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SOME EFFECTS OF LONG CHAIN POLYMETHYLENE BIS-ONIUM SALTS ON JUNCTIONAL TRANSMISSION IN THE PERIPHERAL NERVOUS SYSTEM

BY

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A survey has been made of the effects on junctional transmission of the complete series of polymethylene bis-trimethylammonium (BTM) and bis-triethylammonium (BTE) salts from the decamethylene compounds (BTM 10 and BTE 10) to those with twenty-one methylene groups in the chain. These were tested for their ability to cause contracture of the isolated chick biventer cervicis preparation, and for their ability to block the twitch responses of this preparation, those of the rat isolated diaphragm preparation, and those of the cat tibialis anterior preparation. They were also tested for their ability to block transmission in the cat superior cervical ganglion, to block the actions of acetylcholine on the guinea-pig isolated ileum, and for ability to inhibit the hydrolysis of acetylcholine by acetylcholinesterase. Their electrical conductivity has been measured in aqueous solution. Ability to cause contracture of the chick biventer cervicis is confined to the compounds BTM 10 to 15; BTE 10, 11 and 12 have some weak activity but the other BTE compounds, and the BTM compounds with more than fifteen methylene groups, have virtually no activity. In the BTE series both neuromuscular blocking and ganglion-blocking activities increase with chain length up to a maximum in the region of BTE 15 to 17 and then decline. In the BTM series ganglion-blocking activity increases with chain length in much the same way as in the BTE series, though the maximum activity is at a slightly longer chain length. At the neuromuscular junction an increase in chain length beyond BTM 10 leads to a decline in activity but this returns to some extent at longer chain lengths, reaching a second maximum at BTM 18, above which it declines further. At the ganglion BTE 16 is only slightly more active than BTM 16 and about five-times as active as hexamethonium; at the neuromuscular junction in the cat BTE 16 is about five-times as active as BTM 16 and about eight-times as active as (+)-tubocurarine. The affinity of the BTE compounds for the postganglionic acetylcholine receptors of the guinea-pig ileum reaches a maximum at BTE 14 but does not decline significantly with further increase in chain length. Anticholinesterase activity, likewise, does not alter significantly between BTM 12 and BTM 21 and the activity of the compounds in the BTE series appears to be similar. This property could conceivably be modifying the actions of some of the intermediate compounds but is not likely to be affecting those of the more active ones. The conductivity experiments indicate that micelle formation could be limiting the actions of the compounds with 20 or 21 methylene groups, but is not likely to be affecting those of the other compounds. The results suggest that there is a regular increase with chain length of the affinity of these compounds for the receptors in the ganglia and at the neuromuscular junction but that efficacy in causing contracture is limited to compounds with three methyl groups in the cationic head and a chain of about ten methylene groups. The connexion between this ability to depolarize and the ability to block transmission by desensitization is discussed.

The actions of polymethylene bis-trimethylammonium salts, $R_3N^+.(CH_2)_n.N^+R_3$ ($R=CH_3$, $n=3$ to 13; referred to as BTM *n*), have been described by Barlow & Ing (1948) Paton & Zaimis (1948, 1949), Castillo, Phillips & De Beer (1949) and Jewell & Zaimis (1954). The analogous bis-triethylammonium salts ($R=C_2H_5$; referred to as BTE *n*) have not been examined so systematically. Chou & Elio (1947) studied the ganglion-blocking activity of BTE 2, 3, 5, 7 and 10, and Barlow & Ing (1948a, b) tested these compounds, and also BTE 4, 8, 9 and 13, for neuromuscular blocking activity on the rat diaphragm preparation. From this incomplete survey it appeared that the relationships between chemical structure and biological activity were not the same in the two series and that BTE 13 was the most active of the BTE compounds which had been examined.

The present paper describes a more complete study of the properties of polymethylene bis-triethylammonium salts. Barlow & Vane, in a communication to the British Pharmacological Society (in January 1956), described some of the properties of BTE 13, 14, 15 and 16. The compounds were tested on the frog rectus, rat diaphragm, cat soleus and cat superior cervical ganglion preparations, and also as antagonists of acetylcholine on the guinea-pig isolated ileum preparation. In all these tests it was found that blocking activity increased with chain length. This was subsequently confirmed by Warriner (1960) who also tested BTE 17 and found that in most of the tests this compound was at least as active as, if not more active than, BTE 16. In order that it should be quite clear where maximal activity is obtained, it has, therefore, been necessary to extend the series to include all the compounds from BTE 10 to BTE 21.

The only compound which had been studied in the analogous BTM series with a chain length greater than thirteen methylene groups was BTM 18; accordingly, because of the findings in the BTE series, it has likewise also become necessary to prepare and examine the full range of BTM compounds up to BTM 21.

In addition, the properties of the compounds hexadecamethylenebis(ethyldimethylammonium bromide) (BEDM 16) and hexadecamethylenebis(methyldiethylammonium bromide) (BMDE 16) have been studied in order to compare the effects of replacing methyl groups in the cationic head of BTM 16 by ethyl groups with the effects of comparable changes in BTM 10 (Ginzel, Klupp & Werner, 1951; Barlow, Roberts & Reid, 1953; Thesleff & Unna, 1954; Ariëns, Simonis & De Groot, 1955) and in BTM 6 (Wien & Mason, 1951; Wien, Mason, Edge & Langston, 1952). Some work has also been done with hexadecamethylenebis(dimethylamine hydrobromide) (BDM 16), hexadecamethylenebis(diethylamine hydrobromide) (BDE 16) and hexadecamethylenebis[(hydroxyethyl)dimethylammonium bromide] (BHDM 16).

The compounds used in this study, and their abbreviations, are set out in Table 1.

The compounds have been tested for ganglion-blocking activity on the superior cervical ganglion of the cat, and for neuromuscular blocking activity on the cat tibialis preparation and on the rat isolated phrenic nerve-diaphragm preparation. They have also been tested for ability to cause contracture of the chick biventer-cervicis preparation and to block the twitch response of this muscle to electrical stimulation of the nerve supply. They have been examined for their ability to antagonize the actions of acetylcholine at the postganglionic acetylcholine receptors

TABLE 1

BTM series $[(CH_3)_3\overset{+}{N}.[CH_2]_n.\overset{+}{N}(CH_3)_3]2Br^-$

Abbreviation	*n*	Chemical name
BTM 11	11	Undecamethylenebis(trimethylammonium bromide)
BTM 12	12	Dodecamethylenebis(trimethylammonium bromide)
BTM 13	13	Tridecamethylenebis(trimethylammonium bromide)
BTM 14	14	Tetradecamethylenebis(trimethylammonium bromide)
BTM 15	15	Pentadecamethylenebis(trimethylammonium bromide)
BTM 16	16	Hexadecamethylenebis(trimethylammonium bromide)
BTM 17	17	Heptadecamethylenebis(trimethylammonium bromide)
BTM 18	18	Octadecamethylenebis(trimethylammonium bromide)
BTM 19	19	Nonadecamethylenebis(trimethylammonium bromide)
BTM 20	20	Eicosamethylenebis(trimethylammonium bromide)
BTM 21	21	Heneicosamethylenebis(trimethylammonium bromide)

