REVIEW ARTICLE

QUATERNARY AMMONIUM COMPOUNDS IN MEDICINAL CHEMISTRY. II*

By P. F. D'Arcy, B.Pharm., Ph.D., M.P.S., and E. P. Taylor, B.Pharm., B.Sc., Ph.D., F.R.I.C.

The Research Division, Allen & Hanburys Limited, Ware, Hertfordshire

Chemotherapeutic Activity

Antimicrobial Agents

The field of antimicrobial chemotherapy has grown enormously over the past quarter of a century, and shows every sign of increasing at an even greater rate. The chemotherapeutic application of onium compounds has received a tremendous impetus since the end of the Second World War. Many members of this family have been shown to possess marked *in vitro* antibacterial activity, while others, as has been described, possess useful pharmacological and pharmacodynamic actions; in addition, recent studies have disclosed potent antifungal activity in many onium salts. Whilst the antibiotics and the sulphonamides have been responsible for the major advances in the oral and systemic treatment of bacterial infections, the onium compounds are largely restricted to topical use for local infective conditions because of their poor and unpredictable absorption from the gut, and their toxicity when injected parenterally. At the present time much concern is felt over the increasing development of strains of bacteria resistant to the antibiotics, particularly in hospital wards. The development of bacterial resistance to onium salts is rare, and our own repeated attempts to produce strains of such resistant bacteria have been uniformly unsuccessful. This absence of onium resistant bacterial strains is a definite stimulus to research aimed at the discovery of new, less toxic onium salts possessing more predictable absorption.

In the general field of asepsis, one of the most useful properties of onium salts is their surface activity. Cationic surface-active agents dissociate in aqueous solution into a relatively large and complex cation, which is responsible for the detergent action, and a smaller, usually inactive, anion. The cationic onium group may consist of a comparatively simple aliphatic ammonium, a pyridinium or piperidinium or other heterocyclic group, and usually contains a long chain alkyl group with 8 to 18 carbon atoms. In addition to the emulsifying and detergent properties usually connected with surface-active agents, these cationic compounds often have a marked antimicrobial activity associated with low toxicity and freedom from irritant effects when applied topically. In general, such onium salts are more effective in neutral solution; although reasonably stable to acids, the antibacterial activity of onium salts is appreciably diminished in acid

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conditions. Normally, the antimicrobial properties of cationic onium salts are diminished or abolished in the presence of anionic substances such as soaps. However, a new development has been reported by Rebold and his colleagues (Rebold, Monte Bovi and Medici, 1958) who described the activity of a number of onium compounds, particularly lauryldimethyl-3,4-dichlorobenzylammonium 2-mercaptobenzothiazolate, which are unusual in that they apparently retain full bacteriostatic activity even in the presence of soaps.

One of the earliest successful quaternary ammonium antimicrobials is cetrimide (CTAB), a mixture of dodecyl-, tetradecyl-, and hexadecyl-trimethylammonium bromide. The spectrum of antibacterial activity of this agent embraces both Gram-positive and Gram-negative organisms. Although primarily a general purpose and skin sterilising agent, the use of cetrimide has been recently suggested as an antifungal agent in swimming baths, since *in vitro* studies have shown it to be active against the growth of *Trichophyton* species and *Epidermophyton floccosum* (Garland, 1959). Some related compounds showing similar antimicrobial activity include cetylpyridinium chloride, laurylpyridinium chloride, benzethonium chloride, and more important, benzalkonium chloride [XXX, R = alkyl from octyl to octadecyl]. This latter compound, which like cetrimide is not a single chemical entity, is the active constituent of Roccal, and is a general purpose and skin sterilising agent; it has found specific use against urea-splitting organisms in alleviating and preventing napkin rash and the associated secondary infective conditions. A further useful member of this class is domiphen bromide (Bradosol) [XXXI, R = mainly dodecyl]. Solutions of this salt are non-toxic and non-irritant to skin, and have been used for application to wounds and burns, in obstetrics and urology, and for rapid antibacterial and antifungal disinfection of the patients' skin before operation. In addition, it has a general application, for example the disinfection of linen and utensils. Domiphen has been incorporated into antiseptic throat lozenges, and has shown success in the treatment and prophylaxis of infection of the mouth and throat.

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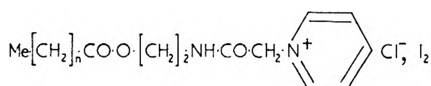
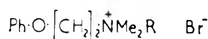
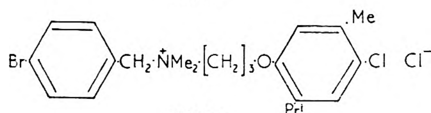


XXXII



XXXI



XXXIII

Two other cationic surface-active agents have rather confusingly been marketed under the same name, although having entirely different chemical structures. Thus Desogen is a 10 per cent solution of a mixture of trimethyl-1-*p*-tolylalkylammonium methanesulphonates, which when diluted, is used as a non-irritant antiseptic for the treatment of infected wounds, and in gynaecology and midwifery; the solution may also be used for storing sterilised surgical instruments. Desogen lozenges are throat lozenges

containing dimethyl-2-*N*-methyldodecanamidoethyl (phenylcarbamoylmethyl) ammonium chloride, used in the treatment of bacterial and monilial infections of the mouth and throat. This salt was originally introduced as a topical and general household disinfectant and detergent.

A new and most interesting development in the application of quaternary ammonium salts has been the formation of a complex of iodine with an onium salt, acylcholaminoformylmethylpyridinium chloride [XXXII, n = 6 to 12]. This complex has been found to be effective as a vaginal douche in monilial infections, and is also used for the local treatment of fungal infections of the scalp and feet. It differs from other surface-active quaternaries in that the antimicrobial activity of the complex is derived almost wholly from the elemental iodine, which is slowly released on contact with skin and mucous membranes. The quaternary constituent of this complex merely provides a wetting action, which facilitates contact of the iodine with the surface areas. Unlike tincture of iodine, this complex does not cause stinging or irritation, and in addition does not stain the skin or clothing (Anon, 1959).

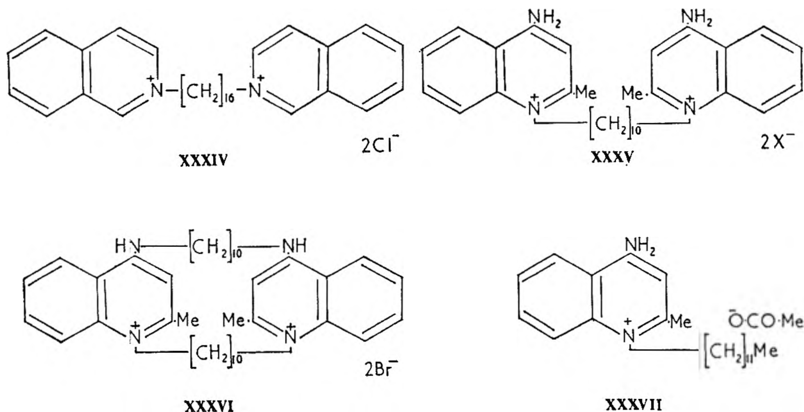
Halopenium [XXXIII] is yet another antimicrobial onium salt, and although originally prepared in 1942, it has only recently been marketed, formulated into a lozenge, for the treatment of *Candida* infections of the mouth and throat.

So far, all the onium compounds mentioned in this section of the review have been monoquaternary salts. Many bisquaternary ammonium compounds also possess marked antibacterial and antifungal activity, although they may be devoid of any appreciable surface-active properties. Examples of this are the polymethylene-bisquinolinium and -bisisoquinolinium salts, of which perhaps the most important is hedaquinium chloride (Teoquil) [XXXIV]. This compound has been shown to possess good antibacterial activity (Collier, Potter and Taylor, 1953) and it is also effective as a fungistatic agent *in vitro* over a wide spectrum of pathogenic fungi (Collier, Potter and Taylor, 1955); additional observations on the biological properties of hedaquinium have been published by Collier, Cox, Huskinson and Robinson (1959). Clinical studies have shown hedaquinium to be effective in the topical treatment of fungal infections, especially those due to *Trichophyton* species, both in man (Colin-Jones, 1958) and in animals (Gold and Jones, 1958; McPherson, 1959a, b). Hedaquinium is probably the most active antifungal agent *in vitro* of the quaternaries, and has been selected as a reference standard in the evaluation of the activity of other potential antifungal agents (Renzi, Garner and Burger, 1958).

Dequalinium (Dequadin) [XXXV] is a further bisonium compound with a very wide antimicrobial spectrum, being active against both Gram-positive and Gram-negative bacteria and many pathogenic fungi (Babbs, Collier, Austin, Potter and Taylor, 1956; Collier and Grimshaw, 1958; Collier and others, 1959). The main indications for the use of dequalinium, which has recently been reviewed (Wilkinson, 1959), are in the treatment of bacterial and fungal infections of the mouth, throat and skin (Coles, Grubb, Mathuranayagam and Wilkinson, 1958); in addition

dequalinium is of considerable value in the treatment of specific fungal infections, notably Lingua nigra—"Black hairy tongue" (Stockdale and Banks, 1959), and of the varied forms of monilial infestation. Roddie (1958) and Levinson (1959) have found dequalinium to be effective in the treatment of trichomoniasis in women, whilst Catterall (1960) has shown that moderately satisfactory results in the treatment of trichomonal urethritis in the male can be obtained by urethro-vesical irrigations with solutions of dequalinium chloride.

The veterinary use of dequalinium for the local treatment of wounds has been favourably described by Fowler and Jones (1957). In addition an interesting development in the possible application of dequalinium has been the demonstration that this agent is effective against *Candida albicans* and *Pityrosporum ovale* (D'Arcy, Cox, Hedge and Wilkinson, 1960; Cox and D'Arcy, 1961). The latter yeast-like organism is associated with dandruff in man, and although there is much contention whether it is the specific infective agent causing dandruff, or whether it is a saprophyte only on human scurf causing allergic skin reactions, it is certain that its continued presence on the human scalp is far from desirable. In initial clinical investigations dequalinium has proved highly effective in seborrhoea and infective dandruff (Colin-Jones, private communication). Other quaternaries have shown some activity against dandruff, and this use has been reported by Lesser (1952) in his excellent review on the cause and treatment of dandruff.

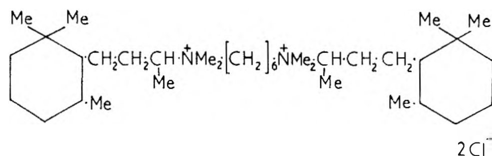


Kasparek and Stark (1960) have described the toxicity of an onium salt [XXXVI], closely related in structure to dequalinium, but as yet no information on its antimicrobial activity has been reported.

Recently, it has been shown that monoquaternary derivatives of 4-aminoquinaldine, which resemble half the dequalinium molecule, especially 4-aminoquinaldinium laurylacetate (Laurodin) [XXXVII], have marked antibacterial and antifungal properties (Cox and D'Arcy, 1959). In a comparative survey of a series of common skin antiseptics under ward conditions, Verdon (1961) has reported the efficacy of a solution of this substance as a standard pre-injection skin disinfectant. Recent studies

by Caldwell, Cox, D'Arcy and Rowe (1961) have shown that the analogous 4-aminoquinolinium salts have also good antimicrobial activity although they are slightly less potent than the corresponding quinaldinium salts.

So far, attention has been drawn only to those quaternary salts that have achieved therapeutic application; but much other work has been reported which indicates that there may be several onium salts yet to be introduced into this field. Thus Gadebusch and Cavallito (1957) have described the broad antimicrobial spectrum of a series of diquaternary salts of α,ω-bis-(2,2'-dipyridylamino)alkanes, while Schnitzer, Grunberg, DeLorenzo and Bagdon (1959) have reported the local antimicrobial activity of an interesting derivative of β-ionone, triclobisonium chloride (Triburon) [XXXVIII].



XXXVIII

Onium salts have obviously an important and widening rôle in the control and treatment of localised infective conditions. Although there are few reviews on this general topic, the antifungal activity of onium salts has been included in a recently published review (Taylor and D'Arcy, 1961).

Surface-active Agents

During the past 20 years, a vast range of synthetic materials, including many onium compounds, has been introduced to displace soap from its formerly unique position as the sole available detergent. Many onium salts have both antimicrobial and detergent properties and this valuable combination of activities is being widely used in cosmetic formulations, although this application is comparatively recent. The use of quaternary ammonium compounds in cosmetics has been reviewed by Lincoln (1954).

Prophylactic deodorants function by killing or inactivating the bacteria which are normally responsible for the decomposition of perspiration into odoriferous products. Arising from this, onium compounds have been found useful in antiperspirant formulations, and are often the only active odour inhibitors in various deodorant preparations. In addition, many skin dusting powders now contain a proportion of an antimicrobial quaternary.

Recently attention has been drawn in the medical press (Anon, 1958; Boheimer, 1958), to the failure of detergents to disinfect. This is of particular importance in connection with the disinfection of hospital wards, bedding and linen, since much concern is now felt over the comparatively widespread development and dissemination of bacterial strains, particularly Staphylococcal, resistant to many of the antibiotics in current use (Gillespie, Simpson and Tozer, 1958; Timbury, Wilson, Hutchison and Govan, 1958; Finland, Jones and Barnes, 1959; Hassall and

Rountree, 1959; Koch, Kastensen and Resnick, 1959; Elias-Jones, Gordon and Whittaker, 1961). It is considered that inefficiently disinfected blankets may serve as a continual reservoir for re-infection (Thomas, Liddell and Carmichael, 1958; Anderson and Sheppard, 1959). A further source of hospital infection is from the noses of healthy individuals, which probably form by far the largest breeding-ground for the pathogenic staphylococci (Williams, Jevons, Shooter, Hunter, Girling, Griffiths and Taylor, 1959), whilst localised infections (for example, boils) in theatre staff may also give rise to post-operative wound infections in patients (Mitchell, Timbury, Pettigrew and Hutchison, 1959). It would seem that onium salts could be useful in this field, both as topical agents for skin application and as general disinfectants for all-purpose cleansing; however, the choice of the correct onium salt is obviously of prime importance since it is well known that many Gram-negative bacilli, especially *Pseudomonas pyocyanea*, are unaffected by some quaternaries (Anon, 1958). The importance of the whole question of the prevention of infection in hospitals is emphasised by the fact that the entire January (1961) issue of the *Journal of Clinical Pathology* was devoted to this subject. The use of onium salts in this context receives favourable comment.

Several good reviews of the general use of onium compounds as germicides are available for more detailed study, for example Lawrence (1950) and de Benneville (1956); Sykes (1958) has fully discussed the whole field of disinfection and sterilisation. Several other publications deal with the evaluation of disinfectant and surface-active agents (Cook, 1959; British Standard 3286, 1960; Anon, 1960), while Hugo (1957), in a most interesting review, discussed the mode of action of antiseptics.

Antiparasitic Agents

Modern improvements in the facilities for world travel have caused a wide distribution of many diseases and infections previously localised in comparatively obscure regions. One of the few tangible benefits of the Second World War was the stimulation of organised effort in the search for new drugs in the chemotherapy of tropical diseases. The ubiquitous biological activity of the onium compounds has made them an obvious choice for detailed examination as antiparasitic agents and many have been shown to have activity.

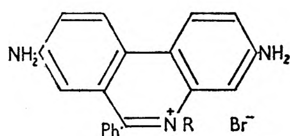
Trypanosomiasis

Trypanosomes have a notable place in the history of chemotherapy, being the causative agents of a group of diseases widely distributed in man and animal species. There are two main forms of trypanosomiasis in man; one is African sleeping sickness, caused by *Trypanosoma gambiense* and *T. rhodesiense*, and the other, prevalent in South America, is Chagas' disease caused by *T. cruzi*. Horses and cattle in certain tropical areas are also subject to trypanosomiasis, due mainly to infections by *T. brucei*, *T. congolense*, *T. vivax*, *T. equiperdum*, *T. equinum* and *T. evansi*. Infection of cattle is often very widespread and large areas, especially in tropical Africa, are held back from full development because of this.

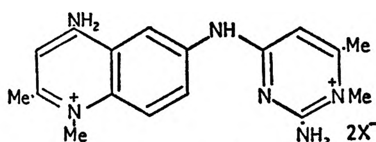
Thus in addition to the obvious rôle played by antitrypanosome agents in the clinical treatment of man and animals, the successful use of these drugs exerts a large economic and sociological influence. The nature of the problems of animal husbandry and management in these tropical areas has made the development of very long acting prophylactic or therapeutic drugs a major consideration in the chemotherapy of this disease. In the treatment of man also, the nomadic habits of many of the indigenous peoples renders continued treatment equally difficult.

One of the earliest observations on trypanocidal activity of onium salts was made by Browning, Cohen, Ellingworth and Gulbransen (1929), who investigated the properties of various styrylquinolines and their derivatives, which although promising in their activity were rejected after field trials. One of the most important steps in the application of onium compounds to the treatment of trypanosomiasis was the introduction of the phenanthridinium compounds. The first compounds of this type were synthesised by Morgan and Walls (1938) and examined by Browning, Morgan, Robb and Walls (1938). In 1948, Browning, Calver and Adamson described the high trypanocidal activity of 2,7-diamino-9-phenyl-10-methylphenanthridinium bromide (Dimidium) [XXXIX, R = Me], the preparation of which had earlier been reported by Walls (1947); this substance was widely used and achieved considerable success in the treatment of cattle trypanosomiasis. In subsequent years, a wide variety of phenanthridinium compounds was investigated for trypanocidal activity, but it was not until 1952 that the remarkable effect of changing the quaternising group of Dimidium from methyl to ethyl was discovered. In this year, Watkins and Woolfe (1952) first described homidium bromide (Ethidium) [XXXIX, R = Et], which although differing from Dimidium only in this one respect, proved much more active in laboratory experiments and in field trials in cattle infected with *T. congolense*.

