 2 g. of each of these in turn. The resultant solution was titrated with 0·1N hydrochloric acid, pH changes being recorded potentiometrically using a glass electrode. The results (Figure 2) show that in both cases the end point is not sharp, and that neither methyl orange nor methyl red can be used to indicate it properly.

Since the unwanted buffering must be caused mainly by the presence of the weak acid, hydrogen cyanide, it was considered that its removal would

not only reduce buffering, but also encourage the completion of the reaction,

$$Hg(CN)_2 + 4I^- + 2H^+ = HgI_4^{--} + 2HCN.$$

Attempts were made, by titration in the presence of small amounts of water soluble carbonyl compounds to find a convenient reagent capable of removing hydrogen cyanide. Of these, acetone and formaldehyde showed the most promise (Fig. 2). Even with excess hydrochloric acid, no odour of hydrogen cyanide was perceptible in the presence of acetone

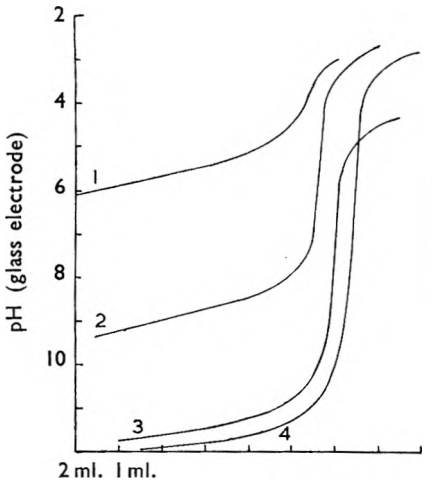

FIG. 1. Titration of oxide in mercuric oxycyanide. Curves originate at 0.5 ml. intervals.

1. 50 ml. mercuric oxycyanide solution.
2. 50 ml. mercuric oxycyanide solution containing 1 g. sodium chloride.
3. 50 ml. mercuric oxycyanide solution containing 1 g. potassium thiocyanate.
4. 50 ml. N/70 potassium hydroxide solution containing 1 g. potassium thiocyanate.

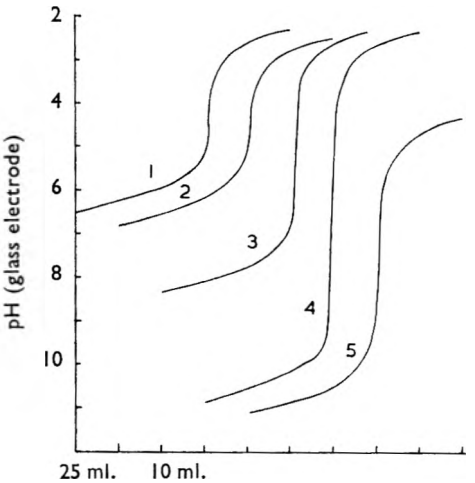

FIG. 2. Titration of cyanide in mercuric oxycyanide. Curves originate at 5 ml. intervals.

1. 50 ml. mercuric oxycyanide solution containing 2 g. potassium iodide.
2. 50 ml. mercuric oxycyanide solution containing 2 g. sodium thiosulphate.
3. 50 ml. mercuric oxycyanide solution containing 2 g. potassium iodide and 2 ml. acetone.
4. 50 ml. mercuric oxycyanide solution containing 2 g. potassium iodide and 2 ml. formaldehyde.
5. 50 ml. mercuric oxycyanide solution containing 2 g. sodium thiosulphate and 2 ml. formaldehyde solution.

or formaldehyde. Formaldehyde was further investigated for this purpose.

The neutralisation of the mercuric oxide was first effected by taking 50 ml. portions of a stock solution of mercuric oxycyanide, and adding 1 g. of potassium thiocyanate and 10 ml. of 0·1N hydrochloric acid. Two g. of potassium iodide and various volumes of 40 per cent solution of formaldehyde (neutralised to phenol red) were then introduced, and the solution titrated with 0·1N hydrochloric acid, pH changes being recorded.

The use of large volumes of formaldehyde solution re-introduced some buffering probably caused by the formate present in the neutral solution;

but too little formaldehyde resulted in a slow removal of hydrogen cyanide and consequently the pH of the solution on the addition of acid fell sharply and subsequently returned slowly to its equilibrium value. A suitable compromise was reached by using 2 ml. of formaldehyde solution free from formic acid (as distinct from one containing neutralised formic acid).

This was obtained by passing formaldehyde solution through a twelve inch column of "De-Acidite G" or "De-Acidite FF" in the basic form. A solution prepared by boiling paraformaldehyde with water and filtering was almost as suitable, and the titration curves obtained using this formaldehyde show little sign of formate buffering (Fig. 3). It is essential that the temperature of the solution should not exceed 20°. At higher temperatures the alkaline formaldehyde reduces mercuric ion to mercury with production of formic acid and consequent invalidation of the determination. With deionised formaldehyde there is no danger of elevating the temperature and this was therefore preferred.

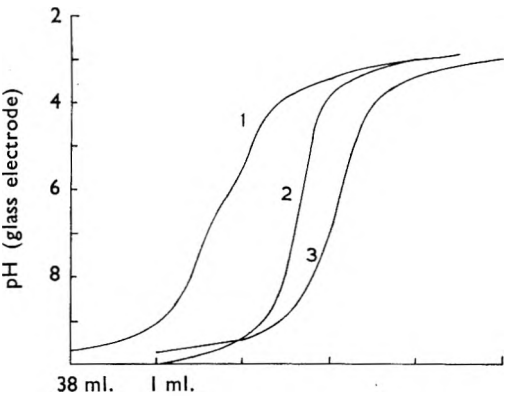

FIG. 3. Cyanide titration—effect of formate ion. Curves originate at 0.5 ml. intervals.

1. 50 ml. mercuric oxycyanide solution, 2 g. potassium iodide, and 2 ml. formaldehyde, neutralised with sodium hydroxide solution.
2. 50 ml. mercuric oxycyanide solution, 2 g. potassium iodide and 2 ml. formaldehyde treated with "De-acidite FF".
3. 50 ml. mercuric oxycyanide solution, 2 g. potassium iodide and the filtrate from 1 g. paraformaldehyde and 10 ml. boiling water.

By carrying out the titration using various weights of potassium iodide, the minimum satisfactory amount was found. No further improvement in the end point occurs on increasing the amount beyond 4 g.

The following process for the determination of mercuric oxycyanide is therefore recommended:

Mercuric oxide. Dissolve about 0·5 g., accurately weighed, in 50 ml. of water and boil gently for ten minutes. Cool to 20° without agitation, add 1 g. of potassium thiocyanate and titrate against decinormal hydrochloric acid, using phenol red indicator, until the full yellow colour of the indicator is obtained. Each ml. of 0·1N hydrochloric acid is equivalent to 0·01083 g. of HgO.

Mercuric cyanide. Continue the titration after the addition of 4 g. of potassium iodide and 2 ml. of *deionised solution of formaldehyde.* Each additional ml. of 0·1N hydrochloric acid is equivalent to 0·01263 g. of $Hg(CN)_2$.

Deionised solution of formaldehyde. Solution of Formaldehyde B.P. from which the formate ions have been removed by treatment with a basic ion exchange resin. 5 ml. of the solution is not coloured red by the addition of 5 drops of solution of phenol red and requires not more than 0·05 ml. of 0·1N hydrochloric acid to produce a red colour.

TABLE II

DETERMINATION OF A COMMERCIAL SAMPLE OF MERCURIC OXYCYANIDE B.P.