BTE series $[(C_2H_5)_3\overset{+}{N}.[CH_2]_n.\overset{+}{N}(C_2H_5)_3]2Br^-$

BTE 10	10	Decamethylenebis(triethylammonium bromide)
BTE 11	11	Undecamethylenebis(triethylammonium bromide)
BTE 12	12	Dodecamethylenebis(triethylammonium bromide)
BTE 13	13	Tridecamethylenebis(triethylammonium bromide)
BTE 14	14	Tetradecamethylenebis(triethylammonium bromide)
BTE 15	15	Pentadecamethylenebis(triethylammonium bromide)
BTE 16	16	Hexadecamethylenebis(triethylammonium bromide)
BTE 17	17	Heptadecamethylenebis(triethylammonium bromide)
BTE 18	18	Octadecamethylenebis(triethylammonium bromide)
BTE 19	19	Nonadecamethylenebis(triethylammonium bromide)
BTE 20	20	Eicosamethylenebis(triethylammonium bromide)
BTE 21	21	Heneicosamethylenebis(triethylammonium bromide)

Hexadecamethylene compounds $[RR'R''\overset{+}{N}.(CH_2)_{16}.\overset{+}{N}RR'R'']2Br^-$

Abbreviation	R	R′	R″	Chemical name
BDM 16	$-H$	$-CH_3$	$-CH_3$	Hexadecamethylenebis(dimethylamine hydrobromide)
BDE 16	$-H$	$-C_2H_5$	$-C_2H_5$	Hexadecamethylenebis(diethylamine hydrobromide)
BEDM 16	$-C_2H_5$	$-CH_3$	$-CH_3$	Hexadecamethylenebis(ethyldimethylammonium bromide)
BMDE 16	$-C_2H_5$	$-C_2H_5$	$-CH_3$	Hexadecamethylenebis(diethylmethylammonium bromide)
BHDM 16	$-CH_2.CH_2OH$	$-CH_3$	$-CH_3$	Hexadecamethylenebis[(2-hydroxyethyl)-dimethylammonium bromide]

in the guinea-pig ileum and for their ability to inhibit the hydrolysis of acetylcholine by acetylcholinesterases. In addition their electrical conductivity in water has been measured in order to obtain information about the possible formation of micelles.

METHODS

Preparations. The chick biventer cervicis preparation was set up as described by Ginsborg & Warriner (1960) and the rat phrenic nerve-diaphragm preparation as described by Bülbring (1946). Both preparations were immersed in Krebs-Henseleit (1932) solution at 37° C and gassed with a mixture of 95% oxygen and 5% carbon dioxide. The bath volume was 50 ml. and the preparation was stimulated, at 12 shocks/min, with short rectangular-wave pulses producing maximal twitches. Doses of drug were added to the preparation by pipette, in a volume not exceeding 0.2 ml., and were allowed to act for 10 min, or until a maximal effect had been clearly seen. The preparation was then washed and allowed to recover for 20 min; the interval between doses was thus 30 min.

The cat anterior tibialis preparation was set up as described by Brown (1938). The peroneal nerve was stimulated, at 12 shocks/min, with short rectangular-wave pulses producing maximal

twitches. The blood pressure was recorded from a carotid artery with a mercury manometer. Injections were made retrogradely into the tibial artery and washed in with 0.9% saline. Subsequent doses were given only when the contractions had returned to normal and usually the interval between doses was at least 30 min. No changes in blood pressure were produced by the compounds when injected in this way in amounts which caused neuromuscular block.

The cat superior cervical ganglion preparation was set up as described by Paton & Perry (1953). The preganglionic sympathetic nerve was stimulated, at 10 shocks/sec, with short rectangular-wave pulses, producing a maximal contracture of the nictitating membrane. Stimulation was maintained for 10 min and then switched off for 20 min. In the earlier experiments the lingual artery and any branches of the external carotid artery were tied, the external carotid artery was then tied peripherally and a remote-control bulldog clip placed centrally, and the artery cannulated between these with a needle cannula attached to a syringe. In later experiments the external carotid artery was tied off and the lingual artery cannulated retrogradely with a needle cannula attached by fine polyethylene tubing to a two-way tap and a syringe. The drug was applied to the preparation either by releasing the bulldog clip or by opening the tap, making the injection from the syringe and washing it in with 0.9% saline and then closing the bulldog or tap. The blood pressure was recorded from a femoral artery with a mercury manometer. Drugs were injected in a volume of about 0.2 ml. and, as with the cat tibialis preparation, the interval between doses was usually at least 30 min. No changes in blood pressure were produced by the compounds when injected in this way in amounts which caused ganglionic block.

Estimation of activity. Because of the hygroscopic nature of the compounds, it was necessary to check the composition of all stock solutions by titrating the ionized bromine present with standard silver nitrate. The discrepancies between the expected values and those found by titration were only small (Table 2) but were sufficient seriously to affect the estimates of conductivity (see below); the results of the biological tests would not markedly be affected by such errors though, in fact, the solutions used in these experiments were always made up from stock solutions which had been checked by titration.

Agonist activity was estimated by comparing the concentrations of the compounds which produced comparable contractures of the chick biventer cervicis. Responses were obtained

TABLE 2

COMPOSITION OF STOCK SOLUTIONS (IN DE-IONIZED WATER)

Note that the concentration expected from the weight of material taken is always greater than the concentration as determined by titration, which is consistent with the idea that the discrepancy is due to hydration of the material during weighing. The difference is greater for members of the BTE series than for those of the BTM series; the former are much more hygroscopic than the latter. In the preparation of the stock solutions of the compounds not included in this table the titration figure alone was taken as indicating the concentration

Compound	Molarity calculated from weight taken ($\times 10^{-3}$M)	Molarity found by titration of Br^- ($\times 10^{-3}$M)
BTM 11	9·79	9·52
BTM 12	12·8	12·6
BTM 13	6·56	6·30
BTM 14	10·7	10·3
BTM 15	8·37	8·16
BTE 11	4·93	4·71
BTE 12	12·7	12·2
BTE 13	9·33	9·07
BTE 14	8·64	8·09
BTE 16	9·19	8·64
BTE 19	7·13	7·00
BTE 20	8·98	8·52

to three or more different doses both of the test drug and of the standard, and the equipotent molar ratio was calculated from the graphs of log dose against response. For the members of the BTM series, BTM 10 was used as standard ; BTE 10 was used for the BTE series. The activity of BTE 10 relative to BTM 10 has already been described (Barlow & Zoller, 1962).

Blocking activity was measured by comparing the concentrations, or doses, of compounds which produced roughly equivalent effects (matching assays only were used because of the large number of compounds involved). The result was expressed as an equipotent molar ratio relative to some standard drug, BTE 16 for the members of the BTE series and BTM 16 for the members of the BTM series. A comparison was then made of BTM 16, BEDM 16, BMDE 16 and BTE 16, and also, on some preparations, of BDM 16, BDE 16 and BHDM 16.

Antagonist activity at the postganglionic acetylcholine receptors of the guinea-pig ileum was estimated by obtaining a range of responses to acetylcholine alone and then responses in the presence of at least two concentrations of antagonist. From the graph of the logarithm of the dose of acetylcholine against the response in the absence of antagonist, and the responses to acetylcholine in the presence of antagonist, the dose ratios for each concentration of antagonist were calculated and hence the affinity constant. Only two estimations were made for each compound as the experiments were only designed to indicate the order of magnitude of the affinity ; the results for the individual compounds, however, do not vary very greatly (see below).

