*Brit. J. Pharmacol.* (1962), **19**, 198–218.

# TRIETHYLCHOLINE COMPARED WITH OTHER SUBSTANCES AFFECTING NEUROMUSCULAR TRANSMISSION

BY

W. C. BOWMAN, B. A. HEMSWORTH AND M. J. RAND

*From the Department of Pharmacology, School of Pharmacy, University of London*

(*Received May* 29, 1962)

Triethylcholine (triethyl-2-hydroxyethyl ammonium) has been compared, in its actions on neuromuscular transmission, with the motor end-plate blocking drugs tubocurarine and decamethonium, with the anticholinesterase neostigmine, and with the closely related drug tetraethylammonium. The experiments were carried out on conscious rabbits and mice, on the tibialis anterior muscle of cats under chloralose anaesthesia and on the isolated phrenic nerve-diaphragm preparation of the rat. Anticholinesterase activity was determined manometrically using the Warburg apparatus. Triethylcholine possessed a slight curare-like action, but this effect was shown to be too weak and transient to contribute to the slowly developing and long-lasting transmission failure which occurs selectively in frequently excited nerve-muscle preparations and in exercised conscious animals. It was confirmed that the site of the blocking action of triethylcholine was pre-junctional. Triethylcholine often produced a slight potentiation of the contractions before blocking them. This effect was not due to a depolarizing or an anticholinesterase action, and it was concluded that the slight initial facilitating action of triethylcholine on neuromuscular transmission was due to an increase in the quantity of acetylcholine released by the nerve impulse. Tetraethylammonium was much more powerful than triethylcholine in this respect. The pre-junctional transmission failure produced by triethylcholine could not be explained simply on the basis that an initial excessive release led to exhaustion of transmitter.

The triethyl analogue of choline (triethyl 2-hydroxyethyl ammonium) has been shown to produce a failure of neuromuscular transmission in mammalian nerve-muscle preparations stimulated rapidly through their nerves. At the time of maximum block, contractions of the muscle produced by close-arterially injected acetylcholine were not reduced in tension and it was concluded that the site of the blocking action was the motor nerve endings (Bowman & Rand, 1961a, b).

Roberts (1962), using the technique of iontophoretic micro-application of acetylcholine and intracellular recording, has recently shown in isolated frog muscle that triethylcholine possesses a curare-like action on the motor end-plates together with a pre-junctional facilitatory action through which the amount of acetylcholine released by a motor nerve impulse is increased. In these respects, therefore, triethylcholine appears to resemble the closely related drug tetraethylammonium (Koketsu, 1958).

The present experiments were designed to determine to what extent, if any, a curare-like action and a pre-junctional facilitatory action contribute to the effects of triethylcholine in the mammal.

## METHODS

Maximal twitches and tetani of the tibialis anterior muscles of cats, anaesthetized with intravenous chloralose (80 mg/kg), were elicited by stimulating the sciatic nerves with rectangular pulses of 50 or 100 $\mu$sec 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 preparation of the tibialis anterior muscle for the close-arterial injection of drugs was carried out by the method described by Brown (1938). In some experiments both tibialis anterior muscles were prepared for close-arterial injection, while in others such injections were made only to the more frequently excited muscle. The muscle contractions were recorded on a kymograph by means of flat steel spring myographs, or on a cathode-ray oscilloscope by means of an RCA 5734 mechano-electric transducer strain gauge. In the latter case, muscle action potentials were simultaneously recorded either by belly-tendon electrodes or by concentric needle electrodes.

Experiments were also carried out on the isolated phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946). In many of these experiments, both hemidiaphragms from the same rat were mounted together in McEwen's (1956) solution at 32° C. The muscles were excited by supramaximal rectangular pulses of 100 $\mu$sec duration applied to the phrenic nerves. One was stimulated 1/sec and the other 1/10 sec. The contractions were recorded on a kymograph. Drugs were added to the bath fluid or were injected into the blood vessels supplying the diaphragm by a method similar to that described by Burgen, Dickens & Zatman (1949).

Determinations of acute toxicity in mice, and of the effects of drugs on muscular strength in conscious rabbits, were carried out by the methods described by Bowman & Rand (1961b).

