THE EFFECTS OF LONG-CHAIN POLYMETHYLENE BISTRIETHYL AND BISTRIMETHYL AMMONIUM SALTS ON JUNCTIONAL TRANSMISSION

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C O N T E N T S

INTRODUCTION

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I. THE DEVELOPMENT OF THE CONCEPT OF CHOLINERGIC TRANSMISSION AT PERIPHERAL SYNAPSES

The nerve or muscle impulse consists of a wave of electrical activity, the action potential, propagated along the fibre by 'local circuit' transmission, and dependent on selective permeability to ions of the cell membrane.

During the early part of this century it was considered that this electrical transmission of the wave of excitation was unbroken across nerve-nerve and nerve-effector cell junctions. However, there is a difference between fibre and synapse - while conduction is equally possible in either direction in nerve or muscle fibre, transmission across a synapse is unidirectional.

The first suggestion that nervous effects might be transmitted by means of a chemical stimulant was made by Elliott in 1904. He had just showed that adrenaline exhibited effects similar to those of stimulation of the sympathetic nervous system, acting at the periphery and only

on muscles which possessed or had possessed sympathetic innervation. He suggested that there might be a structure on the muscle cell whose function was to receive and transform the nervous impulse, and that adrenaline might be the chemical stimulant liberated when the impulse arrived at the periphery (Elliott, 1904).

This idea was taken up by Dixon (1906) who further suggested that parasympathetic nerves might also release a chemical transmitter - for example, stimulation of the vagus might result in the release of a substance 'inhibitin' which caused cardiac standstill.

However, the idea then lay dormant for a period of 15 years. In the interim, Hunt and Taveau (1906) tested a number of esters of choline, and found one of them, acetylcholine, to have 'extraordinary physiological activity'. An injection of 1 ml. of a solution of 1×10^{-9} w/v acetylcholine caused a fall in the blood pressure of a curarised rabbit, and this property made it 'the most powerful substance known in effect on the circulation'. Also in this period Sir Henry Dale was drawn into this field.

He found acetylcholine in a sample of ergot, and was prompted to make a detailed study of its action, and that of some other esters of choline (Dale, 1914). He found that acetylcholine showed all the characteristics of stimulation of the parasympathetic nervous system (muscarine-like properties), and also possessed nicotine-like properties (e.g. stimulation of sympathetic ganglia). Its effects were transient, and he surmised that this was due to its ready hydrolysis to choline and acetic acid. However it had not been shown to occur in the body, and so speculations on its physiological significance were not justified.

Then, in 1921, Loewi performed his classical experiment with the perfused frog heart. He showed that perfusion fluid taken from a heart during inhibition by vagal stimulation and transferred to another heart would cause slowing of this second heart also. Thus some 'vagus substance' must have been released by the vagus nerve in the first heart, there to act on the cardiac muscle (Loewi, 1921). This was followed by a series of experiments in which he showed that this transmitter had the

properties of an unstable ester of choline, which was rapidly destroyed by an esterase present in heart muscle. The action of the transmitter, but not its release, could be prevented by atropine, and its destruction by the esterase could be prevented by eserine.

Thus it had been shown that parasympathetic effects could be transmitted by a substance which showed all the properties of acetylcholine - but acetylcholine had never been demonstrated as a natural body constituent. Then in 1929 Dale and Dudley succeeded in isolating and identifying acetylcholine from horse and ox spleen (Dale and Dudley, 1929). The release of a substance resembling acetylcholine by other parasympathetic nerves was also demonstrated e.g. the chorda-lingual nerve, by observing the effect of stimulating it on the denervated tongue, which has become sensitised to acetylcholine (Dale and Gaddum, 1930), and the vagus nerve to the stomach, by taking venous effluent from the perfused stomach and subjecting it to parallel assays against known acetylcholine solutions (Dale and Feldberg, 1934a). The sum of this evidence made it quite clear that at

post-ganglionic parasympathetic nerve-endings, a chemical transmitter was released, and that this transmitter was acetylcholine (Dale, 1934).

It had long been known that there were a few sites at which sympathetic stimulation was not mimicked by adrenaline, the best known example being the sweat-glands of man and the cat. Dale and Feldberg (1934b) showed (by perfusing the foot with eserinised Locke's solution and testing the effluent) that for the sweat glands of the pads of the cat's foot, the transmitter was acetylcholine. In the previous year, Dale had suggested the terms 'cholinergic' and 'adrenergic' for fibres, since he said that chemical transmission at parasympathetic and sympathetic nerve endings was generally accepted, though the actual identities of the transmitters were not known (Dale, 1933). This work of Dale and Feldberg showed that there could be, in some instances, cholinergic sympathetic fibres.

Chang and Gaddum (1933) tested extracts from many animal tissues for acetylcholine. They confirmed the earlier finding of large amounts in horse and ox spleen and also found

human placenta to be rich in acetylcholine, without being able to explain these high results. Generally, they found that tissues with highest acetylcholine-equivalents were those tissues whose main activities were controlled by nerves which could be supposed to act by liberating acetylcholine. They also found fairly large amounts in horse sympathetic chain - acetylcholine was known to stimulate ganglia, and so could it be involved in normal transmission at ganglia?

Kibjakow (1933) perfused the superior cervical ganglion of a cat with Locke's solution and found that perfusate collected during a period of stimulation of the pre-ganglionic sympathetic nerve, when added again to the perfusion fluid, would itself stimulate the ganglion, as shown by a contraction of the nictitating membrane. He suggested that there was a chemical transmitter released at the ganglionic synapse.

Feldberg and Gaddum (1934), using the same technique, could not repeat Kibjakow's results, but found that if eserine was added to the Locke's solution perfusion fluid, the perfusate

collected during preganglionic stimulation showed acetylcholine-like activity, while perfusate collected before or after stimulation was inactive. The active substance was indistinguishable from acetylcholine in parallel quantitative biological assays. Feldberg, Minz and Tsudzimura (1934) stimulated the splanchnic nerves of a cat and demonstrated salivary secretion and effects on its blood pressure due to released acetylcholine, as well as effects of the adrenaline liberated from the cells of the adrenal medulla. Thus the splanchnic nerve fibres were shown to release acetylcholine as their transmitter substance, and these fibres are also preganglionic sympathetic fibres, the adrenal medullary cells being morphologically equivalent to sympathetic ganglion cells. Feldberg and Vartiainen (1934) showed that in the cat superior cervical ganglion, the released acetylcholine could only have come from the preganglionic nerve-endings, and that the output of acetylcholine was proportional to the number of stimuli.

There was ample evidence, then, that acetylcholine was released at preganglionic

synaptic endings, and that it could be synthesised during prolonged stimulation in order to maintain transmission in the ganglion indefinitely (Brown and Feldberg, 1936). But unlike the slow, steadily maintained response of autonomic structures to nerve stimulation, a single preganglionic impulse evokes a single postganglionic impulse, so that a ganglionic transmitter would have to vanish within the refractory period of the cell. It could only be said that, in the absence of eserine, no acetylcholine could be detected in venous fluid, and that there was a high concentration of cholinesterase, the specific enzyme known to destroy acetylcholine, in the sympathetic ganglion, and that this disappeared on degeneration of the preganglionic nerve (Dale, 1937).

Finally there remained the neuromuscular junction, a rather more awkward proposition for perfusion due to the bulk of the muscle fibres compared to the tiny, scattered motor nerve-endings. Dale, Feldberg and Vogt (1936) did however succeed in perfusing various mammalian voluntary muscles with eserinised Locke's

solution, and obtaining active samples only on stimulation of the motor-nerves, the active substance being indistinguishable from acetylcholine. Acetylcholine was not obtained on direct stimulation of denervated muscle, but was still obtained during motor nerve stimulation when muscle contraction was completely blocked by curarine - its appearance was thus associated with neuromuscular transmission and not with mechanical response.

The next problem was that normal voluntary muscle had never been shown to give quick contractions - twitches - in response to acetylcholine. Only slow contractures had been observed, in some frog or bird muscles and in denervated mammalian muscle (see Dale, 1937).

Dale thought that the reason for this failure was probably the inability to produce an abrupt substantial concentration similar to that produced by sudden release of acetylcholine close to the motor end-plates. Brown, Dale and Feldberg (1936) designed a method of arterial injection close to the desired muscle (cat gastrocnemius) with which they succeeded

in obtaining quick contractions in response to small doses of acetylcholine. These quick contractions superficially resembled rather slow single twitches, but were, as expected, shown by electrical records to be short bursts of asynchronous tetanus, produced by summation of the asynchronous, repetitive, progagated twitches of individual fibres (Brown, 1937). Brown also showed that eserine, by delaying the destruction of acetylcholine, converted the response to a single nerve impulse from a single twitch to a repetitive response, a short waning tetanus.

Thus by 1937 it was concluded that transmission at peripheral synapses was chemical, and that the transmitter released from pre-ganglionic ~~autonomic~~ sympathetic nerve-endings, post-ganglionic parasympathetic nerve-endings and motor nerve-endings was acetylcholine. This impinged on the sensitive post-synaptic structures and was then quickly removed, by diffusion or by a high local concentration of the enzyme cholinesterase. The exception was the post-ganglionic sympathetic nerve-ending

where the transmitter was an adrenaline-like substance (Dale, 1937). This idea of chemical transmission was then generally accepted, except by J.C. Eccles and some of his colleagues, who did not believe that chemical transmission took place at ganglia. In 1944, he still maintained that in this case transmission was due to a direct depolarisation of the ganglion cell by the 'action current' of the preganglionic impulse, with a 'tail action' due to liberated acetylcholine, mainly because he was unable to obtain a potentiating effect of eserine on potentials set up by preganglionic volleys (Eccles, 1944). However, in 1952, he finally withdrew this opposition on the basis of observations made on motoneurones of the mammalian central nervous system (Brock, Coombs and Eccles, 1952).

II. MICROPHYSIOLOGY OF CHOLINERGIC PERIPHERAL SYNAPSES

(a) THE NEUROMUSCULAR JUNCTION

The resting nerve or muscle fibre maintains, by steady metabolic activity, concentration gradients of sodium and potassium across its surface membrane, resulting in a potential difference across the membrane. Excitation occurs when this potential difference is reduced to a critical 'threshold' level, at which point there is an alteration in membrane permeability and an exchange of ions across the membrane, causing a large potential change - the 'spike' or 'action potential'. The action potential is propagated by 'local circuit' transmission, which depends on structural continuity of the nerve or muscle fibre (Hodgkin, 1951; Castillo and Katz, 1956).

At the neuromuscular junction, this continuity is broken, and, as has been shown in the previous section, here transmission is effected by release of acetylcholine from the motor nerve-endings. This acetylcholine acts on special sensitive structures on the muscle

fibre, the motor end-plates, causing an action potential to be set up, and is subsequently destroyed by the enzyme cholinesterase. Technical developments, mainly in the use of electro-physiological methods, have enabled further details of this process to be filled in.

Using a small superficial strip of cat soleus muscle and external electrodes, Eccles and O'Connor (1939) recorded simple spike potentials corresponding to a brief wave of negativity propagated along the muscle fibres. They found that with short volley intervals, a second nerve volley failed to set up a propagated impulse, due to the 'refractory period' of the muscle fibre, but that with one electrode on the localised end-plate zone which exists in this muscle, a smooth wave of negative potential was still recorded. This was also found with deeply curarised muscle - there were no propagated spike potentials, but a negative potential, about 10% of the height of the spike, was obtained at the end-plate. They called this non-propagated local potential

change the 'end-plate potential' (e.p.p.) and concluded that a single nerve volley would also set up an end-plate potential in normal muscle (Eccles and O'Connor, 1939).

It was found that in curarised muscle (Eccles, Katz and Kuffler, 1941) and in normal muscle (Eccles and Kuffler, 1941), when the end-plate potential reached a certain threshold level, it initiated a propagated action potential. It was suggested that this local depolarisation, the e.p.p., could be due to a transient effect of acetylcholine on the muscle membrane. The effects of eserine, increasing and lengthening the e.p.p., and curare, decreasing the e.p.p., confirmed the view that the e.p.p. was due to a depolarising action of acetylcholine, released by nerve impulses, on the motor end-plates (Eccles, Katz and Kuffler, 1942). It was also shown that direct topical application of a drop of acetylcholine to isolated frog muscle, at the end-plate region only, gave rise to propagated action potentials, or with a subthreshold dose of acetylcholine, to a slow negative potential

only (Kuffler, 1943). The latency always obtained between stimulation of the motor nerve and the onset of the e.p.p. (about 1 msec. for the frog end-plate), and unchanged by curare or an anticholinesterase, provided a further argument, if one was necessary, for the intervention of a transmitter at the neuromuscular junction (Hunt and Kuffler, 1950). Thus in 1950 the picture of events at the neuromuscular junction was as follows: nerve impulse ⟶ transmitter (acetylcholine) ⟶ e.p.p. ⟶ muscle impulse ⟶ contraction.

Then it became possible to insert microelectrodes directly into individual cells, and to record resting potentials and changes in potential across the cell membrane (Ling and Gerrard, 1949; Nastuk and Hodgkin, 1950); and also to give doses of drugs close to the sensitive structures themselves, by ionophoretic discharge from micropipettes (Nastuk, 1951, 1953).

With intracellular microelectrodes in frog muscle fibres, small spontaneous discharges were detected, localised to the end-plate regions

and not due to damage of either muscle fibre or motor nerve. These were similar in shape to the end-plate potential, but about $\frac{1}{100}$ of the size, and were found in normal, resting muscle fibres by Fatt and Katz (1952), who called them spontaneous miniature end-plate potentials (m.e.p.p.). They could also be recorded, their polarity being reversed, with external electrodes. For any one end-plate, the mean amplitude of m.e.p.p.'s was fairly constant, but their frequency was not. They were found statistically to occur in a random manner.

The m.e.p.p.'s were shown by pharmacological tests to be caused by local random impacts of acetylcholine on the receptors of the muscle end-plate - they were reduced in size by curarine, and increased in amplitude and duration by prostigmine, as were the end-plate response to a nerve impulse and the depolarisation produced by local application of acetylcholine. Since chronically denervated muscle showed no m.e.p.p.'s, the source of this acetylcholine must be the motor nerve terminals

(Fatt and Katz, 1952).

Fatt and Katz wondered whether each m.e.p.p. might be due to the impact of a single molecule of acetylcholine, which had leaked from the terminal store (Feldberg (1945) had concluded that the acetylcholine store in cholinergic tissues was in a state of flux, being synthesised continually to replace that lost by leakage), but they decided from experimental evidence that this was not the case. Instead, each me.p.p. must have been due to the momentary, synchronous impact of a packet, or 'quantum', of hundreds, or perhaps thousands of acetylcholine molecules (Fatt and Katz, 1952; Katz, 1958). Neither sodium, nor electrical excitability, nor the normal resting potential, but only the structural integrity of the nerve terminals, was essential for the release of these quanta (Katz, 1958).

No means had been found of influencing the size of the quantum which was released - the amplitude of the m.e.p.p. was independent of the state of the nerve-endings and influenced solely by post-synaptic influences - while the frequency of the m.e.p.p.s was

affected by altering the condition of the nerve-endings (Katz, 1958). Particularly, depolarisation of the motor nerve-ending increased the frequency of the me.p.p.s, the frequency going up exponentially by a factor of 10 for a 15 mV lowering of the presynaptic membrane potential (the factor, however, depending on the ionic environment)(Liley, 1956, cited by Katz, 1958).

With lowered calcium and raised magnesium concentrations, the amount of acetylcholine released by a nerve impulse was reduced, and therefore the e.p.p. was reduced in size - and it was possible to show that the fluctuations of the e.p.p. were of a discontinuous step-wise nature, involving quantal steps, each step corresponding to a unit identical with a spontaneous m.e.p.p. (Katz, 1958).

Therefore, Katz and his colleagues reached the following conclusions: that the m.e.p.p. represented the least quantum of action in neuromuscular transmission, that this unit of acetylcholine release remained unchanged whether it occurred spontaneously or during motor nerve activity, and that the impulse,

by depolarising the membrane of the motor nerve terminal, momentarily increased the statistical chances of quantal release by a factor of several hundred thousand, resulting in the output of sufficient quanta of acetylcholine to produce a supra-threshold e.p.p. and hence an action potential.

Acetylcholine was known to be synthesised in the motor nerve (Feldberg, 1943) and to be present in the nerve not in free solution but in some bound form (Feldberg, 1945). Electron microscope studies showed that motor nerve terminals contained, as well as mitochondria, numerous small vesicular bodies - synaptic vesicles (Robertson, 1956). Robertson suggested that these might be analogous to secretion granules, and might contain acetylcholine. The presence of acetylcholine in 'packets' like this would conform very well with the idea of quantal release.

This means that for acetylcholine to be released from the nerve terminals, it must penetrate two barriers, the 'vesicle' capsule and the cell membrane. On the mode of this release there can at present only be speculation.

Katz however has suggested that there are frequent thermal collisions between vesicles and cell membrane, but that only when 'key molecules' on vesicle and cell membrane meet does a local membrane breakdown follow which allows acetylcholine to escape into the synaptic cleft. In the resting state, the probability of such an event is low, but the nerve impulse or applied depolarisation increases the reactivity (i.e. number of 'key molecules') of the nerve membrane, and so increases the probability of successful collisions, and thus results in the synchronous release of many quanta (Katz, 1958, 1962).

Once the transmitter has been released from the nerve-endings, the next stage is probably a simple process of diffusion. By using a micropipette and the technique of iontophoresis, a dose as small as 10^{-16} gm.mol. of acetylcholine was found to give rise to a muscle impulse, if it was given close enough to the end-plate (Katz, 1958). When the diffusion path was reduced to the order of microns an artificial e.p.p. could be obtained,

whose rise and fall was not much slower than that of one naturally produced by the transmitter (Krnjevic and Miledi, 1958, cited by Katz, 1962).

It was shown that the receptors for acetylcholine must be on the external surface of the post-synaptic membrane, since with the ionophoretic micropipette, if acetylcholine was released outside the cell, an impulse was produced, while the same dose, injected into the interior of the cell, produced no effect (Katz, 1958). But the nature of these receptors is still unknown.

However, when acetylcholine reacted with these 'receptors', a depolarisation of the membrane did take place and it was found that this was accompanied, and presumably caused, by a considerable lowering of the membrane resistance of the end-plate (Katz, 1958). This lowered resistance and depolarisation was not due simply to the rapid transfer of acetylcholine ions across the membrane, since the electrical charge transfer which occurred during the local depolarisation was much greater than the total charge carried by the quantity of acetylcholine released by an impulse (Castillo and

and Katz, 1956) and also, intracellular application of acetylcholine was ineffective, as mentioned before (Katz, 1958).

The acetylcholine-receptor reaction must therefore cause an increase in the ionic permeability of the membrane. From determinations of the 'null-point', the level of the membrane potential at which the e.p.p. reverses its sign, it seems that there is an unselective increase in permeability to all ions, or perhaps a combination of high sodium and high potassium permeability (Katz, 1958). The action potential, on the other hand, is due to selective, out-of-phase permeability changes, first to sodium, then to potassium (Hodgkin, 1951). Later results seem to show a simultaneous rise of sodium and potassium permeability, and little participation by chloride and other anions (Katz, 1962). Sodium itself is not essential, and can be replaced by other cations (Katz, 1958).

The present picture of events at the neuromuscular junction then seems to be as follows. Acetylcholine, synthesised in the motor nerve cell, is stored in vesicles at the nerve

terminal, and in the resting state, these vesicles are discharged in a random fashion, releasing quanta of acetylcholine which produce small local depolarisations of the motor end-plate - miniature end-plate potentials. The nerve impulse, by depolarising the membrane, momentarily increases the statistical chances of quantal release, and this released acetylcholine reacts with 'receptors' on the external surface of the end-plate membrane and causes an increased ionic permeability of the membrane, and hence a local depolarisation - the end-plate potential. When this end-plate potential reaches a threshold value, it initiates a propagated action potential, and muscle contraction follows. The acetylcholine is destroyed by cholinesterase which is present in high concentrations on the membrane of the muscle end-plate.

(b) THE AUTONOMIC GANGLION

The story for the ganglion is similar to that for the neuromuscular junction. As described in the first section, it had been

shown that acetylcholine was released from the preganglionic nerve-endings on stimulation, and that it could stimulate ganglia. Paton and Perry (1953) showed, using external electrodes, that close arterial injection of small doses (1 - 10 μg) of acetylcholine caused a transient depolarisation of the superior cervical ganglion cells in the cat, and that this depolarisation was prolonged by eserine. Similar results were obtained for the parasympathetic ciliary ganglion (Perry and Talesnik, 1953).

There had been difficulties involved in the acceptance of the idea of cholinergic transmission in ganglia, mainly due to the interpretation of the complex action potential of the ganglion obtained with extracellular electrodes. However, when intracellular electrodes were used to record the action potential in response to preganglionic stimulation, in the isolated superior sympathetic ganglion of the rabbit, it was clearly shown that a small depolarisation, the synaptic potential, preceded the spike potential, and in fact generated the spike when it depolarised

the membrane to a critical level (Eccles, 1955).

Nishi and Koketsu (1960) used intracellular electrodes to record spike potentials in response to antidromic, direct and orthodromic stimulation in ganglion cells of the isolated frog sympathetic chain. The orthodromic response showed an initial depolarisation phase (the 'synaptic potential'), and a small dip after the spike crest, which was slightly lower than that of the antidromic response. Also by displacing the resting membrane potential, they found the reversal point of the synaptic potential to be about -15 mV. They suggested therefore that the action of the transmitter at the frog sympathetic ganglion was similar to that of acetylcholine at the neuromuscular junction, and that it caused the permeability of the synaptic membrane to increase briefly to certain ions, and hence to short-circuit the active neuronal membrane (Nishi and Koketsu, 1960).

Using a similar preparation and technique, Blackman, Ginsborg and Ray (1963) confirmed Nishi and Koketsu's findings, and by timing an orthodromic stimulus to arrive at

different points on an antidromic response and recording the changes in shape of the spike, they showed that it was likely that the transmitter was responsible for the synaptic potential and that its action modified the shape of the spike action potential. The mode of action of the transmitter was similar to that at the neuromuscular junction, but its duration of action was longer, presumably due to the known absence of cholinesterase in frog sympathetic ganglion cells (Blackman, Ginsborg and Ray, 1963a).

The ganglion blocking drugs mecamylamine and hexamethonium were added to the bath fluid, and they reduced the rate of rise of the 'synaptic step' and increased the 'positive phase' of after hyperpolarisation. Acetylcholine was applied by ionophoresis through a micropipette and found to depolarise the ganglion cell. These facts were consistent with acetylcholine being the transmitter - the action of the blocking drugs was what would be expected from substances which reduced the intensity of action of the transmitter (Blackman, Ginsborg and Ray, 1963a).

The existence of miniature synaptic potentials in frog ganglion cells, mentioned by Nishi and Koketsu (1960), was confirmed by Blackman, Ginsborg and Ray (1963b) - small subthreshold depolarisations, occurring spontaneously, were recorded from about $\frac{1}{3}$ of the cells tested. Since they persisted in concentrations of K^+ which blocked presynaptic conduction, their release must be spontaneous. They were reduced in amplitude by tubocurarine, and would seem to be analogous to the miniature end-plate potentials at the neuromuscular junction, due to the action of 'packets' of acetylcholine. They occurred at a random frequency, usually less than 1/3 sec., and their frequency was increased by increasing the tonicity of the bath fluid, or by increasing the concentration of K^+ above that needed to block presynaptic transmission (probably due to depolarisation of the presynaptic terminals).

Since De Robertis and Bennet, and Taxi, have described 'synaptic vesicles' in presynaptic terminals of the frog sympathetic ganglion (see Blackman, Ginsborg and Ray, 1963b), it seems likely that acetylcholine may be released here

in a quantal fashion as at the neuromuscular junction. No miniature synaptic potentials have as yet been observed in mammalian ganglion cells (Eccles, 1955), but they have been shown to release acetylcholine spontaneously when perfused with high K^+ solutions (Brown and Feldberg, 1936).

Responses to orthodromic stimuli were recorded when transmission was depressed by a high Mg^{++}: Ca^{++} ratio in the bath fluid (Blackman, Ginsborg and Ray, 1963c). The amplitude of successive synaptic potentials varied, and mathematical analysis of the variation showed that it was consistent with the idea that the transmitter was released in the form of quanta identical with those which produced miniature synaptic potentials, and that the depression of transmission was caused by a reduction in the number of quanta released by a stimulus. As expected, the responses fluctuated much less when transmission was depressed with tubocurarine, since its action is believed to be post-synaptic, and it would not affect the number of quanta released.

Though the evidence is not yet quite strong enough for a definite conclusion to be reached, it seems likely that transmission at the sympathetic ganglion, at least in the frog, is very similar to that at the neuromuscular junction i.e. by the quantal release of acetylcholine, the nervous impulse being transmitted by a large increase in the number of quanta released simultaneously, due to its wave of depolarisation. Enzymic destruction of acetylcholine seems however to be less important at the ganglion, where the pattern of cholinesterase distribution is different from that at the neuromuscular junction.

III. MECHANISMS OF BLOCK OF CHOLINERGIC PERIPHERAL SYNAPSES

Following from the preceding description of cholinergic transmission it can be seen that drugs can interfere with transmission at the neuromuscular junction or the ganglionic synapse in the following ways: (1) by preventing or diminishing the release of acetylcholine from the pre-synaptic nerve-endings, (2) by competing with acetylcholine for the receptors on the post-synaptic membrane and thus preventing it from exerting its depolarising action, (3) by altering the condition of these receptors so that they are no longer sensitive to the depolarising action of acetylcholine, or (4) by inhibiting cholinesterase and thus preventing the destruction of acetylcholine.

Although transmission is all-or-none at each individual junction of one nerve-fibre and one muscle fibre, and an antagonist will either block or fail to block any one end-plate, a graded response i.e. a partial paralysis can be obtained if only a certain proportion of the end-plate population is blocked. Except in

experiments using electrical micro-methods, the graded response is the type which is measured.

Groups of drugs are known which interfere with transmission in each of the four ways mentioned above. For reasons of space and time, and because they are less relevant to the experimental investigation to be described, groups(1) and(4) will only be mentioned briefly. The best-known example of group(1) is botulinus toxin; acetylcholine release can also be prevented experimentally by local anaesthetics, hemicholinium compounds, and excess of magnesium or deficiency of calcium ions. Group(4), the anticholinesterases, comprises a large number of compounds of two main types, reversible eg. eserine, and irreversible, those which form a covalent bond with the receptor eg. DFP. The effect of applying an anticholinesterase may be either to facilitate a submaximal stimulus, or to cause block resulting from the prolonged action of excess acetylcholine, depending on dosage, stimulation etc.