(*a*) *Proposed method*

Operator A		Operator B		Operator C		Operator D	
HgO per cent	$Hg(CN)_2$ per cent	HgO per cent	$Hg(CN)_2$ per cent	HgO per cent	$Hg(CN)_2$ per cent	HgO per cent	$Hg(CN)_2$ per cent
15·5	84·0	15·2	84·4	15·1	84·4	15·4	84·0
15·5	84·3	15·3	84·3	15·2	84·4	15·5	83·9
15·6	84·0	15·3	84·1	15·3	84·4	15·6	84·3
15·6	84·3	15·1	84·4	15·4	84·4	15·3	84·0
15·5	84·2	15·0	84·4	15·4	84·0	15·3	84·1
15·2	84·4	15·0	84·4	15·4	84·1	15·3	83·8

Mean of 24 results
= 15·35 per cent HgO
= 84·2 per cent $Hg(CN)_2$

Standard deviation
= 1·174 per cent of mean
= 0·2315 per cent of mean

(*b*) *Method of B.P.* 1953

Operator A		Operator B		Operator C		Operator D	
HgO per cent	$Hg(CN)_2$ per cent	HgO per cent	$Hg(CN)_2$ per cent	HgO per cent	$Hg(CN)_2$ per cent	HgO per cent	$Hg(CN)_2$ per cent
15·2	83·6	15·7	84·2	15·5	84·2	15·6	84·3
15·0	82·5	15·8	84·0	15·4	84·8	15·3	82·6
14·9	83·0	15·6	83·7	15·4	84·4	15·7	84·4
15·0	84·3	15·4	84·4	15·5	84·5	15·8	84·0
15·0	83·8	15·9	84·4	15·5	84·2	15·5	84·5
14·8	83·7	15·5	84·6	15·8	85·2	15·5	84·2

Mean of 24 results
= 15·4 per cent HgO
= 84·0 per cent $Hg(CN)_2$

Standard deviation
= 1·95 per cent of mean
= 0·763 per cent of mean

Comparison of Proposed Method with other Methods

(i) A sample of mercuric cyanide determined by the proposed method gave a result of 99·5 per cent $Hg(CN)_2$ and independently by means of standard ammonium thiocyanate gave 99·5 per cent $Hg(CN)_2$.

(ii) A solution was prepared by dissolving in water accurately weighed amounts of mercuric oxide and mercuric cyanide, which had previously been determined by independent methods. This solution was titrated for both components using the suggested procedure. Each 50 ml. of solution contained, HgO calcd. 0·0507 g., found 0·0500 g.; $Hg(CN)_2$ calcd. 0·3190 g., found 0·3200 g.

(iii) A commercial sample of Mercuric Oxycyanide B.P. was assayed using first, the proposed method, and second, the method of the British Pharmacopoeia, 1953. The results are given in Table II.

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A NOTE ON THE ANAESTHETIC ACTIVITY OF SOME 1:3-PROPANEDIOLS

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The discovery is reported of the anaesthetic activity of 2-alkyl-substituted alkane-1 : 3-diols in mice. The preparation of some hitherto undescribed members of this class is reported.

2:2-Dialkyl-substituted propane-1:3-diols (for example, 2:2-diethylpropane-1:3-diol) are known to possess anticonvulsant activity[1–7], but although the muscle relaxant activity of 2-ethylpentane-1:3-diol has been reported[1], the anaesthetic activity does not seem to have been noted before in this series except in high doses[2]. It was found during another investigation that 2-ethylhexane-1:3-diol (I; R = *n*Pr; R′ = Et; R″ = H) produced general anesthesia after

$$R-CHOH\cdot CR'R''\cdot CH_2OH$$

(I)

intraperitoneal injection into mice. Several other compounds of this type were, therefore, tested and are listed in Table I.

TABLE I

Anaesthetic activity and toxicity of compounds examined

Compound	Reference	Formula (I) R	R′	R″	Dilution (in water) used (vol.)	ED50 (ml. compd./kg. body wt.)	LD50 (ml. compd./kg. body wt.)
2-Ethylpropane-1:3-diol[a]	12, 13	H	Et	H	1/2	3·7[e]	7·5[e]
2:2-Dimethylpropane-1:3-diol	*	H	Me	Me	1/1·5	†	8·0[f]
2-Methylpentane-1:3-diol	*	Et	Me	H	1/10	1·95[g]	4·2[g]
2:2-Diethylpropane-1:3-diol[b]	8, 9	H	Et	Et	1 g./12·5 ml.	0·8‡[e]	1·2‡[e]
2-Methyl-2-propylpropane-1:3-diol[c]	8, 10	H	Me	*n*Pr	1 g./30 ml.	0·4‡[h]	0·66‡[h]
2-Ethylpentane-1:3-diol	*d*	Et	Et	H	1/10	0·75[e]	1·5[e]
Heptane-1:3-diol	*d*	*n*Bu	H	H	1/10	0·5[i]	1·5[i]
2-Ethylhexane-1:3-diol	*	nPr	Et	H	1/30	0·65[j]	1·2[j]
2-Ethylheptane-1:3-diol	*d*	*n*Bu	Et	H	1/100	0·3[j]	0·48[j]

a n_D^{20} 1·4495. b.p. 122 to 123° at 17 mm. *b* m.p. 61°. *c* m.p. 57° after crystallisation from light petroleum (b.p. 80 to 100°) and sublimation. Yale and others[8] quote m.p. 62 to 63°. *d* See text for preparation. *e–j* Conditions of test: *e* five mice at each of three dose levels; *f* ten mice at each of four dose levels; *g* ten mice at each of three dose levels; *h* five mice at each of five dose levels; *i* four mice at each of four dose levels; *j* ten mice at each of five dose levels. * Commercial material. † No anaesthetic effect detected. ‡ g./kg. body weight.

Most of the compounds tested are well-known and some were obtained from commercial sources. A convenient route to propane-1:3-diols, which are not alkyl-substituted at the terminal carbon atoms, is afforded by the reduction with lithium aluminium hydride of substituted malonic esters, and has been previously used for the preparation of (I; R = H; R′ = R″ = Et)[8,9]. 2-Methyl-2-propylpropane-1:3-diol was prepared by the reaction of formaldehyde and sodium hydroxide on 2-methylvaleraldehyde[10]. For propanediols substituted on the 3-carbon atom

(I; R = alkyl) the route chosen (cf.[8]) was the reduction by lithium aluminium hydride of esters of β-hydroxyacids, themselves prepared by the Reformatsky reaction.

The biological testing was carried out by the intraperitoneal injection of aqueous solutions into groups of mice. The criterion of activity was the production of anaesthesia and its maintenance for 30 minutes; from the results obtained, the median effective and median lethal doses were calculated. No ill effects were noted in the mice which recovered. The results are shown in Table I.

Since the number of compounds tested was small, it is not possible to relate the activity to the structure, except to note that the activity and toxicity are greatest in the compounds of higher molecular weight.

It is thought that 1:3-diols might be useful, under carefully controlled conditions, as anaesthetics for laboratory animals.

Preparation of Compounds

2-Ethylpentane-1:3-diol. A mixture of ethyl 2-bromo-*n*-butyrate (48·7 g.), propionaldehyde (17·4 g.), ether (20 ml.), and benzene (80 ml.) was cautiously added to a stirred suspension of zinc powder (22 g.), (previously washed in turn with 2 per cent v/v hydrochloric acid, water, ethanol, acetone, and ether, and dried at 60°), in ether (2 ml.) and benzene (18 ml.). The vigorous reaction was maintained under reflux by the application of heat and the continued addition of the ester–aldehyde mixture; the heating was continued for 30 minutes after the addition was complete. The mixture was poured into ice-cold 10 per cent v/v aqueous sulphuric acid and the organic layer was separated, washed with dilute sodium bicarbonate solution, and dried. The combined products of two such experiments were fractionated (12 in. packed column). No sharply-boiling fraction was obtained, and the crude ethyl 2-ethyl-3-hydroxyvalerate (20·5 g.), b.p. 97 to 105° at 13 mm., was collected separately. This material (19·1 g.) was reduced in ether (250 ml.) with lithium aluminium hydride (7·2 g.) and the product was distilled to give the crude diol (9·8 g.), b.p. 120 to 124° at 13 mm. Redistillation gave *2-ethylpentane-1:3-diol* as a viscous liquid, b.p. 124 to 125° at 14 mm., n_D^{20} 1·4505. (Found: C, 64·1; H, 11·9. $C_7H_{16}O_2$ requires C, 63·6; H, 12·2 per cent.)

