REVIEW ARTICLE

QUATERNARY AMMONIUM COMPOUNDS IN MEDICINAL CHEMISTRY. I*

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Interest in the biological activity of quaternary ammonium salts stems from the elegant work of Crum Brown and Fraser (1868–9), who were the first to record the curariform activity of methiodides of alkaloids such as strychnine, brucine, and atropine. Apart from a few scattered papers, the pharmacological activity of quaternary ammonium salts received little attention for many years. Hunt, Renshaw and their associates, working over a period of some 35 years from 1904 to 1939, published a very large number of papers dealing with the effects of these compounds on the autonomic nervous system; they established three basic types of activity, muscarinic, nicotinic and curariform. References to this work are listed in full in Craig's excellent review, "Curariform activity and chemical structure" (1948).

Concurrently with these studies, quaternary ammonium compounds were emerging into greater prominence by virtue of their activity against micro-organisms. Their germicidal activity was first recognised in 1916, when Jacobs and his colleagues (Jacobs, 1916; Jacobs, Heidelberger and Amoss, 1916; Jacobs, Heidelberger and Bull, 1916) investigated the relation between chemical structure and bactericidal activity of quaternary salts of the hexamethylenetetramine series. The synthesis of the sapamines (acylaminoethyltrialkylammonium salts) by Hartmann and Kägi (1928) is representative of the work that led to the recognition of the exceptional virtues of this class of high molecular weight quaternary ammonium salts (the so called "invert soaps"); the development of these compounds as germicides paralleled the study of their properties as surface-active agents and as textile chemicals.

Nevertheless the potentiality of quaternary agents was not fully realised, and their widespread use was not established, until Domagk (1935) examined certain compounds and only found notable germicidal activity when at least one of the four radicals had a carbon chain of length C_8 to C_{18}. The most important compound to emerge from this work was Zephirol (Zephiran, benzalkonium chloride), which has the structure $RN^+.(Me_2).CH_2.Ph\ Cl^-$ where R is a saturated straight alkyl chain containing from 12 to 18 carbon atoms.

Initially quaternary ammonium germicides were considered to be primarily suited for surgical and medical usage, but later an increasing application to public health and sanitation became a major factor in their commercial development and exploitation. An important stage in the

* The second part of this review, together with all the references, will appear in the April issue of the Journal.

use of quaternaries in chemotherapy followed the investigations of Browning, Cohen, Ellingworth and Gulbransen (1929) on some quaternary derivatives of anil and styrylquinoline, some of which were found to possess high activity against trypanosome infections. Later investigations by Browning, Morgan, Robb andWalls (1938)on certain phenanthridinium compounds synthesised by Morgan and Walls (1938), showed some of these substances to possess a curative action on *Trypanosoma brucei* infections in mice. Subsequent work in this field led to the development of Dimidium, homidium (Ethidium) and finally quinapyramine (Antrycide), which is still one of the most effective compounds used in the treatment of trypanosomiasis.

During the years of the Second World War, and in the immediate post-war period, much fresh light was thrown on the pharmacological properties of the quaternaries. For many years King at the National Institute for Medical Research, London, had been interested in curare, and from tube curare he had isolated a pure crystalline alkaloid, (+)-tubocurarine chloride (King, 1935). Although the complete structure was not elucidated until some years later (King, 1948), it was soon realised that this alkaloid was a bisquaternary ammonium compound. The extensive clinical investigations of West (1932, 1935a, b, c), Griffith and Johnson (1942) and Gray and Halton (1946), on curare have been the major stimulus for a vast amount of subsequent chemical, pharmacological, and clinical study. However, perhaps the most important contribution in this field was the epoch-making discovery, announced simultaneously by Barlow and Ing (1948) at Oxford, and by Paton and Zaimis (1948) at the N.I.M.R., of the neuromuscular and ganglionic blocking activity of the polymethylenebistrimethylammonium salts (the "methonium salts"). This work has provided the stepping stone from which has sprung the majority of the synthetic neuromuscular and ganglionic blocking agents to receive clinical application. Some of these drugs are choline derivatives, and it should be remembered that acetylcholine, the parasympathetic transmitter, is the only quaternary ammonium salt with a physiological rôle in the body.

Investigations into the properties of acetylcholine really began with the work of Hunt and Taveau (1906), and later it was established that this activity could be divided into two principal classes, muscarine-like and nicotine-like. The specificity of the acyl group was investigated by Chang and Gaddum (1933) and others (see Bergel, 1951), who described the biological activities of a series of different acylcholines. Other workers, notably Ing and his colleagues (Ing, 1949; Holton and Ing, 1949; and Ing, Kordik, and Tudor Williams, 1952), investigated the importance of the size of the quaternary grouping in choline derivatives and demonstrated that, while the replacement of one methyl group by another grouping decreased activity, the major decline occurred when a second methyl group was replaced. In addition, the effects of other alterations in the chemical structure of choline have been studied, especially in relation to the relative muscarinic and nicotinic activity of these compounds (see Barlow, 1955).

Quaternary ammonium salts have also found clinical value as antispasmodic and antisecretory agents in the treatment of various irritant gastric conditions. In general this type of activity is related to an atropine-like effect, as for example in lachesine (Ing, Dawes and Wajda, 1945), although there are notable exceptions such as amprotropine phosphate (Syntropan) (Fromherz, 1933, 1934, 1937). Furthermore some quaternaries such as choline theophyllinate (Choledyl) and related compounds have been used for the relief of bronchospasm. Potent anticholinesterase activity is present in certain synthetic quaternary compounds, modelled upon the non-quaternary alkaloid physostigmine, such as neostigmine and others of related structure (Blaschko, Bülbring and Chou, 1949). This type of compound has been applied to the treatment of myasthenia gravis (Walker, 1934, 1935; Schwab, 1960) and also as an antagonist to the neuromuscular blocking action of tubocurarine and curare-like compounds (Riker, 1953). Anticholinesterase activity, to a varying extent, is shown by other quaternaries, for example the heterocyclic polymethylenebisquaternary ammonium salts (Riker, 1953; Barlow and Himms, 1955; Cavallito and Sandy, 1959).

Although the antibacterial application of quaternaries has been known for some time, it is only within the last 10 years or so that their potential as antifungal agents has become apparent. Compounds such as cetrimide, dequalinium, domiphen, and hedaquinium have provided highly successful local antifungal agents. Another development of quaternaries is their use in the formulation of cosmetics (Lincoln, 1954), which is mainly due to the fortunate combination of surface-activity and germicidal action found in these salts.

From this brief introduction, it must be apparent that quaternary ammonium salts have retained the interest of chemist and biologist alike for almost a century. In our opinion, this subject has hitherto not been adequately reviewed, and the present article has therefore been written in an attempt to summarise the more important features of the research carried out during this period. We would however, like to draw attention to the article by Lesser (1949), which reviewed the current knowledge of quaternaries at that time.

It is not possible to discuss the vast number of naturally occurring quaternary salts (such as the alkaloids), and it is therefore proposed to deal only with the more important synthetic compounds that have activity or potential activity in the treatment of human or animal disease. For brevity, the term "onium" will be used throughout to represent quaternary ammonium compounds.

Pharmacodynamic Activity

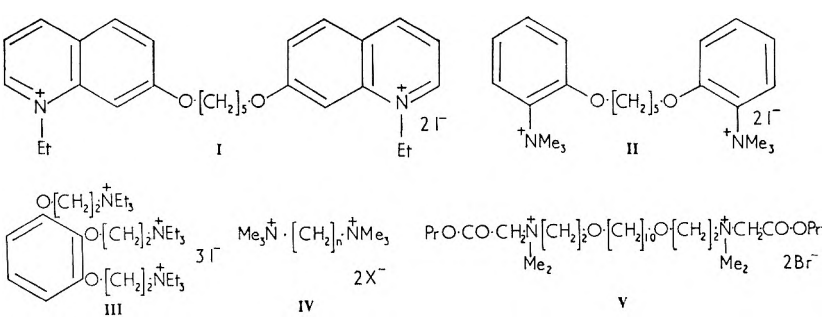
Neuromuscular Blocking Agents

The use of muscle relaxants as an aid to anaesthesia is nowadays accepted procedure; however, it is actually little more than 15 years old. The oldest known relaxant is, of course, curare; reports of its use by South American Indians reached Europe as early as the sixteenth century,

but little was known of its source or mode of action for nearly 300 years. The first clear accounts of the preparation, use and action of this poison were given by travellers such as Humboldt, Schomburgk and Waterton in the early nineteenth century; these are reported by McIntyre (1947) in his fascinating book *Curare*.

Experimental studies with neuromuscular blocking drugs started in 1850, when Pelouze and Bernard showed that the paralysis caused by crude extracts of curare was in fact due to a block at the neuromuscular junction; this work was repeated and extended by Crum Brown and Fraser (1868–69), since when it has been recognised that neuromuscular blocking properties are characteristic of onium salts as a class. However, it was not until much later that the development of satisfactory methods for the biological evaluation of neuromuscular blocking agents enabled further progress to be made. The first reliable estimations were made by Ing and Wright (1931, 1933), who measured the time taken for equimolar solutions of different neuromuscular blocking drugs to produce complete paralysis of the frog sartorius preparation.

Before King's final elucidation of the structure of (+)-tubocurarine in 1948, it was established that the drug was a bis-onium salt and sufficient of the general structure was known to provide a lead for the synthesis of simpler substitutes. In 1946 and subsequently, Bovet and his colleagues (Bovet, Courvoisier, Ducrot and Horclois, 1946; Bovet, Depierre and de Lestrange, 1947) produced a series of quaternaries with marked muscle-relaxing properties; these included 3381.R.P.[I], 3565.R.P.[II], and 3697.R.P.[III], the latter being appropriately named gallamine triethiodide (Flaxedil).



Although gallamine was the first synthetic neuromuscular blocking agent to be accorded widespread clinical use, it is still extensively employed and is included in the British Pharmacopoeia 1958. Many attempts have been made (Riker and Wescoe, 1951; Roberts, Riker and Wescoe, 1951; Pelikan and Unna, 1952) to modify the structure of gallamine to improve its neuromuscular blocking activity and to reduce the incidence of side effects; but none of these related compounds has replaced the parent substance.

A major advance in the development of synthetic neuromuscular blocking agents was the simultaneous, but independent, publication in 1948 of

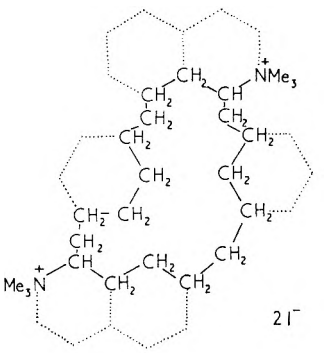
the activity of the "methonium" compounds by Barlow and Ing of Oxford, and Paton and Zaimis working at the National Institute for Medical Research. These two groups of workers investigated the pharmacology of the polymethylenebistrimethylammonium salts and found the decamethylene member, decamethonium, (C_{10}, Eulissin, Syncurine) [IV, n = 10], to be an extremely potent neuromuscular blocking agent, approximately five times more active than tubocurarine in man; but, as with all other such agents, there is considerable difference in the relative potency between various animal species. The methonium compounds also possess ganglionic blocking activity, which is optimal with the hexamethylene analogue, hexamethonium or C_6 [IV, n = 6]; this proved to be the stepping stone from which the bulk of the numerous synthetic hypotensive agents now available have been developed.

Decamethonium differs from tubocurarine in its method of action, since it produces neuromuscular blockade by depolarisation of the muscle end plate, whereas tubocurarine acts by competing with acetylcholine for the end plate receptor. Robson and Keele (1956) have given an excellent summary of the three main mechanisms of neuromuscular blockade (Competitive, Depolarising and mixed Competitive and Depolarising); they have also reviewed the experimental criteria used to differentiate these three mechanisms. It does not fall within the scope of this review article to expand upon this subject.

Decamethonium, like all depolarising agents, is not antagonised by anticholinesterases (for example, neostigmine), which may indeed enhance its action. This lack of a satisfactory antagonist has been a serious disadvantage in its clinical use, and although some successful antagonists were later developed (Phillips and Castillo, 1951; Phillips, 1952; Dallemagne and Phillippot, 1953; Vandam, Safar and Dumke, 1953), decamethonium has never really fulfilled its early promise as an adjuvant to anaesthesia; nevertheless some anaesthetists still favour its use (Hale Enderby, 1959; Fisk, 1961). Decamethonium was omitted from the British Pharmacopoeia, 1958. As with gallamine, numerous efforts have been made to improve the biological properties of decamethonium by modification of its structure, but probably the only compound of this type worthy of mention is diohexadecanium (Prestonal) [V], which has achieved some success abroad, although it is apparently not used to any great extent in this country (Griffith, Cullen and Welt, 1956; Jolly, 1957; Rendell-Baker, Foldes, Birch and D'Souza, 1957). Interest in this type of structure has not, however, entirely flagged since currently Lewis and Stenlake and their associates in Glasgow (Edwards, Lewis, McPhail, Muir and Stenlake, 1960; Edwards, Stenlake, Lewis and Stothers, 1961, and earlier papers) are preparing and studying the properties of numerous poly-onium compounds. While their results are not yet complete, certain trends of considerable interest have developed.

A most interesting compound developed recently is cyclo-octadecane-1,10-bis(trimethylammonium iodide) or cyclomethone (VI); it will be apparent that while this compound is related to decamethonium, the ring is the same size as the central ring of tubocurarine [VII]. Lüttringhaus,

Kerp and Preugschas (1957) in Germany have found that this compound acts like tubocurarine in chicks and equals its potency in producing neuromuscular blockade. The Czechs, Votava and Metysová (1959), however, describe it as a short-acting muscle relaxant, only partially antagonised by neostigmine, and consider it to be of the "mixed" type of relaxant, although more closely related in mechanism of action to decamethonium than to tubocurarine, as evaluated by the different sensitivity of different species of animals.



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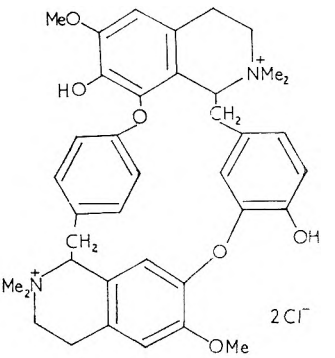

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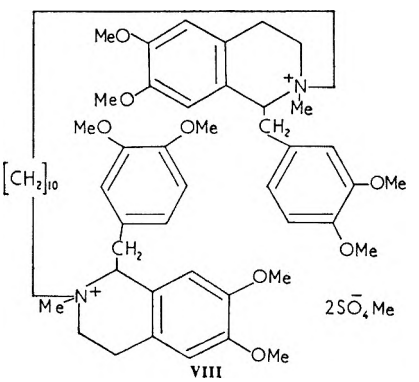


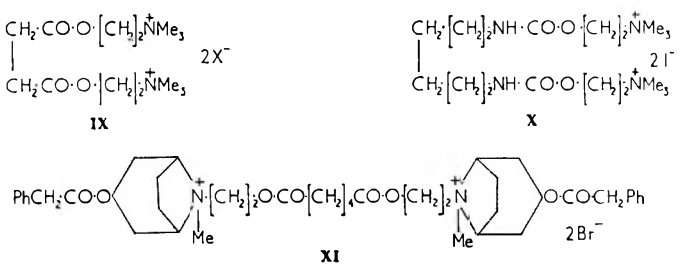
VIII

The replacement of the trimethylammonium groups in decamethonium by heterocyclic quaternary groups was studied by Collier and Taylor (Collier and Taylor, 1949; Taylor and Collier, 1950, 1951), whose results supported the criteria postulated by Craig (1948), particularly in that, ". . . in general all of the really effective curare-like compounds have the nitrogen present in a saturated ring", and " . . . the methoxyl group enhances curare-like activity". Laudexium methylsulphate (Laudolissin), [VIII] emerged from this work. This produces a curare-like paralysis of voluntary muscle, which is somewhat slower in development but of similar duration to tubocurarine itself. Although laudexium achieved a measure of clinical success (see for example Bodman, Morton and

Wylie, 1952; Dundee, Gray and Riding, 1954) it has now been largely superceded by the shorter acting muscle relaxants.

Undoubtedly, the most important development in the use of synthetic neuromuscular blocking agents was the introduction of the short-acting depolarising agent, suxamethonium, B.P. (Scoline, Anectine, Brevedil M.), [IX]. Although suxamethonium had been known for many years, its application to anaesthesia, as a very short-acting muscle relaxant, was not reported until 1949 (Bovet and Bovet-Nitti, 1949). Its very brief duration of action is due to rapid enzymatic destruction, one of the metabolic products being the comparatively inactive monocholine ester of succinic acid (Lehmann and Silk, 1953), and in view of this no antagonist is normally necessary. In addition to its use in minor operations of short duration, the introduction of suxamethonium led to the development of a new technique in the use of relaxants in anaesthesia. Thus suxamethonium is now very widely employed as a relaxant in major surgery in the form of continuous intravenous infusion, using the "drip" technique; the relaxant effect may be halted at any time by merely discontinuing the supply. The use of suxamethonium has been the subject of many excellent reviews (de Beer, Castillo, Phillips, Fanelli, Wnuck and Norton, 1951; Thesleff, 1952; Collier, 1953; Paton, 1953; Randall and Jampolsky, 1953; and Bovet and Bovet-Nitti, 1955).

Practically every conceivable modification of the suxamethonium molecule has been made (see for example Brücke, 1956) but as so often occurs in this field, optimal activity still remains with the parent compound. However, one of these modifications, hexamethylenebiscarbaminoylcholine (Imbretil), [X], which is one of the most potent relaxants known, has achieved some clinical success abroad. (Delaby, Chabrier and Najer, 1953; Brücke and Reis, 1954; Kobinger and Kraupp, 1955; Foldes, Wolfson, Torres-Kay and Monte, 1959; Wiemers and Overbeck, 1960).



Although suxamethonium is probably the most widely used of all the relaxants, nothing is perfect in this imperfect world and suxamethonium is no exception. Not only is there no satisfactory antagonist for those rare occasions on which an antagonist may be required, but, more important, there have been several reports of severe post-operative muscle pains and stiffness after the use of this drug (Churchill-Davidson, 1954; Known, 1959; Leatherdale, Mayhew and Hayton-Williams, 1959; Foster, 1960; Burtles, 1961; Burtles and Tunstall, 1961; Bush and Roth,

1961). Because of this, various workers have attempted to produce a short-acting neuromuscular blocking agent of the curariform type, devoid of these inherent disadvantages. Thus in our laboratories (Collier, Gladych, Macauley and Taylor, 1958, 1959; Brittain, Collier and D'Arcy, 1961), a series of bis-onium salts combining the chemical properties of laudexium and suxamethonium has been prepared and their pharmacology investigated. Although some of these compounds possess curare-like activity none was suitable for clinical use.

Haining and his co-workers in Edinburgh (Haining, Johnston and Smith, 1959) have described a series of bisquaternary tropeines, one of which, *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(3-phenylacetoxytropanium bromide), [XI], combines the short duration of action of suxamethonium with the approximate potency of tubocurarine chloride; this compound is antagonised by neostigmine. It is not yet commercially available, and no indications as to its clinical value were given in the paper on its pharmacological properties (Haining, Johnston and Smith, 1960).

Amongst other onium compounds described by various workers as potential neuromuscular blocking agents are benzoquinonium (Mytolon), (Hoppe, 1950, 1951) and hexafluorenium (Mylaxen), (Cordaro and Arrowood, 1955; Foldes, Molloy, Zsigmond and Zwartz, 1960); however, neither of these drugs has fulfilled its earlier promise.