Groups(2) and(3) will now be considered in more detail.

(a) The Neuromuscular Junction

In the latter half of the last century, Claude Bernard studied the properties of the paralytic South American poison, curare. He injected the drug into a frog in which the vessels of one leg were ligatured, and found that only the ligatured leg responded to stimulation of the nerve, but that the other paralysed leg still responded when the muscle was stimulated directly. Thus neither the muscle itself nor transmission of impulses along the nerve was affected. He concluded that curare must act at the junction between nerve and muscle and until about 1935 it was assumed, without experimental proof, that curare acted by 'inhibiting' the motor end-plate, since it lay between nerve and muscle.

Then Dale, Feldberg and Vogt (1936) showed that acetylcholine was released on stimulation of the motor nerves to voluntary muscle, and suggested that it acted as a transmitter, stimulating the motor end-plate. They also showed that curare did not prevent the release of acetylcholine, and suggested that it 'rendered the receptive element resistant to

the action of acetylcholine'. Curare was found to inhibit the twitch response of a voluntary muscle to injected acetylcholine even more than that to nerve stimulation (Brown, Dale and Feldberg, 1936).

Using external electrical recording techniques, curare was shown to prevent the production of a propagated action potential in the muscle by nerve stimulation (Eccles and O'Connor, 1939), and to diminish the size of the end-plate potential, which was believed to be due to the depolarising action of the transmitter (Eccles, Katz and Kuffler, 1941). It was known that curare was an amorphous mixture of quaternary alkaloids, and it had been recently found that (+)-tubocurarine chloride, crystallised from crude curare, was a bis-quaternary benzyl isoquinoline (King, 1935). Since acetylcholine was also a quaternary ammonium compound, and since it was believed to produce its depolarising action at the motor end-plate by combining with specific receptors on the surface, it was suggested that curare blocked the action of acetylcholine by combining

with these same receptors (Eccles, Katz and Kuffler, 1942). Kuffler (1943) demonstrated antagonism between acetylcholine and curarine applied directly to frog end-plates. Curare could also be antagonised by partial depolarisation of the membrane, by drugs or an applied current, which would sum with the reduced e.p.p. from nerve stimulation, and by anticholinesterases, which presumably increased the amount of acetylcholine available to compete with curare for receptors (Hunt and Kuffler, 1950).

It had been known for a long time that quaternary ammonium compounds could cause neuromuscular block (see Ing, 1936), and after the demonstration that tubocurarine was a bis-quaternary compound, some simple polymethylene bis-ammonium salts were prepared and tested for activity at the neuromuscular junction. The most active blocking agent among these was decamethonium (Barlow and Ing, 1948; Paton and Zaimis, 1948).

It was soon shown, however, that the blocking actions of tubocurarine and decamethonium were not identical. In different species and even in different muscles in the same animal

there seemed to be an inverse relationship between sensitivity to decamethonium and sensitivity to (+)-tubocurarine. Decamethonium possessed stimulant activity lacking in (+)-tubocurarine. On the cat tibialis preparation, decamethonium potentiated the twitch before blocking it, and fasciculations of the muscle could be seen between contractions. It caused a contracture of the frog rectus, of avian muscle and of denervated mammalian muscle. Block by decamethonium was not relieved by anticholinesterases, or any of the other agents which could diminish block by (+)-tubocurarine. A muscle blocked by decamethonium could sustain a tetanus, unlike a muscle blocked by (+)-tubocurarine. Overall, decamethonium seemed to show more similarities to acetylcholine than to (+)-tubocurarine (Paton and Zaimis, 1949; Paton, 1951).

Burns and Paton (1951) then showed that decamethonium caused a depolarisation of the end-plate region of the cat's gracilis muscle, and suggested that this depolarisation, which persisted for the same length of time as the

block, was the cause of the block. They observed that during the action of decamethonium the end-plate region was inexcitable to direct stimulation, that the propagation of a directly excited action potential along the muscle fibre was blocked at the end-plate region, that the propagation threshold at the end-plate was raised, and that artificial repolarisation of the end-plate reversed the block. These features of decamethonium block were not shared by tubocurarine, but they could be reproduced by acetylcholine in the presence of eserine, or by application of a cathode at the end-plate region. Decamethonium block could, in fact, be antagonised by tubocurarine, as would be expected if it acted similarly to acetylcholine, while by the same token it was not antagonised but rather intensified by anticholinesterases. They concluded that decamethonium blocked the neuromuscular junction by persistent depolarisation of the end-plate region, this electrical inexcitability preventing e.p.p.s from exciting the adjacent muscle membrane, whereas tubocurarine blocked by increasing the threshold of

the end-plate to acetylcholine (Burns and Paton, 1951; Paton and Zaimis, 1952).

Neuromuscular blocking drugs subsequently tended to be divided into two classes, competitive or curare-like, and depolarising or decamethonium-like. In support of this it was noted that sensitivity to decamethonium varied from species to species and from muscle to muscle. There seemed to be a reciprocal relationship between the sensitivities to the two types of blocking drugs for any given muscle e.g. decamethonium was more effective in blocking 'red' muscle than 'white' muscle in the cat, while for tubocurarine the opposite was the case (Paton and Zaimis, 1952; Zaimis, 1953).

However, it soon appeared that the action of decamethonium could not be regarded as purely depolarising in all situations. Jenden, Kamijo and Taylor (1951) found that decamethonium had a 'biphasic' blocking action on the isolated rabbit lumbrical muscle. The first phase showed the usual properties of decamethonium block <u>in vivo</u>, but the second

phase, slower in onset, could be reversed by eserine. Zaimis (1953) tested decamethonium on eight different species and found that on some of them (e.g. monkey, rabbit, dog) the block had some characteristics of depolarisation, some of competition and some absent from either type. Successive doses of decamethonium had less and less effect (Burns and Paton (1951) had even found this for depolarisation on the cat gracilis muscle) and the block was antagonised by a tetanus and neostigmine, and potentiated by curare. Zaimis therefore maintained that decamethonium possessed a dual mode of action in some species and that this explained the varying sensitivity of different species to decamethonium (Zaimis, 1953).

Thesleff (1955a) used both external and intracellular recording on the isolated frog sartorius preparation and found that acetylcholine, decamethonium, suxamethonium and nicotine all acted in a qualitatively similar way. They caused a brief depolarisation of the end-plate region, which subsided spontaneously without removal of the drugs, the membrane

potential returning to about its normal value. Neuromuscular block developed during the depolarisation phase, persisted despite repolarisation of the membrane, and only reached its maximum when the membrane potential had returned to its normal value. During the period of neuromuscular block, the end-plate region was insensitive to the depolarising effect of a further dose of acetylcholine or any of the other drugs. Thesleff concluded that at least for the frog, neuromuscular block caused by these four 'depolarising' drugs was not due to persistent depolarisation of the end-plate region or adjacent muscle membrane, but to a decrease in sensitivity of the end-plate to the transmitter (Thesleff, 1955a).

Thesleff (1955b) repeated these experiments on the rat phrenic nerve-diaphragm preparation, and found that acetylcholine, decamethonium and suxamethonium initially depolarised the end-plate region, though to a lesser extent than on the frog, and also the whole muscle membrane showed some degree of depolarisation. During complete neuromuscular block, there was a tendency to

repolarisation, though not to the resting level. However the depolarisation of the membrane obtained by altering the potassium concentration in the bathing fluid, which was required to produce neuromuscular block, was greater than that obtaining during block due to these drugs. Also, during block by the drugs acetylcholine no longer depolarised the muscle membrane and so Thesleff concluded that their action on the rat was perhaps initially by blocking a few fibres by depolarisation, but mainly by decreasing end-plate sensitivity to the transmitter (Thesleff, 1955b).

Using internal recording microelectrodes and ionophoretic application of drugs from micropipettes on the frog sartorius preparations Castillo and Katz (1957a) confirmed that (+)-tubocurarine did not alter the resting potential of the muscle membrane, and that it reduced the transient depolarisation caused by pulses of acetylcholine, or steady depolarisation caused by continuous release of acetylcholine. This effect was obtained only with external application of (+)-tubocurarine, but not when

it was applied inside the muscle fibre. These results support the idea of competition between (+)-tubocurarine and acetylcholine for receptors, but do not prove it to be true, since the traditional test of increasing doses of the 'competing' drugs cannot be used with this local method of application. The time of decay of (+)-tubocurarine's inhibiting action was found to be greater than that of the depolarising action of acetylcholine or carbachol (carbaminoyl choline), a stable choline ester. This slow decay was not likely to be limited by diffusion, but was suggested to be due to slow dissociation of the drug-receptor complex (Castillo and Katz, 1957a).

It had been previously found (e.g. van Maanen, 1950) that the antagonism between acetylcholine and (+)-tubocurarine was competitive when tested quantitatively using contracture of the frog rectus as a measure of drug action. Jenkinson (1960) used the depolarisation of the end-plate region of frog skeletal muscle (recorded with extracellular electrodes) or of individual end-plates (recorded with intra-

cellular microelectrodes) by acetylcholine in the absence or presence of (+)-tubocurarine. He showed that the equation

$$\frac{[A]}{[A']} - 1 = K[I]$$

where $[A]$ = agonist concentration in presence of antagonist

$[A']$ = agonist concentration in absence of antagonist

$[I]$ = antagonist concentration

K = affinity constant of antagonist

held over a wide range of concentrations. This equation expressed the relation between agonist and antagonist where both compete on a one-to-one basis for the same receptors (Gaddum, 1943). Jenkinson therefore said that his results were consistent with the hypothesis that (+)-tubocurarine is a competitive antagonist of acetylcholine at the neuromuscular junction.

Castillo and Katz (1957b) have also used this method of ionophoretic application to compare the time course of action of acetylcholine and of some stable depolarising agents. The time course of an acetylcholine potential was

faster than that of the other drugs - by a factor of about 2 for carbachol, 2 to 3 for nicotine and succinyl choline, and about 10 for decamethonium. In the presence of prostigmine, the difference between acetylcholine and carbachol became insignificant, which suggests that for these two, and probably also for nicotine and succinyl choline, the time course differences depend rather on the kinetics of local diffusion and hydrolysis than of drug-receptor reactions.

Castillo and Katz (1957c) further showed that decamethonium and also choline had three effects (i) weak depolarisation (ii) potentiation of acetylcholine in normal Ringer solution (iii) inhibition of acetylcholine in the presence of prostigmine. Also only inhibition occurs when decamethonium is given with carbachol instead of with acetylcholine. They suggested therefore that (ii) was due to inhibition of acetylcholine-esterase by the drug. To explain the inhibition (iii), they postulated first a drug-receptor reaction similar to that of an enzyme reaction (Michaelis theory):-

$S + R \rightleftharpoons SR \rightleftharpoons SR'$ where S = drug

R = receptor

SR = intermediate inactive compound

SR' = active depolarising compound

(+)-tubocurarine, they suggested, proceeded only to the first step, the inactive compound, whereas for decamethonium and other 'depolarising' drugs, the rate constant for the second reaction, from the inactive to the active form was very slow. Thus, though it could depolarise, it antagonised the depolarisation produced by fast and powerful agents such as acetylcholine and carbachol.

This scheme, however, did not explain the 'desensitisation' which Thesleff (1955a and b) had shown to be the cause of neuromuscular block by acetylcholine or depolarising drugs. Castillo and Katz therefore added a third step to the above equation:-

(1) $Ach + R \rightleftharpoons Ach\,R \rightleftharpoons Ach\,R' \longrightarrow Ach + R'$

where Ach = acetylcholine

and R' = unreactive or 'refractory' receptor.

Recovery of the receptor from R' to R was postulated to be very slow. Thus a high concentration of acetylcholine would result in most of the receptors being converted into the refractory form R' (Castillo and Katz, 1957c).

Katz and Thesleff (1957a) also considered two other hypotheses to explain the relationship between depolarisation and desensitisation: that the two reactions, a depolarising reaction which reached equilibrium rapidly, and a desensitising reaction which proceeded slowly occurred either (2) consecutively or (3) in parallel:-

(2) $S + A \underset{\text{fast}}{\rightleftharpoons} SA \underset{\text{slow}}{\rightleftharpoons} SB$

where S = drug

A = free receptors

SA = effective drug-receptor compound

SB = refractory drug-receptor compound

(3) $S + A \rightleftharpoons \begin{matrix} SA \\ \text{fast} \\ \text{slow} \\ SB \end{matrix}$

These hypotheses led to the predictions that the onset of desensitisation should be faster than the recovery, and that complete desensitisation should only occur when the rate of onset was very much faster than that of the recovery. However, by applying steady 'conditioning' and pulsatile 'test' doses of acetylcholine to receptive spots on frog end-plates, Katz and Thesleff showed that these predictions on desensitisation were not fulfilled. Half-desensitisation was found to develop at a rate equal to, or lower than, that of the subsequent recovery, instead of as predicted, at a rate of development twice that of recovery. These schemes therefore could not be accepted - what was apparently needed was a reaction in which the recovery process B to A, was slowed by the presence of the drug.

They then put forward a further hypothesis (Katz and Thesleff, 1957a) to satisfy this requirement.

(4)

$$\begin{array}{ccc} S + A & \underset{\text{fast}}{\overset{a}{\rightleftharpoons}} & SA \\ \uparrow\downarrow\ (\text{slow})k_2 & k_4 \quad k_3 & \uparrow\downarrow\ k_1\ (\text{slow}) \\ S + B & \underset{b}{\overset{\text{fast}}{\rightleftharpoons}} & SB \end{array}$$

where a and b are affinity constants and k_1, k_2, k_3 and k_4 are rate constants.

Equilibration requires

$$\frac{b}{a} = \frac{k_1 k_2}{k_3 k_4}$$

This scheme could not be rigorously tested, since with the ionophoretic method the values of S are not known, but it would be fitted if $k_1 \gg k_3$, $b \gg a$ and $\frac{b}{a} > \frac{k_1}{k_2}$.

According to this hypothesis, the free receptors are distributed, even in the absence of the drug, between states A and B i.e. some of the receptors are present in the refractory form, and due to their high affinity ($\frac{b}{a} \gg 1$) will preferentially absorb small quantities of applied acetylcholine.

Katz and Thesleff found that a small steady dose of carbachol could facilitate the action of an added pulse of the drug, and they explained this on the basis of the above hypothesis (4). The effect of the initial small dose would be mainly to occupy the B-type receptors, which have a high affinity but no depolarising power. When the second dose was added, a smaller fraction of the drug molecules

would be absorbed by B receptors and a larger fraction would be available to cause depolarisation by combining with A-receptors, than when this dose was given alone. This is of course different from the facilitation of acetylcholine action by cholinesterase inhibitors.

As stated at the beginning of this section, drugs can also interfere with cholinergic transmission by inhibiting cholinesterase and so preventing the destruction of acetylcholine. This can cause either a potentiation of the stimulant action of acetylcholine, or a blocking action due to the prolonged effect of excess acetylcholine. It is not proposed to discuss anticholinesterase activity here, but it must be mentioned that some drugs classified as cholinesterase inhibitors also possess, particularly in high concentrations, some blocking action on the motor end-plate, and similarly many neuromuscular blocking agents also have anticholinesterase activity. This anticholinesterase activity may mask blocking activity or simulate stimulant activity on test preparations,

and it is therefore important to discover from in vitro experiments the anticholinesterase activity of compounds thought to be active at the neuromuscular junction. This possible inhibitory activity on the enzyme also reduces the value of attempts to classify compounds as 'competitive' or 'depolarising' by finding whether they are reversed by a known anti-cholinesterase. For example, Thesleff and Unna (1954) investigated the polymethylene bis-trimethyl ammonium series, and in their results reversal of paralysis by the anticholinesterase Tensilon (Katz and Thesleff, 1957b) does not seem to correlate with ability to cause contracture of the chick gastrocnemius.

If the drug being tested is a more potent inhibitor than the 'standard' anticholinesterase, then obviously it will not be reversed by it. The drug Mytolon (Win 2747) was originally classed as a 'depolarising' blocking agent, since it was not reversed by Tensilon, but it was then found to be a more powerful inhibitor of cholinesterase than Tensilon (Tabachnick et al., 1958). The compound Win 8078 reverses

both the block caused by (+)-tubocurarine and that caused by decamethonium - this may be due to its anticholinesterase activity and its 'curare-like' activity respectively (Blaber, 1960).

(b) The Autonomic Ganglion

In the latter half of the nineteenth century it was shown that the alkaloid nicotine could first cause slowing of the dog's heart, and then prevent vagal stimulation from producing slowing of the heart. The site of this stimulation and then block by nicotine was some connecting link between the vagus trunk and the site of action of atropine, which prevented the initial slowing by nicotine.

In 1889 Langley and Dickinson showed that the site of action of nicotine was the autonomic ganglion. Topical application of nicotine to the superior cervical ganglion of the cat caused first stimulation - pupillary dilatation, as with electrical stimulation of the cervical sympathetic chain - and then block at the ganglion - preganglionic electrical stimulation was ineffective, while post-ganglionic stimulation was still effective. Later, Langley (1901) showed that nicotine still stimulated the sympathetic ganglion after the preganglionic nerve had been cut and allowed to degenerate, and hence deduced that

the site of action of nicotine was the ganglion cell surface and not the preganglionic nerve ending.

The simplest quaternary ammonium compounds were also shown to be active at the autonomic ganglion. Tetramethylammonium was shown to have an action similar to that of nicotine i.e. stimulation followed by block, demonstrated on the blood pressure of the cat; whereas tetraethylammonium possessed only the blocking action, without the preliminary stimulation (Burn and Dale, 1915). Thus already two types of agent blocking at the ganglion had been shown to exist, as was subsequently found to be the case at the neuromuscular junction, but no attempt at analysis of their modes of action was made until much later. By this time, other ganglion blocking agents had been synthesised, in particular the polymethylene bis-ammonium salt hexamethonium (Paton and Zaimis, 1949).

Paton and Perry (1953) studied the effects of acetylcholine and several ganglion blocking drugs on the cat superior cervical ganglion using electrical recording of the action potential from the ganglion with

external electrodes. Small doses of acetylcholine produced only a transient depolarisation of the ganglion, while large doses of acetylcholine, or doses of nicotine or tetramethylammonium, resulted in a considerable depolarisation accompanied by a reduction in the height of the action potential. All other ganglion blockers tested i.e. hexamethonium, pentamethonium, tetraethylammonium, tubocurarine and decamethonium (in large doses), decreased or abolished the spike without any trace of depolarisation. Thus ganglion-blockers could be divided into two groups, depolarising blocking drugs and competitive blocking drugs, this classification corresponding to the previous one of whether or not they produced initial stimulation of the ganglion. This classification was analogous to that put forward for the neuromuscular junction, but Paton and Perry noted that at the ganglion, decamethonium appeared to act as a competitive blocker, whereas at the neuromuscular junction it was considered a depolarising blocker (Burns and Paton, 1951).

However, the action of nicotine was not identical with that of acetylcholine or tetramethylammonium. With tetramethylammonium, recovery from depolarisation and recovery of spike height were almost parallel, but with nicotine the depolarisation was much more transient, and the reduction in spike height lasted much longer than the depolarisation e.g. in one experiment, ganglion negativity had disappeared 3 min. after the injection, while the spike height did not return to normal for 30 min. This suggested to Paton and Perry that nicotine might have a mixed action, initially depolarising and subsequently competitive. To test this, they gave a second identical dose of nicotine shortly after the ganglion depolarisation had disappeared, and found as they expected, that it produced much less depolarisation than the original dose. However, this might equally well be explained, in the light of further findings at the neuromuscular junction, as a desensitisation following the initial depolarisation.

Acetylcholine appeared to occupy a

position between that of tetramethylammonium and nicotine, in that the rate of recovery of spike height was slower than the rate of repolarisation. This could also mean that ganglion block by acetylcholine is at least partly due to receptor desensitisation, as has been shown to be the case at the neuromuscular junction (Thesleff, 1955). If ganglion block by nicotine and acetylcholine were due to desensitisation, that would leave tetramethylammonium in a lonely position - is its blocking action due only to the depolarisation it produces or is there a concurrent desensitisation? Unfortunately no work similar to that of Katz and his colleagues on neuromuscular blocking agents has so far been performed on the ganglion, to verify or refute the existence of desensitisation block at this site.

Ganglion-blocking drugs have been administered to the frog sympathetic ganglion, while intracellular recording was in progress (Ray, 1962; Blackman, Ginsborg and Ray, 1963). The non-depolarising drugs hexamethonium,

mecamylamine and (+)-tubocurarine decreased and then abolished the orthodromic spike leaving only the synaptic potential, while not affecting the antidromic spike or the membrane potential. This is as would be expected on the assumption that they reduced the intensity of transmitter action, and it was therefore tentatively assumed that they acted post-synaptically by combining with specific receptors for acetylcholine (Ray, 1962).

Nicotine, on the other hand, first depolarised the cell and abolished the orthodromic spike, uncovering the synaptic potential, but the depolarisation passed off while the orthodromic spike remained blocked. When low divided doses of tetramethylammonium were given, block could be obtained without depolarisation. Ray concluded that 'depolarising' ganglion blockers do not necessarily block by depolarisation, since block occurs after peak depolarisation has occurred, transmission is not restored after membrane potential is restored and the antidromic response is not blocked at any stage (it can be decreased by depolarisation

e.g. by increasing the Ca^{++} concentration). He did not speculate on other possible modes of blocking action, but it seems likely that events at the ganglion are similar to those at the neuromuscular junction, and that hypotheses put forward for neuromuscular blocking action may be extended to cover the ganglion. Investigations on the ganglion have mainly been hampered by technical difficulties, but it is hoped that in the near future some further work may emerge which will shed more light on events there.

IV. THE PROPERTIES OF ONIUM SALTS

A. THE NEUROMUSCULAR JUNCTION

(a) Complex Onium Salts

In the middle of the 19th century, Crum Brown and Fraser converted a number of alkaloids, all of which had pronounced effects on the central nervous system, from tertiary bases to quaternary metho salts. They found that these quaternary salts had lost almost entirely the typical central actions of their parent bases, and had acquired a new property, the production of a curare-like paralysis of voluntary muscle. By performing experiments similar to those of Claude Bernard on the frog, they showed that the paralysis was like that of curare - nervous conduction and direct excitability of the muscle were unaffected, and so the drug action must be at the neuromuscular junction. They found that the methiodides and methosulphates of strychnine, brucine, thebaine, codeine, morphine, atropine and N-methylconiine all showed this property, though nicotine was anomalous. They also found that this action was not confined to the metho salts of complex alkaloids - it was also shown by their etho salts and even by the simplest quaternary ammonium salt, tetramethylammonium chloride. They

considered that the production of curare-like paralysis was a property of quaternary ammonium salts as a class (Crum Brown and Fraser, 1869 and 1872; see Ing, 1936).

Ing (1936) used the term 'onium salts' for cations of the ammonium, sulphonium, phosphonium type in which the central atom satisfies its maximum covalency and also exerts one electrovalency, and he reviewed the literature concerning the curare-like properties of these salts. Phosphonium salts (Vulpian, 1868; Lindemann, 1898), sulphonium salts (Crum Brown and Fraser, 1872), arsonium and stibonium salts (Rabateau, 1882) and iodonium salts (Gottlieb, 1894) had all been shown to have this activity. Curarine, an active alkaloid from curare isolated by Boehm (1897), was itself shown to be a quaternary base.

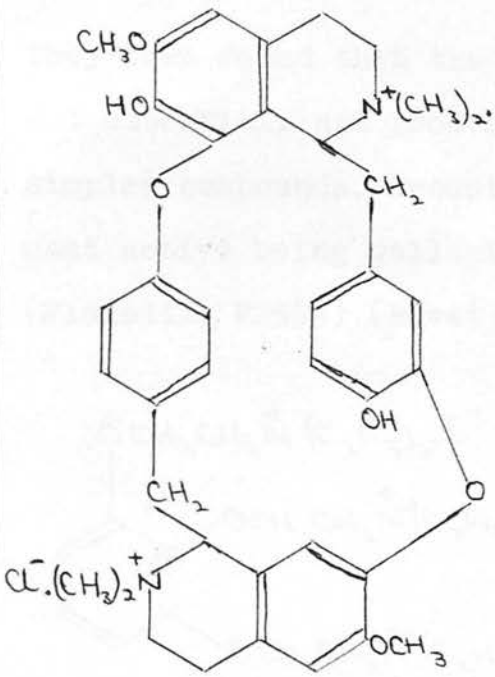
Though there was a large number of compounds known to possess this neuromuscular blocking activity, attempts to compare their activity quantitatively had not been a success due to poor testing techniques. Ing and Wright (1932, 1934) used the isolated frog sartorius preparation, a thin muscle where diffusion factors should be negligible, times of paralysis short, and recovery quick. They

measured the times of paralysis for equimolar concentrations, and found that curare-like activity was remarkably independent of detailed chemical structure i.e. the nature of the central atom and the radicals attached to it. This suggested that the activity of onium compounds depended rather on their ionic character. However, in the simplest compound, tetramethylammonium, successive replacements of methyl groups by ethyl considerably decreased the activity. Taking into account variations in some other simple quaternary salts, it seemed that there might be a certain ionic radius at which there was minimum activity (Ing and Wright, 1934).

It was just at this time that the transmission of nerve impulses from motor nerve to striated muscle by acetyl choline was demonstrated (Dale, Feldberg and Vogt, 1936; Brown, Dale and Feldberg, 1936). Since acetyl choline and curare were also onium salts, it seemed likely that the action of onium salts in general was, like that of curare, an antagonism of the chemical transmitter at the motor end-plate.

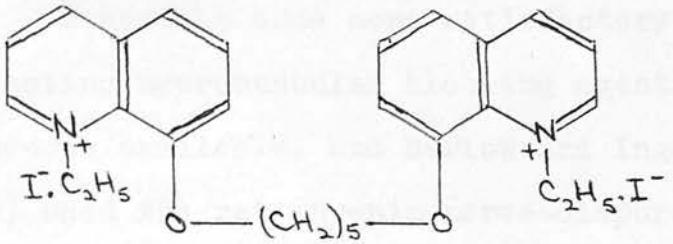
King (1935, 1936) isolated and crystallised an active principle (+)-tubocurarine chloride

from crude tube curare, and found that it was a bis-tetrahydroisoquinolinium salt with 2 quaternary nitrogen atoms. He later elucidated the complete structural formula (King, 1948).