Inhibition of acetylcholinesterases. Purified acetylcholinesterase from ox red cells was obtained from Nutritional Biochemicals Corporation. The effects of the compounds on the hydrolysis of acetylcholine (5.5×10^{-4} M) by this enzyme were studied by standard manometric methods. Two concentrations of each inhibitor were found, one which produced more than 50% inhibition of the control reaction and one which produced less than 50% (the former was always three times the concentration of the latter) ; from the graph of the reciprocal of the rate of the reaction plotted against the concentration of the inhibitor (which should be a straight line if the inhibition is competitive) the concentration which should produce 50% inhibition of the control reaction was calculated. From a study of the effect of substrate concentration on the rate of hydrolysis and the graph of the reciprocal of the rate against

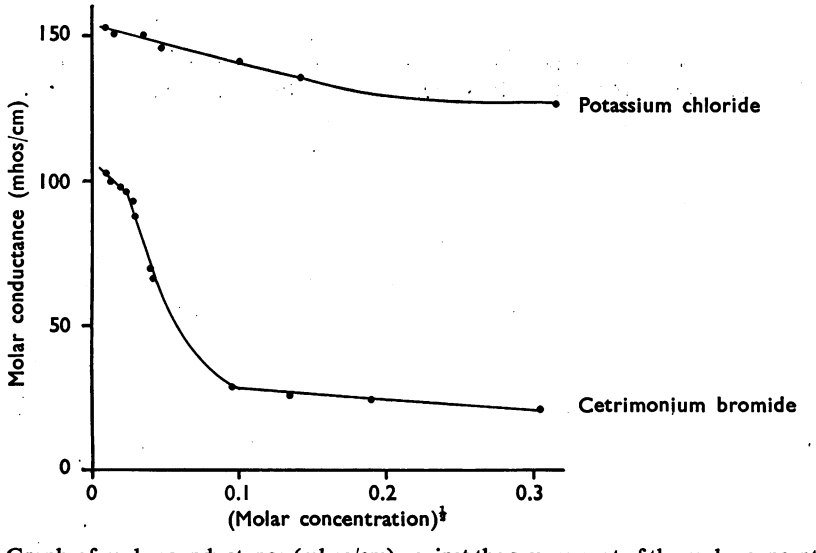

Fig. 1. Graph of molar conductance (mhos/cm) against the square root of the molar concentration: the graph for cetrimonium bromide indicates formation of micelles in concentrations above $(0{\cdot}022)^2$M, that is $4{\cdot}8 \times 10^{-4}$M. The graph for potassium chloride is included for comparison.

the reciprocal of the substrate concentration, the Michaelis constant for this particular enzyme preparation was computed to be 2.6×10^{-4} M. If the inhibition is competitive, the values of the inhibitor constant, K_i, for these compounds can be obtained because $I_{50}/K_i = 1 + S/K_s$ where I_{50} is the concentration of inhibitor causing 50% inhibition of the hydrolysis of a concentration S of the substrate whose Michaelis constant is K_i. In these particular experiments S is approximately twice K_s, so I_{50} should be $3\times K_i$.

Conductivity. Conductivities were measured at $25\pm0.1°$ C with a Philips conductivity bridge (Type PR 9500) and cell (Type GM 4221). Stock solutions of the compounds, standardized by titration of the bromide ion with silver nitrate, were diluted with water from an Elgastat de-ionizer column (whose conductivity was less than 1×10^{-6} mhos/cm), placed in Quickfit test-tubes in the thermostat and stoppered (to exclude carbon dioxide). After 15 min the conductivity cell, which was normally kept in a tube containing de-ionized water, was placed in one of these tubes, left for 2 min and the conductance then read. Two tubes were used for each concentration and the average value of the two conductances (which did not differ greatly) was used in the calculation of the molar conductance. Measurements were made with solutions of 10^{-2}, 10^{-3} and 10^{-4} M and the graph of molar conductance against the square root of the concentration was plotted in order to obtain the limiting conductance. The graph should also indicate whether the compound forms micelles; these will have a lower conductance than the ions and an abrupt change in the shape of the graph should be observed at the concentrations at which micelles are formed (Fig. 1). When there was evidence that a compound was behaving in this way, its conductance was measured at a number of concentrations above and below this "critical micelle-forming concentration."

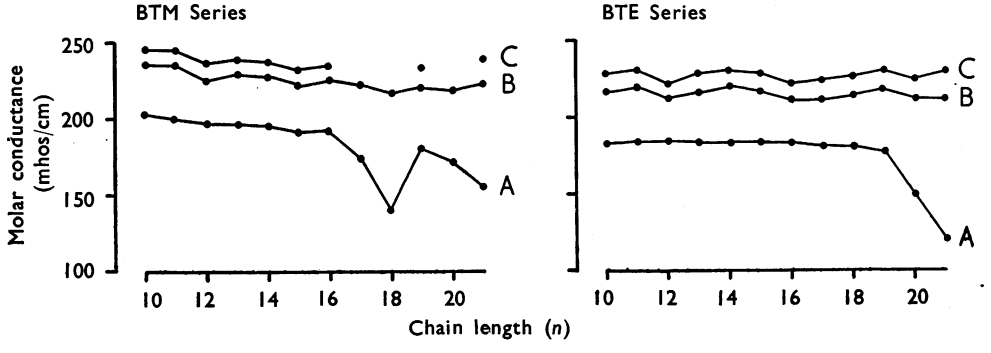

Fig. 2. Graph of molar conductance (mhos/cm) against the chain length in the BTM series (left-hand) and BTE series (right-hand). The concentrations were : A, 10^{-2}M, B, 10^{-3}M and C, 10^{-4}M.

RESULTS

Conductivity Experiments. The molar conductances for the compounds are shown in Table 3 and illustrated graphically in Fig. 2. At concentrations of 10^{-3} M there appears to be virtually no change in the conductance with chain length in either series. This is true for the BTE series at a concentration of 10^{-4} M and probaby also for the BTM series at this concentration, though it is difficult to measure the conductivity of these very dilute solutions accurately. At a concentration of 10^{-2} M there was evidence of micelle formation with BTE 20 and 21, and with BTM 21 (Fig. 3). Abnormally low values were obtained with BTM 17 and 18 but these appeared to be due to the unexpectedly low solubility of these compounds; at 25° C the solutions appeared to be saturated and the conductance measurements

TABLE 3

MOLAR CONDUCTANCES

Figures show the molar conductance in mhos/cm at the concentration indicated

Compound	Molar conductance (mhos/cm) at 10^{-2}M	10^{-3}M	10^{-4}M	Compound	Molar conductance (mhos/cm) at 10^{-2}M	10^{-3}M	10^{-4}M
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
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				

were inaccurate. The reason for the insolubility of these particular compounds, but not of the longer compounds, BTM 19, 20 and 21, is unknown. It is not associated with any significant fluctuation in biological properties nor is it observed in the BTE series.

The conductances of the various hexadecamethylene compounds with different onium groups are not greatly different from each other, though it seems clear that those with the highest values, BTM 16 and BDM 16, are those with the smallest onium groups, as might be expected.

This work showed the need to estimate the concentrations of the compounds by titration. In the first experiments the concentrations were computed from the weight of material taken and a series of unexpected fluctuations in conductance with chain

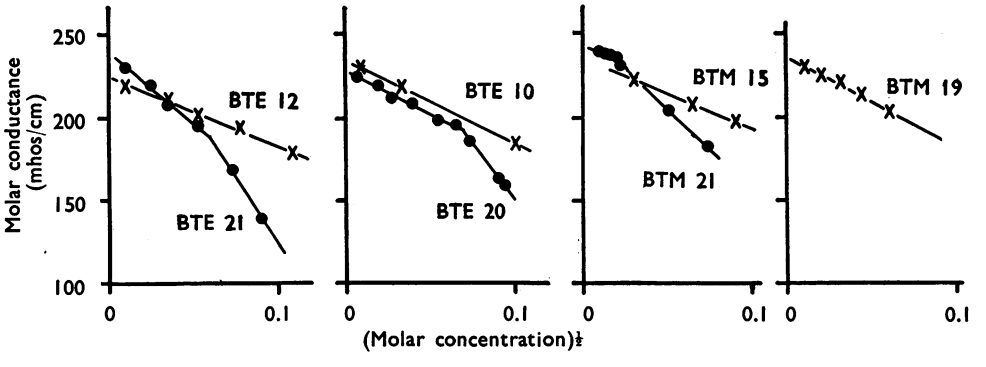

Fig. 3. Graphs of molar conductance (mhos/cm) against the square root of the molar concentration. Note the indication of micelle formation with BTE 20 and 21 and BTM 21 but not with BTM 19 (in concentrations up to 10^{-2}M).

length was found. It also shows that though surface activity increases with the chain length of these compounds, micelle formation is unlikely to limit their biological activity unless they are being used in concentrations greater than about 10^{-3} M.