While it was not intended to include natural compounds in this review, the Erythrina alkaloids are especially worthy of exemption. Although these contain a tertiary nitrogen atom, they possess marked curare-like action (Hanna, Macmillan and McHugo, 1960), which is abolished by quaternisation. This is the only known class of compound that loses curare-like activity on conversion of a tertiary to a quaternary nitrogen. Another natural compound important enough to warrant mention is C-toxiferine I, one of the calabash curare alkaloids. As early as 1953, Waser first suggested that, in view of its extremely high potency and lack of secondary effects, this alkaloid should be investigated in clinical anaesthesia, and two papers have now appeared on this use (Waser and Harbeck, 1959; Foldes, Wolfson and Sorkoll, 1961).

Before concluding the subject of muscle relaxants, their application to the treatment of tetanus should be briefly considered. Curare, in various forms, has been widely used for this purpose since the early 1940's. Woolmer and Cates (1952), and Shackleton (1954) have reported on the treatment of tetanus with suxamethonium; although regarded as a valuable contribution to recovery, the use of this drug presented problems owing to its very short action and the fact that the continuous administration required practically constant medical supervision. Gallamine triethiodide has also been successfully used in the treatment of tetanus (Van Bergen and Buckley, 1952; Parkes, 1954). Honey, Dwyer, Smith and Spalding (1954) have pointed out that a long-acting relaxant is desirable, and tubocurarine has been successfully used by various workers in a number of cases. In addition, Garland (1959) has reported the efficacy of intramuscular injections of laudexium, another long-acting muscle relaxant, in the treatment of this condition.

In spite of the vast amount of work done in the field of neuromuscular blockade, saturation point has not yet been reached and in many research groups there is still enthusiasm for the synthesis and investigation of new neuromuscular blocking agents. This is well exemplified by the various International Symposia held within the last few years, for example in New York 1951, Rio de Janeiro in 1957, and Venice in 1958, and by the numerous books and review articles that have been published (for example Foldes, 1957; Bovet, Bovet-Nitti and Marini-Bettòlo, 1959; Lewis and Muir, 1959; Adriani, 1960; Davis, 1960; Foldes, 1960). In addition, a whole issue of *Anesthesiology* (**20**, (4), 1959) and a complete issue of *Studii si Cercetari de Biochimie* (**3**, (4), 1960) have been devoted to the study of muscle relaxants.

Ganglionic Blocking Agents

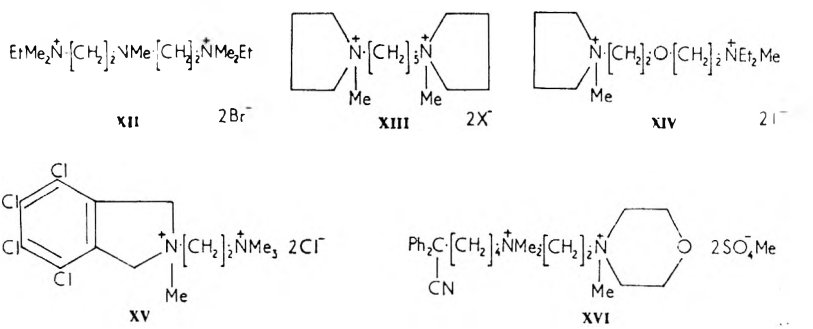
Quaternary ammonium compounds have achieved success as ganglionic blocking agents and since this class of drug still continues to be of importance in the treatment of hypertension, it is inevitable that much of this section must review the use of onium salts as hypotensive agents.

In a classical paper, Burn and Dale (1914) compared the effects of tetramethyl- and tetraethylammonium salts on blood pressure, drawing attention to the almost specific ganglionic blocking action of the tetraethylammonium salt (TEA). Some 30 years later Acheson and Moe (1946) and Acheson and Pereira (1946) re-examined the properties of TEA and showed that it produced ganglionic blockade without the preliminary stimulation normally produced by nicotine, and occasionally by the curare alkaloids. Although this compound has been used in man for the treatment of peripheral vascular disease (Berry, Campbell, Lyons, Moe and Sutler, 1946; Boyd, Crawshaw, Ratcliffe and Jepson, 1948) and of hypertension (Berry and others, 1946; Lyons, Moe, Neligh, Hoobler, Campbell, Berry and Rennick, 1947), it has never achieved wide clinical recognition. None of the numerous congeners of TEA has been found to be better than the parent compound (Moe and Freyburger, 1950).

The introduction of the methonium salts by Barlow and Ing (1948) and by Paton and Zaimis (1948) renewed interest in the ganglionic blocking agents, and initiated a vast new field of research. Paton and Zaimis (1949) showed that the activities of the methonium series varied with the number of methylene groups in the chain. The decamethylene member is a potent neuromuscular blocking agent, while those compounds with a shorter chain, particularly the tetra-, penta-, and hexamethylene compounds, have a marked ganglionic blocking effect. An excellent review on the methonium salts has been published by Paton and Zaimis (1952). The hexamethylene member (hexamethonium, C_6) achieved an initial success in the treatment of hypertension and is still official in the British Pharmacopoeia, although in recent years its use has diminished. A very interesting paper was published by Gill and Ing (1958), in which they discussed the mode of action of hexamethonium on the ganglia. Much work has been undertaken on modifications of the methonium molecules, both in altering the alkyl groups in the quaternary structure, and in replacing one

or more of the methylene groups in the chain by various hetero atoms or groups. Generally little appreciable improvement in activity over that of the parent substances has been achieved by these changes. However, there is one exception, namely when the central methylene group in the pentamethonium series is replaced by an *N*-methyl group, one of the products, 3-methyl-3-azapentamethylene-1,5-bis(ethyldimethylammonium bromide) (azamethonium, Pendiomide) [XII] shows potent ganglionic blocking activity (Bein and Meier, 1950, 1951; Haley, Leitch, McCormick and McCulloh, 1954), although Smirk (1952) has found it to be less effective than hexamethonium. Azemethonium is not commercially available in this country.

An important step in the development of clinically useful ganglionic blocking agents was made by Libman, Pain, and Slack (1952), who replaced the trialkylammonium groups of the methonium salts by heterocyclic nuclei. The activity of one of these compounds, pentamethylenebis-(1-methylpyrrolidinium halide), [XIII] is about five times that of hexamethonium on the nictitating membrane of the cat (Wien and Mason, 1953; Mason and Wien, 1955). This compound, pentolinium (Ansolysen) has largely replaced hexamethonium in the treatment of hypertension; although pentolinium shows some side effects these are less severe than those exhibited by some other hypotensive agents, for example the non-quaternary drug, mecamylamine (Sears, Snow and Houston, 1959).



XII XIII XIV XV XVI

An interesting unsymmetrical compound related to both pentolinium and the methonium salts was described by Frommel and his associates (Frommel, Vincent, Gold, Melkonian, Radouco-Thomas, Meyer, de Quay and Vallette, 1955; Frommel, Vincent, Radouco-Thomas, von Allmen and Vallette, 1955) although this compound, 3-oxapentamethylene-1-1(1-methylpyrrolidinium-5-(methyldiethylammonium) di-iodide, [XIV], has not achieved any notable clinical success. A more important unsymmetrical compound is 4,5,6,7-tetrachloro-2-(2-dimethylaminoethyl) isoindoline dimethochloride (chlorisondamine, Ecolid), [XV]. In animal experiments, this compound has been shown to be a long-acting, orally effective ganglionic blocking agent (Plummer, Trapold, Schneider, Maxwell and Earl, 1955; Maxwell, Plummer and Osborne, 1956). In clinical practice this compound has been shown to be a powerful hypotensive agent with consistent activity when given by mouth, and, moreover,

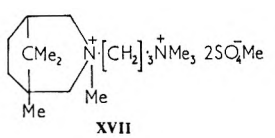
parenteral administration is some fifteen times more effective than oral. Chlorisondamine is more potent than pentolinium and has a longer duration of action, although it may give rise to more severe side effects. (Grimson, Tarazi and Frazer, 1955a, 1955b; Maxwell and Howie, 1955; Winsor, 1955; Smirk and Hamilton, 1956).

Adamson, Billinghurst, Green and Locket (1956) have described another interesting group of unsymmetrical bis-onium salts containing a benzhydrylalkyl structure, and satisfactory clinical trials of two members of this group in the treatment of hypertension were described by Locket (1956). More recently, McKendrick and Jones (1958) have reported a clinical study on the most active member of this series, *N'*-(5-cyano-5,5-diphenylpentyl)-*N'N'N''*-trimethylethylene-1-ammonium-2-morpholinium bismethylsulphate (pentacynium, Presidal), [XVI], in the management of hypertension and have found this agent to be of value; its side effects however, are similar to those of other ganglionic blocking agents. In a later publication, Locket (1958) has evaluated the hypotensive efficacy of compound 189c56, the 4,4-dichlorophenyl analogue of pentacynium, and has reported very favourably on this agent; it is invariably effective when given by mouth, and in a single daily dose it consistently produces the expected degree of hypotension. However, the dosage of this drug would seem to be highly critical since a slight alteration in the controlling dosage leads to a marked effect on the extent and duration of the hypotension.

In recent years there has been a tendency to drift away from the use of quaternaries for treatment of hypertension, notable and successful examples of this being the development of mecamylamine and more recently pempidine (Lee, Wragg, Corne, Edge and Reading, 1958; Spinks and Young, 1958). Indeed, the quaternisation of pempidine (Bretherick, Lee, Lunt, Wragg and Edge, 1959) while increasing the ganglionic blocking activity, greatly diminished the duration of action and increased the oral toxicity. In spite of this trend, however, new quaternaries still emerge. For example, γ-trimethylammoniumpropyl-*N*-methylcamphidonium dimethylsulphate, (trimethidinium, Camphidonium, Ostensin) [XVII], which bears a superficial resemblance to the potent secondary amine, mecamylamine, has been recently clinically evaluated (Dunsmore, Dunsmore, Goldman, Elias and Warner, 1958; Borhani, 1959). Houston and Sears (1960) have shown that while the prolonged action of this drug was a great help, its main drawback, especially in high dosage, was its irregular absorption. This was not a major source of difficulty but it occurred sufficiently often to be a potential hazard to patients. Moreover, trimethidium sometimes gave irregular severe postural hypotension.

A recent development has been the introduction of bretylium tosylate (Darenthin) [XVIII], which is a hypotensive onium salt with a new type of action. It is one of a series of benzyl quaternary ammonium compounds described by Boura and his associates (Boura, Copp and Green, 1959; Boura, Green, McCoubrey, Laurence, Moulton and Rosenheim, 1959); the pharmacology of this compound was investigated by Boura and Green (1959) and others (Boura, Copp, Duncombe, Green and McCoubrey, 1960; Duncombe and McCoubrey, 1960). In animal experiments,

bretylium selectively blocks the peripheral sympathetic nervous system by an action on the adrenergic nerves, in which it selectively accumulates; it does not inhibit the effects of circulating or injected adrenaline or noradrenaline. Bretylium has no apparent effect on the parasympathetic or central nervous systems. Boura, Green, and others (1959) found this drug to be orally effective in man, and recommended its extensive trial in the treatment of hypertension. However, later reports by other workers were not so favourable (Dollery, Emslie-Smith and McMichaels, 1960; Evanson and Sears, 1960; Hurley, Page and Dustan, 1960; Lowther and Turner, 1960). Although by the elimination of parasympathetic blockade, the development of bretylium marked a "break through" in hypotensive therapy, it must be accepted in the face of this evidence that in its present form the drug is unsuitable for long-term treatment of hypertensive patients (Leishman, 1961), despite the fact that some patients who have been submitted to unremitting ganglionic blockade for years are almost lyrical in their enthusiasm for bretylium (Dollery, 1960). Even now there is still interest in the clinical possibilities of bretylium, and recent notes have discussed the mechanism of the acquired tolerance that develops in hypertensive patients who initially respond to the drug (Green, 1961; Laurence and Nagle, 1961; Lowe, 1961; Montuschi, 1961; Zaimis, 1961).



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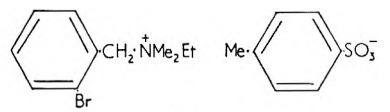

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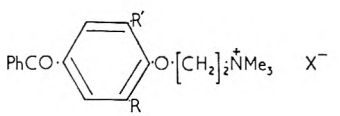
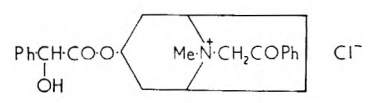


XIX



XX

Boura and his co-workers (Boura, Coker, Copp, Duncombe, Elphick, Green and McCoubrey, 1960) have continued to investigate the hypotensive activity of monoquaternaries, and have recently described the compound 172C58 [XIX, R = R′ = Me], which is a less active hypotensive agent than bretylium; its main value would seem to be as a pharmacological tool, its advantages over bretylium being its more rapid and readily reversible action and its freedom from sympathomimetic properties. The same workers have also investigated the related compound 225C59 [XIX, R = H; R′ = Br], which although possessing similar adrenergic neurone-blocking properties, produces muscarine-like effects at high dosage. Many chemical variations on the compound 172C58, which included branching or lengthening the chain, additional substitution or hydrogenation of the 4-benzoyl group and several variations of the groups in the 2- and 6-positions and in the cationic head, have led to lower activities.

The plethora of commercially available antihypertensive agents reflects both the difficulties encountered in the treatment of hypertension and the absence of the ideal drug. A further onium salt now available is phenacyl homatropinium chloride (phenactropinium, Trophenium) [XX], a quaternary derivative of homatropine; this is a powerful ganglionic blocking agent which was recommended by Robertson, Gillies and Spencer (1957) for use in the production of controlled hypotension during surgery. Eyre-Walker (1961) has reported favourably on its use in gynaecological cases. In addition to its ganglionic blocking and antihypertensive actions, phenactropinium is a powerful inhibitor of cholinesterase and pseudocholinesterase (Lehmann and Patston, 1958), which suggests that it may, under certain circumstances, potentiate the neuromuscular blocking action of suxamethonium.

Nádor and Gyermek (1958) have reported on the activities of a series of quaternary derivatives of atropine, structurally related, to some extent, to phenactropinium. Of these compounds the one most worthy of note is Gastropin, the 4-diphenylmethyl quaternary derivative of atropine, which combines marked ganglionic blocking activity with only slight parasympathetic paralysing action.

It is evident that the number of onium compounds with ganglionic blocking and hypotensive activity is large and continually increasing. Moreover, except in certain limited examples, there is no well defined structure-activity relationship, and it is therefore unlikely that activity in compounds of widely different structure can be interpreted in terms of a single mechanism of action. However, in the case of bisquaternary compounds, the relationship between ganglionic blocking activity and inter-quaternary distance has been discussed by Gill (1959).

The laboratory evaluation of ganglionic blocking agents (Lewis and Muir, 1960) and their clinical use in the treatment of hypertension (Birchall, Weber and Batson, 1956; Liertzer, 1957; Page, 1957; Turner, 1959; Mackinnon and Hammond, 1960; Leishman, 1961; Smirk, 1961; Turner and Lowther, 1961; Wien, 1961) have formed the subject of several excellent reviews within the last 5 years. In addition, the proceedings of a symposium on hypotensive drugs and the control of vascular tone in hypertension has been published (Harington, 1956).

Anti-acetylcholine Agents and Anticholinesterases

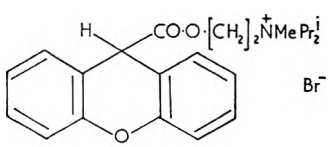
The effects of anti-acetylcholine agents on the body resemble the effects produced by cutting the parasympathetic nerve supply to the various tissues and organs. This anti-acetylcholine effect does not arise from a suppression of acetylcholine formation but from a failure of the liberated acetylcholine to stimulate the effector cell. Drugs of the atropine-alkaloid series are classic members of this group. The anti-acetylcholine action of atropine cannot be fully utilised in clinical practice because of the numerous side-effects of the drug. Attempts have been made to synthesise compounds in which spasmolytic properties predominate specifically over undesirable effects on the cardiovascular system; the resulting group of compounds form the so called "spasmolytic" drugs,

which have wide application in the treatment of gastrointestinal disorders and of certain spastic disorders of the biliary and genito-urinary tracts. It is important at this stage to draw a distinction between the two types of spasmolytic drug, the atropine-like drug (neurotropic), and the papaverine-like (musculotropic); it is in the former class that onium compounds occur.

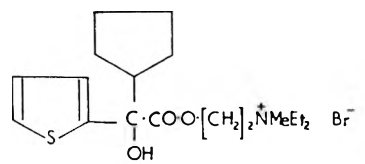
It was amongst tropic esters of amino alcohols that useful synthetic spasmolytics were first discovered, for example the γ-dimethyl-amino-neopentyl ester (amprotropine phosphate, Syntropan) (Fromherz, 1933, 1934, 1937). Compounds based upon atropine such as methylatropinium nitrate (Eumydrine) and mandelyltropine methobromide (Novatropine) followed, but did not achieve any notable success. More successful compounds were the simple quaternary derivatives of hyoscine, for example the methobromide (methscopolamine, Pamine) and the butobromide (Buscopan).

Methanthelinium bromide (Banthine), while once widely used in the treatment of peptic ulcers, has now been largely replaced by the analogue, β-di-isopropylaminoethyl xanthen-9-carboxylate methobromide (propantheline, Pro-Banthine) [XXI], which is more potent, and exhibits less severe, although not less frequent, side effects. Scott and Sutherland (1956) have reported that the pain relieving properties of propantheline rendered it a valuable addition to current methods of treatment of duodenal ulceration.

Another potent gastric antisecretory and spasmolytic agent in man (Kirsner and Palmer, 1953) is penthienate bromide (Monodral) [XXII], the pharmacology of which has been described by Luduena and Lands (1954). A further effective onium salt is oxyphenonium bromide (Antrenyl), [XXIII], which after satisfactory pharmacological investiga-



XXI



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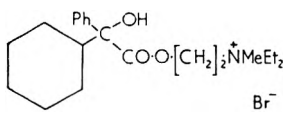

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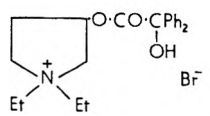
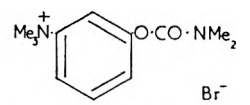


XXIV



XXV

tions (Plummer, Barrett, Rutledge and Yonkman, 1953; Barrett, Rutledge, Plummer and Yonkman, 1953), was shown to have spasmolytic properties comparable to atropine, and to give good results in the treatment of peptic ulcer in man, its freedom from bitterness being a distinct advantage in its use (Rowen, Bachrach, Halsted and Schapiro, 1953).

This compound has also been recommended for pre-anaesthetic medication to reduce salivary secretion and to prevent laryngospasm or bronchospasm (Stephen, Bowers, Nowill and Martin, 1956).

Several other onium salts are to be found in this class of drug, but most of these have achieved only partial or moderate success in their clinical use. Specific examples are, 3-diethylamino-1-phenyl-1-cyclohexyl propan-1-ol ethochloride (tridihexethyl, Pathilon); 3-pyrrolidino-1-phenyl-1-cyclohexyl propan-1-ol methochloride (tricyclamol, Elorine); 4-diphenylmethylene-1,1-dimethylpiperidinium methosulphate (diphemanil, Prantal); 1-(3-hydroxy-5-methyl-4-phenylhexyl) piperidine methobromide (Darstine); and 1-ethyl-3-piperidyl benzilate methobromide (pipenzolate, Piptal). Recently, Sterkel, Brucker and Knight (1958) have described the benzilate of 1,1-diethyl-3-hydroxypyrrolidinium bromide (benzilonium bromide, Portyn) [XXIV] as a potent anti-acetylcholine agent, useful in the treatment of duodenal ulcer; this drug became commercially available only last year.