Heptane-1:3-diol. Ethyl 3-hydroxyheptoate was prepared from valeraldehyde (48 g.), ethyl bromoacetate (95 g.), ether (20 ml.), benzene (100 ml.), and zinc powder (46·7 g.) by a similar process to that used for the preparation of ethyl 2-ethyl-3-hydroxyvalerate above. Ethyl 3-hydroxyheptoate (46 g.) was obtained as a fraction, b.p. 103 to 110° at 10 mm. [Adickes and Andressen[11] quote b.p. 94 to 96° at 5 mm.] On reduction with lithium aluminium hydride (12·7 g.) it gave a fraction (16·5 g.), b.p. 94 to 96° at 1 mm., which, on redistillation, gave the *diol* (14·8 g.) as a viscous liquid, b.p. 95° at 0·75 mm. (Found: C, 63·7; H, 11·8. $C_7H_{16}O_2$ requires C, 63·6; H, 12·2 per cent.)

2-Ethylheptane-1:3-diol. The reaction of valeraldehyde (26 g.), ethyl 2-bromo-*n*-butyrate (58·8 g.), and zinc powder (23 g.), carried out as described above for analogous reactions, gave a fraction (34·8 g.), b.p.

115 to 127° at 12 mm., a sample of which was redistilled to give *ethyl 2-ethyl-3-hydroxyheptoate*, b.p. 124° at 12 mm. (Found: C, 65·5; H, 10·9. $C_{11}H_{22}O_3$ requires C, 65·3; H, 10·9 per cent.) Reduction of the main portion of the fraction (28 g.) with lithium aluminium hydride (6·85 g.) in ether, gave the *diol* (13·5 g.), b.p. 140 to 143° at 12 mm., n_D^{20} 1·4515. (Found: C, 67·9; H, 12·6. $C_9H_{20}O_2$ requires C, 67·5; H, 12·6 per cent.)

Acknowledgement. We should like to acknowledge the able assistance of Mr. H. J. Fearn, who made the animal tests.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Digitalis Glycosides, Colorimetric Estimation of. D. H. E. Tattje. (*Pharm. Weekbl.*, 1958, **93**, 245.) The glycoside or aglycone is dissolved in 4 ml. of ethanol and the solution mixed with 5 ml. of a 0·075 per cent w/v solution of 2:4-dinitrophenylsulphone in ethanol. 1 ml. of 0·15 N aqueous sodium hydroxide is added and the colour measured at 600 mμ, against a reagent blank. When applied to digitoxin and digitoxigenin this test is much more sensitive than the picric acid, 3:5-dinitrobenzoic acid and *m*-dinitrobenzene reactions, but with gitoxigenin the difference is less marked. G. B.

Gentisic Acid, Polarographic Determination of, in Pharmaceutical Preparations. M. Jirka. (*Českoslov. Farm.*, 1957, **6**, 609.) For the determination of gentisic acid in tablets, a sample is ground and extracted three times with water. The combined extracts are filtered and the filtrate is made up to give a solution containing 0·33 per cent of gentisic acid. To 4·9 ml. of carbonate buffer (pH 8·7) 0·1 ml. of this solution is added and the resulting solution is polarographed in a Heyrovsky polarograph, the oxygen being removed by a stream of nitrogen. The height of the anode wave of gentisic acid is directly proportional to the concentration and the concentration in the test solution can be read from a calibration curve. E. H.

Hormones, Assay of Mixtures of, in Solution in Oil. G. Tappi, E. M. Andreoli and E. Frea. (*Pharm. Weekbl.*, 1958, **93**, 231.) The preparation under test is diluted with light petroleum, passed through a chromatographic column packed with magnesium stearate ('Florisil') and the oil washed through with a mixture of light petroleum 3, chloroform 1. Compounds containing no free hydroxyl or keto-groups (such as esters of certain steroid hormones) are eluted with a mixture of equal volumes of chloroform and light petroleum. Ketones are then removed from the column by elution with pure chloroform, and finally alcohols and phenols with chloroform-methanol. The various fractions may be assayed by colorimetric or ultra-violet absorption methods. Details are given of the analysis of a typical preparation, in which a recovery of 94–97 per cent of each hormone may be attained. G. B.

Oestrogens, Bis-phenolic, Belonging to the Stilbene Series, Potentiometric Titration of. K. Backe-Hansen and A. Wickstrøm. (*Medd. Norsk farm. Sels.*, 1957, **19**, 193.) Dienoestrol, hexoestrol and stilboestrol were titrated quantitatively with potassium methoxide in benzene-methanol solution. The end point was determined potentiometrically, and the three compounds behaved as dibasic acids under these conditions. A colorimetric determination of the end point, using azo violet as indicator was satisfactory for dienoestrol. Using 0·1 N tetrabutylammonium hydroxide in place of potassium methoxide, the titrations could be carried out in the same manner, and dienoestrol and hexoestrol could be titrated as monobasic acids in pyridine or acetone. This method was not successful for stilboestrol which behaves under these conditions as a much weaker acid, presumably owing to resonance of the symmetrical conjugated benzene-ethylene-benzene structure. G. B.

Opium Alkaloids, Identification of Natural, and Synthetic Derivatives of, by "Test-tube" Chromatography. R. Fischer and N. Otterbeck. (*Sci. Pharm.*, 1957, **25**, 242.) In "test-tube" chromatography, strips of filter paper are suspended in tubes about 25 cm. long and 1·8 cm. bore. One drop of a chloroform solution of the alkaloidal bases is placed about 1·5 cm. from the bottom edge of the paper, the chloroform allowed to dry, and the paper immersed to a depth of 1 cm. in the solvent. After the solvent has climbed to within 2 cm. of the top edge of the paper strip, the latter is dried. Temperature control is important. The advantage of the technique is that many chromatograms can be run simultaneously, and the experiment is complete in 3 to 4 hours. Using this technique it was possible to identify 19 analgesics (morphine derivatives, opium alkaloids and synthetic substances) by the differences in their R_F values by the simultaneous running of four different solvent mixtures. The only ones which could not be distinguished were codeine and eucodal, for which a suitable microchemical reaction is quoted. D. B. C.

Parathion, Spectrophotometric Determination of. H. Kita, H. Maeda, B. Hanazaki, M. Kawai and T. Takazawa. (*Bull. Tokio Med. and Dental Univ.*, 1957, **4**, 379.) A simple method is described for the determination of parathion in water. Neutralise the solution with dilute hydrochloric acid if alkaline and extract 20 ml. by shaking with an equal volume of benzene for five minutes. Weak solutions are first concentrated. The optical density is measured at 2800 Å in a spectrophotometer and the parathion content determined by reference to a standard curve prepared as follows. Weigh 0·100 g. of parathion, dissolve in 500 ml. of benzene. Take 25 ml. of this solution and make up to 250 ml. with benzene. This solution contains 20 μg./ml. of parathion. Take 0·5, 1, 2, 3, 4 and 5 ml. and dilute to 10 ml. and measure the optical density as before. G. F. S.

Phenothiazine Derivatives, Separation of. A. Calò, A. Mariani and O. M. Marelli. (*Pharm. Acta Helvet.*, 1958, **33**, 126.) The properties of promazine are compared with those of its chlorine analogue chlorpromazine, with a view to finding a method of separation. This latter shows a higher mobility in iontophoresis, and is more sensitive to oxidation with iodic acid giving a more intense colour. The behaviour of promazine is similar to that of other derivatives of phenothiazine without the chlorine in the molecule. D. B. C.

Purine Derivatives, Non-aqueous Titration of. B. Salvesen. (*Medd. Norsk farm. Sels.*, 1957, **19**, 199.) Mixtures of caffeine with sodium benzoate or sodium salicylate may be titrated with perchloric acid in acetic acid solution. The most accurate results are obtained when acetic anhydride in excess of that required to remove water is added. The sample is dissolved in 2–5 ml. of glacial acetic acid, and mixed with acetic anhydride alone or with twice its volume of benzene, before titration. The first end point is detected by the use of 4 drops of a saturated solution of tropeoline OO, and corresponds to the neutralisation of the sodium benzoate or salicylate. On adding 8 drops of methyl violet and continuing the titration, the end point corresponding to neutralisation of caffeine is obtained. Mixtures of caffeine with diphenhydramine hydrochloride may be analysed in the same manner, using 5 ml. of a 5 per cent solution of mercuric acetate in glacial acetic acid, in place of the acid alone. G. B.

