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EFFECTS OF SOME POLYMETHYLENE BIS(HYDROXYETHYL)DIMETHYLAMMONIUM SALTS ON NEUROMUSCULAR TRANSMISSION

BY

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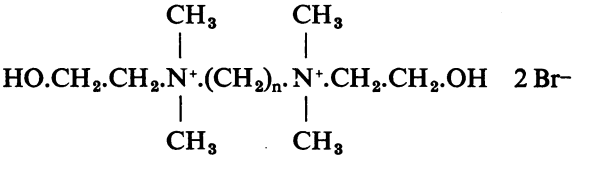
Interest in drugs which interrupt cholinergic transmission by interfering with the acetylation of choline in the nerve endings stems from the development of the hemicholiniums by Schueler (1955) and the subsequent analysis of their mechanism of action (for a review, see Schueler, 1960). Hemicholinium has been found to prevent the synthesis of acetylcholine by organized brain tissue but to be without this effect after disruption of the subcellular particles (MacIntosh, Birks & Sastry, 1956; Gardiner, 1961). It is generally believed that hemicholinium competes with choline for a carrier mechanism in the particle membrane and causes transmission failure at cholinergic junctions by preventing the transport of choline to the site of its acetylation (MacIntosh, 1959). The effects of hemicholinium are reversed by an excess of choline.

Some simple analogues of choline produce effects on neuromuscular transmission and on acetylcholine synthesis and output which closely resemble those of the hemicholiniums (Bowman & Rand, 1961, 1962; Bowman, Hemsworth & Rand, 1962; Bull & Hemsworth, 1963; Bowman & Hemsworth, 1965). The present investigation concerns some bisquaternary choline analogues of the following structures:



where n = 3, 4, 5, 6 or 10. In the text the decamethylene compound is abbreviated to the form C_{10}-dichol.

Burgen, Burke & Desbarats-Schonbaum (1956) studied the ability of choline acetyltransferase from rat brain to acetylate three compounds of this series (where n = 3, 5 or 10) and found that only the decamethylene analogue is acetylated. Barlow (1955) showed that the decamethylene analogue has weak anticholinesterase activity *in vitro*, and Barlow & Zoller (1962) have studied its action on the isolated biventer cervicis preparation of the chick (Ginsborg & Warriner, 1960) and found it to have a stimulant action resembling that of decamethonium.

METHODS

Nerve-muscle preparations. The methods used were identical with those previously described (Bowman & Rand, 1961; Bowman, Hemsworth & Rand, 1962). Maximal twitches and tetani of the tibialis anterior muscles of cats anaesthetized with chloralose (80 mg/kg, intravenously) were elicited by stimulating the sciatic nerves with rectangular pulses of 50 μsec duration and of about twice the strength required to evoke a maximal twitch. In most experiments, the contractions of both tibialis anterior muscles were recorded simultaneously, one being excited once every second through a 1 : 1 isolation transformer, and the other once every 10 sec. The muscle contractions were recorded on smoked paper by means of flat steel spring myographs. Drugs were injected intravenously through a cannula in a jugular vein, or close-arterially to a tibialis anterior muscle by the method described by Brown (1938). Artificial ventilation was applied when necessary.

Experiments were also carried out on the isolated phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946). Both hemidiaphragms from each rat were mounted separately in two 50-ml. organ-baths or were mounted together in the same 100-ml. organ-bath in Tyrode or McEwen (1956) solution at 32° C. The muscles were excited by supramaximal rectangular pulses of 50 μsec duration applied to the phrenic nerves. One was stimulated at 1 shock/sec and the other 6 shocks/min and the contractions were recorded on smoked paper.

Observations of the effects of the drugs were also made after intravenous injection into conscious chicks aged between 2 and 7 days (Buttle & Zaimis, 1949), and on the isolated rectus abdominis muscle of the frog.