Meanwhile other workers were following up leads originally developed by Jensch (1937, 1950) and as a result Curd and Davey (1949, 1950)



XXXIX



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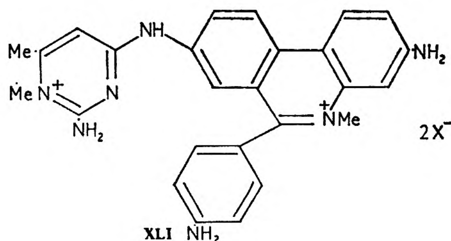


XLI

described the very active compound quinapyramine (Antrycide) [XL]. This drug is very useful since apart from the curative action of its readily soluble methosulphate, the less soluble chloride is more slowly absorbed and therefore possesses potent prophylactic properties. This has particular application to the problem of drug treatment of trypanosomiasis, which is three fold, involving (*a*) the treatment of early cases, (*b*) the treatment of advanced cases and (*c*) prophylaxis. A number of drugs are effective in combating early infections, but only a few penetrate into the central nervous system to kill the parasite. In cattle trypanosomiasis, prophylaxis is of vital importance, since it enables the cattle to be driven from the raising area through the wide belt of tsetse fly infestation to new grazing grounds or to market with safety.

An interesting development has been reported by Watkins and Woolfe (1956), when they first described the use of Prothidium [XLI] for the prophylactic treatment of trypanosome infections in cattle; this drug combines certain structural features of both Dimidium and quinapyramine. Robson (1958) has described a field trial in Zebu cattle in which he compares the prophylactic activity of homidium, quinapyramine and Prothidium. Homidium, although effective, produced severe local reactions, whereas Prothidium, at single dosage, and quinapyramine, at repeated dosage at 2-monthly intervals, gave good protection without severe reactions.

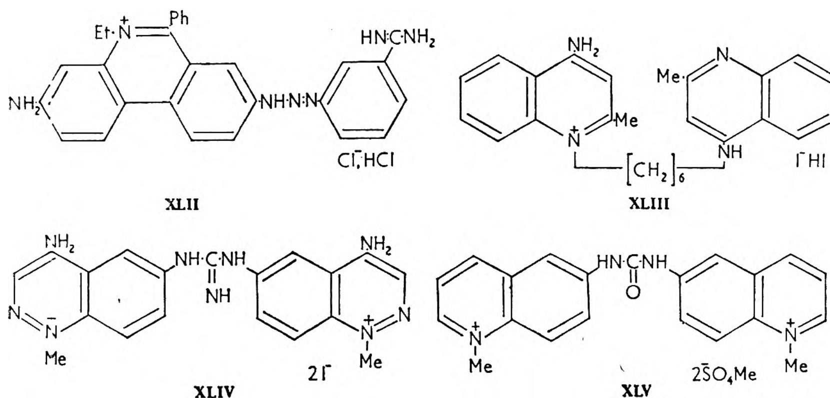
In 1958, Wragg, Washbourn, Brown and Hill described a new derivative of homidium prepared by coupling *m*-amidinobenzenediazonium chloride and homidium chloride. An approximately equal mixture of two isomers was obtained, one purple, the other red, which these authors originally thought to differ only in the position of the diazoamino group. The red isomer was found to be the more active against *T. congolense* infection in mice, both therapeutically and prophylactically. In practice, however, the mixture of the two isomers, which has been assigned the common name of Metamidium chloride hydrochloride, has proved to be the most promising trypanocide since it shows excellent prophylactic properties at a dose one ninth that of the LD50 value. This is of interest, since neither the parent compound homidium nor the non-quaternary drug Berenil, with which Metamidium shows some structural similarities, has any appreciable prophylactic activity at a dose equivalent to one-third of the LD50. Stephen (1960) has shown that Metamidium is a successful prophylactic agent against trypanosomiasis in cattle.

Further chemical study (Berg, 1960) has shown that the formulae provisionally suggested for the two isomers present in Metamidium (Wragg and others, 1958) have to be modified. The red isomer is now shown to be 7-*m*-amidinophenyldiazoamino-2-amino-10-ethyl-9-phenylphenanthridinium chloride hydrochloride [XLII], a structure which was originally assigned to the purple compound. Recent investigations into the structure of the latter have revealed it to be an isomeric aminoazo compound. Concurrently, the original coupling reaction has been studied and conditions have been established under which the red isomer is the main product formed, the ratio of red : purple being as high as 9 : 1; the pure

red product can be readily isolated by standard procedures. This red isomer is now termed isoMetamidium and its trypanocidal activity has been confirmed in cattle, the results of field trials in Africa being encouraging (Berg, 1960).

In the course of their systematic study on the chemotherapeutic activity of polymethylenebisquaternaryammonium salts, Austin, Collier, Potter, Smith and Taylor (1957) described the trypanocidal activity of bisonium derivatives of 4-aminoquinaldine. The hexamethylene member of this series was at first thought to be highly active against *T. congolense* in mice, but it was later shown that this activity was due to the presence of an isomeric impurity, the hydriodide of 6-(4-quinaldylamino)hexyl-4-aminoquinaldinium iodide (Tozocide) [XLIII]. This compound was highly effective in both therapeutic and prophylactic experiments, the latter studies being made with the suramin salt. In field trials in Africa however, the prophylactic activity was disappointing although the therapeutic action was confirmed in cattle. At this point attention may be drawn to the potential prophylactic value of suramin salts of most trypanocidal agents. This concept of fortifying the trypanocidal activity of an agent and simultaneously producing an insoluble form suitable for use in depot therapy was developed by Williamson and Desowitz (1956), following an original study by Guimaraes and Lourie (1951). Nowadays, it would seem to be routine practice to prepare and examine the suramin derivative of any new trypanocidal agent.

A further compound which also showed promising activity in initial studies was the cinnoline compound 528 [XLIV], described by Keneford, Lourie, Morley, Simpson, Williamson and Wright (1948) and by Lourie, Morley, Simpson and Walker (1951). This substance was found to be an active trypanocidal agent against *T. congolense* infections in mice, with a therapeutic index not significantly different from that of quinapyramine; however, it does not appear to have survived the rigours of field trials.



A most interesting feature in the development of many synthetic trypanocidal onium compounds is, that often the final active compound of a series has eventually been shown to have a constitution different from that originally ascribed to it, the activity proving to be due to the presence of

an impurity in the original material submitted for testing. This occurred with quinapyramine, Tozocide and Cinnoline 528; there was also some confusion between the Metamidium isomers.

In addition to this outline of the activity of trypanocidal onium salts, further details are given in excellent general reviews by Walls (1951), Ing (1953) and Davey (1957); furthermore, an excellent general review dealing with the African trypanosomiasis problem as a whole has been published by Nash (1960).

Babesiasis

Babesiasis (piroplasmosis) is an endoparasitic disease of cattle and other mammals (surprisingly enough, man appears to be immune) which is caused by species of *Babesia* and *Theileria*. Bovine redwater, one of the most common forms, is the name given to a condition due to *B. bovis* infestation. Piroplasmosis in its various forms is widely distributed in many animal species throughout the world. It has been observed that many trypanocidal compounds are quite active against babesiasis, in particular against *Babesia* infections in mice (*B. rodhaini*). Thus Beveridge (1956), when investigating the activity of more than 200 phenanthridine and phenanthridinium compounds against *B. rodhaini* in mice, found that the general requirements of chemical structure for babesicidal activity were similar to those for trypanocidal action. Similarly, Taylor, Terry and Godfrey (1956) showed that quinapyramine and homidium were effective against babesiasis in mice, although certain other known trypanocidal agents were found to be ineffective. In our laboratories, Spurling finds Tozocide to be only slightly active against *B. rodhaini* in mice.

The agent currently used for over 25 years in the treatment of British bovine redwater is quinuronium sulphate (Acapron, Babesan, Pirevan, Piroparv) [XLV], first described by Kikuth (1935–36). However, quinuronium has the disadvantage that even in therapeutic doses it produces toxic effects associated with parasympathetic stimulation (Ashley, Berg and Lucas, 1960).

In an excellent review on the chemotherapy of babesia infections, Ryley (1957) described, *inter alia*, the properties of two new babesicidal compounds,4-amino-6-(2-amino-1,6-dimethylpyrimidinium-4-amino)2-phenyl-1-methylquinolinium dichloride (compound 10,073), and 1,1′-dimethyl-4,4′-dimethylamino-6,6′-diquinaldiniumamino dimethylsulphate (compound 14,911). The latter is somewhat similar to quinuronium in constitution, whilst compound 10,073 is related to quinapyramine; both substances are highly active against *B. rodhaini* infections in mice, whilst in splenectomised calves and dogs, compound 10,073 was shown to have excellent activity after a single subcutaneous dose against *B. bovis* and *B. canis* respectively.

Very recently, Berg and Lucas (1961) have described a new babesicidal onium salt, 6-(*m*-amidinophenyldiazoamino)-4-amino-1,2-dimethylquinazolinium chloride hydrochloride, [XLVI], which, it is interesting to note, incorporates the *m*-amidinophenyldiazoamino structure present in iso-Metamidium, the trypanocidal agent which originated from the same

laboratories. This compound is active against *B. canis* infections in dogs.

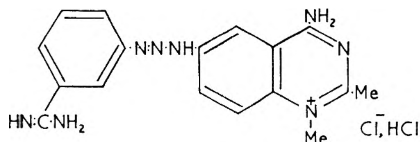
Although it is by no means an ideal drug, quinuronium has not been superceded in general veterinary practice; but, it must be emphasised that in this field much research is being carried out on non-onium babesicidal agents, particularly 3,3′-diamidinocarbanilide di-isethionate (Amicarbalide) (Ashley and others, 1960; Beveridge, Thwaite and Shepherd, 1960; Lucas, 1960).

Rodhain (1951), Ryley (1957) and Canache Mata (1959) have reviewed the progress of babesiasis and its chemotherapy in small laboratory animals, whilst Wright and Woodford (1958) have presented a brief review of bovine piroplasmosis in this country.

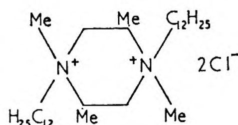
Helminthiasis

In 1947, Stoll surveyed the helminth parasites of man, and came to the startling conclusion that the number of helminthic infections in the world was 2,257,100,000; this figure was greater than the total world population at that time, and illustrates the principle that "wormy" people frequently harbour several different species of helminth at the same time. It is estimated that over 800,000,000 people throughout the world are infected with helminths.

As in other fields, World War II proved to be a stimulus to research, and workers have continued to focus their attention on chemotherapeutic studies of helminthic diseases; several new compounds, including some onium salts, have achieved some success. Although efficacy and safety are of prime importance in an anthelmintic compound, cheapness is also desirable, since in general, in human infections the hosts are often members of the lowest economic group of a poor country. Furthermore, worm infestation of cattle also presents a serious problem to the economy of under-developed areas. Helminth diseases both human and animal, are caused by a wide variety of parasites; these have been enumerated by Brown (1960) in a comprehensive article surveying the actions and uses of anthelmintics. A good anthelmintic must therefore possess a wide spectrum of activity since the worm burden of both man and beast may include several different species of parasite.



XLVI



XLVII

Piperazine and its salts, which have been widely used over the past 10 years, are amongst the best established chemotherapeutic agents in this field. More recently, mono- and bis-quaternary piperazines have been investigated, principally by Harfenist and his colleagues (Harfenist, Fanelli, Baltzly, Brown, Hussey and Chan, 1957; Brown, Hussey, Chan,

Harfenist, Fanelli and Magnien, 1959). The most successful compound reported by this group is the highest-melting isomer of 1,4-(2,5-*trans*)-tetramethyl-1,4-bis-dodecylpiperazinium dichloride [XLVII], which was found to be very effective *in vivo* against the mouse pinworm, *Syphacia obvelata*; so far, there have been no clinical reports of the activity of this drug in man or cattle.

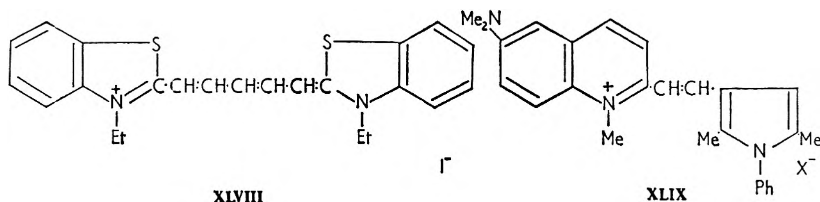
Some polymethylene-bisonium compounds possess appreciable anthelmintic activity, in particular against filarial infections and against the tapeworm, *Hymenolepsis nana.* Thus Hawking and Terry (1959) and Taylor and Terry (1960) have investigated the antifilarial activity of a number of onium salts against *Litomosoides carinii* in the cotton rat; these compounds were mainly polymethylene-bisisoquinolinium salts of chain lengths varying from dodecamethylene to tetracontylene. The octadecamethylene and eicosylene compounds were the most effective in these laboratory studies although later toxicity trials in dogs in our laboratories indicated that these compounds caused severe venous irritation and were therefore unlikely to be of practical value. The eicosylene member was also examined, amongst other compounds, by Sen and Hawking (1960) for *in vitro* cesticidal activity against the tapeworm *Hymenolepsis nana*, and was found to be one of the most active of the compounds studied.

The cyanines constitute an interesting group of onium salts which were originally developed for use in the photographic industry. Two members of this series, dithiazanine iodide (Abminthic, Delvex) [XLVIII] and viprynium salts (Pyrvinium, Vanquin) [XLIX], have quite recently achieved success in the treatment of anthelmintic infections. Cyanine dyes as a class are sparingly soluble and poorly absorbed from the gastrointestinal tract; their high concentrations in the intestine after oral administration exert an inhibitory effect on the anaerobic metabolic reactions of certain intestinal helminths.

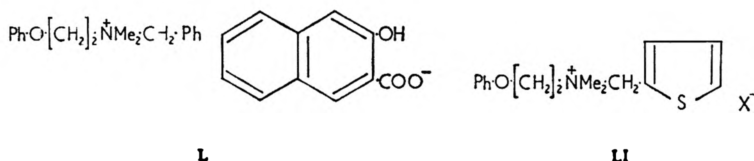
Dithiazanine has achieved clinical success against several worm infestations of man, although in others it is less effective. Thus, it is extremely active against *Strongyloides stercoralis*, and in this respect is superior to many other anthelmintics (Brumpt and Ho-Thi-Sang, 1959; Lloyd, 1959). It is also active in the treatment of trichuriasis in man (Brumpt and Ho-Thi-Sang, 1959; Paine, Lower and Cooper, 1959), and against *Trichuris vulpis*, the canine whipworm (Bueding, Kmetec, Swartzwelder, Abadie and Saz, 1961). Dithiazanine is highly effective against pinworm infections, but since its use is associated with a much higher incidence of gastro-intestinal side-effects than is therapy with piperazine, the latter agent is considered preferable (Brown, 1960). Dithiazanine is inferior, however, to piperazine in the treatment of ascariasis and is ineffective in patients with ancylostomiasis (Brumpt and Ho-Thi-Sang, 1959).

Pyrvinium chloride was shown by Weston, Thompson, Reinertson, Fisken and Reutner (1953), to be effective against pinworm in laboratory animals, whilst Bumbalo, Plummer and Warner (1958), found this drug to be effective in the treatment of enterobiasis in children. However, these authors consider that piperazine is still the drug of choice for the

treatment of this infection. More recently, Beck, Saavedra, Antell and Tejeiro (1959), concluded that both pyrvinium chloride and pyrvinium pamoate are comparable in curative value against enterobiasis in children, but that the pamoate was only about one-quarter as toxic as the chloride in animal tests. A marked advance over the customary multiple dosage was the cure rate of 96 per cent achieved by a single dose of the pamoate; similar results have been recorded by other workers (Biguet, Deblock, Capron and Machez, 1959; Komiya, Kobayashi, Ogawa and Kumada, 1960; Bisley, Davidson, Stewart, Wheatley and Wilson, 1961). Sanders and Hall (1960) have compared dithiazanine iodide and pyrvinium pamoate in the treatment of enterobiasis in both children and adults. Many of those treated with dithiazanine iodide vomited and did not complete the



XLVIII XLIX



L LI

course of treatment, but in those who completed the course, eradiction of the pinworm was generally achieved. In a series of 29 individuals given a single dose of pyrvinium pamoate, vomiting occurred in only one and the treatment eliminated the pinworms in the remaining 28 patients.

One of the most recent developments in the anthelmintic field was the discovery of bephenium (Alcopar) hydroxynaphthoate [L] by Copp, Standen, Scarnell, Rawes and Burrows (1958), which, as will be seen, is structurally related to the hypotensive onium salt bretylium. These authors reported that the bephenium series of compounds was highly active against a wide spectrum of helminths, but was relatively more effective against the mucosa-dwelling species of parasitic nematodes than against those living more freely in the lumen of the gut. Reporting on clinical trials against hookworm infection (*Necator americanus*) of man, Goodwin, Jayewardene and Standen (1958) found that bephenium was particularly suitable for the treatment of patients with advanced anaemia, diarrhoea, and heavy hookworm infection, because of its low toxicity and because no purge was necessary; similar results to these have also been reported by Gillies, Watson-Williams and Worlledge (1961). This drug was also found to be effective against roundworm (*Ascaris* species), which was present as a concurrent infection in many of these patients.

Simultaneously, Rogers (1958) investigated the excretion of bephenium salts in the urine of volunteers. Jayewardene, Ismail and Wijayaratnam (1960), have more recently reported on the use of this drug in the treatment of ascariasis in children, and found that the only disagreeable side-effect directly attributable to the drug was vomiting, which was significantly more in children under four years of age. However, the small dose recommended for the treatment of ascariasis could be safely given to any age group without causing anxiety.