As with hypotensive agents, there is a very large number of drugs, both quaternary and non-quaternary, available for use in the treatment of the various ulcerous conditions which specifically respond to anti-acetylcholine drugs. It would seem that the ideal drug for the treatment of peptic and duodenal ulcers has yet to be found, which is not surprising since, in spite of the high incidence of this complaint and in spite of much intensive research, the cause is still uncertain. The use of anti-acetylcholine drugs in the treatment of these ulcers has been the subject of several reviews (Cayer, 1956; Roth, Wechsler and Bockus, 1956; Texter and Ruffin, 1956; Texter, Smith and Barborka, 1956; Bachrach, 1958), although Hadley (1961) in discussing the medical treatment of peptic ulcer, has stated that "drugs are relatively unimportant".

It would seem that quaternary ammonium anti-acetylcholine drugs have been mainly used in the treatment of gastrointestinal ulcers, and have played little part in the wider spectrum of application common to many of the non-quaternary anticholinergic drugs. For example, anti-acetylcholine drugs have an accepted rôle in relieving the rigidity and tremor of postencephalitic Parkinson's disease (paralysis agitans), and other basal ganglion disorders; in addition these drugs have been found to be successful in controlling or mitigating the Parkinson-like syndrome that may occur with continued high dosage of the phenothiazine tranquillisers. While many successful drugs in the treatment of Parkinsonism have been acid addition salts of tertiary amines, there seems to be little or no evidence that quaternisation of these bases produces agents of any therapeutic value.

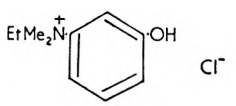
An interesting example of the diversity of action of onium salts is the fact that, although some show potent anti-acetylcholine action, others potentiate the action of acetylcholine by their anticholinesterase activity. The most important natural anticholinesterase is physostigmine (eserine), one of six alkaloids isolated from the seeds of *Physostigma venenosum*, a perennial vine found in West Africa. This alkaloid achieved early notoriety from the use of the seed—the Calabar bean—as an ordeal poison

in native witchcraft trials. As early as 1863, Robertson recorded strong miosis when treating himself with an extract of the bean, and as a consequence suggested its use in ophthalmology. Stedman (1926), and Stedman and Stedman (1929), attempted to discover the part of the molecule which was responsible for activity and found esters of *N*-methylcarbamic acid to be the most effective of the compounds studied. Further investigations on dialkylcarbamic esters were carried out by Aeschlimann and Reinert (1931) who found the most active anticholinesterase to be the quaternary, neostigmine (Prostigmine) [XXV]. This drug was originally introduced into therapeutics for its stimulant action on the intestinal tract; some 4 years later it was found to be markedly effective in restoring muscle strength in myasthenia gravis (Walker, 1935), and superior in its action to physostigmine. Its use has continued in the diagnosis and treatment of this condition. With the introduction of the curare-like neuromuscular blocking drugs into clinical practice, this very versatile onium salt has fulfilled a further rôle as an antagonist to these agents. Much work has since been done on the development of other synthetic anticurare agents; edrophonium (Tensilon) [XXVI] was discovered as a curare antagonist following a systematic analysis of the pharmacological activity of phenolic onium salts closely related to neostigmine (Randall, 1950), and has since been introduced as a therapeutic agent with a selective action on the skeletal myoneural junction. Osserman and Teng (1956) have described the successful use of edrophonium as a diagnostic agent for myasthenia gravis in over 300 patients, and also reported on its value in the differential diagnosis of myasthenic from cholinergic crisis. Recent experimental studies have been made by Nastuk and Alving (1958–59), who investigated the properties of edrophonium and some closely related analogues on activity at the neuromuscular junction, particularly in augmenting muscle tension output, the time course of this augmentation effect, and the anticurare activity.

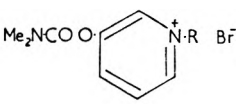
Various heterocyclic analogues of neostigmine have been studied and some have achieved clinical success. Thus benzpyrinium bromide (Stigmonene) [XXVII, R = CH_2-Ph] has been used in the United States as an anticholinesterase drug for the treatment of post-operative abdominal distention and urine retention (Anon, 1951); however, it does not appear to have been used in this country. One of the main disadvantages of neostigmine is its short duration of action, and pyridostigmine (Mestinon) [XXVII, R = Me] was introduced by Tether (1954), who found it to have a rather longer action and to be less apt to cause side-effects on the alimentary canal. Later reports have confirmed that pyridostigmine offers definite advantages over neostigmine in the treatment of myasthenia gravis (Churchill-Davidson and Richardson, 1955; Lange, 1955; Tether, 1956).

Ambenonium chloride (Mytelase) [XXVIII], introduced for the management of myasthenia gravis by Schwab, Marshall and Timberlake (1955), resembles pyridostigmine in that it also is a cholinesterase inhibitor having activity similar to that of neostigmine. As with pyridostigmine, the somewhat more sustained action and lower incidence of gastrointestinal

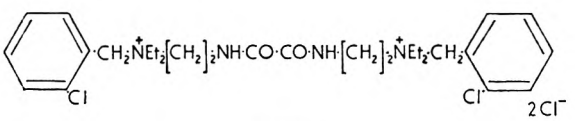
side-effects of ambenonium offer distinct advantages when compared with neostigmine. The pharmacology of ambenonium has been described by Lands, Karczmar and their associates (Lands, Karczmar, Howard and Arnold, 1955; Karczmar, 1957; Lands, Hoppe, Karczmar and Arnold, 1957; Lands, Hoppe, Arnold and Kirchner, 1958). More recently Blaber (1960) has investigated the antagonism of muscle relaxants by ambenonium and its methoxy analogue in the cat, and found that although both compounds possessed anticholinesterase activity, there was no correlation between their relative abilities to antagonise tubocurarine paralysis and their abilities to inhibit muscle cholinesterase *in vitro*.



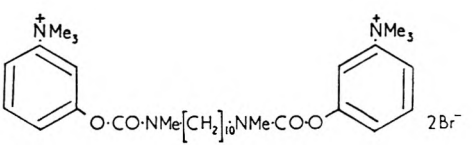
XXVI



XXVII



XXVIII



XXIX

It is evident, therefore, that of the drugs used in the treatment of myasthenia gravis, onium salts derived initially from physostigmine seem to be the only really successful agents; although organophosphorus cholinesterase inhibitors have been investigated in this context, they have been found to be uncertain in their action and liable to frequent and uncontrollable toxic effects. The status of drugs currently used in the treatment of myasthenia gravis has been summarised by Turner (1959), and a most important review on synthetic analogues of physostigmine has been made by Stempel and Aeschlimann (1956). A general review by Holmstedt (1959), although dealing primarily with the pharmacology of organophosphorus cholinesterase inhibitors, does contain a section on quaternary ammonium anticholinesterases.

So far, we have dealt primarily with the use of anticholinesterase onium salts in the management of myasthenia gravis, and as antagonists to curare-like agents. Anticholinesterases are however, of value in the treatment of glaucoma, and both physostigmine and neostigmine have been used for this purpose. Whereas both of these have a short duration of action, decamethylenebis(*m*-dimethylaminophenyl-*N*-methylcarbamate)

dimethobromide (Demecarium bromide) [XXIX], a potent and long-acting anticholinesterase, has recently been found by Krishna and Leopold (1960) to be an effective agent in the control of glaucoma, confirming reports by earlier workers. This drug offers distinct advantages over the established agents, although it does possess certain disadvantages which necessitate caution in its use.

A novel and recent development in the design of synthetic quaternary ammonium anticholinesterases has been described by Thomas (1961a, b), who has reported on the activities of a series of heterocyclic spiran quaternary ammonium salts, in which the two rings are linked together by a common nitrogen atom, thus providing a rigid molecule. Although as yet there is no evidence that compounds of this general type may eventually prove to be of therapeutic value, this structure does offer new and interesting stereochemical possibilities.

RESEARCH PAPERS

THE ALKALOIDS OF THE GENUS *DATURA*, SECTION BRUGMANSIA

PART I. *D. CORNIGERA* HOOK

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From the Department of Pharmacy, University of Nottingham

Received September 15, 1961

Hyoscine has been isolated as the main alkaloid from the leaves, flowers, pericarp and seeds of *Datura cornigera* Hook. Noratropine occurs in the leaves, flowers and pericarp. The following alkaloids have been obtained from the roots, (−)-3α,6β-ditigloyloxytropane, 7-hydroxy-3,6-ditigloyloxytropane, hyoscine, hyoscyamine, atropine, norhyoscyamine, noratropine, 3α,6β-dihydroxytropane and the presence of others indicated. Hyoscyamine is the principal alkaloid of the whole roots but the root-wood, although giving a low yield of total alkaloids, contains a relatively high proportion of noratropine.

THE taxonomy of the *Brugmansia* group of species presents certain difficulties, the group being variously regarded as a section of the genus *Datura* (Bernhardi, 1833; Safford, 1921) or as having generic rank (Persoon, 1805; Lagerheim, 1895; van Zijp, 1920; van Steenis, 1930–31). Its members are widely distributed throughout Central and South America where they have been cultivated as ornamental plants and used by the Indians for their narcotic properties (Safford, 1920). Safford (1921) listed 14 species of this Section and Barclay (1959) has recently described a new species, *D. vulcanicola*. A number of the cultivated forms have been discussed by De Wolf (1956). Satina and Avery (1959) have indicated that more studies are necessary to clarify the taxonomy of the genus and in particular the relationship of the *Brugmansia* group of species to the other Sections. They suggest a fresh approach with new criteria and data because a classification based almost exclusively on morphological descriptions and on phenotypical resemblances and differences is apparently insufficient for the proper determination of these solanaceous species. But, for the present, it is necessary to identify species as adequately as possible by reference to the information available and, for this reason, we have given below, those features of the plant on which we have based our identification. *D. cornigera* was described by Hooker (1846) from a cultivated plant of unknown origin and was later recorded by Hemsley in 1882 in the valley of Mexico (Matuda, 1952) and by Lagerheim (1895) as cultivated around Quito and probably occurring wild somewhere in the forests of Ecuador. The species is closely related to *D. arborea* L.

No chemical investigation of *D. cornigera* appears to have been reported but it is pertinent to record the results of investigations carried out on the so-called *D. arborea*. Kircher (1905) investigated the distribution of alkaloids in *D. arborea*—"also known as *Brugmansia candida*". If the material was *B. candida* Pers., it can be regarded as *D. arborea* Ruiz and

Pavon., a plant having larger flowers than *D. arborea* L., with the margins of the limb between the corolla teeth being entire or rounded. Kircher's description of the plant does not make complete identification possible but the leaves with soft hairs on both sides are not consistent with De Wolf's (1956) characters of *D. arborea* L. The plants were grown in the botanical garden, Marburg and found to contain mainly hyoscine in the leaves and flowers and, hyoscine with some hyoscyamine in the young stems and roots. In contrast Schmidt and Kircher (1906) found the seeds of *D. arborea*, obtained from "foreign" plants, to contain hyoscine and hyoscyamine in the ratio 1 : 4. Montesinos (1939) reported "Datura Arbórea" of Peru to contain in the roots, leaves, flowers and seeds, 0·16, 0·15, 0·116 and 0·12 per cent alkaloids respectively. In the leaves hyoscine was the main alkaloid, together with small quantities of atropine and hyoscyamine. Three varieties of the plant are mentioned differing in the form of the flowers; from the photographs they could be *D. arborea* L., the double-flowered form of *D. cornigera*, often known as *D. knightii* and *D. candida* respectively. Montesinos used chiefly the third variety for his studies. Barriga Villalba, Medina and Albarracin (1945) give the alkaloidal content of the dry flowers, leaves and berries of *D. arborea* as 0·490, 0·287 and 0·063 per cent respectively, with hyoscine as the main alkaloid. In contrast Suárez (1952) found no significant amounts of alkaloid in a cultivated variety of *D. arborea* and, in a phytochemical investigation of a Peruvian sample, Aguero (1943) records only 0·02 per cent of alkaloids. Chlorogenic acid (Politas, 1948), scopoletin and aesculetin (Kala, 1958) have also been reported as constituents of *D. arborea.*

In view of this rather limited knowledge concerning the alkaloidal constituents of the white flowered tree daturas the following investigation on *D. cornigera* has been undertaken as part of a more extensive phytochemical examination of the whole genus.

Plant Material

The original seed samples were obtained in 1953 from Cochabamba, Bolivia and first year plants raised in 1954. Subsequent propagation was by cuttings and, more recently, by seed produced by the latter. Two leaf-varieties were evident among the plants, one with leaves having a sinuate margin and a covering of soft hairs (Type I) and the other with angular-toothed leaves and a rough texture (Type II). The flowers of both varieties were similar in form and in measurements of peduncle, calyx, corolla, corolla lobe, pistil, stamens and anthers. Type I produced more flowers than Type II; no fruits could be obtained from either variety by self-pollination but by cross-pollination of the two varieties, Type I bore fruits with viable seeds. This is consistent with the field studies of Barclay and Schultes who tell us that they find that in the wild state, the *Brugmansia* often require cross-pollination between different clones for the successful production of fruits.

We have identified these plants as *D. cornigera* Hook, a species very closely related to *D. arborea* L. and possibly a cultivar of it. The material

agrees well with Hooker's (1846) description of the species and is identical with his illustration. The plants can be distinguished from the other white-flowered forms of tree daturas by the following points. The deciduous calyx which is not toothed at the apex and the size of the flower are inconsistent with *D. candida* (Pers.) Safford (synonyms *D. arborea* Ruiz and Pavon; *Brugmansia candida* Pers.). The long calyx (up to 19 cm.) tapering to a horn-like recurved point, differentiates the plant from *D. arborea* L. which has a relatively short calyx and no horn-like point and from *D. affinis* Safford (synonym *Brugmansia arborea* Lagerh.) which also has a short calyx (8·5–10·0 cm.) but with five teeth. Hooker records no measurements for the flowers but Lagerheim (1895) in his study of the Ecuadorian species differentiates more fully between *D. cornigera* Hook. and a plant he regarded as *D. arborea* L., but which Safford (1921) subsequently renamed *D. affinis*. Our plants agree well with Lagerheim's description of *D. cornigera* with the exception of lengths of corolla and stamens which are intermediate between the values he records for the two species. Neither of the two types of leaves shown by Type I and Type II is inconsistent with the descriptions of Hooker and Lagerheim but leaf-shape as a distinguishing feature (De Wolf, 1956) would seem to be of doubtful value. Other white-flowered species, also differentiated in Safford's key by the toothed calyx but having other marked differences from *D. cornigera* are *D. suaveolens* Humb. and Bonpl., *D. dolichocarpa* (Lagerh.) Safford and *D. longifolia* (Lagerh.) Safford.

Experimental

Alkaloids of the Aerial Parts

The air-dry, coarsely-powdered leaves and small stems (500 g.) of *D. cornigera* were moistened with water (200 ml.) and stored overnight. Calcium hydroxide (30 g.) was stirred in and the mixture macerated with solvent ether (about 500 ml.) for 3 hr. The supernatant ether was drained off and the remaining alkaloids extracted by percolation with more ether (about 6 litres). Evaporation of the combined ether extracts to about 100 ml. caused deposition of solid material which was removed by filtration through paper. The final concentrate was passed through a column of purified kieselguhr (60 g.) supporting 5N sulphuric acid (30 ml.), ether (460 ml.) being used to elute most of the pigments and ammoniacal chloroform (2 litres) to collect the alkaloids. Removal of the chloroform afforded 1·9 g. of a greenish-brown gummy residue which was then fractionated by partition chromatography.

In a typical small-scale experiment the basic residue (0·12 g.) was treated with ether (2 ml.) and a few drops of chloroform to effect solution and poured on to a column of kieselguhr (20 g.) loaded with 0·5M phosphate buffer solution (10 ml.), pH 6·6. The chromatogram was developed successively with light petroleum (b.p. 60–80°), ether, and chloroform. The eluate was collected in 5 ml. fractions, each titrated with 0·005N sulphuric acid with bromocresol green as indicator and the separated alkaloids recovered as described previously (Evans and Wellendorf, 1959).

A small quantity of alkaloid having a high R_F value by paper chromatography* was recovered from the light petroleum eluate but no crystalline derivatives of it could be prepared. The titration curve obtained from the ether eluate showed a large peak followed by two smaller ones. The base corresponding to the first peak was identified as (−)-hyoscine by the preparation of hyoscine picrate m.p. 187–188°, undepressed on admixture with authentic (−)-hyoscine picrate and hyoscine aurichloride, m.p. and mixed m.p. with authentic material 204°. The oily base derived from the eluate corresponding to the second ether peak, when neutralised with dilute sulphuric and treated with sodium picrate, afforded prisms m.p. 229–230° undepressed on admixture with the alkaloid picrate m.p. 230°, of unknown constitution, previously derived from other species of *Datura* (Evans and Partridge, 1949; Evans and Wellendorf, 1959). No crystalline derivatives could be obtained from the eluate corresponding to the third ether peak. Titration of the fractions of chloroform eluate indicated at least four bases; no crystalline derivatives could be obtained for the first three of these but the last furnished a picrate, needles from aqueous ethanol, m.p. 226° undepressed on admixture with noratropine picrate, m.p. 227°.

TABLE I

DISTRIBUTION OF PRINCIPAL ALKALOIDS IN *Datura cornigera* HOOK.

	I*	II	III	IV	V	VI	VII
Leafy shoots	0·27			0·19		<0·01	0·06
Stems	0·23			0·13		<0·01	0·08
Flowers	0·96			0·57		0·12	0·24
Pericarp	0·50			0·24		0·13	0·11
Seeds—Bolivian sample, 8 years old	0·14			0·11			0·03
Seeds—Nottingham sample, viable	0·65			0·52			0·11
Roots—plants raised in the field from cuttings, 1 year old	0·95	0·03	0·05	0·12	0·48	0·09	0·17
Roots—plants 6 years old, raised under glass	0·24	0·01		0·06	0·06	0·07	0·04
Root-wood—plants 6 years old raised under glass	0·11	<0·01				0·05	0·05

* I, total alkaloids calculated as hyoscyamine; II, (−)-3α,6β-ditigloyloxytropane; III, 7-hydroxy-3,6 ditigloyloxytropane; IV, hyoscine; V, hyoscyamine and/or atropine; VI, norhyoscyamine and/or noratropine; VII, other alkaloids calculated as hyoscyamine. All per cent.

Samples (3 g.) of the ripe seeds produced in Nottingham and of the original seed-sample from Bolivia, 8 years old and no longer viable, were analysed by the method of Evans and Partridge (1952) with the modifications that light petroleum (b.p. 60–80°) replaced carbon tetrachloride and that the eluate was collected in 5 ml. fractions which were titrated individually. No bases were eluted with light petroleum. The ether eluate gave on the titration curve one very small peak which was followed by a main peak corresponding to hyoscine, m.p. and mixed m.p. of the picrate 186–187°. The chloroform contained very little alkaloid. A similar examination of the pericarp (5 g.) gave hyoscine as the main alkaloid, m.p. and mixed m.p. of the picrate 186–187° with two smaller peaks in the chloroform fraction, the second of which appeared to be noratropine,

* All paper chromatograms were prepared by the ascending method using light petroleum (b.p. 60–80°), amyl alcohol, glacial acetic acid and water (1 : 3 : 3 : 3) as the developing mixture.

m.p. of the picrate 224–225°, softening at 218° undepressed on admixture with noratropine picrate, m.p. 226–227°. The alkaloid mixture in the flowers (5 g.) was similar to that of the pericarp. The quantitative results for these determinations, together with similar ones for the leaves and stems are recorded in Table I.

