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FACILITATION OF NEUROMUSCULAR TRANSMISSION BY ANTICHOLINESTERASE DRUGS

BY

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A close correlation has been shown to exist between the *in vitro* anticholinesterase potencies of ambenonium, neostigmine, methoxyambenonium and edrophonium chloride and their abilities to increase muscle contractions produced by close-arterial injections of acetylcholine. No correlation was found between the anticholinesterase potencies of the drugs and their potentiations of the maximal twitch in response to electrical stimulation, or their antagonisms of tubocurarine. It is concluded that some action, in addition to inhibition of cholinesterase, contributed to their facilitation at the neuromuscular junction, and it is suggested that this action may be at the pre-junctional site.

A lack of correlation between the ability of anticholinesterases to facilitate neuromuscular transmission and to inhibit cholinesterase *in vitro* has often been reported (Cowan, 1938; Wescoe, Riker & Brothers, 1949; Randall & Lehmann, 1950; Wescoe & Riker, 1951; Blaber, 1960; Blaber & Bowman, 1962b), and such reports might be used to support the opinion that inhibition of cholinesterase is not the sole mechanism involved in the action of such drugs. However, the lack of correlation might possibly be ascribed to the different conditions prevailing *in vitro* and *in vivo*, and the present experiments were undertaken to test this possibility. The abilities of edrophonium, neostigmine, ambenonium, and methoxyambenonium to potentiate muscle contractions produced both by nerve stimulation and by injected acetylcholine have been studied and compared with the *in vitro* anticholinesterase activities of the drugs.

METHODS

The experiments were carried out with adult cats (2.5 to 4.0 kg of body weight) and White Leghorn hens (1.5 to 2.0 kg), anaesthetized with intravenous chloralose (80 mg/kg).

With cats the tendon of a tibialis anterior muscle and with hens that of the lateral head of a gastrocnemius muscle was attached to a semi-isometric recording lever. The sciatic nerve was cut between ligatures high in the thigh, and shielded platinum electrodes were placed on a branch of the nerve supplying the muscle to be studied. The limb was rigidly clamped in position and twitches of the muscles, recorded on a kymograph, were elicited by electrical stimulation of the nerve with rectangular pulses of 50 μsec duration and of twice the strength required to evoke a maximal twitch; these responses will be called maximal twitches.

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For experiments with denervated muscle, the sciatic nerve was cut high in the thigh during ether anaesthesia after induction with sodium pentobarbitone for hens, and using sodium pentobarbitone alone for cats; nerve degeneration was allowed to proceed for 10 to 12 days in hens and for 19 to 21 days in cats. Direct stimulation of the denervated hen muscle was applied between a platinum wire inserted through the musculo-tendinous junction and an indifferent electrode attached to the drill through the lower end of the femur.

In several experiments muscle action potentials were recorded via concentric needle electrodes, simultaneously with isometric tension changes recorded by means of a mechano-electric transducer strain gauge (RCA 5734). These responses were displayed on a double-beam oscilloscope (Tektronix type 502) and photographed on 35 mm film.

Drugs were injected intravenously through a cannula in a jugular vein or close-arterially into the tibialis anterior muscle of cats (Brown, 1938; Blaber, 1960) or the lateral head of the gastrocnemius muscle of hens (Brown & Harvey, 1938).

Inhibition of cholinesterase was estimated manometrically by the method described previously (Blaber, 1960).

The drugs used were tubocurarine chloride, benzoquinonium chloride, acetylcholine chloride, edrophonium chloride, neostigmine methylsulphate, ambenonium chloride and methoxyambenonium chloride. The doses quoted refer to these salts.

RESULTS

Non-curarized muscles

Brown (1938) first demonstrated a quick contraction of the tibialis anterior muscle of the cat in response to a close-arterial injection of acetylcholine. However, the response to acetylcholine is not strictly comparable to a maximal twitch elicited by indirect electrical stimulation. A maximal twitch consists of a nearly synchronous contraction of all the constituent muscle fibres accompanied by a single summed muscle action potential, whereas the contraction produced by an injection of acetylcholine is accompanied by an irregular burst of muscle action potentials and is therefore a brief asynchronous tetanus (Brown, 1937a). Fig. 1 illustrates the difference between these two types of response.

Potentiation by anticholinesterases of the maximal twitch arises as a result of a conversion of the single muscle response to a brief asynchronous tetanus (Fig. 1, A). Since the response to injected acetylcholine is already an asynchronous tetanus the degree of potentiation by anticholinesterases might be expected to be smaller than with the maximal twitch, and this was in fact found. Nevertheless, providing the acetylcholine response was submaximal, an easily discernible potentiation was produced by some of the anticholinesterases.