Ability to cause contracture of the chick biventer-cervicis preparation. The results of these experiments are summarized in Table 4. Activity appears to be

TABLE 4

ABILITY TO CAUSE CONTRACTURE OF THE CHICK BIVENTER CERVICIS PREPARATION

Equipotent molar ratios (± standard error) relative to BTM 10 for compounds of the BTM series, relative to BTE 10 for the BTE series, and relative to BTM 16 for the hexadecamethylene series. The number of experiments is shown in parentheses. The equipotent molar ratio for BTE 10 relative to BTM 10 was 350 ± 95 (7) (Barlow & Zoller, 1962)

BTM Series		*BTE Series*		*Hexadecamethylene compounds*	
Compound	Molar ratio	Compound	Molar ratio	Compound	Molar ratio
BTM 10	1·00	BTE 10	1·00	BTM 16	1·00
BTM 11	0·71 ± 0·04(6)	BTE 11	0·28 ± 0·05(4)	BEDM 16	17 ± 3·3(3)
BTM 12	0·82 ± 0·03(6)	BTE 12	0·32 ± 0·04(4)	BMDE 16	10,000(1)
BTM 13	0·58 ± 0·03(6)	BTE 13	No contracture	BTE 16	No contracture
BTM 14	0·63 ± 0·07(7)	BTE 14	No contracture	BDM 16	130 ± 20(2)
BTM 15	0·73 ± 0·08(6)	BTE 15	No contracture	BHDM 16	10,000(2)
BTM 16	1·55 ± 0·29(6)	BTE 16	No contracture		
BTM 17	100	BTE 17	No contracture		
BTM 18	No contracture	BTE 18	No contracture		
BTM 19	No contracture	BTE 19	No contracture		
BTM 20	No contracture	BTE 20	No contracture		
BTM 21	No contracture	BTE 21	No contracture		

associated with the presence of a trimethylammonium group and a chain length of between ten and sixteen methylene groups. The BTE compounds are only feebly active. As already reported (Barlow & Zoller, 1962), the equipotent molar ratio for BTE 10 relative to BTM 10 on this preparation is at least 350 and the compound is only a partial agonist. Although BTE 11 and 12 were slightly more active, the longer compounds appeared to have no detectable activity at all. In the BTM series it was observed that the slopes of the log dose/response graphs for the longer

TABLE 5

BLOCKING ACTIVITY ON THE TWITCH RESPONSE OF THE CHICK BIVENTER CERVICIS PREPARATION

Equipotent molar ratios (± standard error) are relative to BTE 16. The number of experiments is shown in parentheses

Compound	Molar ratio
BTE 10	33·0 ± 0·1(4)
BTE 11	9·2 ± 0·4(3)
BTE 12	6·1 ± 0·3(3)
BTE 13	3·3 ± 0·1(3)
BTE 14	1·3 ± 0·1(3)
BTE 15	1·0 ± 0·1(3)
BTE 16	1·0
BTE 17	1·3 ± 0·1(4)
BTE 18	6·1 ± 0·4(3)
BTE 19	69 ± 7·3(3)
BTE 20	300
BTE 21	500

chain compounds were less than those for the shorter homologues (BTM 10 has a very steep log dose/response graph indeed). The compounds BTM 18, 19, 20 and 21 were only extremely feebly active and did not produce maximal contractures even when given in very high concentrations.

The replacement of a methyl group in the cationic head of BTM 16 by hydrogen or ethyl greatly reduced activity and the effects of such structural changes on activity are very similar to those observed by Barlow & Zoller (1962) when similar changes are made in BTM 10.

Ability to block the twitch response of the chick biventer-cervicis preparation. The results of these experiments with the members of the BTE series are shown in Table 5. There is a rise in activity with chain length until a maximum is reached at BTE 15, 16 and 17, above which activity declines sharply (Fig. 4). The members

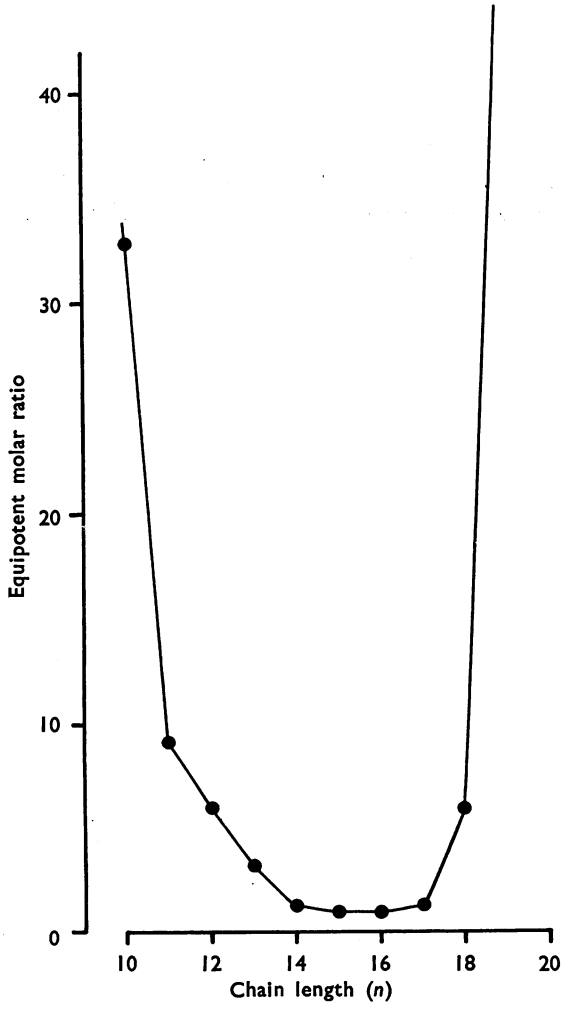

Fig. 4. Ability to block the twitch response of the chick biventer cervicis preparation. Graph of equipotent molar ratio (relative to BTE 16) against chain length (*n*).

TABLE 6

BLOCKING ACTIVITY ON THE RAT DIAPHRAGM PREPARATION

Equipotent molar ratios (± standard error) are relative to BTM 16 and BTE 16 for the BTM and BTE series respectively and relative to BTM 16 for the hexadecamethylene compounds. The number of experiments is shown in parentheses

BTM Series		*BTE Series*		*Hexadecamethylene compounds*	
Compound	Molar ratio	Compound	Molar ratio	Compound	Molar ratio
BTM 10	9·8 ± 1·4(3)	BTE 10	11 ± 1 (2)	BTM 16	1·0
BTM 11	10 ± 1·9(3)	BTE 11	8 ± 1·6(3)	BEDM 16	0·7 ± 0·1(3)
BTM 12	15 ± 1·8(3)	BTE 12	5 ± 2 (2)	BMDE 16	1·0 ± 0·2(3)
BTM 13	8·9 ± 0·6(3)	BTE 13	1·8 ± 0·2(2)	BTE 16	1·0 ± 0·2(5)
BTM 14	8·5 ± 1·5(3)	BTE 14	1·0 ± 0·1(2)		
BTM 15	2·8 ± 0·1(4)	BTE 15	0·7 ± 0·1(3)		
BTM 16	1·0	BTE 16	1·0		
BTM 17	0·6 ± 0·2(3)	BTE 17	1·5 ± 0·5(2)		
BTM 18	1·5 ± 0·2(3)	BTE 18	>3, toxic		
BTM 19	>5, toxic	BTE 19	>3, toxic		
BTM 20	>10, toxic	BTE 20	>5, toxic		
BTM 21	>10, toxic	BTE 21	>5, toxic		

of the BTM series could not be tested for their ability to block the twitch response of this preparation because they caused contracture of the slow fibres.

