REVIEW ARTICLE

THE CHEMOTHERAPY OF HELMINTHIASIS*

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This review deals in the main with helminth infections of the gastro-intestinal tract of man and animals.

The control of helminthiasis in man and animals will not be accomplished through chemotherapeutic measures alone. Such measures are, of course, of immediate value but of supreme importance is the establishment of a high standard of hygiene to minimise spread of infection. Stransky and Reyes[1] quote that while human ascaris infection in Central Europe is not higher than 3·5 per cent, under more primitive conditions, in the tropics, the percentage of infestation may be as high as 90 or more. The establishment of satisfactory hygienic conditions in the animal field will be more difficult. However, other measures of control available to the farmer could include the use of ovicides, continual use of chemotherapeutic agents at a low level as prophylatics, and immunological measures[2].

The progress made in the chemotherapy of human and animal helminthiasis has been slow and the few successes achieved in recent years do not match those made in the bacterial and protozoal fields. Anthelmintics have been in continual use since the days of early man when it was recognised that a diseased condition was associated with infection by helminths. Whereas diseases such as trypanosomiasis, yaws, leprosy, malaria and others are slowly being brought under control in tropical areas helminthiasis is still as pressing a problem as it was 50 years ago. It is a sad reflection that the 2000 years-old filix mas treatment of tapeworm infection is still in use today despite the fact that it is not always successful and the drug has a very small chemotherapeutic index. Some of the older remedies and also some of the present day treatments, especially of the gastrointestinal infections of animals, can only be described as drastic. Substances like lead arsenate, sodium arsenite, nicotine sulphate, carbon disulphide, and carbon tetrachloride, are amongst the more noxious materials on the chemist's shelf. These treatments are possible under conditions where the quantity of drug absorbed into the blood system is small and activity against helminths is by virtue of the generally poisonous nature of the substance. Much larger chemotherapeutic indices are required for drugs for use in blood and tissue diseases. The aim should be, to develop chemical agents which function specifically as anthelmintics with chemotherapeutic indices larger than those of the majority of anthelmintic drugs in present day use.

Many helminth diseases exist for which no anthelmintic treatment is available or the available treatment yields variable results and is often hazardous.

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There are two main contributory factors which are probaby responsible for the slow pace of development of chemotherapeutic measures and which will be discussed in detail. The first of these has been the failure to recognise the harmful effects of the disease and the second has been the difficulty involved in devising satisfactory screening methods.

Is Helminthiasis of Importance?

Helminth disease of man and animals is seldom fatal. It lacks the urgency which is associated with more lethal diseases and it has consequently attracted less research effort from chemotherapeutists. Clinicians, however, now recognise that the health standards of whole populations are affected by helminthiasis, with a consequent lowering of resistance towards other diseases, and that heavy infection, especially of children inevitably leads to malnutrition. Jelliffe[3] states that infestation with the large roundworm, *Ascaris lumbricoides*, is almost universal in many tropical areas and that it is common for children to carry worm burdens of 100 or more. Stoll[4] has calculated that 644 million people are infected with *Ascaris lumbricoides* and 209 million people with the seat-worm *Enterobius vermicularis.* He estimates that 456 million people are infected with human hookworms, 39 millions with the beef tapeworm *Taenia saginata*, 20 million with the dwarf tapeworm *Hymenolepis nana*, 10 millions with the fish tapeworm *Diphyllobothrium latum*, 2½ millions with the pork tapeworm *Taenia solium*, and 27 millions with the pork trichina worm. Infection with blood and tissue worms or flukes is also as widespread.

The extent of infection in the animal world is even more striking. The farmer today is well aware of the damage to the economical and nutritional management of his stock. He realises that the insidious nature of the disease results in lower wool, milk and meat production. In 1937, the British Veterinary Association concluded that parasitic gastro-enteritis in sheep caused losses equal to £348,000 per annum in Great Britain. This figure could probably be multiplied by a factor of 3 according to the post-war value of sheep. A recent estimate by the Northern Ireland Ministry of Agriculture showed that liver fluke was responsible for the loss to them of approximately 360 tons of liver per annum. Foster[5] and Boughton[6] have made estimates of United States losses in the livestock industry through helminth infection. The latter quotes a financial loss of 227,672,000 dollars per year. He also estimates that swine in the United States carry a burden of 12 million lb. weight of *Ascaris lumbricoides.*

Difficulties of Screening

Gastrointestinal helminths in particular, exhibit not only host specificity but they also occupy specific localities within that host, depending on factors like oxygen tension, carbohydrate and vitamin supplies, pH, and nature of the host secretions at that locality. They are particularly sensitive to even small changes in the surrounding conditions and it has been possible only in a limited number of cases to culture helminths *in vitro.*

In vivo testing of related helminths in small animals is not entirely satisfactory as there is a wide variation in response of even very closely related helminths to the same anthelmintic. In the last analysis the drug must be tested against the particular helminth in the particular host. Such a screening was carried out on an extensive scale in man by Caius and Mhaskar[7] but these methods are time-consuming and expensive and must of necessity be limited in extent. However, *in vitro*, and *in vivo* screening tests in small animals, do allow a comparison to be made of the relative anthelmintic values of members of a series of compounds and comparisons with chemicals of known anthelmintic activity can always be made. The tests described below are some of those which are in general use. A more detailed discussion of screening methods and techniques of assessing individual activities of drugs is given by Stewart[8]. Whitten[9] discusses screening methods for compounds with taenicidal activity.

Test A. Trendelburg[10] found that the common earthworm responded to santonin and he then employed it as a screening test for human *Ascaris*. Although many investigators have pointed out the inadvisability of using annelid material for anthelmintic screening purposes, the test is still used. Singh and others[11] state that it may be of particular value as a preliminary screen for taenicidal action.

Test B. Hall[12] tested the activity of drugs against *Ascaris*, hookworm, trichuris and tapeworm in dogs by means of his "critical test". This test has been the basis of subsequent screening techniques using various host animals in which the numbers of worms voided after administration of drugs and numbers of worms remaining on post mortem are counted. Leiper[13] uses domestic hens infected with *Ascaridia*, *Heterakis* and *Capillaria*.

Test C. Lamson and others[14] used intact specimens of pig *Ascaris* which were kept alive for a limited period of time. In this test santonin was inactive.

Test D. Baldwin[15] has used tied off neuromuscular preparations of *Ascaris*. Although the majority of the known anthelmintics had an effect on the neuromuscular apparatus, phenothiazine and gentian violet were found to be inactive. Baldwin's test has been adapted by Chance and Mansour[16] for screening compounds active against liver fluke and by Batham[17] for the screening of compounds with taenicidal activity.

Test E. Whitlock[18] and Rogers[19] have used the trichostrongylid worm *Nippostrongylux muris* for the screening of potential anthelmintics for use in trichostrongyle, *Ascaris* and hookworm infections.

Test F. Erhardt[20] has used cats infected with strongyloides, whipworm, *Ascaris*, cestodes and trematodes for screening purposes.

Test G. Erhardt and Gieser[21] used rabbits infected with *Passalurus ambiguus* for the screening of oxyuricidal compounds.

Test H. Leiper[22] and Vanne[23] have used the free living vinegar eelworm for preliminary screening of potential nematocidal drugs.

Test I. Mice infected with *Aspiculuris tetraptera* or *Syphacia obvelata* or with both worms have been used for screening purposes[24–29]. The test is of particular value for screening compounds with oxyuricidal properties.

Test J. Stewart[8] suggests that a mixed infection of *Heterakis spumosa* and *Nippostrongylus muris* in rats could be a valuable screening test for activity against *Enterobius*, strongyles, hookworms and trichostrongyles.

Test K. Taenicidal activity has been specifically screened by Harwood and Jerstad[30] using *Raillietina cesticillus* in chickens and by Holton[31] using *Hymenolepis diminuta* and *H. nana* in mice.

Test L. Parnell[32] and later Levine[33] have used fresh horse strongyle larvae for the screening of compounds with larvicidal activity. Since this activity is directed towards nematode tissue Levine has expanded the test and has adopted it as a general screening test for anthelmintic activity to which known anthelmintics respond. Parnell and Mackie[34] have tested a large number of chemicals by this method.

But even if a particular chemical were known to have the required anthelmintic properties it cannot be said with certainty that anthelmintic action will follow in the intended host. Some helminths are well protected by mucous and some are more deeply embedded in the gut wall than others and so less prone to attack. Physical factors may therefore determine whether anthelmintic action takes place. These factors can also so modify anthelmintic action that a compound inferior in a screening test, might function better in the host than a more active compound owing to less interference from mucous or greater ease of penetration of the helminth cuticle. The rate of penetration of cuticle by hexylresorcinol, for instance, can be greatly diminished in the presence of mucin and bile salts and can be increased in the presence of small concentrations of a natural soap or a synthetic detergent[19,35,36].

In the assessment of results obtained from screening tests and in the consideration of whether any particular compound is likely to show activity in the host, such physical factors as the relation of solubility and absorption of the drug by the host to the locality of the helminth within the host, particle size of insoluble drugs, solubility and pH dependence, and behaviour in presence of mucous must be taken into account. Chemical behaviour, like possible hydrolysis of the drug, is equally important.

The establishment of satisfactory screening methods and indeed the solution of the problem of the chemotherapeutic approach to helminthiasis will be possible only through the co-ordinated efforts of biochemists, parasitologists, chemists, and veterinary or medical clinicians. Their problems in the helminth field are inter-related.

The recent findings in nutritional and metabolic studies of helminths, lead one to anticipate the establishment of screening tests in which it will be possible to test the action of chemicals against an organism in a habitat approaching that of its natural surroundings. Indeed, such studies might eventually lead to a more rational approach to chemotherapy where one could make attempts to interfere with essential metabolic processes of the helminth. But, while vermicidal activity might well be demonstrated in such *in vitro* tests, vermifuge action would be a more difficult matter to recognise.

Nutritional studies have established that utilisation of glucose and the rapid synthesis and utilisation of glycogen are the common function of

many helminths. Read and Rothman[37,38] and Read and Laurie[30] have studied the effect of diets rich and deficient in glucose and other sugars on the normal development of the tapeworm. Gaafar and Ackert[49] studied the effects of low calcium and phosphorus diets on the growth of *Ascaridia galli* in chicks. Other studies include the effect of exclusion from diet of vitamins A and B complex, proteins and minerals, on helminth growth and the resistance of the host animals to helminth infection[41–43].

The availability of such modern techniques as partition chromatography, radioactive tracer and electrophoretic methods has resulted in increased knowledge concerning the metabolic processes and chemical composition of helminths. The advances in this field have been reviewed by Bueding[44] amongst others, and more recently by von Brand[45]. Helminth oxidative mechanisms are inefficient and a variety of carbohydrate oxidation products results. Bueding[44] states that whereas human *Ascaris* excretes mainly the lower fatty acids, notably *n*-valeric acid, *Moniezia expansa* of sheep and *Fasciola hepatica* excrete mainly the higher fatty acids. Protein and lipid characterisation and metabolism in parasites have not received as much attention as have the carbohydrates. It is known that nematode cuticle is almost completely composed of protein. Recently, Bird[46] has found that the cuticles of three species of nematode *Ascaris lumbricoides* var. *suis*, *Toxocara mystax* and *Strongylus equinus* contain the majority of naturally occurring amino acids and that they show a similarity of number and type in the different helminths. There were, however, slight quantitative differences. The unsaponifiable lipid fraction in many parasites is large. Fairbairn and Jones[47] have confirmed the presence of cholesterol and saturated sterols as well as a substance related to ascaryl alcohol in the body wall of *Ascaris*.

Compounds Used as Anthelmintics

A large number of chemical compounds has been shown to exhibit anthelmintic properties to a widely varying degree. There are possibly more chemical agents available for combating helminth disease of the gastrointestinal tract than there are for combating any other type of disease. This is because a direct method of attack on such helminths has been possible, by the easy method of administration *per os*, and assessing the results by examining the faeces. These chemical agents may be large in quantity but they are poor in quality and relatively few are satisfactory anthelmintics from the point of view of efficiency, or chemotherapeutic index.

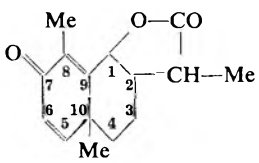
In the course of this review a selection of the accepted and more promising anthelmintics has been made.

The older anthelmintic remedies from plant extracts although sometimes exhibiting a high efficiency, frequently give rise to toxic symptoms. The chemist has been able to extract and characterise the active principles in these remedies so that an exact dosage schedule has been made possible. But variations in the structures of these active materials have not yet produced compounds of improved anthelmintic quality.

The natural products will be considered first.

Santonin

Santonin (1) is the active principle of *Artemesia maritima*, var. *anthelminticum*. Until quite recently it was the drug most widely used in the treatment of human ascariasis. Trendelburg[10] recognised that its anthelmintic properties were probably due to its effect on contraction of worm muscle.

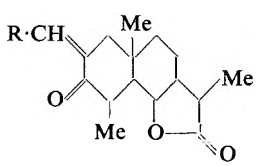


(I)

Caius and Mhaskar[7] tested a number of santonin-like compounds (santoninic, santonic and santonous acids, *desmotropo*santonin and santonone) in humans and concluded that the active centre of the santonin molecule was the ketonic grouping at 7. Baldwin[48] using Test D, in a study of similar santonin-like compounds found that the structures common to the active compounds of the series were an intact γ-lactone ring, a double bond at position 7, and an angular methyl group at position 10. He then proceeded to test a variety of compounds containing the active centres contained in santonin. Activity was shown in substituted alkyl and substituted phenyl acetophenones, various benzophenones and benzylidene acetones amongst which were found compounds with activities approaching those of thymol and β-naphthol. Alkylated resorcinyl ketones, cyclic diketones and α-ketoacids showed little promise.

Lautenschläger[49], von Oettingen[50] and other workers had previously found an activity amongst the lactones and this was confirmed by Baldwin[48]. He found that the γ-butyrolactones were particularly active but not as active as santonin itself and that coumarin and 3-coumaranone exhibited some activity. Recently Nakabyashi and others, have expanded this series. They have studied the relative anthelmintic activities of santonin and coumarin derivatives including octahydro- and thia-derivatives[51] and have concluded that the ketonic character of santonin is of supreme importance.

Cocker and McMurray[52] suggested that the active santonin compounds investigated by Baldwin were all capable of chelation and might function therefore by removing essential metallic ions from enzyme systems of nematodes. They however found little activity in a series of α-tetrahydrosantonin compounds (II) which contained the essential features for activity of the santonin molecule and also were capable of chelation.



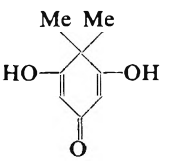
(II)

R = OH; C_6H_5; *p*-MeO-C_6H_4; CH_2—O

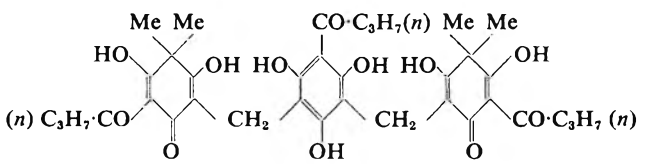
Filix Mas

An ethereal extract of male fern is a preparation widely used for treatment of tapeworm infestation in many animals and also in liver-fluke infestation of ruminants. Its chemical characteristics were first studied by Boehm[53]. The compounds present—filixic acid, aspidin, albaspidin, flavaspidic acid, filicinic acid, aspindol and others—are inter-related and are all phloroglucinol derivatives. Much investigational work has been carried out on the constitution and synthesis of these substances.

A frequently occurring degradation product is filicinic acid (III), which Robertson and Sandrock synthesised in 1933[54]. Flavaspidic acid and albaspidin as a by-product were synthesised by Riedl[55], from butyrylfilicinic acid, formaldehyde and butyrylmethylphloroglucinol. Recently Chan and Hassall[56] have proposed an alternative structure to that suggested by Boehm[57] for filixic acid, which is the major biologically active constituent. The formula they propose (IV), $C_{36}H_{44}O_{12}$, is in better agreement with the experimental analysis and is also in agreement with ultra-violet absorption data.



(III)



(IV)

The whole extract of male fern is still used since it is computed that each constituent contributes towards the anthelmintic activity. Anthonen[58] is of the opinion that pure flavaspidic acid is more effective and less toxic than male fern extract.

Inagaki and colleagues[59], have synthesised various acylphloroglucinols, methylphloroglucinols and their condensation products with formaldehyde with the view to testing their anthelmintic power. A large number of ketonic phloroglucinols has been synthesised by Riedl and others[60].

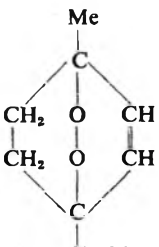
Of great interest is the fact that other tapeworm remedies of natural origin contain phloroglucinol derivatives usually involving a methylene bridge. Such substances are kamala which has been resolved by Khorana and Motiwala[61] into five constituents of which rottlerin and *iso*rottlerin alone showed anthelmintic activity. Birch and Todd[62] have proposed formulae for protokosin, α- and β-kosins which are found in "Kousso". These structures again contain the phloroglucinol unit.

Oil of Chenopodium

The oil from *Chenopodium anthelminticum* (American wormseed plant) was used for anthelmintic purposes by the South American Indians and was introduced into Europe in 1881. Its active constituent, ascaridole, is present to the extent of 45–70 per cent. Bruening[63] recognised it as a

valuable agent for removal of human *Ascaris* and hookworm but he also noted its toxic properties. It was formerly widely used for removal of ascarids from pigs but it has now been superceded for this purpose by the piperazines.

The structure of ascaridole (V) was elucidated by Nelson in 1911[64] and it has been synthesised by photochemical oxidation of α-terpinene by Bodendorf[65] and later workers.



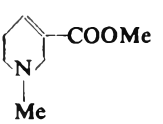
(V)

Caius and Mhaskar[7] reported that reduction of ascaridole increased its anthelmintic powers but its toxicity was thereby also increased. Little investigation of the anthelmintic activity of compounds similar in type to ascaridole has been made. A review of ascaridole and its uses has been given by Schenk[66].

Arecoline (VI)

This alkaloid is the principle constituent of areca nut. It has been used in the form of a variety of salts as an effective taenicidal agent, particularly for dogs.

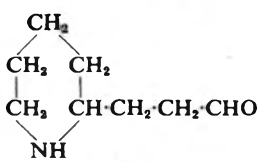
It was first used for this purpose by Lentz[67]. It frequently causes vomiting. The antidote for arecoline poisoning is atropine. Many derivatives have been synthesised, mainly during the earlier work in the elucidation of its structure but their taenicidal activity has not been recorded.



(VI)

Arecoline causes violent peristaltic movements and causes an outpouring of mucous secretion. Its taenicidal action may therefore be purely mechanical in that it causes the tapeworm to become detached and hence expelled from the host.

Similar taenicidal compounds are the pelletierines which occur in pomegranate bark. Four alkaloids are known to be present, viz. pelletierine (VII), *iso*pelletierine, methylpelletierine and pseudopelletierine. These compounds are toxic. Variations in the structures may prove of value in attempts to decrease their toxic nature.



(VII)

Synthetic Compounds

The Phenols

Phenolic substances have consistently been shown to exhibit anthelmintic properties usually of a nematocidal nature. In many instances the anthelmintic activity has paralleled the antibacterial activity. This has led to the belief that the phenols (as also do certain antibiotics) destroy the bacterial flora upon which the helminth may be dependent or with which it is in biological equilibrium. However, direct action by the phenols on the helminth itself has been recorded[14,35]. It is possible that phenols form complexes with essential metabolites through hydrogen bonding.

The anthelmintic properties of phenols were first recognised by Bozzolo in 1879[68], who used thymol for treatment of hookworm infestation.