Neostigmine (50 to 100 μg/kg) and ambenonium (50 μg/kg), administered intravenously in doses sufficient to potentiate the indirectly elicited maximal twitches, also increased the contractions produced by close-arterially injected acetylcholine (Fig. 1). After neostigmine, the contractions produced by injected acetylcholine were increased in tension and were also more sustained. Intravenously injected edrophonium (100 to 150 μg/kg) always potentiated the maximal twitch but never increased the response to injected acetylcholine; in fact this response was usually depressed. Methoxyambenonium, in intravenous doses up to 200 μg/kg, neither increased the maximal twitch nor potentiated the acetylcholine response.

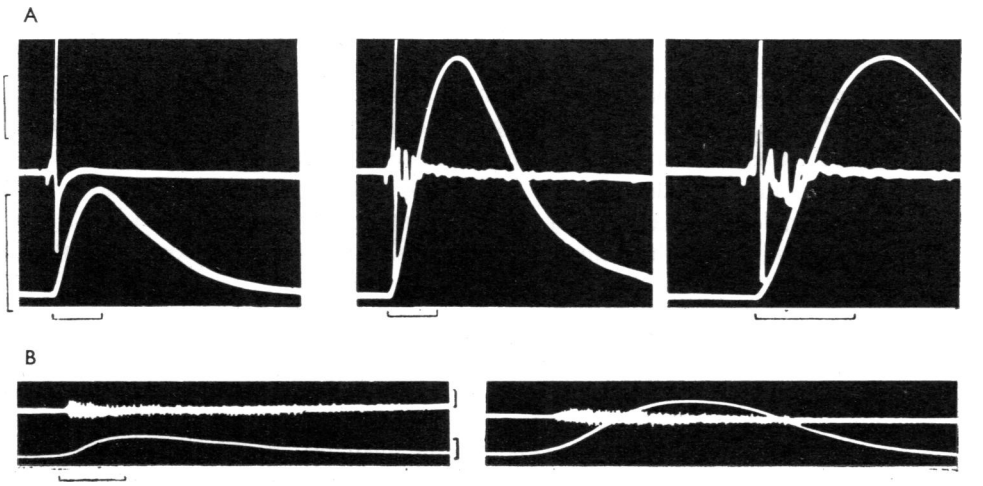

Fig. 1. Muscle action potential (upper trace) recorded with concentric needle electrodes, and isometric muscle tension (lower trace) recorded from the cat tibialis anterior muscle: A, in response to indirect nerve stimulation (0.1 shocks/sec), and B, following a close-arterial injection of 1 μg of acetylcholine. Neostigmine (150 μg, intravenously) was administered between the first and second records of both A and B. Voltage calibrations: A, nerve 5 mV; B, nerve 400 μV. Tension calibrations: A, muscle 1 kg; B, muscle 100 g. Time calibrations: A, 20 msec; B, 200 msec.

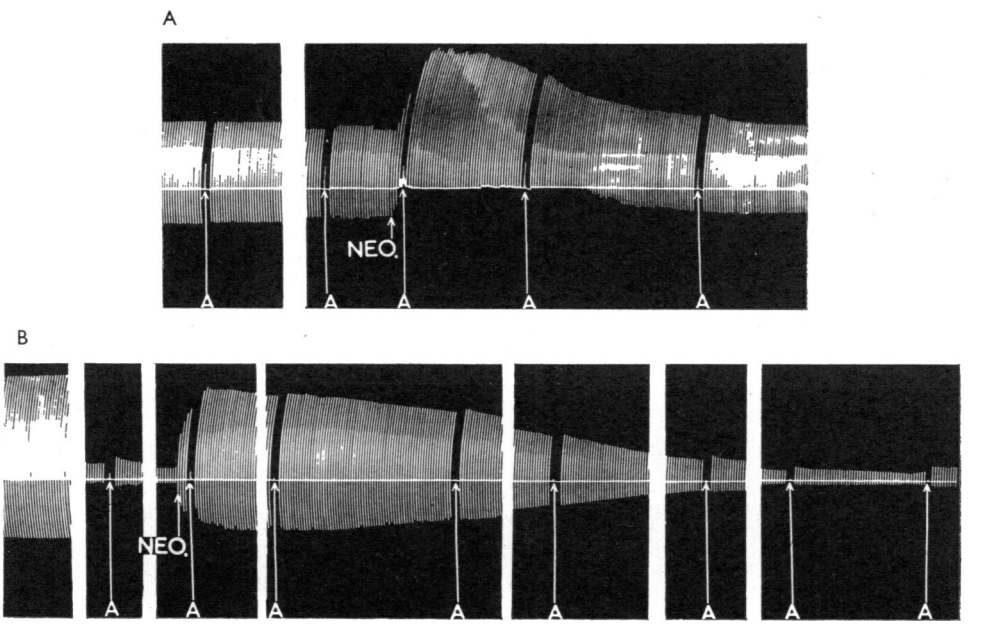

Fig. 2. Maximal twitches of the cat's tibialis anterior muscle elicited indirectly once every 10 sec. A, at A, 2 μg of acetylcholine, and at NEO, 5 μg of neostigmine were administered. B, a constant partial paralysis was maintained by a continuous infusion of tubocurarine (1.8 mg/hr). At A, 100 μg of acetylcholine, and at NEO, 5 μg of neostigmine were administered. All injections were given close-arterially. Note the absence of potentiation of the response to acetylcholine.