Collection and assay of acetylcholine. The effects of the decamethylene analogue on acetylcholine output from the electrically stimulated isolated phrenic nerve-diaphragm preparation of the rat were studied by the method previously described (Bowman & Hemsworth, 1965). Both Tyrode solution and McEwen solution, each containing physostigmine (5 μg/ml.), were used to bathe the hemidiaphragms. Throughout each experiment the hemidiaphragms were continually stimulated at a frequency of 1 shock/sec through their phrenic nerves. The acetylcholine released was collected for 15 min in every hour (so that 45 min elapsed between each collection period) and was assayed on the dorsal muscle of the leech. The decamethylene analogue (50 or 100 μg/ml.) was added to the preparation 5 min after the second of two control collections of acetylcholine. Further 15-min collections were made in the presence of the drug, beginning 40 and 100 min after the initial addition of the compound to the bathing fluid. Choline (50 μg/ml.) was then also added to the bathing fluid in the continued presence of the decamethylene analogue, and 40 min later the acetylcholine output during 15 min was again estimated. Finally, the release during a further 15-min period was estimated after washing out both the decamethylene analogue and the choline. The muscle contractions were recorded throughout on smoked paper. Standard acetylcholine solutions were prepared in Tyrode or McEwen solution containing physostigmine (5 μg/ml.) and where appropriate they contained the decamethylene analogue and choline in the same concentrations as those present in the test samples. These two compounds themselves did not cause contraction of the assay preparation in the concentrations used. The test solutions were acidified to *p*H 4 immediately after collection but were neutralized to the same *p*H as the standards before assay. The active substance in the test solutions was identified as acetylcholine by the tests previously described (Bowman & Hemsworth, 1965).

Acetylcholine synthesis. The effect of the decamethylene analogue on the synthesis of acetylcholine by the choline acetyltransferase in the mitochondrial fraction of rabbit brain homogenate was studied by the method described by Bull & Hemsworth (1963). The mitochondrial fraction of rabbit brain homogenate was prepared by the method described by Hebb (1963). 0.1 ml. of the mitochondrial fraction, equivalent to 20 mg of fresh tissue, was incubated with 0.4 ml. of reaction mixture which had previously been incubated for 10 min at 39° C. This reaction mixture contained (μmoles/ml.): potassium chloride, 218; acetyl phosphate, 12.5; magnesium chloride, 6.4; L-cysteine, 31.3; physostigmine sulphate, 0.18; sodium phosphate buffer (*p*H 6.9), 17.4; as well as 0.34 mg of phosphate acetyltransferase, and 30 to 40 units of coenzyme A. Various amounts of choline and of the decamethylene analogue, contained in a constant volume of 0.1 ml., were added to the system which was then allowed to incubate for 1 hr at 37° C. A similar procedure was followed with heat-inactivated mitochondrial fraction to serve as a control in the biological assay procedure. The acetylcholine synthesized during the incubation was assayed on the frog rectus abdominis muscle against standard acetylcholine solutions containing appropriate amounts of the control. The concentrations of choline, when present in the control solutions and in the solutions to be

assayed, were too small to produce contracture of the rectus muscle. The first addition of the decamethylene analogue itself produced a small contracture of the rectus muscle, but the tissue rapidly became insensitive to this compound, subsequent additions being without effect. Control assays carried out with acetylcholine solutions of known strength and containing both choline and the decamethylene analogue showed that the presence of these two compounds did not affect the accuracy of the assays.

The Tyrode solution had the following composition (g/l.): NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaHPO_4$ 0.1, $NaHCO_3$ 1.0, and glucose 1.0. The McEwen solution used was identical with that described by McEwen (1956). The drugs used were neostigmine methylsulphate (Roche), physostigmine sulphate (B.D.H.), edrophonium chloride (Roche), tubocurarine chloride (Burroughs Wellcome), hexamethonium bromide (May & Baker), decamethonium iodide (L. Light), choline chloride (B.D.H.) and acetylcholine chloride (B.D.H.). The drugs were diluted in 0.9% (w/v) saline and all doses refer to the salts specified here and elsewhere.

RESULTS

Neuromuscular blocking action

The decamethylene derivative (C_{10}-dichol) was the most potent of the compounds studied and was the most clear-cut in its actions. Evidence of a depolarizing action was obtained from experiments in which it produced a contraction of the non-stimulated tibialis anterior muscle of the cat on close arterial injection (0.5 mg), spastic paralysis of the conscious chick on intravenous injection (0.5 mg/100 g of chick) and contracture of the isolated rectus abdominis muscle of the frog (20 to 100 μg/ml.). These results support those of Barlow & Zoller (1962).

In the cat, the neuromuscular blocking action of C_{10}-dichol exhibited two phases. On intravenous injection of 5 to 10 mg/kg, a decrease in the amplitude of the maximal indirectly elicited twitches rapidly followed an initial small potentiation. This first phase of block occurred in both rapidly (1 shock/sec) and slowly (6 shocks/min) stimulated tibialis anterior muscles, and exhibited characteristics of block by depolarization as produced by decamethonium in this muscle (Paton & Zaimis, 1952). Thus, tetanic stimulation (100 shocks/sec for 10 sec) and intravenously injected neostigmine (200 μg/kg) or edrophonium (350 μg/kg) were without any antagonistic action while the previous intravenous injection of tubocurarine (0.1 mg/kg), in a dose too small to produce block, reduced or prevented the first phase of block in response to C_{10}-dichol. The effect of a tetanus was not fully typical of that occurring during depolarization-block produced by decamethonium. During partial paralysis of maximal twitches produced by decamethonium, the tension of a tetanus, though depressed, is well maintained throughout the period of stimulation (Paton & Zaimis, 1952). In contrast, tetanic tension rapidly waned during the block produced by C_{10}-dichol (Fig. 1,*a*).