β-Naphthol was used by Bentley in 1904[69] and further evaluated by Schüffner and Vervoort[70] in hookworm disease. Lamson and others[71,72], investigating the anthelmintic properties of phenols chose hexylresorcinol since this compound had been shown by Leonard[73] to be the most active germicidal agent of the alkylphenols. They found the compound to be 93–96 per cent efficient against *Ascaris* in man and that it was relatively non-toxic although it caused temporary irritation of the mouth. Lamson and others[14], then made a systematic study of more than 150 phenols, using screening Test C in the hope of finding a phenol which would show the same or better anthelmintic properties as hexylresorcinol, and yet be non-irritant. Despite this and much subsequent work, hexylresorcinol remains the phenolic compound of greatest activity against *Ascaris*, *Enterobius*, hookworm and *Trichuris*. It is also reported to have some taenicidal action.

The compounds examined by Lamson and colleagues[14] included the alkylphenols and alkylpolyhydric phenols, chlorinated phenols, naphthols, phenanthrols, hydroxydiphenylmethanes and ethanes, *cyclo*hexyl, benzyl and phenylphenols, etc. They found that in an alkyl series of phenols the amyl, hexyl and heptyl compounds showed maximum activity. In such a series they correlated anthelmintic activity solubility, and melting point of the phenol.

Other phenols of interest which have been announced from time to time are 4-*t*-butyl-2-chlorophenol[74], 2-ethyl-4-chloro-6-hexylresorcinol[75], 4-fluoro-2-propylphenol[76], 6-*t*-butyl-1-chloro-2-naphthol as a taenicidal agent[77], resorcinol ketones as taenicides[78], 1:8-dihydroxyanthraquinone, active against *Trichuris* in dogs[70] and *Trichuris* in sheep[89], 1-bromo-2-naphthol[81] and the hydroxydiphenylmethanes, which are discussed later. Tomita and others[82], in a study of active, chlorinated alkylphenols found all the compounds to be irritant to the tongue. Martin[83] could find no marked relation between structure and anthelmintic action in a series of polyhydric phenols. Fushimi[84] from a study of a large number of phenols established that the pig *Ascaris* screening test (Test C) was of value in the screening of compounds active against human *Ascaris*.

The irritant properties of hexylresorcinol can be greatly reduced by complex formation with piperazine[85] which itself is an active ascaricide (see later). Two compounds are described which are formed in the ratio of 3 piperazine:2 hexylresorcinol molecules and 1 piperazine:2 hexylresorcinol molecules. Both compounds readily break down in presence of acid to regenerate the components.

Phenolic compounds have proved of great value in the chemotherapy of helminthiasis. They are of especial interest in that they show a wide spectrum of activity and are relatively nontoxic. Investigational work into the anthelmintic activity of phenols is by no means a closed chapter and further work may yet result in improved anthelmintics.

Halogenated Hydrocarbons

This group of compounds displays anthelmintic activity particularly

against hookworm and liver fluke and to some degree against *Ascarids*. They are probably all protoplasmic poisons.

Hall[12] found carbon tetrachloride to be very efficient in hookworm infestation of dogs. Tetrachlorethylene was found by Hall and Shillinger[86] to be less effective than carbon tetrachloride but less toxic. Hexachlorethane[87–89] is another drug of this type which is now widely used for treatment of fascioliasis in cattle and it is claimed to be well tolerated by animals. Kudicke and Weise[90] in an examination of a number of halogenated derivatives of the lower hydrocarbons found that the anthelmintic activities were inversely related to the solubilities of the compounds. In general the bromo- compounds were more active than the chloro-analogues. Butyl chloride was found by Wright and Schaffer[91] in their study of chlorinated alkyl hydrocarbons to be highly active against *Ascarids* and hookworms in dogs and by Harwood and others[92], to be moderately active against whipworm in dogs. Its activity against whipworms, hookworms and roundworms of dogs was confirmed by Whitney and Whitney[93].

Other chlorinated hydrocarbons reported to show good *in vitro* activity are 4-hydroxy-4-trifluoromethyl-1 : 1 : 1 : 7 : 7 : 7 : hexafluoroheptane[94] and hexachloropentadiene[95].

Few halogenated aromatic hydrocarbons have been tested for anthelmintic properties. Dickmans[96] showed that *p*-dichlorobenzene was active against hookworms, roundworms and whipworms in dogs but Daubine[97] found it to be inactive against sheep helminths. Gordon[98] however, found that it was active against *Haemonchus contortus* and *Trichostrongyles* of sheep if the oesophageal groove reflex were first stimulated by a dose of copper sulphate. He found that *o*-dichlorobenzene was superior to the *p*-isomer and that bromo-, trichloro- and tetrachlorobenzenes were inactive.

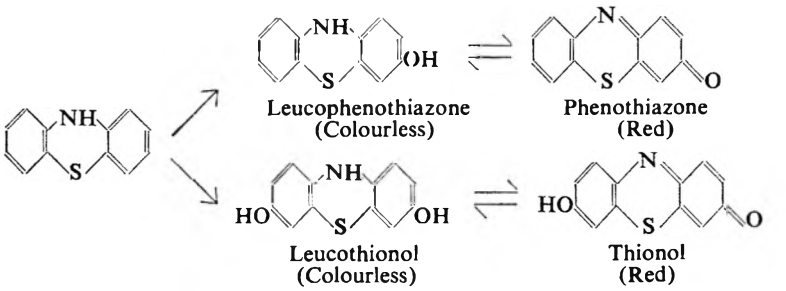
Phenothiazine

The discovery in 1938 by Harwood and others[99], of the valuable anthelmintic properties of phenothiazine proved to be a major contribution to the field of veterinary medicine. Although it is used for the treatment of helminth infection of many animals it is of particular value in nematode infections of sheep and cattle.

Several reviews dealing with the use of phenothiazine as an anthelmintic have appeared[100–103]. The compound is only slightly soluble in water (1 part in 800,000) yet 40 per cent of an oral dose is absorbed and excreted in the urine in the form of a number of soluble phenothiazine derivatives[104]. The metabolism of phenothiazine in the animal body has received considerable study but it has not yet been conclusively established whether phenothiazine itself or a derivative thereof is the active anthelmintic principle.

Phenothiazine is readily oxidised according to the following scheme[100] and such an oxidation-reduction system could conceivably interfere with essential enzymatic processes in the worm. Phenothiazine and its oxidation products are effective inhibitors of many mammalian enzyme systems[105]. It seems probable that phenothiazine causes paralysis of the

muscular systems of helminths and causes failure in the reproductive system. Lazarus and Rogers[196] using ^{35}S labelled phenothiazine found that the main portal of entry of the drug was via the helminth cuticle. Esserman[107] also using ^{35}S labelled drug, found that the intestinal fluid of rats and chickens fed with the labelled drug and also the parasites themselves (*Ascaridia galli*) contained phenothiazine and a complex of phenothiazine with a fatty substance and no oxidation product was detected.



(VIII)

Habermann and Shorb[108] showed that small daily doses of phenothiazine given to sheep in the form of a salt-lick were effective in inhibiting the development of worm larvae in the faeces. This technique of dosing has been examined with great thoroughness and it is now employed as a valuable prophylactic measure in the control of helminthiasis.

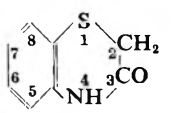
Although of unquestionable value to the farmer, phenothiazine has its limitations. The nematodes of sheep vary in their susceptibility to the action of the drug so that large doses are required for elimination of the more resistant helminths and some species of sheep helminths are totally resistant. Staining of wool by the coloured oxidation products is a serious problem in wool-producing areas.

Despite much chemical work involving the manipulation of the phenothiazine skeleton and formation of phenothiazine derivatives no compound with anthelmintic properties superior to those of phenothiazine itself, has been forthcoming. In general, it can be said that substitution in the phenothiazine molecule has the effect of decreasing the anthelmintic action. Activity is retained, however in those compounds, such as 10-acyl derivatives which can regenerate phenothiazine under *in vivo* conditions. *In vitro* testing of such compounds would indicate that they were inactive. Stable substituents in positions 3, 7 and 10 could reasonably be expected to yield compounds of diminished activity since these would prevent quinone formation (see VIII). An even greater reduction in activity which is unexpected, occurs when substituents are placed at positions 2 and 8 (Leiper and Watkins, unpublished).

Diphenylamine, xanthone, phenothioxine, and phenazine, even in large doses did not give promising results in limited tests against *Ascaris* or *Oesphagostomum* in swine but diphenylamine had some action in sheep, although it proved to be very toxic[109]. Guthrie[110] found that diphenylamine was active against *Trichuris* in dogs. Gordon and Lipson[111] found

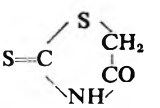
that phenothiazone and thionol were inactive but that phenothioxine had some activity against *Haemonchus contortus* in sheep. Gordon[112] also found that methylene blue, xanthone, trimethylthianthrene were inactive and Whitten[113] found the sulphoxide to be against *H. contortus*.

Mackie and Raeburn[114] found that phenothiazone was lethal and that thionol and phenothiazine sulphoxide were paralysant only in the Chance and Mansour screening test for liver fluke. They found considerable paralysant activity in a series of 6-substituted 2:3-dihydro-3-keto-1:4-benzothiazines (IX).



(IX)

Mackie and colleagues[115,116] tested a series of β-10-phenothiazinylpropionic acid esters and salts and found that a number had a paralysant effect on liver fluke but only the free acid and its sodium salt had any effect on roundworm (Test D). Derivatives of rhodanine (X) were also tested but little activity was obtained in the series. The benzylidene compounds however, had marked activity against liver fluke.



(X)

Rogers and others[117], using Test I have examined the anthelmintic activity of a number of tricyclic compounds in which the NH and S groups of phenothiazine were replaced by other groups such as CH, CO, N, O, S, Se, SO_2. They also tested carbazole and compounds in which its NH grouping had been replaced by other groups, diphenylamine and diphenyl sulphide. The only compounds which they found to be active were phenothiazine and phenoxazine. Other compounds prepared by these workers were 2:3-dihydrobenzo-1:4-thiazine and various alkyl-phenothiazines.

Levine (Test L) has examined various 10-substituted phenothiazines, phenothioxine, xanthone, xanthydrol, phenazine and phenoxazine. The potassium salt of 1:3:7:9-tetrasulphonic-5:5′-phenothiazine dioxide showed an interesting activity. (2:8?)-Phenothiazinedisulphonic acid as the soluble calcium or sodium salt has been reported by Pegreffi and Quesada[118] to be active in a bronchopneumonia caused by larvae and eggs of *Synthetocaulus rufescens*.

The Piperazines

The screening of compounds for filaricidal activity by Hewitt and others[119], using cotton rats infected with *Litomosides carinii* led to the discovery of Hetrazan, also named diethylcarbamazine, 1-diethylcarbamyl-4-methylpiperazine, hydrochloride. This compound which is now widely used in the treatment of filariasis has also been reported to have some action against *Ascaris* in man[120] and also against the *Ascarids* of dogs and cats[121,122].

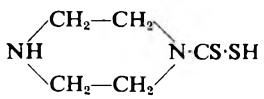
In 1949, Fayard[123] reported that the parent base piperazine was active against *Ascaris* in man. Mouriquand and others[124], found that it was active against *Syphacia obvelata* and *Aspiculuris tetraptera* in mice (Screening Test I) and they extended their studies to *Enterobius* and *Ascaris*

infections in man with successful results. Subsequent work has confirmed the value of piperazine and its salts in ascarid and oxyurid infections of man and animals[125–139].

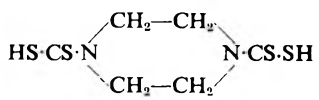
Gordon[131] showed that the piperazines were of limited use only, in helminthiasis of sheep. Marquardt and Fritts[132] found that there was no action against a variety of helminths in sheep.

Brown and colleagues[133] from tests in mice (Screening Test I) and Leiper and Watkins (unpublished) from tests in chickens, have shown that substitution in any position in the piperazine molecule has the effect of decreasing anthelmintic activity. This is reminiscent of the effect on activity, of substitution in the phenothiazine molecule. Harfenist[134], however, has found that some high alkyl substituted piperazines have an activity equal to that of piperazine itself.

Piperazine, which is a strong base, exists as a deliquescent hexahydrate. It is presented on the market in the more palatable form of salts (diphenylacetate, adipate, citrate, tartrate, dilaurate, etc.). Leiper[129] describes an insoluble complex of piperazine and carbon disulphide, which is decomposed by gastric juice into its components, both of which are anthelmintic in action. This compound was shown by Dunderdale and Watkins[135] to be a polymer structurally composed of the units, piperazine-1-carbodithioic acid (XI) and the piperazine salt of piperazine-1 : 4-dicarbodithioic acid (XII).



(XI)



(XII)

Oelkers[136] has noted that piperazine exerts a paralysant action on *Ascaris lumbricoides* and *Enterobius* and also on nerve preparations of earthworms and leeches. It acts as a vermifuge and worms are expelled alive from the host. Norton and Beer[137] find that piperazine produces paralysis of *Ascaris* by blocking the neuromuscular junction.

Piperazine salts in general are very soluble in water and Harned and others[138], have shown that a large proportion of an oral dose of piperazine is excreted in the urine. The high activity against *Ascarids* and particularly against *Syphacia obvelata* and *Enterobius vermicularis* is therefore surprising as it has been generally accepted that chemical agents with low water solubility are more likely to reach the localities of the intestinal helminths. It would be interesting to establish whether the piperazine susceptible helminths are affected by the direct action of possibly only a small fraction of the oral dose which reaches them or whether in fact it is the absorbed material in the blood stream, either as piperazine or a metabolite thereof, which is the active anthelmintic principle. It is interesting to note, in this connection, that phenothiazine which is virtually insoluble in water is particularly active against the sheep stomach worm, *Haemonchus contortus*, which has been conclusively shown by Leiper and Watkins (unpublished) using ^{32}P labelled blood, and by other workers to be

a voracious blood sucker. Here also it is possible that these particular worms receive their dose of drug through the blood stream which is known to contain phenothiazine metabolites. These observations may lead one to speculate whether more attention should be paid to administration of anthelmintics by parenteral methods.

Metallic Compounds

Copper sulphate and sodium arsenite are materials which have been of use in the treatment of sheep helminthiasis. Sodium fluoride[139] was formerly used for removal of *Ascarids* from pigs. Tin compounds also show interesting anthelmintic properties. Guthrie and others[140], showed that tetra*iso*butyl tin was active against *Raillietina cesticullis* in chickens and Guthrie and Harwood[141] showed that tin oleate, tartrate and oxalate were active taenicides. The taenicidal activity of "Stannoxyl"—a mixture of tin, tin oxide and tin salts was examined by Le Gac[142] and by Hirte[143]. Kerr and Walde[144] tested a large number of tin compounds against *Ascaridia galli* and *Raillietina cesticillus* in chickens. The compounds were of the type $R_{4-n}SnX_n$ where R = alkyl, aryl or aralkyl, X = O, S or an inorganic or organic anion and n = 1, 2 or 3. This work was followed by a report by Kerr[145] in which it was stated that di-*n*-butyl tin dilaurate was an efficient taenicide in chickens, of chemotherapeutic index = *ca.* 20. Kerr and Walde[146] have extended their study of tin compounds. They found a consistent activity against *A. galli* and *R. cesticillus* in 112 compounds of the type R_2SnX_2.

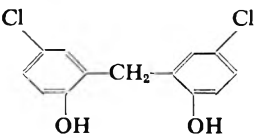
Compounds of the type R_3SnX were also tested and of these tributyl tin-acetate, -chloride and -thiol were found to be very active. Compounds of general formula $RSnX_3$ and R_4Sn were generally inactive. During the course of this work the authors made the observation that the compounds were likely to show a greater anthelmintic efficiency when they were administered to chickens in their feed than when they were administered in capsules. They concluded that distribution of a compound in the gut and possible repeated exposure to a compound is essential for a high degree of activity.

Cadmium compounds were shown by Guthrie[148] to be effective ascaricides in pigs but the soluble compounds proved to be rather toxic. However, cadmium anthranilate, being insoluble and therefore less likely to be absorbed from the gastrointestinal tract, showed less evidence of toxicity. A range of cadmium compounds was investigated by Levine and Ivens[149] (Screening Test L).

Diphenylmethanes

An interesting taenicidal activity has been discovered in halogenated hydroxydiphenylmethanes which compounds also exhibit germicidal properties. Craige and Kleckner[150] showed that 5:5′-dichloro-2:2′-dihydroxydiphenylmethane (XIII) (Dichlorophen, Diphenthane-70, G4) was very active against the tapeworms of dogs, and this compound is now widely used in the taenicidal treatment of a number of animals. Another compound of this series which also shows taenicidal action is

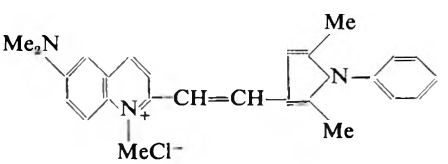
3:3′:5:5′:6:6′-hexachloro-2:2′-dihydroxydiphenylmethane[151] (Hexachlorophene, G 11). The compounds are efficient and not very toxic but the chemotherapeutic doses are large. The worms are killed by the drug and they then disintegrate during their passage through the intestines. A large number of diphenylmethane compounds have been synthesised as potential germicides but the anthelmintic properties of the majority are not known. Kerr and Green[152] studied a series in which the number and positions of chlorine and bromine substituents and of the two hydroxyl groupings were varied and the methylene grouping was also replaced by oxygen. Taenicidal activity apparently increased with increasing halogen content. It was not established whether the presence of hydroxyl groupings was essential for activity.



(XIII)

Cyanine Dyes

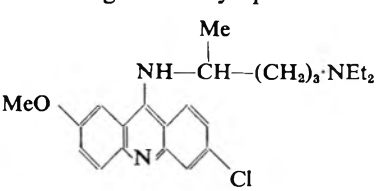
Various cyanine dyes have been shown to possess anthelmintic action. 6-Dimethylamino-2-[2-(2:5-dimethyl-1-phenyl-3-pyrryl) vinyl]-1-methylquinolinium chloride (XIV) was shown by Hales and Welch[153] to be active against *Ascaris*, hookworms and whipworms in dogs. It is also reported[154] that it is as active as the piperazines against *Enterobius vermicularis* in man. Cyanine compounds which have been reported to be of value in the treatment of helminthiasis of sheep and of threadworm in man are various 1:1′-dialkyl-2:2′-quinocarbocyanine salts[155], (1-alkylquinoline-2) (3′-alkylbenzthiazole-2′) trimethine cyanine salts[156] and 1:1′-dialkyl-2:2′-benzthiazole trimethine cyanine salts[157].



(XIV)

Acridines

Culberston[158] in 1940 found that mepacrine (XV) hydrochloride was active against *Hymenolepis fraterna* in mice and Neghme in the same year found that it was active against *Taenia saginata* in man. The compound is now widely used as a taenicidal agent in man and most of the reports of its use are favourable although toxic symptoms occur occasionally.

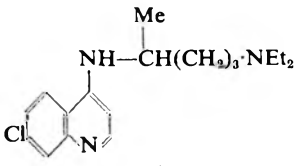


(XV)

Mustakillo and Saikonnen[160] suggest that mepacrine (an electropositive dye according to Keller[161]) abolishes the electrochemical forces responsible

for the attachment of the scolex of the worm to the intestine. The worm is usually expelled alive.

Camero[162] found that chloroquine (XVI) diphosphate was active against *Taenia saginata* and that it was non-toxic.



(XVI)

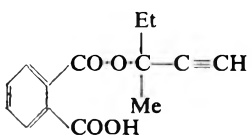
Surrey and others[163], found that compounds whose structures contained hydroxyl groups in the basic side chain of mepacrine had marked anthelmintic activity against *Aspiculuris tetraptera* and *Syphacia obvelata* in mice (Screening Test I). Kotova[164] and Semenova[165] have tested a large number of acridine compounds against *Hymenolepis nana* in mice. 5-Aminoacridine and 5-substituted-aminoacridines were reported to exhibit the greatest activity.