When administered close-arterially in doses sufficient to potentiate the maximal twitch at least as much as when the same dose was given intravenously, only ambenonium (3 μg) potentiated the responses to acetylcholine. Neostigmine (5 to 10 μg ; Fig. 2, A) and edrophonium (10 μg), although markedly increasing the twitch tension, were either without effect on, or depressed, the response to acetylcholine. Methoxyambenonium, in doses up to 20 μg, did not potentiate the twitch or the response to acetylcholine, and larger doses produced neuromuscular block. Some of these results with edrophonium, ambenonium and methoxyambenonium confirm previous reports (Blaber & Bowman, 1959 ; Blaber, 1960).

Effects during partial neuromuscular block

A series of experiments similar to those described above were next carried out, but with the addition that a constant degree of partial block of the maximal indirectly stimulated twitches was produced and maintained by continuous intravenous infusions of tubocurarine or benzoquinonium. Under these conditions, the close-arterial injected doses of acetylcholine had to be increased about twenty times in order to produce a muscle contraction. All the drugs antagonized tubocurarine paralysis when given intravenously or by close-arterial injection. The anticurare actions of intravenously administered neostigmine (Fig. 2, B) or ambenonium were accompanied by potentiation of the contractions due to acetylcholine, but the anti-curare actions of the other drugs were not. When injected close-arterially, only ambenonium potentiated the response to injected acetylcholine.

None of the anticholinesterases, when injected intravenously or close-arterially, antagonized a partial paralysis of the muscle produced by benzoquinonium. However, as in normal muscle and in muscle partially blocked by tubocurarine, intravenously injected neostigmine and intravenously or close-arterially injected ambenonium increased the contractions produced by injected acetylcholine. This effect of ambenonium is compared with that of methoxyambenonium in Fig. 3.

Ambenonium was the most potent agent in potentiating the response to close-arterially injected acetylcholine, since it was the only drug which produced this effect by close-arterial administration. Experiments were therefore carried out to determine the smallest effective intravenous doses of ambenonium. Intravenous doses as low

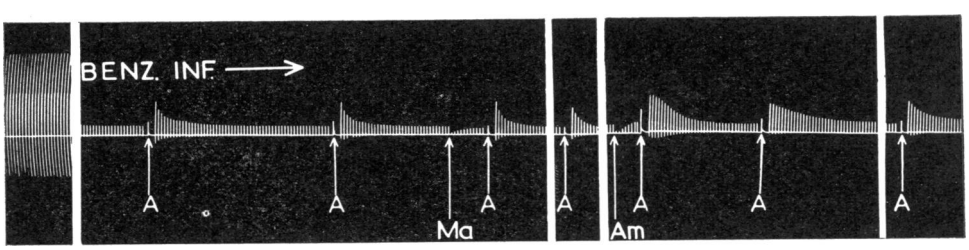

Fig. 3. Maximal twitches of the cat's tibialis anterior muscle elicited indirectly once every 10 sec. A constant partial paralysis was maintained by a continuous infusion of benzoquinonium (3.25 mg/hr). At A, 100 μg of acetylcholine, at Ma, 20 μg of methoxyambenonium, and at Am, 3 μg of ambenonium were injected close-arterially. Only ambenonium potentiated the response to injected acetylcholine, and neither drug increased the response to nerve stimulation.

as 2 μg/kg regularly increased the size of contractions produced by close-arterially injected acetylcholine both in unblocked muscles (Fig. 4, A) and in muscles partially paralysed by tubocurarine or benzoquinonium, even though this dose level was considerably below that necessary to potentiate the maximal twitch or to antagonize tubocurarine. This intravenous dose is similar to the close-arterial dose necessary