The application of bephenium to the veterinary field has been widely studied by various workers. Thus, Rawes and Scarnell (1959), have recommended that this agent be used in the prevention and treatment of nematodiriasis and other forms of parasitic gastroenteritis in the unweaned lamb. Gibson (1960) has compared the efficacy of bephenium hydroxynaphthoate and bephenium embonate with those of two organic phosphorus compounds, Trolene and Neguvon, against *Trichostrongylus axei* in sheep. He supports the general view that both the phosphorus compounds are ineffective against this organism in sheep, and also finds that both the bephenium salts are less efficient than is phenothiazine against this infection. The hydroxynaphthoate is preferable to the embonate in the control of nematodiriasis since it has superior activity against trichostrongylid worms other than *Nematodirus* species. The anthelmintic activity of bephenium hydroxynaphthoate against the more common gastrointestinal *strongyles* found in Nigerian Zebu cattle has been investigated by Armour and Hart (1960). The drug is highly effective against *Cooperia* species and *Oesophagostomum radiatum* at all dosage levels used, but is only fully active against *Haemonchus* species and *Bunostomum phlebotomum* at the highest doses (225 mg./kg.). At this dose, *Trichostrongylus* species were satisfactorily eliminated in most animals, but not in all. In addition to its use in the treatment of human and cattle infections, bephenium hydroxynapthoate has proved to be highly effective in hookworm in dogs, but its introduction into this veterinary usage has been severely hampered by its emetic properties in the dog (Rawes and McIntyre, reported by Burrows, Clapham, Rawes, Copp and Standen, 1960). These latter authors have reported a series of compounds related to bephenium, one of which, 611C55 [LI], has been extensively tested in larger animals in the form of its *p*-chlorobenzenesulphonate. This drug is marginally more effective against *Ancylostoma caninum* and *Uncinaria stenocephala* in the dog, but is only minimally emetic at therapeutic doses. It also appears to be more effective against *Toxocara canis* and *Toxascaris leonina*. In contrast, compound 611C55 is substantially less effective than bephenium against some of the gastrointestinal *Trichostrongyles* in sheep. However, the development of compound 611C55 as an antihookworm drug in the dog is of considerable interest, since it combines high efficiency with only mild emetic properties in this vomit-prone animal; clinical trials of this drug against human hookworm infections are planned.

Several excellent reviews have recently appeared, which although primarily dealing with other compounds, have described the use of a

diverse number of onium salts as anthelmintic agents; the following are particularly worthy of mention: Watkins (1958); Cavier (1960); Brown (1960), and Watson (1960).

Amoebiasis

In view of the extraordinarily wide field of chemotherapeutic activity of the onium salts, it is interesting to note that very little has been reported on the activity of these compounds on two of the major scourges of mankind, malaria and amoebic dysentery. For some time the treatment of malaria with synthetic drugs has been the object of sustained investigation by many groups of workers; some of the recently introduced antimalarials are so highly potent, and have proved so successful, that the possible application of onium salts in this context would appear fruitless. However, it may well be that onium salts have a potential use in the treatment of amoebic dysentery, especially in view of their notably poor absorption from the gastro intestinal tract, which would be a positive advantage in the treatment of this condition. Some preliminary indications of activity of onium salts against *Entamoeba histolytica* have been found in the authors' laboratories; thus dequalinium is slightly effective when administered orally to young rats infected with *E. histolytica*, and Laurodin is also slightly effective but only at near toxic levels. Tetramethylenebis-7-aminoisoquinolinium salts also possess some *in vivo* activity (Austin, Lunts, Potter and Taylor, 1959).

A comprehensive review on the chemotherapy of tropical diseases has been published by Goodwin (1952), in which he deals *inter alia* with amoebiasis as well as a most detailed survey of all other important tropical diseases.

COMMENTS AND CONCLUSIONS

This review has summarised principally the activities of onium compounds in those human and veterinary spheres in which these agents have achieved some measure of experimental or clinical success. It is apparent that onium salts have a wide and diverse spectrum of activity extending from neuromuscular and ganglionic blockade, through anti-acetylcholine and anticholinesterase action, antimicrobial and antiparasitic activity to detergent and cosmetic adjuvant applications; activities that do not seem to have any well defined common mechanism of action. In spite of this versatility, it is obvious that there are many fields in which onium compounds are not effective. So far as is possible to ascertain, onium salts have found little, if any, use as local anaesthetics, antihistamine drugs, antiemetics, analgesics, tranquillisers, or antitussives; neither have they found application as CNS depressants nor as analeptics or CNS stimulants. It is difficult to envisage, with the wide interest that has been shown in onium compounds, that they have not been investigated for these actions; it is more probable that they have been fully examined and found wanting. Absence of specific activity in these instances may arise from, in part, the well known parenteral toxicity of onium salts as a class, and also to

their poor absorption after doses given by mouth, factors that automatically exclude many onium salts from the usual methods of administration. At this point, it may be worthwhile to mention that the poor intestinal absorption of onium compounds is a subject of current investigation; thus, Levine and Pelikan (1961) have studied the absorption of a single selected onium salt, benzomethamine. In unanaesthetised rats, using single- and multiple-loop preparations, they found that absorption was increased above control levels by fasting the animals and by perfusing the intestinal lumen with small quantities of water before the beginning of the experiments. Absorption was unaffected by mild mechanical manipulation of the gut; the amount of benzomethamine absorbed was found to be greatest in the intestinal segments closest to the pylorus. The presence of intestinal mucous material in the drug solutions uniformly decreased the amount of benzomethamine absorbed per quantity administered. It would also seem that where good *in vivo* activity is shown by onium salts, the mechanism of action does not involve the central nervous system. Therefore it may well be that these agents find difficulty in reaching central nervous tissue and would consequently not show any pharmacological effect in conditions which require the drug to act in the brain.

In the major sphere of their biological activity, quaternary ammonium compounds can be roughly classified into those with pronounced pharmacodynamic action, and those with anti-infective properties. In the former class, the future of onium salts, in our opinion, lies (*a*) in the development of new, safe hypotensive and ganglionic blocking agents, more predictable in their therapeutic use, and (*b*) in the discovery of new non-depolarising neuromuscular blocking agents. These latter agents must be short in action, easily controllable, and be capable of reversal by a harmless antagonist. Their fate in the body should not be affected by pathological changes, and their breakdown products should not exert any neuromuscular blocking action.

As anti-infective agents, perhaps the major requirement of onium salts is the discovery of a potent drug with a broad spectrum of antimicrobial action, devoid of toxic effects and unwanted pharmacological activity, but sufficiently well absorbed to produce effective blood and tissue levels of the drug when administered either orally or parenterally. This target, although formidable, should not present an unsurmountable problem, since many quaternary ammonium compounds, at present employed in the treatment of local infective conditions, are markedly effective against a diverse range of microbial species and many are active even in the presence of blood, serum, pus and tissue exudate. To our knowledge, strains of bacteria initially susceptible to onium salts do not subsequently become resistant. Only the potential toxicity and poor absorbability of the onium salts currently available prevent their use in general systemic therapy. In this field it would seem that research should be directed, not necessarily towards the development of more potent antimicrobial agents, but towards the transition of their known local activity to oral and systemic use.

Apart from the field of oral and systemic antimicrobial agents, an almost

equally important application of onium salts would seem to be in the treatment of the tropical diseases, and in this respect there are many indications of the potential efficacy of these salts. Undoubtedly there is still a tremendous need for cheap, long-acting and, if possible, single dose, anthelmintics, trypanocides and other antiprotozoal agents.

One problem to which there is, as yet, no complete solution, is the erection of a barrier between the sick patient and the airborne or dust-carried bacteria. Currently there are many reports of the incidence of bacterial infection in hospital wards, due to a variety of causes, not the least of which is the emergence of strains of bacteria resistant to the common antibiotics. Many of the so-called disinfectant-detergents fail to prevent the dissemination of bacteria, and it would seem that what is required is a really effective, but cheap onium compound, suitable for washing crockery, blankets, walls and floors, and also suitable for spraying into the air of the ward. It is appreciated that many onium salts may either possess undesirable, or lack suitable, properties for general disinfectant use. Thus, many are antagonised by anionic agents such as soaps, others have low solubilities in water, and several are expensive. On the other hand most onium salts are stable in aqueous solution, many are detergents as well as antiseptics, and a large proportion of these are highly active against bacteria in low concentration. One further possible application of onium salts, which has not yet received much attention, lies in the prevention of secondary infection after skin burns. It would seem that many onium salts could have particular application to this use because of their protein precipitating properties, since they would provide a sterile deposit of protein on the raw or exposed tissue surface. It must be emphasised however, that many such onium salts also delay wound healing when applied in high concentration, and ideally therefore a balance between these two contrasting effects must be sought.

The centenary of the first description of the biological activity of the onium salts by Crum Brown and Fraser will occur in less than 10 years time; in view of the very rapid development of medicinal chemistry during the post-war period, it is interesting to speculate on the advances that have yet to be made in the development of onium salts and their application to medicine. In the light of the current trend in research, new quaternary ammonium compounds should certainly have become well established in the treatment of tropical diseases, in the treatment of local, and possibly systemic, infective conditions and ought still to maintain their commanding position in pharmacodynamics.

Since this manuscript was written, we have received a copy of *Progress in Drug Research*, Volume 2, 1960, which contains *inter alia* an excellent article by Cavillito and Gray entitled "Chemical Nature and Pharmacological Action of Quaternary Ammonium Salts".

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RESEARCH PAPERS

SOME FACTORS INFLUENCING THE ABSORPTION OF GRISEOFULVIN FROM THE GASTROINTESTINAL TRACT

By W. A. M. Duncan, G. Macdonald and M. J. Thornton

From Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, England

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Some surface-active agents enhance the absorption of griseofulvin from suspensions administered to rats and man. The method of incorporating the agent into the suspension is important. No similar effect is observed with tablets of griseofulvin in man. The effect of particle size on the absorption of griseofulvin when administered as a suspension and as tablets has been studied in a statistically designed experiment in man. The results show that doses of 0·5 g. griseofulvin of surface area 0·35 m.^{2}/g. or 0·25 g. of material with a surface area of 1·5 m.^{2}/g. give similar concentrations of griseofulvin in the blood.

Many papers have been published on the systemic use of griseofulvin for fungal infections without any conclusion being drawn as to the optimum dosage régime. Atkinson, Bedford, Child and Tomich (1962) in a study of the concentrations of griseofulvin in the blood of man after different dosing schedules showed that a given amount of griseofulvin administered in divided doses would be expected to be more effective than a large single dose. They also observed a variation between patients in the concentrations of griseofulvin in the blood.

As a further study of the absorption of griseofulvin, experiments have been conducted in animals and man to examine the effect of surface-active agents incorporated either in suspensions or in tablets of the antibiotic. We understand that the studies of Atkinson, Bedford, Child and Tomich with preparations of different particle sizes have been extended to include griseofulvin with a surface area of 5·0 m.^{2}/g.

Experimental

Estimation of Griseofulvin

The concentration of griseofulvin in blood was determined by a modification of the spectrophotofluorometric method described by Bedford, Child and Tomich (1959). The oxalated blood sample (1 ml.) was extracted with ether (10 ml.) by shaking for 1 min. after which an aliquot (9 ml.) of the ether extract was evaporated to dryness and the residue dissolved in ethanol (3 ml.). The fluorescence of the alcoholic solution was measured using a Locarte Photofluorometer with an ultra-violet selecting filter on the primary side and a combination of two filters transmitting light between 420 mμ and 470 mμ on the secondary side.

Measurement of Surface Area

Surface area measurements were made using a modification of the air permeability method described by Rigden (1943).

Preparation of Griseofulvin Suspensions

Two formulation techniques were used to prepare the suspensions of griseofulvin. *Technique A.* The griseofulvin and the appropriate surface-active agent were mixed together before the addition of water. *Technique B.* The surface-active agent was dissolved in water to give the desired final concentration and the griseofulvin then added.

Preparation of Tablets

In the experiments to determine the effect of the surface-active agent Perminal BXN on the absorption of griseofulvin, tablets of griseofulvin were prepared in which (*a*) starch was the only excipient and (*b*) the griseofulvin was mixed with Perminal BXN before the addition of the starch paste.

Effect of Particle Size on the Absorption of Griseofulvin

Suspensions of griseofulvin of three surface areas were prepared by different milling techniques. The surface area of the griseofulvin in the finest suspension was believed, from study of photomicrographs, to be greater than 5·0 m.^{2}/g. though no direct measurement was used. This suspension, after dilution, was freeze dried to give a powder of 3·0 m.^{2}/g. from which tablets were prepared.

The suspension of 1·5 m.^{2}/g. griseofulvin was freeze dried to give material with the same surface area from which tablets were prepared.

The coarse suspension of griseofulvin with a surface area of 0·35 m.^{2}/g. was oven-dried to give a preparation with the same surface area.

The tablets prepared from each of the three preparations of griseofulvin contained the same excipients and had a Monsanto hardness of 3–4.

Animals

Male, specific rat-pathogen free, albino rats of the Alderley Park, I.C.I. Ltd. strain (120–150 g.) were used. Animals in groups of 40 were each given oral doses of griseofulvin (50 mg./kg.) presented as a 1 per cent suspension containing 0·5 per cent of a surface-active agent. Eight rats from a group were killed at each selected time interval and their blood separately analysed for griseofulvin.

Experiments in Man

Healthy, adult, male volunteers were used. Suspensions containing 2 per cent griseofulvin and 0·04 per cent of surface-active agent were prepared by formulation technique A; each man received the equivalent of 0·5 g. griseofulvin on each occasion. Blood samples were taken by venepuncture.

To determine whether Perminal BXN incorporated in tablets influenced the absorption of griseofulvin, 1 g. griseofulvin was administered to each man in a cross-over trial once with and once without Perminal BXN.

The effect of particle size on absorption was examined in 30 male volunteers who were each given griseofulvin on three occasions at weekly intervals; the concentration of griseofulvin in their blood was measured

4, 8, 25 and 49 hr. after each administration. Five dose: particle size combinations were investigated as tablets and also as suspensions so that on each of the three occasions only three volunteers received the same dose of a particular surface area either as tablets or as a suspension.

Surface-active Agents

Myrj 52, Arlacel 20, Tween 20, Tween 65 and Tween 80 are produced by the Atlas Powder Company, Wilmington, Delaware, U.S.A. Perminal BXN, Agral 2, Dispersol LN, Lissapol NX and Lubrol W were obtained from Imperial Chemical Industries Limited, Dyestuffs Division, Manchester, England. Lecithin RG was manufactured by The Glidden Company, 1825 N. Laramie Avenue, Chicago, Illinois, U.S.A., Goulac by Production Chemicals (Rochdale) Limited, Manchester, England, Pluronic F68 by Wyandotte Chemicals Corporation, Wyandotte, Michigan, U.S.A., and Aerosol OT by American Cyanamid, Pearl River, New York, U.S.A. Nekal was obtained from Ayerst Laboratories Incorporated, New York, U.S.A.

Results and Discussion

Many observations have been made on the effect of adjuvants on the absorption of drugs from the gastrointestinal tract. Kozlik and Mosinger (1957) showed that the surface-active agent, sodium lauryl sulphate, increased the rate of absorption of glucose from the gastrointestinal tract

TABLE I

THE ARITHMETIC MEAN CONCENTRATIONS OF GRISEOFULVIN (μG./ML.) AND THE STANDARD ERROR, IN THE BLOOD OF RATS AFTER ORAL ADMINISTRATION OF 50 MG. GRISEOFULVIN/KG.; THE DRUG WAS ADMINISTERED AS A SUSPENSION PREPARED BY FORMULATION TECHNIQUE B*

Surface-active agent	Chemical constituent	Hr. after administration				
		2	4	6	7·5	24
Perminal BXN	Butylated sodium naphthalene sulphonate	1·9 ± 0·8	2·5 ± 1·0	2·0 ± 0·2	0·8 ± 0·1	0
Lecithin (Glidden R.G.)	Phosphatidylcholine	1·6 ± 0·8	1·6 ± 1·0	1·3 ± 0·4	1·1 ± 0·3	0·3 ± 0·3
Sod. Lauryl sulphate B.P.	—	0·8 ± 0·5	1·5 ± 0·4	0·7 ± 0·3	0·4 ± 0·2	0·2 ± 0·2
Aerosol O.T.	Dioctyl sodium sulphosuccinate	0·9 ± 0·2	0·8 ± 0·5	0·4 ± 0·3	0·1 ± 0·2	—
Pluronic F68	Polyoxyethylene stearate	1·5 ± 0·6	1·5 ± 0·3	0·8 ± 0·4	0·4 ± 0·2	—
Myrj 52	Polyoxyethylene stearate	0·9 ± 0·1	1·0 ± 0·4	0·6 ± 0·3	0·6 ± 0·5	0·2 ± 0·1
Arlacel 20	Sorbitan monolaurate	0·6 ± 0·3	1·0 ± 0·6	0·8 ± 0·6	0·4 ± 0·4	0·2 ± 0·1
Tween 20	Polyoxyethylene sorbitan monolaurate	1·4 ± 0·6	1·8 ± 0·6	1·6 ± 0·6	0·3 ± 0·3	0
Tween 65	Polyoxyethylene sorbitan tristearate	1·1 ± 0·2	1·1 ± 0·4	1·0 ± 0·6	1·0 ± 0·4	0·1 ± 0·1
Tween 80	Polyoxyethylene sorbitan mono-oleate	1·3 ± 0·4	1·5 ± 0·4	2·3 ± 0·8	1·8 ± 0·7	—
Goulac	Calcium lignosulphonate	1·7 ± 0·5	1·5 ± 0·4	1·4 ± 0·3	2·4 ± 1·0	—
Lubrol W	Cetyl alcohol/ethylene oxide condensate	1·2 ± 0·3	1·0 ± 0·2	2·0 ± 0·9	1·0 ± 0·2	—
Nekal	Propylated sodium naphthalene sulphonate	0·7 ± 0·1	1·2 ± 0·4	1·2 ± 0·5	0·7 ± 0·2	0·2 ± 0·1
Agral 2	Propylated sodium naphthalene sulphonate	0·2 ± 0·3	0·6 ± 0·4	1·1 ± 0·7	0·8 ± 0·5	0
Dispersol LN	Methylene dinaphthalene sodium sulphonate	0·2 ± 0·2	0·8 ± 0·3	1·4 ± 0·3	1·5 ± 0·4	0
Lissapol NX	Polyethylene oxide condensate of nonyl phenol	2·4 ± 1·0	2·6 ± 0·8	2·4 ± 0·3	2·0 ± 0·5	—

* See experimental section.

whereas Nissim (1960) reported that the cation trimethylhexadecyl-ammonium inhibited the absorption of glucose but did not inhibit the absorption of methionine or sodium butyrate in the rabbit. The results of experiments in rats on the influence of surface-active agents on the absorption of griseofulvin from suspensions are shown in Table I. Some surface-active agents affected the concentrations of griseofulvin found in the blood and therefore apparently the rate and extent of absorption of the drug. Perminal BXN and Lissapol NXA, both containing an aromatic nucleus, consistently gave rise to higher concentrations of griseofulvin in the blood than did the other agents examined. As far as could be determined by appropriate control experiments the substance extracted from the blood of these animals for measurement was griseofulvin uncontaminated by the surface-active agent used. It was further observed that the surface-active agent could affect the pattern of absorption as may be seen by comparing the results obtained with Dispersol LN and Aerosol OT. The method of incorporation of the surface-active agent into the suspension of griseofulvin also influenced the apparent absorption of the drug (Table II). In this experiment the standard error of the observed concentrations was found to increase with the mean level of the results and it was appropriate therefore to use the geometric mean rather than the arithmetic mean and to consider the standard error as a percentage rather than in μg./ml. as previously. The standard error of a single observed concentration of griseofulvin was ± 60 per cent and the standard error of the means (Table II), which are based on the results

TABLE II

THE GEOMETRIC MEAN CONCENTRATIONS OF GRISEOFULVIN (μG./ML.) IN THE BLOOD OF RATS AFTER ORAL ADMINISTRATION OF 50 MG. GRISEOFULVIN/KG.