Antibiotics

A large number of antibiotics has been screened for anthelmintic activity, mostly by the mouse test (Screening Test I). The compounds active in such screening tests were—oxytetracycline[166], chlortetracycline[167,168], bacitracin[29,169], erythromycin[170], puromycin (Stylomycin) [171,172]. Some anthelmintic activity has also been found in neomycin, chloramphenicol but not in dihydrostreptomycin. Wells and others[173], found that oxytetracycline was active in *Enterobius vermicularis* infection of man and it is now widely used for this purpose. Hygromycin has been found to be active against *Ascaris lumbricoides* var. *suum* in pigs[147,174]. Puromycin has been reported by Young and Freed[175] to have some anthelmintic effect in *Enterobius vermicularis* infection of man.

Proteolytic Enzymes

Gastrointestinal helminths are able to withstand the action of digestive enzymes. *Ascarids*, for instance, produce an antienzyme, "ascarase" which has an anti-trypsin and anti-pepsin activity, so protecting the helminth cuticle from damage. In extracts of body wall of *A. lumbricoides* a chymotrypsin factor has recently been found which is different from the anti-trypsin factor from the same source[176]. However, a few proteolytic enzymes from plant products are known which are effective in digesting parasites. Examples of these are, ficin, from leche de higueron[177]; papain, from the paw-paw fruit[178]; raigan (*Omphalia lapidescens*)[170] and bromelin[189], from pineapple juice.



(XVII)

(XVIII)

Two recently introduced drugs which have anthelmintic properties of a specific character are 3-methyl-1-pentyn-3-yl sodium phthalate (Whipcide)

(XVII)[181] which is claimed to be very active against whipworm in dogs, and cyanacethydrazide (XVIII)[182] which may prove to be of value in the treatment of lungworm infestation of animals.

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RESEARCH PAPERS

AN INVESTIGATION OF THE CONSTITUENTS OF *DIGITALIS PURPUREA*

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Increase in the concentration of ethanol or the inclusion of methanol, ethylene glycol or glycerol in the alkaline 3:5-dinitrobenzoic acid assay for digitalis glycosides, progressively reduces the maximum optical density of the reaction mixture. The constituents of mixtures of two digitalis glycosides have been separated by paper partition chromatography and recovered. The percentage recoveries obtained lay between 90 and 110 per cent of the quantity taken. The concentrations of four named glycosides in samples of powdered *D. purpurea* have been estimated by chromatographic separation followed by chemical assay. Estimates of the biological activity represented by the concentrations of these four constituents in each of the leaf samples have been made; the total biological activity of each leaf sample estimated in this way was found to be approximately half of the total biological activity of the whole leaf sample, as estimated by direct biological assay.

THE experimental work described in this paper is presented in two parts. Part I deals with the composition of the reaction mixture in the alkaline 3:5-dinitrobenzoic acid reaction[1] used for the assay of digitalis glycosides and aglycones and Part II with the results of a quantitative chromatographic investigation of *D. purpurea*.

Previously published work[2-5] has dealt with the chemical assay, the chromatographic analysis and the biological assay of some of the constituents of *D. purpurea*. Tattje[6], Rigby[7] and Rowson[8] have investigated the effect of varying the concentrations of the reactants in the assay with alkaline 3:5-dinitrobenzoic acid with similar conclusions. Tattje[6] observed the effect of methanol and *n*-propanol and we now describe the effects of methanol, ethanol, ethylene glycol and glycerol in the assay of digitoxin with alkaline 3:5-dinitrobenzoic acid (Part I). The results presented here agree with and extend some of the work reported by Tattje.

The chromatographic investigation of *D. purpurea* has been continued by the introduction of a new non-aqueous solvent system for use in paper partition work. This system, ethylene glycol:chloroform[5] was used because the two primary glycosides of *D. purpurea* were separated as isolated spots before the solvent front reached the bottom of the chromatogram; no such separation was achieved by the commonly used formamide systems.

The ethylene glycol:chloroform system has now been used for the separation, recovery and assay of the constituents of mixtures of digitalis

* Based upon a thesis for Ph.D. at Manchester University, 1956.

glycosides and the work has been extended to the estimation of the concentrations of four named glycosides in samples of powdered *D. purpurea* leaf.

Experimental Methods

Materials

Ethanol, dehydrated alcohol B.P.C.; ethylene glycol, reagent quality; methanol, A.R.; glycerol, A.R.; chloroform, A.R.; 3:5-dinitrobenzoic acid reagent—2·0 per cent w/v solution of 3:5-dinitrobenzoic acid in ethanol; xanthydrol reagent—xanthydrol 0·125 per cent w/v glacial acetic acid to 100 per cent, add 1·0 per cent v/v of concentrated hydrochloric acid to a suitable volume of the xanthydrol solution immediately before use; chromatographic paper, Whatman No. 3 MM filter paper 3 in. × 22·5 in.

Part I

The effect of ethylene glycol on the reaction between alkaline 3:5-dinitrobenzoic acid in ethanol and digitalis glycosides was first examined.

Method

10 ml. of reaction mixture was prepared by mixing 1·0 ml. of a solution in ethanol of a commercial sample of digitoxin containing 0·3 mg. in 1·0 ml., 2·0 ml. of 3:5-dinitrobenzoic acid reagent, a definite volume of ethylene glycol, 1·0 ml. of N NaOH and sufficient ethanol and distilled water to adjust the volume to 10 ml. and the ethanol concentration to between 65 per cent and 70 per cent v/v.

Triplicate mixtures were prepared and the maximum extinction at 535 mμ and 20° measured using a Uvispek spectrophotometer, by comparison with a blank prepared in exactly the same way but in which the 1·0 ml. of digitoxin solution was replaced by 1·0 ml. of ethanol.

The extinction was measured after five minutes and again at one minute intervals until the maximum extinction had been determined.

The effects of methanol and of glycerol were similarly investigated. In addition, the effect of variation of the ethanol concentration of the reaction mixture was investigated. In this series of experiments, the results of which are set out in Table II, no alcohol other than ethanol was present in the reaction mixtures.

Results

The presence of ethylene glycol, methanol or glycerol in the reaction mixture significantly reduces the extinction (Table I).

The concentration of ethanol was varied from 30 per cent v/v to 70 per cent v/v; the maximum extinction produced by 0·3 mg. of the commercial digitoxin in the alkaline 3:5-dinitrobenzoic acid reaction progressively decreased as the ethanol concentration was increased above 40 per cent v/v (Table II).

PART II

The Separation by Paper Partition Chromatography, Recovery and Assay of Some Constituents of D. purpurea

Four commercial samples (I to IV) of powdered *D. purpurea* leaf were investigated. Sample I was a moderately fine powder; samples II and III were moderately coarse powders and sample IV was a coarse powder.

Extraction of the powdered leaf. From each leaf sample a 1 in 10 tincture was prepared by maceration with continuous mechanical agitation for 24 hours with 80 per cent v/v ethanol.

Treatment of the tincture before application to the chromatogram. 18 ml. of tincture was mixed with 6 ml. of distilled water and the diluted tincture was drawn through a 10 cm. × 1 cm. column of chromatographic

TABLE I

THE EFFECT OF METHANOL, ETHYLENE GLYCOL AND GLYCEROL ON THE MAXIMUM EXTINCTION OF AN ALKALINE 3 : 5-DINITROBENZOIC ACID REACTION MIXTURE CONTAINING 0·003 PER CENT W/V OF COMMERCIAL DIGITOXIN

Concentration of ethanol in the reaction mixture per cent v/v	Concentration of methanol in the reaction mixture per cent v/v	Concentration of ethylene glycol in the reaction mixture per cent v/v	Concentration of glycerol in the reaction mixture per cent v/v	Maximum extinction of the reaction mixture
70				0·180
40	30			0·128
30	40			0·137
70				0·184
67		3·25		0·149
63		7·5		0·130
70				0·184
67			3·25	0·128
63			7·5	0·091

alumina (B.D.H.). This procedure removes a large proportion of the pigments from the tincture and approximately 6 per cent of the glycoside content of the tincture[3].

Four 3 ml. volumes of the partially decolourised eluate were separately evaporated almost to dryness in Quickfit boiling tubes at a temperature not exceeding 70°. The residue in each tube (approximately 0·2 ml.) was then transferred to the starting line of a strip of chromatographic paper. 3 ml. of eluate contains the extractive from 0·225 g. of leaf.

Quantitative transfer of the leaf extractive to the starting line of the prepared chromatographic paper. Each chromatographic paper strip was folded along a line 4 in. from one end to form a crease which facilitated the application of the leaf extract. The strips were passed through a 30 per cent v/v solution of ethylene glycol in methanol and then blotted between a fold of Whatman No. 1 paper.

The residual solution in one of the four tubes was drawn into a very fine capillary pipette fitted with a rubber teat and expelled along the length of the starting line of one of the prepared strips. When all the solution had been applied, the pipette was rinsed with boiling chloroform and methanol 1 : 1, and the rinsings expelled into the boiling tube and evaporated at a

temperature between 65° and 70° to a volume of approximately 0·2 ml. This solution was applied to the starting line of the paper strip, using the same pipette.

The process of rinsing, evaporation and application of the residue to the strip was repeated five times.

Test for incomplete transference of the leaf extractive to the chromatographic paper. Quantitative transference of the leaf extractive to the paper was tested by determining whether or not any glycosidal matter

TABLE II

THE VARIATION OF THE MAXIMUM EXTINCTION WITH THE ETHANOL CONCENTRATION OF AN ALKALINE 3 : 5-DINITROBENZOIC ACID REACTION MIXTURE CONTAINING 0·003 PER CENT W/V OF COMMERCIAL DIGITOXIN

Concentration of ethanol in the reaction mixture per cent v/v	Maximum extinction of the reaction mixture
30	0·215
40	0·232
50	0·214
60	0·188
70	0·182

remained in one of the four Quickfit tubes as follows: 2·0 ml. of xanthydrol reagent was added to the tube, the corresponding pipette was rinsed in this and the tube was placed in a beaker of boiling water for five minutes. The absence of a pink or red colour was taken to indicate that no glycosides remained in the tube. If such a colour developed, the corresponding chromatogram was later used to locate the separated constituents of the leaf, i.e., as a qualitative chromatogram. Rinsing of the three remaining tubes, evaporation of the rinsings and application to the paper strips was repeated twice more and one of the tubes was then tested with the xanthydrol reagent as described above.

Development of the chromatograms. The top portion of each chromatogram was trapped inside a fold of dry Whatman No. 3 MM paper and the four chromatograms were suspended from a single tray supported inside a chromatographic chamber containing at the bottom about half an inch depth of chloroform. The chamber was sealed and left undisturbed overnight. The mobile phase, chloroform saturated with ethylene glycol, was then added to the tray and development allowed to proceed at 20° ± 2°, until the solvent front reached to the bottom end of the chromatograms (approximately two hours).

The fold of paper around the end of the chromatogram inside the tray prevents excessive deposition of ethylene glycol on the chromatogram itself; such deposition slows the development.

Localisation and extraction of the separated digitalis constituents. Before extracting the isolated glycosides, the ethylene glycol was removed from the developed chromatograms by drying them at 60° to 65° for 18 hours, since glycol interferes with the alkaline 3:5-dinitrobenzoic acid assay (Part I).

The chromatogram intended for qualitative examination was cut into longitudinal strips which were treated with the following reagents:

trichloroacetic acid and hydrogen peroxide[9], alkaline 3:5-dinitrobenzoic acid and xanthydrol[10], to locate and where possible identify the separated constituents.

Corresponding pieces of paper, each bearing the same constituent, were cut from the three remaining chromatograms. Each piece was separately extracted by elution with 70 per cent ethanol for 48 hours.

During the later stages of the experimental work, an alternative method of extraction was used; the paper strips cut from the chromatograms were extracted by shaking for 30 minutes with three successive 40 ml. volumes of a mixture of chloroform and methanol 1:1.

Whichever method of extraction was used, the extracts from the three paper strips were separately evaporated to dryness under reduced pressure at a temperature not exceeding 60° in 100 ml. Quickfit flasks.

Assay of the recovered glycosides. The blank was prepared by adding 7·0 ml. of ethanol, 2·0 ml. of distilled water and 1·0 ml of N NaOH to one of the flasks. The flask was shaken to dissolve the recovered glycoside and the solution was filtered through a No. 4 sintered glass filter. 2·0 ml. of distilled water, 5·0 ml. of ethanol and 2·0 ml. of 3:5-dinitrobenzoic acid reagent were added to one of the remaining flasks and the recovered glycoside dissolved by shaking the flask. 1·0 ml. of N NaOH was then added and the mauve coloured solution immediately filtered as described above.

The extinction of this solution at 535 mμ relative to that of the blank was measured immediately and at one minute intervals until the maximum extinction (E_1) had been determined.

The extinction of the blank at 535 mμ relative to that of distilled water was then determined (E_2).

The contents of the remaining flask were similarly dissolved, the solution filtered and the maximum extinction at 535 mμ of the filtrate relative to that of distilled water determined (E_3). Theoretically, the extinction E_1 should be identical with the difference between the extinctions E_3 and E_2.

The 3:5-dinitrobenzoic acid reagent is present in the two assay solutions but is absent from the blank. The action of NaOH on the reagent produces a solution with a small absorption at 535 mμ and this necessitates a correction to the above readings. An estimate of the correction to the extinction readings was obtained by measuring the extinction at 535 mμ of a solution containing 2·0 ml. of 3:5-dinitrobenzoic acid reagent, 5·0 ml. of ethanol, 2·0 ml. of distilled water and 1·0 ml. of N NaOH, relative to that of distilled water. Measurements were made at two minute intervals for 20 minutes. The mean of these readings was 0·010 $\pm$ 0·002 and the readings did not increase with time during the 20 minute period.

The corrected duplicate extinctions were interpreted as mg. of the constituent under assay by reference to a calibration curve.

Results

The constituents separated from the leaf extracts and estimated in this way were the desacetyldigilanids A and B, digitoxin and gitoxin. In addition, unidentified compounds remaining on the starting line were

estimated and their concentration expressed as "equivalent mg." of desacetyldigilanid B.

The term "equivalent mg." was used to express the number of mg. of desacetyldigilanid B which produced the same extinction (when assayed

TABLE III

THE QUANTITY OF CERTAIN DIGITALIS CONSTITUENTS ESTIMATED TO BE PRESENT IN ONE G. OF EACH OF FOUR SAMPLES OF POWDERED *Digitalis purpurea* LEAF. THE CONCENTRATION OF UNIDENTIFIED CONSTITUENTS IS EXPRESSED AS MG. OF EITHER DESACETYLDIGILANID B. OR GITOXIN

Leaf sample	Constituents found on the starting line of the developed chromatogram, expressed as "equivalent mg." of desacetyldigilanid B mg.	Desacetyl digilanid A mg.	Desacetyl digilanid B mg.	Digitoxin mg.	Gitoxin mg.	Unidentified constituents expressed as "equivalent mg." of gitoxin mg. (i)	(ii)
I ..	0·65 0·70	1·1 1·0	0·75 0·90	1·1 1·3	0·85 0·65	0·20 0·15	— —
II ..	2·1 2·0	1·3 1·2	1·1 1·0	1·2 1·3	1·0 0·8	0·20 0·20	0·20 0·10
III ..	1·15 0·95	2·45 1·55	1·3 1·95	1·2 1·2	0·3 0·4	0·90 1·0	— —
IV ..	0·6 0·5	0·25 0·40	0·35 0·35	1·3 1·2	1·2 1·55	0·20 0·35	

by the method described on page 229—omitting the ethylene glycol) as the corrected duplicate extinctions obtained from the unidentified compounds remaining on the starting line.

Other unidentified compounds with R_F values lying between 0·4 and 0·7 were estimated and their concentration similarly expressed as "equivalent mg." of gitoxin.

The concentration of each of the leaf constituents estimated has been expressed as the amount present in one gram of powdered leaf (Table III).

TABLE IV

THE RECOVERY OF DIGITALIS GLYCOSIDES FROM DEVELOPED ETHYLENE GLYCOL-CHLOROFORM CHROMATOGRAMS

Mixture number	Name and weight of each glycoside in the mixture applied to the starting line of the chromatogram	mg.	Estimated weight of each glycoside recovered duplicate assays mg. (i)	mg. (ii)
1	Desacetyldigilanid A	0·4	0·39	0·385
	Desacetyldigilanid B	0·4	0·405	0·41
2	Desacetyldigilanid A	0·4	0·405	0·405
	Digitoxin	0·4	0·38	0·39
3	Desacetyldigilanid B	0·4	0·40	0·395
	Gitoxin	0·3	0·28	0·275
4	Digitoxin	0·4	0·37	0·36
	Gitoxin	0·3	0·32	0·325

To obtain an estimate of the errors involved in the assay described above, the process was first used to separate and assay the constituents of different mixtures of known amounts of two digitalis glycosides. The results of this work suggest that the techniques involved in the assay are associated

with an error of not more than ± 10 per cent (Table IV). This error is probably less than the error associated with the results of the assays of the isolated leaf constituents (Table III), since the latter assays are more difficult to perform and are complicated by the presence of plant pigments and constituents other than glycosides.

Brindle, Rigby and Sharma[4] and Rigby[7] biologically assayed several digitalis glycosides, individually and in mixtures, using the guinea pig and the frog. From their results which are expressed as *international units of*

TABLE V

COMPARATIVE ASSAYS OF EACH OF FOUR SAMPLES OF POWDERED *Digitalis purpurea*

Leaf sample	"Assumed activity" of the leaf sample. Units/g. (Results of duplicate assays)	Biological activity of the leaf sample I.U./g. with 95 per cent fiducial limits
I	4·88 4·81	6·6—7·7—8·9
II	5·84 5·64	8·2—9·4—10·7
III	9·07 7·30	12·2—13·7—15·3
IV	2·39 2·72	3·7—4·8—6·1

activity per mg. of glycoside, the number of units of activity represented by the weight of each of the four glycosides, desacetyldigilanid A, desacetyldigilanid B, digitoxin and gitoxin in one gram of each leaf sample was calculated.

The sum of the number of units of activity per g., represented by each of these four constituents, was calculated for each of the four leaf samples, and has been termed the "assumed activity" of the leaf.

The "assumed activity" of each leaf is shown in Table V along with the results of a biological assay of the leaf. The "assumed activity" has been expressed in units.

The 1 in 10 tinctures prepared from the powdered leaf samples as described on page 230 were biologically assayed by the 18 hour frog lymph sac method as described in the British Pharmacopoeia.

DISCUSSION

It has been shown (Table II) that variation of the ethanol concentration in the alkaline 3 : 5-dinitrobenzoic acid reaction with digitoxin significantly affects the extinction of the mixture. The presence of certain other alcohols in the reaction mixture considerably reduced the maximum extinction (Table I). From these data it was calculated that in the presence of equimolar concentrations of different alcohols, the reduction in maximum extinction was progressively greater as the number of hydroxyl groups in the alcohol molecule increased. The decrease in maximum extinction when 0·1 g. molecule of an alcohol was present in 100 ml. of a reaction mixture containing 0·003 per cent. w/v of commercial digitoxin, was calculated to be approximately 2·5 per cent in the case of the two monohydric alcohols, methanol and ethanol, about 25 per cent in the case of the dihydric alcohol ethylene glycol and about 50 per cent in the case of the trihydric alcohol glycerol.

The figures in Table V show that the sum of the biological activities represented by the concentrations of four specified glycosides in any one of the leaf samples investigated is approximately 50 to 60 per cent only of the total biological activity of the leaf sample as estimated by bioassay. In making this comparison, it has been assumed that not more than 15 per cent potentiation of activity occurs when a mixture of the four constituents investigated is assayed biologically. This assumption is based on previously published work[4]. This 40 to 50 per cent discrepancy may be due to several factors. These may be,

(*a*) the loss of active constituents during the partial decolourisation process,

(*b*) the failure to transfer the "decolourised" extract without loss to the starting line of the chromatogram,

(*c*) the adsorption at the starting line, by the paper, of constituents which would normally move down the paper during development of the chromatogram,

(*d*) the distribution of small amounts of a constituent of high R_F over that part of the chromatogram traversed by the constituent during development,

(*e*) the failure to extract a separated constituent completely from the developed chromatogram,

(*f*) the presence of unidentified constituents in the leaf, one or more of which might have considerable biological activity, and

(*g*) the presence of pigments derived from the leaf, in the assay reaction mixture.