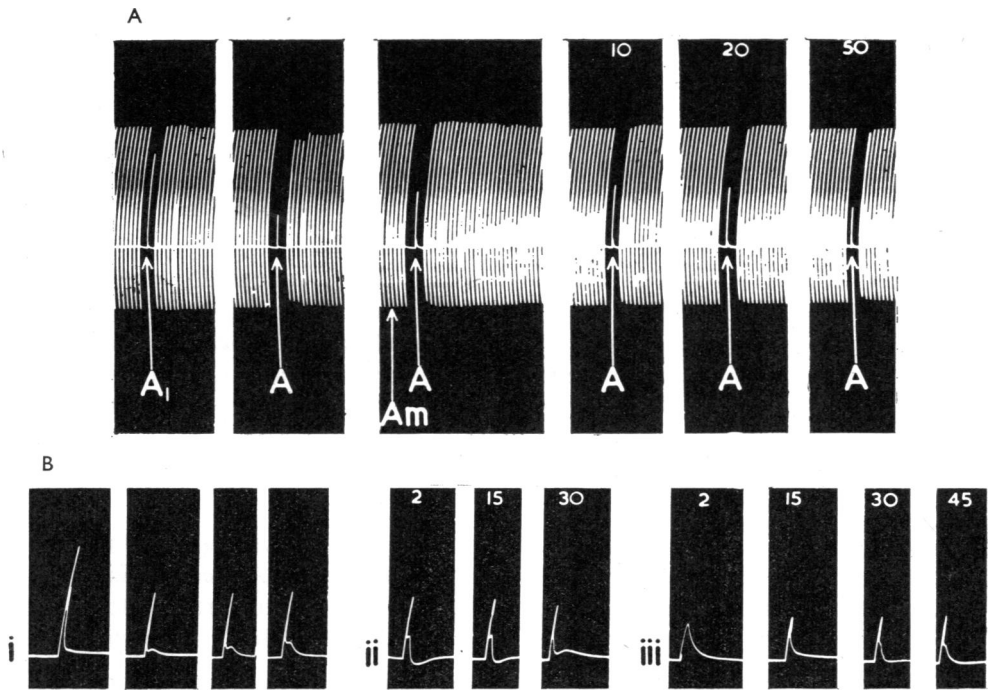

Fig. 4. A, maximal twitches of the cat's tibialis anterior muscle elicited indirectly once every 10 sec. At A_1, 2 μg, and at A, 1 μg of acetylcholine were injected close-arterially. At Am, 1 μg/kg of ambenonium was injected intravenously. Ambenonium increased the response to injected acetylcholine but did not potentiate the twitches. B, chronically denervated cat's tibialis anterior muscle (18 days). First response to 0.0625 μg of acetylcholine and subsequent responses to 0.031 μg of acetylcholine injected close-arterially. Between i and ii, 5 μg, and between ii and iii, 200 μg of ambenonium were injected close-arterially. No potentiation of the responses to acetylcholine was produced. Figures at the top of the records indicate the times in min after the administration of ambenonium.

to potentiate contractions due to acetylcholine, and these results therefore suggest that it is the total blood concentration of the drug rather than the route of its injection which is important for this effect.

Chronically denervated muscles

If inhibition of blood cholinesterase and consequent preservation of the injected acetylcholine were the only factors involved, then potentiation of the response of the denervated muscle to acetylcholine should be produced by doses of the inhibitors equal to those found to be effective in the innervated muscle.

Brown (1937b) showed that the response of denervated mammalian muscle to a close-arterial injection of a small dose of acetylcholine was accompanied by a burst of action potentials. With slightly larger doses the tension response became biphasic, the initial quick contraction being followed by a secondary slowly rising phase during which all propagated action potentials disappeared. The doses of acetylcholine in the present experiments were small enough to ensure that the initial phase of contraction constituted the main part of the response, the secondary phase of contracture being small or non-existent.

No doses of the facilitatory drugs, whether injected intravenously or close-arterially, potentiated the sub-maximal responses to acetylcholine in any dose. Fig. 4, B, illustrates this lack of effect of ambenonium (5 μg, close-arterially). Large doses of ambenonium (200 μg, close-arterially) depressed the response to acetylcholine.

The chronically denervated gastrocnemius muscle of the hen was also used, since the distribution of cholinesterase is different in the multiply-innervated fibres of this muscle (Ginsborg & Mackay, 1961). The hen muscle often began to diminish in responsiveness about 30 min after the start of the experiment, apparently because of poor blood supply through the denervated muscle. Direct electrical stimulation of the muscle with single supramaximal shocks improved the condition of the muscle, and, for this reason, the denervated muscle was stimulated in this way between injections.