Alkaloids of the Roots

Preliminary experiments involving partition chromatography of an ether extract of *D. cornigera* roots at pH 6·6 indicated the presence of at least 10 bases in the mixture. For a more detailed study of the individual alkaloids, powdered roots (600 g.) from plants one year old were moistened with water (300 ml.) and allowed to stand overnight. Calcium hydroxide (30 g.) was mixed in, the powder transferred to a percolator and the basic constituents extracted with ether (9 litres). Concentration of the percolate to about 200 ml. caused the deposition of some solid material; this was removed by filtration and the alkaloids collected in 0·1N sulphuric acid. The acidic solution was repeatedly shaken with chloroform and the separated chloroform extract (1·75 litres) basified with ammonia, washed with water and the solvent removed *in vacuo* leaving a light-brown residue (0·8 g.) designated "Fraction A". The yellow-brown, acid solution was made alkaline by the addition of a strong solution of ammonia and the liberated alkaloids collected in chloroform (2 litres). Removal of the chloroform under reduced pressure afforded a deep-brown syrupy residue (4·7 g.) designated "Fraction B". The marc remaining from the ether extraction was percolated with industrial methylated spirit (5 litres) and the solvent removed *in vacuo* from the extract leaving a brown, semi-solid residue (47·7 g.)—"Fraction C". Paper chromatography of these fractions showed A to contain mainly bases of high R_F values, B intermediate R_F values and C, low R_F values.

Fraction A was dissolved in chloroform (3 ml.) and submitted to partition chromatography on kieselguhr (30 g.) loaded with 0·5M phosphate buffer solution (15 ml.), pH 5·4; light petroleum (b.p. 60–80°), ether, and chloroform were used successively as developing solvents. Two bases were evident in the petroleum ether fraction. The first (initial 60 ml. eluate) was isolated as a colourless gummy mass (0·03 per cent) from the titration liquors and identified as (−)-3α,6β-ditigloyloxytropane by the preparation of the following derivatives: picrate, filamentous needles from aqueous ethanol, m.p. and mixed m.p. with authentic (−)-3α,6β-ditigloyloxytropane picrate, 151° (Found: C, 52·4; H, 5·3. Calc. for $C_{18}H_{27}NO_4,C_6H_3N_3O_7$: C, 52·4; H, 5·5 per cent); chloroplatinate, orange rosettes from dilute hydrochloric acid, m.p. 230° (decomp.). The addition of a saturated solution of ammonium reineckate to a neutral solution of the base afforded a *reineckate*, micro-rosettes from 20 per cent aqueous acetone, m.p. 172–173° after sintering at 167–168° (Found: C, 40·3; H, 5·1. $C_{18}H_{27}NO_4$, $H[Cr(SCN)_4(NH_3)_2]$, H_2O requires C, 40·1; H, 5·2 per cent). The second base, contained in the subsequent 335 ml. light petroleum, was shown to be 7-hydroxy-3,6-ditigloyloxytropane (0·02 per cent) by the following derivatives: picrate, plates from aqueous

ethanol, m.p. and mixed m.p. with authentic material 182–183° (Found: C, 51·2; H, 5·42. Calc. for $C_{18}H_{27}NO_5$, $C_6H_3N_3O_7$: C, 50·9; H, 5·3 per cent); chloroplatinate, orange prisms from 0·1N hydrochloric acid m.p. 251–252° (decomp.). A neutral solution of the base in water, afforded on the addition of a saturated solution of ammonium reineckate, a *reineckate*, micro-rosettes from aqueous acetone, m.p. 194–195° (decomp.) after sintering at 189–190° (Found: C, 40·2; H, 5·23. $C_{18}H_{27}NO_5$, $H[Cr(SCN)_4(NH_3)_2]$ requires C, 40·2; H, 5·06 per cent). The ether eluate showed two small peaks on the titration curve; no crystalline derivatives could be obtained from the eluate corresponding to the first peak but the second (0·004 per cent) afforded a picrate, plates from aqueous ethanol, m.p. 171–172° (Found: C, 55·3; H, 4·9 per cent). The chloroplatinate, m.p. 202–203° (decomp.), formed nodules from dilute hydrochloric acid and the base gave a positive Vitali-Morin reaction and had an R_F value intermediate between that of hyoscyamine and the ditigloyl esters. Insufficient material prevented further work on this alkaloid. The chloroform eluate contained only a small amount of basic material of which no crystalline derivatives could be prepared.

Fraction B was dissolved in ether (about 3 ml.) and submitted to partition chromatography using a column prepared from kieselguhr (60 g.) on which was distributed 45 ml. of 0·5M phosphate buffer solution, pH 6·6. The alkaloid mixtures derived from the light petroleum, ether and chloroform eluates were designated B1, B2 and B3 respectively. Ammoniacal chloroform was used to remove any residual alkaloids from the column—B4. B1 contained a small quantity of basic material with a high R_F value and probably represented residual Fraction A bases. B2 contained a mixture of the principal alkaloids, the separation of which was incomplete. Consequently it was divided into Subfraction B2a—the first 400 ml. of ether eluate and Subfraction B2b—the following 350 ml. eluate. The alkaloid mixture obtained from B2a was resubmitted to partition chromatography at pH 6·6. The first portion of ether eluate contained (−)-hyoscine which was characterised by the picrate, m.p. 184–185°, undepressed on admixture with authentic (−)-hyoscine picrate m.p. 188° and the reineckate m.p. 170–171° (decomp.) after sintering at 169–170°. The subsequent ether eluate (about 250 ml.), represented by a low hump on the titration curve, appeared to contain a single alkaloid having an R_F value similar to that of 3α-tigloyloxytropane (Evans and Wellendorf, 1959). However, no crystalline picrate could be isolated from the fraction and the reineckate, stout needles from aqueous acetone, m.p. 144–145° (decomp.), sintering at 130°, did not appear identical with the reineckate prepared from authentic 3α-tigloyloxytropane which had m.p. 159–160° (decomp.), sintering at 151–152°. From the chloroform eluate, atropine was characterised as the picrate, m.p. after several recrystallisations from aqueous ethanol 170–171°, mixed m.p. with authentic atropine picrate (m.p. 174°) 171–172°. The base derived from Subfraction B2b afforded in neutral solution with sodium picrate, hyoscyamine picrate m.p. and mixed m.p. 165° and, with ammonium reineckate solution, hyoscyamine reineckate m.p. 154–155° (decomp.)

after sintering at 148°. Fraction B3 was collected in three parts, B3a, represented by a small sharp peak on the elution titration curve, B3b the principal portion and B3c, the tail of the B3b peak. B3a on repeated chromatography at pH 6·6, gave three fractions, the largest of which afforded (−)-hyoscyamine which was characterised as the picrate. Paper chromatography indicated the presence of three bases in the Subfraction B3b but repeated chromatography at pH 6·8 and 6·4 did not resolve the mixture. The bases were therefore recovered from the eluates and submitted to counter-current extraction for which the immiscible phases were prepared by shaking together light petroleum (b.p. 60–80°), n-amyl alcohol, glacial acetic acid and water in the ratio 1 : 3 : 3 : 3 and using the upper and lower layers as moving and stationary phases respectively. Fifteen tubes were used and the degree of fractionation of the alkaloids was followed by paper chromatography. The alkaloid acetates from tubes 7 and 8 were combined, the free bases recovered and neutralised with 0·005N sulphuric acid (14 ml.). The addition of sodium picrate solution to the neutral solution gave norhyoscyamine picrate, needles from dilute ethanol, m.p. and mixed m.p. with authentic norhyoscyamine picrate, 220°. (Found: C, 52·4; H, 5·05. $C_{16}H_{21}NO_3,C_6H_3N_3O_7$ requires C, 52·4; H, 4·76 per cent.) From tubes 4 and 5, which paper chromatography indicated to contain two alkaloids, one having a R_F value the same as cuscohygrine, it was possible to isolate only norhyoscyamine. Tubes 1 and 2 also contained the cuscohygrine-like alkaloid (4 mg.) but no crystalline derivatives of it could be prepared. The same base appeared to be the principal constituent of B3c and a neutralised aqueous solution of it readily gave a picrate, prisms from dilute ethanol, m.p. 214–215° (decomp.) after collapsing into a viscous mass at 201–202° (Found: C, 51·8; H, 5·23; N, 11·3. Calc. for cuscohygrine dipicrate, $C_{13}H_{24}N_2O,2C_6H_3N_3O_7$: C, 43·9; H, 4·4; N, 16·4 per cent). Lack of material prevented a more thorough examination of this base.

Fraction C, a dark brown semi-solid was treated with commercial absolute ethanol (50 ml.) and acetone (100 ml.). Insoluble matter, consisting of inorganic material and other impurities, was removed by filtration, the filtrate concentrated to 25 ml. and an equal volume of acetone: ether mixture (50 : 50) added causing the deposition of considerable oily material. Removal of the solvent from the decanted, clear supernatant liquid left a residue (1·9 g.), which by paper chromatography showed the presence of at least two bases of low R_F value. For the attempted characterisation of these, a portion of the residue (0·8 g.) was esterified with tigloyl chloride and the mixture of isolated esters submitted to partition chromatography (Evans and Wellendorf, 1959). The light petroleum eluate from the column contained (−)-3α,6β-ditigloyloxytropane (0·03 g.) as shown by the following characters: picrate, filamentous needles from aqueous ethanol, m.p. and mixed m.p. 151° (Found: C, 52·5; H, 5·49 per cent), mixed m.p. with (+)-3α,6β-ditigloyloxytropane picrate (m.p. 152°) 173–174° after softening at 156° (Evans and Wellendorf, 1958); chloroplatinate, plates from dilute hydrochloric acid m.p. 230–231° (decomp.). Attempts to isolate other tigloyl esters (0·01 g.)

were unsuccessful although paper chromatography indicated their presence. The proportions of the principal alkaloids in the roots were determined by a slight modification of the method of Evans and Wellendorf (1959) and are given in Table I, together with similar figures for the roots of older plants.

Alkaloids of the Root-Wood

Paper chromatography indicated the distribution of alkaloids in the root-wood to differ from that of the whole root. The alkaloids from coarsely powdered root-wood (200 g.) of six-year old plants grown in a temperate greenhouse were extracted as previously described for the whole roots and collected in 0·05N sulphuric acid. Excess ammonia solution was added and the alkaloids extracted with chloroform; removal of the solvent gave a residue (0·75 g.) which was dissolved in ether (3 ml.) and submitted to partition chromatography at pH 6·6 using light petroleum, ether and chloroform in succession as eluants. A small petroleum ether fraction afforded (−)-3α,6β-ditigloyloxytropane (0·002 per cent), picrate filamentous needles, m.p. and mixed m.p. with authentic material 151–152°. No crystalline derivatives could be obtained from the ether eluate but the principal constituent of the chloroform was shown to be noratropine (0·05 per cent); picrate, stout needles from aqueous ethanol m.p. 226–227° (Found: C, 52·4; H, 4·8 per cent); aurichloride, m.p. 162–164°. The high m.p. of the aurichloride compared with that of authentic material (157°) could be ascribed to the presence of some unracemised norhyoscyamine, a possibility supported by the slight laevorotation of a solution of the base in ethanol. The quantitative analytical figures for the root-wood are given in Table I.

Discussion

The occurrence of hyoscine as the principal alkaloid of the leaves, stems and flowers of *D. cornigera* is consistent with other observations (*loc. cit.*) on closely related white-flowered tree daturas. Although we have been unable to confirm the presence of hyoscyamine or atropine in the leaves, their occurrence in small amounts cannot be excluded, as unresolvable mixtures, having R_F values on paper the same as hyoscyamine, have been obtained. An uncharacterised alkaloid, affording a picrate m.p. 230° and previously isolated from the aerial parts of *D. ferox*, Indian henbane and the roots of *D. innoxia* occurs in the leaves together with a small quantity of noratropine. The latter alkaloid is also reported, for the first time, in the flowers and pericarp of a *Datura* species. The isolation of hyoscine as the main alkaloid of *D. cornigera* seeds produced both in this country and in Bolivia supports the view that the alkaloid mixture of the seeds is a reflection of the alkaloid mixture in the aerial parts of the plant at the time of seed formation. Schmidt and Kircher (1906) suggested that their unexpected finding of a 4:1 ratio of hyoscyamine to hyoscine in the seeds from foreign plants of *D. arborea* might be explained by differences in climatic conditions. Subsequent investigations on *D.*

arborea grown in S. America (Montesinos, 1939; Villalba, Medina and Albarracin, 1945) have not yet indicated specimens having hyoscyamine as the principal alkaloid of the aerial parts.

Concerning the nature of the alkaloids of the roots, *D. cornigera* shows resemblances to those members of the Sections Dutra and Stramonium already studied in some detail (Evans and Partridge, 1957; Evans and Wellendorf, 1958, 1959). 7-Hydroxy-3,6-ditigloyloxytropane and (−)-3α,6β-ditigloyloxytropane appear to be confined to the roots. Irrespective of whether the aerial parts of the species contain either hyoscine or hyoscyamine as the major alkaloid, hyoscyamine or atropine usually appear to be the principal constituents of the roots with the exception of two species so far examined in which meteloidine predominates. Recently it has been shown that with a number of species, hyoscyamine produced in the roots, may be converted in the leaves to hyoscine via 6-hydroxyhyoscyamine (Romeike, 1959, 1960; Romeike and Fodor, 1960). A similar mechanism would explain in part the alkaloid pattern of *D. cornigera*. In our observations, the total alkaloidal content of the leafy aerial parts varied very little with age of plant but with the roots, those from 6 year old plants contained less alkaloid on a per cent dry weight basis than those from young plants. No 7-hydroxy-3,6-ditigloyloxytropane could be detected in the older roots but they contained a relatively high proportion of noratropine, which in the root-wood was the principal single alkaloid. The isolation of 3,6-dihydroxytropane gives another example of the presence of free alkamines in the roots of *Datura* species and the complexity of the root alkaloid mixture is further shown by the indication of other alkamines and uncharacterised bases.

A new derivative, suitable for the characterisation of the ditigloyl esters of the root is the reineckate.

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THE MORPHOLOGY AND HISTOLOGY OF SEEDS OF *DATURA CORNIGERA* HOOK

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The macroscopical and microscopical characters of the seeds of *Datura cornigera* Hook. are described and illustrated. A most striking feature is the peculiar spongy parenchyma which constitutes the major part of the testa. The location of alkaloids is surprising, only the collapsed inner part of the testa showed no content of alkaloids. In the outer layers of the endosperm, many cells contained regular crystals of a substance which dissolved in strong sulphuric acid giving an intense orange colour.

The systematics of the section *Brugmansia* are rather complicated, one of the main difficulties being the hybrid nature of most forms. The plants are very often only cultivated as garden ornamentals in their native home, the tropical parts of South America and in greenhouses in temperate countries. A reason making study difficult is the limited ability of many forms to produce mature fruits; according to Lagerheim (1895), even in South America, specimens with fruits are rare, and for several species the fruits are still unknown. The only comprehensive records of South American brugmansias are the monograph by Lagerheim (1895) and the synopsis by Safford (1921).

The general features of the seeds from the section *Brugmansia* have been mentioned by Lagerheim (1895), but no detailed illustrations given. According to him most brugmansias have flattened seeds which on maturity are loose within the fruit. Both *Datura aurea* Lagerh. and *D. arborea* Linn. have seeds which are irregular and thick and not loose within the fruit; *D. aurea* seeds have a smooth testa. Certain similarities exist between the seed structure of *D. stramonium* Linn. and that of the seeds of brugmansias but two characteristic features are different: in the brugmansia seeds the inner walls of the epidermal cells are not thickened to the same degree as in stramonium seeds and a thick middle layer is developed in the testa of several brugmansia seeds. This layer also found in the seeds here in question is composed of a very spongy tissue which is responsible for the surprisingly low weight of these large seeds.

The anatomy of seeds of different *Datura* species has been thoroughly investigated by Timmerman (1927) and Moll and Janssonius (1923) but only the seeds of commerce, i.e., seeds of *D. stramonium*, *D. innoxia* and *D. metel* were included in these studies.

The position of alkaloids in the seeds of *D. stramonium* has always been shown to be in the inner part of the testa only (Siim, 1900; James, 1946).

Plant Material

The seeds of *D. cornigera* Hook. (Fig. 1) used in this investigation are samples from Nottingham University. The origin of the seeds and the

identity of the plants have been described by Evans and Pe Than (1962). Both the original seeds from Cochabamba, Bolivia and seeds from plants cross pollinated in Nottingham were investigated, and showed identity.

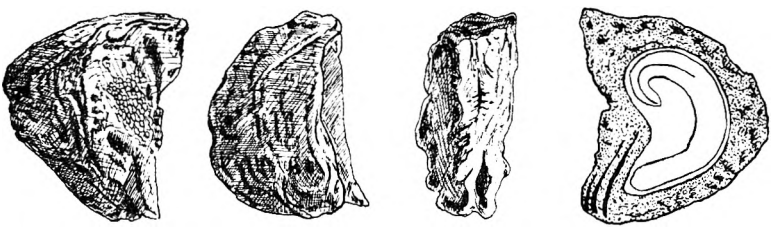

FIG. 1. Seed of *Datura cornigera* Hook. The seeds seen from different visual angles. One seed cut longitudinally, shows the curved embryo in the endosperm and the large spongy parenchyma in the testa. × 2.

The 100-seed weight was calculated to 12·40 g. and average measurements were length, 1·3 cm. width, 1·1 cm. and thickness, 0·6 cm. The greyish-brown seeds are flattened and from the side the outline is triangular to half orbicular. The two faces are quite smooth with only a few wrinkles and furrows, the convex side is deeply furrowed. The hilum is present as a small cavity at one end of the acute edge. The interior is composed of the spongy brown part of the testa which surrounds the oily endosperm embedded within which is a cylindrical embryo, about 2 mm. in diameter. The hypocotyle-radicle is directed towards the hilum; the embryo is coiled but not as strongly as in stramonium seeds.

HISTOLOGICAL CHARACTERS (Fig. 2)

The testa of the *D. cornigera* seed consists of three distinct parts: outer epidermis, a spongy parenchyma and an inner epidermis. The structure of the cells of the outer epidermis may be elucidated by comparing transverse sections, surface preparations and isolated cells. As Timmerman (1927) points out it is rather difficult to macerate the epidermis. In this case the usual Schultze method (nitric acid and potassium chlorate) was too drastic but very fine preparations were obtained with Franklin's method (1937), using glacial acetic acid and 30 per cent hydrogen peroxide at 100° for $\frac{1}{2}$ hr. In transverse section the epidermal cells are about 135 μ in height on the faces but when the testa is wrinkled the height may increase up to 270 μ; the width of the cells is up to 135 μ; the lumen is more or less isodiametric. The outer wall of the epidermal cells consists of three distinct layers. An outer lamella (cuticle) which stains deep red with ruthenium-red, a middle layer of cellulose which stains blue with iodine and 60 per cent sulphuric acid, and an inner part which gives a lignin reaction with phloroglucinol and concentrated hydrochloric acid. Sudan III does not stain any layer. The outer lamella must therefore consist of pectin or a mucilagineous substance. An isolated epidermal cell shows several rounded processes in the upper end and the base shows a similar structure due to folds in the inner part of the lateral walls. The

lignified portion of the wall shows a fine striation due to alternating lignified and cellulose layers. After staining with iodine-sulphuric acid it is difficult to see the blue colour due to the strong brownish colour of the wall but after bleaching with hypochlorite, extremely thin sections show blue coloured lamellae within the cell wall.