Many of the possible sources of error listed above are associated with the techniques employed in the chromatographic assay. The results of the quantitative separation, recovery and assay of the constituents of mixtures of two glycosides (Table IV) suggest that the techniques employed in the assay may give rise to an error of approximately 10 per cent. However, the presence of pigments and other extractive in the partially decolourised leaf extracts may considerably affect (*a*) the adsorption of the glycosides by the paper, especially at the starting line, (*b*) the distribution of the glycosides over the whole chromatogram, and (*c*) the efficiency of elution of the separated glycosides from the developed chromatogram. These factors would inevitably increase the error of the result. More complete decolourisation might reduce the error.

In addition, loss of active constituents from the leaf extracts occurs during partial decolourisation; this loss may be as high as 6 per cent (Brindle, Rigby and Sharma[3]).

In conclusion, it can be said that the chemical assay of certain constituents separated by paper partition chromatography from an extract of *D. purpurea*, cannot, at present, replace the biological assay of *D. purpurea*.

Furthermore, in view of the numerous sources of error listed above and bearing in mind the large number of constituents identified in samples of *D. purpurea* it is not likely that a chemical assay of this type can be achieved.

The chemical assay following chromatographic separation described in this paper can, however, be applied to the estimation of the concentration of one or more of the constituents of a leaf, provided that the limitations of the assay are recognised; for example, the isolation of certain constituents may necessitate the use of a particular chromatographic solvent system or of a suitable reagent for the chemical assay. The work described by Sellwood[11] may be quoted as an important example in this respect.

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COUNTER-CURRENT SEPARATION OF CONSTITUENTS OF *DIGITALIS PURPUREA*

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Mixtures of some glycosides of *Digitalis purpurea* have been partitioned in a mixture of ethyl acetate, benzene and water by counter-current distribution. The "A" and "B" series of primary glycosides were separated from the corresponding secondary glycosides. The partition ratio of each of the glycosides has been determined and used to calculate the theoretical distribution curve: this closely resembled the experimentally determined curve. Deviation of the "experimental" from the "theoretical" curve for digitoxin has been attributed to the presence of gitoxin in the sample.

Counter-current distribution has been used for the separation of many mixtures of closely related compounds, for example, antimalarial drugs[1], fatty acids[2], penicillins[3], phospholipids[4] and alkaloids of rauwolfia[5]. We have now used it to separate mixtures of digitalis glycosides.

Experimental Methods

Materials. The apparatus used was a modification of the automatic apparatus originally designed by Craig and others[6]. It carried 50 tubes, allowing 49 stages and could conveniently be modified so that recycling of the contents of the tubes could be carried out making an infinite number of stages possible. The glycosides investigated were the desacetyl-digilanids A and B, kindly supplied by Professor A. Stoll and commercial samples of digitoxin and gitoxin. The solvents used were ethyl acetate, distilling between 76° and 77°, benzene, distilling between 79° and 81°, and ethanol, dehydrated alcohol, B.P.C. The 3:5-dinitrobenzoic acid reagent was a 2 per cent w/v solution of 3:5-dinitrobenzoic acid in ethanol.

Methods

Preliminary. Before selecting the solvent system described below, a series of paper chromatograms was prepared using several of the solvent systems commonly employed for the separation of digitalis constituents. A list of some of these systems is included in the paper by Brindle and others[7]. Whatman No. 3 MM paper was used.

Silberman and Thorp[8] in their paper partition experiments used ethyl acetate 86 volumes, benzene 14 volumes and distilled water 50 volumes, to the separated organic phase of which they added up to 7·5 per cent v/v of ethanol.

The use of ethanol in this way cannot be simulated in the counter-current experiments and for this reason ethanol was omitted from the solvent system in our paper partition chromatograms. The approximate R_F values of the four digitalis glycosides when chromatographed on paper

at 20° ± 2° with the above solvent system containing no added ethanol, are desacetyldigilanid B 0·10, desacetyldigilanid A 0·26, gitoxin 0·84, and digitoxin 0·92. This solvent system was also used in the counter-current apparatus.

Counter-current distributions. The appropriate volumes of the solvents were mixed in 2 litre glass-stoppered bottles and shaken occasionally during 48 hours. The upper organic phase was then separated from the lower aqueous phase.

1*st experiment.* Approximately 10 mg. of each of the three glycosides desacetyldigilanid A, desacetyldigilanid B and digitoxin were shaken for 2 hours with a mixture of 30 ml. of the upper phase and 30 ml. of the lower phase of the solvent system, in a 100 ml. glass-stoppered measuring cylinder.

The mixture was passed through a No. 4 sintered glass filter and the volume of the upper phase adjusted to 40 ml. and that of the lower phase to 50 ml. by the addition of the corresponding phase of the solvent system. The whole 90 ml. was then placed in the first tube of the distribution apparatus. 50 ml. of the lower phase of the solvent system was placed in each of the remaining 49 tubes and the reservoir flasks were filled with the upper phase. The shaking period was 5 minutes and the settling time 20 minutes. Separation of the two phases in the tubes was fairly rapid and emulsions did not form.

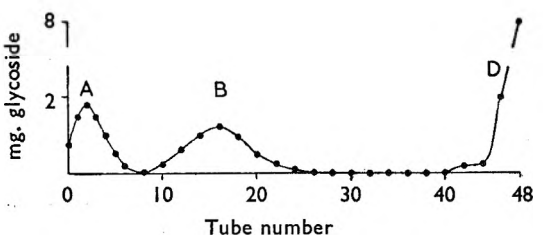

FIG. 1. Experimental distribution curve for desacetyldigilanids (A) and (B) and digitoxin (D). Forty-eight stages completed.

The extraction was allowed to proceed until 48 transfers had been completed when each of the two phases in each of the 49 tubes was assayed as described below.

Assay of tube contents. After separation of the constituents of a mixture of digitalis glycosides in the automatic counter-current distribution apparatus the contents of the tubes were assayed. 10 ml. samples of each phase were separately evaporated to dryness on a steam bath. To each dish was added 4 ml. of ethanol and 2 ml. of distilled water, the residue in the dish was dissolved and the solution transferred to a 10 ml. standard flask. 2·0 ml. of dinitrobenzoic acid reagent was added and the flask transferred to a water bath at 20° for 15 minutes; 1 ml. of N NaOH was then added and the volume adjusted to 10 ml. with ethanol. After immediately filtering through sintered glass, the solution was transferred to an absorptiometer cup and the maximum extinction determined at approximately 535 mμ by comparison with a blank. The blank solution contained 2·0 ml. of dinitrobenzoic acid reagent, 2·0 ml. of distilled water, 1·0 ml. of 1·0N NaOH and sufficient ethanol to adjust the volume to 10 ml.

The weight of glycoside in each tube was calculated from the volume of each phase and by reference to an extinction calibration curve for the particular glycoside identified.

From these results, the distribution curve, Figure 1, showing the weight of glycoside in successive tubes was plotted.

Second experiment. Gitoxin was included in the primary solution in addition to the three glycosides employed in the first experiment. Forty-nine transfers were completed, the solvent system, shaking and settling times being the same as in the first experiment. The 100 assays were carried out as described above and the distribution curve, Figure 2, was constructed from the results.

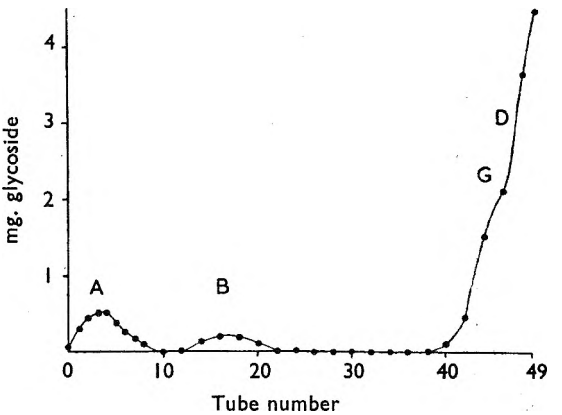

FIG. 2. Experimental distribution curve for desacetyldigilanids (A) and (B), digitoxin (D) and gitoxin (G). Forty-nine stages completed.

The construction of theoretical distribution curves. Theoretical distribution curves for the digitalis glycosides were calculated after determining the partition ratio of the digitalis glycosides in the ethyl acetate 86: benzene 14: water 50 solvent.

Approximately 5 mg. of glycoside was shaken with a mixture of 20 ml. of each phase of the solvent system in a stoppered glass tube. The solution was filtered to remove any undissolved glycoside and 5 ml. of each phase assayed as described on page 238. The estimated partition ratio for each of the four glycosides is, desacetyldigilanid A 0·68, desacetyldigilanid B 0·12, digitoxin 84·8, and gitoxin 14·1.

Calculation of Theoretical Distribution Curves

Martin and Synge[9] showed that the process of counter-current distribution could be expressed mathematically as a binomial expansion:

$$\left[\frac{1}{1+kx}+\frac{kx}{1+kx}\right]^n \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where k = partition ratio, x = ratio of the volumes of the two phases of solvent and n is the number of stages effected. The general term of the expansion of this is:

$$T_r = \frac{n!}{r!(n-r)!} \cdot \frac{k^r x}{(1+kx)^n} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where T_r = the fraction present in the rth tube, of the total material distributed through n tubes.

A simplified method of calculating the theoretical distribution in this way was devised by Liebermann[10]. On dividing T_r by T_{r-1} the expression

$$T_r = \frac{T_{r-1}\, kx(n + 1 - r)}{r} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

is obtained. This may be expressed as

$$\log T_r = \log T_{r-1} + \log kx + \log \left(\frac{n + 1 - r}{r}\right) .. \quad .. \quad .. \quad (4)$$

The logarithm of the zero term is obtained by substituting $r = o$ in equation (2) and expressing logarithmically to give

$$\log T_o = -n \log (1 + kx) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

From equations (4) and (5) and using the partition ratios a theoretical distribution curve for the glycosides desacetyldigilanids A and B, digitoxin and gitoxin was calculated for 49 stages. The curve is shown in Figure 3.

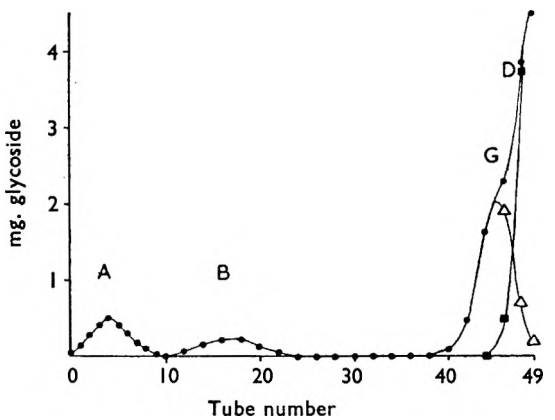

FIG. 3. Theoretical distribution curve for desacetyldigilanids (A) and (B), digitoxin (D) and gitoxin (G). Forty-nine stages calculated. ● total glycoside; ■ digitoxin; △ gitoxin.

RESULTS

In the first experiment (Fig. 1), digitoxin was completely separated from the two primary glycosides which were themselves well separated. A qualitative paper chromatographic examination was unnecessary.

In the second experiment, gitoxin was not separated from digitoxin (Fig. 2). The total amount of glycoside in each of the tubes 38 to 49 inclusive was therefore calculated as "equivalent mg." of digitoxin in order to construct Figure 2. The contents of several of these tubes were examined qualitatively by evaporating 25 ml. of the upper phase in the tube to about 0·25 ml. volume and applying this to the starting line of a formamide:chloroform chromatogram. Both gitoxin and digitoxin were detected in most of the tubes. An approximate estimate of the proportions of the two glycosides in each tube was obtained by comparison of the intensities of the reactions of the isolated glycosides with an alkaline solution of the dinitrobenzoic acid reagent on the developed paper chromatograms (Table I).

DISCUSSION

The primary purpurea glycosides desacetyldigilanids A and B could be separated from one another and from secondary glycosides by

discontinuous counter-current distribution in only 49 stages, in the ethyl acetate : benzene : water solvent (Figs. 1 and 2). Under the same conditions, gitoxin cannot be separated from digitoxin (Fig. 2). The four

TABLE I

QUALITATIVE PAPER CHROMATOGRAPHIC ANALYSIS OF THE CONTENTS OF SELECTED FRACTIONS FROM THE COUNTER-CURRENT SEPARATION OF A MIXTURE OF FOUR DIGITALIS GLYCOSIDES. AN APPROXIMATE ASSESSMENT OF THE CONCENTRATION OF GITOXIN AND DIGITOXIN IN THE LATER FRACTIONS IS INDICATED

Number of the tube in the Craig apparatus	Glycosides found to be present in the contents of the Craig tube
4	Desacetyldigilanid B
17	Desacetyldigilanid A
40	Gitoxin +
42	Gitoxin + +
44	Gitoxin + + +
46	Gitoxin + + + and Digitoxin +
48	Gitoxin + + and Digitoxin + +
49	Gitoxin + and Digitoxin + + +

glycosides are distributed in the counter-current experiments in the same order as that in which they are separated by paper partition chromatography with the same solvent.

The similarity of Figures 1, 2 and 3 shows that the theoretical and the experimental distribution of the glycosides are also similar.

Larger amounts of solutes can be investigated by counter-current distribution than is possible by paper partition chromatography. Furthermore, deviations of the experimental from the theoretical distribution curve may be an indication that a hitherto undetected solute may be present in the primary mixture of solutes. The presence of an impurity in a solute might also be recognised in the same way; for example, in Figure 1 the experimental curve for digitoxin exhibits a kink which, by reference to Figure 3, was thought to mean that some gitoxin was present in the commercial sample of digitoxin. This was confirmed by running a large amount (approximately 0·2 mg.) of the commercial sample of digitoxin, on a formamide : chloroform chromatogram, when a spot corresponding in R_F value to gitoxin was detected. This spot produced a blue fluorescence with the trichloroacetic acid : hydrogen peroxide reagent[11].

Attempts are at present being made to effect a complete separation of the two secondary glycosides either by using a different solvent system or by recycling, using the ethyl acetate : benzene : water solvent system.

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THE DETERMINATION OF BETA-SUBSTITUTED GLUTARIMIDES IN BLOOD: TIME-CONCENTRATION CURVES AFTER INTRAVENOUS ADMINISTRATION OF TWO BARBITURATE ANTAGONISTS

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A simple and rapid spectrophotometric procedure has been developed which is capable of estimating 2 mg. per cent of β-substituted glutarimides in blood with an accuracy of 90 per cent. Within 10 minutes of intravenous administration in guinea pigs 90 per cent of two barbiturate antagonists, β-methyl-β-ethyl glutarimide and *β-spirocyclo*pentane glutarimide were removed from blood. Some discussion follows on whether the compounds were rapidly metabolised in blood or distributed evenly throughout the body tissues. The phase of rapid removal is followed by a period of moderately slow removal, apparently due to excretion.

The barbiturate-antagonistic activity of the drug β-methyl-β-ethyl glutarimide (bemegride) has recently received considerable attention in the clinical literature. Its ability to shorten pentobarbitone anaesthesia is still detectable in rats up to 48 hours after a single oral dose of 80 mg./kg.[1], but apart from one reported metabolite[2], nothing is known of its distribution or metabolic fate. A method for the quantitative estimation of the chemically related sedative, α-ethyl-α-phenyl glutarimide (glutethimide) in blood and urine has been published[3], and is applicable to other substituted glutarimides such as bemegride. However, it did not give good recoveries of added glutarimide from blood in this laboratory. A simple spectrophotometric procedure has enabled us to obtain information on the concentrations of two barbiturate antagonists, bemegride and *β-spirocyclo*pentane glutarimide (N.P.122)[4], in the blood of guinea pigs at intervals after intravenous administration.

Materials and Methods

In the undissociated form in aqueous solution bemegride exhibited an ultra-violet absorption peak at 205 mμ. This was too close to the lower spectral limit of our spectrophotometer (Hilger-Uvispek) to be reproducible quantitatively and led us to investigate the dissociated form. By determining the absorption spectra of bemegride in various buffers it was seen that dissociation occurred largely between pH 10 and 11·75 (pKa approx. 11·3). Above pH 12 the compound was apparently fully dissociated and showed a strong absorption peak at 230 mμ. Furthermore the optical density at 230 mμ was almost exactly double the optical density at 220 and 240 mμ. Although this characteristic pattern of absorption was not always fully recognisable in the glutarimide recovered from blood, there was sufficient approximation, as will be shown later, to justify using the data as an aid to identification of the absorbing

substance. Bemegride was unstable in solution at pH 12 but losses could be minimised by working at low temperature. In all that has so far been said the behaviour of N.P.122 was identical to bemegride, though each had its specific extinction coefficient at 230 mμ. The same analytical procedure was therefore, after suitable calibration, applicable to either compound.

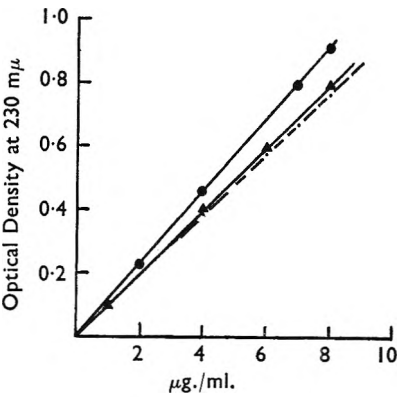


FIG. 1. Calibration curves for the glutarimides in cold 0·04N NaOH. ● Bemegride; ▲ *β-spiro-cyclo*pentane glutarimide; — — bemegride after standing for one hour at room temperature.

Two to 5 ml. aliquots of heparinised blood containing at least 100 μg. of glutarimide were extracted with 30 ml. of chloroform for 30 minutes with mechanical shaking and the two layers separated by centrifuging. Ten ml. aliquots of the chloroform layer were pipetted into stoppered cylinders (25 ml.) and chilled to 0°. These were extracted with five successive 10 ml. volumes of cold 0·04N NaOH in a room at 0°. The aqueous layers were removed by aspiration. The combined alkaline extracts were centrifuged under refrigeration to remove suspended chloroform and the absorption of the clear aqueous layer immediately determined at 230, 220 and 240 mμ against a blank similarly prepared from glutarimide-free blood. Concentrations of the drug were ascertained from a calibration curve showing the absorption at 230 mμ of varying concentrations of the pure compound in cold 0·04N NaOH. When estimating very low blood levels (less than 4 mg. per cent) it was desirable to concentrate the chloroform extract. This was conveniently achieved by blowing a stream of warm air on to the extract until a 20 to 25 ml. aliquot was reduced to 10 ml. This procedure occasionally led to the formation of emulsions during the subsequent alkaline extraction but these were readily broken in the refrigerated centrifuge.

Information on the levels in blood after intravenous administration of the compounds was obtained using female guinea pigs (700 to 1100 g.) which had been fasted overnight. It was necessary to expose the jugular vein for administration and, since barbiturates interfere in the assay, anaesthesia for this purpose was obtained with intraperitoneal urethane assisted if necessary with a little ether. Bemegride was given at a dose of 30 mg./kg. in saline. N.P.122 is less soluble than bemegride and is a powerful, long-acting convulsant. It was given at a level of 15 mg./kg. in saline. The animals were killed by decapitation at intervals up to 4 hours after the injection and blood was collected into a beaker containing heparin. Suitable aliquots were assayed by the above procedure and optical densities of the final solutions were determined at 230, 220 and 240 mμ.

Results

The calibration curves in Figure 1 are those for bemegride and N.P.122 in cold 0·04N NaOH. The broken line shows the optical densities of the same bemegride solutions after one hour at room temperature. During this period the temperature of the solutions rose from 10° to 18·5°.