Reserpine, Ajmaline and Serpentine, Determination of, in Mixtures. F. Machovičová, V. Parrák, O. Lišková and J. Ružičková. (*Československ. Farm.*, 1957, **6**, 584.) Reserpine, serpentine and ajmaline are separated by chromatography on Whatman No. 1 paper impregnated with formamide-methanol (1 : 1). R_F values are given for the three alkaloids with chloroform and chloroform-benzene (4 : 1 and 1 : 1) as mobile phases. The spots are detected by observing the paper in ultra-violet light; the intensity of fluorescence can be increased by exposing the paper to hydrochloric acid vapour and then immersing it in 3 per cent hydrogen peroxide. Reserpine and ajmaline are quantitatively eluted from the paper with chloroform, and serpentine with methanol. The alkaloids in the eluates are estimated colorimetrically by a method based on the formation of addition compounds between the alkaloids and methyl orange. The intensity of the colour produced is measured in a Hilger absorptiometer with filters 604 and H503. E. H.

Strychnine and Brucine, Determination in the Seeds, Tincture and Extract of Strychnos. R. Fischer and S. Gharbo. (*Pharm. Zentralh.*, 1958, **97**, 101.) This determination depends upon chromatographic separation of strychnine and brucine and titrimetric estimation. It was found that when both alkaloids were adsorbed on alumina containing 5 per cent water, the strychnine could be eluted with trichlorethylene containing 0·5 per cent acetone leaving the brucine behind. It was found best to pass the strychnine eluate through another column containing dry, neutral alumina; both columns could then be eluted with methanol and the alkaloids titrated in the usual way. The extract of the powdered drug was prepared by rubbing the drug down with ammonia or caustic soda solution and dry sodium sulphate, packing in a Soxhlet extractor over a layer of alumina and extracting with chloroform for two hours. The tincture was purified by passing it through a short alumina column, eluting with methanol and evaporating, and taking up in trichlorethylene. Elution in this case was done with tetrachlorethylene containing 1 per cent acetone. The extract was diluted with ethanol and treated similarly to the tincture. The accuracy of the method was checked in each case by adding known quantities of pure alkaloids to the assayed galenical preparations and reassaying. D. B. C.

Thymol in Thyme Oil, Colorimetric Determination of. L. Fibranz, M. I. Blake and C. E. Miller. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 133.) A colour reaction with *p*-dimethylaminobenzaldehyde was found to be suitable for the rapid determination of thymol in thyme oil. The U.S.P. reagent did not give reproducible results, but a 0·125 per cent solution in concentrated sulphuric acid was found to be satisfactory. In the suggested method the sample is diluted with chloroform and 1 ml. of the solution, corresponding to about 80 μg. of thymol, is shaken with 10 ml. of reagent for 10 seconds, allowed to stand for 5 minutes, and the colour measured in a photoelectric colorimeter with a blue filter, against a reagent blank. The result is read from a standard curve. Assays by this technique gave results in agreement with the U.S.N.F. X method, and with non-aqueous titration in dimethylformamide. It was established that the small quantities of linalol, α-pinene, *p*-cymene, geraniol and borneol likely to be present in the oil do not affect the result, but abnormally high concentrations of these compounds might interfere, since they give a colour with *p*-dimethylaminobenzaldehyde reagent. G. B.

CHEMOTHERAPY

Antibiotic Salts of Reduced Toxicity, A New Class of. F. A. Alves M. F. C. A. N. Graça and H. L. Baptista. (*Nature, Lond.*, 1958, **181**, 182.) Amino acid salts of dihydrostreptomycin were prepared by neutralisation of a concentrated aqueous solution of the base with amino acid (3 molecules to each molecule of base), or by double decomposition between solutions of dihydrostreptomycin sulphate and the barium salt of the appropriate amino acid. The salts were isolated by freeze-drying or by concentrating solutions under reduced pressure at room temperature, dissolving the resulting syrup in methanol and precipitating with acetone. The following salts were prepared: glycinate, DL-leucinate, L-glutamate, L-methionate, *N*-acetyl-DL-methionate and *S*-ethyl-L-cysteinate. With the exception of this last compound (the anion of which has itself some tuberculostatic activity) all these amino acid salts were less toxic, as determined by intravenous injection into mice, than the sulphate or glucuronate. The leucinate and methionate are less toxic than the pantothenate and are being studied further. G. B.

Lymphotrophic Antibiotics. P. Málek, M. Herold, J. Hoffman and J. Kolc. (*Nature, Lond.*, 1958, **181**, 706.) Streptomycin, viomycin and streptothrycin have been combined with carboxylic, sulphonic or phosphorylated high molecular weight compounds to give macromolecular salts termed 'antibiolymphins', and their pharmacological properties studied. They are classified under three headings: (1) salts of antibiotic bases with polyacrylic acids (streptolymphin I, neolymphin I); (2) salts of antibiotic bases with sulphonic or phosphorylated polysaccharides (streptolymphin II, neolymphin II); (3) salts of antibiotic bases with natural polycarboxyl acids from a series of polyuronic substances and polysaccharide derivatives containing carboxyl groups (streptolymphin III, neolymphin III). The physiological behaviour of antibiolymphins lies between that of pure crystalloid and pure colloid, as shown by infusion into a peripheral lymph vessel of the hind extremity of a dog and examination of percentage of substance appearing in the thoracic drainage. Antibiolymphins are absorbed from the injection locus primarily by the lymphatic system; and high concentrations of long duration (up to 72 hours) may be produced in the lymph nodes of the drainage channels. Blood levels are generally lower than found with the normal antibiotic equivalent, but are maintained for much longer times. The acute toxicity is less than that of the parent antibiotic, the LD50 in rats and mice being raised five times with streptolymphin I and ten times with neolymphin I. J. B. S.

PHARMACY

Adrenaline, Stability of, and its Stabilization in Eye Lotions. J. Král. (*Sci. Pharm.*, 1958, **26**, 1.) The influence of the composition and the conditions of storage of some eye lotions on the stability of adrenaline contained therein was investigated. These eye lotions contained the most commonly used medicaments, e.g., zinc sulphate, boric acid, mercuric oxycyanide, silver proteinate, borax and silver proteinate diacetyl tannate, together with stabilisers such as sodium metabisulphite (0·1 to 0·8 per cent) and disodium ethylenediamine tetra-acetic acid (up to 1 per cent). Buffers were also included from pH 2 to 6. Trials were also carried out on plain solutions stored under various conditions, e.g., in the light, in the dark, exposed to air, air excluded, at room temperature

and at 32°. The most unstable eye lotions were those containing borax and silver proteinate diacetyltannate. Both required 0·2 per cent sodium metabisulphite, and the former a buffer at pH 6. D. B. C.

Antoxidant from Yeast, Isolation and Chemical Studies. M. Forbes, F. Zilliken, G. Roberts and P. György. (*J. Amer. chem. Soc.*, 1957, **80**, 385.) A new antoxidant, capable of preventing the occurrence of a haemorrhagic liver necrosis which follows dietary deficiencies in rats, has been isolated in crystalline form, $C_{16}H_{12}O_5$ from various yeasts. It was obtained by extractions with 90 per cent ethanol, removal of sterols by solution in methanol, extraction with ether and chromatography on Florisil. Yields of the order of 0·5 to 2 mg. per pound were obtained. The substance is optically inactive; the molecule contains one methoxyl group and no C-methyl groups. Of the other four oxygen functions one is phenolic and the remaining three ether links, since diazomethane gave a dimethoxy derivative. Fusion with potassium hydroxide at 200° gave an aromatic carboxylic acid without hydroxyl substituents, whilst microhydrogenation with PtO_2 as catalyst in acetic acid showed an uptake of 9 moles of hydrogen, indicating the presence of two aromatic nucleii, one being unsubstituted and the other containing the hydroxyl and methoxyl substituents. J. B. S.