The onset of recovery from the first phase of block produced by C_{10}-dichol occurred at about the same time in both muscles and, in the muscle stimulated once every 10 sec, recovery was usually complete within 15 to 20 min after injection. However, with the more rapidly stimulated muscle the recovery curve was usually interrupted by a second phase of block during which the twitches either diminished again in size, as in Fig. 1,*b*, or became temporarily constant at a reduced level, as in Fig. 2. Recovery from the second phase of block was prolonged and occurred 1 to 2 hr after injection when the stimulation frequency was maintained at 1 shock/sec; recovery could be hastened by reducing the frequency of stimulation to 6 shocks/min. The second phase of block was not always

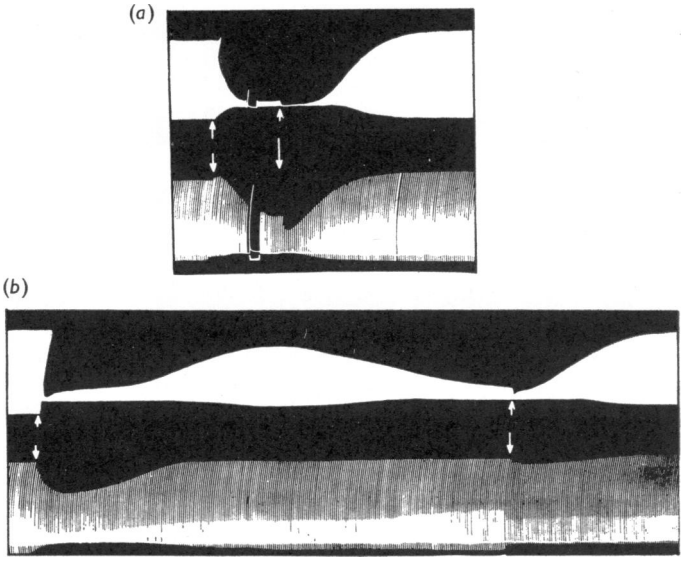


Fig. 1. Cat, 3.4 kg. Maximal twitches of right and left tibialis anterior muscles elicited indirectly by 1 shock/sec and 6 shocks/min respectively. The two panels are continuous. At the first pair of arrows in each panel 5 mg/kg of C_{10}-dichol and, at the second pair of arrows in each panel, 5 mg/kg of choline, were injected intravenously. The small horizontal bar in (*a*) denotes the duration of a tetanus (100 shocks/sec for 5 sec). The kymograph speed was increased during the tetanus.

definitely distinguishable after the first injection of C_{10}-dichol but was invariably evident after the second.

Choline (5 mg/kg, intravenously) slightly enhanced the first phase of block which occurred in both muscles (Fig. 1,*a*) but antagonized the second phase of block which occurred selectively in the more rapidly stimulated muscle (Fig. 1,*b*). Choline injected during the first phase of block always completely prevented the development of the second phase of block in the rapidly stimulated muscle so that recovery from the first phase was complete (Fig. 1,*a*).

The second phase of block further differed from the first as follows: it was temporarily reversed after a brief period of tetanic stimulation of the motor nerve (100 shocks/sec for 10 sec); neostigmine (200 μg/kg) and edrophonium (350 μg/kg) injected intravenously produced some antagonism although their effect was less striking than their anti-curare action; and tubocurarine (100 to 200 μg/kg, intravenously) augmented the second phase of the block.