Ability to block transmission in the rat phrenic nerve-diaphragm preparation. The results of these experiments are summarized in Table 6. Blocking activity in

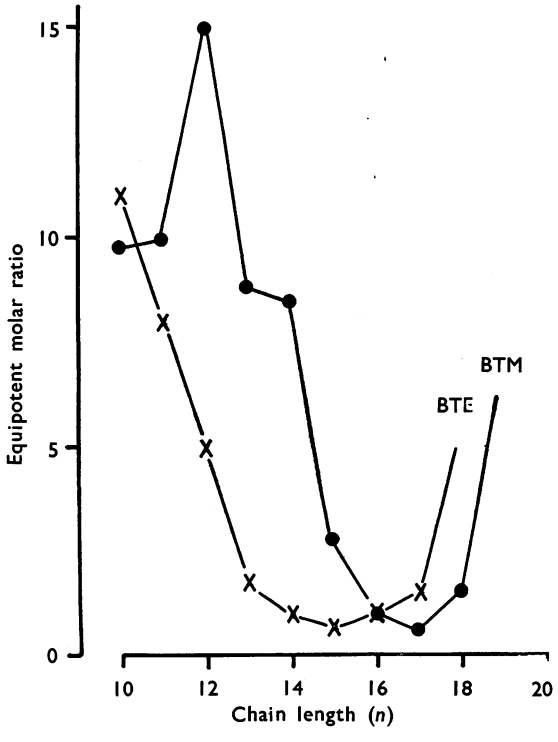

Fig. 5. Neuromuscular blocking activity on the rat diaphragm. Graph of equipotent molar ratios (all relative to BTE 16) against chain length. The ratios for the members of the BTM series were all calculated relative to BTM 16 but this has been found to have the same activity as BTE 16.

both the BTM and BTE series was found to increase with chain length up to a maximum at BTM 17 in the former and about BTE 15 in the latter (Fig. 5). The very long chain compounds, although less active as neuromuscular blocking agents, appeared to have other actions on the preparation which were irreversible. The effects of chain length on activity were remarkably similar in both series and similar also to the results obtained with the BTE series when tested for ability to block the twitch responses of the chick biventer-cervicis preparation.

There was little difference between the blocking activities of BTM 16, BTE 16, and the intermediate compounds, BEDM 16 and BMDE 16. There was also surprisingly little difference between BTM 10 and BTE 10. A much bigger difference was found by Barlow *et al.* (1953) but in the present work the compounds were allowed to act for a much longer period of time. The earlier results may indicate differences in rates of activity rather than in intensity of activity.

Ability to block transmission in the cat tibialis preparation. The results of these experiments are shown in Table 7 and Fig. 6. The determination of the activity of

TABLE 7

BLOCKING ACTIVITY ON THE CAT TIBIALIS PREPARATION

Equipotent molar ratios (± the standard error) are relative to BTM 16 and BTE 16 for the BTM and BTE series respectively, and relative to BTE 16 for the hexadecamethylene compounds. The number of experiments is shown in parentheses

BTM Series		*BTE Series*		*Hexadecamethylene compounds*	
Compound	Molar ratio	Compound	Molar ratio	Compound	Molar ratio
BTM 10	0·4 ± 0·1(3)	BTE 10	13 ± 3 (2)	BTM 16	5·5 ± 0·5(2)
BTM 11	0·9 ± 0·7(2)	BTE 11	9 ± 3 (2)	BEDM 16	2·9 ± 0·4(2)
BTM 12	1·3 ± 0·3(2)	BTE 12	6 ± 2 (2)	BMDE 16	2·5 (1)
BTM 13	1·6 ± 0·4(2)	BTE 13	3·5 ± 1·5(2)	BTE 16	1·0
BTM 14	1·6 ± 0·2(3)	BTE 14	1·9 ± 0·6(2)	(+)-Tubo-curarine	8·0 (1)
BTM 15	1·3 ± 0·2(3)	BTE 15	0·9 ± 0·1(4)		
BTM 16	1·0	BTE 16	1·0		
BTM 17	0·9 ± 0·1(3)	BTE 17	1·0 ± 0·1(4)		
BTM 18	0·8 ± 0·2(3)	BTE 18	1·6 ± 0·4(2)		
BTM 19	1·3 ± 0·0(2)	BTE 19	2·8 ± 0·3(2)		
BTM 20	1·7 ± 0·4(2)	BTE 20	31 ± 9·5(2)		
BTM 21	3·0 ± 0·1(2)	BTE 21	35 (1)		

the very long chain compounds was complicated by their long duration of action and an accurate estimate of the equipotent molar ratio is not really possible. Nevertheless, for the BTE series activity clearly increases up to a maximum at BTE 15, 16 and 17 and then declines. The results for this series are remarkably similar to those obtained on the rat diaphragm preparation and on the twitch responses of the chick biventer-cervicis preparation.

The results for the BTM series are quite different. There is a decline in activity from BTM 10 to BTM 13, then a gradual return in activity up to BTM 18, followed by a further decline. The activity of BTM 18 relative to BTM 10 is similar to that found by Paton & Zaimis (1949) but the variation of the activity with chain length follows a different pattern from what might have been expected.

The very long chain members of the BTE series are more active than their BTM analogues and the replacement of methyl groups in BTM 16 by ethyl groups leads

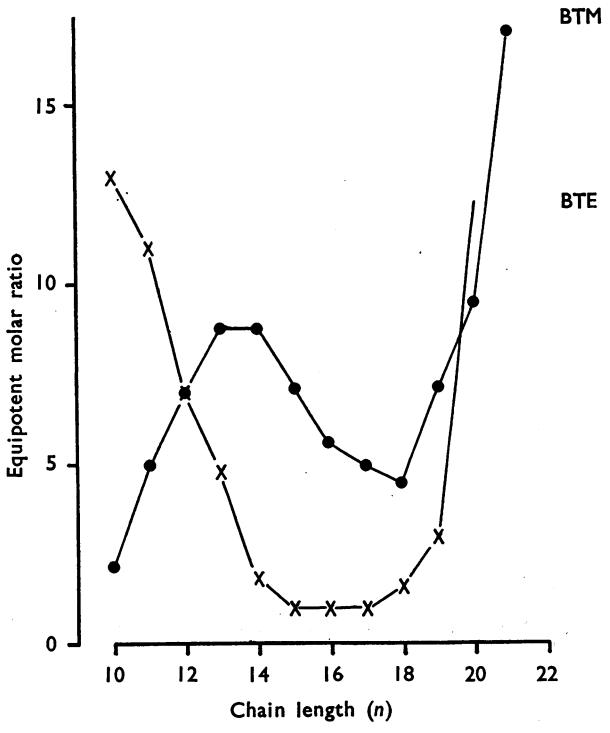

Fig. 6. Neuromuscular blocking activity on the cat tibialis. Graph of equipotent molar ratios (all relative to BTE 16) against chain length (*n*). The ratios for the members of the BTM series were all measured relative to BTM 16 but have been converted to values relative to BTE 16 from the known ratio for BTM 16 relative to BTE 16.

to a fairly regular increase in activity. BTE 16 appears to be considerably more active than (+)-tubocurarine chloride.