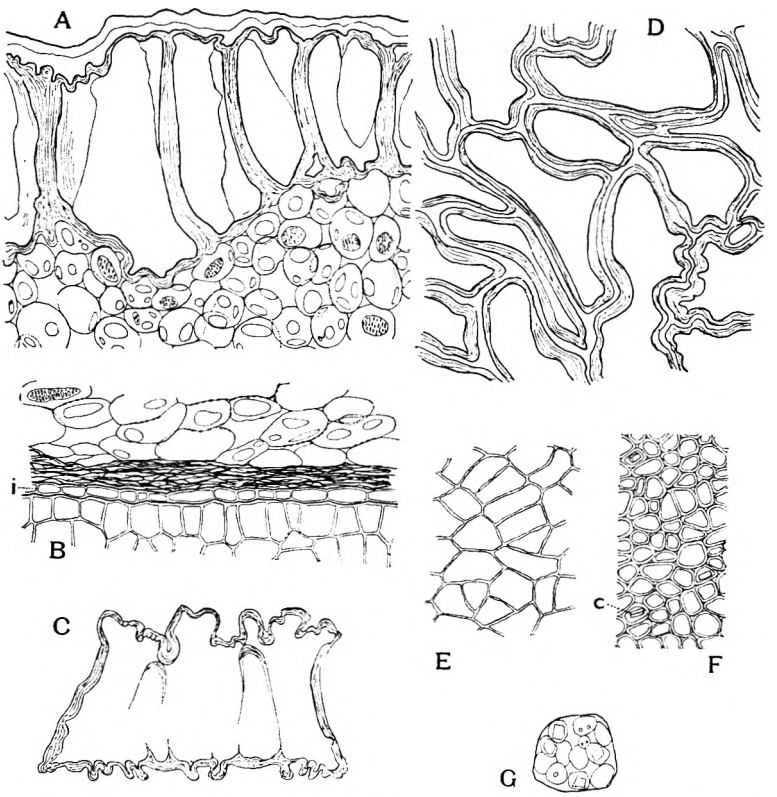

FIG. 2. Seed of *Datura cornigera* Hook. A, transverse section through the outer epidermis and the outer part of the spongy parenchyma. B, transverse section through the inner part of the testa and the endosperm. C, isolated epidermal cell (from a maceration). D, surface view of the outer epidermis. E, surface view of the inner epidermis. F, surface view of the outer layer of the endosperm. G, single cell with aleurone grains from the endosperm. All figures × 105 except G: × 275. i = inner epidermis of the testa; c = crystal in the endosperm. A, B and D from preparations cleared with chloral hydrate reagent.

A surface view of the epidermis shows very sinuous lateral walls and below the surface the middle lamella is quite distinct. Numerous processes are seen, often looking like very small single cells.

The dominant tissue in the testa is the enormously thick lignified layer which occupies all the space between the outer and the inner epidermis. The single cells are sphaeric in outline and joined to one another by numerous poral connections. These connections are very easy to observe

due to the large single pores between two neighbouring cells: the pores show reticulated membranes. Close to the inner epidermis the cells become more closely packed and the cell-walls show many small simple pores. The last 3–10 layers outside the inner epidermis are completely collapsed. The inner epidermis is formed by a 15–20 μ high layer of, in transverse section, rectangular cells. In surface view they are polygonal in shape, with somewhat uneven thickened walls. The reason this layer should be called "inner epidermis" of the seed testa and not "perisperm" as stated by many earlier authors (see Moll and Janssonius, 1923) is the existence of a cuticularised membrane on the inner walls of this layer. In unripe seeds of *D. stramonium* a similar cuticle separates the inner epidermis of the integument from the tissue in the nucellus (see Netolitzky, 1929).

The cells of the endosperm have hyaline walls and contain abundant protein and globules of oil. The aleurone grains are of variable size, especially small grains are found in the outer cell-layers which also contain single crystals of a colourless highly refractive substance soluble in concentrated sulphuric acid with an intense orange colour. The embryo also contains protein and oil.

Location of Alkaloids

A fresh seed is very easy to divide into the different tissues. The epidermis scales off and the spongy parenchyma splits away exposing the collapsed cells on the surface of the endosperm. These few cell-layers may then be scraped off with a fine knife and the whitish-grey endosperm split open to remove the white embryo. The five "seed-fractions" are then each extracted with a few ml. ethanol and a few drops of strong ammonia. The liquid extracts are filtered, evaporated to dryness and the residues each dissolved in a few drops of chloroform. Spotted on to filter-paper and sprayed with Dragendorff's reagent only four extracts gave a strong positive reaction; the collapsed cell-layer showed no reaction for alkaloids. Submitted to paper chromatography the four extracts gave spots with the same R_F value as hyoscine (see Evans and Pe Than, 1962). The amount of seeds in my possession did not permit further chemical investigations.

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THE EFFECT OF DEFICIENCY AND SMALL EXCESS OF THIAMINE ON THE RAT PHRENIC NERVE DIAPHRAGM PREPARATION

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Preparations made from thiamine deficient rats showed subnormal twitch tensions in response to nerve stimulation but gave normal twitch tensions when directly stimulated. Tensions developed during tetanus were subnormal whether elicited directly or indirectly. Sensitivity to an excess of potassium ion was reduced but that to tubocurarine remained unchanged. Excess of thiamine, in near physiological concentrations, was almost devoid of curare-like action except in the presence of an excess of thyroid hormone.

It has been suggested (von Muralt, 1947) that thiamine may play an important part in the transmission of excitation from the motor nerve terminals to voluntary muscle, and may also influence the contractile process in muscle fibres, because Minz (1938) found that cholinergic fibres liberate thiamine as well as acetylcholine when excited electrically. Our purpose has therefore been to make investigation of the possible functions of thiamine at the neuromuscular junction and in the contraction of muscle fibres using the rat phrenic nerve diaphragm preparation.

Experimental

Methods

Female rats of a single Wistar strain, weighing 200–250 g., were used. Those adrenalectomised drank 0·4 per cent NaCl in tap water, were fed diet 41 b of Stein, and were used for experiments on the fourth to sixth postoperative day. All other rats were fed a basic diet of: corn starch, 60; casein, 18; corn oil, 6; dried whole liver powder, 5; dried yeast, 7 and U.S.P. XII salt mixture No. 2, 4 per cent. This diet was prepared in bulk and was stored at room temperature in sealed containers: the thiamine content, estimated by the thiochrome method, was 137 μg./100 g. at the time of use. Thiamine deficient diet was prepared by mixing sodium metabisulphite into the standard diet to 0·6 per cent w/w, at room temperature; this was used in the interval of two to eight weeks after its preparation. The treatment with metabisulphite reduced the thiamine content to less than 1 μg./100 g. in 2 weeks. Weighed amounts of basic and sulphite-treated diets were mixed to stiff pastes, daily, with water and were supplied in narrow troughs fixed to the sides of the cages. Animals fed on these diets each received 0·5 ml. cod liver oil orally, weekly, by pipette.

Rats fed on the sulphited-treated diet ceased to gain weight in the third or fourth week, then lost weight and developed severe polyneuritis and bradycardia in the fifth or sixth week. They were used for experiments only when signs of advanced thiamine deficiency had become evident.

Adrenalectomy. Bilateral adrenalectomy was performed as described by Venning, Kazmin and Bell (1946) under pentobarbitone anaesthesia.

Phrenic nerve diaphragm peparations were made as described by Bülbring (1946) and were suspended in a bath containing a measured volume (80–90 ml.) of aerated Tyrode's fluid at 27 $\pm$ 1° which contained twice the glucose stated in the original formula. Contractions of the diaphragm were induced either by stimulation of the phrenic nerve or, in curarised preparations, directly. Rectangular pulses of 100 μ-sec. duration were delivered at intervals of 10 sec. (twitches) or at a frequency of 100/sec. (tetanus) from a voltage source 1·5 times that required to induce maximum tension. This source did not exceed 10 volts during nerve, or 100 volts during direct stimulation.

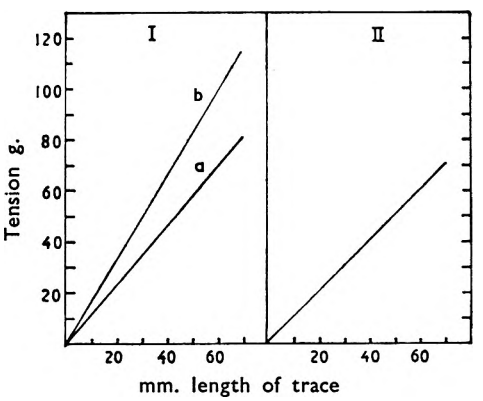

FIG. 1. The graphs show regression lines which are calibration curves relating length of trace in mm. to g. hung from each of two isometric levers (I and II) at a distance of 1 cm. from the fulcrum. a and b refer to two different magnifications used with lever I.

The design of experiments. A. Calibration curves for two Palmer isometric levers used are shown in Fig. 1. Preparations were suspended from these levers from a point 1 cm. from the fulcrum and were subjected to an initial tension of 10 g. In each experiment maximum tensions developed in response to single shocks and tetanus delivered through the phrenic nerve were measured, as was the effect of 5 min. of exposure to an additional 0·3 ml. 15 per cent KCl per 80 ml. bath fluid on twitch tension. These measurements were then repeated after full curarisation during direct stimulation (Figs. 2, 3). *B.* Preparations were suspended from heart levers and maximum twitch tension was elicited every 10 sec. in response to nerve stimulation. A fixed dose of tubocurarine, sub-maximal in effect, was added to the bath fluid every 40 min. for a contact period of 10 min. When a constant response to the fixed dose of tubocurarine became evident another drug was added to the bath fluid 5 min. before the standard dose of tubocurarine was due, and was washed out together with the tubocurarine 15 min. later. Return was then made to tubocurarine alone. Change in twitch height after 10 min. exposure to

tubocurarine has been expressed as a per cent of initial twitch height. Modification of response to tubocurarine by a second drug has been referred to the mean of flanking responses to tubocurarine alone.

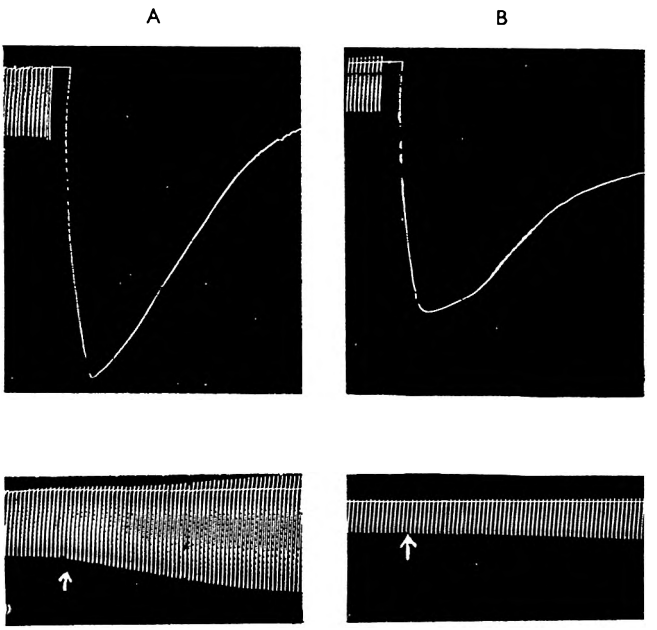

FIG. 2. The tracings were made by the contractions of a nerve diaphragm preparation from a normal rat. The diaphragm was attached 1 cm. from the fulcrum of an isometric lever and was subjected to an initial tension of 10 g. Above, maximum twitch and tetanus, below, the effect of added KCl (at arrow), 0·3 ml. 15 per cent, to 80 ml. bath fluid. A, stimulation through the nerve; B, direct stimulation, fully curarised.

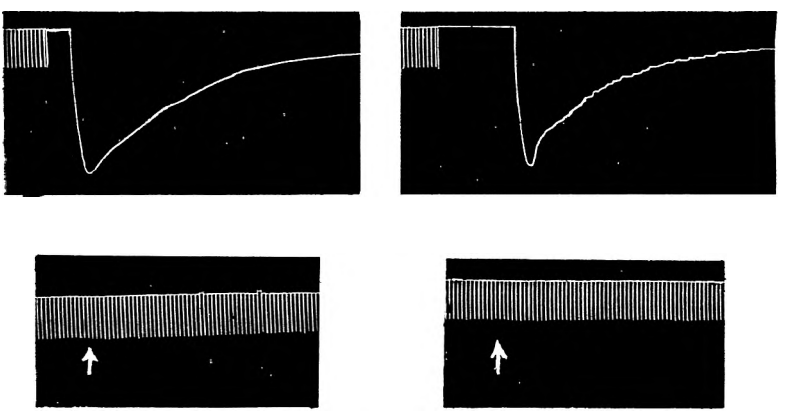

FIG. 3. As Fig. 2, except that the preparation used was taken from a rat in advanced deficiency of thiamine.

RESULTS

The Effect of Deficiency of Thiamine on Tensions Developed During Twitch and Tetanus by Rat Diaphragms in Reponse Both to Direct and Indirect Stimulation

Single preparations taken from each of six normal rats, and six made from four rats in advanced thiamine insufficiency, were used in experiments which yielded the data summarised in Tables I and II. The maximum

TABLE I

A COMPARISON OF TENSIONS DEVELOPED DURING MAXIMUM TWITCH AND TETANUS, AND OF SENSITIVITY TO K^+ EXCESS, IN CURARISED DIAPHRAGM PREPARATIONS MADE FROM NORMAL AND THIAMINE DEFICIENT RATS AND STIMULATED DIRECTLY

	g. tension developed in response to direct stimulation			
Wt. of rat g.	Maximum twitch	Maximum tetanus	Maximum twitches	
			Normal	Added K^+
Thiamine deficient—				
150	13·5	15·0	13·5	13·5
160	20·0	21·0	18·5	18·5
165	13·0	16·5	13·0	13·0
165	14·0	30·5	12·5	12·0
165	16·0	35·0	16·0	16·0
155	17·0	36·5	17·0	17·0
Normal—				
170	18·0	72·0	17·5	19·0
180	26·0	111·0	25·0	29·0
165	15·0	48·0	14·0	22·0
170	17·0	70·0	17·0	23·0
150	13·0	57·5	13·0	15·5
175	16·0	50·5	14·0	15·5
Thiamine deficient—				
159 ± 2·1	15·6 ± 1·2	25·9 ± 3·8	15·1 ± 1·0	15·0 ± 1·0
Normal—				
168 ± 4·3	17·5 ± 1·9	68·2 ± 9·4	16·8 ± 1·8	22·3 ± 2·1
t calc. (n = 10)—	1·27	9·95	1·26	4·09

twitch tensions given by thiamine deficient preparations equalled the normal only when curarised muscles were stimulated directly (Table I). Twitch tension was found subnormal in the deficient preparations when these were excited through the nerve (Table II). The maximum tensions developed during tetanus, and the ratio of maximum tetanus/twitch tensions were subnormal in thiamine deficient preparations whether excited by direct stimulation or through the nerve (Tables I and II). Whereas normal preparations responded to the excess concentration of potassium ions used by increase in twitch tension, whether stimulated directly or through the nerve, the thiamine deficient preparations did not (Tables I and II). The diaphragms taken from the rats deficient in thiamine looked thinner, and had less adherent fatty tissue than did those from normal rats.

The Effect of Deficiency and Excess of Thiamine on the Response of the Rat Diaphragm to Nerve Stimulation, and on Sensitivity to Tubocurarine

Four nerve diaphragm preparations made from normal rats, weighing 185 ± 7 g., developed maximum twitch tensions in response to single

shocks to the phrenic nerve which were recorded as contractions measuring 36·0 ± 3·8 (4) mm. on a smoked drum by semi-isometric levers. Four similar preparations made from animals of the same age group which were in an advanced stage of thiamine deficiency (body weight 110 ± 5 g.) gave maximum twitch heights of 29·0 ± 4·8 (4) mm. on the same lever systems; in addition they developed less tension (*t* calc. = 2·4, P = <0·05) during a maximal twitch than did normal diaphragms.

TABLE II

A COMPARISON OF TENSIONS DEVELOPED DURING MAXIMUM TWITCH AND TETANUS, AND OF SENSITIVITY TO K^+ EXCESS, IN PHRENIC NERVE DIAPHRAGM PREPARATIONS MADE FROM NORMAL AND THIAMINE DEFICIENT RATS AND STIMULATED THROUGH THE PHRENIC NERVES

Wt. of rat g.	g. tensions developed in response to nerve stimulation			
	Maximum twitch	Maximum tetanus	Maximum twitches	
			Normal	Added K^+
Thiamine deficient—				
150	13·5	24·0	13·5	13·5
160	22·5	42·0	20·5	20·5
165	14·0	29·0	15·5	15·5
160	14·0	29·5	14·0	14·0
165	18·0	39·0	18·0	18·0
155	17·0	37·5	17·0	18·0
Normal—				
170	27·0	87·0	26·0	35·0
180	34·0	122·0	31·0	35·5
165	21·0	70·0	22·0	31·0
170	18·5	54·0	18·0	22·5
150	23·0	82·0	19·5	30·0
175	23·0	91·5	23·0	28·0
Thiamine deficient—				
159 ± 2·1	16·5 ± 1·4	33·5 ± 1·2	16·6 ± 0·8	16·7 ± 0·9
Normal—				
168 ± 4·3	24·4 ± 2·2	84·4 ± 3·0	23·3 ± 1·9	30·3 ± 2·0
t calc. (n = 10)—	2·98	15·9	3·14	4·85

The neuromuscular blocking action of tubocurarine did not differ in these two groups of nerve diaphragm preparations. Tubocurarine, 0·85 ± 0·08 μg./ml. reduced the maximum twitch tension by 63·6 ± 3·9 per cent of the control values in a 10 min. period of contact with the normal diaphragms. A concentration of 0·87 ± 0·02 μg. tubocurarine/ml. reduced the maximum twitch height by 65·5 ± 3·9 per cent of control values, when preparations from thiamine deficient animals were similarly examined.

Thiamine hydrochloride, 5 to 20 μg., added to bath volumes of fluid ranging from 60 to 100 ml. was without effect on twitch tensions whether the nerve diaphragm preparations had been taken from normal rats or from those deficient in thiamine. These concentrations of thiamine did in some cases cause a slight increase in the blocking action of tubocurarine in preparations made from both normal and from thiamine deficient animals (Table III); this, since it occurred in seven out of eight preparations, was significant by *t* test. The calculated values of *t* are shown at the foot of Table III.