Of all the inhibitors, only neostigmine potentiated the response of the hen denervated muscle to close-arterial injections of acetylcholine. Neostigmine was effective in intravenous doses from 4 to 100 μg/kg and in close-arterial doses of 4 μg and above. Fig. 5 compares the effects of close-arterial doses of neostigmine

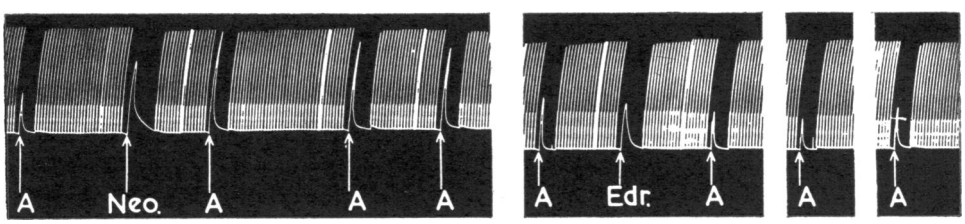

Fig. 5. Chronically denervated hen gastrocnemius muscle stimulated directly once every 10 sec except when injections were made. At A, 0.1 μg of acetylcholine, at Neo, 5 μg of neostigmine, and at Edr, 5 μg of edrophonium were injected close-arterially. Note that neostigmine and edrophonium themselves caused contracture of the denervated muscle. Neostigmine potentiated the response to acetylcholine and edrophonium slightly depressed it.

and edrophonium on the subsequent acetylcholine responses. Neostigmine and edrophonium themselves increased the tension of the denervated muscle.

Anticholinesterase potency

Fig. 6 is a graphical representation of *in vitro* determinations of the anticholinesterase potency of the compounds against the enzyme present in cat muscle. Ambenonium was by far the most potent inhibitor, being about 35 times more potent

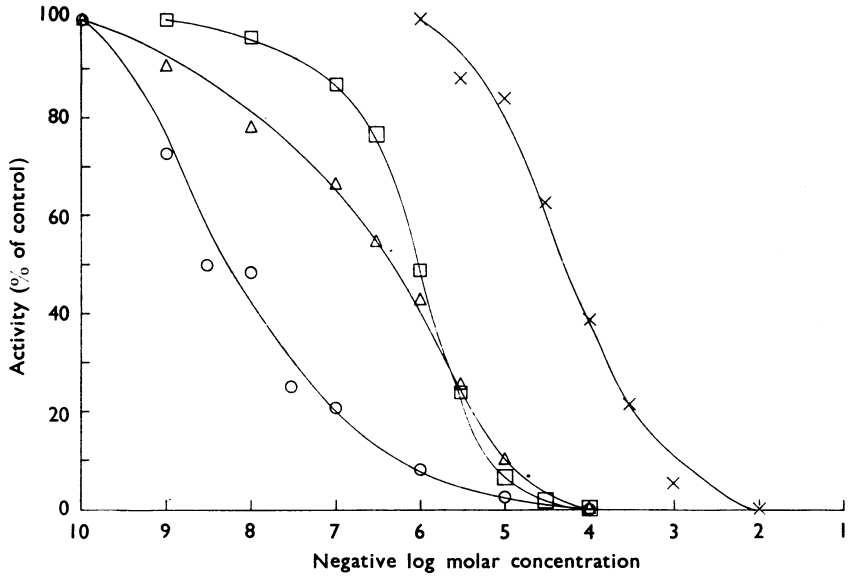

Fig. 6. Inhibition of cholinesterase activity of homogenates of cat tibialis anterior muscle. ○, ambenonium; △, neostigmine; □, methoxyambenonium; and X, edrophonium.

than neostigmine at the 50% level of inhibition. Methoxyambenonium was 150 times less active than ambenonium at all concentrations. Edrophonium was 7,000 times less active than ambenonium and 100 times less active than neostigmine at the 50% level.

DISCUSSION

The small quantity of acetylcholine released from the motor nerve endings rapidly reaches the motor end-plates in a high concentration. Even if not destroyed by cholinesterase, it is clear from the calculations of Ogston (1955) that this acetylcholine would rapidly diffuse away from the junctional region. In contrast, acetylcholine injected close-arterially in amounts sufficient to cause a mechanical response is contained in a relatively large volume and reaches the muscle much more diffusely. Furthermore, the amount of acetylcholine contained in the injected volume is very much greater than that released by a single nerve impulse. One explanation of the finding that in some circumstances potentiated maximal twitches were accompanied by unaltered, or even depressed, responses to acetylcholine might therefore be that, while preservation of the relatively small amount of acetylcholine released by a nerve impulse led to repetitive firing of the muscle fibres, preservation of the much larger injected amounts exceeded the threshold for block by depolarization. However, if this were the explanation, depressed responses to acetylcholine would be more likely to occur in the presence of the more potent anticholinesterases. In fact, contractions in response to injected acetylcholine were potentiated by the more potent and depressed by the weaker cholinesterase inhibitors. Furthermore, similar results

were obtained during partial neuromuscular block produced by tubocurarine, yet under these conditions block by depolarization is unlikely to occur.