The estimated accuracy of the analytical procedure as determined by the recovery of glutarimides added to guinea pig blood *in vitro* in known amounts is shown in Table I.

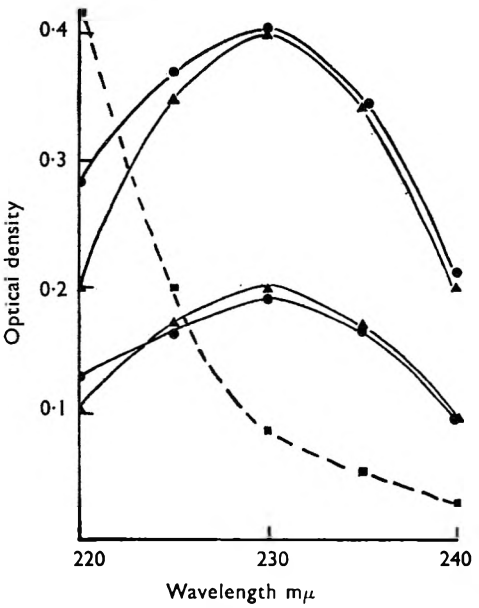


FIG. 2. Absorption curves of the glutarimides in 0·04N NaOH. ▲ Bemegride, 1·75 μg., and 3·5 μg./ml. respectively; ● bemegride recovered from blood; ■ blood blank.

Two of the curves in Figure 2 are those obtained by the extraction of bemegride from blood and for comparison the curves of pure bemegride solutions of 3·5 and 1·75 μg./ml. in 0·04N NaOH are included. The broken line is a typical absorption curve of a blood blank. It can be seen that the ratio of the optical density at 230 mμ to that at 220 mμ ($O.D._{230}/O.D._{220}$) is not constant at a value near 2 after the recovery of bemegride from blood. This is probably due to differences in the blank absorption which is changing rapidly over this wavelength range. The ratio $O.D._{230}/O.D._{240}$ on the other hand is constant at a value of approximately 2. It was a routine step during analysis to check that there was in fact an absorption peak at 230 mμ and that $O.D._{230}$ was approximately twice the value of $O.D._{240}$. Values of the ratio $O.D._{230}/O.D._{240}$ recorded during this investigation are set out in Table II for bemegride and N.P.122 before and after recovery from guinea pig blood.

To reduce the results from the different animals to a common basis the blood concentration of the drug as μg./ml. was multiplied by the animals' blood volume (7·2 ml./100 g.)[5]. The figure so obtained, expressed in milligrams, represented the total glutarimide present in the blood and this was calculated as a percentage of the original dose. Figure 3 shows the percentage remaining at various intervals after administration. Each point on the graph represents the observation from a single animal. Both compounds left the blood at the same rate and only 10 per cent of the initial dose remained in the blood after ten minutes. This amount could still be detected after 30 minutes and in the case of bemegride 5 per cent was present after four hours.

The injections of glutarimide were given over a period of less than one minute and at the conclusion the animals were beginning to convulse.

TABLE I

STANDARD RECOVERIES FROM BLOOD

Amount added mg./100 ml.	Amount recovered per cent	
	Bemegride	N.P.122
125	99·0	94·0
"	94·4	96·4
50	90·0	—
"	89·6	—
25	94·0	92·7
"	93·8	93·1
12·5	91·4	—
"	88·0	—
5·0	90·9	91·3
"	93·1	87·6
2·5	92·2	90·1
"	91·7	90·0
Mean	92·3	91·9
Standardard deviation between duplicates	1·78	1·57

Guinea pigs given bemegride convulsed severely for 5 to 6 minutes and this was followed by a further 5 to 6 minute period of twitching. After this the animals became quiet. There appeared to be a relation between

TABLE II

THE ABSORPTION RATIOS OF THE GLUTARIMIDES

Ratio $O.D._{280}/O.D._{240}$				
Bemegride			N.P.122	
Pure soln.	ex blood *in vitro*	ex blood *in vivo*	Pure soln.	ex blood *in vivo*
1·97	2·08	1·81	2·08	2·03
1·97	2·06	2·13	2·02	1·98
2·06	1·88	1·84	2·02	2·08
2·14	2·22	1·82	2·05	2·00
1·99	1·96	2·12	1·94	2·02
2·03	1·95	1·73	1·95	1·97
2·12	1·90	1·96	1·91	1·88
2·09	1·89	1·93	2·00	—
1·96	1·99	1·80	1·80	—
2·12	2·07	1·99	2·17	—
*Means** 2·045	2·000	1·913	1·994	1·994

* A difference of 0·1 in the mean of the ratios is significant at the $p < 0·05$ level.

the decrease in blood concentration of bemegride and the decrease in convulsions. Observation of animals given N.P.122 were taken only up to 30 minutes after administration and they convulsed throughout this period.

DISCUSSION

The strong ultra-violet absorption band at 230 mμ appears to depend primarily on an intact glutarimide ring, secondly, ionisation of the –OC–NH–CO–grouping of the ring, and finally the type and position of the ring substituents.

Although not reported here it was shown during the preliminary investigation of this project that several β-substituted glutarimides had identical absorption curves in the 200 to 250 mμ range. Alkaline hydrolysis, however, led to cleavage of the ring at the imide linkage, yielding first the substituted glutaramic acid, which in equivalent concentration showed

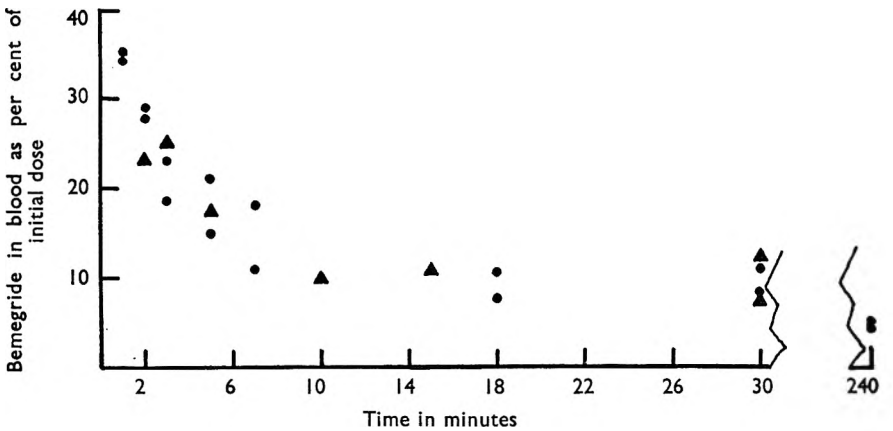
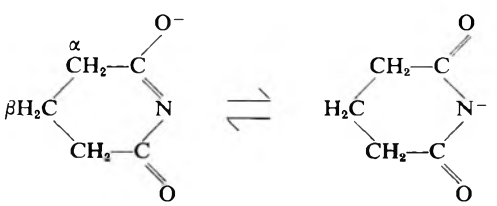


FIG. 3. The concentration of glutarimides in blood after intravenous administration. ● Bemegride; ▲ β-*spiro-cyclo*pentane glutarimide.

approximately 1/10th of the absorption of the parent compound at 230 mμ, and finally the substituted glutaric acid which showed no absorption in this region. However, two alpha substituted glutarimides, α-*n*-butyl- and α-phenyl-α-ethyl glutarimide, which had intact ring structures had no definite absorption peak at 230 mμ in alkaline solution and an αβ-substituted compound (α-methyl bemegride) had absorption characteristics only slightly, if at all different from, those of bemegride. Very little is known about the mechanism of ionisation of the glutarimides, but two forms are possible and it seems likely that they occur together in some sort of equilibrium:



Alpha substituent groups by virtue of their proximity would exert a greater influence on the mechanism of dissociation than β substituent groups—and so, depending on their structure may be the final factor controlling the optical absorption of the glutarimides at 230 mμ.

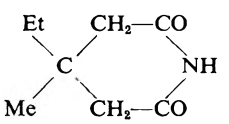
From consideration of the curves in Figure 2 and the means of the ratios $O.D._{230}/O.D._{240}$ in Table II, it seems clear that the absorption of the blood extracts at 230 mμ was due to the glutarimides.

Losses due to the instability of the glutarimides in 0·04N NaOH were minimised by holding solutions at temperatures less than 10°. The loss of bemegride in a solution which stood for one hour at room temperature and rose from an initial 10° to 18·5° was approximately 15 per cent. Curry has shown the loss to be about 50 per cent per hour in 0·5N NaOH at 37°[7].

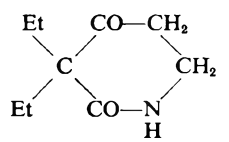
The rapid rate at which both compounds left the blood of guinea pigs after intravenous administration seems worthy of further comment. This disappearance was too rapid to be due to excretion alone. Therefore the glutarimides were either metabolised in the blood or stored within the organism. If a rapid metabolism caused the disappearance from the blood then this probably involved cleavage of the imide linkage which reduces or abolishes absorption at 230 mμ. The two likely metabolites resulting from biological cleavage of the bemegride ring are the β-methyl-β-ethyl glutaramic and β-methyl-β-ethyl glutaric acids, and it is interesting to note that while both have been claimed to be non-active as barbiturate antagonists[4,6] recent work in these laboratories by Mr. A. W. Macfarlane has established the presence of weak activity in the former.

On the other hand the glutarimides are lipid soluble and this together with their slight ionisation at biological pH suggests that they would pass rapidly through most biological membranes. Information on the tendency for the drugs to accumulate in various tissues awaits the development of a sufficiently sensitive tissue assay. However, the sedative "Dihyprylone" (3:3-diethyl-2:4-piperidinedione) which is chemically related to bemegride has recently been shown to be uniformly distributed in all tissues after oral ingestion[8] and the blood levels of bemegride after 10 minutes, when equilibrium had been established, were on the basis of Ancill's data[5] compatible with an even distribution through the entire animal body.

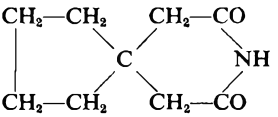
The blood levels of bemegride after four hours suggest a moderately slow rate of excretion. This may explain its ability to shorten barbiturate sleep 48 hours after a single oral dose in rats[1], and indicates that the drug is active at low blood levels.



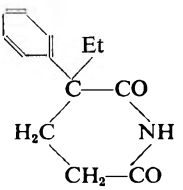
Bemegride: β-methy-β-ethyl glutarimide
(4:4-ethyl-methyl-2:6-piperidinedione)



Dihyprylone: 3:3-diethyl-2:4-piperidinedione



N.P.122: *β-spiro-cyclo*pentane glutarimide



Glutethimide: α-ethyl-α-phenyl glutarimide

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THE INFLUENCE OF β-TETRAHYDRONAPHTHYLAMINE AND A DERIVATIVE ON THE CENTRAL EFFECTS OF 5-HYDROXYTRYPTAMINE, RESERPINE AND IPRONIAZID

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N-Methyl-*N*-propyl-β-tetrahydronaphthylamine, a specific antagonist of 5-hydroxytryptamine on the isolated rat uterus preparation, fails to inhibit 5-hydroxytryptamine potentiation of the hypnotic action of hexobarbitone in mice. The potentiating actions of reserpine and iproniazid are likewise unaffected. The action of β-tetrahydronaphthylamine as an antagonist of the potentiating action of 5-hydroxytryptamine on hexobarbitone sleeping time in mice has been confirmed, and it has also been shown to be an effective antagonist of the sleep prolonging effects of reserpine. β-Tetrahydronaphthylamine also shows a partial antagonism to iproniazid potentiation of hexobarbitone hypnosis. These results provide an example of the lack of parallelism between the central and smooth muscle actions of 5-hydroxytryptamine antagonists.

STUDIES of 5-hydroxytryptamine antagonists have indicated that there are drugs such as β-tetrahydronaphthylamine (β-tetra), which while altering the central actions of 5-hydroxytryptamine (5-HT), do not antagonise its actions on isolated smooth muscle preparations. 5-Hydroxytryptamine causes depression when injected into the lateral cerebral ventricle of cats[1], and causes prolongation of hexobarbitone hypnosis in mice[2]. β-Tetra antagonises both these effects[3]. On the other hand it has little action against 5-HT on the isolated rat uterus preparation[3,4]. These findings have been confirmed in this laboratory. β-Tetra has a slight anti-5-HT action on perfused rabbit ear vessels[3].

While investigating the pharmacological actions of a series of β-tetrahydronaphthylamine derivatives synthesised by Craig, Moore and Ritchie (unpublished) we have observed that *N*-methyl-*N*-propyl-β-tetrahydronaphthylamine (M-P. β-tetra) specifically inhibited the action of 5-HT on the rat uterus preparation in a concentration of $1 : 10^{-7}$. The present paper describes the effects of this derivative and of β-tetra on the duration of 5-HT potentiated hexobarbitone hypnosis in mice.

The effects of these two substances on the sleep-prolonging effects of reserpine have been investigated since Shore and others[5] have suggested that this action of reserpine is mediated through 5-HT. The effects of β-tetra and M-P. β-tetra on the potentiation of hexobarbitone hypnosis brought about by iproniazid, which produces an increase in endogenous brain 5-HT[6], have also been studied.

METHODS

Hexobarbitone (90–100 mg./kg.) was administered by intraperitoneal injection to albino mice of either sex weighing between 20 and 22 g. The

* Drug Houses of Australia Research Fellow in Experimental Pharmacology.

doses used were 30 mg./kg. of 5-HT (as the creatinine sulphate), 1 mg./kg. of reserpine and 18 or 20 mg./kg. of iproniazid (Marsilid) as the phosphate. Both the 5-HT and the reserpine were given by intraperitoneal injection, while iproniazid was administered subcutaneously. An interval of one hour was allowed after the injection of 5-HT or iproniazid before the administration of hexobarbitone; with reserpine, however, a two hour

TABLE I

THE EFFECT OF β-TETRAHYDRONAPHTHYLAMINE HYDROCHLORIDE UPON THE SLEEPING TIME DUE TO HEXOBARBITONE, POTENTIATED BY 5-HYDROXYTRYPTAMINE CREATININE SULPHATE (5-HT), RESERPINE AND IPRONIAZID
(Room temperature 22°)

Drug	Dose in mg./kg.	Route	Mean sleeping time	Difference in sleeping times	t_8
Hexobarbitone ..	100	I.P.	26·4 ± 2·9 (22)		
				26·0 ± 1·5	17·6
Hexobarbitone + 5-HT	100 30	I.P. I.P.	52·5 ± 5·0 (11)		
				22·1 ± 1·2	14·0
Hexobarbitone + 5-HT + β-tetra	100 30 25	I.P. I.P. I.P.	26·0 ± 1·5 (12)		
Hexobarbitone ..	100	I.P.	22·3 ± 1·0 (10)		
				23·6 ± 3·6	6·5
Hexobarbitone + reserpine	100 30	I.P. I.P.	45·9 ± 4·9 (10)		
				20·1 ± 2·2	8·9
Hexobarbitone + reserpine + β-tetra	100 1 25	I.P. I.P. I.P.	25·6 ± 5·7 (8)		
Hexobarbitone ..	100	I.P.	23·1 ± 1·0 (24)		
				73·7 ± 8·6	8·6
Hexobarbitone + iproniazid	100 20	I.P. S.C.	96·8 ± 13·0 (12)		
				56·2 ± 11·0	5·1
Hexobarbitone + iproniazid + β-tetra	100 20 25	I.P. S.C. I.P.	40·6 ± 7·6 (11)		
Hexobarbitone ..	90	I.P.	12·8 ± 1·5 (11)		
				9·0 ± 0·9	10·0
Hexobarbitone + iproniazid	90 18	I.P. S.C.	22·0 ± 1·8 (11)		
				4·4 ± 0·7	5·8
Hexobarbitone + iproniazid + β-tetra	90 18 1	I.P. S.C. I.P.	17·6 ± 1·9 (11)		

The number in brackets after each mean sleeping time gives the number of animals in the group from which the tabulated mean value was calculated. The values appearing in the column headed "difference in sleeping times" refer to the differences between the mean sleeping times appearing in the preceding column immediately above the tabulated values, and those in the preceding column immediately below it. The *t*-test shows significant ($P < 0{\cdot}001$) differences between the mean values compared.

interval was found preferable. The tests were performed in a thermostatically controlled cabinet adjustable from 5 to 40° ± 0·1°, usually set at 22°.

The sleeping time was measured as the time from the loss of the righting reflex to its reappearance within 30 seconds of placing the mouse on its back. Ten or more mice were used in each test group except in two experiments.

Each comparison between the hypnotic drug and potentiators or antagonists was made at the same time since the control sleeping time with hexobarbitone varied from day to day.

Preliminary experiments were performed to determine doses of β-tetra and M-P. β-tetra which did not affect the sleeping time of the anaesthetised mice in the absence of other drugs. The doses of these two compounds refer to the weights of the salts, hydrochloride and hydriodide respectively.

Means, standard errors and the significance of the differences between mean values were calculated for all experiments.

RESULTS

The results of experiments with β-tetra are shown in Table I, and those for M-P. β-tetra are shown in Table II.

5-HT increased the sleeping time of mice anaesthetised with hexobarbitone from 26·4 minutes to 52·5 minutes. This prolongation was not

TABLE II

THE EFFECT OF *N*-METHYL-*N*-PROPYL-β-TETRAHYDRONAPHTHYLAMINE HYDROBROMIDE UPON THE SLEEPING TIME DUE TO HEXOBARBITONE, POTENTIATED BY 5-HYDROXYTRYPTAMINE CREATININE SULPHATE (5-HT), RESERPINE AND IPRONIAZID
(Room temperature 22°)

Drug	Dose in mg./kg.	Route	Mean sleeping times Min. ± SE.	Difference in sleeping times Min. ± SE.	t_8
Hexobarbitone ..	100	I.P.	26·4 ± 2·9 (22)	26·0 ± 1·5	17·6
Hexobarbitone + 5-HT	100 30	I.P. I.P.	52·5 ± 5·0 (11)		not significant
Hexobarbitone + 5-HT + M-P. β-tetra	100 30 25	I.P. I.P.	47·7 ± 3·7 (9)		
Hexobarbitone ..	100	I.P.	22·3 ± 1·0 (10)	23·6 ± 3·6	6·5
Hexobarbitone + reserpine	100 1	I.P. I.P.	45·9 ± 4·9 (10)		not significant
Hexobarbitone + reserpine + M-P. β-tetra	100 1 25	I.P. I.P. I.P.	39·3 ± 6·3 (10)		
Hexobarbitone ..	100	I.P.	23·1 ± 1·0 (24)	73·7 ± 8·6	8·6
Hexobarbitone + iproniazid	100 20	I.P. I.P.	96·8 ± 13·0 (12)		not significant
Hexobarbitone + iproniazid + M-P. β-tetra	100 20 25	I.P. S.C. I.P.	76·3 ± 21·54 (11)		

See Table I for explanation of numerals in brackets and for interpretation of values appearing in column headed "difference in sleeping times". The *t*-test shows significant ($P > 0{\cdot}001$) differences between the mean values compared, except where the words "not significant" appear. The experiment with M-P. β-tetra with each of the three potentiating agents were performed at the same time as the experiments with β-tetra which are shown in Table I.

altered significantly by M-P. β-tetra. β-Tetra reduced the sleeping time of mice treated with 5-HT to 26·0 minutes a value which was not significantly different from the control time of 26·4 minutes for hexobarbitone alone. In the doses used, neither β-tetra nor M-P. β-tetra when injected alone before hexobarbitone administration gave a value for sleeping time different from that of the hexobarbitone controls.

Reserpine also increased the sleeping time from the control value approximately twofold. β-Tetra antagonised this potentiation completely, reducing the sleeping time of reserpine treated mice to 25·6

minutes compared with the control value of 22·3 minutes. M-P. β-tetra did not reduce significantly the reserpine potentiation.

Iproniazid produced a fourfold increase in sleeping time of hexobarbitone treated mice. This was reduced to 40·6 minutes by β-tetra and this value differed significantly ($P > 0.001$) from that obtained for the group receiving iproniazid alone. It appears that this compound does not completely antagonise the action of iproniazid. From Table II it will be seen that M-P. β-tetra has no effect on the iproniazid potentiation.