Ability to block transmission in the cat superior cervical ganglion preparation. The results of these experiments are shown in Table 8 and Fig. 7. As in the experiments with the cat tibialis preparation, difficulty was experienced in estimating the equipotent molar ratios of the very long chain compounds because of their long duration of action.

The results for the BTE series are clearly similar to those obtained on the other preparations, and activity was maximal in BTE 15, 16 and 17. A rather similar variation of activity with chain length was found in the BTM series on this preparation though the maximum in activity appeared to be at a slightly longer chain length than in the BTE series (this was also found in the experiments on the rat diaphragm).

The compound BTM 16 appeared to be slightly less active than BTE 16 and the intermediate compounds, BEDM 16 and BMDE 16, had similar activity. The compound BTE 16 was considerably more active than hexamethonium (BTM 6). In these experiments BTM 10 appeared to have a rather higher degree of ganglion-blocking activity than might have been expected.

TABLE 8

BLOCKING ACTIVITY ON THE CAT SUPERIOR CERVICAL GANGLION PREPARATION

Equipotent molar ratios (± the standard error) are relative to BTM 16 and BTE 16 for the BTM and BTE series respectively, and relative to BTE 16 for the hexadecamethylene compounds. The number of experiments is shown in parentheses

BTM Series		*BTE Series*		*Hexadecamethylene compounds*		
Compound	Molar ratio	Compound	Molar ratio	Compound	Molar ratio	
BTM 10	8·5 ± 1·5(2)	BTE 10	20 ± 0·1(3)	BTM 16	1·3 ± 0·2(2)	
BTM 11	16 ± 3·5(2)	BTE 11	20 ± 0·1(2)	BEDM 16	1·6	(1)
BTM 12	14 ± 1·0(2)	BTE 12	10 ± 0·1(2)	BMDE 16	1·5	(1)
BTM 13	8·5 ± 1·5(2)	BTE 13	4·5 ± 0·5(2)	BTE 16	1·0	
BTM 14	4·5 ± 0·5(2)	BTE 14	2·3 ± 0·3(2)	BTM 6	5·0	(1)
BTM 15	1·4 ± 0·1(2)	BTE 15	1·5 ± 0·1(2)			
BTM 16	1·0	BTE 16	1·0			
BTM 17	1·0 ± 0·1(2)	BTE 17	0·8 ± 0·3(2)			
BTM 18	0·9 ± 0·1(2)	BTE 18	2·5 ± 0·5(2)			
BTM 19	2·0 ± 0·1(2)	BTE 19	10 ± 0·1(2)			
BTM 20	4·5 ± 1·5(2)	BTE 20	18 ± 2·5(2)			
BTM 21	11 ± 0·1(2)	BTE 21	80 ± 20 (2)			

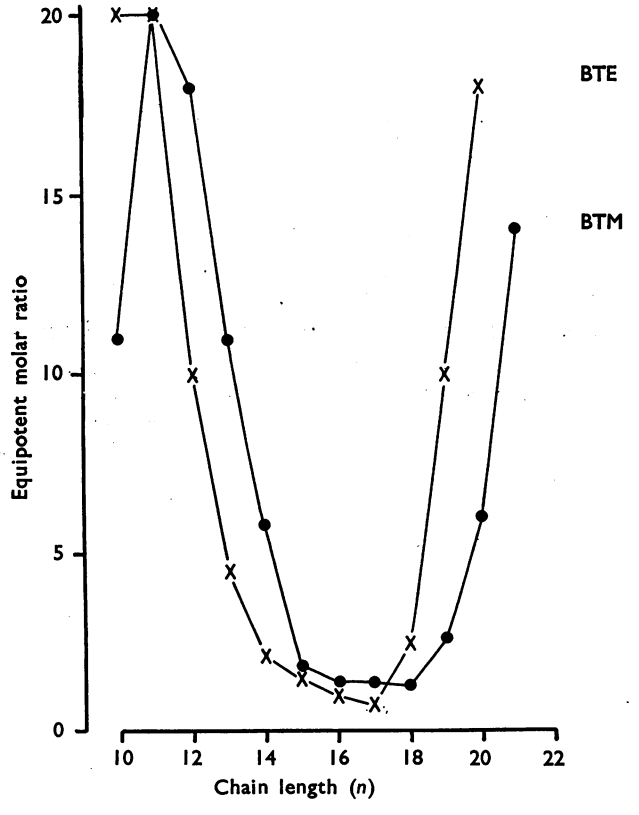

Fig. 7. Blocking activity on the cat superior cervical ganglion preparation. Graph of equipotent molar ratios (all relative to BTE 16) against chain length (*n*). The ratios for the members of the BTM series were all measured relative to BTM 16 but have been converted to values relative to BTE 16 from the known ratio for BTM 16 relative to BTE 16.

TABLE 9

MOLAR AFFINITY CONSTANTS FOR THE POSTGANGLIONIC ACETYLCHOLINE RECEPTORS OF THE GUINEA-PIG ILEUM

Results are the means of two estimates

Compound	Affinity constant (M)
BTE 10	Agonist
BTE 11	10^5
BTE 12	10^6
BTE 13	$1{\cdot}3 \times 10^6$
BTE 14	$1{\cdot}6 \times 10^7$
BTE 15	$2{\cdot}5 \times 10^7$
BTE 16	$5{\cdot}0 \times 10^7$
BTE 17	$4{\cdot}0 \times 10^7$
BTE 18	$4{\cdot}0 \times 10^7$
BTE 19	$2{\cdot}5 \times 10^7$
BTE 20	$3{\cdot}0 \times 10^7$
BTE 21	$2{\cdot}5 \times 10^7$

Ability to block the postganglionic acetylcholine receptors of the guinea-pig ileum. The results of these experiments with the BTE series are shown in Table 9. Above BTE 14 there appears to be remarkably little variation in activity with chain length.

Ability to inhibit the hydrolysis of acetylcholine by acetylcholinesterase. The results of these experiments are shown in Table 10. Although Paton & Zaimis (1949) and Bergmann & Segal (1954) found an increase in anticholinesterase activity with chain length in the BTM series up to BTM 12 (the longest chain compound

TABLE 10

CONCENTRATION OF BTE COMPOUNDS PRODUCING 50% INHIBITION OF THE HYDROLYSIS OF ACETYLCHOLINE ($5{\cdot}5 \times 10^{-4}$M) BY THE ACETYLCHOLINESTERASES OF OX RED CELLS

The mean value is shown (± the standard error) with the number of experiments in parentheses

Compound	Concentration ($\times 10^{-6}$M)
BTM 12	$2{\cdot}9 \pm 0{\cdot}3$(6)
BTM 13	$2{\cdot}6 \pm 0{\cdot}3$(3)
BTM 14	$2{\cdot}7 \pm 0{\cdot}4$(3)
BTM 15	$2{\cdot}8 \pm 0{\cdot}2$(3)
BTM 16	$3{\cdot}3 \pm 0{\cdot}3$(5)
BTM 17	$4{\cdot}0 \pm 1{\cdot}1$(2)
BTM 18	$5{\cdot}0 \pm 0{\cdot}9$(4)
BTM 19	$3{\cdot}8 \pm 0{\cdot}6$(2)
BTM 20	3·5 (1)
BTM 21	$2{\cdot}5 \pm 0{\cdot}7$(3)

tested), it appears that beyond this point the affinity for the enzyme does not alter appreciably. Similar results were obtained with the members of the BTE series by Warriner (1960), using the acetylcholinesterase of dog caudate nucleus and 6×10^{-3} M-acetylcholine as substrate. The reaction was inhibited by from 50 to 65% by BTE 10 to 17 (inclusive) in concentrations of 3×10^{-5} M.