Numerous workers have described the weak *in vitro* inhibition of cholinesterase by edrophonium compared with neostigmine, but Smith, Cohen, Pelikan & Unna (1952) and Wilson (1955) found a ratio of potency for these two drugs which approximately corresponds to their action in facilitating neuromuscular transmission in mammals. Wilson (1955) used a colorimetric technique which allowed a rapid estimation of cholinesterase inhibition, and he suggested that the anticholinesterase potency of edrophonium *in vivo* may be greater than indicated by the *in vitro* values obtained by other workers. If the enzyme-edrophonium reaction is a dynamic one and dissociation and re-association occur continually, then considerable destruction of acetylcholine might occur *in vitro* because of the long time that the relatively high concentration of substrate is in contact with the enzyme-inhibitor complex. A similar argument might be put forward to explain the present finding that, although edrophonium potentiated the twitch, it never increased the response to injected acetylcholine. Thus, injected acetylcholine is in contact with cholinesterase for a much longer period than the few milliseconds during which the transmitter released from the nerve remains at the motor end-plates. Consequently injected acetylcholine may be hydrolysed despite the presence of edrophonium and its effect would not be increased.

The results of both Smith *et al.* (1952) and of Wilson (1955) were obtained with electric eel cholinesterase, and Blaber & Cuthbert (1962) and Blaber & Bowman (1962b) have emphasized the fallacies that may arise when the results obtained with cholinesterase from one species are used to interpret the *in vivo* effects of anticholinesterase drugs in another. A study of the literature strongly suggests that the *in vitro* ratios of potency for neostigmine and edrophonium are closer using cholinesterase from the lower orders of animals, and that this effect is mainly due to the weaker activity of neostigmine in the lower orders rather than to a more powerful activity of edrophonium. Published ratios of *in vitro* potency for these two drugs are: edrophonium : neostigmine, 1 : 4.3 and 1 : 3.4 for electric eel cholinesterase (Smith *et al.*, 1952 ; Wilson, 1955, respectively) ; 1 : 10 for frog rectus abdominis muscle (Smith *et al.*, 1952) ; 1 : 46.6 for bovine erythrocytes (Smith *et al.*, 1952) ; 1 : 114 for hen gastrocnemius muscle (Blaber & Bowman, 1962b) ; 1 : 110 for human erythrocytes (Hobbiger, 1952) ; and 1 : 100 for cat tibialis anterior muscle (present study). It therefore appears that the unusually close ratio of *in vitro* potency for these two drugs found by Wilson (1955) was a consequence of species difference. Support for this view lies in the fact that Smith *et al.* (1952), although they used the more common manometric technique, obtained similar results to those of Wilson (1955) with electric eel cholinesterase. The argument outlined above for edrophonium cannot be applied to the other drugs used in this study since all of them are known to dissociate slowly from the enzyme and then only upon dilution.

It therefore seems justifiable to view the potentiation of the response to injected acetylcholine as an indication of the degree of cholinesterase inhibition *in vivo*, and in fact this effect could be correlated with the *in vitro* determinations of anticholinesterase activity. In the cat only ambenonium, the most potent inhibitor when

injected close-arterially, potentiated the response to acetylcholine by this route; and, for the intravenous route, only ambenonium and neostigmine, the two most potent inhibitors when injected intravenously at the increased dose level necessary to potentiate the twitch, increased the response to acetylcholine. When the close-arterial dose of ambenonium (3 μg) was given intravenously, the maximal twitches were unaffected but the response to acetylcholine was still potentiated showing that for the latter effect the amount of drug was important rather than the route by which it was given.

If it is assumed that the extracellular fluid is 15% of the body weight, the close-arterial dose of ambenonium when distributed uniformly in this volume would be sufficiently concentrated to produce 70 to 80% inhibition of the true cholinesterase in the cat. Inhibition by ambenonium of the pseudo-cholinesterase requires 1,400 to 2,000 times the concentration needed to inhibit the true cholinesterase (Lands, Karczmar, Howard & Arnold, 1955) and therefore no inhibition of the plasma cholinesterase could have occurred in these experiments. The intravenous dose of neostigmine (150 μg) when distributed in the extracellular fluid was also sufficient to inhibit 70 to 80% of the true cholinesterase of the cat, but the close-arterial dose (5 μg) would only have produced about 30% of inhibition. Since true cholinesterase is present in the erythrocytes and at the motor end-plates, inhibition at either of these sites might have contributed to the potentiation of the response to injected acetylcholine.