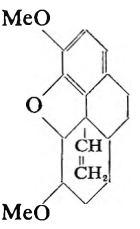
Menaphthone Sodium Bisulphite Injection, Stability of. Shu-yuan Yeh and G. A. Wiese. (*Drug Standards*, 1958, **26**, 22.) On prolonged storage, solutions of menaphthone sodium bisulphite become yellow and a precipitate forms, decomposition being accelerated by heat and light. Solutions are most stable below pH 2·5. Sodium chloride, added to the solution, improves its stability, and the addition of 0·2 per cent of sodium bisulphite is advantageous in preventing breakdown to menaphthone and sodium bisulphite. When solutions containing sodium bisulphite are stored under an inert gas the yellow colour and precipitate do not appear unless the ampoules are stored in sunlight. However, these conditions increase the rate of conversion to inactive 2-methyl-1:4-naphthaquinone-3-sulphonate. Solutions containing 0·2 per cent of sodium bisulphite and 0·7 per cent of sodium chloride, sterilised by filtration and filled into ampoules under air were shown by accelerated decomposition studies to have a shelf life of about $2\frac{1}{2}$ years at 20° or $1\frac{1}{2}$ years at 30°. G. B.

PHARMACOGNOSY

Digitalis Leaves, Distinction between various Types by Ring Chromatography. K. Rada. (*Pharm. Zentralh.*, 1958, **97**, 163.) The separation of glycoside complexes in purified extracts of the leaves of five species of *Digitalis* was attempted. These were *D. purpurea*, *D. ambigua*, *D. lanata*, *D. ferruginea* and *D. thapsi*. Using the same solvent mixture (xylene-methylethyl ketone 1:1) and Whatman No. 1 filter paper impregnated with formamide, zones were obtained by means of which various species of *Digitalis* could be distinguished by their R_F values and fluorescence in ultra-violet light. The method could be used for the investigation of a mixture of powdered leaves containing *D. purpurea*, *D. lanata* and *D. ferruginea*. D. B. C.

Morphine, Biogenesis of. A. R. Battersby and B. J. T. Harper. (*Chem. Ind.*, 1958, 364.) The biogenesis of morphine has been investigated by feeding

plants of *Papaver somniferum* with α-^{14}C-DL-tyrosine, and subsequent isolation of all the major alkaloids by countercurrent distribution. The morphine, diluted with inactive morphine and purified as picrate, had a constant activity of $4{\cdot}29 \times 10^5$ dis. p.m./mM. The recovered morphine was degraded via codeine methyl ether methiodide, α-codeimethine methyl ether and tetrahydrocodeimethine methyl ether. Hofmann degradation of the latter gave inactive trimethylamine (purified as picrate) and the morphenol derivative (I), which on hydroxylation with osmium tetroxide and cleavage with periodate gave formaldehyde, isolated as its dimedone derivative, which had activity $2{\cdot}02 \times 10^5$ dis. p.m./mM. It is concluded that half the activity of the original morphine is located at position 16 in accord with the biogenetic theory which incorporates one molecule of tyrosine at this point. J. B. S.



(I)

PHARMACOLOGY AND THERAPEUTICS

Chlorpheniramine Maleate in Blood Transfusions. M. Hobsley. (*Lancet*, 1958, **1**, 497.) A controlled trial in 200 blood transfusions failed to demonstrate the efficacy of chlorpheniramine maleate (10 mg. in each bottle of blood) in preventing pyrexial reactions during transfusions. No statistical evidence of such an effect was obtained and there is no argument in favour of routine antihistamine medication of the blood. The addition of any material to a bottle of blood must carry with it some danger of contamination, and this consideration must outweigh the possible avoidance of a small number of minor thermal reactions. This finding does not affect the validity of the practice of using chlorpheniramine in transfusions to patients with a history of allergy, in order to reduce the number of allergic reactions. S. L. W.

2-Hydrazine-benzazoles, Structure and Pharmacological Activity of. I. V. Panov and N. P. Bednyagina. (*Farmakologiya i Toksikologiya*, 1957, **20**, No. 6, 25.) The biological actions of 2-hydrazino-derivatives of benzothiazole (II), benzoxazole (III), benzimidazole (IV) and 1-methyl-benzimidazole (V) are compared with those of 1-hydrazinophthalazine (hydrallazine) in experiments on the isolated frog heart, on the vessels of the isolated posterior extremities of the frog, and on the blood pressure of the dog. Compounds II, III and V have no effect on blood pressure and no vasoconstricting action. In contrast to hydrallazine, compound IV, in intravenous doses of 0·5 mg./kg., produces a marked hypertensive effect in the dog. E. H.

5-Hydroxytryptamine and Reserpine, Differentiation between the Sedative Actions of. W. Kobinger. (*Acta pharm. tox. Kbh.*, 1958, **14**, 38.) The sedative actions of reserpine and 5-hydroxytryptamine (5-HT) have been compared in motility experiments using mice, in which the motility has been artificially increased by (+)-methylamphetamine or cocaine. It has been found that 5-HT counteracted the increased motility produced by (+)-methylamphetamine, the effect being linearly related to the logarithm of the dose. Reserpine had no action. Both 5-HT and reserpine antagonised the hyperactivity induced by cocaine and again both showed a relationship between sedative action and the log dose; but there was a significant difference between the regression lines. The results indicate different modes of action of 5-HT and reserpine on the central nervous system of mice. G. F. S.

Laminarin Sulphate, Physiological Activity of. W. W. Hawkins and V. G. Leonard. (*Canad. J. Biochem. Physiol.*, 1958, **36**, 161.) This paper gives a detailed account of the anticoagulant activity of laminarin sulphate. Some studies on its absorption, excretion and its activity as an antilipaemic agent are also made. In all instances its actions were compared with those of heparin. The immediate effect on the blood clotting efficiency, as measured 30 minutes after intravenous injection into dogs, indicated that laminarin sulphate is one-third as potent as heparin. The thrombin time was affected more than any other measure of the clotting efficiency of the plasma. This indicates that laminarin sulphate, like heparin, acts in an important capacity in the terminal stage of clotting as an antithrombin. It also shows anticoagulant activity when administered subcutaneously and intramuscularly. After the intravenous administration of a moderate amount of laminarin sulphate to the dog, considerable anticoagulant material passed into the urine. When large oral doses were given to the rat, absorption from the intestine was very small and much of the material passed into the faeces. When it was given intravenously, laminarin sulphate acted like heparin in clearing alimentary lipaemia in the dog and the rat. Further work on its antilipaemic properties is in progress. M. M.

Linoleic and Stearic Acids: Effect on Cholesterol-induced Lipid Deposition in Human Aortic Cells. D. D. Rutstein, E. F. Ingenito, J. M. Craig and M. Martinelli. (*Lancet*, 1958, **1**, 545.) Factors affecting the intracellular deposition of lipid were studied in human aortic cells in tissue-culture in a medium containing human blood-serum. When cholesterol in ethanol is added to the culture medium, the amount of lipid deposited in the cells is proportional to the amount of cholesterol added. Thus, at a concentration of cholesterol of 1 mg./100 ml. there is little evidence of intracellular lipid deposition; at 3 mg./100 ml. deposition regularly appears, with an increase in size of the cells: and at 5 mg./100 ml. there is considerable deposition and an enormous increase in size of cells. There is a similar proportional deposition when cholesterol bound to beta-lipoprotein is added to the culture medium. Thus, at a concentration of cholesterol of 30 mg./100 ml. there is little evidence of deposition; at 50 mg./100 ml. deposition regularly appears, with increase in size of cells; and at 70 mg./100 ml. there is considerable deposition and the cells are enormously increased in size. The deposition caused by adding cholesterol disappears in 5 days if the medium containing cholesterol is replaced by normal medium. The deposition is completely inhibited by simultaneous addition of linoleic acid (1 mg./100 ml.); it is increased by the simultaneous addition of stearic acid (1 mg./100 ml.). Thus, in tissue cultures of human aortic cells deposition of lipid can be induced by cholesterol. This is reversible and can be inhibited by an unsaturated fatty acid and potentiated by the corresponding saturated fatty acid. Details of method of preparation of the culture medium and cholesterol solution are given. S. L. W.