TABLE III

THE EFFECT OF THIAMINE HYDROCHLORIDE IN THE BATH FLUID ON THE SENSITIVITY OF PHRENIC NERVE DIAPHRAGM FROM NORMAL RATS AND FROM RATS DEFICIENT IN THIAMINE ON THE BLOCK PRODUCED BY A FIXED DOSE OF TUBOCURARINE

	Per cent inhibition of twitch tension resulting from 10 min. exposure to a fixed concentration of tubocurarine			
	In the absence of thiamine			In the presence of thiamine 5–10 μg./100 ml.
μg. tubocurarine used	Control 1	Control 2	Mean control value	
Normal rats –				
I 75	60·0	57·5	58·8	54·5
II 75	61·5	60·0	60·8	65·5
III 75	56·8	62·2	59·5	66·6
IV 75	60·5	57·1	58·8	67·5
Rats deficient in thiamine—				
I 75	62·0	71·0	66·5	75·0
II 60	64·0	68·0	66·0	69·0
III 100	69·0	78·0	73·5	77·0
IV 60	50·0	61·0	55·5	59·0

Significance of differences have been examined by *t* tests in which each preparation has served as its own control.
Values of *t* calc. for effect of thiamine:—
Preparations from normal rats, $t = 2{\cdot}00$, n = 3, $P = <0{\cdot}1$.
Preparations from deficient rats, $t = 3{\cdot}74$, n = 3, $P = <0{\cdot}05$.
Together, $t = 2{\cdot}54$, n = 6, $P = <0{\cdot}05$.

The Effect of an Excess of Triiodothyronine on the Sensitivity of the Phrenic Nerve Diaphragm Preparation to the Blocking Action of Tubocurarine

Pickens and Lockett (1961) have shown a reduction in the quantity of acetylcholine liberated per nerve impulse from the phrenic nerve diaphragm preparation when this preparation is bathed in a fluid containing 0·05 μg. triiodothyronine per 100 ml. It was therefore of interest to discover whether the small reduction of acetylcholine output occasioned by this excess of triiodothyronine was detectable as an increased sensitivity to tubocurarine, and, if so, whether the effects of triiodothyronine and thiamine excess could summate.

TABLE IV

THE EFFECT OF L-TRIIODOTHYRONINE, 0·05 μg./100 ml. AND TRIIODOTHYRONINE WITH THIAMINE HYDROCHLORIDE, 5–10 μg./90 ml., ON THE SENSITIVITY OF PHRENIC NERVE DIAPHRAGM PREPARATIONS FROM NORMAL AND ADRENALECTOMISED RATS TO TUBOCURARINE

Drugs		Effect of tubocurarine alone (decrease in twitch tension as per cent initial twitch tension)	Change in per cent response to curare induced by other drugs	*t* calc.
Tubocurarine μg./ml.	Other drugs			
Normal rats—				
0·75 ± 0·08 (6)	triiodothyronine (T_3)	59·2 ± 6·3	+6·2 ± 3·8	2·8
Adrenalectomised rats—				
0·73 ± 0·09 (7)	thiamine (2) cocarboxylase (5) thiamine or cocarb.	55·3 ± 5·4	+1·9 ± 1·18	0·86
	+ T_3	63·7 ± 4·3	−0·3 ± 2·39	0·33
Thiamine deficient rats—				
0·86 ± 0·05 (3)	thiamine	74·1 ± 10·9	+3·1 ± 0·07	3·12
	thiamine + T_3	70·2 ± 16·8	+6·5 ± 1·95	3·33

t calculated as in Table I.

Comparison has therefore been made of the intensity of the neuromuscular block caused by a fixed dose of tubocurarine in the absence of other drug and in the presence of triiodothyronine (0·05 μg./100 ml.) alone and with thiamine hydrochloride (5 to 10 μg./100 ml.). The results of these experiments are seen in Table IV. Triiodothyronine caused just significant potentiation of the blocking action of tubocurarine in preparations from normal rats and appeared to sum in effect with thiamine in this respect in preparations made from thiamine deficient animals. It was not found possible to demonstrate potentiation of the action of tubocurarine by thiamine and triiodothyronine in those preparations made from adrenalectomised salt maintained animals.

Discussion

The results summarised in Table I show that curarised voluntary muscle from thiamine deficient animals is capable of producing a short lived twitch of normal tension but cannot develop or sustain normal tensions when in tetanus. This reduction in power is most probably to be attributed to reduction in muscle adenosine triphosphate by reason of a depression of activity in the Krebs cycle for lack of co-carboxylase. The fact that thiamine deficient muscle was abnormally insensitive to an excess of extracellular potassium ion may indicate a greater than normal stability of membrane polarisation. The results shown in Table II contribute one additional fact. Twitch tension in response to nerve stimulation is reduced during thiamine insufficiency, but that in response to direct stimulation is not. This observation may possibly be explained by the facts that the quantities of acetylcholine and of acetylcoenzyme A (Bhagat and Lockett, 1962) found in nerve tissue are reduced in late thiamine deficiency. It therefore follows that there may be a reduction in the amount of acetylcholine liberated per nerve impulse from the motor nerves to voluntary muscle during thiamine deficiency. Any such reduction cannot, however, have been great, since it was insufficient to increase sensitivity to tubocurarine (Table III).

It was possible to demonstrate a slight curare-like action of quantities of excess thiamine which could be considered to approach physiological, augmented by the presence of an excess of triiodothyronine. The curare-like actions of thiamine are well known, but have been elicited in cats, for instance, only by the intravenous injection of huge quantities of the compound 20 mg./kg. (Ngai, Ginsburg and Katz, 1961). A number of other studies have been made of the pharmacological effects of large amounts of thiamine at the myoneural junction of voluntary muscle, for both acetylcholine and thiamine are quaternary compounds which have a free hydroxyl group. Torda and Wolff (1944) found that very large concentrations of thiamine (5×10^{-3} M, upward) induced contracture in frog skeletal muscle, and Cheymol, Bourillet, Levassort and Kerp (1957) showed that exceedingly high concentrations of thiamine (5×10^{-2}) could anul the effects of stimulation of the phrenic nerve on isolated rat diaphragms.

Our experiments indicate that loss of muscular power during deficiency in thiamine is very largely attributable to lack of muscle co-carboxylase, and that possible physiological excesses of thiamine would be likely to affect neuromuscular transmission only in the presence of hyperthyroid state.

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THE BIOLOGICAL ASSAY OF OXYTOCIN IN THE PRESENCE OF ERGOMETRINE

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The oxytocin content of a solution of oxytocin and ergometrine can be assayed with great accuracy by the chicken blood pressure method provided the rooster preparation is highly sensitive to oxytocin. The hormone can also be assayed with reasonable accuracy by measuring the response of the milk-ejection pressure in the mammary gland of the lactating rabbit. However, the rat uterus *in vitro* method yields erroneously high results due to the additional uterotonic effect of the ergometrine.

THE place of oxytocin as well as that of ergometrine is firmly established in obstetrical practice. Recently, ampoules containing 5 I.U. of synthetic oxytocin and 0·5 mg. of Ergometrine Maleate B.P. became available under the name "Syntometrine", and clinical investigations (Embrey, 1961) showed this combination to have merits which are of considerable interest in the management of the third stage of labour and in the prophylaxis and therapy of maternal blood loss.

As a statutory requirement, the potency of pharmaceutical preparations containing oxytocin must be controlled by biological methods of assay and declared in International Units (I.U.) with reference to the Third International Standard for Oxytocic, Vasopressor and Antidiuretic Substances. The British Pharmacopoeia (1958) prescribes two assay procedures, one based on the uterotonic action of oxytocin, the other on its avian depressor effect. The United States Pharmacopeia (1960) gives only the avian blood pressure lowering test. Since ergometrine influences both uterine contractility and arterial blood pressure, it seemed likely that its presence in a solution of oxytocin would interfere with these two methods of assay.

To clarify this question, experiments were performed to test the accuracy of the rat uterus and avian blood pressure methods for the biological assay of oxytocin in the presence of ergometrine. In addition, the milk-ejection pressure test was evaluated, for, although this method is not included in the pharmacopoeias, it is a convenient and specific procedure for the assay of oxytocin.

METHODS

In vitro preparations of rat uteri were used following the procedure described by Holton (1948) and applying the four-point-assay scheme of Schild (1942).

The blood pressure of white Leghorn roosters was measured by the method of Coon (1939) using the experimental design of Thompson (1944).

The milk-ejection pressure of anaesthetised lactating rabbits was measured as described previously (Berde and Cerletti, 1960), the experimental arrangement being an adaptation of that employed by Cross and

Harris (1951/52) and by Van Dyke, Adamsons and Engel (1955). Here also the four-point-assay scheme was used.

As Syntometrine ampoules contain synthetic oxytocin, "Syntocinon", this was used as the reference standard in many of the assays. In others, the Third International Standard for Oxytocic, Vasopressor and Antidiuretic Substances was taken as the reference standard.

TABLE I

THE APPARENT OXYTOCIN CONTENT OF SYNTOMETRINE IN DIFFERENT TESTS (THE ACTUAL SYNTOCINON CONTENT OF SYNTOMETRINE IS TAKEN AS 100 PER CENT)

Test	Value (per cent)	Note
Rat uterus *in vitro*	119 ± 5	11
	100 ± 15	5
	138 ± 30	5
	142 ± 27	8
	171 ± 38	12
	151 ± 28	10
Chicken blood pressure—high sensitivity	100·5 ± 2·2	12
	100·9 ± 2·8	12
	102·6 ± 3·6	12
	99·4 ± 3	10
	101 ± 4·8	10
low sensitivity	86 ± 3	10
	71 ± 8	6
	90 ± 4	6
	76 ± 11	5
Rabbit mammary gland *in situ*	107 ± 11	6
	98 ± 10	4
	102 ± 9	10
	92 ± 7	5
	104 ± 9	5

4-point-assays; doses of unknown; doses of unknown; 4-point-assays

RESULTS

The results are summarised in Table I which gives the means and the standard errors of the assays, the actual Syntocinon content of Syntometrine being taken as 100 per cent.

The oxytccin contents as determined by the rat uterus method are obviously far too high.

Chicken blood pressure assays yield excellent results provided the rooster preparation is highly sensitive to oxytocin, that is to say provided 20 mU. oxytocin i.v. elicits a blood pressure fall of 30 to 50 mm. Hg. If, however, the sensitivity is low, thus, if 40 to 100 mU. oxytocin are required to provoke a fall of 30 to 50 mm. Hg, the measured content is lower than the actual Syntocinon content of the Syntometrine.

The oxytocin contents assayed by the rabbit mammary gland method are in agreement with the actual contents of the test solutions.

DISCUSSION

Bearing in mind the well-known uterotonic effect of ergometrine, it is hardly surprising that the rat uterus *in vitro* should prove unsuitable for the biological assay of an oxytocin solution containing ergometrine. Indeed, it has been reported (Pennefather, 1961) that the isolated rat uterus can be used for the estimation of ergometrine. In our experience

this method of assay is not particularly suitable for ergometrine, since the sensitivity of the rat uterus to this oxytocic tends to fluctuate and the dose-response relationship is not always satisfactory. Be that as it may in the assay of Syntometrine, ergometrine is present in a concentration of 10–40 μg./litre, and in this concentration is liable to elicit contractions of the isolated rat uterus or to reinforce contractions due to oxytocin, thereby yielding erroneously high values for the oxytocin content. The inconsistent influence of ergometrine is reflected by the high standard errors, which greatly exceed the standard errors for assays on solutions containing oxytocin only. On *in situ* uterine preparations of, for example, the rabbit, the summation of the oxytocic effects of the two drugs is evident.

The excellent results obtained with highly sensitive chicken blood pressure preparations show that amounts of ergometrine as small as, for example, 2 μg., do not counteract the vasodilatation provoked by oxytocin. In higher doses, however, the pressor effect of ergometrine attenuates the fall of pressure due to oxytocin. In fact, 10 to 20 μg. ergometrine—without oxytocin—may actually elevate the blood pressure of roosters by 20 to 40 mm. of Hg.

A satisfactory rise of milk-ejection pressure within the mammary gland of the lactating rabbit is elicited by only a few milliunits of oxytocin, so that the oxytocin content of Syntometrine can be assayed with the usual accuracy of this method. The small amount of ergometrine accompanying the effective dose of oxytocin is without any apparent effect.

The studies reported in this paper show that the chicken blood pressure test is the most accurate method for the biological assay of oxytocin in a solution containing oxytocin and ergometrine, provided the rooster preparation is highly sensitive to oxytocin. If this method is not practicable the assay on the rabbit mammary gland is an acceptable, although less accurate, alternative. The isolated rat uterus gives erroneously high values and should not be used for this purpose.

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THE HYDROLYSIS OF ETHYL BENZOATE, DIETHYL PHTHALATE AND BENZOCAINE IN CETRIMIDE SOLUTIONS

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The effect of cetrimide on the alkaline hydrolysis of emulsions and solutions of ethyl benzoate and diethyl phthalate and solutions of benzocaine has been investigated. In the initial stages of the reaction, the hydrolysis rate of emulsions increases with cetrimide concentration while that of solutions decreases. In the later stages the rate of hydrolysis decreases with increasing cetrimide concentration.

Recent studies have shown that surface-active materials are capable of stabilising insoluble organic liquids against attack by atmospheric oxygen, and that solutions are less readily oxidised than emulsions (Carless and Nixon, 1957, 1960).

Complexing (Higuchi, 1955, 1956, 1957) and the addition of organic solvents (Ikeda, 1960) have been shown to confer protection against hydrolysis but little work has been reported on the influence of surface-active agents. McBain and Bolam (1918) showed that soap solutions could protect dissolved material against hydrolysis. More recently Riegelman (1960) and Nogami, Awazu, Watanabe and Sato (1960) have studied the effect of surface-active agents on the hydrolysis of drugs in solution. These results and those presented here indicate that stabilisation is effected when dissolved material is transferred from the aqueous phase to the micellar "phase" of the dispersion.

The literature on the alkaline hydrolysis of fats and oils is extensive (Clayton, 1954). It is believed that the reaction takes place at the oil-water interface. To avoid complications arising from the surface-active nature of the saponification products, King and Mukerjee (1938) studied the alkaline hydrolysis of amyl acetate emulsified in various surface-active materials. However the solubilising action of soap on the dispersed oil does not appear to have been considered. The alkaline hydrolysis of solutions and emulsions of esters in cetrimide is now reported.

Experimental

Materials

Cetrimide B.P. containing 96·8 per cent alkyltrimethylammonium bromides calculated as $C_{14}H_{29}(CH_3)_3N,Br$. Benzocaine B.P. recrystallised from 80 per cent ethanol m.p. 91°, ethyl benzoate b.p. 212°, n^{22}° $1{\cdot}504_5$, diethyl phthalate b.p. 292°, n^{22}° $1{\cdot}500_4$.

Determination of Solubility

Varying concentrations of cetrimide solution were added to the required amount of ester in glass-stoppered cylinders. The cylinders were immersed in a water bath at 35° and rotated until equilibrium was reached. This

took 6–8 hr. for liquid esters but several days for benzocaine. The solubility point was estimated visually.

Determination of Hydrolysis

Ester and cetrimide solution were equilibrated at 35° in a controlled temperature bath. A turbine stirrer was used to maintain uniform dispersion. Sodium hydroxide solution sufficient to provide 100 per cent in excess of that needed for complete hydrolysis was added, and the zero time noted. Samples were withdrawn at definite time intervals and the remaining alkali titrated with hydrochloric acid using phenolphthalein as indicator. Cetrimide does not interfere with the indicator. Preliminary work showed that hydrolysis was independent of stirring at the rates used and reproducible within ± 5 per cent. The amount of cetrimide did not affect the size of the emulsion droplets with the concentrations of esters used.

TABLE I

SOLUBILITIES OF ESTERS IN CETRIMIDE SOLUTIONS AT 35°

Ester	moles/litre	Cetrimide moles/litre
Ethyl benzoate ..	0·05	0·038
Diethyl phthalate ..	0·05	0·096
Diethyl phthalate ..	0·1	0·187
Benzocaine	0·05	0·068

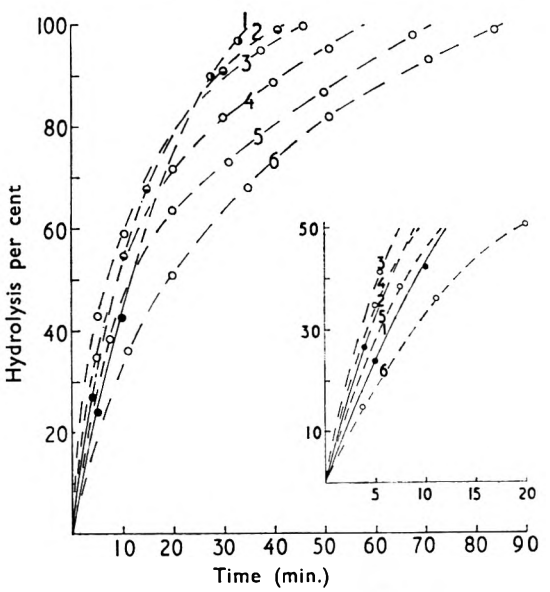

Fig. 1. Alkaline hydrolysis of ethyl benzoate (0·05 moles/litre) in cetrimide solutions, at 35°.

Cetrimide concentration (moles/litre)

1. 0·002. 2. 0·02. 3. 0·04. 4. 0·1. 5. 0·2. 6. 0·4.

—●— Emulsion ----○---- Solution.

◑ ◒ are to distinguish curves 1 and 2.

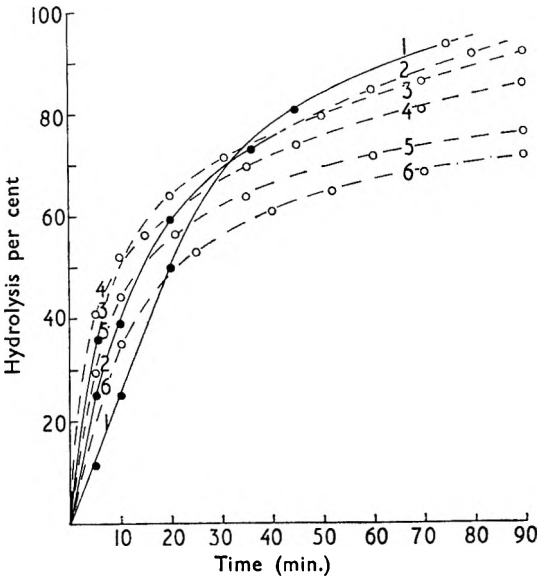

FIG. 2. Alkaline hydrolysis of diethyl phthalate (0·05 moles/litre) in cetrimide solutions at 35°.
Cetrimide concentration (moles/litre)
1. 0·002. 2. 0·02. 3. 0·05. 4. 0·096. 5. 0·14. 6. 0·35.
—— ● —— Emulsion. ----○---- Solution.

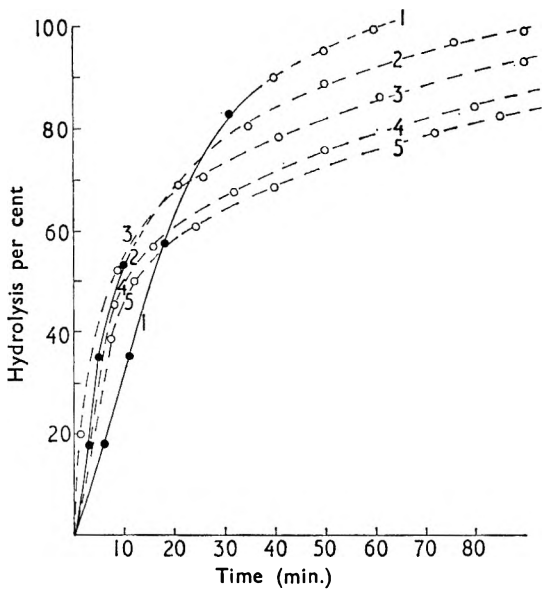

FIG. 3. Alkaline hydrolysis of diethyl phthalate (0·1 mole/litre) in cetrimide solutions at 35°.
Cetrimide concentration (moles/litre)
1. 0·02. 2. 0·08. 3. 0·187. 4. 0·35. 5. 0·43.
—— ● —— Emulsion ---- ○ ---- Solution.