These results suggested that the first phase of block produced by C_{10}-dichol was due to depolarization of the motor end-plates while the second phase, occurring only during higher frequencies of stimulation, appeared to be due to an action at the nerve endings resembling that of triethylcholine (Bowman & Rand, 1961). Fig. 2 illustrates an experiment in which the sensitivity of the muscle to close-arterially injected acetylcholine was tested throughout the blocking action of C_{10}-dichol in a tibialis anterior muscle stimulated once every second. During the first phase of block, contractions produced by injected acetylcholine were

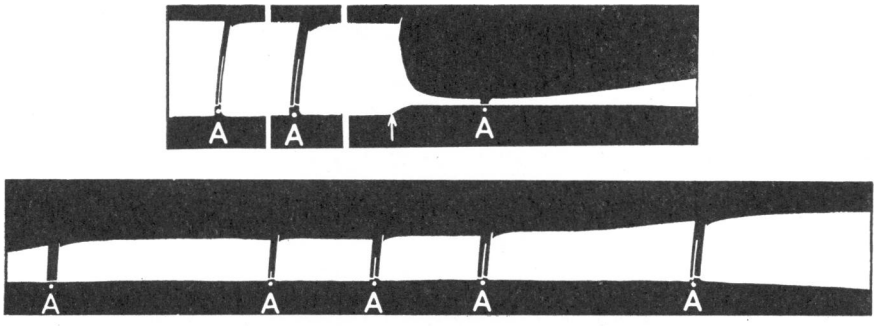


Fig. 2. Cat, 4.0 kg. Maximal twitches of a tibialis anterior muscle elicited indirectly once every second. The lower tracing is a continuation of the upper. At A, electrical stimulation was temporarily stopped and 5 μg of acetylcholine were injected close-arterially. At the arrow, 5 mg/kg of C_{10}-dichol were injected intravenously. The horizontal line corresponds to 10 min.

completely abolished, confirming that this part of the blocking action was exerted postjunctionally. The twitches then partially recovered to become constant in height at about 50% of the original amplitude for a further 30 min. Although the twitches did not increase during this period, the responses to injected acetylcholine became progressively larger, showing that the sensitivity of the postjunctional apparatus was returning to normal. It therefore appeared that partial transmission failure continued because a prejunctional blocking action began to balance the diminishing block by depolarization.

The isolated phrenic nerve-diaphragm preparation of the rat is much less sensitive to block by depolarization than the tibialis anterior muscle of the cat and consequently the

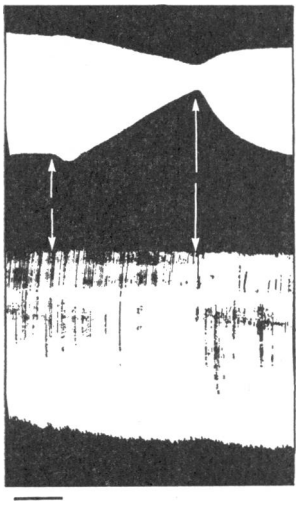


Fig. 3. Maximal twitches of both hemidiaphragms from the same rat mounted in the same 100-ml. organ-bath containing McEwen solution. Contractions are downwards. In the upper tracing the phrenic nerve was stimulated once every second, and in the lower once every 10 sec. At the first pair of arrows 100 μg/ml. of C_{10}-dichol and, at the second pair of arrows, 50 μg/ml. of choline were added to the bath. The horizontal bar corresponds to 10 min.

first phase of block was less obvious in this tissue. In concentrations of 50 to 100 μg/ml., C_{10}-dichol produced a slight potentiation of the twitches followed by a slowly developing depression which occurred selectively in the diaphragm stimulated once every second. The twitches were restored by the addition of choline (Fig. 3). The initial abrupt block of both muscles observed in the cat tibialis anterior muscle, and attributed to depolarization, did not occur at this dose level and the general picture therefore closely resembled that produced by triethylcholine in the rat diaphragm (Bowman & Rand, 1961).

During the experiments on the isolated diaphragm, it was observed that the block produced by C_{10}-dichol when the muscle was bathed in Tyrode solution was always slower in onset and less in extent than that produced in a muscle bathed in McEwen solution. Choline was also less effective as an antagonist when Tyrode solution was used. Tests showed that the difference was chiefly due to the magnesium content of Tyrode solution. Magnesium ions are known to depress acetylcholine release (Del Castillo & Engbaek, 1954) and it therefore seems likely that the magnesium content of Tyrode solution reduced the safety factor in release of acetylcholine, thus conserving the preformed transmitter in the nerve endings for a longer period in the presence of C_{10}-dichol.

It was also regularly observed that the right hemidiaphragm was more susceptible to the blocking action of C_{10}-dichol than the left, but no explanation of this difference was apparent.