In a further experiment with iproniazid, when the dose of hexobarbitone was reduced to 90 mg./kg., iproniazid (18 mg./kg.) increased the sleeping time from 12·8 to 22 minutes. A small but significant ($P > 0.001$) antagonism towards this potentiation was shown even by such a small dose of β-tetra as 1 mg./kg.

Discussion

The methyl-propyl derivative of β-tetrahydronaphthylamine while showing a specific anti-5-HT action on the rat uterus preparation fails to exhibit any action against the potentiation of hexobarbitone hypnosis in mice by 5-HT. These results provide a further example of the lack of parallelism between the central and plain muscle effects of 5-HT antagonists. The effect is the reverse of that seen with β-tetra, where the central effect predominates. The latter compound not only antagonises the action of 5-HT on the duration of hexobarbitone hypnosis, but is also effective against the enhancement produced by reserpine. In this respect β-tetra is similar to lysergic acid diethylamide (LSD). It differs, however, from LSD in that it also antagonises the actions of iproniazid which LSD does not[7].

β-Tetra is an extremely active pyretic drug[8]. We have found that M-P. β-tetra lacks this action. Lessin and Parkes[9] and Fastier, Speden and Waal[10] have found that many drugs which prolong sleeping time also lower body temperature. The possibility that the antagonism of the potentiation of sleeping time by drugs is associated with a pyretic effect is suggested by the present results and merits further investigation.

Acknowledgements. We are indebted to Mr. J. Trahar of Roche Products Ltd., of Sydney, for a donation of Marsilid, and to Mr. A. E. Peake of Abbott Laboratories Ltd. for the 5-hydroxytryptamine creatinine sulphate.

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A NOTE ON THE INFLUENCE OF THE MEDIUM ON THE TOXICITY OF ANTIBIOTICS

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The strain of mice used has been found to affect the results when testing antibiotics for freedom for undue toxicity. The results confirm those of Maffii and colleagues in some instances, but not in others, being related to the strain of mice used. No protective action was seen with cortisone.

ANTIBIOTICS are tested for freedom from undue toxicity by intravenous injection in five mice. The sample passes the test if no mouse dies within 24 (B.P.) or 48 (U.S.P.) hours. The United States Pharmacopeia advises saline as the medium for bacitracin, chlortetracycline or procaine penicillin, but recommends the solution of penicillin or streptomycin in water. Water or saline is official for the antibiotics in the B.P. Recently, however, Maffii, Semenza and Soncin[1] have found that BZL mice tolerated the test dose of tetracyclines and of streptomycin only in solution of saline but died after receiving this dose in water. Water alone was not toxic.

In view of the practical significance of these findings we describe tests, some of which confirm the work of Maffii and his colleagues while others differ. Male mice of 23 $\pm$ 2·18 g. body weight were taken at random from two mixed batches (MLI, ML2) and from three pure strains. Among the latter were two sublines of RIII, a "high cancer" strain known also for its high sensitivity to stress. A fresh 0·2 per cent solution of the sample under test was made up and 0·5 ml. injected intravenously within 5 seconds. The mice were observed for 48 hours. Fragility tests were done according to Harris[2]; the concentration of sodium chloride in which moderate haemolysis occurred was recorded.

The results in Table I show that no mouse died after injection of antibiotics in solution of saline, while the response to water alone or to antibiotics in aqueous solution varied with the stock or strain or even with the subline of the test animal. The findings obtained with strain ML1, for instance, agreed with those reported previously[1] on streptomycin and tetracyclines. Penicillin had the least and procaine penicillin the most toxicity. ML2 strain mice, however, died after injection of water alone, and to these mice aqueous solutions of penicillin or dihydrostreptomycin appeared to be more toxic than to the strain ML1.

There was no evidence that the toxicity of solutions in water as contrasted with solutions in saline was due to deviation from the optimal osmotic pressure. A rise in concentration of an aqueous solution of dihydrostreptomycin from 1 : 500 to 1 : 250 did not reduce but apparently increased the toxicity. Both these concentrations were tolerated when given in saline solution. Moreover, the toxicity of penicillin was not directly related to the fragility of mouse red blood cells, or to the animal's

senstitivity to water alone. One of the RIII sublines was very sensitive to water alone but surprisingly resistant to penicillin in solution of water.

No protective action was seen with cortisone. There was no difference in the toxicity of an aqueous solution of procaine penicillin to 13 ML2 mice pretreated with 0·25 mg. of cortisone acetate in alcoholic saline on

TABLE I

DEATHS AFTER INTRAVENOUS INJECTION*

Mouse strain	Average R.B.C. fragility NaCl per cent	Water	Saline	Penicillin G		Dihydrostreptomycin				Chlortetracycline		Procaine penicillin G	
				in water 1 mg.	in saline 1 mg.	in water 1 mg.	in water 2 mg.	in saline 1 mg.	in saline 2 mg.	in water 1 mg.	in saline 1 mg.	in water 1 mg.	in saline 1 mg.
ML1	0·39 ± 0·030	0/10	0/10	1/12	0/12	2/10	—	0/10	—	4/10	0/10	4/5	0/5
ML2	0·44 ± 0·028	3/5	0/5	6/12	0/12	7/10	8/9	0/10	0/6	3/5	0/5	14/15	0/15
	ML2—ML1: P 0·03												
suisse	0·41 ± 0·023	3/5	—	2/5	0/5	—	—	—	—	—	—	—	—
	suisse—ML1: P 0·3												
RIII	0·41 ± 0·020	4/5	0/5	0/5	—	—	—	—	—	—	—	—	—
	RIII—ML1: P 0·2												
RIII/a	0·40 ± 0·023	0/5	—	1/5	—	—	—	—	—	—	—	—	—
	RIII/a—ML1: P 0·6												
C57 black	0·43 ± 0·020	2/5	—	3/5	—	—	—	—	—	—	—	—	—
	C57—ML1: P 0·04												

* All injections were in 0·5 ml.

3 days preceding the test and 13 mice pretreated with the medium alone. The mortality after injection of water alone was only 33 per cent less in 13 mice pretreated with cortisone than in 13 control mice.

It would appear that different factors are involved in determining the animal's resistance to water alone on the one hand and to aqueous solutions of antibiotics on the other. Prescott, Kaufmann and James[3] have shown recently that the toxicity of isoniazid to DBA mice is less when it is in solution with glycerol than in solution with water. This result and the work referred to and presented here would suggest the necessity for detailed specification in the standardisation procedure by the pharmacopoeias stating the appropriate medium for each drug requiring tests for undue toxicity.

We are indebted to Dr. M. V. Piekovski for the inbred mouse strains.

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EFFECTS OF RESERPINE AND HYDRALLAZINE ON ISOLATED STRIPS OF CAROTID ARTERIES

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Spirally cut sections of horse carotid artery have been found suitable for the qualitative testing of vasoconstrictors and their antagonists. With this preparation reserpine and hydrallazine non-specifically antagonise the contractions induced by a number of stimulant drugs, including adrenaline, noradrenaline, histamine, 5-hydroxytryptamine, acetylcholine, barium chloride and potassium chloride. Measurements of "self protection" by means of Furchgott's method but using strips of horse carotid arteries have shown that hydrallazine may possess an affinity for adrenergic receptors.

The hypotensive action of hydrallazine appears to be predominantly peripheral. That of reserpine seems to consist of two effects, one on the central nervous system and the other directly on the blood vessels. Of the two effects it is difficult to assess precisely which is the more important.

Hydrallazine, dihydrallazine and some related compounds cause vasodilatation in the rat isolated perfused hindquarters and the rabbit ear and antagonise the vasoconstriction produced in these preparations by adrenaline, noradrenaline, histamine, barium chloride and 5-hydroxytryptamine.[1] Both hydrallazine and dihydrallazine relax isolated aortic strips from cats and rabbits, and antagonise the contractions caused by adrenaline, noradrenaline, histamine and 5-hydroxytryptamine but not those caused by barium chloride[1]. Recently, reserpine has also been shown to have a direct action upon arterial smooth muscle[2].

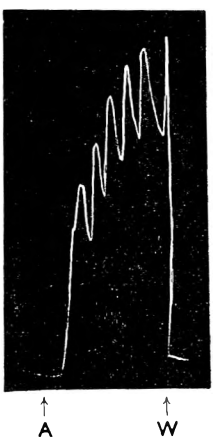

Fig. 1. Spontaneous rhythmic activity in a strip of horse carotid artery: At A, 0·66 μg. Hm for 6 minutes. At W, wash out.

Preparations from the aortae of cats and rabbits were unsatisfactory because the magnitude of the contraction or relaxation was small. Furthermore, the response was slow and recovery prolonged, so that the time interval between doses was often as much as two hours, and many of the strips were refractory to stimulant drugs, including acetylcholine, 5-hydroxytryptamine and histamine. We have now used spirally cut strips of horse carotid artery to study the effects of hydrallazine and reserpine on arterial smooth muscle.

Materials and Methods

The composition of the Tyrode's solution was as follows (g./litre). NaCl 8·0, KCl 0·198, $CaCl_2$ 0·2, $MgCl_2$ 0·1, NaH_2PO_4 0·05, $NaHCO_3$ 1·0, glucose 1·0.

In studying drug antagonisms, the following drugs were used, dissolved in Tyrode's solution. Acetylcholine chloride (ACh), (−)-adrenaline hydrochloride (Ad), (−)-noradrenaline bitartrate (NA), 5-hydroxytryptamine creatinine sulphate (5-HT), histamine acid phosphate (Hm), barium chloride ($BaCl_2$), potassium chloride (KCl), atropine sulphate (atropine), mepyramine maleate (mepyramine), phentolamine, (+)-lysergic acid diethylamide tartrate (LSD), 1-hydrazinophthalazine hydrochloride (hydrallazine), and reserpine in the form of a buffered solution in ascorbic acid-sodium ascorbate.

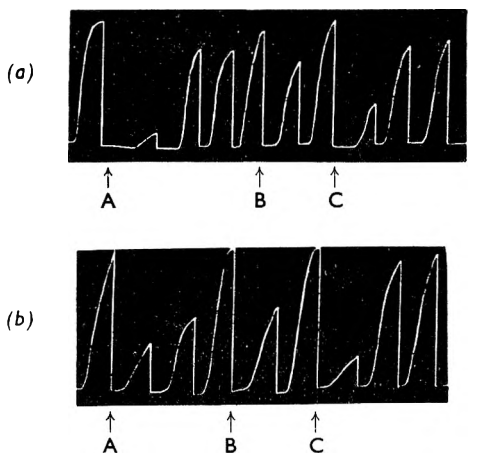

FIG. 2. (*a*) Noradrenaline-phentolamine antagonism in horse carotid artery strips. All contractions due to 0·5 μg. NA. At A, 0·03 μg. phentolamine. At B, 0·01 μg. phentolamine. At C, 0·02 μg. phentolamine. (*b*) Acetylcholine-atropine antagonism in horse carotid artery strips. All contractions due to 0·2 μg. of ACh. At A, 0·02 ng. atropine. At B, 0·01 ng. atropine. At C, 0·04 ng. atropine.

Lengths of common carotid artery were removed from horses immediately after death and placed in Tyrode's solution. 10 cm. lengths were cut spirally (Furchgott and Bhadrakom[3]), and pieces about 2 cm. long were set up in oxygenated Tyrode's solution at 36°; bath volumes varied from 10 to 75 ml. Before drugs were added to the bath a tension of 10 g. was applied for 1 hour. The drugs were then added and left in contact with the tissue for 5 minutes. In some experiments the contraction was complete in 4 minutes, in others, 6 minutes. When this happened the period of contact between the drug and the tissue was decreased or increased. Some strips showed rhythmic activity in the presence of Ad or Hm and these were rejected. An example is shown in Figure 1. After the drug was washed out, the lever returned slowly to the base-line. With ACh contractions, relaxation on washing took place in 5 to 20 minutes, but with the other drugs the relaxation took 30 minutes or more. Standard reproducible submaximal responses were obtained to ACh, NA, Ad, Hm, 5-HT, KCl and $BaCl_2$. Once these had been obtained, reserpine or hydrallazine was added 20 minutes before the next addition. Total time of contact of reserpine or hydrallazine with the tissue was 25 minutes.

The artery strips always contracted after the addition of ACh but were less sensitive to the other drugs, and sometimes did not respond unless high doses were used. Stored at 4°, artery strips retained their sensitivity to drugs for about 3 days. In one or two cases sensitivity was maintained for much longer.

Drug concentrations are expressed as the final concentration per ml. of the bath fluid.

Results

ACh (0·1 ng. to 2·0 μg.), Ad (10·0 ng. to 5·0 μg.), NA (10·0 ng. to 5·0 μg), 5-HT (40 ng. to 3·0 μg.), Hm (0·1 to 5·0 μg.), $BaCl_2$ (0·1 to 0·5 mg.) and KCl (3·0 to 5·0 mg.), all caused contractions of the artery strips. Large, reproducible contractions were obtained with small doses of drugs. There appeared to be a linear relation between the logarithm of the dose and the magnitude of the response.

Contractions due to ACh were antagonised by atropine (0·001 to 0·015 ng.), those due to Hm by mepyramine (0·001 to 0·01 ng.), 5-HT by LSD (0·02 to 1 ng.), Ad and NA by phentolamine (0·01 to 0·015 ng.). Some of these antagonisms are shown in Figure 2. Drug antagonisms were specific, atropine did not antagonise Hm induced contractions and mepyramine did not antagonise contractions due to ACh. Reserpine (1·0 to 25·0 μg.) and hydrallazine (25·0 to 500·0 μg). antagonised the contractile responses to all of the spasmogens tested. No evidence of specificity or selectivity was obtained. Recovery with hydrallazine was usually rapid and complete (Fig. 3) but in a few experiments it was incomplete. After reserpine it was rare for the tissue to recover, although occasionally recovery was seen after 6 to 12 hours (Fig. 4). This points to the strong affinity of this drug for arterial smooth muscle. In some experiments using smaller doses of hydrallazine (5·0 to 10·0 μg.) there was a slight potentiation of the contractions due to ACh and Hm.

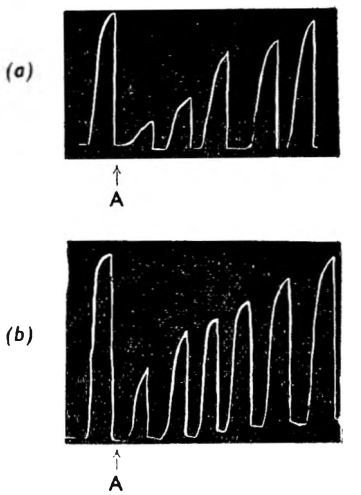

FIG. 3. (*a*) Effect of hydrallazine upon noradrenaline-induced contractions of horse carotid artery strips. All contractions due to 0·66 μg. of NA for 5 minutes. At A, 26·5 μg. hydrallazine 20 minutes before NA. (*b*) Effect of hydrallazine upon acetylcholine-induced contractions of horse carotid artery strips. All contractions due to 0·13 μg. of ACh for 5 minutes. At A, 100 μg. of hydrallazine 20 minutes before ACh.

Furchgott[4], in experiments with strips of rabbit aorta has shown that the presence of a high concentration of a stimulating drug during exposure to a specific antagonist, can protect against the effects of the antagonist. Such "self protection" implies that the antagonist is blocking the same receptors with which the stimulant drug combines, since these have been saturated by the high concentration of the stimulant drug and are, therefore, not available to the antagonist. For example, atropine will specifically antagonise contractions caused by ACh but if the initial concentration of ACh is high, then atropine is less effective. This has been taken

to imply that atropine is acting on the same receptors and that ACh is protecting itself. If it can be assumed that when there is no "self protection," there is no specificity, then this method can be used to differentiate between drugs which act on specific receptors and those which do not. A few experiments have been carried out on this assumption.

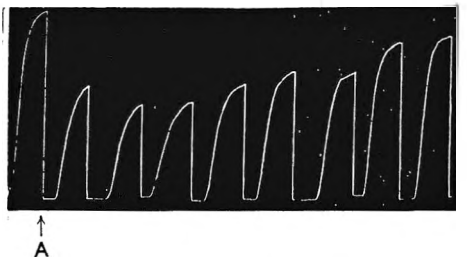

FIG. 4. Effect of reserpine upon acetylcholine-induced contractions of horse carotid artery strips. All contractions due to 0·05 μg. of ACh for 5 minutes. At A, 12·5 μg. reserpine 20 minutes before ACh.

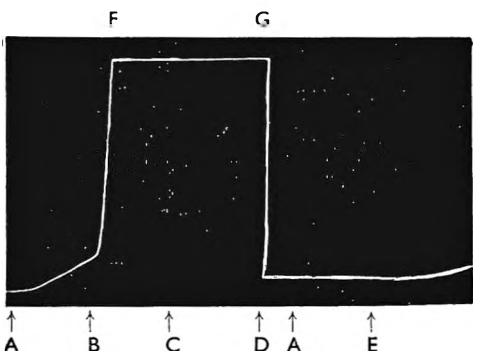

FIG. 5. Failure of a high dose of histamine to protect against hydrallazine. At A, 1 μg./ml. Hm for 5 minutes. At B, 10 μg./ml. Hm. for 15 minutes. At C, 25 μg./ml. hydrallazine for 20 minutes. At D, wash out. At E, small contraction due to 1·2 μg./ml. Hm for 5 minutes. Due to the magnitude of the contraction following 10 μg./ml. Hm a "stop" had to be placed on the lever to prevent this leaving the drum. This accounts for the plateau FG.

Artery strips were stimulated using small concentrations of ACh, Hm, Ad or NA (1·0 to 5·0 μg.). The low concentration of the stimulant was allowed to act for 5 minutes and then a second, higher dose of 0·5 mg. to 1·0 mg. of the same drug was added. The second dose was left in the bath for 15 minutes and reserpine (5·0 to 25·0 μg.) or hydrallazine (25·0 to 500·0 μg.) added and left in contact with the tissue for a further 20 minutes. After washing out, the addition of the smaller dose was repeated. The second contraction was usually much reduced (Fig. 5). This was very clear with ACh and Hm but the effect of a second dose of Ad or NA was not reduced to the same extent (Fig. 6), thereby showing some affinity of hydrallazine for adrenergic receptors.

DISCUSSION

Strips of horse carotid artery have been shown to be suitable for demonstrating the activity of drugs which cause contraction of smooth muscle. We have observed that horse arterial muscle reacts in a typical fashion towards ACh, Ad, NA, Hm, 5-HT, $BaCl_2$ and KCl and the specific antagonists of the first five drugs. Reserpine and hydrallazine relaxed arterial smooth muscle irrespective of the nature of the stimulant drug used and were capable also of causing direct relaxation of artery

strips. The experiments described point to non-specific drug effects rather than to an action upon specific receptors, although there is some evidence for affinity towards adrenergic receptors. Reserpine may act by virtue of an interference with the metabolic processes which underly the contraction of intestinal smooth muscle[5–7]. It seems not unlikely that reserpine and hydrallazine are acting in a similar manner on arterial smooth muscle.

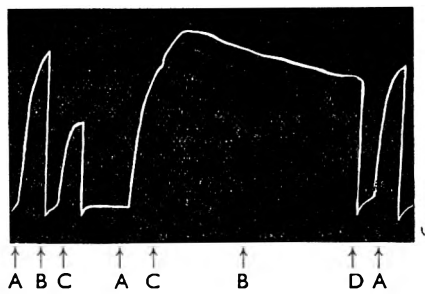

FIG. 6. Self protection by a high dose of adrenaline against hydrallazine At A, 1 μg./ml. Ad. for 5 minutes. At B, 20 μg./ml. hydrallazine for 20 minutes before Ad. At C, 10 μg./ml. Ad for 15 minutes. At D, wash out.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Dienoestrol and Diethylstiboestrol, Colorimetric Determination of. G. Tokár and I. Simonyi. (*Magyar Kémiai Folyóirat.*, 1956, **62**, 320. *Hung. Tech. Abstr.*, 1957, **9**, 111.) A colorimetric method for determining dienoestrol, dienoestrol diacetate and diethylstilboestrol dipropionate is based on the fact that nitration products of the compounds produce intense colour reactions with alkali solutions. The coloured solutions obey the Beer-Lambert law, and thus they are suitable for the quantitative determination of the two hormones.