In a few experiments with BTE 12 and BTE 18 it was clear that they did not differ much from the BTM compounds in their ability to inhibit the hydrolysis of 5.5×10^{-4} M-acetylcholine by the acetylcholinesterase of ox red cells.

DISCUSSION

These results seem to show that, at the neuromuscular junction and at the ganglion, the chief difference between the properties of the members of the BTM series and those of the BTE series is that the BTE compounds are essentially antagonists, whereas many members of the BTM series are agonists, even though the result of this agonist activity may be a depolarization or desensitization and consequent block of transmission.

The fairly regular increase in both neuromuscular blocking and ganglion-blocking activity up the BTE series must indicate that affinity increases with chain length. For the active centres of acetylcholinesterase, such an increase in the affinity of the members of the BTM series (up to BTM 12) was observed by Bergmann & Segal (1954). The increase was even more regular in the series of simple alkyltrimethylammonium salts, where it indicated an increase in the free energy of adsorption of roughly 300 cal/mole/methylene group; the changes in the affinities of the BTM compounds appeared to indicate free energy changes of the same order. The greater variation in the rate of change of affinity with chain length in the bis-onium salts could be ascribed to the variation in the contribution to adsorbability of the second onium group, which should depend upon the ease with which it can interact with charged groups on the receptor ("anchoring sites"; Gill & Ing, 1958). Barlow & Himms (1955) observed a similar regular increase in the affinity of polymethylene bisquinolinium salts for the acetylcholinesterase of dog caudate nucleus.

The increase with chain length of the affinity of the members of the BTE series for the receptors in the neuromuscular junction and ganglion, for the postganglionic acetylcholine receptors in the guinea-pig ileum, and for the active centres of acetylcholinesterase, however, appears to have a limit. For the neuromuscular junction and ganglion, an increase in chain length above seventeen or eighteen methylene groups leads to a decline in activity, but for the receptors in the guinea-pig ileum, and for the active centres of acetylcholinesterase, there is no real evidence for a decline in activity even with twenty-one methylene groups in the chain. For the receptors in the ileum the affinity remains more or less constant from BTE 14 to BTE 21 and for the enzyme it is more or less constant from BTE 12 to BTE 21.

The simplest explanation for this limit in affinity is that there is nothing further which is accessible for the drug to become attached to. The decline in activity with the very long chain compounds at the neuromuscular junction and at the ganglion is more difficult to explain. It seems most unlikely that it is due to micelle formation, except possibly in the experiments on the rat diaphragm. This can be seen from Table 11 which shows the concentrations of the compounds used in the various tests. The concentration of BTM 21 which was needed in these experiments, for instance, was about 10^{-4} M, and the critical micelle-forming concentration was 3×10^{-4} M. Certainly the "toxic" actions of these long chain compounds on this preparation could be due to their physiochemical properties. The BTE compounds, for example, were "toxic" on the rat diaphragm but not on the chick biventer preparation, on which they were tested in much lower concentrations. Possibly the series should be extended still further to see if the affinity for the postganglionic acetylcholine receptors of the guinea-pig ileum and for the active centres of acetyl-

TABLE 11

COMPARISON OF MOLAR CONCENTRATIONS USED IN THE VARIOUS EXPERIMENTS

The figures are only approximate and indicate the concentrations likely to produce some measurable response. The asterisk indicates that the concentration has been calculated on the assumption that the dose is diluted 100-fold (Barlow & Hamilton 1962). The figures for the inhibition of cholinesterase by the BTM compounds refer to the enzyme from ox red cell and a substrate concentration of $5{\cdot}5 \times 10^{-4}$M-acetylcholine. The figures for the BTE compounds, marked with a query, seem to be likely from Warriner's (1960) experiments with the enzyme from dog's caudate nucleus and a substrate concentration of 6×10^{-3}M-acetylcholine and from a few experiments with BTE 12, the enzyme from ox red cell, and $5{\cdot}5 \times 10^{-4}$M-acetylcholine

	Compound					
	BTM 12	BTM 16	BTM 21	BTE 12	BTE 16	BTE 21
Contracture of chick biventer	8×10^{-8}	1×10^{-7}	—	1×10^{-5}	—	—
Block of chick biventer	—	—	—	3×10^{-6}	5×10^{-7}	5×10^{-4}
Block of rat diaphragm	1×10^{-4}	1×10^{-5}	1×10^{-4}	5×10^{-5}	1×10^{-5}	5×10^{-5}
Block of cat tibialis*	7×10^{-7}	5×10^{-7}	2×10^{-6}	7×10^{-7}	1×10^{-7}	3×10^{-6}
Block of cat superior cervical ganglion*	2×10^{-6}	1×10^{-7}	1×10^{-6}	1×10^{-6}	1×10^{-7}	8×10^{-6}
50% inhibition of acetylcholinesterase	3×10^{-6}	3×10^{-6}	3×10^{-6}	3×10^{-6}(?)	3×10^{-6}(?)	3×10^{-6}(?)
Critical micelle-forming concentration	—	—	3×10^{-4}	—	—	4×10^{-3}

cholinesterase declines at still longer chain lengths. It seems reasonable to expect that the surface activity of these compounds should ultimately limit their action, even if they do not produce toxic effects; possibly the drugs become strongly adsorbed at a variety of other sites besides the receptors themselves.

The figures in Table 11 indicate that some of the results may be complicated by the anticholinesterase activity of the compounds, most probably those with between twelve and fifteen methylene groups, in which anticholinesterase activity has reached a maximum but neuromuscular blocking activity and ganglion-blocking activity have not. This would seem to be quite likely in the experiments on the rat diaphragm; it seems much less likely to affect those on the chick biventer. On the cat tibialis and superior cervical ganglion preparations it is difficult to estimate the concentration of the drug at the receptor and the values in Table 11 have been calculated on the assumption that the doses administered are diluted 100-fold (compare with Barlow & Hamilton, 1962). This may lead to an underestimate of the concentration but, nevertheless, it seems unlikely that the neuromuscular blocking and ganglion-blocking properties of the most active compounds, those with about sixteen methylene groups, are markedly affected by the actions of the compounds themselves on acetylcholinesterase.

The results on the chick biventer show that, for these bis-onium salts, ability to cause contracture, that is to depolarize, is associated with a chain length of from ten to fifteen methylene groups and a cationic head containing three methyl groups

(the importance of the substitution in the cationic head has already been discussed by Barlow & Zoller, 1962). These results are quite consistent with the findings of Paton & Zaimis (1949), who observed that ability to cause contracture of the frog rectus preparation increased from BTM 10 to BTM 12 (the longest compound studied). They should also be compared with the results of Bovet, Bovet-Nitti, Guarino, Longo & Marotta (1949), who observed that the activity on the frog rectus of dibasic esters of choline increased with chain length and was greatest in the sebacic ester, $Me_3N^+CH_2CH_2O.OC.(CH_2)_8.CO.OCH_2CH_2.N^+.Me_3$, the longest compound tested. This substance could be compared with BTM 16, although the two ester groups are not strictly comparable geometrically with four methylene groups.