The anticholinesterase activities of triethylcholine and neostigmine were compared manometrically using the Warburg apparatus. Cat tibialis anterior muscles, or frog rectus abdominis muscles, were homogenized in an all-glass homogenizer to give a concentration of 150 mg/ml. in 0.04 M sodium bicarbonate (adjusted to *p*H 7.6 by the addition of hydrochloric acid) and 2.0 ml. of this homogenate was used for each estimation. The inhibitors were dissolved in 0.04 M sodium bicarbonate. Acetylcholine chloride was used as the substrate, the final concentration being 0.0138 M (Aldridge, 1950). The total volume of fluid in each flask was 3.0 ml. The flasks were gassed with a mixture containing 95% nitrogen and 5% carbon dioxide for 10 min and the manometers were read at 10-min intervals for 30 min.

Triethylcholine, tetraethylammonium, choline, acetylcholine and tubocurarine were used as the chlorides, decamethonium as the iodide and neostigmine as the methylsulphate. The doses quoted are in terms of these salts.

## RESULTS

### *Curare-like action of triethylcholine*

In previous experiments on the cat (Bowman & Rand, 1961b) the effect of close-arterially injected acetylcholine in producing contractions of the tibialis anterior muscle was tested after triethylcholine had produced a well-developed depression of the indirectly excited maximal twitches ; the acetylcholine contractions did not differ from those elicited before triethylcholine was injected. Further experiments have now been carried out in which the response to acetylcholine was tested at more frequent intervals after the injection of triethylcholine. Fig. 1 shows that soon after the intravenous injection of triethylcholine (40 mg/kg) there was a small decrease in the responses of both the rapidly and slowly stimulated muscles to close-arterial injection of acetylcholine. The maximal decrease in the response to acetylcholine occurred within 1 to 2 min after the injection of triethylcholine, and 10 to 15 min

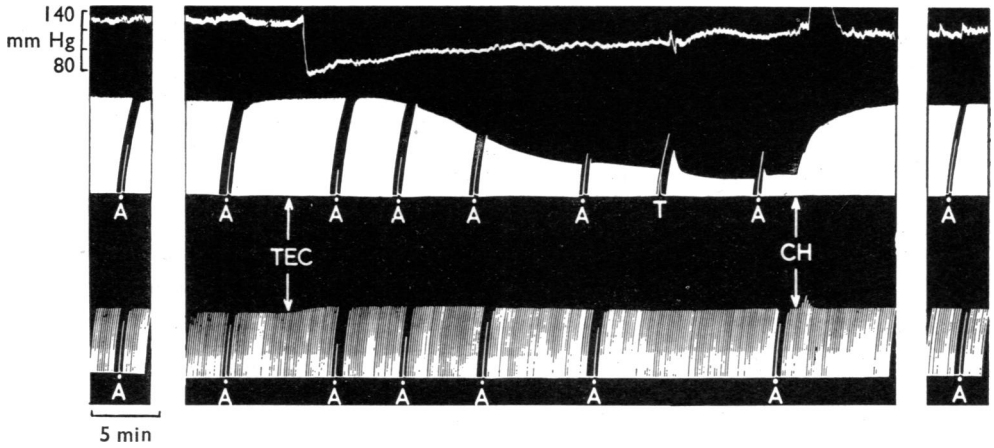


Fig. 1. Cat 3.2 kg. Upper record, blood pressure; middle and lower records, maximal twitches of right and left tibialis anterior muscles elicited indirectly 1/sec and 1/10 sec respectively. At *A*, 4 $\mu$g acetylcholine injected close-arterially to the muscle indicated. Electrical stimulation of the appropriate nerve was temporarily stopped during acetylcholine injections. At *TEC*, 40 mg/kg triethylcholine, and at *CH*, 5 mg/kg choline injected intravenously. At *T*, a tetanus of the right tibialis anterior was elicited indirectly (100/sec for 5 sec).

later the acetylcholine-responses had fully regained their original height. The time-course of the effect of triethylcholine in depressing the responses to acetylcholine was quite different from that of its effect on the maximal motor nerve twitches. During the time that the decrease in twitch tension was developing in the more rapidly stimulated muscle, the responses to acetylcholine were already returning towards the control level so that, as previously reported, the acetylcholine response was unaffected at the time of maximum block of the twitches. Furthermore, although triethylcholine depressed the responses to acetylcholine to about the same extent in both muscles, the decrease in twitch tension occurred only in the more rapidly stimulated muscle.