Surface-active agent	Formulation technique*	Hr. after dosing				
		2	4	6	7·5	24
Lecithin Glidden R.G. ..	A	1·49	1·39	1·20	1·00	0·23
Perminal BXN..	A	1·79	1·91	1·84	0·74	—
Lecithin Glidden R.G. ..	B	1·06	1·44	0·90	0·53	—
Perminal BXN..	B	0·72	1·31	1·63	1·24	—

* See experimental section.

from 8 animals, was therefore ± 18 per cent. The concentration of griseofulvin in the blood was maximal between 2 and 6 hr. after dosing, after which it decreased and had virtually reached zero by 24 hr. As there was no significant difference in the mean concentrations at 2, 4 and 6 hr. for any particular agent and method of incorporation, the average concentration over this period was used to assess the statistical significance of the observed differences between the agents and the formulation technique.

Technique A gave rise to higher concentrations of griseofulvin in the blood than technique B for both lecithin and Perminal; the mean difference in concentration, for both substances, was 40 per cent which is significant at the 95 per cent level. Using the formulation technique A, in which the surface-active agent and the griseofulvin were mixed together

before addition of water, the mean concentration with Perminal was 36 per cent higher than with lecithin; this difference was also statistically significant (95 per cent confidence limits 4 per cent and 79 per cent). To establish whether or not this effect in rats would be observed in man an experiment was conducted using suspensions of the same preparation of griseofulvin containing either Lecithin Glidden R.G. or Perminal BXN formulated according to technique A (Table III). Over the period 2 to

TABLE III

THE CONCENTRATIONS OF GRISEOFULVIN (μG./ML.) IN THE BLOOD OF MAN AFTER ORAL ADMINISTRATION OF 0·5 G. THE GRISEOFULVIN WAS FORMULATED BY TECHNIQUE A* AS SUSPENSIONS INCORPORATING EITHER PERMINAL BXN OR LECITHIN GLIDDEN R.G.

Volunteer code	Surface-active agent	Hr. after dosing			
		2	4	7	24
A	Perminal BXN	0·9	1·1	0·8	0·1
B	,,	1·0	1·2	0·7	0·0
C	,,	1·4	2·1	1·4	0·5
D	,,	1·4	1·7	1·2	0·0
E	,,	0·7	1·2	1·2	0·6
F	,,	0·9	1·2	0·7	—
A	Lecithin Glidden R.G.	0·7	1·0	0·9	0·0
B	,,	1·0	0·9	0·6	0·0
C	,,	0·6	0·4	0·2	0·0
D	,,	1·0	1·3	1·2	0·0
E	,,	0·7	0·5	0·8	0·0
F	,,	1·0	1·3	0·9	0·0

* See experimental section.

7 hr. after dosing the mean concentration when using Perminal was 50 per cent higher than with lecithin. This was comparable to the increase shown in rats, though it was not itself quite significant at the 95 per cent level. Three subjects had detectable amounts of griseofulvin in their blood 24 hr. after receiving the Perminal preparation but not with the lecithin preparation.

TABLE IV

THE CONCENTRATIONS OF GRISEOFULVIN IN THE BLOOD (μG./ML.) OF MALE VOLUNTEERS AT INTERVALS AFTER RECEIVING 1 G. GRISEOFULVIN AS TABLETS FORMULATED EITHER WITH OR WITHOUT PERMINAL BXN

Volunteer code	Occasion One					Occasion Two				
	Formulation	Hr. after dosing				Formulation	Hr. after dosing			
		2	4	7	24		2	4	7	24
G	Perminal BXN	0·4	1·1	1·5	0·3	No agent	0·1	0·6	0·9	0·3
H	,,	1·4	1·2	1·2	0·1	,,	0·5	0·7	0·9	0·4
I	,,	1·7	2·2	2·0	0·3	,,	0·9	1·5	1·7	0·3
J	,,	0·7	0·9	0·6	0·1	,,	0·1	0·9	1·2	0·4
K	,,	0·8	0·7	0·9	0·2	,,	0·7	0·4	0·8	0·5
L	,,	1·2	1·5	1·3	0·2	,,	1·0	0·8	0·9	0·7
M	No agent	0·6	0·6	1·3	0·4	Perminal BXN	0·4	0·6	1·3	0·4
N	,,	0·3	0·2	0·1	0·0	,,	0·7	0·4	1·0	0·4
O	,,	0·1	1·4	1·7	0·5	,,	0·0	1·1	0·8	0·2
P	,,	0·6	0·5	0·5	0·2	,,	0·5	0·7	1·6	0·5
Q	,,	0·9	1·5	1·2	0·3	,,	0·8	1·3	1·5	0·6
R	,,	1·2	0·8	0·6	0·0	,,	0·8	0·7	1·0	0·5

These experiments indicate that Perminal BXN and Lecithin (Glidden R.G.) when incorporated into suspensions of griseofulvin most probably

exert the same influence on the intestinal absorption of the antibiotic in both man and rats. How these agents promote the absorption of griseofulvin is unknown.

The effect of incorporating Perminal BXN into tablets of griseofulvin was determined in a cross-over trial with 12 adult male volunteers. The results (Table IV) were subjected to an analysis of variance using "available griseofulvin" values which were calculated from the area under the blood concentration: time curve over the period 0–24 hr. No statistically significant difference was observed between the tablets containing Perminal BXN and those without a surface-active agent.

Although the tablets of griseofulvin contained the same relative proportion of Perminal BXN as the suspensions we were unable to demonstrate any enhancement of absorption compared with tablets without a surface-active agent. No explanation is advanced for the difference in results between suspensions and tablets.

TABLE V

THE MEAN "AVAILABLE GRISEOFULVIN" VALUES (μG. GRISEOFULVIN/ML. BLOOD HR.) CALCULATED OVER THE PERIOD 0–25 HR. FROM THE RESULTS FOR 30 MALE VOLUNTEERS AFTER RECEIVING DIFFERENT FORMULATIONS OF GRISEOFULVIN. THE STANDARD ERROR OF THESE VALUES WAS 1·01

Formulation	Dose	Surface-area (m.^{2}/g.) 0·35	1·5	3·0	5·0
Suspension	0·5 g.	12·2	17·4	—	20·5
Tablets	0·5 g.	15·6	21·0	15·0	—
Suspension	0·25 g.	—	11·4	—	14·4
Tablets	0·25 g.	—	13·4	10·2	—

In the experiments on the absorption of griseofulvin by man the volunteers apparently differed in the extent to which they either absorbed or excreted griseofulvin: in the experiments with rats (Tables I and II) the concentrations of griseofulvin found in the blood varied with the occasion. To study the effect of particle size on the absorption of griseofulvin by man it was therefore necessary to design an experiment in which allowance could be made in the statistical analysis for any possible effect caused by different volunteers having different absorption patterns, and for any bias in the results obtained on the different occasions. As a precaution the volunteers were randomly assigned a code number so that any unexpected effect attributed to age or other variable would affect all treatments equally. From the results the "available griseofulvin" values (that is, the area under the blood concentration : time curve) for the periods 0–25 hr. (Table V) and 0–49 hr. (Table VI) were calculated. These sets of results were each analysed statistically as it was considered possible that the effect of the 5·0 or the 3·0 m.^{2}/g. griseofulvin might be to maintain a higher concentration of griseofulvin in the blood for a longer time. However, both sets of calculations presented the same result so that the following comments apply to both.

The analysis confirmed apparent differences in the results obtained on the separate occasions and also revealed a difference in the mean "available griseofulvin" values from one volunteer to another. Within the scope of this experiment it is difficult to assess the true magnitude of this volunteer effect but the statistical approach used in the analysis of this experiment indicated that the highest "available griseofulvin" value obtained, on a standard formulation, was about three times greater than the lowest. A difference of this kind between patients is obviously of clinical importance and emphasises the necessity of ensuring that each patient receives an adequate dosage of the antibiotic to allow for his particular absorption characteristics.

TABLE VI

THE MEAN "AVAILABLE GRISEOFULVIN" VALUES (μG. GRISEOFULVIN/ML. BLOOD HR.) CALCULATED OVER THE PERIOD 0–49 HR. FROM THE RESULTS FOR 30 MALE VOLUNTEERS AFTER RECEIVING DIFFERENT FORMULATIONS OF GRISEOFULVIN. THE STANDARD ERROR OF THESE VALUES WAS 1·46

		Surface-area (m.^{2}/g.)			
Formulation	Dose	0·35	1·5	3·0	5·0
Suspension	0·5 g.	17·0	22·4	—	27·8
Tablets	0·5 g.	20·8	28·2	20·4	—
Suspension	0·25 g.	—	13·4	—	17·8
Tablets	0·25 g.	—	17·2	14·2	—

When the griseofulvin preparations of different particle size were administered as suspensions a linear relationship was observed between the logarithm of the surface area and the mean "available griseofulvin" value. This observation does not disagree with that reported to us by Atkinson, Bedford, Child and Tomich although they reported a slightly greater effect. This linear relationship, however, was not observed with tablets as the tablets of griseofulvin with a surface area of 3·0 m.^{2}/g. gave unexpectedly low values. Photomicrographs of the disintegrated tablets did not indicate any extensive aggregation of particles, so this result would appear to be due to some unknown factor in the preparation of the dried powder from the suspension. However, the results obtained with griseofulvin of surface area 1·5 and 0·35 m.^{2}/g. bear the same relationship to each other whether administered as tablets or as suspensions. Although the ratio of the "available griseofulvin" values obtained with 1·5 m.^{2}/g. material to those obtained with 0·35 m.^{2}/g. griseofulvin was less than two, 0·25 g. of the finer material gave almost the same "available griseofulvin" value, a reflection of similar blood levels, as 0·5 g. of the coarser material. No information is available on the concentrations of griseofulvin in the blood which are required for a therapeutic effect nor is it known whether peak or protracted concentrations are important. However, this experiment shows that a similar clinical response should be obtained with 1·5 m.^{2}/g. griseofulvin, administered orally, at half the dosage required for griseofulvin with a surface area of 0·35 m.^{2}/g.

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THE EFFECT OF *ORTHO* SUBSTITUTION ON THE PHARMACOLOGY OF BENZOYLCHOLINE

BY J. THOMAS* AND D. BUCKLEY

From the Department of Pharmacy and the Department of Pharmacology, University of Manchester*

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The effects of mono- and di-*ortho* substitution of benzoylcholine on actions on the rat phrenic diaphragm and frog rectus abdominis preparations have been examined. *Ortho* substitution of benzoylcholine does not necessarily lead to a reduction in activity and may increase potency. It appears that mono-*ortho*-iodo substitution provides most stability combined with pharmacological activity.

The enzymatic cleavage of esters by the esterase group of enzymes is one of the more important routes of drug metabolism (Brodie, Gillette and La Du, 1958, Brodie, Mackel and Jondorf, 1958) and consequently methods of changing the rate of ester hydrolysis are of potential importance in drug design. The stability of an ester towards hydroxide ion-catalysed hydrolysis is dependent on the steric and electronic characteristics of the component acid and alcohol (Hammett, 1940). Levine and Clarke (1955), Glick (1938, 1939, 1941, 1942) and Fu, Birnbaum and Greenstein (1954), have shown that the same factors are also important in *in vitro* and presumably in *in vivo* enzyme catalysed hydrolysis of esters.

It is possible to modify the stability of benzoic acid esters towards hydroxide ion-catalysed hydrolysis by substitution in the aromatic nucleus (Kindler, 1928; Evans, Gordon and Watson, 1937). Substituents in the *ortho* position accelerate less or retard more the rate of hydrolysis of the ester group than the same substituents in the *meta* and *para* positions (Ingold, 1953). This is an example of the *ortho* effect. (For a full discussion of the *ortho* effect see Stoker, 1959.)

To investigate the possibility of using *ortho* substitution as a general method of stabilising ester groups in drug molecules Thomas and Stoker (1961) synthesised a series of mono- and di-*ortho* substituted benzoylcholine derivatives. The hydrolysis of benzoylcholine is catalysed both by hydroxide ions and cholinesterase (Mendel, Mundell and Rudney, 1943) and was, therefore, a suitable molecule to use to compare the effect of *ortho* substitution on the relative rates of chemical and enzymic catalysed hydrolysis. It was found that *ortho* substitution in benzoylcholine produced compounds which were either more rapidly hydrolysed by cholinesterase than the unsubstituted ester, more slowly hydrolysed or completely stable towards the esterase. The stability of the esters towards both hydroxide ion- and cholinesterase catalysed hydrolysis followed roughly that predicted from theoretical considerations of the "*ortho* effect" and the mechanism of hydrolysis. The "stable" substituted benzoylcholines were shown to be inhibitors of cholinesterase. All the *ortho* substituted benzoylcholines were more powerful inhibitors of acetylcholinesterase than benzoylcholine itself. The inhibition studies

were taken to indicate that the *ortho* groups did not prevent the adsorption of benzoylcholine onto the "active site" of the enzymes. To examine further the potential usefulness of *ortho* substitution in drug design the activities of the mono- and di-*ortho* substituted benzoylcholines have now been examined on more complex biological systems than the isolated enzyme preparations used previously.

The pharmacology of benzoylcholine has been studied by Carr and Bell (1947), Bovet and others (1949), Akcasu, Sinha and West (1952), who have shown that it exhibits a number of different actions. For the present purpose it was important to select biological systems which were relatively simple and preferably those on which benzoylcholine had only one type of action, which could be examined quantitatively. These considerations led to the use of the frog rectus abdominis and the rat diaphragm-phrenic nerve preparations. Ormerod (1956) used these two preparations in comparative assays of a series of *meta* and *para* substituted benzoylcholine compounds without reporting any difficulty.

EXPERIMENTAL

Chemical. All compounds were prepared as described by Thomas and Stoker (1961).

Pharmacological

Assays with the frog isolated rectus abdominis muscle. The isolated muscle was suspended in oxygenated Starling frog ringer at room temperature (19–22°). Relaxation was slow and incomplete and the muscle had to be stretched between each addition of drug. Assays were based on the comparison of log dose-response line for the unknown with that of the standard benzoylcholine; two points on each line were determined, each representing the mean of four doses (Ormerod, 1956). The ability of the unknown to cause contracture of the rectus abdominis preparation was computed as a percentage of that of benzoylcholine (Buckley, 1961). The assays were repeated in the presence of eserine 1×10^{-4}, to which the tissue was exposed for 30 min. before any effects were recorded. The organ bath was calibrated at 4·0 ml.; drug solutions in the physiological saline were added in 1·0 ml. volumes from tuberculin syringes. Contact time was exactly 90 sec. after which the muscle was washed repeatedly. At the 5th min. the muscle was gently stretched; the tissue was then allowed to rest until the 10th min. when the next dose was introduced.

In some experiments the effect of subthreshold doses of the compounds on acetylcholine-induced contractures of the muscle was observed. Here the dose of drug was introduced 30 sec. before that of acetylcholine, the resultant effect being recorded for exactly 90 sec. Control effects of acetylcholine were recorded both before and after recording the effect of the drugs on the acetylcholine response. The effect of tubocurarine on the contractures elicited by the compounds was similarly studied.

Assays with the isolated phrenic-diaphragm preparation of the rat. The preparation (Bülbring, 1946) was suspended in a modified Tyrode solution (Taugner and Fleckenstein, 1950), at 29° and aerated with 95 per cent

oxygen and 5 per cent carbon dioxide. The muscle segment was stimulated indirectly *via* the phrenic nerve with single, supramaximal square wave pulses of 5–10 V and 0·5 m-sec. duration at a frequency of 6/min. Direct stimulation of the muscle was by pulses of up to 40 V and 5–20 m-sec. duration; the rate was 6/min. In each experiment the organ bath was calibrated to 14 ml. with the preparation in position. Doses of drug were delivered in 1 ml. volumes by means of tuberculin syringes. Drugs were dissolved in the Taugner-Fleckenstein solution. The concentration (mol./ml.) in organ bath fluid of each compound, which when acting for 3 min. would cause a 50 per cent reduction in the height of contraction was determined. This concentration was calculated from observations of the mean effects of four different doses of each compound on four different rat diaphragm preparations. The method was based on the assumption that over the range of concentrations used the effect increased linearly with the dose. A test was incorporated into the statistical analysis to show that this was so.

TABLE I

THE RELATIVE POTENCIES OF *o*-SUBSTITUTED BENZOYLCHOLINE DERIVATIVES IN ELICITING CONTRACTURE OF THE FROG RECTUS ABDOMINIS MUSCLE

Compound	Potency relative to Benzylcholine (BCH) = 100 per cent		Confidence limits (P = 0·95)		Concn. used in the expts. mol./ml. × 10^{-7}			
					With eserine (10 µg./ml.)		Without eserine	
R-benzoylcholine R =	With eserine (10 µg./ml.)	Without eserine	With eserine (10 µg./ml.)	Without eserine	Low dose	High dose	Low dose	High dose
	(assigned)				(means from all expts)			
H (benzoylcholine) ..	100	100	—	—	0·6	1·2	1·6	3·2
o-Methyl- ..	28 30 28	100 92 89	23·9–32·8 28·7–30·8 23·0–35·0	92·0–110 79·5–106 80·4–97·6	2·4 2·4 1·2	4·8 4·8 2·4	1·5 1·5 2·0	3·0 3·0 4·0
o-Methoxy- ..	43 44	121 127	39·6–46·1 40·8–46·5	116–127 119–136	2·0 1·6	4·0 3·2	1·6 1·2	3·2 2·4
o-Chloro- ..	84 89	120 132	71·3–96·6 77·3–101	104–143 116–150	1·0 1·0	2·0 2·0	1·6 1·2	3·2 2·4
o-Nitro-	19 24 21	* 102 (approx.)	14·3–25·5 22·7–24·9 19·8–22·8	† Results of 4 expts. 97; 89; 101; 119	3 2 2	6 4 4	1·12 to 1·28	2·24 to 2·56
o-Bromo- ..	104 111	202 196	95–113·6 103–119	175–232 182–210	0·5 0·5	1·0 1·0	0·72 0·72	1·42 1·42
o-Iodo-	207 214 227	477 437 453 422	197–218 210–217 201–246	439–533 395–483 402–527 382–463	0·25 0·2 0·2	0·5 0·4 0·4	0·3 0·4 0·5 0·3	0·6 0·8 1·0 0·6
2,6-Dimethoxy- ..			No stimulant action					
2,6-Dichloro- ..			Slight stimulant action					

* Mean of four expts. † (Expts. not valid—see text.) Confidence limits not calculated.