The increase in agonist activity, at the receptors in the chick biventer responsible for contracture, from BTM 10 to BTM 13 could be ascribed either to increased efficacy or to increased affinity. Unfortunately the ability of the BTE compounds to block the contracture was not measured but, by analogy with the activity of the BTE compounds at the receptors involved in the twitch response and at the receptors in the other preparations studied, it would seem likely that the affinity of the BTM compounds for the receptors in the neuromuscular junction (and in the ganglion) should increase with chain length. It is conceivable, therefore, that the efficacy does not increase between BTM 10 and BTM 13, as only a twofold increase in affinity would suffice to account for the change in activity. With chain lengths greater than thirteen methylene groups efficacy should decline considerably, because activity is declining but affinity should be increasing. It follows, therefore, that efficacy, in the process which leads to depolarization and contracture, depends upon the presence of a chain length of about ten methylene groups and a cationic head containing three methyl groups.

It is not clear how far ability to cause contracture is related to ability to produce block by desensitization. It is difficult to explain desensitization on the simple hypothesis of the interaction of drugs with one species of receptor, and Katz & Thesleff (1957) have considered other hypotheses, of which the most general can be represented:

$$\begin{array}{lll} & \overset{K_a}{} & \\ \text{Drug (S)} + \text{Receptor (type A)} & \rightleftharpoons & \text{Complex SA} \rightarrow \ldots . \text{response} \\ \quad \Updownarrow & \overset{K_b}{} & \Updownarrow \\ \text{Drug (S)} + \text{Receptor (type B)} & \rightleftharpoons & \text{Complex SB} \end{array}$$

The effects produced by a compound will depend not only upon its affinity constant, K_a, for the sensitive receptor, but also upon its affinity constant, K_b, for the refractory receptor (it may also be necessary, if desensitization takes time to develop, to consider the rate constants for the conversion of the complex SA to the inactive complex SB). In the BTM series K_a would be expected to increase with chain length: activity on the chick biventer increases from BTM 10 to BTM 13 but activity on the cat tibialis decreases markedly over the same range. This could be taken to indicate that the increase in K_a, which does not appear to be accompanied by any marked drop in efficacy, is not offset by an increase in K_b. Above BTM 13 the decline in efficacy, along with the increased affinity (K_a) for the sensitive receptors, should lead to blocking activity of a different type from that of BTM 10.

The results with the long chain BTM compounds are very similar to those seen in series of derivatives of BTM 10 in which the size of the substituents on the onium groups is increased (Thesleff & Unna, 1954; Van Rossum & Ariëns, 1959). The replacement of only one methyl group at each end of the molecule causes a marked drop in ability to cause contracture of avian muscle or the frog rectus, but further increases in the size of the substituents lead to the production of antagonists, whose neuromuscular blocking action is different from that of BTM 10. Thesleff & Unna (1954) described these compounds as having a curare-like action, but Van Rossum & Ariëns (1959) have described the antagonism as noncompetitive. In view of this it would be interesting to make a detailed analysis of the effects of BTM 18 in antagonizing acetylcholine at the receptors in slow fibres and the neuromuscular junction.

TABLE 12

MELTING POINTS AND ANALYSES

Melting points were taken on a Kofler hot stage and are uncorrected : microanalyses for C and H are by Dr. J. W. Minnis, Department of Biochemistry, University of Edinburgh

BTM Series $[(CH_3)_3\overset{+}{N}.(CH_2)_n.\overset{+}{N}(CH_3)_3]2Br^-$

	Melting Point	Found			Calculated		
n	(°C)	C	H	Br^-	C	H	Br^-
11	245–6	46·8	8·92		47·2	9·34	
12	228·5–230·5	48·4	9·31		48·4	9·50	
13	219–220			34·4			34·7
14	221–3			33·8			33·7
15	219–221	51·7	9·76	32·8	51·6	9·93	32·7
16	228–9			32·2			31·9
17	224–6	53·2	10·2	31·0	53·5	10·2	31·0
18	232–3	53·9	10·4	30·3	54·3	10·3	30·2
19	230–3	55·1	10·2	29·4	55·1	10·4	29·4
20	239–240	56·2	10·3	28·6	55·9	10·5	28·4
21	234–6	56·8	10·3	27·9	56·6	10·6	28·0

BTE Series $[(C_2H_5)_3\overset{+}{N}.(CH_2)_n.\overset{+}{N}(C_2H_5)_3]2Br^-$

10	240–2	52·2	10·2	31·9	52·5	10·1	31·9
11	219–221	53·6	9·95	30·9	53·5	10·2	31·0
12	175–6	54·8	10·4		54·4	10·3	
13	181–182·5	55·3	10·2		55·1	10·4	
14	165–6	55·5	10·1		55·9	10·5	
15	169–171	56·4	10·7		56·6	10·6	
16	171–4	57·1	10·6		57·4	10·7	
17	164–5	58·3	10·5	26·4	58·0	10·8	26·6
18	157–9	58·6	10·7		58·6	10·8	
19	160·5–162	59·5	10·7		59·3	10·9	
20	164–6	60·0	10·7		59·9	11·0	
21	163–5	59·9	10·9		60·4	11·1	

Hexadecamethylene compounds $[RR'R''\overset{+}{N}.(CH_2)_{16}.\overset{+}{N}RR'R'']2Br^-$

	Melting	Br^-	
$R\,R'\,R''\,\overset{+}{N}.$	Point (°C)	Found	Calculated
$(CH_3)_2HN^+$ (BDM 16)	205–206	33·5	33·7
$(CH_3)_2(C_2H_5)N^+$ (BEDM 16)	193·5–194·5	30·2	30·2
$(CH_3)(C_2H_5)_2N^+$ (BMDE 16)	184–185	28·3	28·7
$(C_2H_5)_2HN^+$ (BDE 16)	170·5–171·5	30·2	30·1
$HOCH_2\,CH_2.N^+(CH_3)_2$ (BHDM 16)	147–147·5	28·4	28·4

CHEMICAL APPENDIX

Compounds. Polymethylene dibromides were prepared from undecylenic acid by procedures described by Ashton & Smith (1934), Chuit (1926) and Chuit & Hausser (1929) but the reduction of the intermediate esters was invariably performed with lithium aluminium hydride, which was much more convenient and gave much higher yields than the use of sodium and alcohol as originally described. The melting points of the pure polymethylene dibromides were exactly the same as those obtained by Chuit (1926) and Chuit & Hausser (1929).

The polymethylene dibromides were dissolved in ethanol and refluxed overnight with a large excess of the appropriate base. The excess of base and solvent was removed under vacuum and the residual gum was induced to crystallize by the addition of ethylmethylketone and/or ether and a trace of ethanol. The compounds were recrystallized from a suitable combination of these solvents. They were all hygroscopic and required vigorous drying before analysis. Some difficulty was experienced with the microanalyses for this reason and consequently samples of approximately 100 mg were used for the gravimetric determination of ionized halogen. The proportional changes during the weighing of these large samples were much smaller than with micro-quantities and the method was found to be so satisfactory and convenient that in a few instances it was used alone as an indication of purity. Melting points were taken on a Kofler hot stage and were uncorrected: they are shown together with the analytical figures in Table 12.

We wish to thank Dr W. E. Brocklehurst, Dr K. A. Scott and Mr J. G. Swanton for assistance with some of the experiments, Dr B. L. Ginsborg for helpful discussion, and Professor W. L. M. Perry for his encouragement. One of us (A. Z.) thanks the Faculty of Medicine for the award of a scholarship and we thank the Moray Fund of the University of Edinburgh for a grant to purchase the conductivity bridge. We wish particularly to acknowledge the importance of the work of J. R. Vane and of J. Warrimer on this problem; this has not been published because it was incomplete but our knowledge of their results has been a very great help to us.

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