Bowman & Rand (1961b) reported that an intramuscular injection of triethylcholine was more effective than an intravenous injection in producing muscular weakness in exercised animals. The effects of an intramuscular injection of triethylcholine on the responses of the tibialis anterior to nerve stimulation and to close-arterially injected acetylcholine are shown in Fig. 2. In comparison with the effects of an equally sized intravenous injection of triethylcholine the depression of the responses to nerve stimulation was slower in onset, greater in extent and more prolonged. However, the response to acetylcholine was less affected than it was after intravenous injection of triethylcholine.

The responses of the isolated diaphragm of the rat to intravascular injection of acetylcholine were slightly depressed by triethylcholine (Fig. 3a, b). In contrast to the effect in the cat, the depression of the acetylcholine contractions was not transient, but persisted until the triethylcholine was washed out of the bath when full recovery quickly occurred (Fig. 3b). As in the cat, the depression of the

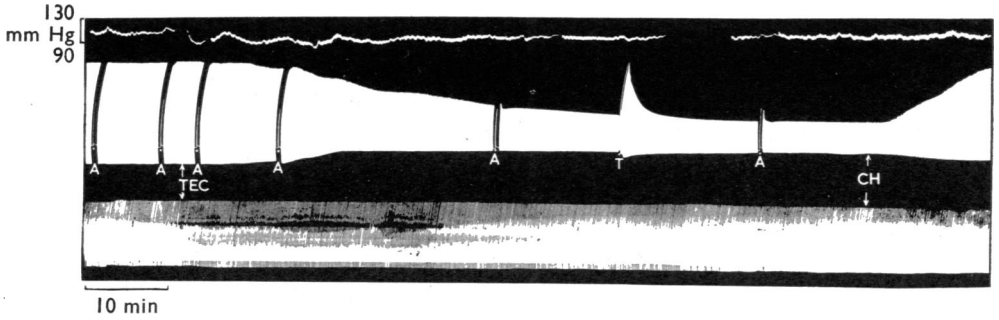


Fig. 2. Cat 3.6 kg. Records as in Fig. 1. At *A*, 5 μg acetylcholine injected close-arterially to the right tibialis anterior muscle. At *TEC*, 25 mg/kg triethylcholine, and at *CH*, 5 mg/kg choline injected intramuscularly into the muscles of the fore-limbs. At *T*, a tetanus of the right tibialis anterior was elicited indirectly (100/sec for 5 sec).