This procedure was adopted to eliminate the bias which would arise from the "staircase" effect occurring on repetition of the same dose of

any of the drugs on a single tissue. Only four doses were applied to any one preparation since it was thought that the final result of an experiment would be significantly influenced by preliminary dosage of the tissue.

The four doses were chosen on the basis of preliminary experimentation. They were in constant ratio and were expected to produce between 25 and 80 per cent blockade in 3 min. The four doses were applied each to four rat phrenic-diaphragms in randomized order in a Latin square design.

Data derived from the four tissues were combined; effects due to order of doses and differences between tissues were eliminated from the calculation of the error by variance analysis (Buckley, 1961).

Results and Discussion

The detailed results obtained with the frog rectus abdominis and rat diaphragm-phrenic nerve preparation are given in Tables I and II respectively. The results from Thomas and Stoker (1961) of antiacetylcholinesterase activity as well as hydrolysis rates are presented together with the two sets of results obtained in the present investigation in Table III.

TABLE II

THE RELATIVE ACTIVITIES OF BENZOYLCHOLINE AND ITS *o*-SUBSTITUTED DERIVATIVES ON THE RAT PHRENIC-DIAPHRAGM PREPARATION

Compound R-benzoylcholine R =	Conc. mol./ml. × 10^{-8} for 50 per cent block in 3 min.	Confidence limits (P = 0·95) mol./ml. × 10^{-8}	Concns. used in expts. mol./ml. × 10^{-8}	Per cent potency relative to benzoylcholine = 100 per cent
H 1.	2·36	2·10 – 2·66	1·66 – 2·86	100
2.	2·38	2·15 – 2·65	1·66 – 2·86	
mean	2·37	2·12 – 2·65		
o-Methyl-	1·20	1·15 – 1·26	0·82 – 1·38	198
o-Methoxy-	2·35	2·25 – 2·45	1·66 – 2·86	100
o-Chloro-	1·73	1·60 – 1·89	1·13 – 1·93	139
o-Nitro-	1·62	1·58 – 1·66	1·37 – 2·33	148
o-Bromo-	2·19	1·99 – 2·41	1·37 – 2·33	108
o-Iodo-	0·96	0·89 – 1·04	0·68 – 1·16	247
2,6-Dimethoxy-	2·17	2·05 – 2·30	1·63 – 2·76	109
2,6-Dichloro-	0·59	0·56 – 0·63	0·45 – 0·80	402

1. The response to indirect stimulation was unaffected when each of the compounds was applied directly to the phrenic nerve.
2. The muscle responded to direct stimulation after the response to phrenic stimulation had been blocked by each of the compounds.
3. Neostigmine did not affect the blocking action of the compounds.

All the *ortho* substituted benzoylcholine derivatives tested inhibit the response of the rat diaphragm-phrenic nerve preparation to indirect supramaximal stimulation. This preparation responded to direct muscle stimulation when the response to indirect stimuli had been blocked by the compounds. When applied directly to the nerve, concentrations of fifteen times those which were effective in the muscle bath failed to prevent

contraction in response to phrenic nerve stimulation. Thus the compounds were considered to be acting at the neuromuscular junction.

All the mono-*ortho* substituted compounds caused contraction of the isolated frog rectus abdominis muscle. 2,6-Dichlorobenzoylcholine caused slight contracture but 2,6-dimethoxybenzoylcholine caused none. Both di-*ortho* substituted compounds did, however, block the response of the frog rectus abdominis to submaximal doses of acetylcholine. Doses of curare which block the response of the frog rectus abdominis to submaximal doses of acetylcholine also block the contracture of this tissue elicited by benzoylcholine compounds. It appears that *ortho* substitution does not prevent the adsorption of these compounds to receptors of the isolated frog rectus and rat diaphragm preparations. Thomas and Stoker (1961) reached the same conclusions with respect to the *in vitro* interaction of the compounds with cholinesterase and acetylcholinesterase.

TABLE III

SUMMARY OF THE RESULTS OF THE STUDIES ON BENZOYLCHOLINE AND SOME MONO-*o* AND DI-*o*-SUBSTITUTED BENZOYLCHOLINE DERIVATIVES

Compound	Action on frog rectus abdominis				Potency on rat diaphragm relative BCH = 100 per cent	* Relative hydrolysis rates		* I 50 Values g. moles/litre acetylcholinesterase
	Relative potency as stimulant (BCH = 100 per cent)							
R-benzoylcholine R =	+ Eserine (10 µg./ml.)		(*a*)	(*b*)		Hydroxide	Enzymic	
H (benzoylcholine) ..	100	100	+	−	100	1·00	1·00	$2{\cdot}90 \times 10^{-3}$
o-Methyl-	28 30 28	100 92 89	+	−	198	0·65	0·24	$9{\cdot}35 \times 10^{-4}$
o-Methoxy-	43 44	121 127	+	−	100	0·77	0·73	$4{\cdot}37 \times 10^{-4}$
o-Chloro-	84 89	120 132	+	−	139	1·40	1·30	$3{\cdot}29 \times 10^{-4}$
o-Nitro-	19 24 21	102 (approx.)	+	−	148	1·40	0·19	$3{\cdot}38 \times 10^{-4}$
o-Bromo-	104 111	202 196	+	−	108	1·02	0·70	$6{\cdot}90 \times 10^{-4}$
o-Iodo-	207 214 227	477 437 453 422	+	−	247	0·74	0·24	$2{\cdot}00 \times 10^{-4}$
2,6-Dimethoxy-	No stimulant action			+	109	0·00	0·00	
2,6-Dichloro-	Slight stimulant action			+	402	0·00	0·00	$4{\cdot}63 \times 10^{-4}$

* *Via* Thomas and Stoker (1961). (*a*) = action of tubocurarine on the response to the compound; (*b*) = action of compound on the response to acetylcholine; + = response inhibited, − = unaffected.

It may be seen from Table III that mono-*o*-methyl groups and di-*ortho* substituents will stabilise benzoylcholine towards enzymic and hydroxide ion-catalysed hydrolysis. When examined as inhibitors of the rat phrenic nerve-diaphragm preparation these compounds were found to be from

one to four times as potent as benzoylcholine. Thus it is possible to use *ortho* substitution to stabilise benzoylcholine without incurring any loss of activity on the isolated rat diaphragm preparation.

On the frog rectus abdominis preparation, both in the absence and presence of eserine the di-*ortho* substituted compounds had very slight (2,6-dichlorobenzoylcholine) or no stimulant action (2,6-dimethoxybenzoylcholine). In the absence of eserine only the mono-*o*-methyl compound was less active than benzoylcholine in causing contracture of the frog rectus abdominis. This reduction of activity (about 10 per cent) would not significantly affect the use of an *o*-methyl group as stabilising substituent. The other mono-*ortho* substituted compounds were more potent than benzoylcholine. *o*-Iodobenzoylcholine was five times as potent and the *o*-bromo compound was twice as potent as benzoylcholine. The nitro derivative was about as potent as benzoylcholine on the frog rectus but the assay was invalidated on the basis of the significant deviation from parallelism which existed between the standard and unknown dose response lines. Thomas and Stoker (1961) noted that *o*-nitrobenzoylcholine was anomalous in its behaviour with the cholinesterases.

Since it has been shown that eserine (10 μg./ml.) inhibits the cholinesterase of frog rectus muscle (Hobbiger, 1950), then in its presence, effects due to the anticholinesterase action and different rates of hydrolysis are excluded from the observed activity of the compounds. The activities obtained in the presence of eserine may be regarded as the result of the effect of the substituents on the inherent activity of benzoylcholine and the ability of the molecule to reach the site of action. The results obtained when eserine was used are given in Table I from which it may be seen that mono-*o*-chloro and *o*-bromo groups have the least effect on the inherent activity of benzoylcholine. Mono-*o*-iodobenzoylcholine is twice as active as the unsubstituted ester while *o*-nitro, *o*-methoxy and *o*-methyl groups significantly reduce the potency of benzoylcholine in the presence of eserine. Considered together, the results obtained using the frog rectus abdominis preparation as test object indicate that the mono-*ortho*-halogen groups are the least likely to diminish the activity of benzoylcholine.

The following conclusions are reached.

Ortho substitution may be used to stabilise benzoylcholine without necessarily removing the biological actions of the ester. In some cases activity is enhanced considerably. From the present investigation it appears that mono-*ortho*-iodo substitution provides the greatest measure of stability combined with pharmacological activity. However because of the complex nature of the *ortho* effect it is not possible to predict which group would be the most efficient in any particular case.

The fact that with the di-*ortho* substituted esters the action of benzoylcholine on the frog rectus is completely removed while other actions such as anticholinesterase and inhibition of the rat diaphragm-phrenic nerve preparation are enhanced leads to the suggestion that *ortho* substitution may be used to increase the specificity of action of a compound.

ORTHO SUBSTITUTION

Finally *ortho* substitution could in principle be used in drug latentation (Harper, 1959). This has been defined as "the chemical modification of a biologically active compound to form a new compound which, upon *in vivo* enzymatic attack, will liberate the parent compound". The modified compound is described as the "transport form" while the modifying groups are termed "carrier groups". In the case of carboxylic acids or alcohols the carrier group may be an esterifying group. Since *ortho* substitution can modify the rate of release of the active drug from the transport molecule, then it could be used to improve the usefulness of a carrier group.

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HYDROLYTIC DESTRUCTION OF THIAMINE, ESPECIALLY IN THE PRESENCE OF CYANOCOBALAMIN

BY JENNIFER HEATHCOTE* AND B. A. WILLS

From the Research and Control Department, Glaxo-Allenburys (S.A.) (Pty.) Limited, Durban, South Africa

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The hydrolytic destruction of thiamine hydrochloride in various buffer solutions at pH 4·5 at 100° has been investigated. The rate of hydrolysis approximated to that of a first order reaction, the salts of weak organic acids acting as catalysts so that buffer salts could not retard the decomposition. It was confirmed that the hydrolytic products of thiamine affect the stability of cyanocobalamin, and that low concentrations of ferric ions can protect cyanocobalamin against the effects of thiamine breakdown products, but this occurs without appreciably affecting the stability of thiamine itself.

THE formulation of stable solutions containing both thiamine hydrochloride and cyanocobalamin, possibly with other components of the vitamin B complex, presents a number of problems. Thiamine hydrochloride is stated by the British Pharmacopoeia to be stable in solutions at pH values below 5 but to deteriorate rapidly in neutral or alkaline solutions, especially on contact with air. The dependence of stability on pH has been demonstrated by Farrer (1941) and Beadle, Greenwood and Kraybill (1943). Booth (1943) and Farrer (1945) showed that the stability of the vitamin in solution was affected by certain buffer salts; and the same authors (Booth, 1943; Farrer, 1947) found that some metal ions at trace concentrations also influenced stability. The rate of decomposition of thiamine has also been held to be dependent on its initial concentration (McIntire and Frost, 1944).

In solutions containing thiamine, cyanocobalamin, and other vitamins of the B complex, rapid destruction of cyanocobalamin occurs and has been ascribed to the effects of nicotinamide and the products of hydrolytic breakdown of thiamine (Blitz, Eigen and Gunsberg, 1954; Feller and Macek, 1955). Mukherjee and Sen (1957, 1959) reported that cyanocobalamin could be protected against this destruction by ferric chloride in low concentrations; similar protection by ferric salts had been reported by Skeggs (1952) and Newmark (1958).

It was thought that the stability of cyanocobalamin in the presence of thiamine might be improved if the hydrolytic breakdown of the thiamine could be retarded by the use of a suitable buffer salt at optimal concentration, and that stability might be also increased by ferric ions in low concentration. We undertook to determine whether such stabilisation was possible and to provide some information on the mechanisms of the reactions involved in the decomposition of the vitamins.

* Present address: Department of Pharmacy, University of Ife, Ibadan Branch, Ibadan, Nigeria.

In most of the previous investigations with thiamine alone, concentrations of thiamine were lower than in many vitamin preparations. The studies were often extended over a wide range of pH, and decomposition from boiling or exposure to higher temperatures was often determined. The conditions we selected were: one pH value of 4·5; the use of therapeutic concentrations of 10 mg. of thiamine hydrochloride per ml. and 100 μg. of cyanocobalamin per ml.; the use of acetate, succinate, glutamate and tartrate buffers, which were considered sufficiently non-toxic for use in either oral or parenteral preparations, at a concentration of 0·15M; exposure of the solutions to a temperature of 100° for fixed periods.

First, the effect of different anions on the rate of hydrolytic breakdown of thiamine alone was investigated. Then the effects of anion concentration and ferric chloride on the rates of destruction of both thiamine and cyanocobalamin were studied. The results were considered in terms of first order reaction kinetics and the specific reaction rates (velocity constants) obtained were compared with those calculated from previously reported values.

Experimental

Analytical reagent grade chemicals were used except thiamine hydrochloride and cyanocobalamin, which were of the British Pharmacopoeia, 1958. Water was freshly distilled and free from carbon dioxide. The solutions were filled into ampoules, which were sealed and heated in a boiling water bath for 1, 2 and 4 hr., after which the ampoules were rapidly cooled. Thiamine was determined gravimetrically by precipitation with silicotungstic acid; cyanocobalamin was determined microbiologically by the *Escherichia coli* cup-plate assay of Robinson, Fitzgerald and Grimshaw (1956), the assessment of potency and validity of the assay being as described by these authors.

In the experiments in which ferric chloride was added to the vitamin solution, succinate and acetate buffer solutions could not be used because they precipitated iron. The glutamate buffer could be used in the experiments only at the lower concentration because of the limited solubility of glutamic acid

Results and Discussion

The destruction of thiamine in the four 0·15M buffer solutions, and also in 0·15M and 0·30M tartrate buffer solutions in the presence of cyanocobalamin, is shown in Table I, together with the pH determined before and after the longest heat treatment and the calculated specific reaction rates for the hydrolytic reaction. Determinations of cyanocobalamin remaining in those solutions containing both vitamins are listed in Table II, together with the calculated specific reaction rates for the decomposition of cyanocobalamin. The specific reaction rates were calculated by the standard graphical method, which also verified that the destruction of both vitamins approximated to reactions of first order.

In all solutions, including the unbuffered control solutions, the pH shift on heating was small and often was negligible.

Destruction of Thiamine

It is well known that thiamine hydrochloride is destroyed on heating by hydrolytic cleavage to yield the pyrimidine and thiazole moieties (Rosenberg, 1942). Since the hydrolysis of thiamine is a first order reaction, the buffer salt emerging unchanged at the end of the reaction, a

TABLE I

DESTRUCTION OF THIAMINE AT 100° IN SOLUTIONS CONTAINING 10 MG. THIAMINE HYDROCHLORIDE PER ML. WITH OR WITHOUT ADDITION OF 100 μG. CYANOCOBALAMIN PER ML. AND 250 P.P.M. FERRIC CHLORIDE

Vehicle	Additions	Thiamine hydrochloride per cent remaining after heating at 100°			pH shift		k_1 thiamine
		Time in hr.					
		1	2	4	Initial	Final	
No buffer ..	Thiamine	96·3	95·3	91·0	4·45	4·30	$4{\cdot}09 \times 10^{-4}$
0·15M Acetate ..	,,	94·0	89·7	79·0	4·50	4·42	$9{\cdot}8 \times 10^{-4}$
0·15M Tartrate ..	,,	93·0	89·3	78·5	4·56	4·44	$1{\cdot}01 \times 10^{-3}$
0·15M Succinate	,,	92·8	88·9	78·9	4·35	4·32	$1{\cdot}00 \times 10^{-3}$
0·15M Glutamate	,,	93·5	88·1	78·8	4·50	4·40	$1{\cdot}02 \times 10^{-3}$
No buffer ..	Thiamine, cyanocobalamin	97·2	96·3	89·9	4·50	4·30	$4{\cdot}22 \times 10^{-4}$
0·15M Tartrate ..	,, ,, +Fe	93·2	87·8	76·1	4·50	4·40	$1{\cdot}08 \times 10^{-3}$
0·3M Tartrate ..	,, ,,	90·0	86·8	75·2	4·56	4·42	$1{\cdot}29 \times 10^{-3}$
0·3M Tartrate ..	,, ,, +Fe	92·6	85·0	73·8	4·54	4·50	$1{\cdot}25 \times 10^{-3}$

general acid-base catalysis is suggested. At the pH of the solutions used, concentrations of hydrogen and hydroxyl ions are small, so that any increase in the reaction rate is dependent on the catalytic coefficients of the undissociated acid and the basic anion, and on the concentration of the anion. Since catalytic coefficients of acids are related to their dissociation constants, a similar contribution to catalytic power would be

TABLE II

CYANOCOBALAMIN DESTRUCTION AT 100° IN SOLUTIONS AT PH 4·5 CONTAINING 10 MG. THIAMINE HYDROCHLORIDE PER ML. AND 100 μG. CYANOCOBALAMIN PER ML. WITH OR WITHOUT THE ADDITION OF 250 P.P.M. FERRIC CHLORIDE

Vehicle	Additions	Cyanocobalamin, per cent remaining after heating at 100°			k_1 cyanocobalamin
		Time in hr.			
		1	2	4	
No buffer	Thiamine, cyanocobalamin	93·2	64·4	36·7	$3{\cdot}62 \times 10^{-3}$
0·15M Tartrate ..	,, ,, +Fe	77·2	52·8	46·0	$3{\cdot}45 \times 10^{-3}$
0·3M Tartrate ..	,, ,,	72·0	47·5	27·7	$5{\cdot}67 \times 10^{-3}$
0·3M Tartrate ..	,, ,, +Fe	89·6	86·0	68·4	$1{\cdot}60 \times 10^{-3}$

expected from the use of acids with approximately equal dissociation constants. This was found (Table I); similar total specific reaction rates were calculated among the four buffer solutions derived from weak acids of pKa values between 4 and 5. Increase in the anion concentration in the two tartrate solutions led to an appreciable increase in the specific reaction rate.