Reserpine, Determination of, in Non-aqueous Media. J. Bayer. (*Magyar Kémiai Folyóirat.*, 1956, **62**, 355. *Hung. Tech. Abstr.*, 1957, **9**, 110.) Reserpine can best be dissolved in glacial acetic acid and in chloroform. The base content of crystalline reserpine dissolved in anhydride-free acetic acid can be determined by means of a 0·1N acetic acid solution of perchloric acid, in the presence of a solution of crystal violet indicator in acetic acid: error of the determination is less than ±0·3 per cent. The microdetermination of reserpine includes the titration of the chloroform solution of the crystalline material by means of a 0·005N chloroform solution of *p*-toluenesulphonic acid, in the presence of *p*-aminoazobenzene indicator dissolved in chloroform: error of the procedure is less than ±0·6 per cent. The accuracy of both procedures considerably surpasses that of previous methods (±2 to 6 per cent).

Reserpine, Reserpic Acid and Yohimbine, Paper Chromatographic Separation of. F. Machovičová. (*Československ. Farm.*, 1957, **6**, 310.) Reserpine is separated from reserpic acid and yohimbine on paper saturated with a 40 per cent methanolic solution of formamide, with benzene + chloroform (1 : 1) saturated with formamide as the mobile phase at 18°. Reserpine has an R_F value of 0·40 but reserpic acid and yohimbine remain stationary. The method is suitable for the identification of reserpine in tablets. For the separation of reserpic acid from yohimbine, the system *n*-butanol-pyridine-water (4 : 1 : 5), or ethyl acetate-pyridine-water (2 : 1 : 2), is used with the same paper. The three alkaloids can be separated by a combination of the two methods. The spots are detected by observing the paper in ultra-violet light. E. H.

Tropic and Mandelic Acids and their Esters, Colorimetric Determination of. I. Simonyi and G. Tokár. (*Magyar Kémiai Folyóirat.*, 1956, **62**, 348. *Hung. Tech. Abstr.*, 1957, **9**, 110.) A colorimetric method has been evolved for determining tropic acid, mandelic acid and their esters (atropine, scopolamine, homatropine). The principle of the procedure is that the nitrated products of tropic acid and mandelic acid give intense colour reactions with hot alkali solutions. The coloured solutions obey the Beer-Lambert law, thus the reaction is suitable for the quantitative determination of the above compounds.

ORGANIC CHEMISTRY

Central Nervous System Depressants, A New Class of. B. M. Bloom, J. F. Gardocki, D. E. Hutcheon and G. D. Laubach. (*J. Amer. chem. Soc.*, 1957, **79**, 5072.) 2(1-Naphthylamino)-2-oxazoline, a new structural type of central nervous system depressant has been synthesised as the hydrobromide from β-bromoethylamine, by condensation with α-naphthyl isocyanate to yield 1-(1-naphthyl)-3-(2-bromoethyl)-urea, and intramolecular cyclization of the latter in boiling water. Treatment of an aqueous solution with ammonium hydroxide gave the free base. The latter with dry hydrogen chloride in methylene chloride gave the corresponding hydrochloride, which reverted to 1-(1-naphthyl)3-(2-chloroethyl)-urea in boiling 2-propanol. Marked quieting and muscle relaxation followed oral or parenteral administration of the oxazoline in cats, dogs and monkeys. It is also more potent than either reserpine or chlorpromazine in potentiating anaesthesia by 21-hydroxypregnanedione sodium succinate. J. B. S.

BIOCHEMISTRY

BIOCHEMICAL ANALYSIS

Carbutamide, Estimation of, in Blood. R. H. Thompson. (*J. clin. Path.*, 1957, **10**, 369.) An accurate method is described for the measurement of blood levels of carbutamide in blood. Add 0·1 ml. of blood to 7·9 ml. of water, add 2 ml. of 15 per cent trichloroacetic acid, mix and centrifuge or filter after a few minutes. Transfer 5 ml. of the filtrate to a test-tube, add 0·5 ml. of 0·1 per cent sodium nitrite solution, shake and stand for five minutes. To the mixture add 0·5 ml. of 0·5 per cent ammonium sulphamate solution, mix, allow to stand for two minutes, then add 1 ml. of a 0·5 per cent solution of *N*-(naphthyl) ethylenediamine solution and mix again. After 15 minutes compare the colour in a colorimeter against a standard prepared by taking 5 ml. of a solution of carbutamide containing 1 mg./ml. Correct for a reagent blank of 4 ml. of distilled water and 1 ml. of 15 per cent trichloroacetic acid through the diazotisation and coupling procedure. G. F. S.

Carbutamide and Sulphonamides, Estimation of, in Blood. D. G. Moss. (*J. clin. Path.*, 1957, **10**, 371.) A method is described for the simultaneous estimation of sugar and sulphonamides in blood. Wash 0·2 ml. of blood into 3·5 ml. of isotonic sodium sulphate/copper sulphate solution (a mixture of 320 ml. of 3 per cent sodium sulphate and 30 ml. of 7 per cent copper sulphate), add 0·3 ml. of a 10 per cent sodium tungstate solution and centrifuge. Use 1 ml. of the supernatant for estimation of the blood sugar by the modified Schaffer–Hartman method. Use a further ml. for estimation of the hypoglycaemic sulphonamide carbutamide as follows. To 1 ml. add 0·2 ml. of N hydrochloric acid and 0·1 ml. of a 0·1 per cent solution of sodium nitrite. Mix well and add 2 ml. of a 1 per cent solution of *N*-sulphatoethyl-*m*-toluidine. A standard for comparison is prepared by taking 1 ml. of a diluted stock standard solution to contain 15 mg. of carbutamide in water. The orange colour formed is measured after 15 minutes in a spectrophotometer on a colorimeter. The method may be used as a routine method for the estimation of other sulphonamides. G. F. S.

Adrenaline and Noradrenaline, Fluorimetric Estimation of, in Plasma. H. Weil-Malherbe and A. D. Bone. (*Biochem. J.*, 1957, **67**, 65.) A further investigation is made into the specificity of the fluorimetric estimation of adrenaline and noradrenaline, based on the condensation of these amines with ethylenediamine, as applied to plasma. Also a comparison is made between this method and the fluorimetric method of Lund which depends on the formation of 3:5:6-trihydroxyindoles. The catechol compounds were extracted from bovine and human plasma by passage through columns of alumina and elution with dilute acid. The eluates were separated into basic and non-basic fractions by passage through cation exchange resins. The purified basic fraction was studied by paper chromatography, paper electrophoresis or bioassay. At every stage of the procedure samples were analysed by the two fluorimetric methods. It was found that the basic fraction accounted for the catechol compounds present in the alumina eluates. The estimates of adrenaline and noradrenaline obtained by direct fluorimetric assay agreed with results obtained by electrophoresis and by chromatography. No 3-hydroxytyramine was detected. A high degree of correlation was found consistently, at every stage of the procedure, between the two fluorimetric methods. The biological estimation of noradrenaline, using the ascending colon of the rat, was inconclusive since the concentration, as determined by the fluorimetric analysis, was on the threshold of the sensitivity of this preparation. Little biological activity was found in the test for adrenaline, using the isolated uterus of the rat, despite the chemical evidence for the presence of this amine. Possible reasons for this discrepancy are discussed. M. M.

Parathion, Paraoxon and *p*-Nitrophenol in Organic Tissue Material, Determination of. O. Karlog. (*Acta pharm. tox. Kbh.*, 1957, **14**, 92.) The method described is intended for use in forensic chemistry and for determining the concentration of residues on vegetables. Grind 100 g. of tissue in a Waring blender and transfer to centrifuge tubes, add 2 N sulphuric acid until acid, 75 ml. of ethanol and 50 ml. of ether. Shake for 5 minutes and centrifuge in a refrigerated centrifuge. Extract the residue twice more and transfer the pooled extract to a separating funnel and add 5 ml. of 2 N sulphuric acid with 100 ml. of water. Shake and separate. Shake the ether phase twice more with ether, wash the pooled ether extract three times with 100 ml. of water to which 5 ml. of 2 N sulphuric acid has been added. Dry the ether extract with anhydrous sodium sulphate and evaporate to dryness at a temperature below 70°. To the residue add 5 ml. of acetonitrile and heat to 70°. Add 6 ml. of water, cool, filter and centrifuge. The residue is treated twice more. The acetonitrile extract is purified by column chromatography as described by Erwin and others (*J. Agric. Food Chem.*, 1955, **3**, 676) using 40 per cent acetonitrile as the developing liquid. The eluate is extracted with ether after adding an equal volume of water. Then 5 ml. of 2 N sulphuric acid is added and the mixture shaken three times with 50 ml. of ether. Wash the total ether extract twice with 25 ml. of water containing 5 ml. of 2 N sulphuric acid. Dry the ether extract with anhydrous sodium sulphate and evaporate at not more than 70°. Dissolve the purified residue in a suitable amount of ether and apply to paper strips using a Carlsberg pipette. Carry out descending paper chromatography with a mixture of water and acetonitrile as the stationary phase and light petroleum saturated with water and acetonitrile as the moving phase. The paper hangs for at least 12 hours in the vessel before the chromatogram is developed. After reading the R_F values the individual spots are eluted by washing three times with 50 per cent ethanol, and the amount of *p*-nitrophenol is determined spectrophotometrically. G. F. S.

CHEMOTHERAPY

1-*iso*Nicotinylamido-2 : 5-dimethylpyrrol (G.144), Tuberculostatic Activity of. J.-M. Gazave, N.-P. Buu-Hoi and N.-D. Xuong (*Thérapie*, 1957, **12**, 486.) When tested *in vitro* against *Mycobacterium tuberculosis* strain H37RVD, *iso*nicotinylamido-2 : 5-dimethylpyrrol (G.144), a structural analogue of isoniazid, appeared to be only slightly less active than isoniazid. The LD50 determined by oral administration to mice was three times greater for G.144 than for isoniazid. G.144 is less soluble than isoniazid, and might be expected to have a more prolonged effect. G. B.

PHARMACY

Lissapol-Cirrasol Cleansing of Blankets. B. R. Frisby. (*Brit. med. J.*, 1957, **2**, 506.) Hospital blankets, unless specially laundered, are bacteriologically contaminated and potentially dangerous, particularly from their harbouring of penicillin-resistant strains of *Staph. aureus*, which are not removed by ordinary laundering. Provided they can be washed frequently, e.g., after every patient, the lissapol-cirrasol technique (Blowers and Wallace, *Lancet*, 1955, **1**, 1250) is adequate to keep ordinary woollen blankets clean. Many hospital laundries may not be equipped, however, to deal with the increased washing required for this technique. Blankets made of terylene may help in the solution of this problem. These may be boiled like sheets and the resulting counts of the clean blankets are under 20 per plate, with no *Staph. aureus*. They produce very little fluff, are light and warm, and have stood up very well to an average of 14 boilings. There must still, however, be adequate facilities for providing each patient with a clean set of blankets. S. L. W.

Tablet Making, Influence of Physical and Mechanical Factors in. P. L. Seth. (D.Sc. (Nat.) Thesis, Zurich, 1956.) This printed thesis describes work done under Prof. Munzel. The author analyses the tabletting process and selects certain aspects for investigation. He extends the work of Munzel and Kagi in separating tabletting additives into two classes—"lubricants" (materials facilitating the smooth ejection of the formed tablet by reducing the tendency to stick to the die) and "glidants" (materials improving the flow properties of the granules). Several types of natural and treated starches are assessed empirically for their glidant properties by measuring the improvement of flow of a standard lactose granulation to which varying proportions of the material under test have been added. Using the flow rate of the standard granule as a reference, the glidant properties of the starches are found to be twice as effective as those of talc. Of the starches and their derivatives the best results are obtained with potato starch, but as a general group they are found to possess little or no lubricating properties. A modified Brinell press is used to measure the influence of increasing compacting pressure on the corresponding ejection force and a linear relation is obtained over the small range considered. The mechanism of the action of the lubricant is discussed. Using the same press and two granule formulations ((*a*) starch/lactose, (*b*) phenacetin) the effects of increasing compacting pressure and increasing moisture content of the granules on tablet strength (Monsanto "hardness") and disintegration times are reported. Some attempt to investigate the effect of storage condition is also made. Studies are made of the conditions for preparing tablets of lactose which meet the requirements of homeopathic specifications; of the difference in properties of tablets compressed with eccentric and rotary types of tablet machines and of the effect of altering tablet shape and size on the strength and disintegration times. D. T.

PHARMACOLOGY AND THERAPEUTICS

5-Hydroxytryptamine, Release of, by Benzoquinolizine Derivatives with Sedative Action. A. Pletscher. (*Science*, 1957, **126**, 507.) It is known that reserpine causes a release of 5-hydroxytryptamine (5-HT) from the brain, intestine and platelets. After one large dose of reserpine the 5-HT content of these tissues decreases to approximately one tenth of the normal level and remains low for several days. Among the rauwolfia alkaloids, only those with a tranquillising action show this effect. It has now been found that various benzoquinolizine derivatives also release 5-HT. In mice and rabbits, these compounds produce sedation without hypnosis. Among the derivatives examined, 2-oxo-3-*iso*butyl-9:10-dimethoxy-1:2:3:4:6:7-hexahydro-11bH-benzo[a]quinolizine (compound I) showed the most marked sedative and 5-HT-releasing activity. After injection of 40 mg. of compound I per kg. the maximum depletion of brain 5-HT occurred within 30 minutes. During the 4 hours after the injection of compound I there was a considerable increase in the urinary excretion of 5-hydroxyindoleacetic acid, the major metabolite of 5-HT. Pretreatment with *iso*propyl*iso*nicotinic acid hydrazide prevented this depletion of 5-HT. Despite these similarities between compound I and reserpine there were some differences; chiefly in the time required to reach a maximum depletion of brain 5-HT, in the maximum degree of depletion obtainable, and in the time necessary for recovery of the 5-HT levels. These benzoquinolizine derivatives are thus a second group of substances which, like the centrally acting rauwolfia alkaloids, cause both sedation and 5-HT depletion of the brain. Further investigation may lead to an explanation of the role of 5-HT in brain function. M. M.

Morphine and Amiphenazole, Antagonism Between. J. Mercier and F. H. Shaw (*Thérapie*, 1957, **12**, 493.) Experiments were carried out in unanaesthetised rabbits to determine the effect of amiphenazole on the motor and respiratory depression induced by doses of 10 mg. or more of morphine per kg. body weight. In all cases amiphenazole was shown to antagonise the action of morphine on the respiration. The main effect of amiphenazole was on the respiratory rhythm, although in about half the rabbits the amplitude was also increased. Electroencephalographic studies in dogs showed that amiphenazole eliminates the slowing of the trace due to morphine, and antagonises its depressant effect on the reactivation of the reticular system. G. B.

Novobiocin and Erythromycin in the Treatment of Burns. E. J. L. Lowbury. (*Lancet*, 1957, **2**, 305.) This is an investigation carried out over a period of 3 months to assess the therapeutic value of concurrent systemic administration of novobiocin and erythromycin in patients whose burns were colonised by *Staphylococcus aureus* sensitive to these antibiotics, and to test the effect of using this mixture of antibiotics on the incidence and emergence of staphylococci resistant to either or both of them. Adult dosage was erythromycin 300 mg. six-hourly, novobiocin 500 mg. 12-hourly. The majority of patients were treated by the "closed" method, penicillin cream being applied at every change of dressings, until the time of skin-grafting or until the burn was healed. The "exposure" method was used on some burns, with repeated local insufflation of penicillin-lactose powder. The results showed that *Staph. aureus* was cleared

from a higher proportion of covered burns treated with the combined antibiotics (34 out of 49, 68 per cent) than from comparable burns receiving no chemotherapy (3 out of 31, 10 per cent). The therapeutic effect of the mixture was significantly greater than that of either of the two drugs used alone. Patients treated by the exposure method usually did not lose their staphylococci when receiving erythromycin and novobiocin. The incidence of erythromycin-resistant staphylococci fell during the first 4 weeks of a period when the two drugs were being used in combination. Some degrees of resistance began to appear to novobiocin in the second week, and there was a gradual increase in the numbers of staphylococci resistant to erythromycin and slightly resistant to novobiocin from the 5th week onwards. In several trials which showed a selective prophylactic or therapeutic action against *Staph. aureus* the results of skin-grafting were better in treated patients than in the controls. The local pathogenicity of *Staph. aureus*, however, is insufficient to warrant routine chemotherapy against the organism in burns. S. L. W.

Oximes and Atropine in Sarin Poisoning. B. M. Askew. (*Brit. J. Pharmacol.*, 1957, **12**, 340.) It has been shown previously that two oximes, mono*iso*nitrosoacetone (MINA) and diacetylmonoxime (DAM), exert a marked protective effect in rats poisoned with *iso*propyl methylphosphonofluoridate (sarin). Although less active in other species, it seemed possible that they might be effective in sarin poisoning when administered in conjunction with atropine. A third oxime, pyridine-2-aldoxime methiodide (PAM) has also been studied. It was found that when treatment commenced 15 minutes before the administration of the sarin, atropine enhanced the protective effect of MINA and DAM 2 to 3 times and of PAM 9 to 10 times, in mice and rats. In mice, rats and guinea pigs, atropine doubled the protective effect of all 3 oximes when given 30 seconds after sarin. Atropine given to monkeys 1 minute after sarin raised the LD50 approximately 3 times. When given in conjunction with MINA or DAM, the LD50 of sarin was raised 7 to 14 times. DAM is less toxic than either MINA or PAM and can therefore be given in somewhat higher doses. However, it is only a poor reactivator of cholinesterase inhibited by sarin; a property of considerable importance in anticholinesterase poisoning. It is therefore likely to be of less importance than MINA or PAM as an antidote in anticholinesterase poisoning. M. M.

Pethidine-Levallorphan Mixtures, Analgesic Action of. A Herxheimer and C. Sanger. (*Brit. med. J.*, 1957, **2**, 802.) A comparison was made between the analgesia produced by intravenous injections of (*a*) pethidine 22·5 mg., (*b*) pethidine 22·5 mg. + levallorphan 0·075 mg. (dose ratio 300:1), (*c*) pethidine 22·5 mg. + levallorphan 0·15 mg. (dose ratio 300:2), and (*d*) physiological saline solution. The effects of these drugs on ischaemic muscle pain and on the thermal pain threshold were measured in 5 normal men. The pethidine-levallorphan mixtures produced no less analgesia than did pethidine; the mixture containing the smaller amount of levallorphan (*b*) produced significantly greater analgesia than did pethidine. This might be due to the fact that the lower dose of levallorphan might cause vasodilatation and/or sweating, and so raise the threshold, while with the higher dose the antagonism for pethidine becomes manifest. On the other hand there is some indication that levallorphan is a weak analgesic, and the rise in threshold may be due to an analgesic action added to that of pethidine. S. L. W.