Methylpentynol Carbamate and Liver Function. A. A. Bartholomew, P. Chappell, E. Marley and J. S. W. Chambers. (*Lancet*, 1958, **1**, 346.) Methylpentynol carbamate was administered to 12 patients in doses of 1 to 3 g. daily by mouth for from 2 to 7 weeks. No abnormalities were discovered in tests for bile salts or pigments, urinary urobilin, serum-bilirubin, alkaline phosphatase, total plasma-proteins, serum-albumin and serum-globulin, zinc-sulphate or cephalin-cholesterol reactions, prothrombin time, haemoglobin values, blood film, and white cell count and differential. Mild disturbances of

hepatic excretory function (urinary urobilinogen) and protein metabolism (thymol turbidity and flocculation) were observed. An increase in the E.S.R. was also noted and a reducing substance found in the urine of one patient. Side-effects included anorexia, nausea, and vomiting, alterations in the mental state, and the appearance of such signs as nystagmus, pupillary anomalies, ptosis, loss of tone in the lower facial musculature, dysarthria, and ataxia. None of these abnormalities seem likely to develop on a daily oral dose of 1 g. or less.

S. L. W.

Methylpentynol, Effect of, in Man. S. E. Dicker and H. Steinberg. (*Brit. J. Pharmacol.*, 1957, **12**, 479). Methylpentynol has a sedative effect and has been used to allay anxiety without affecting motor performance. Experiments in student volunteers have shown that methylpentynol in a dose of 150 mg. decreases autonomic responses caused by the anticipation or performance of a difficult test. These were shown by an increase in pulse rate and blood flow. The subjects were asked to carry out two psychological tests, a test for motor co-ordination where they had to steer a pointer over as many dots as possible on a rotating drum, and a steadiness test where they had to hold a stylus in a hole without touching the side. During the tests the subjects after methylpentynol performed worse and minded less. G. F. S.

Nalorphine and Amiphenazole, Antagonism of, to Morphine. N. A. Kruglov. (*Farmakologiya i Toksikologiya*, 1957, **20**, No. 6, 40.) Experiments on rats, in which sensitivity to pain is measured by the method of D'Amour and Smith, show that nalorphine, in doses of 2·5 to 5 mg./kg. has about the same analgesic action as morphine. With morphine an increase in the dose to 10 to 20 mg./kg. increased the depth and duration of analgesia, but with nalorphine there was no such increase. Results of comparisons of the analgesic actions of these compounds depend on the dosage levels used. Studies on rabbits show that amiphenazole effectively counters the respiratory depression produced by morphine, but at this level it evokes toxic symptoms. Its LD50 for white mice is 270 mg./kg. It enhances the analgesic action but increases the toxicity of morphine; a dose of 50 mg./kg. given 10 minutes before the test reduces the LD50 of morphine from 630 to 340 mg./kg. E. H.

Organophosphates, Protection against the Lethal Effects of, by Pyridine-2-aldoxime Methiodide. F. Hobbiger. (*Brit. J. Pharmacol.*, 1957, **12**, 438.) The interaction between cholinesterase and organophosphates leads to the formation of an inhibited enzyme. Pyridine-2-aldoxime (P-2-AM) is a powerful reactivator of phosphorylated true cholinesterase. The value of P-2-AM and the other oximes has been studied in experimental organophosphate poisoning in mice. The results have shown that in the mouse P-2-AM, the most active oxime, was less effective *in vivo* than was indicated in the *in vitro* experiments. *In vivo* the injection of 25 mg./kg. of P-2-AM, 5 minutes before ethyl pyrophosphate (TEPP), diethyl *p*-nitrophenylphosphate (E 600), 3-(diethoxyphosphinyloxy)-*N*-trimethylanilinium methylsulphate (Ro 3–0340), and 3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3–0422) resulted in only 10 to 24 per cent reactivation. The concentration in the blood of 1 to 4 $\times$ 10^{-4} M of the oxime, to be expected, was sufficient *in vitro* to restore within a few minutes 90 per cent of the activity of true cholinesterase. P-2-AM was more efficient when given 30 minutes after the organophosphate. The effect of 25 mg./kg. P-2-AM on the phosphorylated true cholinesterase in the brain was

negligible. The protection by 25 mg./kg. P-2-AM against lethal doses of TEPP, E 600, Ro 3–0422 and Ro 3–0340 was greater than that obtained with 30 mg./kg. atropine sulphate, but the degree of protection was determined by the organophosphate itself. Protection against lethal doses of di*iso*propyl phosphorofluoridate (dyflos), di*iso*propyl *p*-nitrophenylphosphate (D 600) and 3-(di*iso*-propoxyphosphinyloxy)-pyridine (Ro 3–0351) was negligible. The antidotal effect of P-2-AM was potentiated by atropine. Screening of new oximes for antidotal properties should include as many oximes as possible, and by doing so insecticides could be selected which are less dangerous than those widely used today. G. F. S.

Penicillin, Environmental. J. C. Gould. (*Lancet*, 1958, **1**, 489.) Penicillin was detected in the environment in a general hospital. The amount recovered was greatest nearest those rooms where penicillin was being handled or administered. Air may be contaminated in two ways, either directly, by droplet nuclei (the dried residue of droplets atomised from syringes and sprays), or indirectly by the raising of dried spilt penicillin as dust. The concentration of penicillin is sufficient when inhaled in air, dust and fomites to inhibit penicillin-sensitive *Staphylococcus aureus* and to leave the nares free for colonisation with penicillin-resistant strains. Environmental penicillin is an important factor in the colonisation of hospital nasal carriers with penicillin-resistant *Staph. aureus*, and in the cross-infection of persons receiving or not receiving therapeutic antibiotic. The environment of a penicillin factory was shown to be similar to that of the hospital, and all the carrier strains of *Staph. aureus* were penicillin-resistant. S. L. W.

Phenethyldiguanide; Hypoglycaemic Action. R. I. Nielsen, H. E. Swanson, D. C. Tanner, R. H. Williams and M. O'Connell. (*Arch. int. Med.*, 1958, **101**, 211.) This is a study of the effect of phenethyldiguanide (PEDG) on blood sugar in intact and eviscerated animals, on the hepatic glucose output and on the degradation of insulin ^{131}I. Ten guinea pigs received 10 mg./kg. of PEDG subcutaneously twice daily, and a further 10 received an equal volume of saline by the same route. At the end of six weeks there was no difference in the weights of the animals in the two groups; the ratio of organ weight to body weight was the same in both groups and no histological changes were seen in the liver, kidney, spleen or pancreas. The compound was found to be effective orally, intramuscularly, intraperitoneally and subcutaneously, but the latter route gave the most consistent response. The response threshold was abrupt; 15 mg./kg. produced little or no hypoglycaemia, whereas 20 mg./kg. produced a marked response in 2 hours, killing approximately 50 per cent of the animals within 4 to 12 hours. Although the hypoglycaemia was accompanied by a marked fall in hepatic glucose output, it was found that hypoglycaemia was also produced in the absence of a functioning liver and was still present in the absence of the intestine and pancreas, so that the effect cannot be due to a decreased absorption from the intestine nor to the stimulation of insulin secretion. It was found that the degradation of insulin ^{131}I was inhibited by prior administration of the compound, but it was not felt that this action is of significance in the production of hypoglycaemia. S. L. W.