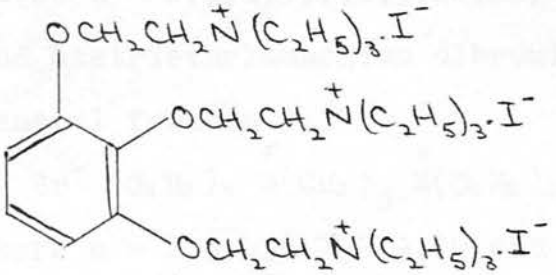
(+)-tubocurarine

This finding together with the use of curare as a muscle relaxant during general anaesthesia (Griffith and Johnson, 1942) prompted the development of synthetic curare-like agents. Bovet and his co-workers investigated a number of onium salts, and succeeded in obtaining compounds with activity similar to that of curare. One of the first was a bis-quinolinium salt, 3381 RP (Bovet et al. 1946).



3381 RP

They then found that the quinolinium ring was not essential, and produced a number of simpler compounds, aromatic choline ethers, the most active being gallamine triethiodide (Flaxedil, F2559) (Bovet et al, 1947).



Flaxedil

The paralysis caused by gallamine could be easily reversed by eserine, and it was suitable for clinical use due to its absence of secondary effects. A large number of other compounds was synthesised in an attempt to relate chemical structure to curare-like activity, and to find more clinically useful drugs (see Bovet, 1951). In general, it was found that the oxygen bridge present in (+)-tubocurarine, gallamine etc. was not essential, and that activity was greatest when two quaternary ammonium groups were present.

(b) Simple bis-onium salts

Meanwhile some more satisfactory methods of testing neuromuscular blocking agents had become available, and Barlow and Ing (1948a and b) used the rat phrenic nerve-diaphragm preparation (Bülbring, 1946; Chou, 1947) and the rabbit head-drop test of Holaday to test simple polymethylene bis-onium salts. They tested bistrimethylammonium dibromides of the general formula

$$Br^- (CH_3)_3 \overset{+}{N} (CH_2)_n \overset{+}{N}(CH_3)_3 Br^-$$

where n = 2,3,4,5,7,8,9,10,11, 12 and 13 (BTMn) and bistriethylammonium dibromides of the general formula

$$Br^- (C_2H_5)_3 \overset{+}{N}(CH_2)_n \overset{+}{N}(C_2H_5)_3 Br^-$$

where n = 2,3,4,5,7,8,9,10 and 13 (BTEn) and also some bis-strychninium, bis-quinolinium and bis-(phenyldimethylammonium) dibromides. On the rat diaphragm, the BTM series showed a gradual rise in activity from BTM3-9, BTM 9, 10, 11 and 12 being about equiactive, and BTM 13 slightly less active. In the BTE series, there was a steady rise in activity from BTE 4-13, BTE 13 being slightly more active than BTM 9. In the rabbit head-drop test, however, there was a pronounced maximum in the

BTM series at BTM 10, this being three times as potent, weight for weight, as (+)-tubocurarine while BTE 13 was 40% active as (+)-tubocurarine. None of the members of the other three series was as active as the most active BTM compounds. It was suggested that the high activity of BTM 10 might be due to the possession of two quaternary nitrogen atoms separated by ten carbon atoms, since in (+)-tubocurarine the shortest route between the nitrogens traversed ten atoms (nine carbon, one oxygen) (Barlow and Ing, 1948).

The trimethylammonium series had also been studied by Paton and Zaimis (1948a and b) and BTM 10 was introduced into clinical use as a substitute for curare with the name of decamethonium. In 1949 Paton and Zaimis published an extensive study of the properties of the BTM series (n = 5 - 12, 18) testing neuromuscular blocking activity on the cat anterior tibialis preparation. They found a distinct maximum at BTM 10, and though (when the drugs were tested by other methods on other species) the potency varied, the maximum was always at BTM 10.

It was noted that with BTM 10 there was a preliminary potentiation of the twitch and fasciculations of the muscle before block occurred, and it was later shown that BTM 10, like acetylcholine, could depolarise the motor end-plate (Burns and Paton, 1951; see preceding section).

The series of compounds was also tested on the superior cervical ganglion preparation, where it was found that there was a maximum in ganglion-blocking activity at BTM 6; on the frog rectus, where some members were found to cause a contracture, with an apparent maximum at BTM 12 (members immediately above BTM 12 were not tested; some of the lower members acted as antagonists to this contracture production); on the guinea-pig gut, where weak muscarine-like activity, increasing to BTM 12, was found; and on cholinesterase, where they had inhibitory activity against true cholinesterase, increasing with chain length (Paton and Zaimis, 1949). Thus it can be seen that these onium compounds have a considerable range of activity, at the same sites as acetylcholine.

(c) Compounds related to decamethonium

Many compounds related to decamethonium have been synthesised and tested in an effort to elucidate structure-action relationships. As well as being tested for blocking activity, many have also been tested for 'acetylcholine-like' activity as possessed by decamethonium, the criterion usually being the ability to produce a contracture of the frog rectus abdominis muscle. Actually activity on the frog rectus is not maximal at BTM 10 - BTM 11 and 12 are both more active, and though BTM 18 is less active, it is not known where the actual maximum lies (Paton and Zaimis, 1949). Any changes in the onium groups of BTM 10 e.g. replacement of methyl groups by ethyl or amino, replacement of quaternary ammonium by quaternary phosphonium, seem to result in a decrease in 'acetylcholine-like' activity, and usually in neuromuscular blocking activity as well (Barlow, 'Introduction to Chemical Pharmacology').

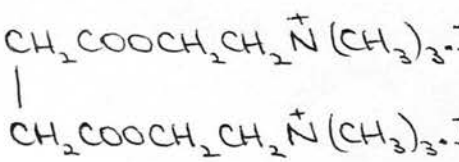
The chain joining the onium groups can be altered e.g. by replacement of part of the chain by a benzene ring, or of a methylene group by an ether oxygen atom, and compounds are still obtained whose activity is similar to, and of

the same type as that of BTM 10 (Barlow, 'Introduction to Chemical Pharmacology'). The compound 'Prestonal' is interesting because it is said to block at least partly by desensitisation (Frey, 1956), though it has a much longer chain than BTM 10 and also has a pair of methyl groups replaced by larger propoxycarbonylmethyl groups - this type of replacement usually leads to abolition of 'acetylcholine-like' activity.

When parts of the methylene chain are replaced by ester groups, compounds of the general formula shown below are obtained, and these are more active than BTM 10 on the frog rectus.

$$(CH_3)_3\overset{+}{N}CH_2\ CH_2COO(CH_2)_nCOOCH_2CH_2\overset{+}{N}(CH_3)_3$$

The compound of greatest interest is suxamethonium (succinylcholine) where n = 2 - this represents a doubling of the acetylcholine molecule.



Suxamethonium

The higher members are still more active on the frog rectus but not in the rabbit head-drop test. Suxamethonium is the compound used clinically,

where its brief duration of action, due to hydrolysis by cholinesterase, makes it very suitable (Bovet et al, 1949; Castillo and de Beer, 1950).

(d) Replacement of methyl by ethyl groups

Changes in activity of onium compounds as N-methyl groups are replaced by ethyl have also been studied. Holton and Ing (1949) and Ing (1949) investigated analogues of acetylcholine itself, and Wien and Mason (1951, 1952) tested polymethylene bis-onium compounds with chain lengths of 4,5,6, and 7. Analogues of decamethonium were studied by Barlow, Roberts and Reid (1953) and a considerable number of bis-onium compounds were studied by Ginzel, Klupp and Werner (1951) and Thesleff and Unna (1954), who noted that not only the activity but also the apparent mode of action could be changed by replacement of methyl groups by ethyl. No consistent pattern of activity change with alteration of N-substituents has emerged from these investigations, but it must be remembered that compounds were tested at a variety of different sites, and that sometimes stimulant and sometimes inhibitory activity was measured.

B. THE AUTONOMIC GANGLION

In addition to blocking transmission at the neuromuscular junction, onium salts can also affect transmission at autonomic ganglia. Burn and Dale (1915) found that tetramethylammonium (TMA) on the cat usually caused a fall in blood-pressure with slowing of the heart, but when this muscarinic effect was abolished by atropine, TMA caused a rise in blood-pressure, followed by a fall. This was annulled by a large dose of nicotine, and so the pressor effect of TMA was a nicotine-like stimulation of sympathetic ganglion cells, followed by a nicotine-like paralysis of ganglia. Tetraethylammonium (TEA), on the other hand, showed no muscarinic actions and no pressor effect on the blood-pressure, but it did block the pressor action of TMA - hence it showed the secondary paralytic phase of nicotine action, without the stimulant action.

Reid Hunt and Renshaw (1925) tested tetramethylammonium, tetramethylphosphonium, tetramethylstibonium and trimethylsulphonium, and found that while they all showed muscarinic effects, only the nitrogen, sulphur and

phosphorus compounds showed nicotine-like properties (tested on cat blood pressure). Reid Hunt (1926) tested tetramethylammonium and a large number of compounds related to it, with one or more of the methyl groups substituted by aliphatic or aromatic groups. He observed that the most marked stimulant nicotine-like action was obtained with compounds containing methyl groups, while the paralysing action of nicotine was obtained with many of the compounds, not limited to those containing methyl groups.

Acheson and Moe (1945, 1946) found that TEA caused a fall in blood-pressure in cats and dogs, and on investigation showed that it was not due to an effect on the heart, on the blood-vessels or on the vasomotor centres. The depressor effect was however dependent on a vasoconstrictor discharge, and they showed that it was due to blocking of the sympathetic ganglia - the nictitating membrane no longer responded to preganglionic electrical stimulation, but still responded to post-ganglionic stimulation. TEA did not possess the other actions of most onium salts - muscarinic, atropine-like,

nicotinic, or curare-like - its predominant action was a ganglion-blocking effect.

Some bis-triethylammonium compounds - BTE 2, 3, 4 and 10-were compared with TEA on the perfused cat superior cervical ganglion by Chou and de Elio (1947). Like TEA, they blocked ganglionic transmission without preliminary stimulation. Activity dropped from TEA to BTE 2 and 3, then started to rise again, BTE 10 being twice as active as TEA.

Paton and Zaimis (1949) studied the actions of members of the bis-trimethylammonium series (BTM 2 - 13, BTM 18) on the cat superior cervical ganglion preparation and on the Trendelenburg preparation for parasympathetic ganglia, as well as at the neuromuscular junction and various other sites. They found that BTM 5 and 6, which could cause a fall in blood-pressure, showed blocking activity at ganglia. Like TEA they blocked ganglia without prior stimulation, though their effect was more prolonged. They were twenty times as active as TEA, BTM 6 being slightly more active than BTM 5.

Since ganglion-blocking agents can be used to treat successfully some cases of high blood-pressure, there has been an extensive search for clinically suitable drugs. The clinical disadvantages of hexamethonium itself are its equal effect on parasympathetic as well as sympathetic ganglia, and its unreliable oral absorption. Since quaternary salts have low lipid solubility, poor oral absorption is almost an inevitable corollary. Consequently, clinically useful drugs are more likely to be found among compounds containing secondary or tertiary amino groups, and having a suitable dissociation constant. However, considerable work has still been done on bis-quaternary compounds.

The effect of alteration of substituents in the onium group depends on the length of the polymethylene chain - the particular onium group which confers optimum activity is different at different chain lengths. Various alterations in the chain have been made, but generally the most active compounds, whose activity is never much greater than that of hexamethonium, have a chain length similar to

hexamethonium. Some asymmetrical compounds were found to be more active than their symmetrical analogues, and also some dissociation of activity at sympathetic and parasympathetic ganglia was obtained. A compound which was about eight times as active as hexamethonium was Chlorisondamine, a short-chain asymmetric compound, with one aliphatic onium group and one large halogenated aromatic onium group (see Barlow 'Introduction to Chemical Pharmacology).

C. LONG-CHAIN POLYMETHYLENE BIS-TRIMETHYL AND BIS-TRIETHYLAMMONIUM SALTS

It can be seen from the foregoing review that a considerable amount of work has been done on polymethylene bis-trimethyl and bis-triethylammonium salts. In particular, the shorter-chain BTM salts (BTM 3 - 13 and 18) have been extensively studied by Paton and Zaimis (1949), and there appeared to be a maximum in ganglion blocking activity at BTM 6 and in neuromuscular blocking activity at BTM 10. In the BTE series, the ganglion-blocking properties of BTE 2, 3, 5 and 10 were described by Chou and de Elio (1947) and the neuromuscular blocking properties of BTE 2 - 5, 7 - 10 and 13 were described by Barlow and Ing (1948). Both types of activity appeared to increase with chain length.

The longer-chain BTE compounds (BTE 10 - 16) were tested on the cat superior cervical ganglion and on the rat diaphragm by Barlow and Vane (unpublished), and both types of blocking activity still seemed to be increasing. Mawer (unpublished) tested BTE 10 - 16 on the

rat diaphragm and found maximal blocking activity at BTE 15. BTE 9 - 17 were studied on a variety of preparations by Warriner (1960) and she found that ganglion-blocking activity on the cat superior cervical ganglion was maximal at about BTE 16 and neuromuscular blocking activity on the cat tibialis maximal at about BTE 15. Atropine-like activity increased to BTE 17, while anticholinesterase activity did not vary much with chain length. Conductivity and surface tension were also measured to see if there was any gross change in conformation in solution with chain length, but there was no evidence of this.

It was therefore considered desirable to repeat and extend Warriner's experiments, using BTE 9 - 21, to confirm the position of maximum activity at about BTE 15 - 16, and also to study the long-chain BTM compounds, BTM 10 - 21, only a few of which had been tested previously. Also cross-sections at C10 and C16 were investigated, to find the effect of stepwise replacement of methyl groups by ethyl.

The compounds were tested for ganglion-blocking activity on the cat superior cervical ganglion preparation, and for neuromuscular blocking activity on the cat tibialis preparation and the isolated rat phrenic nerve-diaphragm preparation. It was also desired to test them for acetylcholine-like 'depolarising' ability, and for this the chick biventer preparation (Ginsborg and Warriner, 1960) was chosen. This muscle contains two types of fibre, one which responds to acetylcholine and 'depolarising' drugs like decamethonium by a contracture, and the other which gives a twitch in response to nerve stimulation and is blocked by neuromuscular blocking agents of either type. Ginsborg (1960) has shown that the contracture-producing fibre is multiply-innervated i.e. is supplied by a number of axons and has neuromuscular junctions distributed at many points on its surface, while the twitch fibre is supplied by a single axon with a focal end-plate. The end-plates in the multiply-innervated fibre are so close that an agent which causes depolarisation at the neuromuscular junction could be expected to produce a

relatively uniform depolarisation of the surface of this fibre, and so it seems likely that there is some association between ability to depolarise and ability to cause contracture of these fibres. This avian muscle was chosen in preference to amphibian muscle such as the frog rectus, which has similar diffusely-innervated 'slow' fibres responding with a contracture to 'depolarising' drugs, because of its greater sensitivity to these drugs and also because blocking of the twitch fibres can be observed concurrently on the same preparation.

It was considered that these long-chain molecules might be able to fold or coil in solution, and that if this occurred suddenly at a certain chain-length, this might affect activity. Also, as polar molecules with a long hydrocarbon moiety, they might tend to form micelles, and this again could affect activity, by reducing the effective number of molecules in the solution. A physical measurement which would provide evidence, if any, of micelle formation or change in configuration in solution is conductivity, and measurements of conductivity were therefore performed.

EXPERIMENTAL SECTION

I. PREPARATIONS

1. MEASUREMENT OF CONDUCTIVITY

The conductivities of the solutions were measured using a Philips conductivity bridge (type PR 9500) and conductivity cell (type GM 4221). This consists essentially of a Wheatstone bridge using an alternating current, the resistance of a solution being measured between two parallel platinum plates mounted in the conductivity cell.

The experiments were carried out using a series of stoppered Quickfit tubes suspended in a thermostatically-controlled constant-temperature bath, maintained at 25 $\pm$ 0.1°C.

All solutions were made up with water from an Elgastat deioniser column, whose conductivity was around 1 x 10^{-6} mhos/cm. Resistances were measured for each compound at a minimum of 3 concentrations and dilutions from the stock solutions (about 1 x 10^{-2} M) were made up immediately prior to use, with deionised water whose resistance had just been measured.

The experimental procedure was as follows. The conductivity cell was kept in a tube of deionised water, and was rinsed and allowed to remain in one for 15 minutes between each test solution. 3 tubes were filled with test solution, and the cell placed in one of them

while the other two were kept stoppered to decrease uptake of carbon dioxide from the atmosphere. They were left in the bath for 15 minutes so that temperature equilibration could take place and ions adsorbed on the platinum black electrodes could equilibrate. After equilibration one tube was unstoppered and the cell was transferred to it.

It was left for 2 minutes and then the resistance read on the appropriate scale of the machine (position of minimum deflection). This process was repeated with the second tube as a check, and the average of the two values taken.

The conductivity cell constant, given as 1.33 at 25°C, was checked using standard potassium chloride solutions, from the expression:-

$$K = \frac{R}{r}$$

where K = cell constant
R = specific resistance
r = measured resistance

and was found to be 1.289 at 25 $\pm$ 0.1°C.

Where $\kappa = \frac{1}{R}$ = specific conductivity, the expression becomes $\kappa = \frac{1}{Kr}$

In the case of dilute solutions, consideration must also be given to the conductivity of the solvent

$\therefore \kappa = \frac{1}{K}\left(\frac{1}{r} - \frac{1}{r_o}\right)$ where r = measured resistance of solution

r_o = measured resistance of solvent

Now molar conductivity $= \lambda = \frac{1000\kappa}{x}$

where x = concentration in gm. moles/litre

$\therefore \lambda = \frac{1000}{x} \cdot \frac{1}{K} \cdot \left(\frac{1}{r} - \frac{1}{r_o}\right)$ (Findlay, 1942)

Molar conductances were calculated for various concentrations of potassium chloride and cetyltrimethylammonium bromide (CTAB). Standard curves were drawn of molar conductance against square root of molar concentration (λ against $\sqrt{x}$), since this should give a straight line if the Debye-Huckel equation is followed. This equation

$\lambda = \lambda_o - k\sqrt{x}$ where k = a constant for each substance

x = ionic concentration in moles/litre

λ_o = molar conductivity at infinite dilution

relates the molar conductivity at infinite dilution to the molar conductivity at any ionic concentration and was derived taking into account the asymmetry effect and the electrophoretic effect in the interionic attraction of electrolytes. The constant k is a complex expression, and the equation is only valid at high dilutions.

It was desired to obtain values for the molar conductance of each bis-onium compound at 3 standard concentrations - 1.0×10^{-2}M, 1.0×10^{-3}M and 1×10^{-4}M - so that these could be compared. When the solutions were made up by weighing out the exact quantity of solid required to make a 1.0×10^{-2}M solution, and dissolving this accordingly the values of λ were found to fluctuate somewhat. Since the crystals of these compounds tend to take up water easily and this might be introducing an error, during the weighing-out process, to the concentration factor in the above expression, it was decided to make up solutions of approximately 1×10^{-2}M, and to obtain their precise normality by titration. The compounds are all bromides, and so the bromide was measured by Volhard estimation - precipitation of the bromide by standard silver nitrate in dilute nitric acid, and back titration of the excess silver nitrate by standard ammonium thiocyanate, using a ferric indicator (Vogel's 'Quantitative Analysis', p.318-9). Having thus calculated the normality of the solution, the molarity was obtained by halving it, since the compounds are dibromides (with the exception of CTAB, a monobromide, also

estimated by this method). Further concentrations were obtained by diluting accurately these stock solutions of known normality. Graphs were drawn of λ against $\sqrt{x}$, and from these the values of λ for the exact desired molarities were obtained. These graphs also gave evidence, by their shape, of micelle formation, if any.

2. THE CHICK BIVENTER CERVICIS PREPARATION

(Ginsborg and Warriner, 1960)

Three-week old chickens were anaesthetised with ether. The back of the neck was plucked and the skin incised along the midline from the skull to below the base of the neck, exposing the 2 biventer cervicis muscles. A thread was tied round the upper belly of one muscle, which was then cut free from its attachment to the skull. The tendon and the lower belly of the muscle were dissected free, and removed together with the lower tendon, which attaches the muscle to the supraspinous ligament.

A loop of thread was tied round the lower tendon and this loop was put round the hook at the bottom of the electrode assembly. The thread on the upper end of the muscle was passed through the electrode and attached to a light semi-isometric lever writing on a smoked drum. The electrode was lowered until it was in contact with the tendon surrounding the nerve. The 50 ml. organ-bath contained Krebs-bicarbonate solution at 37°C, gassed with 95% oxygen and 5% carbon dioxide, via a sintered polythene distributor. The nerve was stimulated maximally at a frequency of 12 shocks/minute.

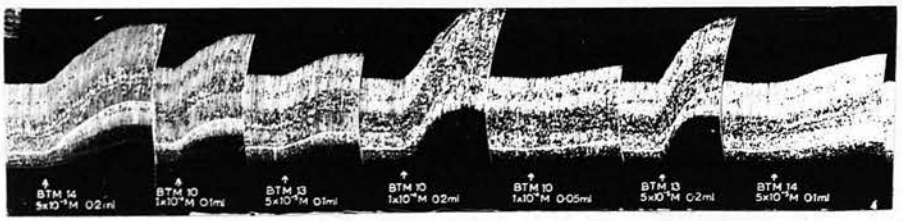


Figure 1: Contracture production on the chick biventer preparation: estimation of equipotent molar ratios compared to BTM 10. The following doses were given:

(1) BTM 14 0.2 ml. of a 5 x 10^{-5}M solution

(2) BTM 10 0.1 ml. of a 1 x 10^{-4}M solution

(3) BTM 13 0.1 ml. of a 5 x 10^{-5}M solution

(4) BTM 10 0.2 ml. of a 1 x 10^{-4}M solution

(5) BTM 10 0.05 ml. of a 1 x 10^{-4}M solution

(6) BTM 13 0.2 ml. of a 5 x 10^{-5}M solution

(7) BTM 14 0.1 ml. of a 5 x 10^{-5}M solution

3. THE RAT PHRENIC NERVE-DIAPHRAGM PREPARATION

(Bülbring, 1946)

Rats of either sex weighing 150-250 gms. were used. They were killed by a blow on the head and bled out. The thorax was opened and the sternum and some ribs on the left side removed, exposing the diaphragm and the left phrenic nerve running up towards the thymus. A thread was tied round the phrenic nerve at the level of the thymus and it was cut and carefully dissected free down to the diaphragm. An incision was made through the left abdominal muscles along the costal margin, and a fan-shaped piece of diaphragm, together with 2 or 3 ribs, was removed - about 3 mms. wide at the tendinous end and about 12 mms. wide at the costal margin. This was placed in Krebs-Henseleit solution in a Petri dish, and a thread was tied round the tendinous end of the muscle.

The preparation was mounted on a hook pushed through the costal margin and suspended in a 50 ml. bath containing Krebs-Henseleit solution, with double glucose, at 37°C. The solution was gassed with 95% oxygen and 5% carbon dioxide via a sintered polythene distributor. The thread from the muscle was attached to a spring lever writing on a smoked drum, and the nerve

was passed through the electrode. The nerve was stimulated maximally at a frequency of 12 shocks/minute.

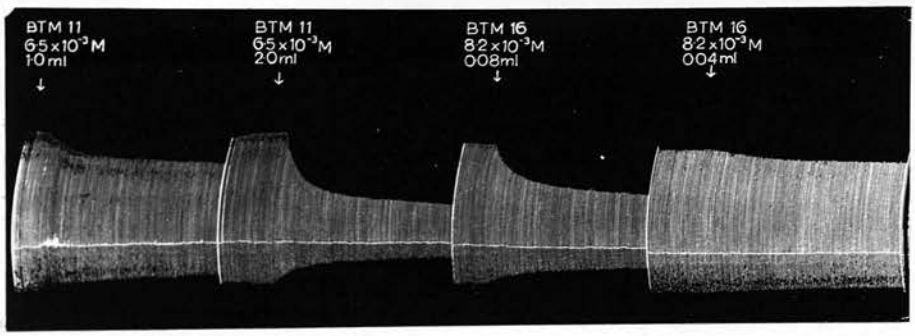


Figure 2: Neuromuscular blocking activity on the rat phrenic nerve-diaphragm preparation: estimation of equipotent molar ratio compared to BTM 16. The following doses were given:

(1) BTM 11 1.0 ml. of a 6.5×10^{-3} M solution

(2) BTM 11 2.0 ml. of a 6.5×10^{-3} M solution

(3) BTM 16 0.08ml. of a 8.2×10^{-3} M solution

(4) BTM 16 0.04ml. of a 8.2×10^{-3} M solution

4. THE CAT ANTERIOR TIBIALIS PREPARATION

(Brown, 1938).

Cats of either sex, weighing from 2 to 5 kg, were used. They were anaesthetised with ethyl chloride, and warm 1% chloralose, 80 mg/kg. was injected into the right femoral vein. A tracheal cannula was inserted.

The left sciatic nerve was located between the ischial tuberosity and the great trochanter of the femur and was crushed by tying a thread round it, to deafferent the limb. The sciatic nerve, dividing into peroneal and medial popliteal nerves, was located in the popliteal space, and a Sherrington-type electrode placed on the peroneal nerve. These incisions were then sewn up again.

A hook was tied into the tendon of the anterior tibialis muscle close to its attachment on the inside of the foot, and the tendon insertion together with a knob of bone was cut out. The tendon was dissected free back to the muscle, cutting the annular ligament. The flexor tendon to the foot was cut.

The lower end of the fibula was cut off, exposing the flat part of the head of the tibia, and a drill-bar was screwed into this,

perpendicular to the outside of the leg. Another drill-bar was inserted, parallel to the first, into the flat part of the femur above the joint.

The saphena vein was then ligated and divided, the fascia over the anterior tibialis muscle divided and the tendon raised. The anterior tibial artery was located lying beneath the muscle, separated and cannulated retrogradely, using the same needle cannula - 2-way tap set-up as was used in the superior cervical ganglion preparation. Saline was injected at intervals from a syringe inserted into the 2-way tap, to prevent clotting. It will be noted that this is a modification of Brown's method - the vessels leading from the anterior tibial artery to the extensor longus digitorum and to the peroneal group were not tied, nor was a thread passed round the anterior tibial artery at the level of the interosseous membrane. The anterior tibialis has not therefore had its blood supply completely isolated, but the method used was considered adequate for present purposes.

The animal was then fixed to the myograph stand by means of the drill-bars, with the tibia vertical, and the tendon hook was

attached to a flat spring isometric recording myograph lever, writing on a smoked drum. A blood pressure record was taken by means of a cannula in the left or right carotid artery, and a mercury manometer. All exposed tissue was kept moist with liquid paraffin, and the animal kept warm by means of a lamp from above.