Axelsson & Thesleff (1959) have shown that the supersensitivity to acetylcholine which develops in chronically denervated muscle is due to the whole of the muscle fibre membrane becoming as sensitive to acetylcholine as the end-plate region. However, the cholinesterase activity remains confined to the site of the motor end-plates (Couteaux, 1942) and will have little effect on acetylcholine which depolarizes the membrane elsewhere. Since the end-plate in a focally innervated mammalian muscle fibre is only a small fraction of the total membrane surface, the denervated muscle behaves as though it lacked cholinesterase. In the denervated muscle injected acetylcholine could not be potentiated even though the inhibition of the blood enzyme by ambenonium or neostigmine must have been the same as in the innervated preparation. Therefore the inhibition of end-plate cholinesterase appears to be essential for the potentiation of the response to acetylcholine in the innervated muscle. However, for the end-plate cholinesterase to be inhibited to the required extent (which appears to be 70 to 80%), it seems that the effective concentration of the reversible inhibitors must be present throughout the whole of the extracellular fluid. It is merely coincidental that this concentration will also be sufficient to inhibit the true cholinesterase present in the erythrocytes. The results with avian muscle may also be explained in this way. Ginsborg & Mackay (1961) have shown that 50% of the muscle fibres in the hen gastrocnemius muscle are multiply-innervated, each such fibre having approximately eighty end-plates. When this muscle is chronically denervated its cholinesterase will therefore still be widely distributed over the membrane surface. Neostigmine, the most potent of the drugs as a cholinesterase inhibitor in the hen (Blaber & Bowman, 1962b), potentiated the response to acetylcholine after denervation. From the results of Blaber & Bowman (1962b) it

may be calculated that the close-arterial dose of neostigmine (5 μg), when distributed throughout the extracellular fluid, is sufficient to inhibit completely the cholinesterase of the hen. In contrast to its effect in the cat, ambenonium given intravenously or close-arterially did not potentiate acetylcholine contractions in the hen. However, this drug, like other bisquaternary compounds, is inactive against the cholinesterases in the hen (Blaber & Cuthbert, 1962 ; Blaber & Bowman, 1962b).

All the drugs used are reversible inhibitors, and the inhibition will therefore reverse as the drug is diluted throughout the extracellular fluid. Thus, most of them, when injected close-arterially, would be rapidly below a concentration sufficient to inhibit cholinesterase.

In this series of experiments, the acetylcholine was always injected close-arterially into the muscle, thus lessening the effects of blood cholinesterase. The further from the muscle the acetylcholine is injected the more important will become the effect of hydrolysis by the blood enzymes, and an increased dose of acetylcholine will be necessary to produce the same mechanical response as that produced by a close-arterial injection. It would therefore be expected that inhibition of blood cholinesterase would potentiate to a greater extent a more distant injection of acetylcholine than a close-arterial one. Brown, Dale & Feldberg (1936) showed that eserine produced the greatest potentiation when acetylcholine was injected arterially distant from the muscle.

Although potentiation of the response to acetylcholine could be correlated with the *in vitro* anticholinesterase action of the drugs, potentiation of the twitches elicited by nervous stimulation could not. Thus the response to acetylcholine could be potentiated in the absence of any potentiation of the maximal twitch, or in the absence of antagonism to tubocurarine. Furthermore, the maximal twitch may be potentiated, or tubocurarine antagonized, in the absence of potentiation of the acetylcholine response. These results therefore suggest that some action in addition to inhibition of cholinesterase may contribute to the facilitation of neuromuscular transmission by anticholinesterase drugs. An additional action exerted at the motor end-plate would be expected to affect in the same way the responses to injected acetylcholine and to acetylcholine released from the nerve. This was not so, and the results therefore support the conclusions of others (Riker, Roberts, Standaert & Fujimori, 1957 ; Riker, Werner, Roberts & Kuperman, 1959 ; Werner, 1960 ; Blaber & Bowman, 1959, 1962a, 1963) that the additional action of the drugs is exerted at the pre-junctional site, probably by increasing the amount of transmitter released by a nerve impulse. Hubbard & Schmidt (1961) have recently supplied direct evidence that neostigmine acts on motor nerve endings. This drug reversibly increased the refractory period and the negative after-potentials of the nerve terminals in the isolated rat phrenic nerve-diaphragm preparation. The increase in refractory period implied a prolongation of the spike potential in the nerve terminals which might be expected to prolong the period of release of transmitter.

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REFERENCES

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol. (Lond.)*, **147**, 178–193.

BLABER, L. C. (1960). The antagonism of muscle relaxants by ambenonium and methoxyambenonium in the cat. *Brit. J. Pharmacol.*, **15**, 476–484.

BLABER, L. C. & BOWMAN, W. C. (1959). A comparison between the effects of edrophonium and choline in the skeletal muscles of the cat. *Brit. J. Pharmacol.*, **14**, 456–466.

BLABER, L. C. & BOWMAN, W. C. (1962a). The interaction between benzoquinonium and anticholinesterases in skeletal muscle. *Arch. int. Pharmacodyn.*, **138**, 90–104.