Specific reaction rates for the destruction of thiamine were calculated from the results of Farrer (1945), which relate to the use of phosphate-citrate and borate-succinate buffers where the reaction was of first order, and Booth (1943); here the rates of hydrolytic destruction in phthalate and phosphate-phthalate buffers were found to approximate to a second order reaction. The data reported by Beadle (1943) and Mukherjee and Sen (1959) were not sufficient for such calculations.

Beadle (1943) found that the nature of the buffer salt used in solutions of thiamine affected the rate of destruction, both acetate and phosphate retarding the rate considerably compared with other salts. We found little evidence of such retardation by acetate. Another difference between our results and those previously reported is in the effect of metals on the rate of decomposition of thiamine. Farrer (1947) reported that various metals at low or trace concentrations affected the rate of hydrolysis, which was occasionally retarded but more usually accelerated. We found ferric chloride, 250 p.p.m., in tartrate buffer solutions not to affect the rate of thiamine destruction significantly.

Farrer (1948), using 5–100 μg. of thiamine per ml. in phosphate-citrate buffer (0·2M/0·1M) at pH 4·5 and heating at 100°, concluded that the rate of decomposition of thiamine was dependent on its initial concentration. His results may be explained by the fact that the lower the thiamine concentration, the higher would be the relative concentration of buffer anion, with resultant increase in the rate of reaction.

Destruction of Cyanocobalamin

Destruction of cyanocobalamin when heated in buffered, weakly acid solution is accelerated by reducing agents. Macek (1961) explained this in terms of oxidation-reduction reactions of cobalt co-ordination complexes. Among agents capable of reducing cyanocobalamin in multivitamin preparations, the best known is ascorbic acid, but the thiazole moiety resulting from hydrolytic cleavage of thiamine exerts a similar effect (Blitz, Eigen and Gunsberg, 1954). This was confirmed by Mukherjee and Sen (1957, 1959) who also showed that, in this instance, low concentrations of ferric chloride could protect cyanocobalamin. Skeggs (1952) had previously shown that the stability of cyanocobalamin solutions was appreciably improved by saccharated iron oxide, and later Newmark (1958) demonstrated that cyanocobalamin, in the presence of ascorbic acid and other constituents of multivitamin solutions, could be successfully stabilised by a number of soluble iron salts, some of which were of sufficiently low toxicity to include in pharmaceutical preparations.

Gambier and Rahn (1958) concluded that if the ratio of thiamine to cyanocobalamin in solutions was less than 120 to 1, decomposition of cyanocobalamin was minimal, provided a critical pH was maintained and heat was avoided in the preparation of injection solutions.

Our results confirm that ferric chloride, 250 p.p.m., added to a solution of thiamine and cyanocobalamin in a tartrate buffer protects the cyanocobalamin from destruction, without affecting the decomposition of thiamine. It can be seen from Table II that the tartrate buffer enhanced

the destruction of cyanocobalamin, compared with the unbuffered solution, an effect explained not by an action of tartrate on cyanocobalamin stability, but by a greater concentration of thiamine hydrolytic products caused by the tartrate. The decomposition of cyanocobalamin approximated to a first order reaction. The reaction between the decomposition products of thiamine (probably the thiazole moiety) and cyanocobalamin was effectively retarded by ferric ions, which implies that the decomposition involves a reduction reaction as postulated by Mukherjee and Sen (1957, 1959) and Macek (1961).

Thus the rate of hydrolytic decomposition of thiamine by the addition of buffer salts at pH 4·5 could not be retarded. Conversely, these buffer salts accelerated the decomposition of thiamine, an effect attributed to both the undissociated acid and its anion and presumably dependent on their catalytic coefficients and on the anion concentration. This is of little practical significance with solutions of thiamine alone, which are adjusted to a more acid reaction to give better stability, but it is significant in multivitamin liquid preparations that are frequently adjusted to a pH of between 4 and 5.

The nature of the buffer salts examined did not appear to influence the stability of thiamine hydrochloride solutions to boiling and presumably would similarly fail to affect stability during prolonged storage at more moderate temperatures. This behaviour may be found when acids of similar dissociation constants and hence similar catalytic coefficients are employed as buffering agents.

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ESTIMATION AND URINARY EXCRETION OF TETRAHYDROAMINOACRIDINE

By P. N. Kaul*

From the Department of Pharmacology, University of Melbourne

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Two methods for the quantitative determination of tetrahydroaminoacridine in aqueous solutions and in urine are described. Four metabolites have been isolated from the rat urine. Two of these, constituting the major proportion of the total metabolites, have also been isolated from human urine and partially characterised.

1,2,3,4-Tetrahydro-5-aminoacridine (THA) was synthesised by Albert and Gledhill (1945) and later shown by Shaw and Bentley (1949) to be a morphine antagonist. It possesses anticholinesterase activity (Shaw and Bentley, 1953) and is a decurarising agent (Gershon and Shaw, 1958). Clinically, THA has been used with morphine in the treatment of intractable pain of terminal carcinoma (Stone, Moon and Shaw, 1961).

Although the pharmacology of THA has been studied at some length, largely in this laboratory, little is known about the metabolic fate of the drug. Studies on the metabolism of a compound like THA are particularly important, for the metabolic products and pathways may lead to an explanation of its pharmacological actions.

This paper reports methods for estimation of THA and some preliminary investigations on the metabolism of the orally and parenterally administered compound by means of urinary excretion studies in the rat and in man.

Experimental

Materials

THA (Monsanto), usually available as a faint yellow powder, was purified to a colourless crystalline product by extracting the base from an aqueous solution at pH 10 into light petroleum or benzene, drying the organic extract over anhydrous sodium sulphate and crystallising the base as hydrochloride. Chloroform and ethylene dichloride were of A.R. grade. Methyl orange reagent consisted of a mixture of equal parts of 0·5 per cent methyl orange solution in water and 0·5 M boric acid solution (pH 5), filtered and kept at 40° to prevent crystallisation.

Methods of Estimation

Method 1. Methyl orange under suitable conditions forms complexes with several organic bases. This property has been used to estimate cinchona alkaloids (Brodie and Udenfriend, 1945) and for example, morphine (Woods, Cochin, Fornefeld and Seevers, 1954). A modification of the original method of Brodie and Udenfriend was adapted for the quantitative determination of THA as follows.

* Present address, Hindustan Antibiotics Ltd., Pimpri, Near Poona, India.

To an impure aqueous solution or urine (10 ml.) containing 0·2 to 3·0 μg./ml. of THA, in a 50 ml. glass-stoppered centrifuge tube, was added saturated sodium carbonate solution (0·2 ml.) to produce a pH not lower than 10, and ethylene dichloride (26 ml.). The mixture was shaken for 7 min. on a mechanical shaker. The tube was then centrifuged at 2,000 r.p.m. for 5 min. A 25 ml. aliquot of the ethylene dichloride extract was transferred to another tube containing methyl orange reagent (0·5 ml.). The tube was shaken mechanically for 7 min. and centrifuged. A 24 ml. aliquot of the coloured organic layer was transferred to a third tube containing 0·5 N hydrochloric acid (4 ml.). The tube was shaken mechanically for 5 min., the acid layer was separated and its extinction at 508 mμ was read against 0·5 N hydrochloric acid on the spectrophotometer (Beckman DU).

Method 2. THA in aqueous solutions shows a characteristic absorption spectrum in the ultra-violet region, with peaks at 323 and 335 mμ. The ratio of the extinctions at these wavelengths characterises THA, whereas the extinction at 323 mμ may be used to measure its concentration in a solution. The procedure adopted for purification and assay was as follows.

To an impure aqueous solution or urine (10 ml.) in a 50 ml. glass-stoppered centrifuge tube were added concentrated ammonia solution (0·2 ml.) and chloroform (23 ml.). The tube was shaken for 7 min. and centrifuged. A 20 ml. aliquot of the organic layer was shaken for 5 min. with 0·5 N hydrochloric acid (5 ml.). After centrifugation, the extinction of the acid layer was measured at 323 mμ against 0·5 N hydrochloric acid saturated with chloroform.

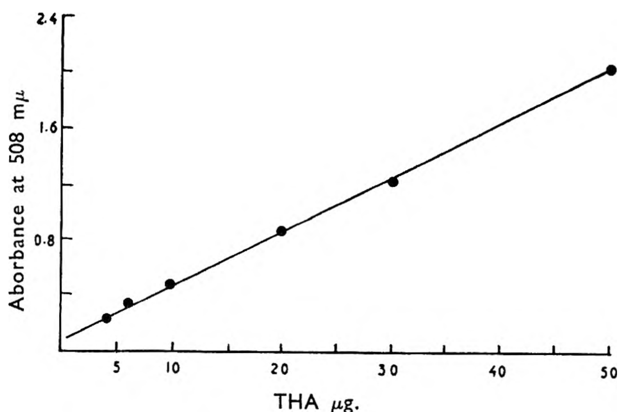

FIG. 1. THA-methyl orange complex.

Suitability of Methods

Fig. 1 shows that the THA-methyl orange complex obeys Beer's law over a wide range of concentrations. The reagent blanks read 0·03 ± 0·01 O.D. units at the maximum wavelength whereas 0·1 μg./ml. THA read

0·12 units. The urine blanks prepared according to the method of Woods and others (1954), which involves trichloroacetic acid treatment, read 0·05 ± 0·01 while with the present method the blanks were 0·08 ± 0·01.

The urine blanks by method 2 read 0·017 ± 0·003 units at 323 mμ, whereas 5 μg./ml. THA read 0·304 units. This corresponds to a 62 per cent recovery based on direct ultra-violet measurements of pure aqueous solutions.

The metabolites of THA are extractable under the same conditions as the parent compound. They also show a similar absorption spectrum and may be estimated by measuring the extinction at 323 mμ. The total measurable substances in urine have been referred to as "THA".

Application and Results

Urinary Excretion

Rat. A 20 mg./kg. dose of THA was given subcutaneously to 6 rats weighing 192 ± 2 g. Estimations, by method 2, of "THA" in the urine of each rat were made on samples collected 5, 24, and 36 hr. after the injections. Table I, giving the percentages of the excreted "THA", shows that about 47 to 63 per cent of the injected dose is excreted over 36 hr.

TABLE I

Urinary Excretion of "THA" in Rats

Wt. of rat g.	Per cent of the injected dose excreted during				
				Total	
	0–5 hr.	5–24 hr.	24–36 hr.	Estimated	Absolute*
190	27·5	9·2	2·5	39·2	63·2
194	10·0	23·8	3·0	36·8	59·3
190	15·6	13·8	1·9	31·3	50·5
190	13·0	17·4	1·7	32·1	51·9
190	19·4	15·2	1·0	35·6	57·4
192	17·7	10·1	1·6	29·4	47·4

*Absolute value is the actual amount present in a sample assayed. Based on addition and recovery experiments, the estimated value is only 62 per cent of the actual amount present.

Man. Five normal human subjects were given 30 mg. of THA by intramuscular and oral routes, and the urinary excretion of "THA" was followed with time, method 2 being used. Table II gives the per cent of the given dose excreted during the time shown.

TABLE II

Excretion of "THA" in Human Urine

Subjects	Vol. voided	Total hr. of collection	Dose and route	Absolute* per cent dose as "THA"
1	1,570	24	30 mg. I.M.	16·8
2	730	24	,, Oral	7·9
3	1,203	23	,, ,,	6·8
4	1,651	24	,, ,,	5·3
5	470	12	,, ,,	4·7

* See Table I.

Isolation of the Metabolites

Rat urine samples collected after subcutaneous administration of 15 mg./kg. were pooled, adjusted to pH 10 and repeatedly extracted with chloroform. The organic extracts were combined and evaporated under reduced pressure on a rotary vacuum evaporator to a small volume suitable for paper chromatography. The concentrate was fractionated on 3 mm. paper by descending partition chromatography, with butanol: acetic acid: water (4 : 1 : 5) as the solvent system.

The chromatogram showed at least four spots which fluoresced under ultra-violet light. On spraying the paper with iodoplatinate reagent, all the four spots acquired a brown colour characteristic of alkaloidal or heterocyclic nitrogen function. The spots were tentatively labelled as metabolites 1 to 4 (M_1, M_2, M_3 and M_4) in order of their increasing R_F values. None of these four spots corresponded to the authentic THA spotted alongside the metabolite mixture obtained from the urine.

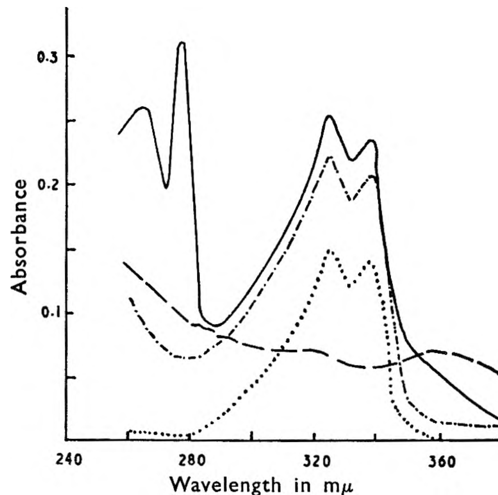

FIG. 2. Ultra-violet absorption spectra of THA and its metabolites. THA . . . ; M_1— — ; M_3—·—·— ; M_4———.

From a series of unsprayed papers, the corresponding spots were eluted with methanol and their ultra-violet absorption spectra recorded. Fig. 2 shows the spectra of M_3 and M_4 to be similar to that of THA. These two metabolites constitute most of the total metabolites isolated.

The spectrum of the M_4 metabolite shows pH-dependent reversible absorption. At basic pH, hypsochromic and hypochromic shifts occur, indicating the presence of an ionizable nitrogen.

Two metabolites were isolated from the urine of general-surgical subjects receiving intramuscular THA plus morphine. Their partition ratios, R_F values and the electronic absorption patterns suggest that these two substances corresponded to M_3 and M_4 isolated from the rat urine.

Fig. 3 shows an infra-red spectrum of the metabolite M_4 (from rat) compared with that of THA. A strong C = O band at about 5·8 μ is conspicuous.

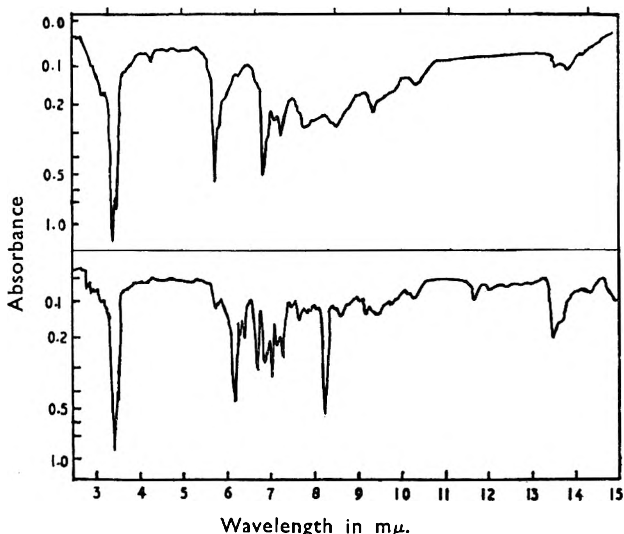

FIG. 3. Infra-red absorption spectra of THA (lower) and M_4 metabolite (upper).

DISCUSSION

The THA-methyl orange complex obeys Beer's law over a wide range of concentrations and therefore this method appears to be sensitive and suitable for solutions from which THA can be isolated before analysis. Woods and others (1954) reported that the morphine complex with methyl orange did not obey Beer's law and suggested the inclusion of a standard morphine sample with each analysis. However, in the present studies morphine was run as a check and it also obeyed Beer's law. Deviation from this law appears to be due to traces of methyl orange carried over while transferring the aliquot of the organic layer, containing the complex, into the acid. A complete separation of the two phases by centrifugation before transfer and careful transfer produces a linear concentration-absorption plot. As little as 0·5 μg./ml. can be determined with 5 per cent accuracy.

Removal of the protein matter from urine with trichloroacetic acid according to the method of Woods and others (1954) appears to be necessary to obtain lower blank values in the methyl orange method.

From the results in Table II it appears that a lower percentage of the administered dose of THA is excreted by the subjects receiving the drug orally. This may indicate that the absorption of oral THA is less complete than the intramuscularly administered drug. Application of the methods to the estimation of THA in blood was unsuccessful.

The ultra-violet absorption spectra of M_3 and M_4 show a close resemblance to that of THA. This may mean that the ring system of the THA

molecule remains almost unchanged. However, a strong absorption band at 5·75 μ in the infra-red spectrum of M_4 reveals the presence of a cyclic carbonyl group in this metabolite. One possible route of bio-origination of this C = O function would be through an oxidative deamination of THA which in biological systems would involve pyridoxal as the co-factor.

Acknowledgement. I am indebted to Professor F. H. Shaw for providing me the funds and facilities for this work and for his interest in it. I am grateful to Miss Anna Winiarski for technical assistance.