The peroneal nerve was stimulated maximally at a rate of 12/minute.

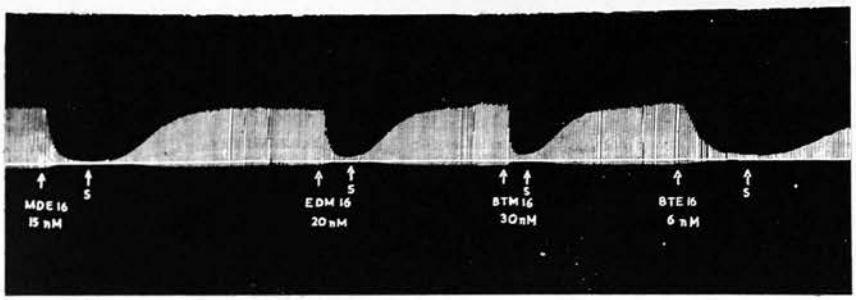


Figure 3: Neuromuscular blocking activity on the cat tibialis preparation: estimation of equipotent molar ratios compared to BTE 16. The following doses were given:

(1) MDE 16 15 nM

(2) EDM 16 20 nM

(3) BTM 16 30 nM

(4) BTE 16 6 nM

Doses were given in approximately 0.2 ml. of solution.

S = 0.2 ml. 0.9% saline

5. THE CAT SUPERIOR CERVICAL GANGLION PREPARATION

(Trendelenburg, 1954; Paton and Perry, 1953)

Cats of either sex, weighing from 2 to 5 kg., were used.

In the earlier series of experiments, the cat was anaesthetised with ether in a box, then transferred to a warm table. The trachea was cannulated and was connected to a Wolff bottle containing ether. The right femoral vein was then exposed and cannulated and a 1% solution of chloralose, at 38°C, was given slowly, in a dosage of 80 mg/kg.

In the later series of experiments, the cat was anaesthetised with ethyl chloride on a mask, the right femoral vein exposed and warm 1% chloralose, 80 mg/kg., given intravenously by syringe. The tracheal cannula was then inserted. This method was found to be quicker and to keep the animal in better condition.

The tissues overlying the common carotid artery on one side, usually the right, were cleared, removing the lymph node and exposing the bifurcation of the external carotid artery and the lingual artery. A length of the

pre-ganglionic sympathetic nerve was also exposed and freed, separating it from the vagus nerve. A thread was put through the right nictitating membrane. The cat's head was immobilised by tying it to a transverse bar placed between the jaws and clamped firmly to the table.

In the earlier series of experiments, the lingual artery and any branches of the external carotid were tied. The external carotid was tied peripherally, a remote-control bulldog clip placed centrally, and the artery cannulated between them, using a Luer syringe needle adapted as a cannula. The cannula and bulldog clip were clamped firmly in position. Injections could be made by inserting a syringe into the needle cannula, opening the bulldog clip and emptying the syringe. Injections of 0.2 ml. 0.9% saline were given at intervals to prevent clotting in the cannula.

In the later series of experiments, the external carotid artery was tied off and the lingual artery cannulated retrogradely, using a needle cannula connected by a short length of fine polythene tubing to a Record needle. This was attached to a Record 2-way tap, into which syringes could be inserted. No remote-control

bulldog clip was thus needed. The 2-way tap was firmly clamped, and saline injections given at intervals.

The preganglionic sympathetic nerve was divided and the central end laid over a probe electrode, which was also firmly clamped. The incision in the neck was then filled with liquid paraffin.

A blood pressure cannula was inserted in the left femoral artery and a record taken by means of a mercury manometer and lever writing on a smoked drum, using heparinised saline as a connecting bridge.

The nictitating membrane thread was passed over a pulley and attached to a light frontal writing lever, writing on the smoked drum, with a magnification of about 10 times. The preganglionic sympathetic nerve was stimulated maximally at a rate of 10/second to produce a maximal contraction of the nictitating membrane. Stimulation was intermittent, approximately 10 mins. on and 20 mins. off.

Further doses of chloralose (about ¼ the original dose) were given every few hours to maintain anaesthesia. If a strychnine-like sensitivity to the chloralose developed, it was dispelled by a small dose of nembutal.

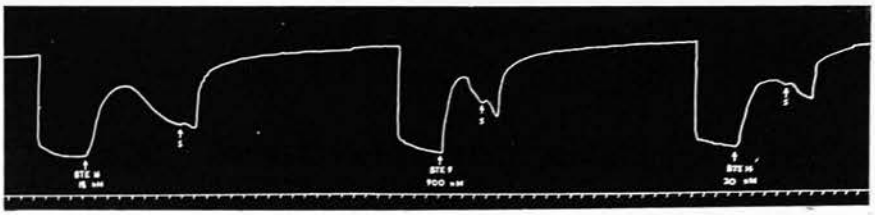


Figure 4: Ganglion-blocking activity on the cat superior cervical ganglion preparation: estimation of equipotent molar ratios compared to BTE 16. The following doses were given:

(1) BTE 16 18 nM

(2) BTE 9 900nM

(3) BTE 14 20 nM

Doses were given in approximately 0.2 ml of solution.

S = 0.2 ml of 0.9% saline

II. METHODS

The experiments were designed to measure changes in activity (agonist activity or antagonist activity) throughout the two series of compounds.

1. AGONIST ACTIVITY

This was measured as contracture production on the chick biventer preparation, and was only given by a certain number of the compounds.

Contractures were measured as the maximum deviation from the base-line. Each compound was compared with the standard (decamethonium). Three or more different doses of either drug were given, and log dose-response curves were plotted (the time required for each dose precluded the performance of a full 4-point assay; however, in trial runs it was found that responses were reproducible). From the log. dose-response curves, the equipotent molar ratio of the compound relative to decamethonium was calculated (i.e. the number of molecules of the test compound which produced the same effect as 1 molecule of decamethonium). Where

the log dose-response curves were not parallel, the comparison was made at an arbitrary standard response level. A few of the less active compounds were tested by matching doses only. Each compound was tested on several preparations.

Standard errors of the means of the equipotent molar ratios were calculated for some of the compounds and the means for the different compounds tested for significant difference, using 'Student's t test'.

For some of the compounds, the log dose-response curves were much less steep than that for decamethonium. To see if the compounds were acting as partial agonists, addition experiments were performed. Concentrations of the test compound and of decamethonium were found which produced the same effect. The two substances were then added simultaneously in half these concentrations, and if the contracture produced was less than that obtained previously the test compound was concluded to be a partial agonist (Stephenson, 1956). It does not follow, however, that if the contracture was the same size the compound was not a partial agonist. It is extremely likely that in this preparation the response is obtained with only a small

proportion of receptors occupied.

Decamethylene bis-acetoxyethyldimethyl-ammonium was also tested, in the presence of eserine, to see if it was appreciably hydrolysed by acetylcholinesterase under these conditions, as it is in vitro (Barlow, 1955).

The drugs were added directly to the organ-bath from a pipette in a small volume (usually 0.2 ml.). They were left in contact with the muscle until the effect had reached a maximum, which usually took about 10 min., then washed out and the preparation allowed to recover for 20 min. or until it had recovered fully, if this took longer. The interval between doses was thus usually 30 min.

2. ANTAGONIST ACTIVITY

This was measured as production of block of the muscle contraction resulting from nerve stimulation. Block was measured as the percentage reduction in height of the contraction (twitch or sustained contraction). Each compound was compared with a standard, and doses of the test compounds were found which

gave degrees of block similar to that obtained with a certain dose of the standard i.e. pairs of doses of test and standard were obtained which gave matching responses. From these equipotent doses, the equipotent molar ratio for each compound relative to the standard was calculated.

(a) Whole Animal Experiments

Due to the large number of compounds to be estimated on a limited number of animals and the length of time required for each dose, only one dose of each test compound was usually given in one experiment. Preliminary experiments were first carried out to find the appropriate concentration range for each compound. Since the sensitivity of these cat preparations increased markedly during the course of an experiment, it was desirable to have a dose of test compound always adjacent to a dose of standard. Doses were therefore given in the following pattern: S A B S C D S etc. where S = standard and A, B, C and D are test compounds. It was intended to have as a standard response 50% block of the contraction, but due to the

varying sensitivity of a preparation, it was not always possible to achieve this. Each compound was tested on at least two animals.

Drug doses were given by filling a Record syringe appropriately, inserting it into one limb of the two-way tap, opening the tap and injecting the drug via the arterial needle cannula. Drugs were given in doses of about 0.2 ml. and were immediately washed in with 0.2 ml. saline, from a syringe fitted in the other limb of the tap. The effect was allowed to reach a maximum, and then a further 0.2 ml. saline was injected. Sometimes this caused a slight further block, showing incomplete wash-out of the drug from the cannula etc. but this effect was never large.

With the cat superior cervical ganglion preparation, intermittent stimulation was used. The dose of drug was given 3 min. after starting stimulation (by which time the contraction had reached a steady level), the effect was allowed to reach a maximum and then start to decline, and then the stimulation was switched off - a total of about 10 min. stimulation.

The preparation was given about 20 min. recovery time, or longer if necessary for full recovery, saline being injected at intervals.

With the cat tibialis preparation, continuous stimulation was used. The dose of drug was given as described above, and time for full recovery from the effect of the drug was allowed, as judged by the record on the drum, saline being given at intervals.

(b) Isolated Tissue Preparations

(i.e. chick biventer and rat phrenic-diaphragm)

The doses of drugs were added directly to the organ-bath in a small volume (usually less than 0.2 ml). They were left in contact with the muscle until the block had reached a maximum (about 10 min.) then washed out, and the preparation left until full recovery had occurred - at least 20 min. The interval between doses was thus about 30 min.

On the rat phrenic nerve-diaphragm preparation sufficient doses were given, when possible, to prepare log dose-response curves, and equipotent molar ratios were calculated from these.

III. RESULTS

1. CONDUCTIVITY

As mentioned in the Methods section, the molarity of each solution was checked by Volhard estimation of bromide. Table 1 shows the molarity calculated from the weight of material taken compared with the molarity found by titration. For the members of the BTE series the observed value for the molarity is always lower than the calculated value, usually by about 3%, and this discrepancy is presumably due to the absorption of water by the solids while being weighed out. For those members of the BTM series for which a comparison was made, the discrepancy is less: these compounds are less hygroscopic than the members of the BTE series: BTM 17, 18 and 20, in fact, were very difficult to dissolve - solutions which were almost saturated had to be used. No explanation for this relative insolubility could be found.

Graph 1 shows the standard curve of molar conductance against square root of concentration for potassium chloride. This

TABLE 1.- Conductivity. Difference between concentration calculated from weight of material taken and concentration calculated from bromide ion found by titration.

Compound	Molarity calculated	Molarity found	Compound	Molarity calculated	Molarity found
			BTE 9	9.33×10^{-3}M	9.16×10^{-3}M
BTM 10	-	1.14×10^{-2}M	BTE 10	-	9.89×10^{-3}M
BTM 11	9.79×10^{-3}M	9.52×10^{-3}M	BTE 11	4.93×10^{-3}M	4.71×10^{-3}M
BTM 12	1.28×10^{-2}M	1.26×10^{-2}M	BTE 12	1.27×10^{-2}M	1.22×10^{-2}M
BTM 13	6.56×10^{-3}M	6.30×10^{-3}M	BTE 13	9.33×10^{-3}M	9.07×10^{-3}M
BTM 14	1.07×10^{-2}M	1.03×10^{-2}M	BTE 14	8.64×10^{-3}M	8.09×10^{-3}M
BTM 15	8.37×10^{-3}M	8.16×10^{-3}M	BTE 15	-	9.49×10^{-3}M
BTM 16	-	3.71×10^{-3}M	BTE 16	9.19×10^{-3}M	8.64×10^{-3}M
BTM 17	-	2.03×10^{-3}M	BTE 17	-	7.67×10^{-3}M
BTM 18	-	8.25×10^{-4}M	BTE 18	-	8.86×10^{-3}M
BTM 19	-	3.54×10^{-3}M	BTE 19	7.13×10^{-3}M	7.00×10^{-3}M
BTM 20	-	9.10×10^{-4}M	BTE 20	8.98×10^{-3}M	8.52×10^{-3}M
BTM 21	-	5.62×10^{-3}M		8.55×10^{-3}M	8.12×10^{-3}M
			BTE 21	-	7.90×10^{-3}M

Concentration of drug in solution: The concentration based on the titration was taken as correct: it is invariably slightly lower than that calculated from the weight of material taken and the difference is ascribed to uptake of water by the sample during weighing.

is linear at low concentrations and deviates from linearity as the concentration increases (above about 4×10^{-2}M, in this estimation), when the Debye-Hückel equation no longer holds.

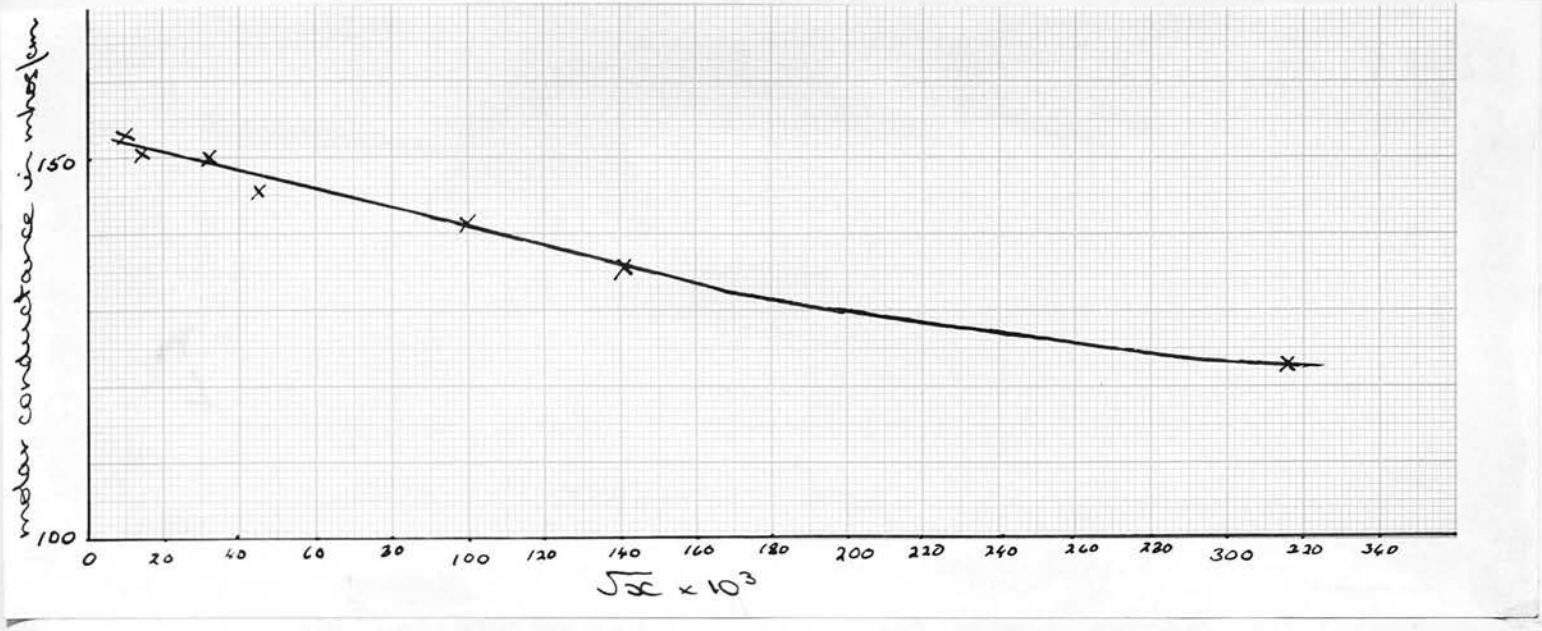
Graph 2 shows the standard curve of molar conductance against square root of concentration for cetyl trimethyl ammonium bromide (CTAB), a surface-active compound which is known to form micelles. The curve shows three characteristic slopes:

(1) at low concentrations it is linear, as for strong electrolytes,

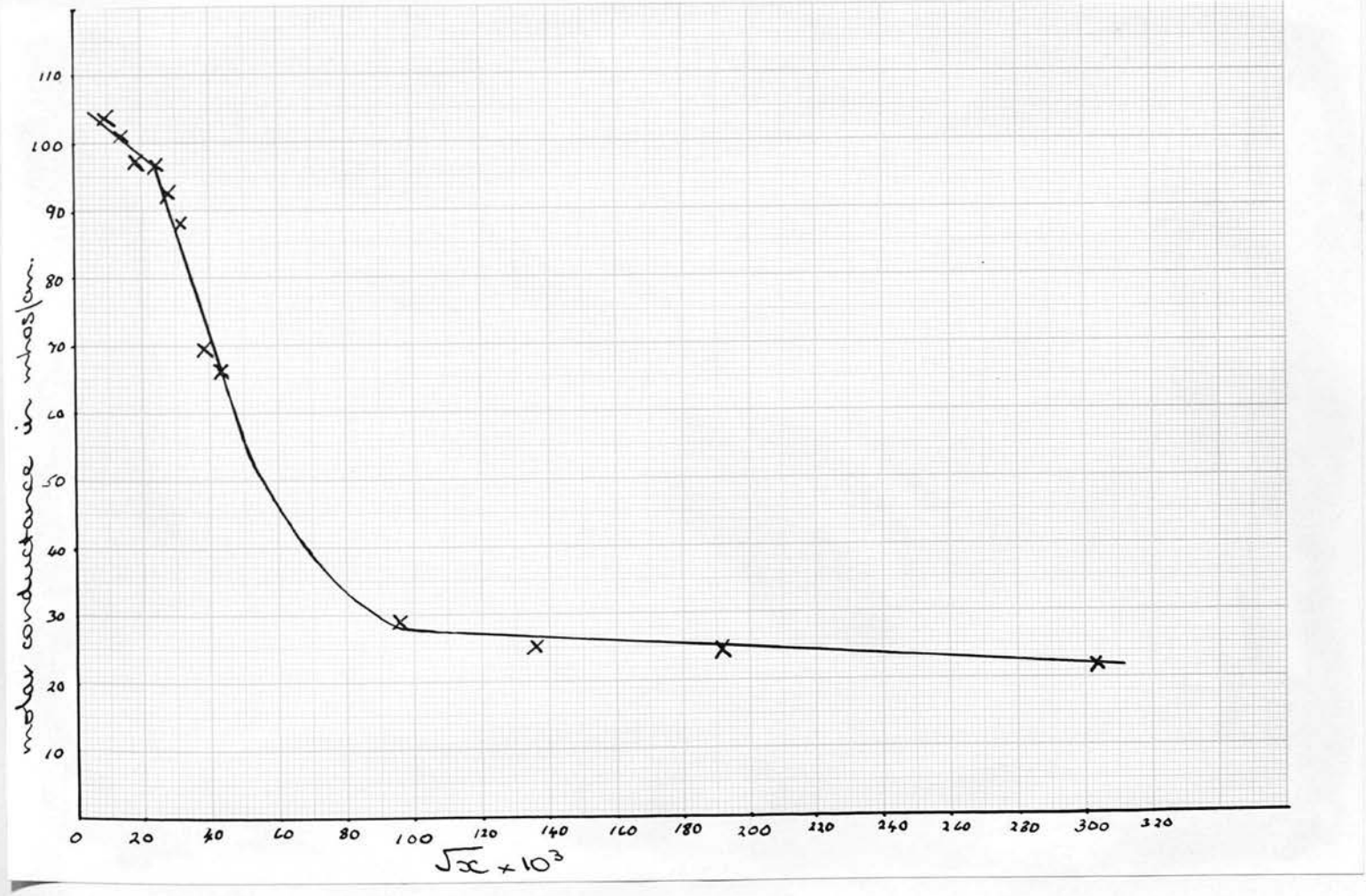
(2) it falls steeply - this is due to micelle formation,

and (3) it flattens out again at high concentrations.

The concentration at the 'break-point' between parts (1) and (2) is called the critical micelle concentration i.e. the lowest concentration at which micelles just begin to form, and for CTAB it is here found to be 5.8×10^{-4}M. In the literature, the curve given for cetyl trimethyl ammonium chloride (CTAC) is identical in shape to that found for CTAB, the critical



Graph 1: Molar conductance against square root of molar conductance ($\sqrt{x}$) for potassium chloride

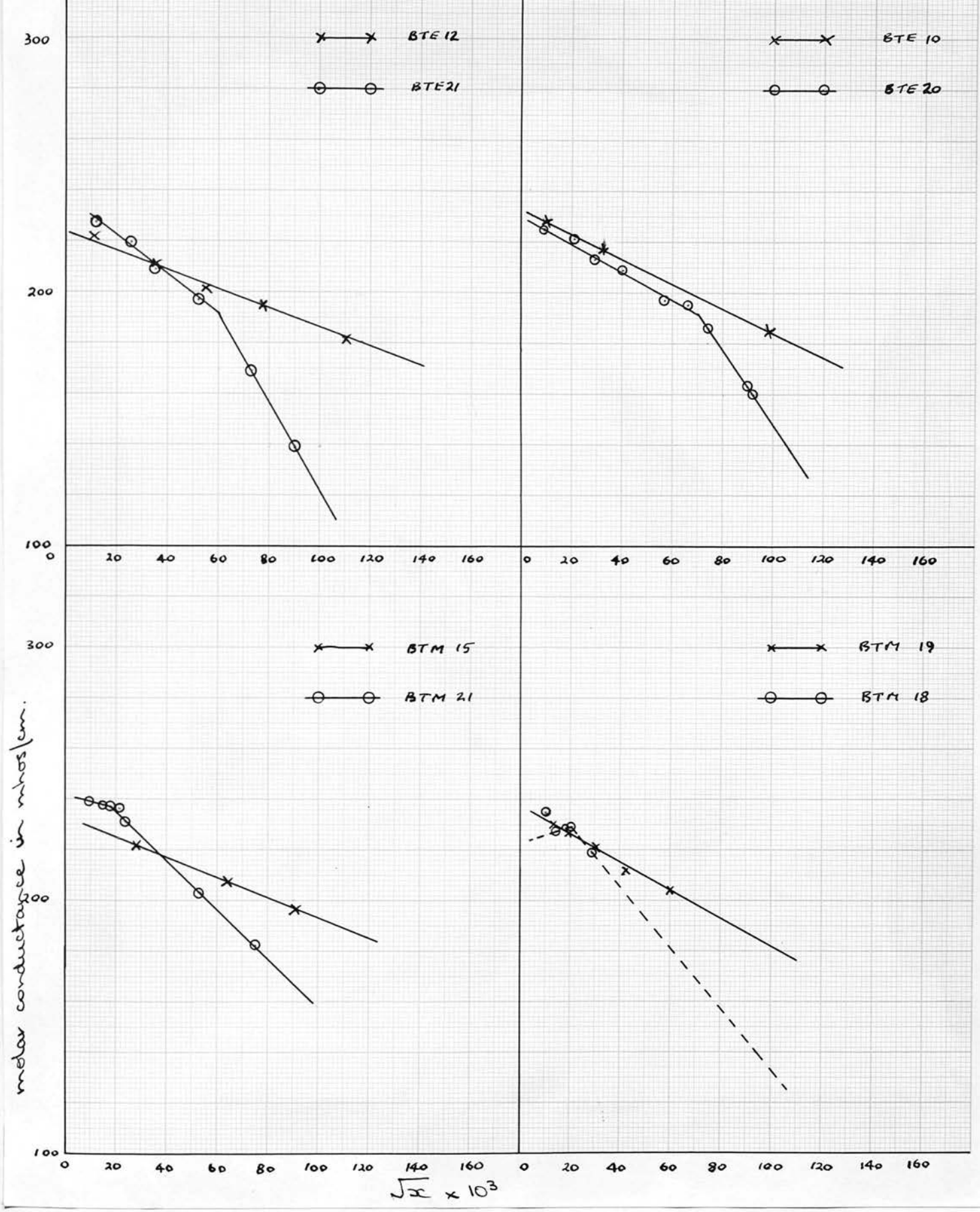


Graph 2: Molar conductance against square root of molar conductance ($\sqrt{x}$) for cetyl trimethylammonium bromide

micelle concentration for CTAC being given as 1.3×10^{-3}M (Ralston et al., 1947).

Graph 3 shows curves of molar conductance against square root of concentration for some of the test compounds. Those for BTM 15 and 19, and BTE 10 and 12 are typical linear graphs as for strong electrolytes. All the curves were of this type except for those of BTE 20 and 21, BTM 21 and BTM 17, 18 and 20. BTE 20 and 21 and BTM 21, as shown in the graphs behaved like CTAB in its two lower concentration ranges. Thus it seems that micelle formation does occur with BTE 20 and 21 and BTM 21 as the concentration is increased, the critical micelle concentrations being 5.0×10^{-3}M, 3.6×10^{-3}M and 3.2×10^{-4}M respectively. Therefore, if these compounds are used in biological experiments at concentrations higher than these critical micelle concentrations, the activity may be affected by micelle formation, which will reduce the effective number of molecules present.

BTM 17, 18 and 20 are the compounds which were difficult to dissolve, and they showed anomalous curves, of which that for BTM 18 is typical. As the concentration decreased, the



Graph 3: Molar conductance against square root of molar concentration ($\sqrt{x}$)

molar conductance first rose sharply, then abruptly levelled off or actually decreased again. For BTM 18 and 20 it was found to have increased again at about 1 x 10^{-4}M, but at this low concentration error due to uptake of carbon dioxide may be considerable. It seems unlikely that micelle formation could be the explanation for these curves, as BTM 19 shows no evidence of micelle formation. The abnormal low solubility and the abnormal conductance curves seem to be linked, but unfortunately no explanation can be provided.

Table 2 and Graphs 4, 5 and 6 show the molar conductances at three standard concentrations for the compounds of the two series, and for some compounds with a chain length of 16 methylene groups but with different substituent groups on the nitrogen atoms. Graph 4 shows that molar conductance does not vary much with chain length in the BTM series - it decreases slightly and gradually with increasing chain length - with the exception of the anomalous results for BTM 17, 18 and 20, and micelle formation at the higher concentrations for BTM 21.

TABLE 2.- Results of Conductivity Measurements.