BLABER, L. C. & BOWMAN, W. C. (1962b). A comparison between the responses of avian and mammalian muscles to substances which facilitate neuromuscular transmission. *Arch. int. Pharmacodyn.*, **138**, 185–208.

BLABER, L. C. & BOWMAN, W. C. (1963). Factors affecting the repetitive discharges produced in nerve and muscle by anticholinesterase drugs. *Int. J. Neuropharmacol.*, in the press.

BLABER, L. C. & CUTHBERT, A. W. (1962). Cholinesterases in the domestic fowl and the specificity of some reversible inhibitors. *Biochem. Pharmacol.*, **11**, 113–124.

BROWN, G. L. (1937a). Action potentials of normal mammalian muscle. Effects of acetylcholine and eserine. *J. Physiol. (Lond.)*, **89**, 220–237.

BROWN, G. L. (1937b). The actions of acetylcholine on denervated mammalian and frog's muscle. *J. Physiol. (Lond.)*, **89**, 438–461.

BROWN, G. L. (1938). The preparation of the tibialis anterior (cat) for close-arterial injections. *J. Physiol. (Lond.)*, **92**, 22–23*P*.

BROWN, G. L., DALE, H. H. & FELDBERG, W. (1936). Reactions of the normal mammalian muscle to acetylcholine and to eserine. *J. Physiol. (Lond.)*, **87**, 394–424.

BROWN, G. L. & HARVEY, A. M. (1938). Reactions of avian muscle to acetylcholine and eserine. *J. Physiol. (Lond.)*, **94**, 101–117.

COUTEAUX, R. (1942). La cholinestérase des plaques motrices après section du nerf moteur. *Bull. biol.*, **76**, 14–57.

COWAN, S. L. (1938). The action of eserine-like and curare-like substances on the responses of frogs' nerve-muscle preparations to repetitive stimulation. *J. Physiol. (Lond.)*, **93**, 215–262.

GINSBORG, B. L. & MACKAY, B. (1961). A histochemical demonstration of two types of motor innervation in avian skeletal muscle. *Bibl. anat. (Basel)*, **2**, 174–181.

HOBBIGER, F. (1952). The mechanism of anticurare action of certain neostigmine analogues. *Brit. J. Pharmacol.*, **7**, 223–236.

HUBBARD, J. I. & SCHMIDT, R. F. (1961). Stimulation of motor nerve terminals. *Nature (Lond.)*, **191**, 1103–1104.

LANDS, A. M., KARCZMAR, A. G., HOWARD, J. W. & ARNOLD, A. (1955). An evaluation of the pharmacologic actions of some bis-quaternary salts of basically substituted oxamides (WIN 8077 and analogues). *J. Pharmacol. exp. Ther.*, **115**, 185–198.

OGSTON, A. G. (1955). Removal of acetylcholine from a limited volume by diffusion. *J. Physiol. (Lond.)*, **128**, 222–224.

RANDALL, L. O. & LEHMANN, G. (1950). Pharmacological properties of some neostigmine analogues. *J. Pharmacol. exp. Ther.*, **99**, 16–32.

RIKER, W. F., ROBERTS, J., STANDAERT, F. G. & FUJIMORI, H. (1957). The motor nerve terminal as the primary focus for drug induced facilitation of neuromuscular transmission. *J. Pharmacol. exp. Ther.*, **121**, 286–312.

RIKER, W. F., WERNER, G., ROBERTS, J. & KUPERMAN, A. (1959). Pharmacologic evidence for the existence of a presynaptic event in neuromuscular transmission. *J. Pharmacol. exp. Ther.*, **125**, 150–158.

SMITH, C. M., COHEN, H. L., PELIKAN, E. W. & UNNA, K. R. (1952). Mode of action of antagonists to curare. *J. Pharmacol. exp. Ther.*, **105**, 391–399.

WERNER, G. (1960). Neuromuscular facilitation and antidromic discharges in motor nerves: their relation to activity in motor nerve terminals. *J. Neurophysiol.*, **23**, 171–187.

WESCOE, W. C. & RIKER, W. F. (1951). The pharmacology of anti-curare agents. *Ann. N.Y. Acad. Sci.*, **54**, 438–459.

WESCOE, W. C., RIKER, W. F. & BROTHERS, M. J. (1949). Studies on the inter-relationships of certain cholinergic compounds. I. The pharmacology of 3-acetoxy phenyltrimethylammonium methylsulphate. *J. Pharmacol. exp. Ther.*, **97**, 190–207.

WILSON, I. B. (1955). The interaction of Tensilon and Neostigmine with acetylcholinesterase. *Arch. int. Pharmacodyn.*, **104**, 204–213.