The decamethonium-like and triethylcholine-like actions of C_{10}-dichol were also evident after intravenous injection into conscious chicks. The first effect of an intravenous dose of 0.5 to 2 mg/100 g of chick was a spastic paralysis (Fig. 4,*a*) like that produced

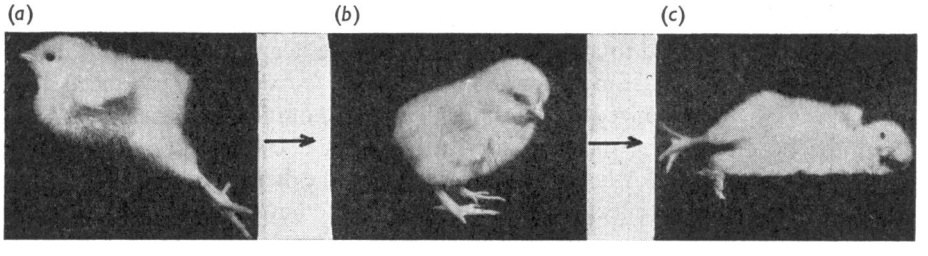


Fig. 4. Conscious chick, 75 g. (*a*) Spastic paralysis 30 sec after an intravenous dose of 1.5 mg of C_{10}-dichol; (*b*) 5 min later; (*c*) fatigue after twenty trials to elicit its righting reflex.

by decamethonium (Buttle & Zaimis, 1949). On recovering from the spasticity, the chick stood up and was apparently normal (Fig. 4,*b*); if undisturbed, the chick exhibited no further effects of the drug. However, if the chick was then repeatedly placed on its back to elicit its righting reflex, a pronounced muscular weakness developed after fifteen to twenty trials (Fig. 4,*c*) which was similar to that produced by triethylcholine (Bowman & Rand, 1961). A similar procedure carried out after recovery from spastic paralysis produced by decamethonium did not result in muscular weakness.

The remaining derivatives studied (n = 6, 5, 4 or 3) did not produce effects characteristic of depolarizing drugs. They did not produce contraction of the non-stimulated tibialis anterior muscle of the cat in doses up to 10 mg injected close-arterially, or contracture of the frog rectus abdominis muscle in concentrations up to 1 mg/ml. In the chick the smallest

effective doses of the hexa- (10 mg/100 g), penta- (18 mg/100 g) and tetramethylene (25 mg/100 g) derivatives produced an immediate flaccid paralysis superficially similar to that produced by tubocurarine (Buttle & Zaimis, 1949). The trimethylene derivative was without immediate effect on the chick in doses up to 50 mg/100 g and no triethylcholine-like effect developed after eliciting the righting reflex.

In the cat, large intravenous doses of the hexamethylene (75 mg/kg) and pentamethylene (100 mg/kg) derivatives produced a small and slowly developing depression of the twitches of the more frequently stimulated muscle (1 shock/sec). This effect was partially reversed by choline and therefore showed some similarity to that of triethylcholine. The tetra- and trimethylene derivatives were without effect on the twitches of the cat tibialis anterior muscles in intravenous doses up to 400 mg/kg.

In isolated diaphragm preparations of the rat, the hexa- and pentamethylene derivatives produced effects resembling that of the decamethylene derivative illustrated in Fig. 3. Concentrations producing comparable effects were 1.25 mg/ml. for the hexamethylene derivative and 1.4 mg/ml. for the pentamethylene derivative. The transmission failure, which occurred selectively in the more rapidly stimulated muscle, was reversed by choline. At a concentration of 4 mg/ml. the tetramethylene derivative produced only a slight depression of twitch tension which was ineffectively reversed by choline. The trimethylene derivative was without effect in concentrations up to 10 mg/ml.

The effects of C_{10}-dichol and of the hexamethylene analogue on the rat diaphragm were compared with the actions of decamethonium and hexamethonium. In all concentrations (10 to 50 μg/ml.) decamethonium caused a rapidly developing neuromuscular block in both the rapidly and the more slowly stimulated muscle. The more rapidly stimulated muscle was generally affected to a greater extent but some degree of block was also always detectable at slow rates of stimulation. The neuromuscular block produced by decamethonium was not antagonized by choline. Hexamethonium, however, acted like the deca- and the hexamethylene dicholine derivatives. In a concentration of 125 μg/ml., hexamethonium caused a slowly developing block in the hemidiaphragm stimulated at 1 shock/sec but was without effect on the muscle stimulated at 6 shocks/min. This neuromuscular block was always reversed by the addition of choline (10 to 20 μg/ml.) to the bathing fluid.

Release of acetylcholine

Physostigmine (5 μg/ml.) was added to the organ-bath in experiments where the acetylcholine output from the diaphragm was estimated and, in the presence of physostigmine the characteristics of the block produced by C_{10}-dichol were altered. When C_{10}-dichol (50 to 100 μg/ml.) was added to the bath 15 min after physostigmine (5 μg/ml.), it caused an immediate 20 to 50% depression of the muscle twitches followed by, or merging with, a more slowly developing block in transmission (Fig. 5). The secondary phase of block occurred only in the rapidly (1 shock/sec) stimulated muscle and was similar to the block observed when no anticholinesterase was present. However, in the presence of physostigmine, choline did not antagonize the block and was either without effect (Fig. 5) or enhanced it.