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ENZYME INHIBITING ACTION OF TETRAHYDROAMINOACRIDINE AND ITS STRUCTURAL FRAGMENTS

BY P. N. KAUL*

From the Department of Pharmacology, University of Melbourne

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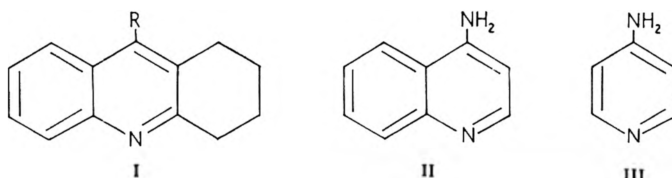
Tetrahydro-5-aminoacridine and four compounds representing its structural fragments have been compared as inhibitors of acetylcholinesterase and of monoamine oxidase. The entire structure of tetrahydro-5-aminoacridine appears to be essential for optimal inhibition of the esterase, less than 10^{-6} M concentration showing a 50 per cent inhibition of the enzyme, with the inhibition constant K_i as 1×10^{-4}. For optimum inhibition of monoamine oxidase, the 4-aminoquinoline part of the acridine molecule appears to be a structural requirement. 4-Aminoquinoline shows a stronger monoamine oxidase inhibition than any known therapeutically used inhibitor. It gives a 50 per cent inhibition of the oxidase in 10^{-6} M concentration, with K_i as $1{\cdot}1 \times 10^{-5}$.

1,2,3,4-TETRAHYDRO-5-AMINOACRIDINE (THA) has been developed in this laboratory as a partial antagonist of morphine (Shaw and Bentley, 1949). It has been used clinically with morphine for the treatment of intractable pain of terminal carcinoma (Stone, Moon and Shaw, 1961). Shaw and Bentley (1953) have demonstrated that THA strongly inhibits cholinesterase. Based on this observation, Gershon and Shaw (1958) showed that THA acts as a decurarizing agent.



I, $R=NH_2$=Tetrahydroaminoacridine
I, $R=NHC_4H_9$=*N*-Butylaminotetrahydroacridine
I, R=H=Tetrahydroacridine
II=4-Aminoquinoline
III=4-Aminopyridine

A consideration of the molecular structure of THA (I) suggested that a fractionation of the total structure into simpler available units retaining one or more features of the original molecule, might yield structures with optimum inhibition on acetylcholinesterase (AChE) and on monoamine oxidase. With this in mind, THA, tetrahydroacridine, 4-aminoquinoline, 4-aminopyridine and *N*-butylaminotetrahydroacridine were tested for acetylcholinesterase inhibitory action and the first four compounds were also tested for monoamine oxidase inhibition. Procaine hydrochloride provided a control inhibitor of AChE.

* Present address, Hindustan Antibiotics Ltd., Pimpri, Near Poona, India.

EXPERIMENTAL

Materials

THA (Monsanto), *N*-butylaminotetrahydroacridine, tetrahydroacridine, 4-aminoquinoline, 4-aminopyridine and procaine hydrochloride were all recrystallised twice and used as buffered solutions; acetylcholinesterase, crystalline (Nutritional Biochemical Corporation); sodium bicarbonate, A.R.; buffer-indicator mixture, 0·001 per cent phenol red in 0·05 M phosphate buffer, pH 8, as described by Caraway (1956); acetylcholine chloride solutions, freshly prepared before use or overnight solutions stored at 0°; 5-hydroxytryptamine (5-HT) creatinine sulphate; various reagents for colorimetric estimation of 5-HT, prepared as described by Udenfriend, Weissbach and Clark (1955). As a source of monoamine oxidase, the liver was removed from decapitated rats and instantly homogenised at 0° in pH 7·4 phosphate buffer (U.S.P.), maintaining a dilution of 1 in 20.

Methods

Determination of esterase activity. The rate of hydrolysis of acetylcholine was used as an index of enzyme activity. The photometric method of Caraway (1956) was employed with modifications. To the enzyme solution (0·1 ml., 340 units/ml.) in a corex cuvette were added an inhibitor solution (0·5 ml.) and the buffer-indicator mixture (2 ml.). After allowing to stand for 2 min. acetylcholine chloride solution was added (0·4 ml.). The contents were mixed instantly and the zero time reading of absorption at 558 mμ was immediately recorded. Subsequent absorptions were measured at definite time intervals to obtain the rate data. The decrease in the initial extinction gives a measure of the hydrolysis.

Determination of oxidase activity. The rate of oxidation of 5-HT in an incubated enzyme-substrate system was measured as an index of the enzyme activity. The unoxidised 5-HT remaining in the incubated mixture was estimated by the method of Udenfriend and others (1955) with modifications.

To the enzyme solution (1·5 ml.) in each of a series of 50 ml. glass-stoppered centrifuge tubes was added the inhibitor solution (1·5 ml.), and the tubes were placed in a water bath at 37·5 $\pm$ 0·5°. After incubation for 15 min. the 5-HT solution (1 ml.) was added to each tube at a noted time. At definite time intervals, the oxidation reactions were arrested by adding 3 drops of octanol to the incubating mixture, followed by an immediate addition of butanol-saturated borate buffer (pH 10, 2 ml.), butanol (15 ml.) saturated with sodium chloride and the borate buffer, and sodium chloride (2 g.). The tubes were stoppered, shaken for 7 min. and centrifuged. The lower aqueous layer was removed by aspiration with a suitable syringe. The butanol extract was washed with 5 ml. of pH 10 borate buffer, diluted with 10 ml. of heptane and shaken for 5 min. with 3 ml. of 0·2 N hydrochloric acid. The contents were centrifuged.

A 2 ml. aliquot of the acid layer was treated at 50° for 7 min. with nitrosonaphthol reagent (1 ml.) and nitrous acid reagent (1 ml.). The coloured solution was washed with ethyl acetate (10 ml.) to remove excess of any unreacted reagents. The extinction of the coloured aqueous phase

was determined at 540 mμ against the reagent blank run under identical conditions but with no 5-HT added.

Use of fixed-volume stop-syringes facilitated the transfers of organic solvents and the single extractions conserved time.

The reactions were run in the presence of varying inhibitor concentrations at two different substrate concentrations. Each series was run at a constant temperature ($\pm 1°$).

Results

Acetylcholinesterase Inhibition

Optimum substrate concentration. Fig. 1 shows the substrate concentration-enzyme activity curve. A concentration of $2{\cdot}8 \times 10^{-3}$ M acetylcholine appears to be the optimum and this agrees with the value cited in literature (Augustinsson, 1948). In the inhibition experiments the two substrate concentrations used were $5{\cdot}56 \times 10^{-3}$ M $[s_1]$ and $2{\cdot}78 \times 10^{-3}$ M $[s_2]$.

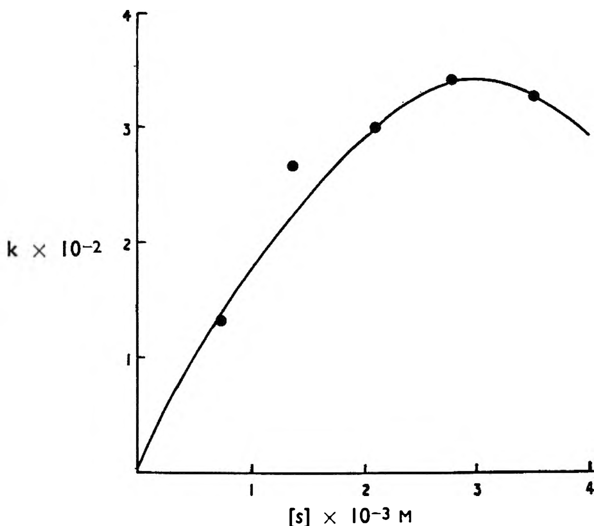


FIG. 1. Effect of the concentration of acetylcholine chloride on the activity of acetylcholinesterase.

Inhibition studies. Rate constants of hydrolysis of acetylcholine at two substrate concentrations in the presence of varying concentrations of inhibitors are shown in Table I. The respective inhibition constants derived by the method of Dixon (1953) are also included.

Table II gives the relative concentrations of the various compounds required to produce a 50 per cent inhibition of AChE and also their pKa values obtained from the literature.

Monoamine Oxidase Inhibition

Optimum substrate concentration. Fig. 2 shows that the rat liver monoamine oxidase has two optimum substrate concentrations. The one at

TABLE I

RATES OF HYDROLYSES OF ACETYLCHOLINE IN THE PRESENCE OF VARYING CONCENTRATIONS OF INHIBITORS

Inhibitor compound	Inhib. conc. (M)	Velocity const. k(x 10^{-2} min.$^{-1}$ at		K_i (Dixon)
		$[s_1]$	$[s_2]$	
Tetrahydroaminoacridine	1×10^{-6}	0·75	1·00	
	2×10^{-6}	0·41	0·54	
	3×10^{-6}	0·28	0·41	
	5×10^{-6}	0·20	—	
	8×10^{-6}	—	0·15	1×10^{-4}
Tetrahydroacridine	1×10^{-6}	2·00	2·20	
	2×10^{-6}	1·50	1·70	
	3×10^{-6}	1·00	1·30	
	5×10^{-6}	0·73	0·80	4×10^{-4}
N-Butylaminotetrahydroacridine ..	1×10^{-6}	2·60	2·80	
	2×10^{-6}	1·70	1·80	
	3×10^{-6}	1·60	1·50	
	5×10^{-6}	1·30	1·20	$1·7 \times 10^{-3}$
4-Aminoquinoline	2×10^{-5}	2·20	2·30	
	4×10^{-5}	1·40	1·40	
	6×10^{-5}	1·00	1·00	
	10×10^{-5}	0·68	0·68	14×10^{-2}
4-Aminopyridine	1×10^{-4}	2·90	3·00	
	2×10^{-4}	2·60	2·90	
	3×10^{-4}	—	2·50	
	5×10^{-4}	2·10	2·10	5×10^{-1}
Procaine hydrochloride	2×10^{-5}		4·60	
	4×10^{-5}		4·20	
	6×10^{-5}		4·10	
	10×10^{-5}		3·80	

TABLE II

INHIBITION OF AChE BY VARIOUS COMPOUNDS

Substance	Concentration required to produce 50 per cent inhibition (M)	pKa
Tetrahydroaminoacridine ..	$<1 \times 10^{-6}$	9·4
Tetrahydroacridine	2×10^{-6}	5·0 (approx.)
Butylaminotetrahydroacridine	3×10^{-6}	9·4 (approx.)
4-Aminoquinoline	4×10^{-5}	9·17
4-Aminopyridine	$>5 \times 10^{-4}$	9·17
Procaine	$>1 \times 10^{-4}$	—

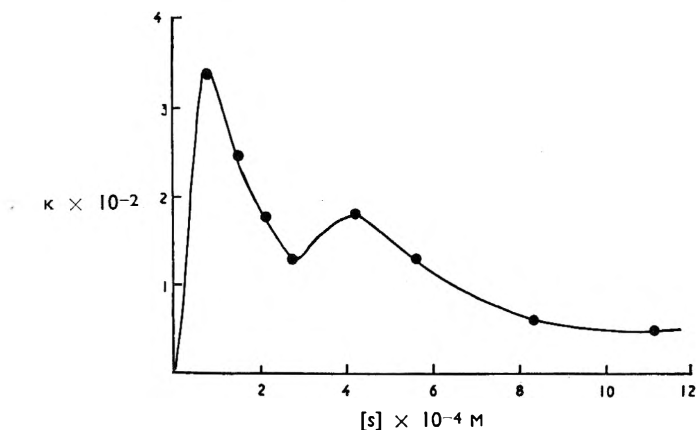


FIG. 2. Effect of the concentration of 5-HT on the activity of monoamine oxidase.

7×10^{-5} M is too low to work with in the present method of assay. Therefore the second concentration, $4{\cdot}2 \times 10^{-4}$ M, was used as the optimum for these studies.

Stability of the oxidase. A 1 in 20 homogenate of the rat liver in pH 7·4 phosphate buffer, when stored overnight at 5°, lost only 5 per cent of its monoamine oxidase activity. It is safe to work with the same liver preparation for one day, though a homogenate maintained overnight in a frozen condition can be used the following day. Fresh liver chilled at 0° also maintains its monoamine oxidase activity for several days.

TABLE III

PER CENT INHIBITION OF MONOAMINE OXIDASE AT 20 MIN.

Inhibitor conc. (M)	Per cent inhibition caused by			
	THA	TACR*	4-AQ†	4-AP‡
$1{\cdot}5 \times 10^{-4}$	54–57	89–94	100	7·5–10
$1{\cdot}0 \times 10^{-4}$	18·4	85	91·8	5·4
$3{\cdot}3 \times 10^{-5}$	0·0	42·8	87·8	0·0

* Tetrahydroacridine. † 4-Aminoquinoline. ‡ 4-Aminopyridine.

Inhibition of the oxidase. Table III shows inhibition per cent of the enzyme caused by various concentrations of the inhibitors at 20 min.

Inhibition of the enzyme by varying concentrations of THA and 4-aminoquinoline at two different substrate concentrations were studied to obtain rate data for the estimation of the inhibition constant (K_i). The K_i values as determined by the method of Dixon (1953) were found to be 2×10^{-4} and $1{\cdot}1 \times 10^{-5}$ for THA and 4-aminoquinoline, respectively.

DISCUSSION

From the data in Table I and II it appears that THA is the most potent inhibitor of AChE in the related small series of compounds. *N*-Alkylaminotetrahydroacridine is more basic than THA, though in the *N*-butyl derivative the steric hindrance arising from the bulky group may counteract the basicity increasing effect of the alkyl group. Tetrahydroacridine itself is a much weaker base than its 5-amino derivative (THA), and therefore also weaker than the *N*-butylamino derivative; yet it shows an activity nearly as great as THA and certainly much greater than the *N*-butyl compound. It is possible that such a decreased activity of the *N*-butyl derivative of THA is due to the steric hindrance of the butyl group which may produce a poor-enzyme-inhibitor interaction.

4-Aminoquinoline is as basic as 4-aminopyridine, but the two compounds show widely different potencies of inhibition. Again, both these compounds are nearly as basic as THA, but they both show very weak AChE inhibiting potency compared to that of THA. All these facts suggest that the maximum inhibition of AChE requires an optimum structure, in this case THA, and not the basicity alone. Indeed some basicity is necessary, because at least one nitrogen needs to be ionized to ensure interaction of the inhibitor with the ionic site of the enzyme.

The modified procedure of assay for monoamine oxidase activity described above enables 36 to 48 analyses to be made each working day. Of the compounds examined, 4-aminoquinoline is a structure required for maximum inhibition of the oxidase. It is possible that THA owes its oxidase inhibiting potency mainly to the 4-aminoquinoline part of the molecule. However, the significance of the reduced ring cannot be overlooked. This is obvious from the inhibiting potency shown by the tetrahydroacridine.

Detailed toxicological studies of 4-aminoquinoline are needed to evaluate the usefulness of this agent as a psychomotor stimulant like other monoamine oxidase inhibitors already in use.

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SPECTROPHOTOFLUOROMETRIC DETERMINATION OF EMETINE IN ANIMAL TISSUES

By B. Davis, M. G. Dodds and E. G. Tomich

From Glaxo Research Limited, Greenford, Middlesex

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A simple rapid spectrophotofluorometric method for assaying emetine in animal tissues is described. Tissue emetine concentrations have been determined in rats at intervals up to 14 days after single doses of emetine hydrochloride or emetine bismuth iodide: the hydrochloride was administered orally or subcutaneously and the bismuth iodide complex orally. Subcutaneously administered emetine hydrochloride produced the highest concentrations. Given orally, emetine hydrochloride produced higher tissue levels than did emetine bismuth iodide.

Emetine is a valuable drug with a low therapeutic index. For 50 years it has found worldwide use in the treatment of amoebiasis, yet references to studies of tissue distribution are few (Gimble, Davison and Smith, 1948; Parmer and Cottrill, 1949; Radomski, Hagan, Fuyat and Nelson, 1952), probably because the assay methods available have been somewhat laborious. We are currently investigating the biological disposition of emetine and some derivatives; to facilitate these studies we have developed a simple spectrophotofluorometric assay. Details of the assay, and the results of some rat experiments in which it was used, are reported here.

Materials and Methods

The materials used in the experiments were emetine hydrochloride B.P., emetine bismuth iodide B.P., N sodium hydroxide, anaesthetic ether B.P. and a KCl/HCl solution of pH 2. Before using the last reagent, which is made by mixing 50 ml. M KCl with 53 ml. 0·2 N HCl and adjusting the volume to 1 litre with distilled water, it is essential to extract it with ether to remove extraneous fluorescent material.

The emetine contents were 70·1 per cent (B.P. assay) and 71·7 per cent (calculated on Kjeldahl nitrogen) for the hydrochloride, and 27·8 per cent (B.P. assay) and 28·6 per cent (calculated on Kjeldahl nitrogen) for the bismuth iodide complex. In calculating dosages it was assumed that the hydrochloride and bismuth iodide complex contained 71 and 28 per cent respectively of emetine base.

Fluorescence Characteristics of Emetine

A solution of emetine hydrochloride (0·05 μg./ml.) in KCl/HCl solution was scanned on a Farrand spectrophotofluorometer. Single peaks were present in the activating and analysing spectra at 290 mμ and 320 mμ, respectively (uncorrected values). At these wavelengths the emetine solution fluoresces three times as strongly as distilled water or the KCl/HCl solution itself; a full scale deflection is obtained on the most sensitive

scale of the microammeter at a concentration of 0·1 μg./ml. The intensity of fluorescence increases linearly over the concentration range 0·01–1·00 μg./ml.: it increases also with decreasing pH, being maximal and constant over the range 1–3. The intensity of fluorescence diminishes with rising temperature: over the range 15–30° each degree rise results in 0·5 per cent less intensity.

Assay of Emetine in Rat tissues

Rats were killed with coal-gas and their hearts, lungs, livers, kidneys and spleens were removed and weighed. Each organ was homogenised in a Townson and Mercer micro wet grinder, and the brei was diluted with distilled water to give a suspension containing 1 g. wet tissue per 10 ml. One ml. of suspension was shaken vigorously for 30 sec. in a shake-tube with 0·5 ml. distilled water, 0·5 ml. N NaOH and 10 ml. ether. In the recovery experiments standard emetine solutions were used in place of the distilled water. Of the ether phase (total volume after shaking, 9·7 ml.) 8 ml. was removed and shaken for 30 sec. with 10 ml. KCl/HCl solution. The ether layer was removed by aspiration and discarded. The fluorescence of the KCl/HCl solution was compared with that of a standard solution of emetine hydrochloride in KCl/HCl solution at the same temperature.