Results

The solubilities of esters in cetrimide are given in Table I. The influence of cetrimide concentration on the hydrolysis of emulsions and solutions of ethyl benzoate and diethyl phthalate is shown in Figs. 1–3. Fig. 4 shows the hydrolysis of benzocaine solubilised in various cetrimide solutions. With increase in cetrimide concentration the initial rate of hydrolysis of emulsions increased, while that of solutions decreased. In the final stages the rate of hydrolysis decreased progressively with increase in the amount of cetrimide.

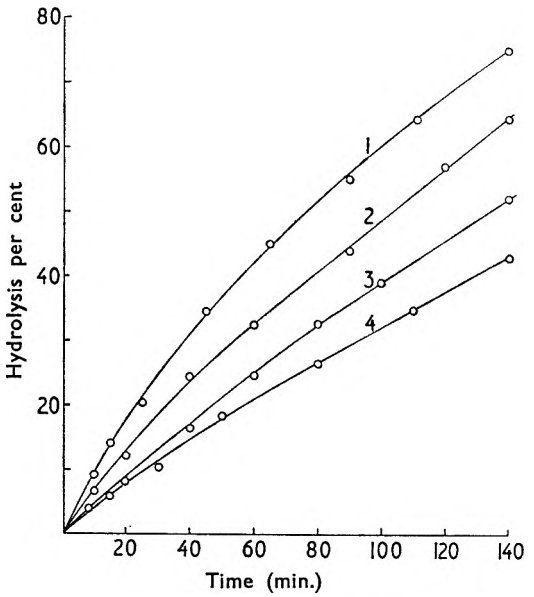

Fig. 4. Alkaline hydrolysis of benzocaine (0·05 moles/litre) in cetrimide solutions at 35°.

Cetrimide concentration (moles/litre)
1. 0·07. 2. 0·1. 3. 0·2. 4. 0·4.

Discussion

The rate of hydrolysis both of the solid ester, benzocaine, and of the liquid esters, ethyl benzoate and diethyl phthalate, varies with the concentration of cetrimide.

Where the esters are in solution the rate of hydrolysis decreases as the concentration of surface-active material is increased. A property of solutions of surface-active materials is their ability to take insoluble materials into solution. It is accepted that this phenomenon is associated with the presence of colloidal aggregates of soap molecules or micelles. The solute distributes itself between the micelles and the aqueous "phase" and since it is preferentially soluble in the micellar pseudo-phase an increase in the concentration of surface-active material will increase the amount of solute in this phase relative to that in true solution. Hydrolysis

will occur most readily in the aqueous "phase" where the ester molecules are easily accessible to attack by hydroxyl ions. Hence increasing the concentration of cetrimide alters the distribution of ester in favour of the micelles and the rate of hydrolysis decreases. Besides the reduction in the amount of ester available in true solution it is possible that the charged atmosphere around the micelles may act as a partial barrier to the penetration of hydroxyl ions.

Using liquid esters it was possible to study the effect of cetrimide on the hydrolysis both of emulsified and solubilised esters. In emulsions the initial rate of hydrolysis increases with cetrimide concentration reaching a maximum when the amount of cetrimide present is sufficient to solubilise all the ester. The solubility of esters increases with cetrimide concentration and the change in rate of hydrolysis is due therefore to an increase in the proportion of ester in solution relative to that in the emulsion droplets. The more rapid hydrolysis of solubilised compared with emulsified ester is the result of the greatly enlarged "interface" available both for attack by hydroxyl ions and for diffusion of ester molecules from the micelles into the true aqueous "phase." Reaction at the emulsion droplet-water interface will contribute to the overall effect but is not responsible for the increase in hydrolysis rate with increasing cetrimide concentration. Addition of cetrimide in excess of that needed for complete solution causes a reduction in the initial rate of hydrolysis for the reasons discussed above.

As the hydrolysis proceeds and ester is removed, emulsions become solutions and solutions become progressively less saturated. In the final stages the rate of reaction decreases systematically with increase in cetrimide concentration causing the hydrolysis curves to cross over as shown in Figs. 1–3.

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THE NEUROMUSCULAR BLOCKING ACTION OF SUXAMETHONIUM ON THE RAT DIAPHRAGM

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Experiments are described using the rat isolated phrenic nerve diaphragm preparation, in which suxamethonium produced a neuromuscular block consisting of an initial phase of fairly sharp onset followed by a prolonged phase, which first remained at a steady level and then slowly decreased in intensity over several hours. Suxamethonium block is antagonised by potassium and intensified by tubocurarine in both phases. It would therefore appear that the depolarising action of suxamethonium is complicated by some measure of competitive inhibition in the isolated nerve-muscle preparation as in the intact animal.

The nature of the neuromuscular block produced by suxamethonium showed a marked species variation. Zaimis (1953) reported some competitive features of the block in monkeys, dogs, rabbits and hares. However, on isolated rat and kitten phrenic nerve diaphragm preparations, Stovner (1958) showed that the neuromuscular block produced by succinylmonocholine was more competitive in nature than that produced by suxamethonium.

There is much clinical evidence for the existence of a mixed neuromuscular block produced by suxamethonium. Grant (1952), Ruddell (1952), Hodges (1953), Guerrier and Williams (1954), and Brennan (1956) have reported reversal of prolonged suxamethonium paralysis by neostigmine in man and in other intact animals.

Experimental

Method

The details of the dissection and assembly of the apparatus were those described by Bülbring (1946) and modified by Chou (1947). The fan-shaped muscle strip was stimulated indirectly through the phrenic nerve at 6/min. with supramaximal rectangular pulses of 0·1 to 0·3 m-sec. duration. Muscle contractions were recorded by a spring-loaded lever. The muscle was immersed in a bath containing Tyrode solution modified by halving the concentration of calcium chloride (bringing the concentration nearer to the ionised concentration in the blood) (McDowall, Miechowski and Shafei, 1949), and reducing the concentration of magnesium chloride from 0·01 to 0·0025 per cent (Taugner and Fleckenstein, 1950). The fluid was aerated with 95 per cent oxygen and 5 per cent carbon dioxide. The capacity of the bath was 75 ml. and the temperature was maintained constant in all experiments at $37° \pm 0·25°$. Doses referred to are in terms of suxamethonium bromide, potassium chloride and tubocurarine chloride.

Results

Different preparations showed a wide variation in sensitivity to suxamethonium. In comparison with tubocurarine the preparations were relatively insensitive to suxamethonium.

The effect of potassium chloride on the neuromuscular block produced by suxamethonium. The two phases of the neuromuscular block produced by the addition of 600 μg. of suxamethonium to the bath fluid are shown in Fig. 1a. There was an initial sharp onset of block during the first 8 to 10 min., followed by a fairly steady prolonged phase and then the block slowly diminished in intensity over several hours.

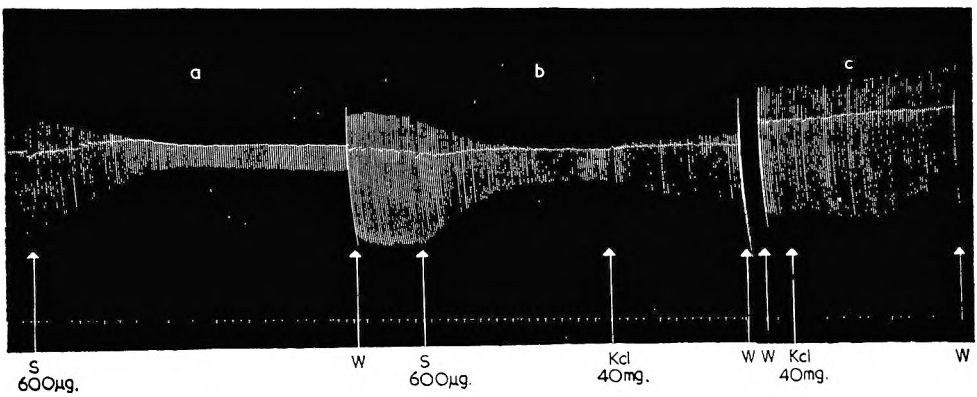

FIG. 1. The antagonism by potassium chloride of the neuromuscular block produced by suxamethonium. Rat isolated diaphragm preparation stimulated through phrenic nerve, 6/min. At S, suxamethonium added to bath fluid for 20 min. At W, preparation washed with Tyrode solution. In (b) potassium chloride added to bath fluid 11 min. after the addition of suxamethonium. Time 30 sec.

Potassium chloride added to the bath fluid during the prolonged phase reduced the block (Fig. 1b). The suxamethonium was left in the bath for 20 min. and in Fig. 1b the potassium was added to the bath fluid 11 min. after the addition of suxamethonium.

The antagonism by potassium, added to the bath fluid during the prolonged phase of suxamethonium block is shown again in Fig. 2c and the antagonism when potassium was added during the initial phase of the block in Fig. 2b. The addition of potassium chloride alone to the bath fluid resulted in increased response of the diaphragm strip to indirect stimulation (Figs. 1c and 2d).

The effect of tubocurarine on the neuromuscular block produced by suxamethonium. When tubocurarine was added to the bath fluid either during the initial or prolonged phases of neuromuscular block produced by suxamethonium, the block was intensified (Figs. 3d and 3b). The suxamethonium was in the bath for 15 min. In Fig. 3d the tubocurarine was added 1 min. after the addition of the suxamethonium to the bath fluid, and in Fig. 3b the tubocurarine was added 9 min. after the addition

of the suxamethonium. Control results with suxamethonium and tubocurarine alone are shown in Figs. 3a and 3c.

Discussion

The original observation by Wilson and Wright (1936), that potassium antagonised the action of curare has since been used to distinguish between drugs which produced neuromuscular block by competition with

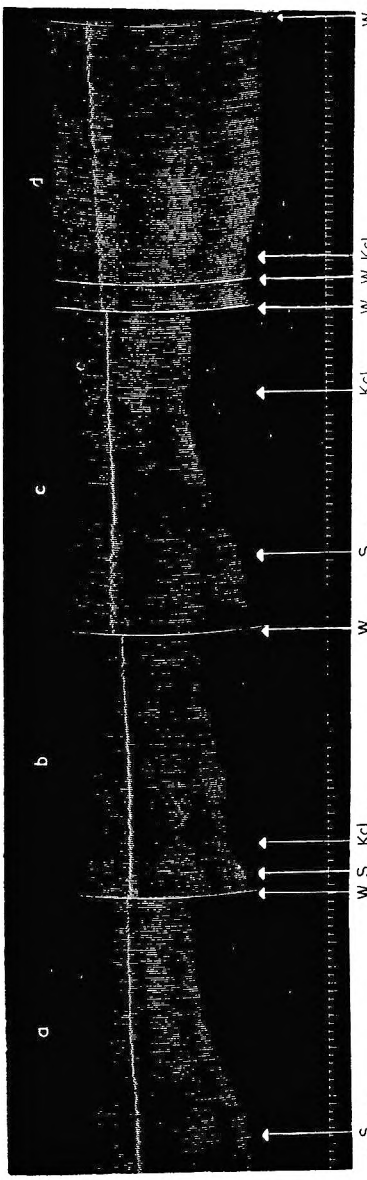

Fig. 2. The antagonism by potassium of the neuromuscular block produced by suxamethonium, when potassium added during the initial phase (b) or during the prolonged phase of block (c). Rat isolated diaphragm preparation stimulated through phrenic nerve, 6/min. At S, suxamethonium added to bath fluid for 15 min. In (b) potassium added to bath fluid 2 min. after suxamethonium. In (c) potassium added to bath fluid 10 min. after suxamethonium. At W preparation washed with Tyrode solution. Time, 30 sec.

acetylcholine, like tubocurarine, and drugs which produced block of the neuromuscular junction by depolarisation (Paton and Zaimis, 1949; Jenden, Kamijo and Taylor, 1951; Bowman, 1958). The antagonism by potassium of both the initial phase (Fig. 2b) and the prolonged phase (Figs. 1b and 2c) of the suxamethonium block indicates therefore the presence of a competitive feature in this block.

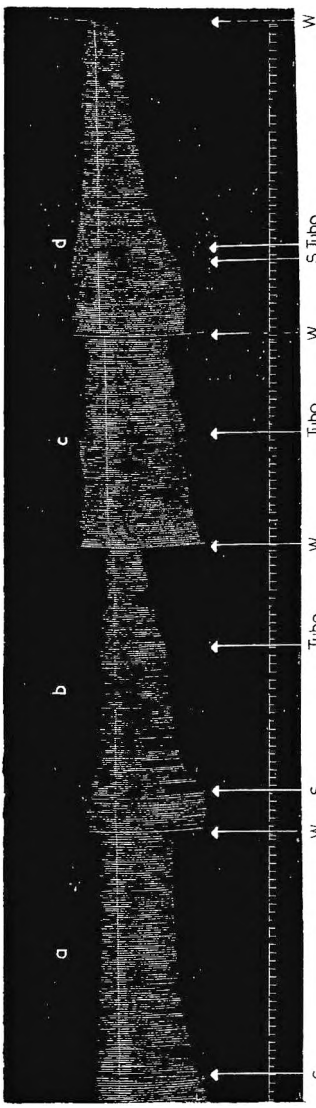

FIG. 3. The intensification by tubocurarine of the neuromuscular block produced by suxamethonium; tubocurarine added during the initial phase (d) and during the prolonged phase of block (b). Rat isolated diaphragm preparation stimulated through phrenic nerve, 6/min. At S, suxamethonium added to the bath fluid for 15 min. In (b) at Tubo., 40 µg. tubocurarine chloride added 9 min. after suxamethonium. In (d) 40 µg. tubocurarine added 1 min. after suxamethonium. At W preparation washed with Tyrode solution. Time, 30 sec.

There are several reports of antagonism existing between depolarising and competitive drugs. The administration of tubocurarine after depolarising drugs like decamethonium and suxamethonium inhibited the

effect of these drugs (Castillo, Phillips and de Beer, 1949; Paton and Zaimis, 1949; Vogel and Steinke, 1956; Dillon, Sabawala, Taylor and Gunter, 1957). The effect of other competitive blocking agents on a block produced by tubocurarine was additive (Winter and Lehman, 1950; Wescoe and Riker Jr., 1951). When tubocurarine was added to the bath fluid either during the initial or prolonged phase of neuromuscular block produced by suxamethonium, the block was intensified (Figs. 3d and 3b). Again this indicates a competitive element.

The observations reported here may be related to the hydrolysis of suxamethonium. Whittaker and Wijesundera (1952) showed that horse serum cholinesterase hydrolysed suxamethonium in two stages: (i) fairly rapidly to succinylmonocholine and choline and (ii) much more slowly to succinic acid and choline. Human plasma cholinesterase acted similarly (Tsuji, Foldes and Rhodes Jr., 1955). Low and Tammelin (1951) reported the breakdown of suxamethonium *in vitro* by both true and pseudocholinesterases.

The competitive features reported here may be connected therefore with the formation of succinylmonocholine, which produces a neuromuscular block showing several competitive features (Stovner, 1958).

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THE EFFECTS OF ANTI-INFLAMMATORY DRUGS ON SOME ASPECTS OF INTERMEDIARY METABOLISM

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The effects of salicylate, 2,4-dinitrophenol, hydrocortisone, dexamethasone, phenylbutazone and chloroquine diphosphate on the incorporation of radioactivity from [^{14}C]glucose and [1,4-^{14}C]succinate into the soluble intermediates of rat liver preparations have been studied. It is concluded that the increased incorporation of radiocarbon into oligosaccharides, phosphates and malic and fumaric acids bears no relation to anti-inflammatory activity in the salicylate group of drugs.

The anti-inflammatory compound, γ-resorcylic acid, produces several effects on intermediary metabolism such as an increased incorporation of radioactivity into the oligosaccharide, phosphate and organic acid fractions of rat liver preparations incubated with labelled substrates (Huggins, Bryant and Smith, 1961). The present work is concerned with the possible relation of these effects with anti-inflammatory activity. The effects of other anti-inflammatory drugs, salicylate, hydrocortisone, dexamethasone, phenylbutazone and chloroquine diphosphate, and of a related phenolic substance, 2,4-dinitrophenol, which is devoid of experimental anti-inflammatory properties, have therefore been studied in the same biochemical systems.

Experimental

The techniques used for the liver preparations and for the radioactive experiments were those described by Huggins, Bryant and Smith (1961). The concentrations of the drugs, after admixture with the tissue preparations and incubation media, were 5 mM for salicylate, 0·5 mM for 2,4-dinitrophenol, 10 μg./ml. for hydrocortisone, phenylbutazone and chloroquine diphosphate and 0·5 μg./ml. for dexamethasone (Moses and Smith, 1961).

TABLE I

Metabolism of [^{14}C]glucose by rat liver homogenate in the presence or the absence of anti-inflammatory drugs

The ^{14}C present in each intermediate is expressed as a percentage of the total ^{14}C incorporated from the labelled substrate into the sum of all the soluble intermediates; ^{14}C in the residual substrate is excluded from all calculations

Soluble intermediate	None*	Resorcylate*	Salicylate	DNP	Hydrocortisone	Dexamethasone	Phenylbutazone	Chloroquine
Alanine	13	7	10	7	8	10	8	11
Lactic acid	14	0	0	3	20	16	24	20
Malic acid	0	0	0	0	2	1	2	2
Oligosaccharides	36	67	53	55	42	43	41	39
Phosphates	37	16	27	29	22	24	19	22
Unidentified compounds	0	10	10	6	6	6	6	6

* Data from Huggins, Bryant and Smith (1961).

RESULTS

The percentages of radiocarbon from [^{14}C]glucose which were incorporated into the soluble metabolic intermediates of a homogenate of rat liver, in the presence or the absence of the drugs, are given in Table I.

Salicylate and 2,4-dinitrophenol (DNP) resembled γ-resorcylate in causing substantial increases in the formation of radioactive oligosaccharides and a decreased incorporation of isotope into the phosphate fraction. The latter effect was also shown by the other anti-inflammatory drugs but these had much smaller actions on the oligosaccharide fraction. γ-Resorcylate, salicylate and DNP reduced the formation of radioactive lactic acid whereas the other drugs tended to produce the reverse effect.

TABLE II

METABOLISM OF [^{14}C]GLUCOSE BY A SOLUBLE FRACTION FROM RAT LIVER IN THE PRESENCE OR THE ABSENCE OF ANTI-INFLAMMATORY DRUGS

(Results expressed as in Table I)

Soluble intermediate	None*	Resorcylate*	Salicylate	DNP	Hydrocortisone	Dexamethasone	Phenylbutazone	Chloroquine
Alanine	16	10	19	19	18	23	24	29
Aspartic acid	16	13	20	16	18	21	24	26
Lactic acid	37	26	34	39	30	17	12	4
Malic acid	0·7	1·0	0·2	0·8	0·9	1·5	1·0	1·3
Oligosaccharides	1·6	2·1	1·2	1·3	3·7	5·3	4·0	5·4
Phosphates	24	48	23	21	24	28	29	31
Unidentified compounds	4·7	0	2·6	2·9	5·4	4·2	6·0	3·3

* Data from Huggins, Bryant and Smith (1961).

The results in Table II show that only γ-resorcylate caused an increase in the incorporation of ^{14}C into the phosphate fraction derived from [^{14}C]glucose in the soluble liver preparation. Hydrocortisone, dexamethasone, phenylbutazone and chloroquine enhanced the formation of the labelled oligosaccharide fraction whereas the other drugs were inactive. No consistent effects on the incorporation of isotope into the lactic acid were observed.