C_{10}-Dichol reduced the amount of acetylcholine released during nerve stimulation; the results are shown in Fig. 6. When C_{10}-dichol had been in contact with the hemidia-

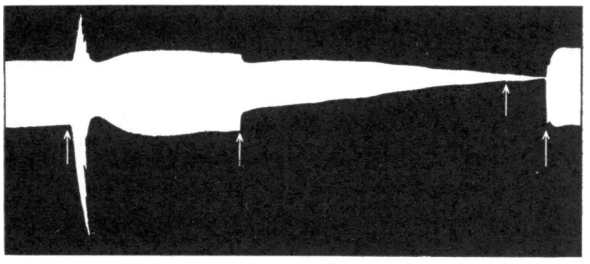


Fig. 5. Maximal twitches of rat hemidiaphragm mounted in a 50-ml. organ-bath in Tyrode solution. Contractions are downwards and are elicited by stimulation of the phrenic nerve once every second. At the first arrow, 5 μg/ml. of physostigmine, at the second arrow, 100 μg/ml. of C_{10}-dichol, and, at the third arrow, 50 μg/ml. of choline were added to the organ-bath. At the fourth arrow the bath fluid was replaced with fresh Tyrode solution. The horizontal bar corresponds to 10 min.

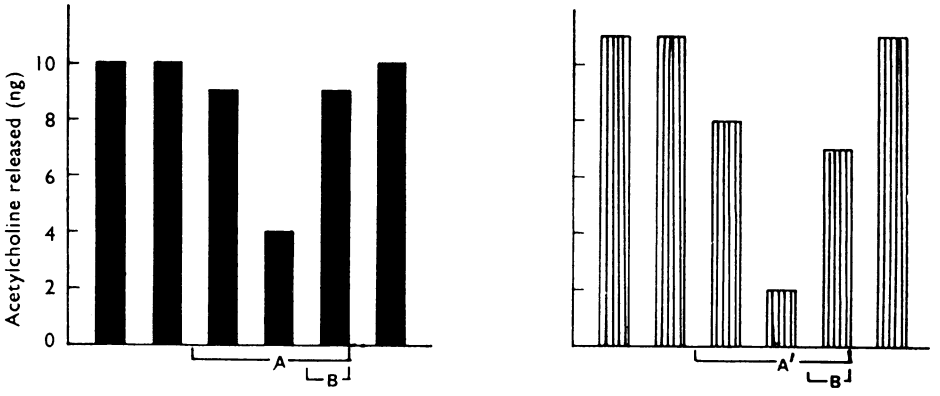


Fig. 6. Each column represents the acetylcholine (as cation) released during a period of 15 min (ordinates in ng). Throughout each experiment, the hemidiaphragms were stimulated through their phrenic nerves at a frequency of 1 shock/sec. The 15-min collection periods were commenced every hour. The first two columns in each histogram represent the release during control collections of acetylcholine before the addition of drugs. The third, fourth and fifth columns represent the acetylcholine released in the presence of C_{10}-dichol (A, 50 μg/ml.; A′, 100 μg/ml.) which was initially added 40 min before the collection period represented by the third column. Choline (B, 50 μg/ml.) was also present during the collection period represented by the fifth column, having been initially added 40 min before it commenced. The sixth column represents the release after washing out the C_{10}-dichol and the choline. Each column represents the mean release, to the nearest whole number, of results obtained on eight diaphragms (that is a total of sixteen similar experiments). Results obtained using Tyrode and McEwen solution were pooled.

phragm for a period of 40 min the acetylcholine output was reduced by 10 to 27% depending on the amount of C_{10}-dichol added to the bathing fluid. After nerve stimulation for a further hour in the presence of C_{10}-dichol the acetylcholine output was depressed by 60 to 80%. When choline (50 μg/ml.) had been present with the C_{10}-dichol for a period of 40 min the acetylcholine output was restored to 65 to 90% of the control output, although the twitches were not increased. After washing out all drugs from the organ-bath, the acetylcholine output (5 hr after the initial collection) was restored to the control level.

Bowman & Hemsworth (1965) found that in control experiments under identical conditions the acetylcholine output remained constant over a period of at least 8 hr.