A B

Compound	λ_A	λ_B	λ_C	Compound	λ_A	λ_B	λ_C
				BTE 9	185	220	231
BTM 10	204	236	246	BTE 10	184	217	228
BTM 11	201	235	245	BTE 11	185	219	230
BTM 12	196	225	235	BTE 12	185	212	221
BTM 13	196	229	239	BTE 13	185	217	228
BTM 14	195	227	237	BTE 14	184	220	232
BTM 15	192	222	231	BTE 15	184	217	229
BTM 16	193	225	235	BTE 16	183	213	222
BTM 17	175	221	-	BTE 17	182	213	224
BTM 18	138	216	-	BTE 18	180	215	226
BTM 19	180	220	233	BTE 19	177	218	232
BTM 20	173	217	-	BTE 20	148	213	225
BTM 21	157	224	239	BTE 21	118	212	230

C

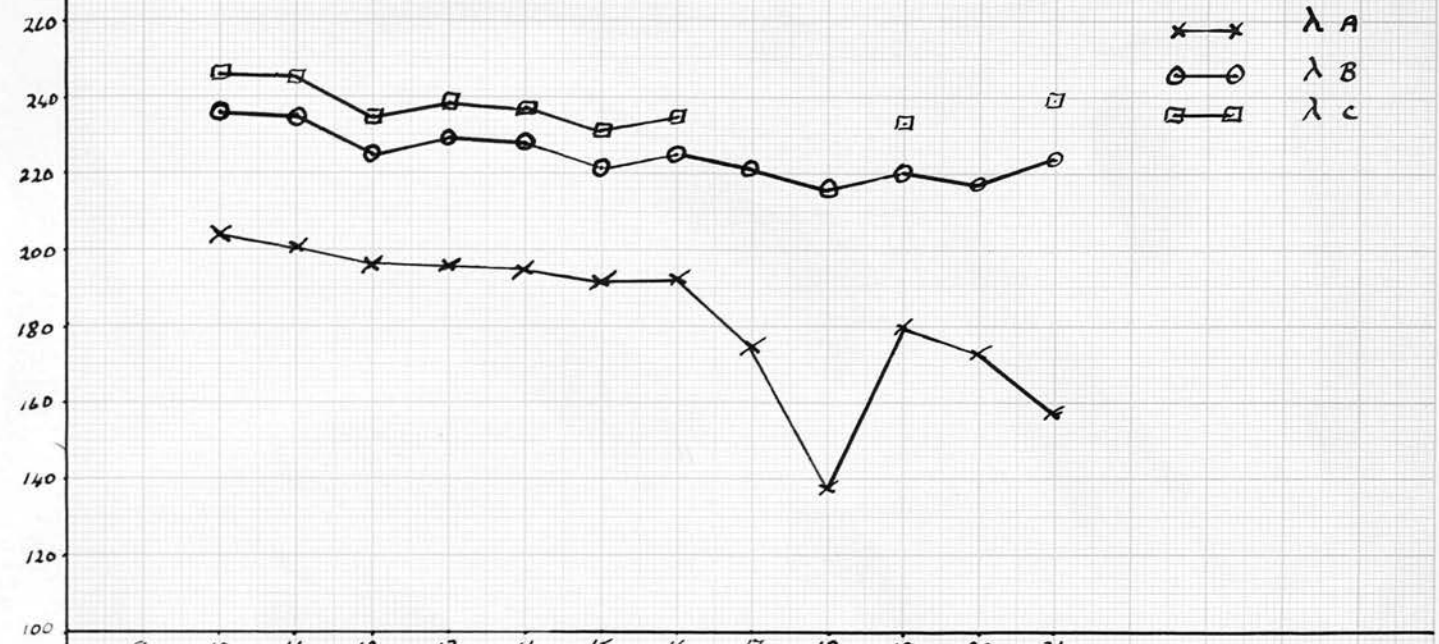
Compound	λ_A	λ_B	λ_C
BTM 16	193	225	235
BEDM 16	185	213	221
BMDE 16	185	212	220
BTE 16	183	213	222
BHDM 16	185	211	220
BDM 16	192	220	229
BDE 16	179	207	216

where λ_A = molar conductance in mhos/cm at 1.000×10^{-2}M

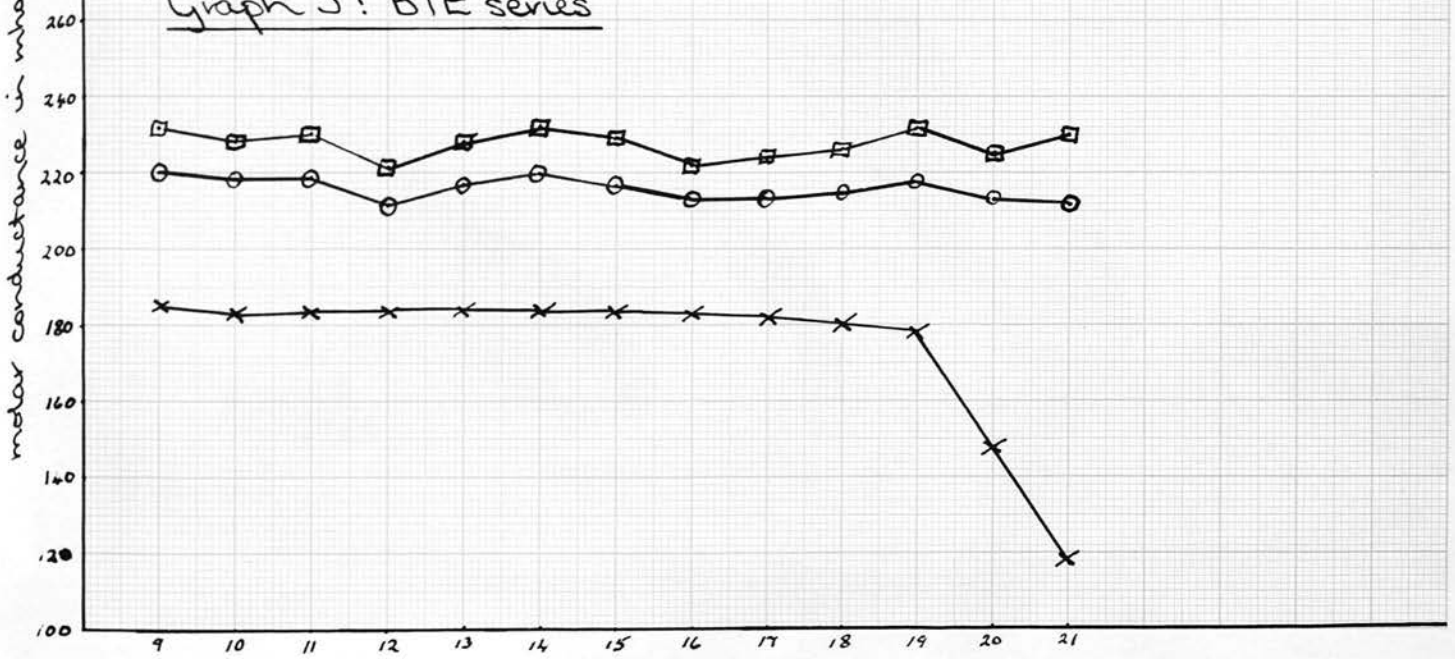
λ_B = molar conductance in mhos/cm at 1.000×10^{-3}M

λ_C = molar conductance in mhos/cm at 1.000×10^{-4}M.

Graph 4 : BTM series



Graph 5 : BTE series



molar conductance in mhos/cm.

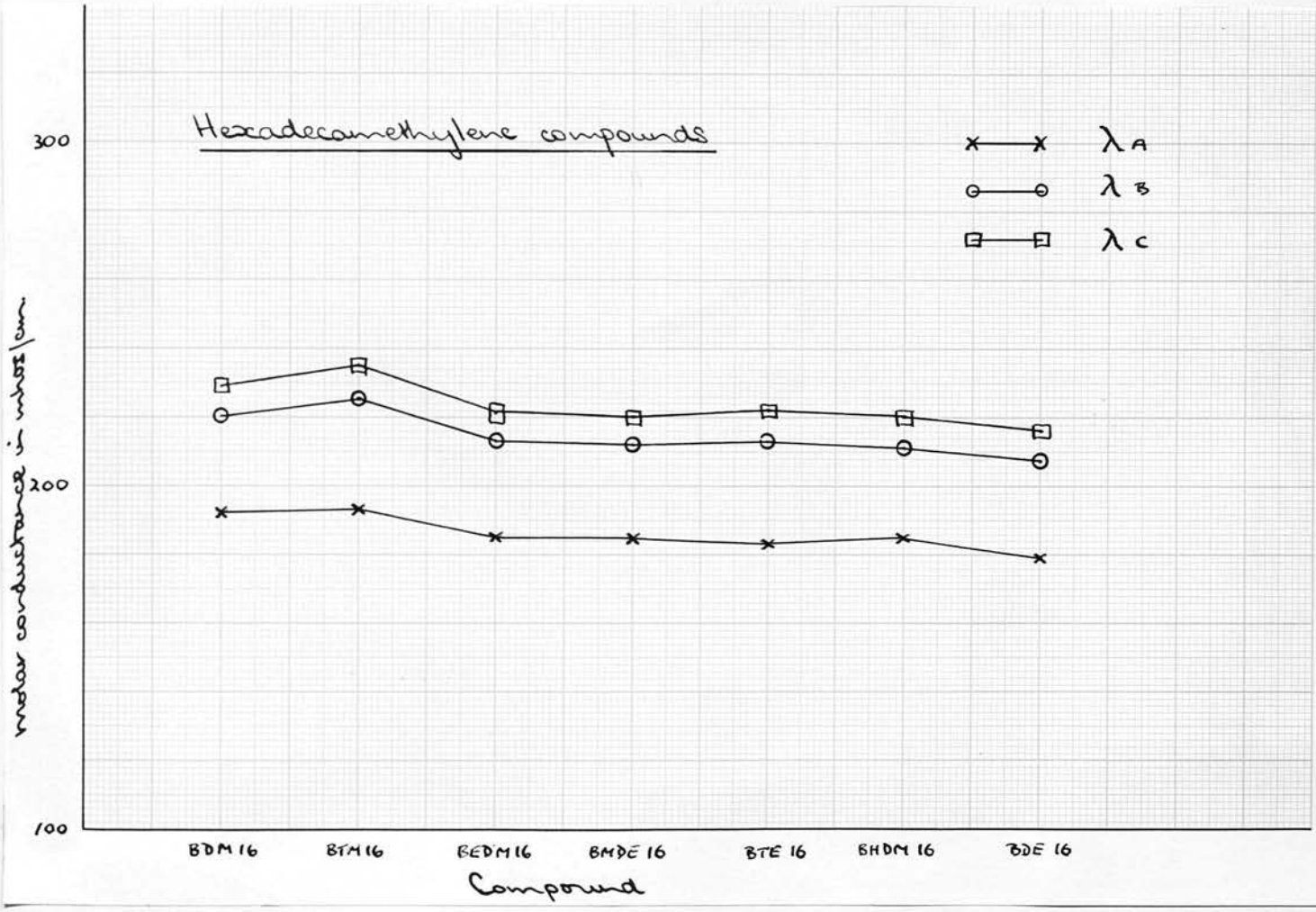
Chain length, n = number of carbon atoms

Molar conductance against chain length at three standard molar concentrations

λ_A at 1.000×10^{-2} M

λ_B at 1.000×10^{-3} M

λ_C at 1.000×10^{-4} M



Graph 6: Alteration in molar conductance with variation in substituent groups on nitrogen atoms

λ_A at 1.000×10^{-2} M
λ_B at 1.000×10^{-3} M
λ_C at 1.000×10^{-4} M

Graph 5 shows that molar conductance is practically constant for the BTE series until micelle formation appears at the highest concentrations for BTE 20 and 21. The slight fluctuations in the values at the two lower concentrations while those at the highest concentrations are steady is probably only due to experimental error (though they do tend to run parallel, and in particular there is a slight drop in the results at concentrations B and C for the compounds in both series with a chain length of 12 methylene groups).

The results for the BTE series, except for that obtained for BTE 15, are similar to those of Warriner (1960), who used uncorrected molarities. She obtained an unexpectedly high value and stated that it required to be checked. In this investigation the value for BTE 15 is quite normal, in accordance with its position in the series, and this suggests that the previous result was erroneous. The results for both series suggest that at no chain length is there any significant alteration in the configuration of the molecule e.g. folding, which

might affect the pharmacological properties. Activity may only be affected by micelle formation in the highest members of the series, at relatively high concentrations.

Graph 6 shows that the molar conductance of compounds with chain-length of 16 methylene groups does not change much with the variations in substituents on the nitrogen atoms tested here. The conductances of the bis-dimethyl and the bis-trimethyl compounds are almost identical, there is a slight drop in conductance when 1 or 2 methyl groups are replaced by ethyl, and a slight further drop when the third methyl is replaced by ethyl. The values for the bis-hydroxyethyl dimethyl compound and the bis-ethyl-dimethyl compound are identical, as of course there is very little difference in their structures, and there is another slight drop in conductance from the bis-triethyl compound to the bis-diethyl compound. It seems therefore that increasing the bulk of the cationic head tends to lower the conductance of the molecule slightly - the conductances for all members of the BTE series are lower than those for the corresponding members of the BTM series, in

agreement with this. This result would be expected as increasing the bulk of the molecule will tend to decrease its mobility in solution. The difference between the values for corresponding members of the two series becomes less as the chain-length increases, presumably as the overall length of the molecule becomes a more important factor than the bulk of the cationic head. On this explanation, however, the lower conductance of the bis-diethyl compound than the bis-triethyl compound is unexpected.

2. ACTIVITY AT THE NEUROMUSCULAR JUNCTION

A. Chick Biventer Preparation

(1) Contracture Production.- The activity of the compounds in producing contracture of the chick biventer preparation is summarised in Tables 3 and 4 and Graphs 7, 8, 9 and 10. The values are expressed as mean equipotent molar ratios ± standard error (EPMR ± SE), and also as mean log equipotent molar ratios ± standard error (log EPMR ± SE), since the distribution of ratios is probably logarithmic (Gaddum, 1945).

In the BTM series (Table 3A) contractures were obtained easily with BTM 10 - 16. Each compound was compared to BTM 10 = 1.0 and it was found that BTM 11 is more active than BTM 10 and BTM 12, and BTM 13 is the most active. The activity decreases from BTM 14-16. The means of the equipotent molar ratios were tested for significant difference using Student's 't' test, at the 0.05 level of probability. The values for BTM 10, 11, 12 and 13 are significantly different, and so are the values for BTM 15 and 16, but there is no significant

TABLE 3 - Activity of Compounds on the Chick Biventer Preparation: Agonist Activity.

A

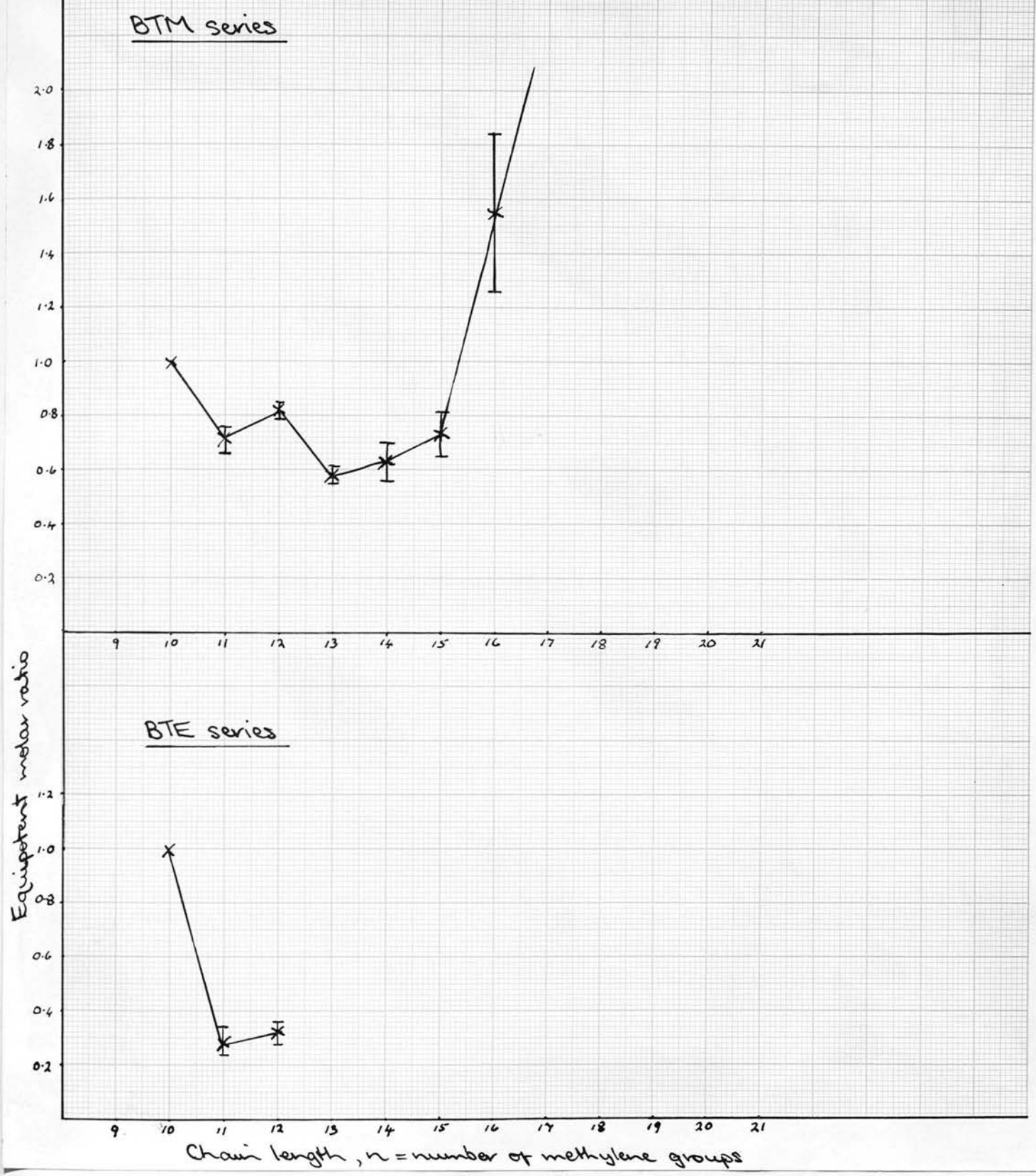
Compound	EPMR±SE	log EPMR±SE	No. of expts.
BTM 10	1.00	0.00	-
BTM 11	0.71±0.04	-0.15±0.02	6
BTM 12	0.82±0.03	-0.09±0.02	6
BTM 13	0.58±0.03	-0.24±0.02	6
BTM 14	0.63±0.07	-0.22±0.04	7
BTM 15	0.73±0.08	-0.15±0.05	6
BTM 16	1.55±0.29	0.15±0.08	6
BTM 17	~ 100		
BTM 18	no contr.		
BTM 19	no contr.		
BTM 20	no contr.		
BTM 21	no contr.		

B

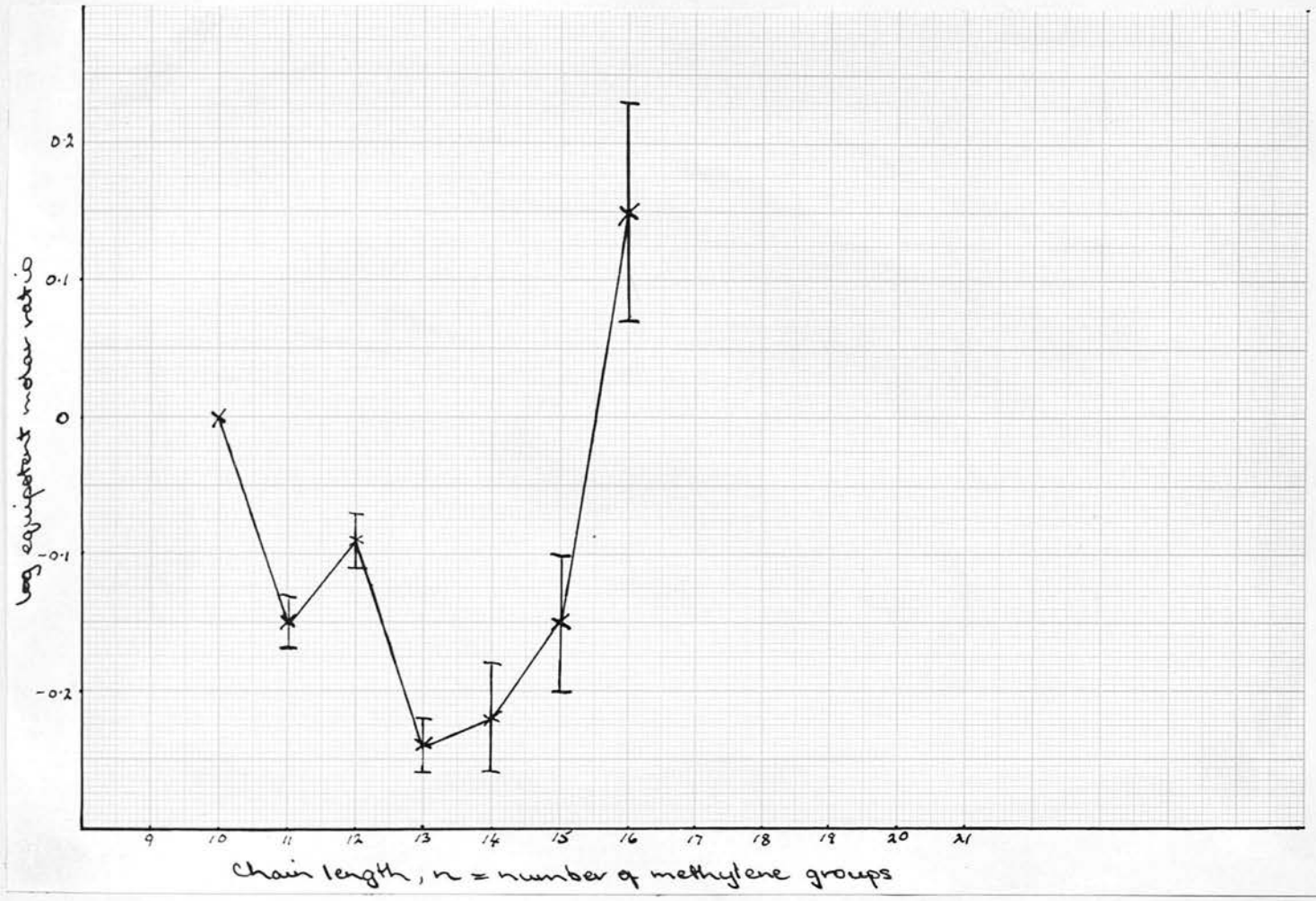
Compound	EPMR±SE	No. of expts.
BTE 9	no contr.	
BTE 10	1.00	-
BTE 11	0.28±0.05	4
BTE 12	0.32±0.04	4
BTE 13	no contr.	
BTE 14	"	
BTE 15	"	
BTE 16	"	
BTE 17	"	
BTE 18	"	
BTE 19	"	
BTE 20	"	
BTE 21	"	

The equipotent molar ratio (e.p.m.r.) indicates the number of molecules of the compound needed to produce the same effect as one molecule of standard. The mean is given together with the standard error (s.e.) and the number of experiments.

Standard: A. BTM 10 = 1.0 , B. BTE 10 = 1.0



Graph 7: Equipotent molar ratio against chain length: contracture production on chick biventer



Graph 8: Variation in activity with chain length for BTM series: log equipotent molar ratio for contracture production on chick biventer

difference between the values for BTM 13, 14 and 15. However, it seems likely that BTM 13 is in fact the most active, since the lack of significant difference from BTM 14 and 15 is mainly due to the high standard error of the means for the latter two. This resulted from deviations from parallelism between the log dose-response curves for BTM 10 and the higher members of the series. The log dose-response curves became flatter, compared to that for BTM 10, as the chain length increased, and the determinations of equipotent molar ratio were made at an arbitrary standard response level.

The higher members of the series, BTM 17 - 21, could produce small contractures when high doses were given, but the log dose-response curves were very flat and maximal contractures could never be obtained - evidently they are only partial agonists.

In the BTE series (Table 3B) contractures could only be obtained with BTE 10, 11 and 12. This confirms Warriner's purely qualitative results (1960). BTE 11 and 12 have similar

activity, about three times that of BTE 10, with which they were compared.

Where the composition of the onium groups in the decamethylene compounds is altered (Table 4A: Barlow and Zoller, 1962), replacement of methylgroups by ethyl decreases the activity in a stepwise fashion. Replacement of one methyl group by hydroxyethyl or acetoxyethyl has more effect than replacement by ethyl. Removal of one methyl group, producing a tertiary compound, reduces the activity to the greatest extent.

The log dose-response curve for BTE 10 was much flatter than that for BTM 10, and addition experiments indicated that it is a partial agonist (the value in Table 4A was obtained using only threshold responses). Addition was demonstrated for BEDM 10, BMDE 10, BHDM 10 and BDM 10, but does not, of course, necessarily mean that they are not partial agonists.