The mean percentage recovery of emetine hydrochloride added in amounts equivalent to 1–100 μg. emetine base/g. wet tissue was 99·3 ± S.E. 2·1 (30 observations). There were no differences in the recoveries from various tissues; as might be expected, there was greater variation at the lower emetine concentrations. Thus the mean percentage recovery at a concentration of 1 μg. emetine base/g. wet tissue was 104·3 ± 6·0 (10 observations), whereas that at 5–100 μg./g. was 96·9 ± 0·9 (20 observations). The fluorescence obtained by extracting tissues from undosed rats was not more than that given by the KCl/HCl solution itself.

Tissue Emetine Concentrations in Dosed Rats

Two groups of female rats of the WAG strain (body weight range 130–170 g.) received single oral doses of either emetine hydrochloride (10 mg./kg. ≡ 7·1 mg. emetine base/kg.) or emetine bismuth iodide (30 mg./kg. ≡ 8·4 mg. emetine base/kg.). A third group received single subcutaneous injections of emetine hydrochloride (10 mg./kg.). The hydrochloride was administered in aqueous solution and the bismuth iodide complex as an aqueous suspension in gum tragacanth, 1 in 200. At various times, from 2 hr. to 14 days after dosing, 3 rats from each group were killed and their tissue emetine levels were determined. The organs of the rats dosed orally were assayed individually, but from those injected subcutaneously the corresponding organs from 3 rats were bulked before assay.

The fluorescent spectra of the tissue extracts were indistinguishable from those of emetine hydrochloride. Furthermore, when the fluorescent substance isolated from the tissues of rats dosed with emetine hydrochloride was examined by the chromatostrip method of Stahl (1958), one

spot only was obtained, and its R_F value agreed with that of emetine. The amoebicidal activity of the fluorescent substance was demonstrated in vitro using *Entamoeba histolytica.*

Results

The concentrations and amounts of emetine found in the different organs at various times after dosing are given in Table I. The total amounts of emetine found in the organs at various times after dosing are shown in Fig. 1, the values being expressed as percentages of the doses administered.

TABLE I

TISSUE DISTRIBUTION OF EMETINE IN RATS AT VARIOUS TIMES AFTER SINGLE DOSES OF THE HYDROCHLORIDE OR BISMUTH IODIDE COMPLEX

Treatment and route	Time after dose	Emetine concentration (µg. base per g. wet tissue)*					Emetine content (per cent of dose)*					Total emetine content in tissues examined (per cent of dose)*
		Heart	Kidney	Liver	Lung	Spleen	Heart	Kidney	Liver	Lung	Spleen	
Emetine hydrochloride 10 mg./kg. (≡7·1 mg. base/kg.) Subcutaneous	2 hr.	11·1	38·0	22·3	63·9	59·5	0·55	4·62	13·55	4·77	2·02	25·5
	4 ,,	9·0	49·5	38·2	98·0	99·3	0·43	5·68	22·20	8·53	2·90	39·7
	8 ,,	7·4	34·7	35·8	79·4	116·0	0·35	3·66	18·20	7·25	3·52	33·0
	1 day	7·5	33·2	30·9	47·4	94·0	0·37	3·23	15·60	5·23	3·78	28·2
	2 days	8·2	24·9	18·9	47·5	85·0	0·38	2·74	10·20	4·89	2·34	20·6
	3 ,,	8·4	20·8	20·8	44·4	33·5	0·38	2·13	9·70	3·56	1·76	17·5
	7 ,,	5·0	12·4	7·2	23·0	39·5	0·22	1·45	4·82	2·26	1·71	10·5
	14 ,,	1·2	2·6	2·1	7·1	12·6	0·06	0·36	1·38	0·74	0·66	3·2
Emetine hydrochloride 10 mg./kg. (≡7·1 mg. base/kg.) Oral	4 hr.	2·3	8·5	19·4	11·1	8·3	0·12	0·90	10·70	1·06	0·29	13·0
	8 ,,	3·4	14·9	23·2	19·1	15·5	0·16	1·51	11·00	1·43	0·65	14·7
	1 day	3·3	12·4	15·9	25·1	24·1	0·16	1·40	7·95	2·03	1·09	12·6
	2 days	4·7	19·8	23·9	30·0	36·8	0·21	2·24	12·40	2·32	2·11	19·3
	3 ,,	5·9	20·2	17·4	30·7	50·8	0·26	2·26	8·03	2·30	2·17	14·9
	7 ,,	1·8	9·1	5·4	11·5	27·9	0·08	1·01	3·49	0·75	1·52	6·9
	14 ,,	1·0	3·7	2·3	5·5	17·4	0·05	0·44	1·35	0·38	0·82	3·0
Emetine bismuth iodide 30 mg./kg. (≡8·4 mg. base/kg.) Oral	4 hr.	1·0	2·6	14·5	3·8	2·6	0·04	0·27	8·10	0·26	1·20	8·8
	8 ,,	3·3	13·1	21·3	20·5	13·6	0·13	1·14	9·01	1·55	0·49	12·3
	1 day	2·3	9·8	16·7	19·1	22·5	0·10	1·01	8·67	1·43	0·94	12·1
	2 days	4·6	16·6	21·2	29·5	42·6	0·18	1·65	10·31	2·02	1·66	15·8
	3 ,,	4·3	16·5	12·1	24·4	40·0	0·17	1·70	6·23	1·62	1·72	11·5
	7 ,,	2·0	10·3	4·7	13·6	25·6	0·08	1·07	2·54	1·02	1·12	5·8
	14 ,,	0·7	1·9	1·9	4·4	10·6	0·03	0·21	1·12	0·31	0·48	2·1

* Group mean values (3 rats per group.)

The concentrations of emetine in the tissues were higher after subcutaneous injection than after oral administration. Only small differences were seen between the two oral preparations, but the total tissue content of emetine was consistently lower after dosing with the bismuth iodide complex than with the hydrochloride.

Discussion

The assay method described is simple and rapid; it gives reproducible results provided that precautions are taken to eliminate extraneous fluorescence.

The method can also be used for determining emetine in muscle and brain (in which we found only traces) and in urine and blood. With blood, the aqueous and ether phases do not separate as rapidly as with

tissue extracts. Because they produce high blank values, it is essential that droplets of diluted blood are not removed with the ether extract. Preliminary experiments indicated that emetine disappeared rapidly from the blood stream in rats, being almost undetectable 10 min. after a single intravenous dose of emetine hydrochloride (10 mg./kg.).

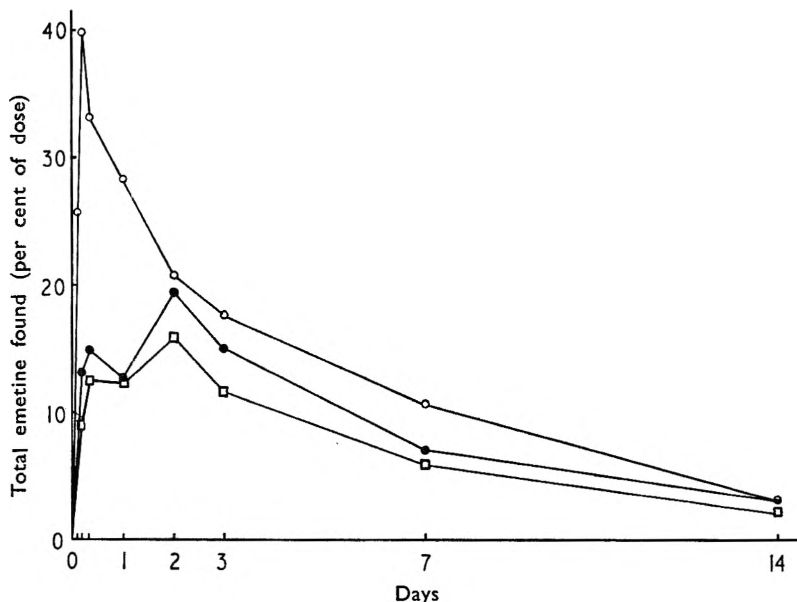

FIG. 1. Total amounts of emetine found in the heart, kidneys, liver, lungs and spleen of female rats at various times after single doses of the hydrochloride or bismuth iodide complex of emetine.

—○— emetine hydrochloride (10 mg ≡ 7·1 mg base/kg.)—subcutaneous
—●— emetine hydrochloride (10 mg. ≡ 7·1 mg. base/kg.)—oral
—□— emetine bismuth iodide (30 mg. ≡ 8·4 mg. base/kg.)—oral
(values are means for groups of 3 rats)

The results reported here agree well with those of Parmer and Cottrill (1948) and are in general agreement with those of Gimble, Davison and Smith (1948). When differences exist, they probably derive from the use of different species of animal and different routes of administration: Parmer and Cottrill used rabbits and intramuscular injection; Gimble, Davison and Smith used rats and the intraperitoneal route.

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LETTERS TO THE EDITOR

Anticonvulsant Activity of Procaine and its Five Congeners against Experimentally Induced Convulsions

SIR,—The concept of a single fundamental mechanism of excitation in nervous and cardiac tissue has been stressed by Harris and Kokernot (1950). On the basis of this hypothesis the anticonvulsant activity of procainamide was studied by Arora and Kapila (1959), but subsequent detailed studies revealed that procainamide had a low therapeutic index. Considering the effectiveness and toxicity of procainamide, it was thought that a study of the action of the parent substance (procaine) and some of its other congeners might prove them to be better anticonvulsant agents.

The anticonvulsant activity of procainamide hydrochloride, procaine hydrochloride and its four congeners with methyl substitution in the benzene ring was studied against maximal electro-shock and also leptazol-induced seizures (Arora, Sharma and Kapila, 1958) in adult albino rats and was compared with phenytoin sodium. The neurotoxicity (Swinyard, 1949) and acute toxicity were also tested and the ED50, TD50, LD50, (Litchfield and Wilcoxon, 1949), and the therapeutic indices and protective indices of the drugs were calculated.

These compounds were effective only against maximal electro-shock seizures. Procaine and its congeners were effective within 10 min. and the effect lasted for 2–3 hr., while the effect of phenytoin sodium lasted much longer. Toxicity studies revealed that procaine hydrochloride and its congeners caused clonic convulsions before death.

Procainamide hydrochloride had a low therapeutic index (2·8) compared with that of phenytoin sodium (9·4) (Table I).

Procaine hydrochloride was effective in low doses (ED50=5·56 mg./kg.) and had a therapeutic index of 36. However, neurotoxicity was observed at low doses and so the protective index was only 5. Procaine and its methyl substituted congeners had higher protective and therapeutic indices compared with phenytoin sodium (Table I).

TABLE I

ANTICONVULSANT ACTIVITY, TOXICITY, PROTECTIVE INDICES AND THERAPEUTIC INDICES OF PROCAINAMIDE, PROCAINE, PHENYTOIN AND PROCAINE CONGENERS IN RATS

Drugs	ED50 against maximal electric shock seizures* mg./kg.	TD50 mg./kg.	Protective index TD50/ED50	LD50 mg./kg.	Therapeutic index LD50/ED50
Procainamide hydrochloride	80·0 (62·98–101·6)	115·3 (96·49–137·78)	1·4	225	2·8
Procaine hydrochloride	5·56 (4·09–7·56)	28·18 (21·51–36·91)	5	200	36
2-Diethylaminoethyl-2,3-dimethylbenzoate hydrochloride	18·0 (10·28–31·50)	146·0 (127·0–168·0)	8·1	300	16·7
2-Diethylamino-2,3,5,6-tetramethylbenzoate hydrochloride	8·61 (5·69–13·02)	50·93 (34·66–74·86)	5·9	200	23·2
2-Diethlaminoethyl-2,5-dimethylbenzoate hydrochloride	9·34 (5·66–15·41)	142·0 (121·36–166·14)	15	350	37·4
2-Diethylaminoethyl-3,5-dimethylbenzoate hydrochloride	5·07 (2·53–10·14)	144·5 (120·92–172·67)	28·5	325	64·1
Phenytoin sodium	16·29 (10·84–24·46)	72·44 (65·26–80·40)	4·4	153	9·4

* Figures in parenthesis are the 95 per cent confidence limits.

Of the methyl substituted procaine congeners, 2-diethylaminoethyl-3 5-dimethylbenzoate hydrochloride was most effective having a therapeutic index of 64·1 and a protective index of 28·5.

2-Diethylaminoethyl-2,3-dimethylbenzoate hydrochloride and 2-diethylaminoethyl-2,3,5,6-tetramethylbenzoate hydrochloride had higher protective indices, but lower therapeutic indices compared with procaine hydrochloride.

Compared with procaine hydrochloride, 2-diethylaminoethyl-2,5-dimethylbenzoate hydrochloride has slightly higher therapeutic index (37·4), but a much higher protective index.

The present study reveals that methyl substitution at position 3 and 5 in the benzene ring of procaine yields a compound which has much higher protective and therapeutic indices compared with the parent substance procaine.

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Department of Pharmacology, KANTI KAPILA.
All-India Institute of Medical Sciences, R. B. ARORA.
New Delhi-16, India.
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Orally Effective Hypoglycaemic Agents from Plants

SIR,—Hypoglycaemic agents from *Allium cepa* Linn. (the domestic onion), *Ficus bengalensis* Linn. and *Eugenia jambolana* Lam. have already been reported by us (1961). The present communication describes two more orally effective hypoglycaemic principles extracted from *Allium sativum* Linn. (garlic) and from an Indian indigenous plant, *Ficus religiosa* Linn.

TABLE I

BIOLOGICAL ASSAY OF ORALLY EFFECTIVE HYPOGLYCAEMIC AGENTS FROM *Allium sativum* AND *F. religiosa.* COMPARED WITH TOLBUTAMIDE

		Blood sugar response mg./100 ml.			
Substance administered	Dose	Initial average values for six rabbits	4 hr. pool average values for six rabbits	Mean reduction per cent	Mean hypoglycaemic potency as per cent of tolbutamide
Tolbutamide (Albert David and Co.)	0·5 g.	100	74·98	25 ± 2·1	100
Total ethyl ether (34–36°) extract from 50 g. dry garlic powder	0·5 g.	117·3	100·03	14·72 ± 3·5	58·88
Total water extract from 50 g. dry root bark powder of *F. religiosa*.	2·5 g.	117·9	95·52	18.97 ± 4·34	75·9

The hypoglycaemic effect of the different fractions of garlic extracts was reported by Laland and Havrivold (1933). *F. religiosa* Linn. is used throughout India as a natural remedy for diabetes mellitus as mentioned by Chopra (1933).

Sun dried cloves of garlic and the root bark of *F. religiosa* were thoroughly dried and extracted separately with different solvents in soxhlets. Only the water extract of *F. religiosa* and the ethyl ether extract of garlic were found to exhibit sufficient hypoglycaemic activity, on oral administration to rabbits, compared to a standard dose of tolbutamide (0·5 g./rabbit). The results of the biological assay of these extracts on groups of normal male albino rabbits weighing 2 kg. with fasting 18 hr. blood sugar levels of 100–125 mg./100 ml. are given in Table I. The biological assay was made by a procedure similar to that of Marks (1926). Blood sugar was determined by the micromethod of Folin and Malmros (1929).

Both these extracts were found to be effective in controlling the hyperglycaemic response of glucose feeding (1 g./kg.) in glucose tolerance experiments on normal fasting rabbits, as will be evident from Fig. 1. The drugs concerned were fed 1 hr. before the administration of glucose to facilitate their absorption.

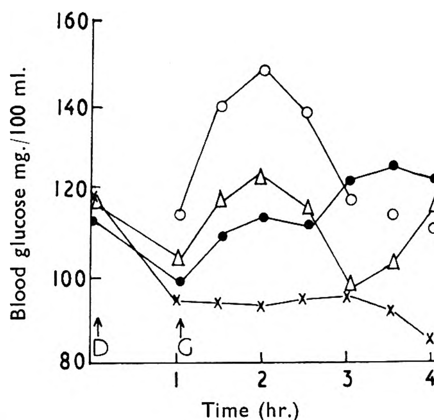

FIG. 1. Effects of the hypoglycaemic agents on the glucose tolerance of normal fasting rabbits. (X——X) tolbutamide; (●——●) *Allium sativum*; (△——△) *Ficus religiosa*; (○——○) control. Dose same as given in the Table I. Each curve is a mean of results from six rabbits. D = drug, G = glucose.

The results, Table I and Fig. 1, show that, *Allium sativum* and *F. religiosa* contain orally effective hypoglycaemic agents.

A detailed report on the separation and isolation of the orally effective hypoglycaemic agents from these natural sources will be published elsewhere.

H. D. BRAHMACHARI.
K. T. AUGUSTI.

Department of Biochemistry,
Birla College,
Pilani (India.)

January 29, 1962.

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The Effect of pH on the Stability of Penicillin-induced Spheroplasts

SIR,—Edebo (1961) has made a detailed study of the effect of pH on the osmotic stability of lysozyme-induced protoplasts and spheroplasts. Protoplasts of *Bacillus megaterium*, prepared as described by Weibull (1953), were found to be more stable on the acid than on the alkaline side of neutrality, and did not burst below pH 5 when the osmotic pressure of the medium was suddenly reduced. Spheroplasts of *Escherichia coli*, obtained by the lysozyme-versene technique of Repaske (1958), and dialysed to pH values less than pH 5–5·5, showed greater resistance to disintegration than spheroplasts at higher pH; in addition, the optical density was higher in samples of lower pH.

In our experiments, spheroplasts of *E.coli*, formerly NCTC 5934, were induced by treatment of the organism with penicillin in 10 ml. nutrient broth containing 0·33M sucrose and 0·25 per cent w/v $MgSO_4 7H_2O$, at pH 7·2. After incubation for 5 hr. at 37°, tubes were centrifuged at a low speed, to avoid disintegration of the spheroplasts, and the spheroplast residue suspended in 10 ml. sucrose-Mg^{++}-broth, containing penicillin, at a pH value within the pH range 5–8. Tubes were re-incubated at 37°, and samples from each examined by interference microscopy at frequent intervals. It was found that the spheroplasts were less susceptible to disintegration at acid pH values. These results confirm those obtained by Edebo (1961) with lysozyme-induced spheroplasts and are also in agreement with the finding that penicillin-induced spheroplasts of *Aerobacter aerogenes* are more stable at acid pH values than at alkaline pH (Gebicki and James 1960).

Department of Pharmacy, The University, Nottingham. — W. B. HUGO.

Welsh School of Pharmacy, Welsh College of Advanced Technology, Cardiff. — A. D. RUSSELL.

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