Acetylcholine synthesis

Both hemicholinium (Gardiner, 1961) and triethylcholine (Bull & Hemsworth, 1963) have been shown to inhibit the synthesis of acetylcholine by organized nerve tissue, and C_{10}-dichol was therefore tested for this effect. Table 1 gives the results of a typical experiment demonstrating the inhibiting action of this compound on the synthesis of acetylcholine by the mitochondrial fraction of rabbit brain homogenate.

TABLE 1

INHIBITION BY C_{10}-DICHOL OF THE ACETYLCHOLINE SYNTHESIZED BY THE MITOCHONDRIAL FRACTION OF RABBIT BRAIN HOMOGENATE

2·74 mg of C_{10}-dichol dibromide is equimolar to 0·8 mg of choline chloride

Choline chloride added (mg)	Acetylcholine synthesized (μg/g of fraction)	Inhibition (%) in the presence of C_{10}-dichol dibromide	
		2·74 mg	10·96 mg
0·2	75	53	77
0·8	125	32	58
3·2	155	13	23

When 0.8 mg of choline chloride was used as substrate, an equimolar concentration of C_{10}-dichol dibromide (2.74 mg) caused a 32% inhibition of acetylcholine synthesis. A fourfold increase in the concentration of C_{10}-dichol or a reduction in the concentration of choline increased the amount of inhibition, while an increase in the concentration of choline decreased the inhibition. The largest inhibition of acetylcholine synthesis produced was 77% when the molar ratio of choline to C_{10}-dichol was 1 : 16 (that is, 0.2 mg of choline chloride : 10.96 mg of C_{10}-dichol dibromide).

DISCUSSION

The results indicate that the neuromuscular blocking action of C_{10}-dichol is the result of two distinct actions, a postjunctional depolarizing action and, when the frequency of stimulation is high, an action on the nerve endings through which the output of acetylcholine is impaired. *In vitro* studies suggested that this latter effect may be explained by an inhibitory action on acetylcholine synthesis resembling those of hemicholinium and triethylcholine, rather than by an effect on the transmitter release mechanism.

Physostigmine was found to alter the pattern of action of C_{10}-dichol in the rat diaphragm. In its presence neuromuscular block produced by C_{10}-dichol occurred abruptly, and the antagonistic action of choline on the depressed contractions was absent, although the acetylcholine output was restored. These changes in the characteristics of the block may be explained by the excess acetylcholine, accumulating in the presence of physostigmine, synergizing with the depolarizing action of C_{10}-dichol. Thus the initial block by depolarization is enhanced, and the increased output of acetylcholine produced by choline is preserved and augments, rather than antagonizes, the second phase of block. Physostigmine may also delay the inhibiting action of C_{10}-dichol on acetylcholine output. After 40 min in the presence of C_{10}-dichol together with physostigmine, acetylcholine output was depressed

by only 10 to 27%. Yet experiments on the diaphragm in the absence of physostigmine showed that the depression of twitch tension was well developed after this period, and choline completely reversed the effect.

During natural muscular activity, the inhibitory effect on acetylcholine synthesis may be a more important aspect of the action of C_{10}-dichol than is indicated by the present experiments. Hemicholinium-like action is more pronounced the higher the frequency of stimulation (Evans & Wilson, 1964). Natural muscular movements do not resemble maximal twitches and are equivalent to higher frequencies of stimulation than 1 shock/sec. Furthermore, the choline : C_{10}-dichol ratio *in vivo* probably favours the inhibitory action on synthesis. The results showed that inhibition increased with increase in the ratio of the concentrations of C_{10}-dichol to choline. When equimolar concentrations of choline and C_{10}-dichol were used, inhibition was only 32%. The concentration of free choline in cat plasma is of the order of 0.7 μg/ml. (Bligh, 1952). The effective intravenous dose of C_{10}-dichol (5 to 10 mg/kg) would produce a concentration of about 30 to 60 μg/ml. assuming it to be equally distributed throughout the extracellular fluids, and on this basis the molar ratio of choline to C_{10}-dichol would be of the order of 1 : 12.5–25 which caused about 70% inhibition in the *in vitro* experiments.