***N*-Phthalyl Glutamic Acid Imide (K 17), Antithyroid Activity of.** J. McC. Murdoch and G. D. Campbell. (*Brit. med. J.*, 1958, **1**, 84.) This tasteless, white, crystalline substance is an imide of glutamic acid and a derivative of

piperidine. It has been reported to have a "calming psychological effect," and has been used extensively on the Continent, in doses of 25 to 40 mg. two to four times daily, as a daytime sedative, and in doses of 50 to 100 mg. as a night-time hypnotic. It would appear to be non-toxic. On the basis of observations that it causes a marked reduction of the raised metabolic rate in hyperthyroidism, the effects of the drug on the thyroid uptake of radioactive iodine were assessed in 9 euthyroid patients. The results showed that it has a mild but definite antithyroid activity when given in doses of 200 mg. or more. The mode of action is at present unknown and it would seem unjustifiable to use it for long-term sedation or hypnotic therapy pending a study of its long-term effects in a larger number of patients. S. L. W.

17α-Propyl-4 : 5β-dihydro-19-nortestosterone, a New Hypotensive Steroid, Pharmacology of. F. M. Sturtevant. (*J. Pharmacol.*, 1957, **121**, 369.) This is a new antihypertensive steroid with no endocrinological, anti-inflammatory or anaesthetic properties. The compound significantly lowered the blood pressure of metacorticoid, metarenal and adrenal-regeneration hypertensive rats in acute and chronic states. Metacorticoid and metarenal hypertensive rats were prepared from month old male rats implanted with deoxycortone in wax or subjected to a two-stage uninephrectomy and contralateral kidney-wrap with latex. Adrenal-regeneration hypertensive rats were prepared by a one-stage right nephrectomy-right adrenalectomy-left adrenal enucleation. They were prepared three months before the tests began. In the 4 : 5-dihydro series, the only 17α-alkyl derivatives that were significantly active were the propyl, *iso*propyl and methyl compounds, while the ethyl and hydrogen compounds were inactive. In the 4-dihydro series the 17α-ethyl and α-propyl compounds were active. The former compound is the anabolic agent norethandrolone. G. F. S.

Secergan, a Quaternary Phenothiazine Compound having Anticholinergic and Ganglionic-blocking Actions. S. Wiedling. (*Acta pharm. tox. Kbh.*, 1958, **14**, 112). Secergan, 10-(α-dimethylaminopropionyl)-phenothiazine methobromide, has a powerful antagonistic action to spasms of smooth muscle induced by acetylcholine, nicotine, 5-hydroxytryptamine and histamine, but only a weak action against barium, adrenaline or noradrenaline. It has a hypotensive action in the anaesthetised rabbit due to a ganglionic-blocking action and the peripheral actions of adrenaline and noradrenaline are potentiated. G. F. S.

***Viscum album* L., Phytochemical and Pharmacological Studies.** G. Samuelson. (*Svensk Farm. Tidskr.*, 1958, **62**, 169.) *Viscum album* L., the mistletoe, is reported to contain both hypotensive principles and a cardiotoxic substance known as viscotoxin. By using suitable solvents, ion exchange resins and paper electrophoresis, the author isolated a small quantity of viscotoxin in a very pure form. Preliminary studies showed that it is a polypeptide which, contrary to previous reports, contains only eleven amino acids (not seventeen) and no sugar group. Tryptophan was also absent. The toxicity on mice was 1·14 ±0·77 mg./kg. intraperitoneally and 2·61 ± 0·18 mg./kg. subcutaneously. Two hypotensive fractions and one hypertensive fraction were also isolated and shown to contain ninhydrin-reacting substances. No choline or arginine could be found in the plant. J. W. F.

APPLIED BACTERIOLOGY

β-Glycyrrhetic Acid and Some of its Derivatives, Bacteriostatic Action of. R. Benigni and E. Franco. (*Pharm. Weekbl.*, 1958, **93**, 114.) 18α-Glycyrrhetic and glycyrrhizic acids and the related triterpenoid compounds ursolic, asiatic and oleanolic acids were shown to be inactive against all the organisms used in the tests, and monoammonium glycyrrhizinate showed only slight activity against *Escherichia coli*. Under the same conditions β-glycyrrhetic acid was shown to be active against *Staphylococcus aureus*, *Corynebacterium diphtheriae*, *Bacillus anthracis*, *Pseudomonas aeruginosa* and *Mycobacterium paratuberculosis*; it was slightly less effective against *Streptococcus faecalis*, *Strep. haemolyticus*, *Shigella flexneri* and *E. coli*. The maximum activity, determined by measurement of the zones of inhibition, was observed with a concentration of 0·1 per cent. The potassium derivative of β-glycyrrhetic acid was less active than the free acid against *Staph. aureus*, *Strep. faecalis*, *Corynebact. diphtheriae* and *B. anthracis*. γ-Glycyrrhetic acid was active against a few species including *Staph. aureus*, *B. anthracis* and *Myco. paratuberculosis*. The variable results which have been reported from the use of glycyrrhetic (glycyrrhetinic) acid in dermatology may have been due to variations in the sensitivity of the infecting organisms to β-glycyrrhetic acid, the identity and purity of the preparations used, and the use of alkaline materials in the ointment bases, causing the formation of the less active salt. G. B.

Phenolic Compounds, Interaction of, with Bacteria. A. H. Beckett, S. J. Patki and A. E. Robinson. (*Nature, Lond.*, 1958, **181**, 712.) Addition of hexylresorcinol to washed suspensions of *E. coli* caused the release of 'cell exudate', which shows absorption in the ultra-violet (max. 260 mμ). Rapid release (10 min.) of cell exudate was proportional to hexylresorcinol concentrations when these were low, but a limiting value was reached when the drug concentration was increased to a bactericidal level. On the other hand, hexylresorcinol taken up by the bacteria was proportional to the concentration of the drug, no limiting value being reached, so that at a bactericidal concentration the amount of hexylresorcinol found per bacterium is greater than that required to form a close packed monomolecular layer surrounding the organism. These results support the accepted view that phenolic compounds act at the cytoplasmic membrane. Addition of cetomacrogol reduced the uptake of hexylresorcinol from solutions by *E. coli* at all concentration levels of drug, and the antibacterial activity of the latter was completely abolished. It is suggested that the non-ionic agent (cetomacrogol) prevents interaction of the drug with the cytoplasmic membrane. J. B. S.

***Plantago lanceolata*, Antibacterial Properties of Extracts of.** M. Felková. (*Pharm. Zentralh.*, 1958, **97**, 61.) The antibacterial activity of alcoholic extracts of fresh leaves of *Plantago lanceolata*, collected throughout the vegetative season, is compared using as test organisms *Staphylococcus areus*, *Streptococcus β-haemolyticus*, *Bacillus subtilis*, and *Escherichia coli*. The zones of inhibition on agar cultures were compared under controlled conditions, a blank being performed with the solvent. Activity was significant and varied considerably throughout the year although there was a peak in all cases just before the flowering season. This peak represented greatest activity except for *Staph. areus* against which activity became greatest later in the season. The least affected organism was *E. coli*. The results with extracts made with physiological saline were negative except against *Staph. areus*. D. B. C.

PHARMACOPOEIAS AND FORMULARIES

THE BRITISH PHARMACOPOEIA 1958*

By Teodor Canbäck, Stockholm

The British Pharmacopoeia of 1958 is in many respects a heavy book. It consists of about one thousand pages. It has taken me about four weeks to read it through and it will probably take me another year to get *well* aquainted with it. By the way, is not pharmacopoeia today a she? It appears in a fresh new dress every five years and in between make-ups are applied. The last make-up of the 1953 edition included quite a few new drugs. One of them, phenylbutazone, has already been dropped, the remainder have been included in the 1958 edition.

The official British attitude towards new miracle drugs seems in the past to have been rather more sceptical than for instance the North American attitude. In this edition, however, some of the most modern drugs have received official recognition: acetazolamide, chlorcyclizine hydrochloride, chlorpromazine hydrochloride, erythromycin, hyaluronidase, hydrocortisone and its acetate, lucanthone hydrochloride, neomycin sulphate, noradrenaline acid tartrate, phenoxymethylpenicillin, piperazine adipate and phosphate, polymyxin B sulphate, prednisolone and its acetate, prednisone and its acetate, sodium radioiodide, sodium radiophosphate, etc.