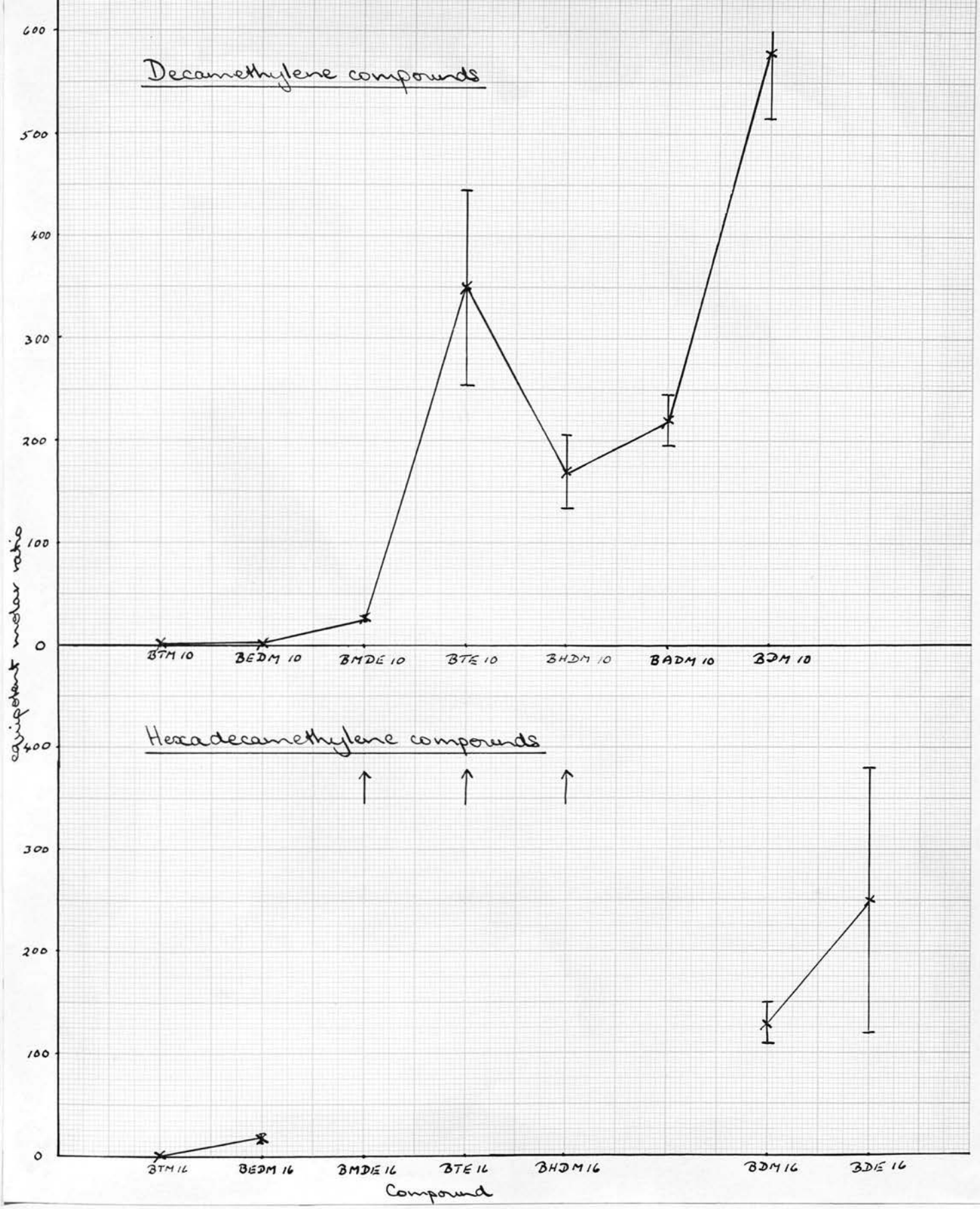
The effects of BADM 10, which is hydrolysed by acetylcholinesterase <u>in vitro</u> (Barlow, 1955), were not potentiated by eserine (in concentrations up to 10^{-4}M), and so it does not appear

TABLE 4.- Activity of Compounds on the Chick Biventer Preparation: Agonist Activity.

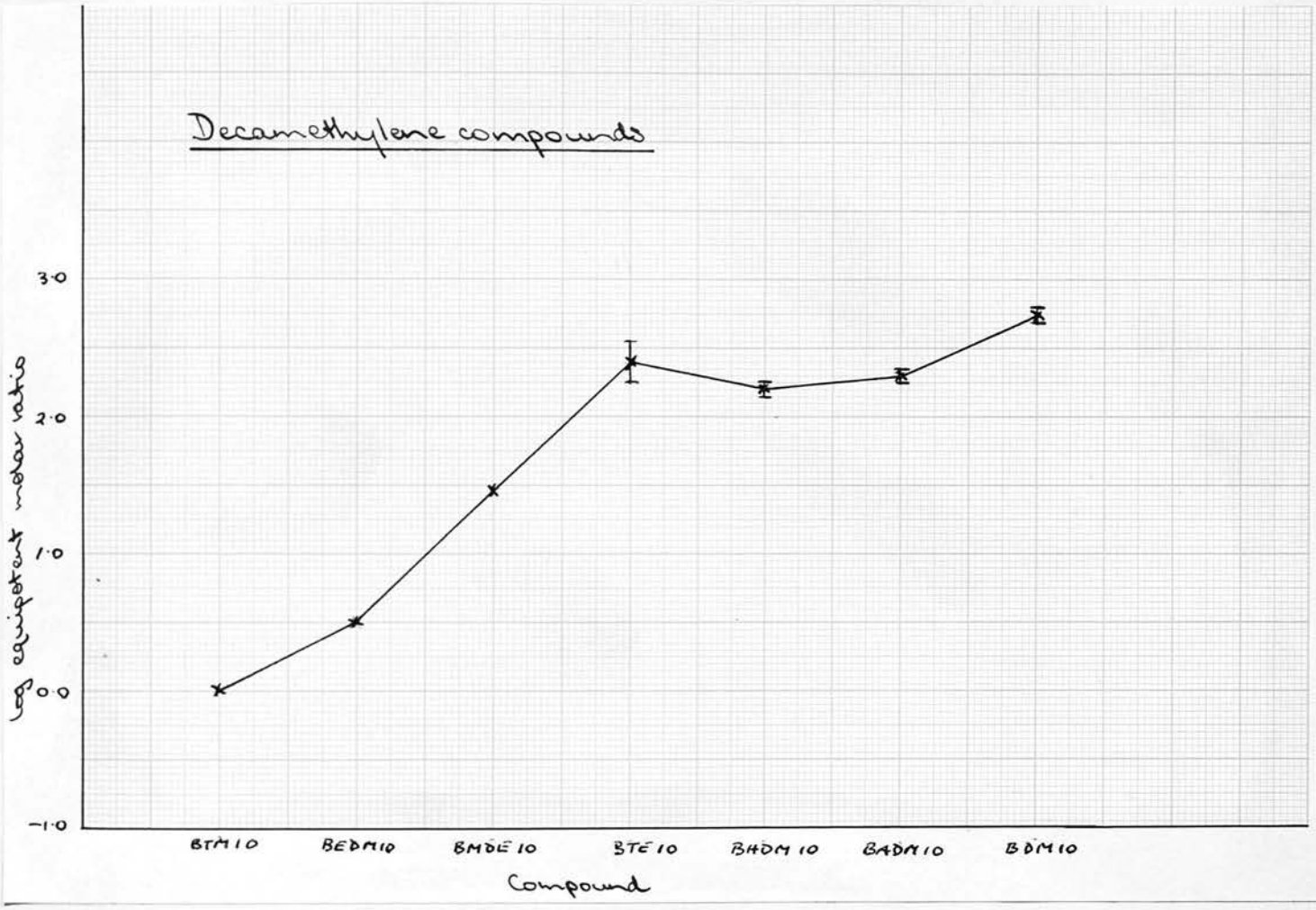
A				B		
Compound	EPMR±SE	log EPMR±SE	No. of expts.	Compound	EPMR±SE	No. of expts.
BTM 10	1.00	0.00	-	BTM 16	1.00	-
BEDM 10	3.23±0.19	0.50±0.03	10	BEDM 16	17 ±3.3	3
BMDE 10	28 ±1	1.45±0.02	7	BMDE 16	~ 10,000	1
BTE 10	350 ±95	2.41±0.16	7	BTE 16	no contr.	
BDM 10	580 ±66	2.75±0.05	6	BDM 16	130 ±20	2
BHDM 10	170 ±35	2.21±0.06	7	BHDM 16	~ 10,000	2
BADM 10	220 ±25	2.32±0.05	9			
				BDE 16	250 ±130	3

Standard: A. BTM 10 = 1.0, B. BTM 16 = 1.0.

The equipotent molar ratio (e.p.m.r.) indicates the number of molecules of the compound needed to produce the same effect as one molecule of standard. The mean is given together with the standard error (s.e.) and the number of experiments.



Graph 9: Variation in activity for compounds with different onium groups: equipotent molar ratio for contracture production on chick biventer



Graph 10: Variation in activity for compounds with different onium groups: log equipotent molar ratios for contracture production on chick biventer

to be destroyed to any extent by the cholin-esterases in this preparation. This justifies the inclusion of the results for BADM 10 in Table 4A, since they were obtained without eserine being present.

When the composition of the onium group in the hexadecamethylene compounds is altered (Table 4B), replacement of one methyl group by ethyl reduces the activity, and replacement of more methyl groups virtually abolishes ability to produce a contracture altogether. Replacement of one methyl group by hydroxyethyl also virtually abolishes activity. On the other hand, removal of one methyl group from BTM 16 or one ethyl group from BTE 16 to give tertiary compounds results in an apparent retaining of the ability to produce contracture. However, this contracture is delayed in onset, increases slowly and apparently continuously, and recovery after washing is very slow indeed. This suggests a different mode of action probably a direct action on the cell and possibly a penetration of the cell membrane by the unionised form of the tertiary compounds.

The concentration of BTM 10 in the bath fluid in these experiments was usually about 1×10^{-7}M. With BTM 10 there was seldom any blocking of the twitch concurrently with the contracture, but as the chain length increased there was an increasing component of twitch block together with the contracture. If the drug was left in for a long time the contracture would begin to pass off without washing out, even with BTM 10.

(2) Blocking activity.- Blocking activity on the chick biventer twitch was determined for the BTE series, most of which did not produce a contracture. Contracture was obtained sometimes, but not always, with BTE 10, 11 and 12, and also sometimes with BTE 17, 20 and 21. Whether or not contracture was obtained seemed to depend to some extent on the tension of the muscle - it is possible that under the right conditions some degree of contracture might have been obtained with all the BTE series, but they would certainly only have been partial agonists.

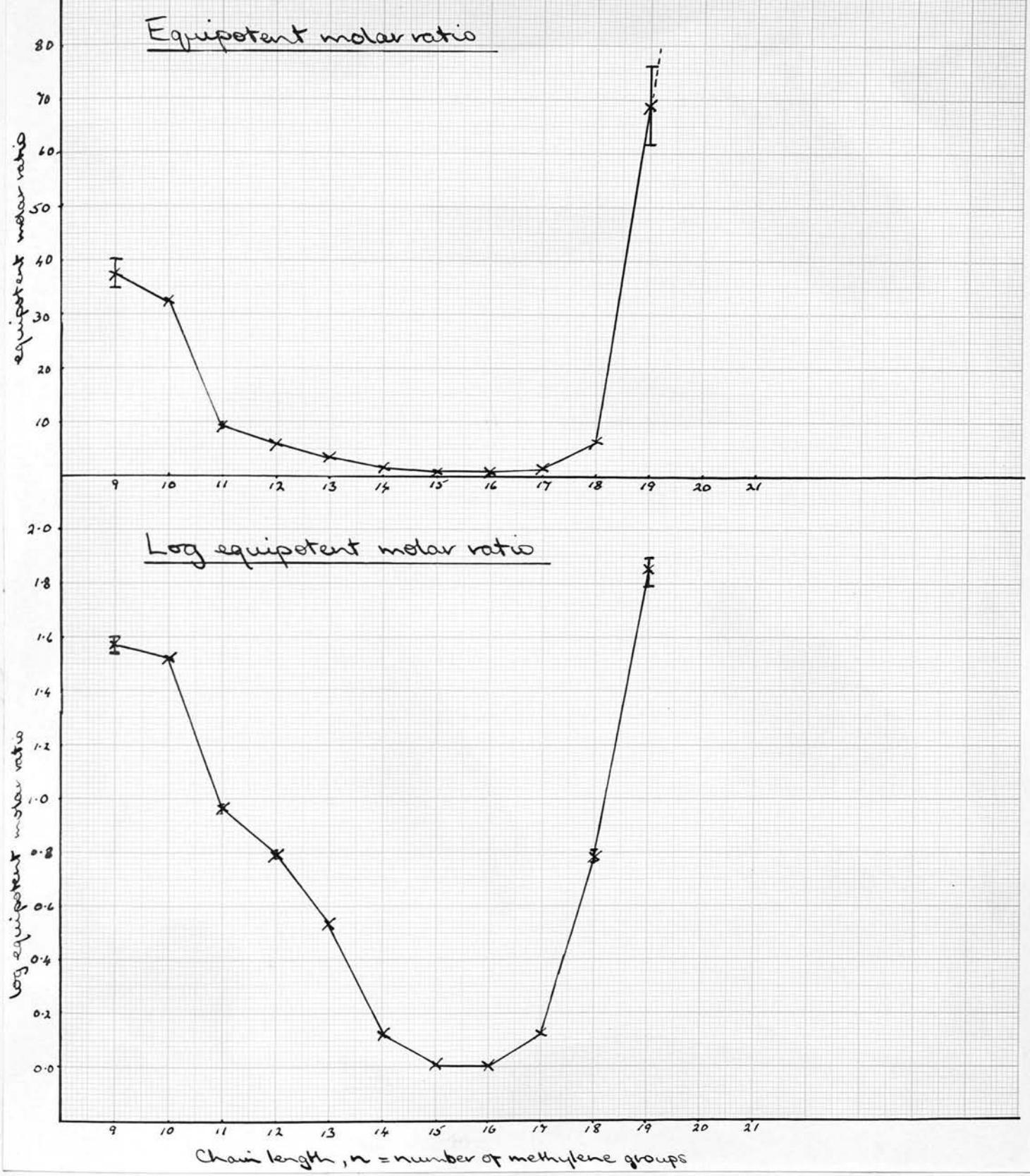
The results are summarised in Table 5 and Graph 11, expressed as equipotent molar ratios compared with BTE 16: the mean is shown with the standard error. Blocking activity increases from BTE 9 to a maximum at BTE 15 - 16, then decreases again, BTE 20 and 21 being almost inactive as blocking agents.

The usual concentration of BTE 16 in the bath fluid required to produce incomplete block was about 5×10^{-7}M.

TABLE 5.- Activity of Compounds on Chick Biventer Preparation: Antagonist Activity.

Compound	EPMR ± SE	log EPMR ± SE	No. of expts.
BTE 9	37 ± 2.6	1.57 ± 0.03	3
BTE 10	33 ± 0	1.52 ± 0	4
BTE 11	9.2 ± 0.4	0.96 ± 0.02	3
BTE 12	6.1 ± 0.3	0.79 ± 0.02	3
BTE 13	3.3 ± 0	0.52 ± 0	3
BTE 14	1.3 ± 0	0.11 ± 0	3
BTE 15	1.0 ± 0	0.00 ± 0	3
BTE 16	1.0	0.00	-
BTE 17	1.3 ± 0	0.11 ± 0	4
BTE 18	6.1 ± 0.4	0.78 ± 0.03	3
BTE 19	69 ± 7.3	1.84 ± 0.05	3
BTE 20	> 300	> 2.5	
BTE 21	> 500	> 2.7	

Equipotent molar ratio (EPMR) ± standard error relative to BTE 16.



Graph 11: Variation of activity with chain length for BTE series: blocking activity on chick bwenter

B. Rat Phrenic Nerve-Diaphragm Preparation

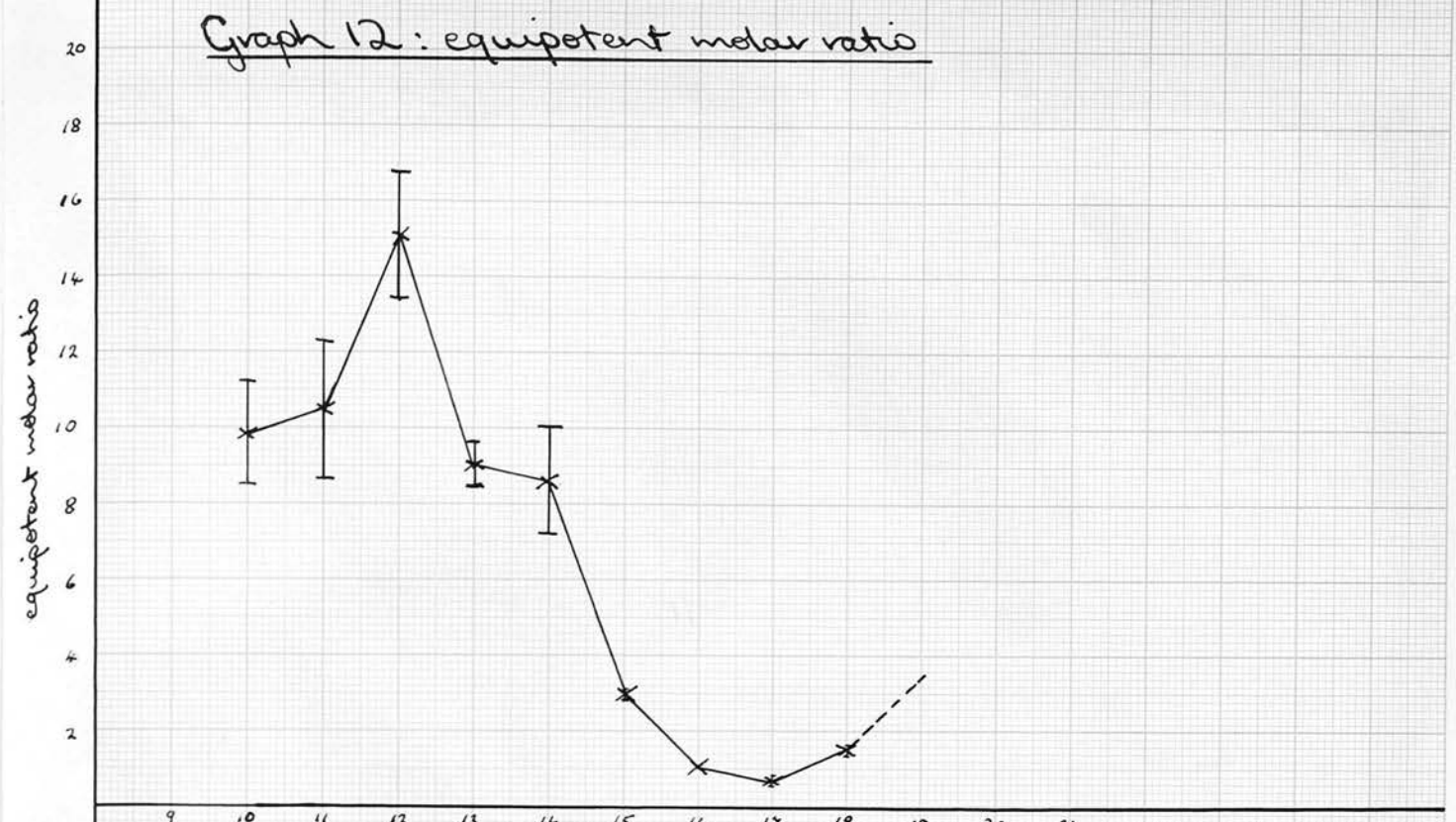
Blocking activity on the rat diaphragm twitch was determined for the BTM series. The results are summarised in Table 6, Graphs 12 and 13. They are expressed as equipotent molar ratios compared with BTM 16: the mean is shown with standard error. These values were obtained from log dose-response curves. Log equipotent molar ratios are also shown.

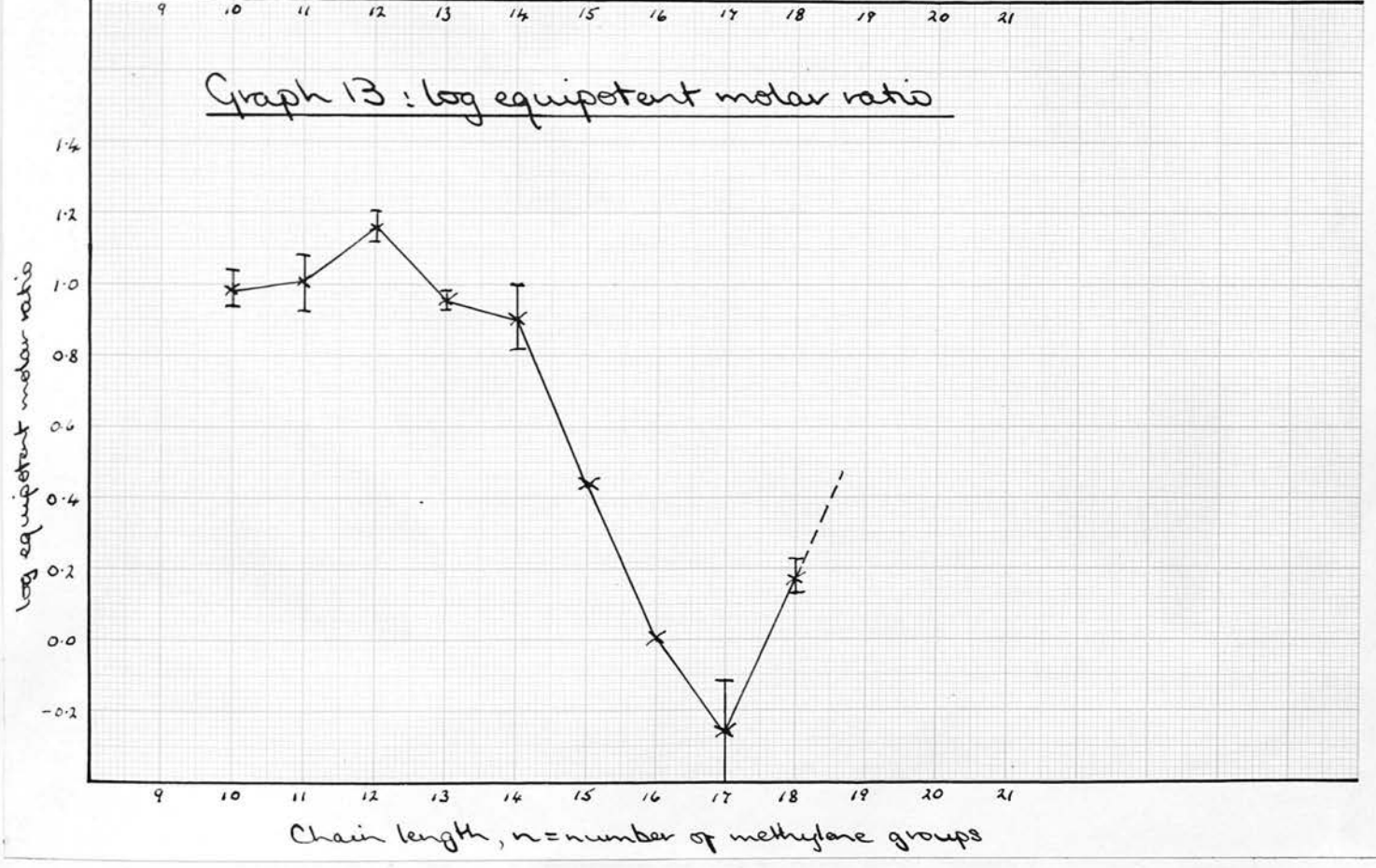
Blocking activity decreases from BTM 10 - 12, then increases again, with a maximum at BTM 17. The rat diaphragm is less sensitive to these compounds than the other test preparations used: the usual concentration of BTM 16 in the bath fluid required to produce partial block was about 1×10^{-5}M. The longer members of the series, BTM 19 - 21, are feeble blocking agents and the high bath concentrations required proved to be toxic to the muscle; they caused an irreversible shortening of the muscle fibres.

TABLE 6 .- Rat phrenic diaphragm preparation: antagonist activity of compounds.

Compound	EPMR ± SE	log EPMR ± SE	No. of expts.
BTM 10	9.8 ± 1.4	0.98 ± 0.06	3
BTM 11	10 ± 1.9	1.00 ± 0.08	3
BTM 12	15 ± 1.8	1.16 ± 0.05	3
BTM 13	8.9 ± 0.6	0.95 ± 0.03	3
BTM 14	8.5 ± 1.5	0.91 ± 0.09	3
BTM 15	2.8 ± 0.1	0.44 ± 0.02	4
BTM 16	1.0	0.00	-
BTM 17	0.6 ± 0.2	-0.27 ± 0.15	3
BTM 18	1.5 ± 0.2	0.17 ± 0.05	3
BTM 19	toxic	-	-
BTM 20	toxic	-	-
BTM 21	toxic	-	-

Equipotent molar ratio (EPMR) ± standard error relative to BTM 16 = 1.0.





Variation of activity with chain length for BTM series: neuromuscular blocking activity on rat diaphragm

C. Cat Anterior Tibialis Preparation

The neuromuscular blocking activity of the compounds on the cat tibialis is summarised in Tables 8, 9 and 10 and Graphs 16, 17 and 18.

In the BTM series, where each member was compared with BTM 16 (Table 8), activity is initially high at BTM 10, declines to BTM 14, then increases again to a second maximum at BTM 18 (though this is lower than that at BTM 10). After BTM 18 activity decreases again. Paton and Zaimis (1949) also found that BTM 11 and 12 were less active than BTM 10, but they found that BTM 18 was less active than BTM 12, contrary to the present result.

In the BTE series, where each member was compared with BTE 16 (Table 9) activity increases from BTE 9 steadily to a maximum at BTE 16 - 17. This confirms Warriner's results (1960). It was considered that one value for each compound would be sufficient if the previous findings were confirmed. This means, however, that standard errors and log values could not be calculated for these compounds.

TABLE 8 .- Cat anterior tibialis preparation: antagonist activity of compounds.