Probably all quaternary ammonium compounds exert some postjunctional action on the motor end-plates, but in addition many have been shown to possess hemicholinium-like action (Schueler, 1960; MacIntosh, 1961; Bowman & Rand, 1962). Hemicholinium itself and, to a smaller extent, triethylcholine possess curare-like motor end-plate blocking activity in large doses (Martin & Orkand, 1961; Bowman & Rand, 1961; Bowman *et al.*, 1962), whereas other drugs, such as C_{10}-dichol and some analogues of suxamethonium (for example bis-2-dimethylaminobutyl succinate bismethochloride, unpublished experiments) have been found to possess depolarizing activity in addition to their action on acetylcholine synthesis. With some drugs, the prejunctional action is the more pronounced, but with others there may be little difference between the doses required to produce the two effects, or the action on the motor end-plates may be the stronger. It may well be that many of the drugs known to block postjunctionally could also be shown to inhibit acetylcholine synthesis if studied under the correct conditions. Bhatnager (quoted by MacIntosh, 1961) has in fact shown that tubocurarine and hexamethonium may reduce the synthesis of acetylcholine by minced brain, and Beani, Bianchi & Ledda (1964) have shown that tubocurarine inhibits the release of acetylcholine from motor nerve endings. In the present experiments hexamethonium was found to produce a triethylcholine-like block of the twitches of the rat diaphragm, and we have obtained similar results with bretylium, an analogue of choline which produces muscular weakness as a side-effect in man (Evanson & Sears, 1960; Campbell & Montuschi, 1960). Hughes (Demonstration to British Pharmacological Society, July 1964) has also demonstrated this effect of bretylium on the isolated rat diaphragm.

The characteristics of a prejunctional block superficially resemble those of block by competition with acetylcholine. Thus a tetanus and anticholinesterase drugs exert some antagonistic action, and tubocurarine augments the paralysis. Numerous reports have indicated that the blocks produced in man by the depolarizing type of muscle relaxant (for example decamethonium, suxamethonium and carbolonium) often change in type after prolonged administration (Grant, 1952; Guerrier & Huxley-Williams, 1954; Hodges,

1955; Argent, Dinnick & Hobbiger, 1955; Hodges & Foldes, 1956; Brennan, 1956; Foldes, Wnuck, Hodges, Thesleff & de Beer, 1957; Churchill-Davidson & Christie, 1959; Wiemers & Overbeck, 1960). Because, at a late stage of the block produced by these depolarizing drugs, a tetanus and neostigmine exert some antagonism, while tubocurarine augments the paralysis, many anaesthetists have concluded that the depolarization phase in man may give way to a competitive phase. The block produced by C_{10}-dichol in the cat appears to resemble that sometimes produced by depolarizing muscle relaxants in man, and it is therefore equally possible, as previously suggested (Bowman, Callingham & Goldberg, 1961; Bowman, 1962), that prolonged administration of depolarizing relaxants in man occasionally leads not to a competitive phase of block but to inhibition of acetylcholine synthesis as the result of a weak hemicholinium-like action. If this is so, choline might be the most effective antagonist, although if an anticholinesterase drug had been injected before choline probably no reversal of the neuromuscular block would be observed, even though acetylcholine output may have been restored.

In numerous experiments on the cat tibialis anterior muscle we have failed to detect a change in the type of block produced by decamethonium, suxamethonium or carbolonium after prolonged administration during stimulation at a frequency of 1 shock/sec, possibly because the powerful postjunctional action masked any prejunctional effect, and owing to the difficulty of assaying acetylcholine in the presence of powerful depolarizing drugs, we have not yet been successful in testing the effects of the depolarizing muscle relaxants on acetylcholine synthesis or release. There may be a true species difference between the cat and man with regard to their susceptibility to a prejunctional action, or the difference might be explained by the different conditions under which the drugs are studied. For example, the patterns of motor nerve activity occurring during natural movements in man may favour a prejunctional action of the depolarizing muscle relaxants more than the single synchronous volleys induced by electrical stimulation in the experiments on cats.

SUMMARY

1. The effects of the polymethylene bis(hydroxyethyl)dimethylammonium salts containing 10, 6, 5, 4 and 3 methylene groups have been studied on nerve muscle preparations from cats and rats, on the isolated rectus abdominis muscle of the frog and after intravenous injection into conscious chicks.

2. The decamethylene analogue was the most potent compound and produced an initial block of neuromuscular transmission, which exhibited the characteristics of block by depolarization, followed by a secondary longer-lasting block which occurred selectively in rapidly stimulated nerve-muscle preparations and which exhibited the characteristics of block produced by inhibition of acetylcholine synthesis.

3. The effects of the decamethylene analogue on the output of acetylcholine from the isolated phrenic nerve-diaphragm preparation of the rat and on the synthesis of acetylcholine by the mitochondrial fraction of rabbit brain homogenate were studied. Both output and synthesis were inhibited by this compound and these effects were reversed by an excess of choline.

4. The results are discussed in relation to the possibility that large amounts of the commonly used depolarizing neuromuscular blocking agents occasionally cause inhibition of acetylcholine synthesis in man.

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