5-Phenylthiazolidine-2:4-dione (2:4-Dihydroxy-5-phenylthiazole), Pharmacology of. A. Shulman. (*Aust. J. exp. Biol. med. Sci.*, 1957, **35**, 289.) The object of this paper is to consider the hypnotic properties of 5-phenylthiazolidine-2:4-dione (P.T.D.) and to present evidence suggesting its possible value as an antiepileptiform agent and to indicate that its associated administration will both antagonise and potentiate selected pharmacological actions of its parent substance, 2:4-diamino-5-phenylthiazole hydrochloride (amiphenazole). Evidence is also presented from both *in vivo* and *in vitro* experiments which indicates that amiphenazole may be metabolised to P.T.D., possibly through the intermediate substance 2-amino-4-hydroxy-5-phenylthiazole. It was found that P.T.D. produces narcosis in mice which is reversed by the barbiturate antagonist bemegride. It also potentiates the hypnotic action of pentobarbitone sodium in mice. P.T.D. protects mice against the convulsant action of leptazol. Further, it antagonises the respiratory stimulant and convulsive properties of its parent substance amiphenazole, in mice. This is followed by an increased depth of narcosis and an increased incidence and rapidity of death, thus indicating that the latter substances may be metabolised to the former during this process of antagonism. A similar interaction between convulsant and hypnotic barbiturates has been noted. As there appears to be a possibility that amiphenazole may be metabolised in the body to yield P.T.D., which has been shown to potentiate barbiturate narcosis, it would be advisable to be more cautious in the use of amiphenazole in the treatment of barbiturate overdosage. Nevertheless very large doses of amiphenazole have been given in combination with bemegride in the treatment of barbiturate intoxication without harmful effects. M. M.

Piperazine in the Treatment of Ascariasis. L. G. Goodwin and O. D. Standen. (*Brit. med. J.*, 1958, **1**, 131.) Various piperazine salts were given as a single large oral dose in the treatment of 770 cases of ascariasis in the Gambia. With piperazine citrate, adipate and phosphate a dose equivalent to 3 g. of piperazine hexahydrate gave complete clearance in 76 per cent of cases: a 4-g. equivalent gave complete clearance in 82 to 89 per cent of cases. Piperazine sebacate and stearate gave clearance in 86 per cent of cases given a 3-g. equivalent, but were objectionable on grounds of taste or bulk. It was concluded that the efficiency of piperazine preparations is directly related to the content of base and is little influenced by the nature of the salt. Piperazine citrate syrup was found to be stable at high temperatures, to be as effective as solid citrate, adipate or phosphate, and to be of more practical value than tablet preparations in mass treatment owing to rapidity of dosage, rigidity of control, and palatability. No toxic side-effects were observed after a single dose of 4 g. of piperazine. S. L. W.

Piperazine in the Treatment of Hookworm. L. G. Goodwin and O. D. Standen. (*Brit. med. J.*, 1958, **1**, 135.) Of 2 children, aged 8 and 11 respectively, infected with hookworm, one was treated with 900 mg. of piperazine adipate three times daily for 7 days, and the other with 1 g. of piperazine phosphate three times daily for 7 days. Neither patient showed any significant decrease in egg count at the end of the course of treatment, but on subsequent treatment with a single dose of 2 ml. of tetrachlorethylene egg counts were reduced to nil in both cases in 24 hours. A third child aged 6 months, not given piperazine treatment, was also cleared of ova after a single dose of 0·25 ml. of tetrachlorethylene. S. L. W.

Piperazine in the Treatment of Tapeworms. L. G. Goodwin and O. D. Standen. (*Brit. med. J.*, 1958, **1**, 133.) One group of 15 patients in Tanganyika harbouring *T. saginata* was given a dose of piperazine citrate equivalent to 3 g. of hexahydrate, and on the following morning were given 5·3 ml. of extract of male fern, followed 2 hours later by 2 oz. of magnesium sulphate. A second group of 15 patients was treated with extract of male fern and purge alone. The number in which tapeworm heads were found in the stools was greater in the first group but the difference was not statistically significant. Piperazine used alone was found to be inefficient. It would be of advantage to investigate the effect of giving the dose of piperazine only 5 to 6 hours before giving the male fern. A systematic method of examining stools for tapeworm heads is described.

S. L. W.

Preludin, Euphoriant Effects of. J. B. Randall. (*Brit. med. J.*, 1957, **2**, 508.) A clinical trial of Preludin in 147 patients suffering from mild depressive and psychoneurotic conditions showed that it has effects similar to those of amphetamine. It appeared that a dose of 25 mg. twice daily produced euphoriant and stimulant effects more powerful than those produced by 5 mg. of amphetamine twice daily. Of the 147 patients, 87 appeared to be improved, 17 were unchanged, and 43 complained of symptoms indicating that the drug had an adverse effect. Fifty-four out of 84 cases of depression were significantly improved, as were 17 of 33 cases suffering from hysteria, 9 out of 13 obsessional states, 4 out of 6 personality disorders, and 3 out of 8 anxiety states. In addition to anorexia, some patients complained of insomnia, excitement, palpitations, trembling, dryness of the mouth, abdominal pain, and sensations of increased energy. Mild depersonalisation also occurred, and elevation of mood, increased mental activity and concentration were reported. In 2 cases appetite was increased, 2 further patients reported symptoms of hunger, and in 1 case there was a definite increase in weight. Weight reduction was not a significant finding as there was very little voluntary reduction of food intake. There is no doubt that the drug is pharmacologically active, and it would appear that it should be classified under the same pharmaceutical schedule as the amphetamines. S. L. W.

Propoxyphene Hydrochloride, Comparison of, with Codeine as an Analgesic. C. M. Gruber. (*J. Amer. med. Ass.*, 1957, **164**, 966.) Propoxyphene hydrochloride (α-(+)-2-propionoxy-4-dimethylamino-1 : 2-diphenyl-3-methyl butane hydrochloride) was compared for its effectiveness as an analgesic with codeine phosphate in 101 patients suffering from chronic pain due to arthritis, malignancy, neuritis, fractures or peripheral vascular disease. Five medicaments in identical capsules were administered orally 4 to 6 times daily; the capsules contained (1) a placebo, (2) 32·5 mg. of codeine, (3) 32·5 mg. of propoxyphene, (4) 65 mg. of codeine, and (5) 65 mg. of propoxyphene. Each medicament was given for 3 consecutive days, the placebo being given on the middle 3 days (7th, 8th and 9th). In equal doses by weight propoxyphene hydrochloride and codeine phosphate were equally effective in reducing pain and discomfort: both drugs were more effective in doses of 65 mg. than in doses of 32·5 mg., and both drugs were appreciably more effective than the placebo. Codeine in doses of 65 mg. produced a significant number of gastrointestinal side-effects; these reactions were much less frequent with the same dose of propoxyphene. In comparison with the placebo, codeine and propoxyphene produced comparable increases in the number of central nervous system side-effects. S. L. W.

Tolbutamide, Clinical Experience with. G. Walker, J. D. H. Slater, E. K. Westlake and J. D. N. Nabarro. (*Brit. med. J.*, 1957, **2**, 323.) This paper presents the results of a trial of tolbutamide in 72 diabetic out-patients observed for up to one year. The patients were not grossly obese and had "mild" diabetes that could not be satisfactorily controlled by diet alone. Their age varied between 22 and 78 (mean 53 years) and the duration of the diabetes between 1 and 30 years (mean 6·6 years). Of the 72 patients, 41 had previously been treated by diet alone, 19 were transferred from carbutamide, and 12 from insulin. Patients transferred from carbutamide were initially given the same dose of tolbutamide. Those who had been taking insulin were given reduced doses for a few days and were then started on tolbutamide. Patients who had had neither carbutamide nor insulin were observed on a strict diet for a month and if their mid-morning blood sugar remained above 200 mg./100 ml. they were given tolbutamide: the starting-dose for these patients and for those transferred from insulin was 0·5 g. two or three times daily, taken with the main meals. The dose was subsequently adjusted to a maximum of 4 g. daily according to the blood sugar response. Ketonuria was regarded as an indication for stopping the drug and resuming insulin. Nineteen of the patients stopped treatment for the following reasons; resistance (requiring insulin), 2; failure to respond, 6; rash, 2; abdominal symptoms, 4; not required, 1; irregular attendance, 2; returned to insulin at own request, 2. Of the 53 patients who had not been treated with carbutamide, 7 showed no response, 33 showed an immediate response and 13 a delayed response: 20 of the patients (61 per cent) who showed the immediate response and 9 (69 per cent) who showed the delayed response appear to be satisfactorily controlled by tolbutamide. All patients who had responded to carbutamide did so to tolbutamide but one became resistant to it. Of 26 patients complaining of diabetic symptoms (thirst, polyuria, and pruritus vulvae), 23 responded to tolbutamide and their symptoms were relieved as the blood sugar fell. In 22 of the 53 still taking tolbutamide control is unsatisfactory as judged by the mid-morning sugar. The authors conclude that about 50 per cent of patients with "mild" diabetes that cannot be controlled by diet alone will benefit from the drug, but that as its mode of action and possible long-term ill-effects are not yet known it should be used cautiously. S. L. W.

Tolbutamide in the Treatment of Diabetes. W. J. H. Butterfield, I. K. Fry, C. Hardwick and H. E. Holling. (*Brit. med. J.*, 1957, **2**, 325.) There is no single test which will serve to distinguish which patient will ultimately be satisfactorily controlled by tolbutamide, and a therapeutic trial is necessary for each patient. (Details are given of a scheme which has been found useful in testing the response of various types of patient to tolbutamide.) The rapidity of response to tolbutamide varies from patient to patient, so that a therapeutic trial should last for 3 weeks and should be cut short only if it appears the diabetes is getting out of control. Tolbutamide is less likely than insulin to produce a hypoglycaemic attack as its full effect takes place about 4 hours after administration, by which time the next meal is due. If satisfactory control is achieved the patient may be maintained indefinitely on up to 3 g. of tolbutamide daily. There should be no relaxation of dietary restrictions. Side-effects are few, skin rashes being the most common complication. Blood dyscrasias are rarely seen, and renal damage has not been encountered. It is not possible at present to assess the value of tolbutamide in the prevention of diabetic complications such as arterial disease, retinitis, neuritis, and renal disease, but it seems unlikely

that it will be more effective than insulin. It will not prevent ketosis or the exacerbation of the diabetic state associated with infection or trauma. S. L. W.

Voacamine, an Alkaloid from *Voacanca africana*, Pharmacology of. A. Quevauviller and O. Blanpin. (*Thérapie*, 1957, **12**, 635.) The cardiotonic action of voacamine is due to a direct action on the heart muscle. Its toxicity (determined in animal experiments) is 100 to 250 times less than that of digitoxin, and it kills by causing respiratory failure. The camphorsulphonate is about half as toxic as the equivalent amount of sulphate. Voacamine has a depressant effect on the central nervous system. It has a hypotensive action due to a parasympathomimetic and sympatholytic effect, and causes contraction of the smooth muscle of the blood vessels and intestine. The action of voacamine resembles that of the total alkaloids of *Voacanca africana*, with the exception of certain digitoxin-like effects, which may be due to the presence of voacorine.

G. B.

APPLIED BACTERIOLOGY

Antimycotic, 2:2-Dihydroxy-5:5-dichlorodiphenylsulphide, Evaluation of. L.-G. Allgrén and Å. Frisk (*Svensk farm. Tidskr.*, 1957, **61**, 637.) The fungistatic effect of 2:2′-dihydroxy-5:5′-dichlorodiphenylsulphide was determined by a serial dilution method, using *Candida albicans* and *C. tropicalis* as test organisms. The substance showed a satisfactory fungistatic action. Doses of 1·0–1·5 mg./g. injected subcutaneously in the form of an oily suspension were lethal to mice. Rabbits received 0·25 g./kg. or more intramuscularly without untoward effect. In clinical investigations, uncoated tablets gave rise to gastric upset, especially when taken on an empty stomach. Intramuscular injections (in oil) caused local pain, and suppositories caused considerable anal irritation and pain. A slight improvement was observed in 5 of 17 cases, mainly of bronchial infections, treated with the drug. The best result was obtained in a case of *Candida* cystitis. The determination of 2:2′-dihydroxy-5:5′-dichlorodiphenylsulphide in serum was carried out by extracting with ether and purifying the extract. The difference in optical density at 323 mμ between acid and alkaline solutions was measured and the concentration calculated. Recovery on extraction was 90–100 per cent, and the determination was reasonably accurate with concentrations above 1 mg./ml. G. B.

Bacteria in Droplets, Action of Disinfectants on, as Compared with Large Volumes. R. C. Valentine. (*J. gen. Microbiol.*, 1957, **17**, 474.) Most work on disinfectant actions of chemicals has been restricted to the use of relatively large volumes of bacterial suspensions; little work has been done on bacteria suspended in droplets because most methods of determining viability are not applicable to such small samples. The urea method of Valentine and Bradfield (*J. gen. Microbiol.*, 1954, 11, 349) provided a suitable method of estimating viability in droplets. The organism used was a paracolon bacterium and test suspensions were prepared from 18 hour growths. Droplets were formed in a platinum loop, which was then held just above the suspension from which the droplet had been taken, the end of the tube having been closed. It was found that the bactericidal action of low concentrations of copper sulphate was much less in the drops than in the bulk suspension, provided the suspension was unwashed. The difference in killing in droplets and in bulk became smaller when suspensions were washed and became negligible when the washing was repeated several times. Phenol was found to be equally toxic to washed or

unwashed suspensions. At phenol concentrations of 0·1 and 0·2 per cent, there was markedly more killing in the drops than in the bulk suspension, but this effect was not observed at higher phenol concentrations. The results with copper sulphate were explained by the concentration of protein at the surface of droplets of unwashed suspensions. This protein decreases the toxicity of copper ions. Phenol, however, itself lowers surface tension and becomes concentrated at the surface of the droplet. From a calculation of the time required for half the bacterial population of a droplet to pass to, and be held in, the surface layer it is suggested that the organism used, although it is non-flagellate, appears to move about five times faster than would be expected from Brownian movement alone. B. A. W.

Nystatin Sensitivity of *Candida* Strains. R. F. Jennison and P. Stenton. (*J. clin. Path.*, 1957, **10**, 219.) In view of reports of the use of nystatin in the treatment of clinical conditions and in prophylaxis of *Candida* infections in patients treated with broad-spectrum antibiotics and cortisone, it was desirable to develop a rapid routine test for sensitivity to this antibiotic. Two methods were studied: a serial dilution technique and a blotting paper disc method. Serial dilutions of nystatin were made in a broth containing 3 per cent glucose and Andrade's indicator at pH 7·2. Penicillin and streptomycin were included in order to inhibit growth of any bacterial contaminants. Development of turbidity was taken as the first indication of growth. Although pH 7·2 is high for growth of *C. albicans*, the use of a solution of acid reaction was precluded by the instability of nystatin. An inoculum of 0·02 ml. of a 48 hour broth culture was added to 5 ml. volumes of broth which were incubated at 37°. Results of turbidity and change of indicator colour were read after 2, 3 and 5 days. The paper disc method employed 8 mm. diameter discs (Ford Mill 428) impregnated with differing amounts of nystatin (*iso*propanol solution) and dried. Blood or nutrient agar plates were seeded with 6 hour *Candida* culture and incubated overnight at 37° after introduction of the discs. Zones of inhibition were difficult to measure except by matching against reference white circles painted on black cards. Results are given for the sensitivity of 76 strains of *C. albicans* and for 14 strains of other species of *Candida*; some of these were stock strains and others had been isolated from cases of vaginal infection. All strains were sensitive to nystatin, the mean inhibitory concentration at 48 hours in broth being 3·2 μg./ml. It was found that paper discs impregnated with 12·5 μg./ml. were stable on refrigeration for several months and gave an average zone of inhibition of 16 mm., which was not dependent upon the type of culture medium used. B. A. W.

LETTER TO THE EDITOR

The Mechanism of Histamine Release from Mast Cells

SIR,—In the Symposium on Histamine[1] in honour of Sir Henry Dale (April, 1955), I drew attention (p. 401) to the importance of the lipid components of the mast cell, stressing in particular a similar behaviour of mast cells and the myelin of nerve sheaths, both of which undergo rapid hydration in the presence of alkali. Since then I have tested several lecithinases on mast cells—the α-toxin of *Cl. welchii*[2], purified bee venom and its heat-stable, non-enzymatic derivative, Melittin[3], and a powerful lecithinase which is present in the venom of the Indian cobra (*Naja naja*)[4]. All bring about disruption of mast cells parallel to their ability to attack lecithin prepared from nerves or egg yolk; Melittin appears to act as a histamine-liberator by virtue of its basicity and surface active properties. But this does not explain why a histamine-liberator, such as compound 48/80, specifically destroys the mast cells in the rat, leaving the myelin in the nerve sheaths intact; indeed, the few mast cells which are present in the nerve sheaths entirely escape the action of the liberator[5], which itself has no specific action on lecithin in model systems.

Professor Uvnäs[6] now offers a hypothesis which may resolve this difficulty. He suggests that there is normally an enzyme, a lecithinase, at the mast cell membrane and that this enzyme is prevented by an inhibitor from attacking the cell envelope. Compound 48/80, or other histamine-liberator specific for the species, removes the inhibitor and thus permits the lecithin of the membrane to be destroyed.

Perhaps we can theorise over this interesting new viewpoint a little further. Through the kindness of Dr. R. M. C. Dawson of Cambridge, chromatographic analysis for phospholipids has been carried out on a variety of tissues rich in mast cells, and upon mast cell granules isolated from the connective tissue of rats. In addition to lecithin, which is known to be present in some quantity in the mast granules of cattle[7], Dr. Dawson has found comparatively large amounts of phosphatidyl serine in all and, in the rat, some phosphatidyl ethanolamine also.

It will be recalled that in the original demonstration of the release of histamine from mast cells in the rat[8], the following morphological observations were made:

(i) The fluorescent diamidines can be seen to concentrate in the granules of the mast cells which then undergo a series of changes accompanying the release of histamine.

(ii) Pre-treatment with an antihistamine drug does not prevent the diamidine from reaching the granules but it does suppress the subsequent swelling, degranulation and disruption of the cell.

(iii) Histamine-release from the granules in the rat is accompanied by a curious vacuolation of the intergranular cytoplasm. Hill[9] has recently described this as a 'honeycomb' appearance.

The question thus arises, can the new hypothesis of Uvnäs help us to explain these morphological findings in terms of pharmacology?

If we modify the hypothesis slightly and postulate that the mast cell enzyme is a Type-C phospholipase, attacking the ester linkage between the phosphoric

acid and the alcohol (choline, serine, ethanolamine), then we may infer from known evidence

that the newly formed primary phosphatide (i.e., of the type $R'—O—\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}—OH$)

will render the film leaky to histamine[10];

that an antihistamine drug will protect the newly formed film against the tendency of histamine to lower the surface tension[10];

and that if the substrate should include phosphatidyl ethanolamine, then the free ethanolamine so formed will give rise to an extraordinary cytoplasmic vacuolation. By a curious coincidence, this was also described as a 'honeycomb" appearance by its discoverers[11].

If a peritoneal 'window' of a freshly exteriorised portion of rat mesentery is coated with normal saline and a fragment of cover slip is applied, saline dilutions of the various split products of the phospholipids can be drawn across the microscope field and their effects on the mast cells observed. It is then evident that neither choline (10^{-3} as the chloride, prepared from lecithin) nor serine (10^{-3} from phosphatidyl serine) cause more than slight swelling of the mast cells, whereas ethanolamine, or the synthetic dimethylethanolamine (10^{-5} in saline), bring about a rapid swelling, vacuolation and even disruption of the cells. The microscopic appearances, in fact, closely resemble the 'explosions' which are seen when mast cells are treated with sodium hydroxide[12]. The point to be stressed, however, is that here the active base, ethanolamine, is formed intracellularly from a normal body constituent. Indeed, we are faced with the possibility that a single histamine-liberator applied extracellularly can produce *two* histamine-liberators intracellularly—a primary phosphatide (from lecithin, phosphatidyl serine or phosphatidyl ethanolamine) and free ethanolamine also when phosphatidyl ethanolamine is present.

Professor Uvnäs is to be congratulated on his ingenious explanation of the release of histamine from mast cells and more especially for giving us a hypothesis which can be tested in many fields of biological research.

My thanks are due to Dr. M. G. Macfarlane, London, for *Cl. welchii* α-toxin; to Dr. E. Habermann, Würtzburg, for purified bee venom and Melittin, and to Dr. R. Hirt, Bern, for samples of synthetic primary and secondary phosphatides.

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