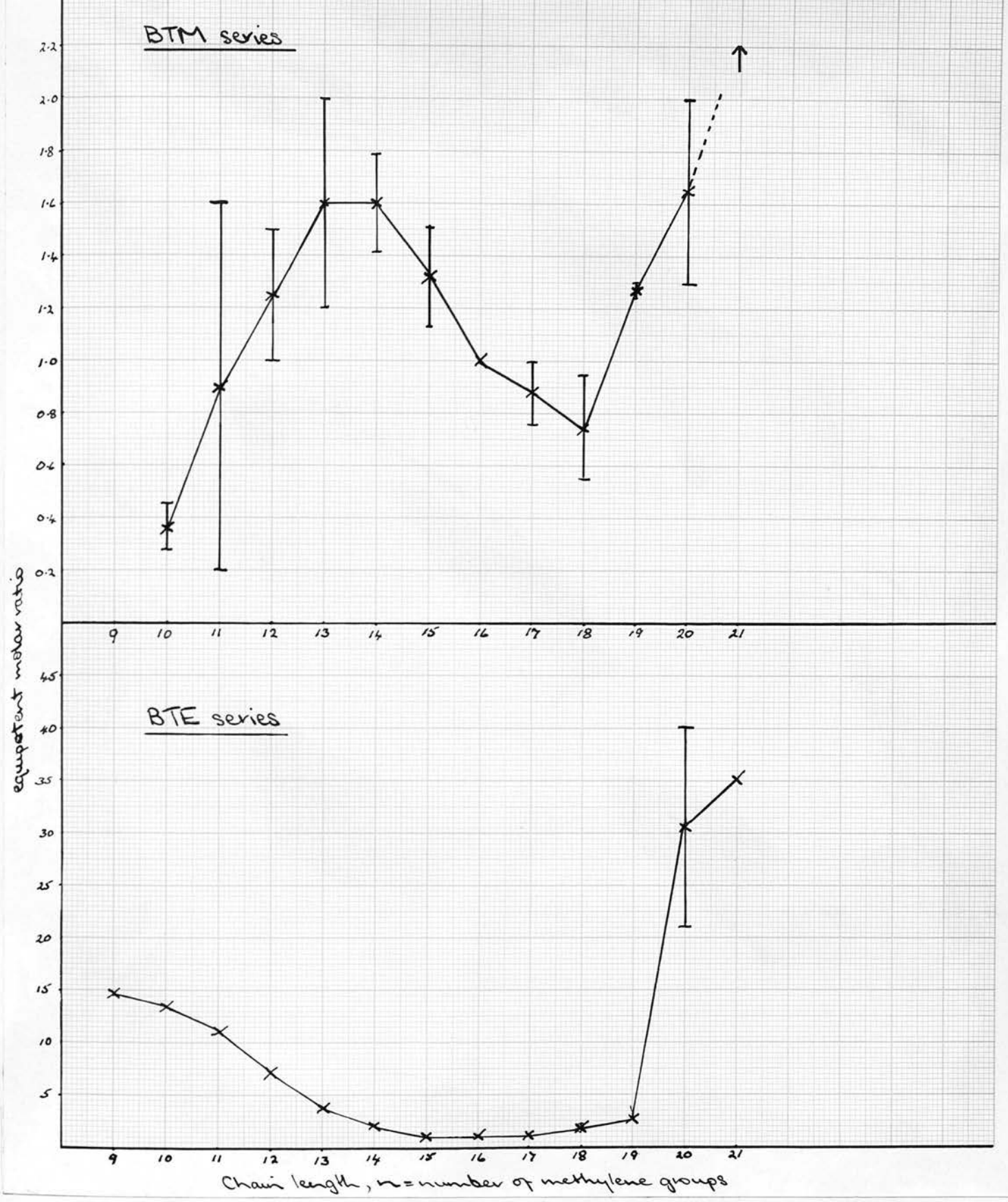
Compound	EPMR ± SE	log EPMR ± SE	No. of expts.
BTM 10	0.4 ± 0.1	-0.47 ± 0.12	3
BTM 11	0.9 ± 0.7	-0.25 ± 0.45	2
BTM 12	1.3 ± 0.3	0.09 ± 0.09	2
BTM 13	1.6 ± 0.4	0.19 ± 0.11	2
BTM 14	1.6 ± 0.2	0.21 ± 0.05	3
BTM 15	1.3 ± 0.2	0.11 ± 0.06	3
BTM 16	1.0	0.00	-
BTM 17	0.9 ± 0.1	-0.06 ±0.06	3
BTM 18	0.8 ± 0.2	-0.15 ± 0.11	3
BTM 19	1.3 ± 0.0	0.10 ± 0.01	2
BTM 20	1.7 ± 0.4	0.21 ± 0.10	2
BTM 21	3.0 ± 0.0	0.48 ± 0.00	2

Equipotent molar ratio (EPMR) ± standard error relative to BTM 16.

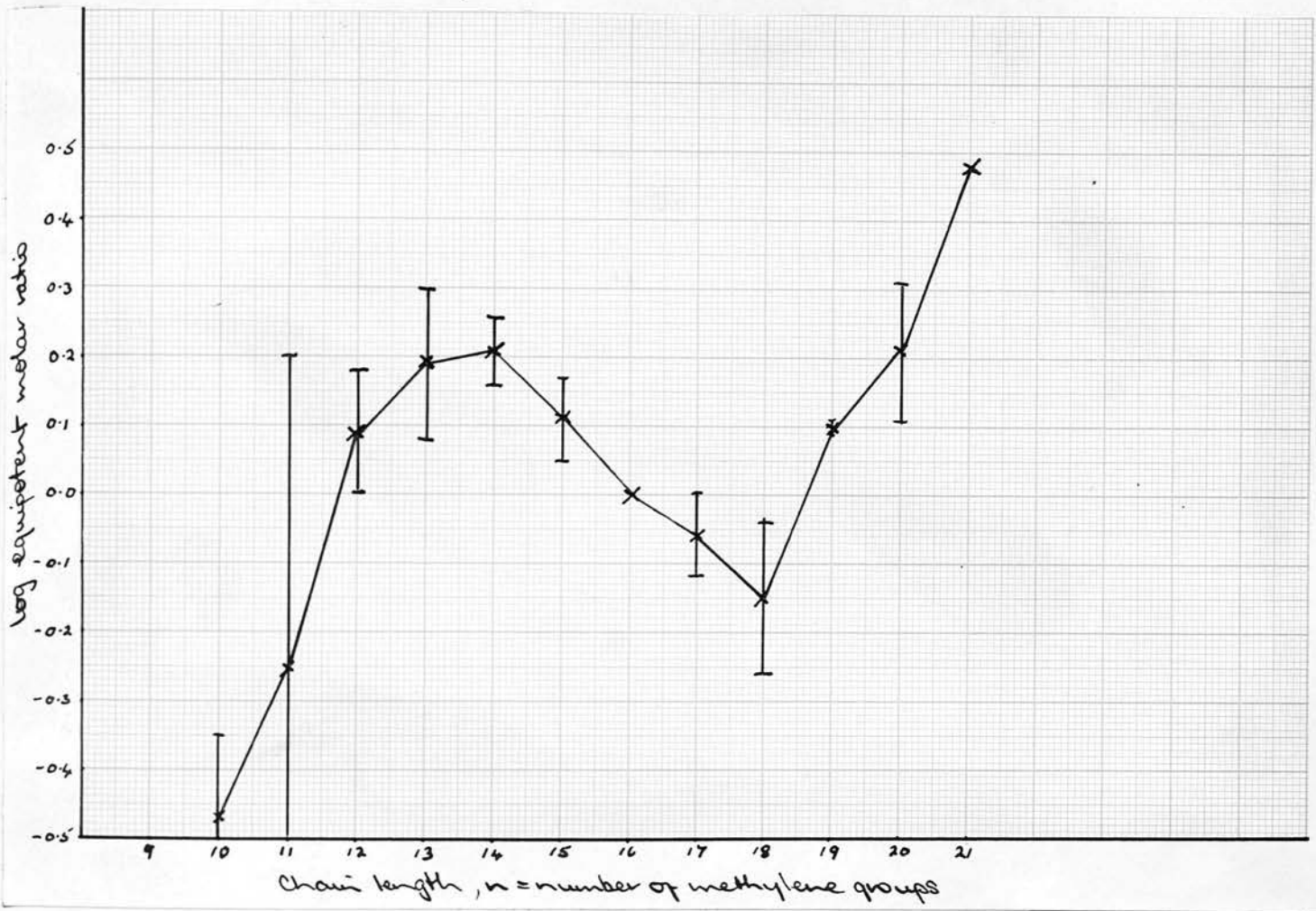
TABLE 9 .- Cat Tibialis: Antagonist activity of compounds.

Compound	EPMR: J.W.	No. of expts.	EPMR: A.H.	No. of expts.	Av. EPMR ± S.E.	log EPMR + S.E.
BTE 9	13	4	20	1	14	
BTE 10	13	2	-	-	13	
BTE 11	10	2	12	1	11	
BTE 12	6.7	2	8.0	1	7.1	
BTE 13	3.0	2	5.0	1	3.7	
BTE 14	1.5	2	2.5	1	1.8	
BTE 15	0.7	3	1.2	2	0.9	
BTE 16	1.0	-	1.0	-	1.0	0.00
BTE 17	1.0	4	1.0	1	1.0	
BTE 18			1.6	2	1.6 ± 0.4	0.19 ± 0.11
BTE 19			2.8	2	2.8 ± 0.3	0.44 ± 0.04
BTE 20			31	2	31 ± 9.5	1.46 ± 0.14
BTE 21			35	1	35	

Equipotent molar ratio (EPMR) ± standard error relative to BTE 16



Graph 16: Variation of activity with chain length: equipotent molar ratios for neuromuscular blocking activity on cat tibialis



Graph 17: Variation of activity with chain length for BTM series: log equipotent molar ratio for neuromuscular blocking activity on cat tibialis

Unfortunately, an anomalous result was obtained for BTE 10, three determinations with different solutions being inconsistent, but all higher than expected. Above BTE 17, activity decreases again, falling sharply at BTE 20.

When the composition of the onium group in the hexadecamethylene compounds is altered, (Table 10: each compound was compared with BTE 16) activity decreases stepwise from BTE 16 to BTM 16, by an overall factor of 5. This means that in fact BTE 16 is slightly more active than BTM 10. It was also about 8 times as active as (+)-tubocurarine.

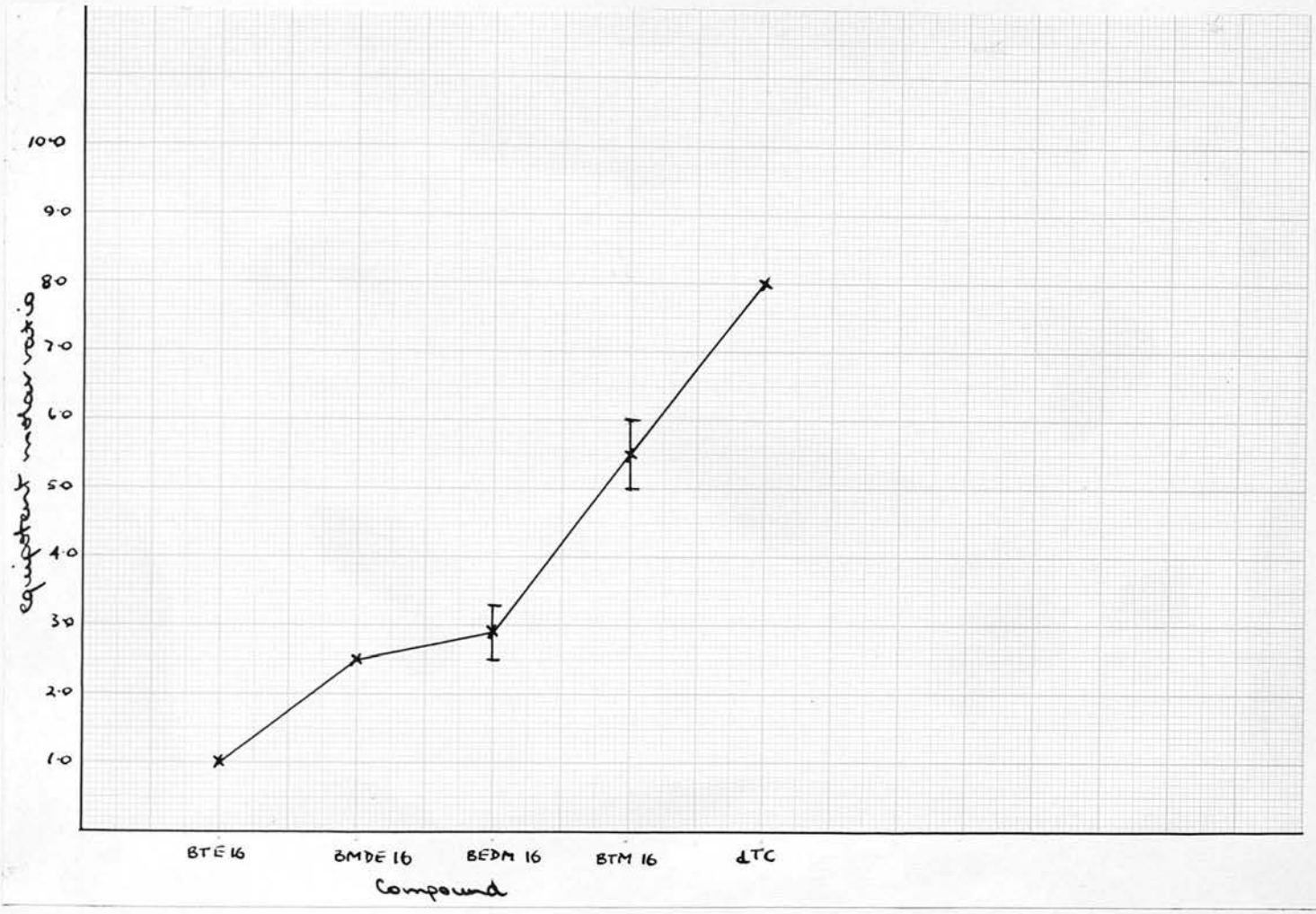
The dose of BTE 16 injected was usually about 0.2 ml. 5×10^{-5}M i.e. 10 nanoMoles, and assuming an approximately 100-fold dilution in the blood as it reaches the ganglion (c.f. Barlow and Hamilton, 1962), this gives a similar concentration (1×10^{-7}M) to that found to block the chick biventer.

With doses sufficient to produce complete block on the tibialis twitch, no effect on the blood-pressure was ever observed.

TABLE 10 .- Cat Tibialis: Antagonist activity of compounds.

Compound	EPMR ± SE	log EPMR ± SE	No. of expts.
BTE 16	1.0	0.00	-
BMDE 16	2.5	0.40	1
BEDM 16	2.9 ± 0.4	0.46 ± 0.06	2
BTM 16	5.5 ± 0.5	0.74 ± 0.04	2
dTC	8.0	0.90	1

Equipotent molar ratio (EPMR) ± standard error relative to BTE 16



Graph 18: Variation of activity for hexadecamethylene compounds with different onium groups and for (+)-tubocurarine: equipotent molar ratio for neuromuscular blocking activity on cat tibialis

3. ACTIVITY AT THE AUTONOMIC GANGLION

Cat Superior Cervical Ganglion Preparation

The ganglion-blocking activity of the compounds on the cat superior cervical ganglion preparation is summarised in Tables 11, 12 and 13 and Graphs 19, 20 and 21).

In the BTM series (Table 11) where each compound is compared with BTM 16, BTM 11 is less active than BTM 10(which is in turn less active than BTM 6, hexamethonium) but activity then increase again to a maximum at BTM 16 - 18. Above this it starts to fall off again, but slowly, BTM 21 being slightly more active than BTM 11 and 12. The range of activity for all the compounds of this series, however, is only ten-fold.

In the BTE series (Table 12: each compound is compared with BTE 16) activity increases from BTE 9 to a maximum at BTE 17. The figures agree quite well with those of Warriner (1960) for BTE 12 - 17. Above BTE 17, activity drops off again, rather more sharply than in the BTM series. The overall range of activity for the BTE series is nearly a hundred-fold.

TABLE 11.- Cat superior cervical ganglion preparation: antagonist activity of compounds

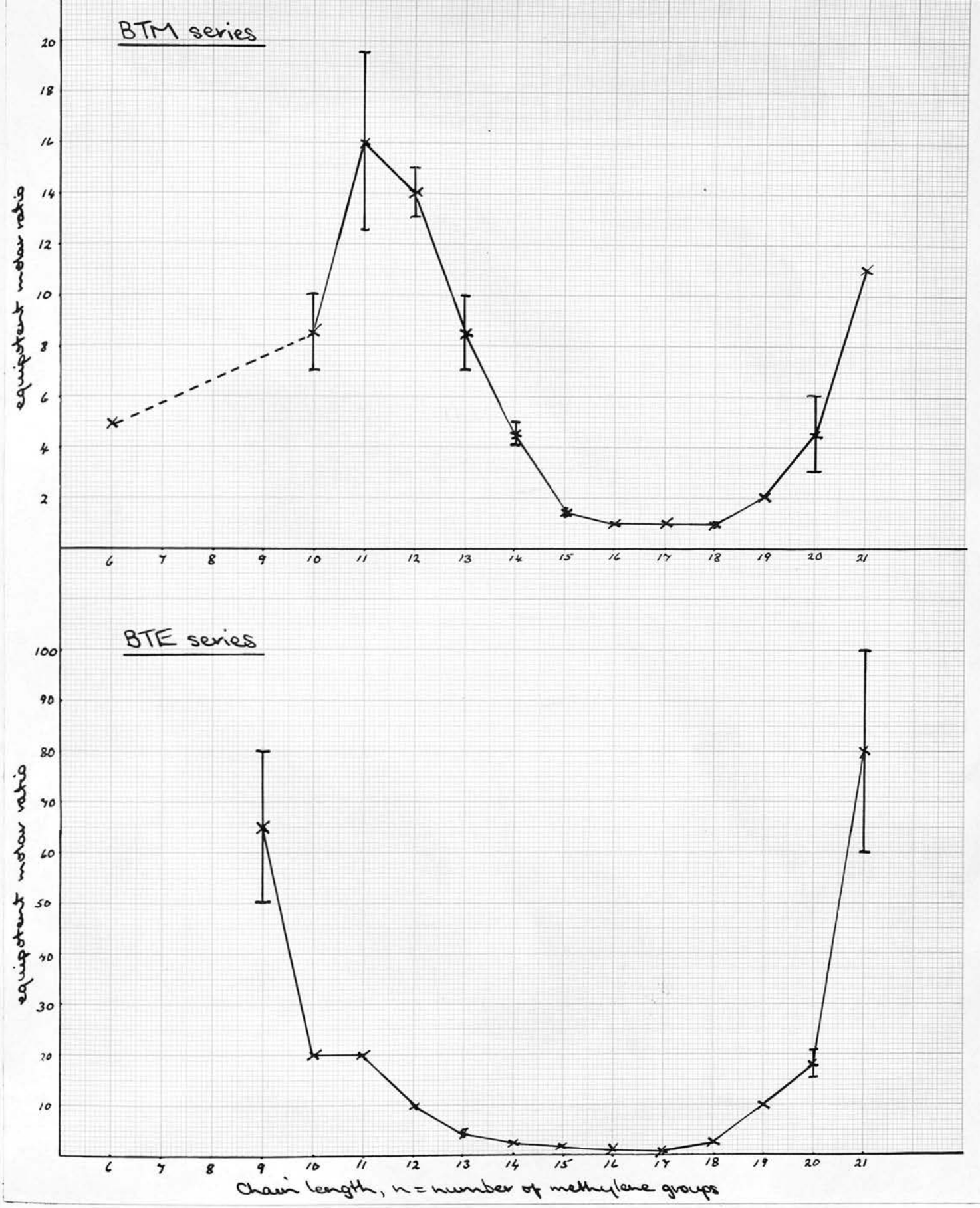
Compound	EPMR ± SE	log EPMR ± SE	No. of expts.
BTM 10	8.5 ± 1.5	0.92 ± 0.08	2
BTM 11	16 ± 3.5	1.21 ± 0.10	2
BTM 12	14 ± 1.0	1.15 ± 0.03	2
BTM 13	8.5 ± 1.5	0.92 ± 0.08	2
BTM 14	4.5 ± 0.5	0.65 ± 0.05	2
BTM 15	1.4 ± 0.1	0.15 ± 0.03	2
BTM 16	1.0	0.00	-
BTM 17	1.0 ± 0	0.00	2
BTM 18	0.9 ± 0.1	-0.05 ± 0.05	2
BTM 19	2.0 ± 0	0.30 ± 0	2
BTM 20	4.5 ± 1.5	0.63 ± 0.15	2
BTM 21	11 ± 0	1.04 ± 0	2

Equipotent molar ratio (EPMR) ± standard error relative to BTM 16.

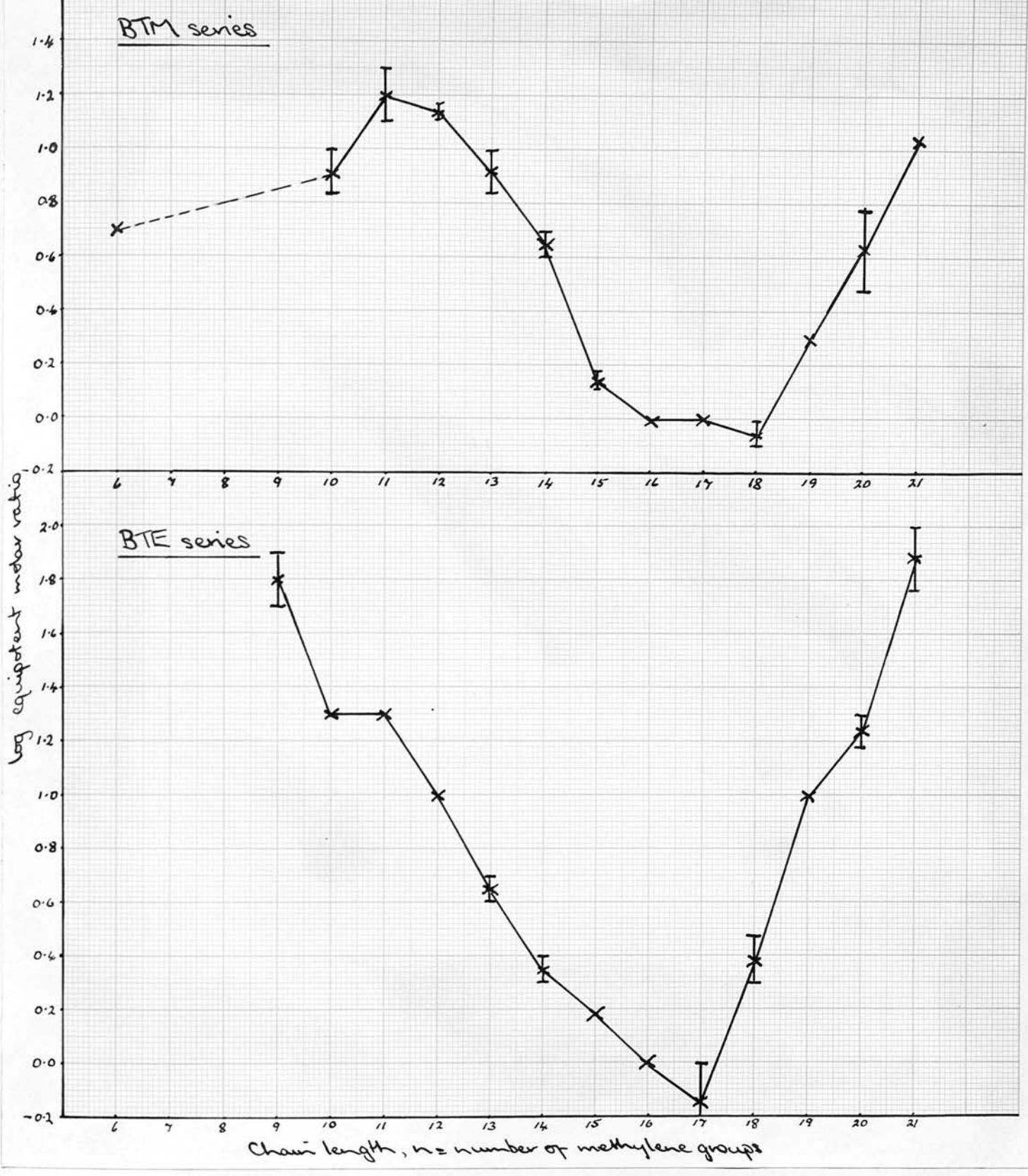
TABLE 12.- Cat superior cervical ganglion preparation: antagonist activity of compounds

Compound	EPMR: J.W.	No. of expts.	EPMR ± SE: A.H.	log EPMR ± SE	No. of expts.
BTE 9			65 ± 15	1.80 ± 0.10	2
BTE 10			20 ± 0	1.30 ± 0	3
BTE 11			20 ± 0	1.30 ± 0	2
BTE 12	7.5	4	10 ± 0	1.00 ± 0	2
BTE 13	4.0	1	4.5 ± 0.5	0.65 ± 0.05	2
BTE 14	2.0	1	2.3 ± 0.3	0.35 ± 0.05	2
BTE 15	1.0	1	1.5 ± 0	0.18 ± 0	2
BTE 16	1.0	-	1.0	0.00	-
BTE 17	1.0	2	0.8 ± 0.3	-0.15 ± 0.15	2
BTE 18			2.5 ± 0.5	0.39 ± 0.09	2
BTE 19			10 ± 0	1.00 ± 0	2
BTE 20			18 ± 2.5	1.24 ± 0.06	2
BTE 21			80 ±20	1.89 ± 0.11	2

Equipotent molar ratio (EPMR) ± standard error relative to BTE 16.



Graph 19: Variation of activity with chain length: equipotent molar ratios for ganglion-blocking activity on cat superior cervical ganglion



Graph 20: Variation in activity with chain length: log equipotent molar ratios for ganglion-blocking activity on cat superior cervical ganglion

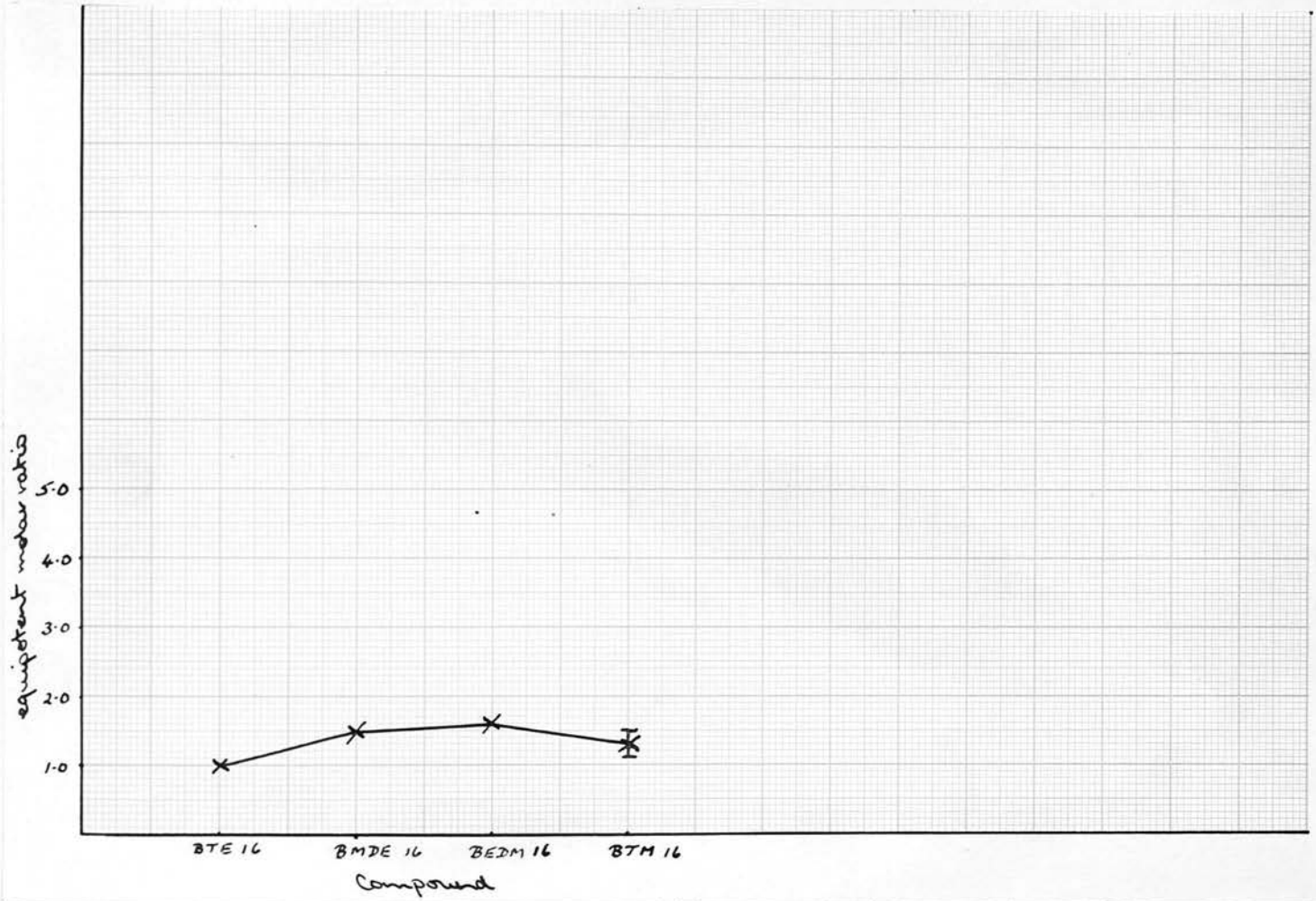
When the composition of the onium group in the hexadecamethylene compounds is altered (Table 13: each compound is compared with BTE 16) there is very little alteration in activity as methyl groups are replaced by ethyl groups. All the C16 compounds are about five times as active as hexamethonium (BTM 6), and their action is more prolonged.