TABLE III

METABOLISM OF [1,4–^{14}C]SUCCINATE BY RAT LIVER MITOCHONDRIA IN THE PRESENCE OR THE ABSENCE OF ANTI-INFLAMMATORY DRUGS

(Results expressed as in Table I)

Soluble intermediate	None*	Resorcylate*	Salicylate	DNP	Hydrocortisone	Dexamethazone	Phenylbutasone	Chloroquine
Alanine	1	1	1	1	2	1	3	0
Asparagine	5	0	0	0	6	12	2	10
Aspartic acid	25	7	10	10	23	37	20	18
Glutamic acid	2	1	3	2	10	0	4	0
Glutamine	19	0	0	1	0	0	0	0
Citric acid	1	4	7	9	3	4	6	3
Fumaric acid	5	21	23	22	8	0	6	3
Malic acid	7	63	55	54	19	4	28	12
Lactic acid	16	1	3	1	11	9	14	12
Phosphates	12	0	0	0	14	19	13	23
Unidentified compounds	7	2	0	0	4	14	4	19

* Data from Huggins, Bryant and Smith (1961).

Table III shows the distribution of radiocarbon from [1,4-^{14}C]succinate among the soluble intermediates of mitochondrial suspensions from rat liver, in the presence or absence of the drugs. The most prominent action of γ-resorcylate, the accumulation of ^{14}C into the fumaric and malic acids, was shared by salicylate and DNP. However, this effect was much less pronounced with hydrocortisone and phenylbutazone and was absent in the dexamethasone and chloroquine experiments. Further differences between γ-resorcylate, salicylate and DNP on the one hand, and the steroids, phenylbutazone and chloroquine, on the other were concerned with the incorporation of isotope into the lactic acid, aspartic acid, asparagine and phosphate fractions. The first group caused a decreased formation of these labelled intermediates but the second group had little effect. All the drugs reduced the incorporation of ^{14}C into glutamine.

DISCUSSION

Three separate effects of γ-resorcylic acid on intermediary metabolism were distinguished by Huggins, Bryant and Smith (1961). These were the increased incorporation of radioactivity from labelled glucose into an oligosaccharide fraction of a rat liver homogenate; into the phosphate compounds formed in a soluble preparation from rat liver and from labelled succinate into the fumaric and malic acids in rat liver mitochondria. The present results show that none of these biochemical effects appear to be connected with anti-inflammatory activity. The relevant data is summarised in Table IV where it is seen that although

TABLE IV

EFFECTS OF ANTI-INFLAMMATORY DRUGS AND DNP ON THE INCORPORATION OF ^{14}C FROM [^{14}C]GLUCOSE OR [1,4-^{14}C]SUCCINATE INTO SOME FRACTIONS OF THE SOLUBLE INTERMEDIATES OF RAT LIVER PREPARATIONS

The results are expressed as percentages of the corresponding control values

Drug	[^{14}C]Glucose		[1,4-^{14}C]Succinate
	Oligosaccharides in homogenates	Phosphates in soluble fraction	Malic plus fumaric acids in mitochondria
Resorcylate	186	200	700
Salicylate	152	96	650
Dinitrophenol	154	88	630
Hydrocortisone	117	100	225
Dexamethasone	119	117	33
Phenylbutazone	114	121	280
Chloroquine	104	129	125

salicylate resembles γ-resorcylate in causing at least a 50 per cent increase in the accumulation of ^{14}C in the oligosaccharides and into fumaric and malic acids, none of the other anti-inflammatory drugs shared these effects. More pertinently, DNP, which does not possess experimental anti-inflammatory activity (Marks, Smith and Cunliffe, 1961) also produced similar effects to γ-resorcylate in these systems. Of the drugs tested only γ-resorcylate caused a doubling of the incorporation of radiocarbon into the phosphate compounds of the soluble liver preparation. It is most unlikely that this action bears any relevance to the anti-inflammatory properties

of the dihydroxybenzoate since the closely related salicylate is without effect. The effect on the formation of radioactive phosphates in the soluble liver preparation appears to be restricted to γ-resorcylate whereas the other effects on oligosaccharides and the tricarboxylic acids are shared by compounds such as salicylate and DNP, which also possess phenolic hydroxyl groups. It must be concluded that none of the above effects can be related with anti-inflammatory activity in general.

The anti-inflammatory drugs used in the present work do not share common actions on intermediary metabolism. This is not surprising in view of their diverse chemical structures and it seems unlikely that they produce their beneficial effects in rheumatism by the same mechanism. The failure of the powerful uncoupling reagent, 2,4-dinitrophenol, to exhibit experimental anti-inflammatory properties (Adams and Cobb, 1958; Marks, Smith and Cunliffe, 1961) shows that this major action on cellular metabolism is not related to anti-inflammatory activity. However, both salicylate and γ-resorcylate inhibit glutamic-pyruvic transaminase activity (Steggle, Huggins and Smith, 1961) whereas DNP is inactive. The possible relation of this biochemical effect to anti-inflammatory activity in the salicylate group of drugs remains to be explored. In this laboratory it has been found that hydrocortisone, dexamethasone, phenylbutazone and chloroquine do not influence glutamic-pyruvic transaminase activity *in vitro* (Steggle and Smith unpublished data) but possess a common action on maltose formation from glucose in chopped liver preparations (Moses and Smith, 1961). These last four compounds also show consistent effects in increasing the incorporation of ^{14}C into oligosaccharide substances in the present experiments and it is possible that this action may bear some relevance to their anti-inflammatory properties.

Acknowledgements. We are grateful to Miss B. Fenton for valuable technical assistance, to the Empire Rheumatism Council for a grant towards the cost of the work and to the Wellcome Trust for a grant for the purchase of a Spinco preparative ultracentrifuge.

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A NOTE ON THE PEROXIDE VALUE OF LANOLIN

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The peroxide value of lanolin is reduced to a low figure by heating at 100° for short periods. This effect is sufficient to explain the low peroxide value of wool wax extracted from wool scour liquors, and may be utilised to prepare low-peroxide lanolin for special purposes, such as the manufacture of penicillin ointment.

Clark and Kitchen (1961) noted that wool wax on the fleece is highly autoxidised and has a high peroxide value, whereas the wax recovered by centrifuging wool scour liquors is invariably of low peroxide value. From peroxide values of waxes recovered from scour liquors after various treatments, they concluded that peroxides are reduced by chemical or biological reducing agents in the scour liquor, or both.

It is shown here that simple heating of wool wax causes reductions in peroxide value of similar magnitude to those measured by Clark and Kitchen.

Results and Discussion

Peroxide values, measured by the A.O.C.S. (1960) method, and expressed as ml. of 0·002N thiosulphate per g. of sample, are shown in Fig. 1 for several different samples of wool wax after heating for various times at 100°. Comparatively short heating times are sufficient to reduce the peroxide values below 10.

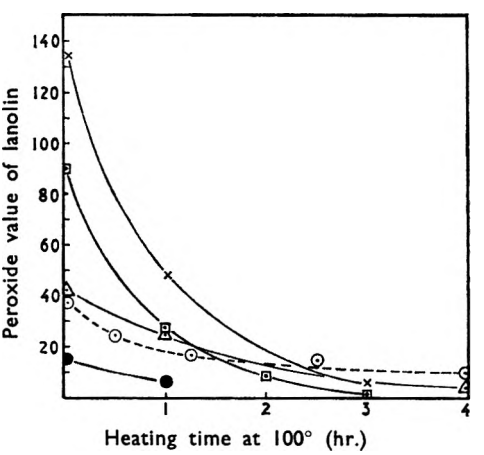

Fig. 1. The effect of heating on the peroxide value of lanolin.

⊙ Surface layer of unbleached lanolin. × Surface layer of wool wax obtained by acid cracking. △ Wool wax extracted from Merino fleece with solvent. ⊡ Lanolin bleached with sodium chlorite. ● Data of Clark and Kitchen (1961).

THE PEROXIDE VALUE OF LANOLIN

In the usual process for recovering wool wax, a scour liquor, originally at 50°, is heated almost to the boil, centrifuged and recycled to the scour bowl. Wool wax removed in the centrifuge is subjected to only one heating cycle, but we have found this wax to be a special fraction containing only unoxidised material so it would not be expected to have a high peroxide value. The remainder of the wax, including all the oxidised material, is not removable from the liquor by ordinary centrifuging, and passes the heating-centrifuging cycle many times before the liquor is discarded. A typical sample of liquor will therefore contain wool wax which has had an exposure of 1 hr. or more to temperatures of 90–100°. This heated wax is recoverable from the liquor by the ether extraction of Clark and Kitchen, and in accordance with the results given in Fig. 1 would be expected to have a low peroxide value.

On this basis the normal thermal treatment of scour liquors is sufficient to explain the low peroxide value of wool wax recovered from them. Reduction by chemical or biological agents, or both, in the liquor may take place, but its effect on peroxide value is probably small compared with the effect of heat.

If a lanolin of low peroxide value is required for a particular purpose, for example in the preparation of penicillin ointments (Diding and Sandell, 1949), it can be obtained from any otherwise suitable grade by a simple heating procedure.

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BOOK REVIEWS

TOBACCO. EXPERIMENTAL AND CLINICAL STUDIES. By P. S. Larson, H. B. Haag and H. Silvette. Pp. xii + 932 (including Index). Bailliere, Tindall and Cox Ltd., London, 1961. 160s.

This is a remarkable volume. Its authors are two professors and a visiting professor of pharmacology at the Medical College of Virginia, and they have dedicated the volume to their wives with ". . . admiration for their incredible patience." The dedication is well deserved. The scope, as the subtitle suggests, is confined to the laboratory and the patient. There is no discussion of the agricultural and industrial aspects of the subject, although much of medical interest might be discovered by examining the sociology of tobacco. Within their chosen limits, the authors have set out to be comprehensive, and they seem to have succeeded. There are 109 pages of references, at about 60 per page. The original printing omitted McArthur to McVay and these are inserted as an extra page. Brief inspection did not show other inaccuracies, and showed a much wider reading of world literature than is sometimes apparent in American works. In the text the well-known facts about nicotine are all collected and fully documented. So is a good deal of material of more doubtful value, such as the use of a chamber pot filled with burning coals and tobacco as a treatment for piles. Whether the fumes of nicotine provide more effective treatment than simply sitting on hot coals is not discussed, but it is expressly not the purpose of this book to adjudicate. The treatment has been reported; here is the reference. In a few years time, this sort of book will be unnecessary, because an adequately programmed computer will be able to extract whatever information is required from its stores. However the output of the computer is unlikely to be as elegant and dignified as this 930 page double-column volume. For anyone who wants to find all the literature on medical aspects of tobacco, it will be invaluable. Anyone who wants discriminating guidance will find his time endlessly wasted, if only because there are so many amusing by-ways to pursue when all the clinical and experimental opinions are gathered in one compendium.

MILES WEATHERALL.

PROCEEDINGS OF THE FOURTH INTERNATIONAL CONGRESS ON CLINICAL CHEMISTRY. EDINBURGH, 1960. Pp. xvi + 212. E. and S. Livingstone, Ltd., Edinburgh, 1961. 35s.

The present volume contains the full text of the papers read at the four plenary sessions together with short abstracts of a catholic array of communications. The main function of the latter is to provide a useful forewarning of their eventual appearance as full papers in the literature. In contrast the detailed accounts of the symposia and their discussions are self-contained chapters and present valuable reviews of the selected topics.

In the session devoted to plasma protein turnover in disease, McFarlane surveys the use of isotopes in clinical research on proteins, being mainly concerned with problems of permissible doses of radioactive isotopes, the choice of amino-acid, the isolation of the labelled protein and the calculation of rates of synthesis and catabolism. Schwartz and Jarnum contribute a critical account of turnover studies with ^{131}I—labelled proteins in various diseases such as cirrhosis, nephrosis, protein-losing gastro-enteropathy and the collagen diseases. The symposium entitled "mechanisms of urine production" includes a detailed paper by Berliner on the renal transport and excretion of potassium,

a more general treatment of modern concepts of renal function by Black and a discussion of the mechanisms of urinary concentration and dilution in the mammalian kidney by Wirz.

The third section is concerned with a subject, enzymes in clinical chemistry, that has achieved much prominence in recent years. King presents a short historical introduction and Webb produces some trenchant comments on the necessity for standardised conditions under which enzyme activities should be measured. Although it is possible to devise methods for measuring the absolute amounts of enzymes in tissues, the clinical interpretation of the results must always be empirical. Two particularly interesting and inter-related papers are by Bruns and by Wroblewski and Gregory. The first concerns the differentiation and measurement of organ-specific enzymes in serum. Here the eventual aim is to distinguish damage or perhaps even different types of damage, to specific organs from the pattern of abnormalities observed in the serum enzymes. The other paper deals more directly with isoenzymes, which are individual plasma or tissue enzyme activities which have been shown to be due to two or more similar but chemically, immunologically, and electrophoretically distinct components. Thus, the lactic dehydrogenase activity of human plasma consists of the sum of five iso-enzymes of lactic dehydrogenase. Individual human tissues, such as cardiac muscle and liver, each have a different and characteristic iso-enzyme composition. Hence, the serum pattern of iso-enzymes in a particular patient may be characteristic of an organ or the pathological process, or both.

The remaining session consists of a group of papers dealing with congenital abnormalities of metabolism. Harris gives a useful general account of the genetic factors with special reference to serum cholinesterase. Kretchmer deals with the disorders or carbohydrate metabolism, such as glycogen storage disease, and Sobotka with those of lipid metabolism including the various lipid storage diseases and gargoylism. Beutler contributes a description of the various metabolic abnormalities of red cells and discusses the mechanism of the sensitivity of the erythrocyte to primaquine.

The book is both interesting and informative and is recommended to all those who are involved with the growing impact of basic research upon the medical sciences.

M. J. H. SMITH.

LETTERS TO THE EDITOR

Effect of Pyrogallol and Catechol on Isolated Smooth Organs

SIR,—Pyrogallol, when given intravenously to dogs, has a secondary excitatory effect on duodenal motility, apparently cholinergic in nature (Izquierdo and Izquierdo, 1961). This result led us to study the action of pyrogallol on isolated smooth organs of rabbits, guinea-pigs and rats. We also studied the effect of catechol, another inhibitor of catechol-*o*-methyltransferase, which according to Sjöstrand (1960) has excitatory effects on the guinea-pig ileum, due to an action on the muscle itself and on the intramuscular ganglion.

Our material and methods were the usual: a 3 ml. bath, thermo-regulated at 37 ± 0·5°, filled with Tyrode solution, and a frontal lever (1 : 10). Drugs used were pyrogallol (Merck), catechol (Poulenc Frères), adrenaline hydrochloride (Parke Davis), ascorbic acid (Roche).

In the first trials we dissolved pyrogallol in 1 per cent ascorbic acid. However, this solvent had an excitatory action on tonus and motility of its own. This led us to use solution of pyrogallol in Tyrode prepared just before adding it to the bath, so as to prevent oxidation.

TABLE I

Preparation	Pyrogallol		Catechol	
	Concentration mg.	Effect	Concentration mg.	Effect
Rabbit:				
duodenum	1	—	3	—
uterus	1	+0		+ tachyphylaxis
Guinea-pig:				
ileum	2	0 antihistaminic effect	1	+
uterus	0·5	+		
Rat:				
duodenum	0·2	—	0·4	—
uterus	0·1 to 0·03	—	0·08	—

— Relaxes. + Contracts. 0 No effect. +0 Eventually contracts.

According to the results expressed in Table I, both pyrogallol and catechol have a similar adrenaline-like action. Pyrogallol enhances the effect of adrenaline on the rabbit duodenum and antagonizes that of histamine on the guinea-pig ileum. These effects are in agreement with those observed on the *in situ* duodenum of dogs (Izquierdo and Izquierdo, 1961).

Though both polyphenols have little activity, the rat uterus appears to be the most sensitive organ to them. No excitatory "cholinergic-type" effect was seen on isolated smooth organs.

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January 3, 1962.

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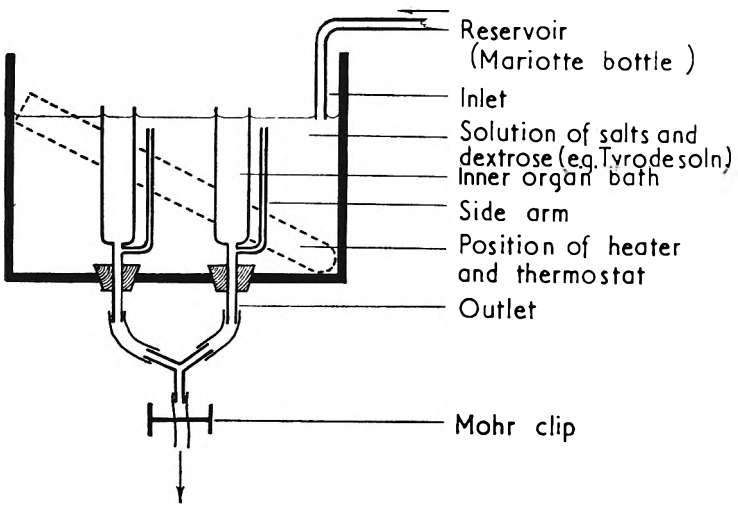
A Modified Dual Unit Organ Bath for Isolated Tissues

SIR,—Isolated tissue preparations are widely used for the investigation, estimation and standardisation of the pharmacological activity of natural and chemically synthesised compounds. Of these preparations, the tissues isolated from warm-blooded vertebrates must be suspended in a solution of salts and dextrose (e.g., Tyrode solution) which is adequately oxygenated or aerated, and maintained at an optimal temperature for the particular tissue. An apparatus incorporating these requirements which is simple but adequate for most routine purposes has been described by Burn (1952). It is often desirable or necessary to experiment with two isolated tissues simultaneously; to answer this need a modified dual unit organ bath has been developed having distinct advantages over those in conventional use.



The apparatus (Fig. 1) consists of a perspex container, 18 cm. long × 10 cm. wide × 12 cm. deep, mounted on a metal frame. The two cylindrical inner organ baths have an internal diameter 1·5 cm. and are 8 cm. in length; in addition, there is a side arm of internal diameter 0·3 cm. and 7 cm. long extending from the base of each organ bath. The perspex container and organ baths are filled with Ringer's solution, the level of this solution being maintained by means of a Mariotte bottle acting as a reservoir. The height of the solution in the container is so adjusted, that, on draining the solution from the organ baths, they are automatically re-filled by the side arm, which in turn activates the filling of the perspex container from the reservoir to the predetermined level. Using this procedure, the volume of solution in the organ baths remains constant during the experimental use of the apparatus.

A heater and thermostat are placed directly into the Ringer's solution in the perspex container; this ensures negligible variation of the required temperature of this solution, even when the organ baths are drained and re-filled at frequent intervals. With this apparatus it is usual to oxygenate or aerate the Ringer's solution both in the organ baths and the perspex container.

Simple experiments have shown that when dyes are introduced into the oxygenated solution in the organ baths, there is no diffusion into the side arms even when the dyes are allowed to remain in the organ baths for periods up to 2 hr.

The apparatus is simple, compact, presents no cleaning difficulties, and facilitates an easy and efficient working procedure; it can be used with equal success for either one or two simultaneous isolated tissue experiments.

We would like to thank Dr. P. F. D'Arcy for his helpful advice in this work.

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Research Division,
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January 23, 1962.

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