Also included are some older well-known substances such as carbromal, dapsone, dexamphetamine sulphate, mustine hydrochloride, phthalylsulphathiazole, reserpine and solapsone.

An onlooker may wonder if there is not a slight change in the principles of selecting drugs now compared with the situation one or two editions back. A certain broader point of view of the advisability of giving standards for new drugs in an early phase of their life seems now to be prevailing. If this be the case the Commission is, in the reviewer's opinion, to be congratulated as it is,—still in the reviewer's opinion—far more important to have standards for a drug as soon as possible and while its use is still booming, than to discuss at length its merits compared with other drugs. The possibilities of publicising a new drug being what they are today, and the possibilities of stopping the trade with unwanted drugs being what *they* are, standards given for new substances nevertheless have an enormous influence today.

Concerning the attitude towards the chemical tests and assays it is safe to say that many of the historic tests for metals, chloride, sulphate, readily carbonisable substances (with concentrated sulphuric acid) have disappeared. The arsenic test is still there. The assays have in many instances been revised and older extraction methods have been replaced by titration in glacial acetic acid with perchloric acid. Included is also lithium methoxide N/10 for the titration of weak acids. A close comparative study of the 1953 and 1958 editions reveals that the laboratory side of the revision work was a dominating part. It is today a necessity to have access to good laboratory facilities for pharmacopoeial work.

As the multitude of new compounds in certain groups like the sulphonamides, antibiotics, antihistamines, and amines of different types is still increasing the compilers of pharmacopoeias are facing a serious problem: it is becoming more and more difficult to design identification tests for substances within those and similar groups, e.g., tests which really identify the substance. Often only one or

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two out of many hundreds of similar compounds are included in a pharmacopoeia. It is usually not difficult to design a good-looking test for a single substance. The danger is, however, that many of the compounds not included in the pharmacopoeia will give the same result when tested with the official method. This *fact* may lead to remarkable consequences if a case should come up in a court where the substance responded to the official tests but nevertheless was not the official substance. As examples of such substances the barbituric acid derivatives may be cited. Of these pentobarbitone sodium with a m.p. of about 128° for the acid is poorly identified. On the market are a number of substance all called pentobarbitone sodium with m.p. for the acid between 126° and 133°. The *p*-nitrobenzyl derivatives of these substances, however, differ as much as between 140° and 160°. In this case very little harm is done as the different substances probably have about the same action, but the example may illustrate the difficulties in designing adequate tests.

A legal situation of the kind outlined above recently occurred in the U.S.A. in connection with the increased production of Salk vaccine. The manufacturing firm was sued for millions of dollars by more than forty victims or their representatives. The court, however, seems to have decided that the cases against the manufacturer were invalid, as the produced vaccine actually met the official tests although the vaccine contained live virus.

Another general remark about pharmacopoeias is in connexion with the probable free trade market in Europe. The work on the unification of standards and tests cannot longer be looked upon as a game for internationally minded pharmacists, it is today a necessity for the manufacturer of basic drugs. The point cannot be stressed firmly enough that groups which choose to work for themselves play a fast and loose game with the export and import trade of drugs in their country. In this connexion the new British Pharmacopoeia is an excellent example of what can be achieved when common sense is accepted as a basis for the work.

It is out of question to review and discuss all the basic changes which have been made in the standards and the tests. However, a few may be of such general interest that it may be justified to comment upon them. The assay for adrenaline in Lignocaine and Adrenaline Injection is based on the oxidation of adrenaline to adrenochrome by means of ferric ions. The amount of adrenochrome produced is measured spectrophotometrically at 540 mμ. Experience has taught me that this method is definitely unsuitable. It works well as long as there is no destruction of adrenaline but a solution in which the adrenaline is destroyed to 50 per cent still contains 100 per cent adrenaline when assayed by this method. The only chemical method which is acceptable today is one based on the measurement of the fluorescence of a rearrangement product of adrenochrome formed from the adrenaline present.

The monograph on Injections is an excellent one and well worth detailed study. The appendix on Biological Assays and Tests is rewritten and consists with its eight subappendices of no less than fifty-three full pages. Noteworthy is that in the test for pyrogens the rise of temperature is interpreted in such a way that sometimes only three animals are needed; twelve animals will always be enough to ascertain a result.

Concluding this short review it shall be stated that the British Pharmacopoeia of 1958 is an excellent pharmacopoeia worthy of its predecessors. Again we have seen a demonstration of the high standard of British Pharmacy.

LETTER TO THE EDITOR

Sensitizing properties of *Haemophilus pertussis* vaccine

SIR,—We have recently reported that injections of *Haemophilus pertussis* vaccine potentiate the production of anaphylactic shock in the albino rat. Such treatment also renders the animal hypersensitive to exogenous histamine and 5-hydroxytryptamine[1]. It has now been found that rats treated with *H. pertussis* vaccine are not hypersensitive to adrenaline, carbachol, reserpine, or potassium chloride, and similar results have been reported in mice[2,3]. Thus the decrease in the resistance produced in animals by *H. pertussis* vaccine is confined so far to histamine, 5-hydroxytryptamine and the products of the antigen-antibody reaction. It is not certain whether the mechanism of the sensitizing property of *H. pertussis* vaccine is similar to that of its immunizing property, as detected by the mouse protection test. Both the histamine-sensitizing factor and the immunizing factor lose potency on storage at 37° for 30 days, though the loss in potency of the former factor is more marked[4].

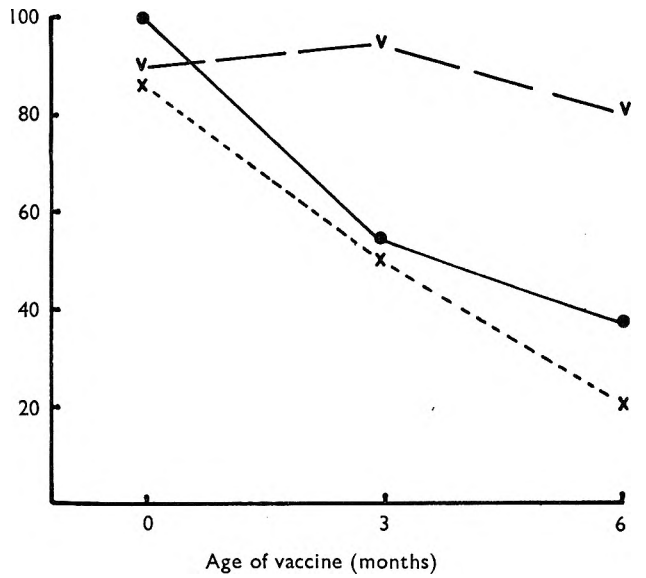

FIG. 1. The influence of age on three sensitizing properties of *H. pertussis* vaccine in rats. The responses recorded are the antigen-antibody reaction (●——●) expressed as the percentage mortality rate, and the sensitivity to histamine (× - - - ×) and to 5-hydroxytryptamine (v——v), both expressed in the form of the reduction in the LD50 value as a percentage of the control value for untreated rats. Note that the sensitizing properties of *H. pertussis* vaccine to histamine and to anaphylaxis are greatly reduced by storing the vaccine for 6 months.

We have now studied the effect of storage at 4° on the sensitizing property of *H. pertussis* vaccine. Control studies using a fresh supply of *H. pertussis* vaccine to test the sensitivity of the animals were carried out at each time period. It was found that such injections always produced the same degree of hypersensitivity to histamine, 5-hydroxytryptamine and the products of the antigen-antibody reaction. But vaccines stored for 3 or 6 months showed a

decline in their power to produce hypersensitivity to histamine and to anaphylaxis but not to 5-hydroxytryptamine (Fig. 1). Since the immunizing factor does not appear to deteriorate on prolonged storage at 4°, the factors in *H. pertussis* vaccine which render rats hypersensitive to histamine and anaphylaxis probably differ from those producing immunization or rendering the animals hypersensitive to 5-hydroxytryptamine.

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