The usual dose of BTE 16 injectedwas about 0.2 ml. 5×10^{-5}M i.e. 10 nanoMoles. Even with dosessufficient to produce complete block of the response of the nictitating membrane, there were usually no effects on the blood-pressure. However on two occasions a rise in blood-pressure was obtained after fairly large doses of BTM 10 - possibly due to adrenaline release from the adrenals. There was no evidence of stimulation of the ganglion by any of the compounds.

TABLE 13.- Cat superior cervical ganglion preparation: Antagonist activity of compounds

Compound	EPMR ± SE	No. of expts.
BTE 16	1.0	-
BMDE 16	1.5	1
BEDM 16	1.6	1
BTM 16	1.3 ± 0.2	2
BTM 6	5.0	1

Equipotent molar ratio (EPMR) ± standard error relative to BTE 16



Graph 21: Variation of activity for hexadecamethylene compounds with different onium groups: equipotent molar ratio for ganglion-blocking activity on cat superior cervical ganglion

DISCUSSION

DISCUSSION

Assessment of the Information Available from the Results

It is necessary to consider how far the results obtained really indicate the true activity of the compounds on junctional transmission and how far they may be affected by other properties. With these chemically inert molecules it is unlikely that there will be any extensive metabolism of the drugs in the experiments. Even differences in rates of elimination of the drugs from the blood, which would complicate the experiments using intact preparations, should not seriously influence the results because both in the cat tibialis and cat superior cervical ganglion experiments the injections were made very close to the site of action of the drug.

In calculating the results it has been assumed that the time course of the effects produced by the drugs is the same for all. If two drugs do not produce effects with comparable time courses it is not possible to compare their activities and express the result as an equipotent molar ratio. It is also only

possible to do this if their log dose-response curves are parallel. With the longer members of the series neither of these criteria appears to hold. There is a gradual increase in the length of action of the compounds as the chain length increases, but this does not become really marked until the chain length is around 18 or 19 methylene groups and the activity is beginning to decline. The same seems likely to be true for the log dose-response curves - on the chick biventer the curves begin to be noticeably flatter than that for BTM 10 at about BTM 15 - 16, but agonist activity is already declining at this chain length; on the rat diaphragm the log dose-response curves of the compounds appear to be more or less parallel until BTM 18 is reached and at this point the antagonist activity starts to decline.

On the cat preparations comparisons were made only at one dose level so it is not possible to say whether the log dose-response curves for the standard and test drugs were parallel or not. However the level of response at which the comparisons were made varied considerably from experiment to another yet the equipotent molar

ratios obtained were reasonably consistent. This suggests that the log dose-response curves must have been at least approximately parallel.

The most serious factor likely to complicate the results is their modification by inhibition of acetylcholinesterase. A full study of the effects of these compounds on acetylcholinesterase has unfortunately not been made but the experiments of Paton and Zaimis (1949) and of Bergmann and Segal (1954) on the trimethyl series indicate that inhibitory activity increases with chain length up to BTM 12, and that BTM 18 is less active than BTM 12. From Bergmann and Segal's results it would appear that $-\log k_i$ for BTM 12 is 6.20 i.e. that a concentration of $10^{-6.20}$ of this compound would produce 50% inhibition of the rate of hydrolysis of acetylcholine when the enzyme is half-saturated. This suggests that anticholinesterase properties might be complicating the results if the concentration of BTM 12 used in the experiments is greater than about 10^{-6}M. Between BTM 12 and BTM 18 the anticholinesterase activity is not known but one experiment with BTM 14 indicated that this

compound is less active than BTM 12. The situation is unsatisfactory (and will be remedied when time permits) but it seems reasonable to suppose that if compounds are active in pharmacological experiments in concentrations less than 10^{-6}M, anticholinesterase activity is not likely to complicate the results. As shown in Table 14 , only the less active compounds are present in concentrations where anticholinesterase activity might be a complicating factor, except on the rat diaphragm preparation. In this experiment, acetylcholinesterase inhibition might well be occurring, and in fact in one experiment potentiation of the twitches by a small dose of BTM 11 was observed.

Compounds of the BTE series were studied by Warriner (1960) who obtained results which indicate that - log k_i for BTE 12 is 5.86 and that the activity does not alter much between BTE 12 and BTE 17. These results are further grounds for assuming that activity in the BTM series does not alter greatly between BTM 12 and BTM 18 (though this is no excuse for not doing the experiments). They also indicate that

TABLE 14.- Composite table: comparison of concentrations of certain compounds active on pharmacological preparations with concentrations showing anticholinesterase activity and micelle formation.

Test object	Molar concentrations of compounds required to produce an effect					
	BTM 12	BTM 16	BTM 21	BTE 12	BTE 16	BTE 21
Chick biventer: (a) contracture (b) block	8×10^{-8}M	1.5×10^{-7}M	-	1×10^{-5}M 3×10^{-6}M	- 5×10^{-7}M	- 5×10^{-4}M
Rat diaphragm: block	1.5×10^{-4}M	1×10^{-5}M	1×10^{-4}M			
*Cat tibialis: block	7×10^{-7}M	5.5×10^{-7}M	1.7×10^{-6}M	7×10^{-7}M	1×10^{-7}M	3.5×10^{-6}M
*Cat superior cervical ganglion: block	1.8×10^{-6}M	1.3×10^{-7}M	1.4×10^{-6}M	1×10^{-6}M	1×10^{-7}M	8×10^{-6}M
Acetylcholin-esterase: 50% inhibition	10^{-6}M	10^{-6}M	10^{-6}M	10^{-6}M	10^{-6}M	10^{-6}M
Critical micelle concentration	-	-	3×10^{-4}M	-	-	4×10^{-3}M

*These figures have been calculated assuming that the dose injected is diluted approximately 100-fold. The results of Barlow and Hamilton (1962) suggest that this is reasonable for the cat superior cervical ganglion and the dilution in the tibialis might be expected to be of the same order, if not greater.

anti-acetylcholinesterase activity is not likely to complicate effects produced by the BTE compounds in concentrations less than 10^{-6}M, and as shown in Table 14, again only the less active compounds are present in concentrations greater than this.

Another factor which may influence the results for the longer compounds is the formation of micelles. This would lead to a discrepancy between the true amount of compound present in solution and the total amount present (both in solution and as micelle). The conductivity experiments indicate that the critical micelle concentration is around 3×10^{-4}M for BTM 21 and $4\text{-}5 \times 10^{-3}$M for BTE 20 and 21. In fact concentrations as high as this were never used in the pharmacological experiments (Table 14).

It seems possible however that the surface activity of some of the compounds on the cells and for receptors may account for certain of their pharmacological properties. For instance in the experiments on the rat diaphragm with the higher members of the BTM series (BTM 19 - 21), effects were only produced by concentrations

which caused an irreversible shortening of the muscle fibres. These compounds were only feeble neuromuscular blocking agents and this toxic action could easily be ascribed to, and is definitely associated with, their surface activity. The same effect was obtained with cetyltrimethylammonium bromide.

In general, then, it seems likely that the results observed are due to the effects of the compounds on the receptors and not to other causes.

Comparison of the Activities of the Compounds

In the results section the activities of the compounds are all expressed relative to a standard chosen from within the particular series being studied. The purpose of this was to try to avoid comparing drugs which might be acting in different ways. It is, however, interesting to compare the activity of all the compounds together.

Values for the agonist activity of the compounds on the chick biventer, all expressed relative to BTM 10, are shown in Table 15. These show quite clearly that agonist activity depends greatly on the presence of a trimethyl-ammonium group and, to a lesser extent, on chain length.

Replacement of even one methyl group in BTM 10 by ethyl in each cationic head reduces activity to about one-third and the replacement of a further methyl group markedly reduces activity. Replacement of one methyl group in each cationic head by hydrogen (as in BDM 10) also markedly reduces activity. Yet nevertheless bistriethyl compounds will cause contracture if the chain length is between 10 and 12 methylene

TABLE 15

Agonist activity : composite table showing equipotent molar ratios of all compounds relative to BTM 10, producing contracture of chick biventer

n	EPMR relative to BTM 10 = 1.0	
	BTM series	BTE series
9		no contr.
10	1.00	350
11	0.71	98
12	0.82	11.0
13	0.58	no contr.
14	0.63	no contr.
15	0.73	no contr.
16	1.55	no contr.
17	~100	no contr.
18	no contr.	no contr.
19	no contr.	no contr.
20	no contr.	no contr.
21	no contr.	no contr.

Compound	EPMR relative to BTM 10=1.0	Compound	EPMR relative to BTM 10=1.0
BTM 10	1.00	BTM 16	1.55
BEDM 10	3.23	BEDM 16	26
BMDE 10	28	BMDE 16	>10,000
BTE 10	350	BTE 16	no contr.
BHDM 10	170	BHDM 16	>10,000
BADM 10	220		
BDM 10	580	BDM	200
		BDE	390

groups. It is interesting that the bis-hydroxyethyldimethylammonium compound, an analogue of choline, is much less active than the ethyldimethylammonium compound; the introduction of the hydroxyl group in fact reduces agonist activity 50-fold or more. What is even more surprising is that the acetoxy compound is less active still.

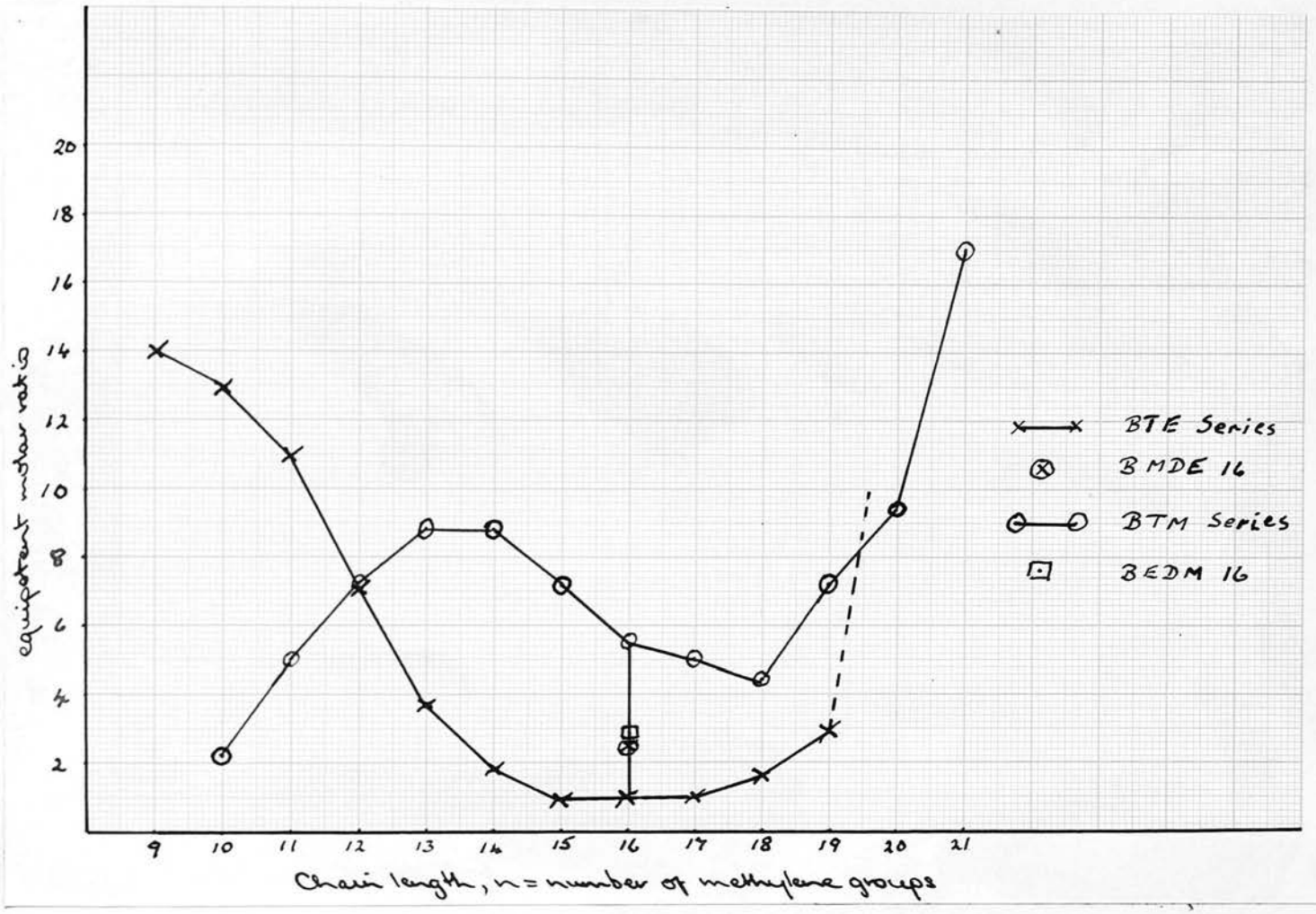
Similar, though more drastic, effects are found when methyl groups in BTM 16 are replaced by ethyl. Replacement of one methyl group reduces activity to about one-twentieth, and further replacement virtually abolishes activity. The bis-hydroxyethyldimethylammonium compound is also virtually inactive - introduction of the hydroxyl group here reduces activity even more than in the decamethylene compounds.

In conclusion then, it seems possible that for compounds of this type to be active they must be long (within limits) and thin molecules.

So far as antagonist activity on the neuromuscular junction is concerned, the results (Table 16A, Graph 22) suggest that the BTM compounds may be acting in a different

TABLE 16 .- Antagonist activity: composite table showing equipotent molar ratios (EPMR) all relative to BTE 16 = 1.0.

n	EPMR relative to BTE 16 = 1.0			
	A: Block on Cat Tibialis		B: Block on Cat Superior Cervical Ganglion	
	BTM series	BTE series	BTM series	BTE series
9		14		65
10	2.2	13	11	20
11	5.0	11	21	20
12	7.2	7.1	18	10
13	8.8	3.7	11	4.5
14	8.8	1.8	5.9	2.3
15	7.2	0.9	1.8	1.5
16	5.5	1.0	1.3	1.0
17	5.0	1.0	1.3	0.8
18	4.4	1.6	1.2	2.5
19	7.2	2.9	2.6	10
20	9.4	31	5.9	18
21	17	35	14	80
BEDM 16 = 2.9; BMDE 16 = 2.5			BEDM 16 = 1.6; BMDE 16 = 1.5	



Graph 22: Composite graphs of variation in activity with chain length for all compounds: equipotent molar ratios for neuromuscular blocking activity on cat tibialis

way from the BTE compounds, particularly those with chain lengths of around 10 - 14 methylene groups. Above this chain length it is unlikely that the BTM compounds are acting like BTM 10. This point is illustrated in Table 17 in which the ability to produce block on either the rat diaphragm or the cat tibialis is compared with the ability to produce contracture on the chick biventer (all expressed relative to BTM 10). If this ratio is greater than one, then the compound is present at the neuromuscular junction in a concentration which should be capable of acting like decamethonium (assuming that the chick biventer gives a reliable measure of decamethonium-like action and that results from it can be transferred to other tissues). Thus if the ratio is greater than one, the compound may be (but need not necessarily be) acting like decamethonium, whereas if the ratio is less than one, the compound is in all probability not acting like decamethonium but rather like the BTE compounds.

If these conclusions are correct and the longer members of the BTM series are not acting like BTM 10, it is in fact more sensible to

TABLE 17A Comparison of blocking activity on the cat tibialis and contracture production on the chick biventer.

n	Block on cat tibialis		Contracture production on chick biventer	EPMR on tibialis
	EPMR rel. to BTM 16 = 1.0	EPMR rel. to BTM 10 = 1.0	EPMR rel. to BTM 10 = 1.0	EPMR on biventer
10	0.4	1.0	1.0	1.0
11	0.9	2.3	0.7	3.3
12	1.3	3.3	0.8	4.1
13	1.6	4.0	0.6	6.6
14	1.6	4.0	0.6	6.6
15	1.3	3.3	0.7	4.7
16	1.0	2.3	1.6	1.4
17	0.9	2.3	> 10	< 1.0
18	0.8	2.0	-	-
19	1.3	3.3	-	-
20	1.7	4.3	-	-
21	3.0	7.5	-	-

TABLE 17B Comparison of blocking activity on the rat diaphragm and contracture production on the chick biventer.

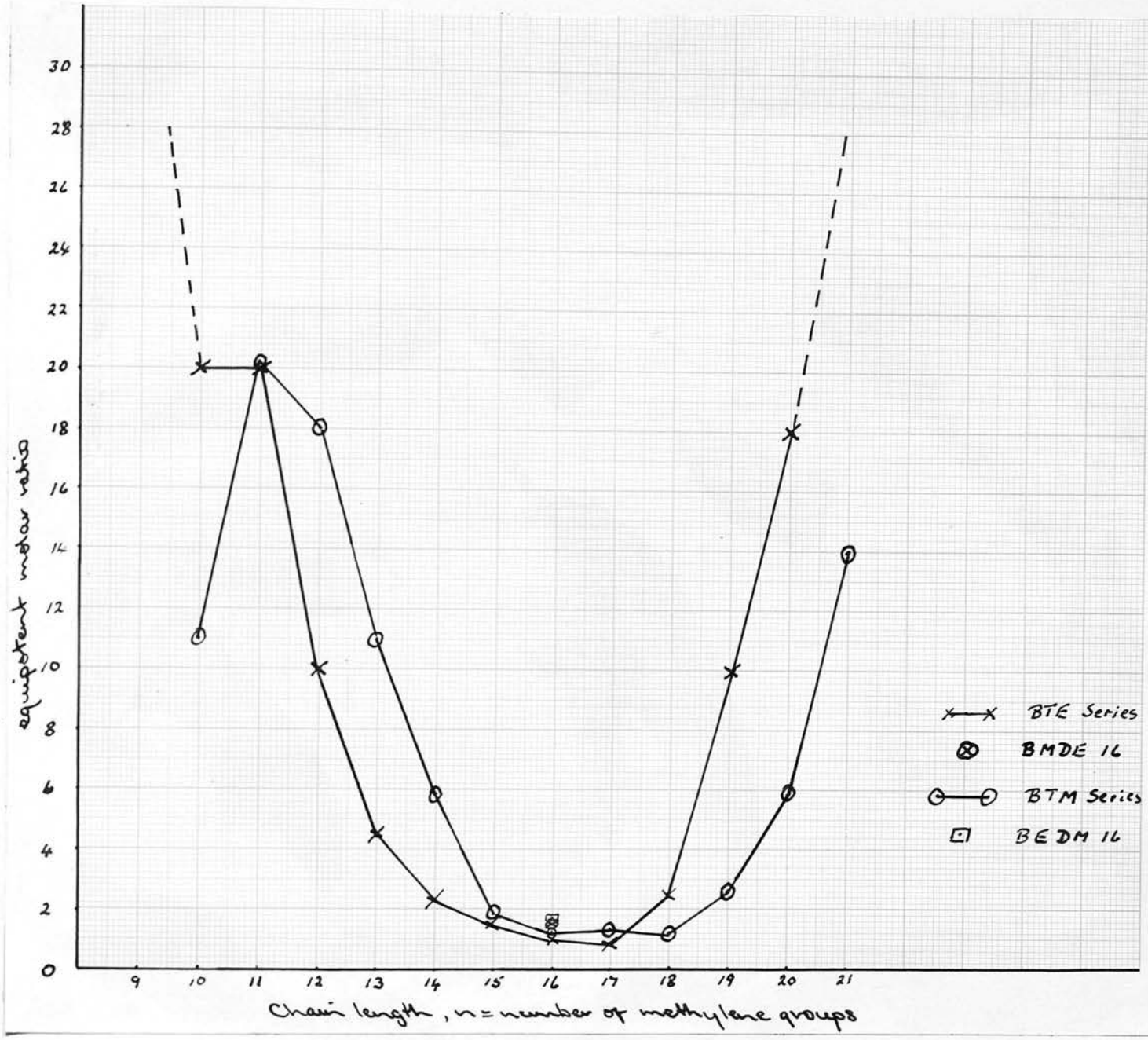
n	Block on rat diaphragm		Contracture production on chick biventer	EPMR on diaphragm / EPMR on biventer
	EPMR rel. to BTM 16 = 1.0	EPMR rel. to BTM 10 = 1.0	EPMR rel. to BTM 10 = 1.0	
10	9.8	1.0	1.0	1.0
11	10	1.0	0.71	1.4
12	15	1.5	0.82	1.9
13	8.9	0.9	0.58	1.5
14	8.5	0.9	0.63	1.5
15	2.8	0.3	0.73	0.4
16	1.0	0.1	1.55	0.6
17	0.6	0.1	> 10	< 1
18	1.5	0.2	-	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-

compare these with a compound such as BTE 16 rather than with a standard within their own series.

The longer BTE compounds i.e. those unlikely to be acting like BTM 10 are more active than the analogous BTM compounds and indeed BTE 16 appears to be about 8 times as active as (+)-tubocurarine and 2 times as active as decamethonium on the cat (if a comparison between these two differently acting drugs has any meaning). It is extremely interesting that activity in the BTM series increases again after declining in the region of BTM 12 - 14. This pattern of activity is not what would be expected from extrapolation of the results of Paton and Zaimis (1949) obtained with BTM 12 and 18, but it is consistent with all the work with the compounds of the BTE series. For the longer compounds there is very little difference in the variation of activity with chain length in the two series, with the exception that the maximum in activity occurs at a slightly shorter chain length in the BTE series (BTE 16 - 17) than in the BTM series (BTM 18).

The correctness of the results obtained in this work is to some extent borne out by the experiments with the compounds in which the methyl groups in BTM 16 are replaced by ethyl groups. The activity increases with increase in the number of groups replaced.

The likelihood that the pattern of activity for the longer members of the BTM series is genuine and that the shorter, decamethonium-like members are the unusual ones is further supported by the results on the ganglion. At this site there is practically no difference in the variation of activity with chain length in the two series (Table 16B, Graph 23) except that again the maximum in activity occurs at a slightly longer chain length in the BTM series (BTM 18) than in the BTE series (BTE 17). Also BTE 16 and BTM 16 are almost equal in activity, and the two intermediate compounds, as ethyl groups are replaced by methyl, have very similar activities too. Probably the slight differences in activity between the four compounds, particularly as the 'mixed' compounds appear to be slightly less active than either BTE 16 or BTM 16, are covered by the limits of experimental error of the method.



Graph 23: Composite graphs of variation in activity with chain length for all compounds: equipotent molar ratios for ganglion-blocking activity on cat superior cervical ganglion

Variation of Activity with Chain Length

The picture that emerges from this work is that of series of compounds which have marked affinity for the acetylcholine receptors in both ganglia and the neuromuscular junction. A few results obtained by Warriner (1960) on the rat ileum and in the present work on the guinea-pig ileum suggest that the longer members of the BTE series at least may also have considerable affinity for muscarinic receptors - atropine-like activity was obtained with BTE 16 - 18 at about 1 x 10^{-7}M, the activity having increased steadily to this level. Paton and Zaimis (1949) did not think that the BTM series possessed any remarkable atropine-like activity, but it may be that the higher members in this series, which they did not test, are also active. The action of these compounds at all these acetyl-choline receptors appears to be purely antagonistic (with the exception of the compounds closely related to BTM 10) and though it might be expected that the antagonism is competitive it has never actually been adequately tested. This is something which should be done. The affinity of the compounds, which leads to their

antagonist activity, seems to be fairly non-specific for all acetylcholine receptors, unlike that of some other compounds which are only active at one site.

In a study of the action of shorter BTM compounds and also of alkyltrimethylammonium salts on acetylcholinesterase, Bergmann and Segal (1954) observed a logarithmic increase in affinity of the alkyltrimethylammonium salts. They suggested that this might be due to a step-wise increase in free energy change on adsorption of the methylene chain on the protein surface, and calculated that the free energy change was about 300 cals. per methylene group. For the BTM compounds, the increase was less regular but was comparable over part of the series. A similar situation appears to be observed with the longer bis-onium compounds at the receptors in the ganglia and the neuromuscular junction. At the ganglion for instance between BTE 11 and BTE 16 and between BTM 12 and BTM 16 the activity increases more or less in a geometrical progression. At the neuromuscular junction the same holds for the compounds from BTE 11 to BTE 15 (Table 16). It might be speculated therefore that the bis-onium compounds combine

with the acetylcholine receptors by virtue of their charged ammonium group, and that the increase in affinity and hence in antagonist activity with increase in chain length is due to the adsorption of each additional methylene group on the receptor protein or adjacent protein surface. Ability to act as an antagonist might depend only on the bulk of the molecule, whereas agonist activity requires a thin molecule whose chain-length is within certain limits.

The reason for the decline in activity above BTE 17 and BTM 18 is not known though it could well be that it is associated with the increase in the surface activity of the compounds. The activity is not likely to be limited by micelle formation but may be limited by some additional surface effect on the cells. It is even possible that the activity may be limited by the sheer bulk of these molecules. After all, the dimensions of the synaptic cleft at the neuromuscular junction appear to be of the order of 300 Å and these longest compounds are between 30 and 35 Å in length when extended!

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