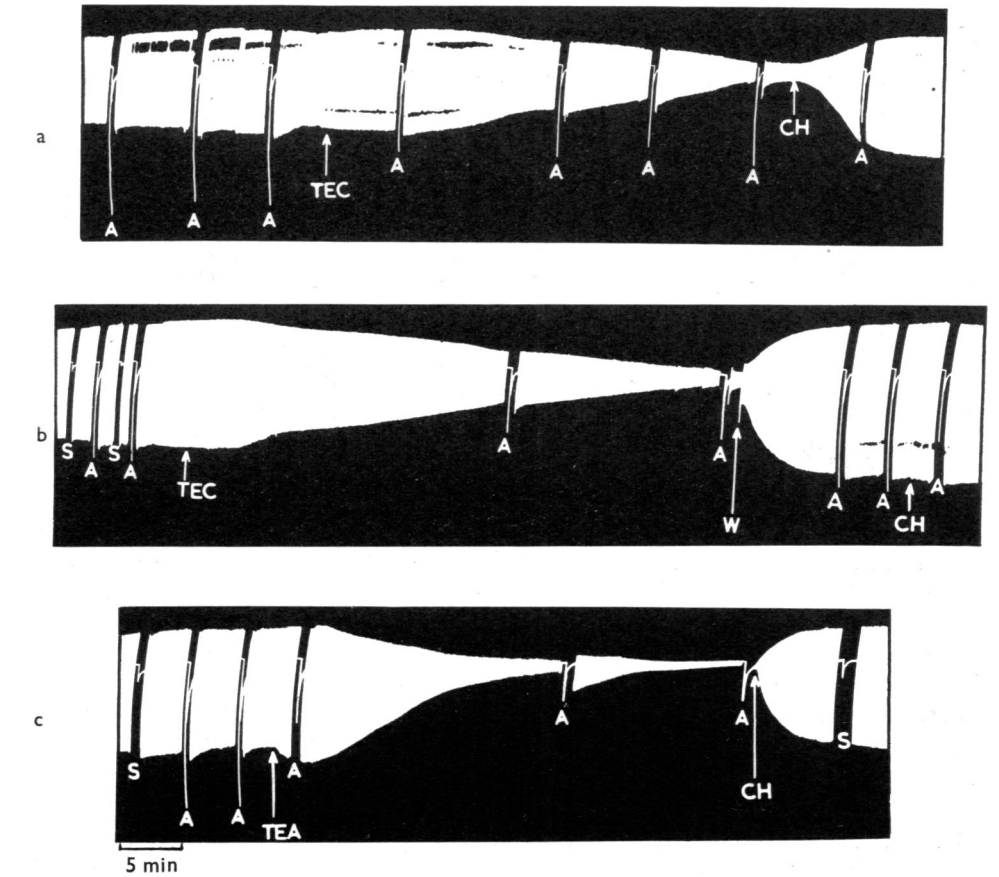


Fig. 3. Records from three different phrenic-nerve diaphragm preparations of rats. Maximal twitches were elicited indirectly once every second except during intravascular injections. At *A*, 10 μg acetylcholine, and at *S*, the same volume of saline injected intravascularly. At *TEC*, triethylcholine (250 μg/ml.); at *TEA*, tetraethylammonium (150 μg/ml.); and at *CH*, choline (60 μg/ml.) added to the bath. At *W* in *b*, the bath fluid was replaced with fresh McEwen's solution without stopping electrical stimulation. Note that the addition of choline in *b* did not affect the twitches, but diminished the response to acetylcholine. (Contractions downwards.)

responses to acetylcholine produced by triethylcholine did not follow the same time-course as the depression of the responses to nerve stimulation.

The effects of triethylcholine on the response to close-arterially injected acetylcholine and on indirectly elicited twitches were compared with the effects of the motor end-plate blocking drugs tubocurarine and decamethonium. Both of these drugs, but particularly tubocurarine, were more effective in blocking the twitches of the more rapidly stimulated muscle (Fig. 4), confirming the results of Preston & van Maanen (1953) and Wislicki (1958). However, increased susceptibility of

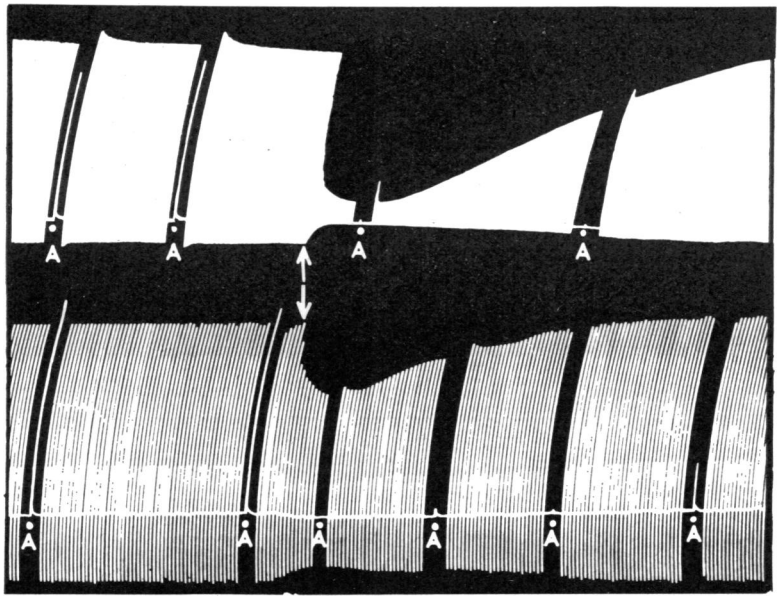


Fig. 4. Cat 2.9 kg. Maximal twitches of the right (upper record) and left (lower record) tibialis anterior muscles elicited indirectly 1/sec and 1/10 sec respectively except during acetylcholine injections. At *A*, 8 $\mu$g acetylcholine injected close-arterially to the muscle indicated. At the arrows, 250 $\mu$g/kg tubocurarine injected intravenously.

rapidly stimulated nerve-muscle preparations to block by tubocurarine was less pronounced than it was with triethylcholine (Bowman & Rand, 1961b). When partial neuromuscular block of the indirect twitches was produced by tubocurarine or by decamethonium, both in the cat and in the isolated rat diaphragm, the contractions of the muscle produced by injected acetylcholine were completely abolished. The time of the maximum depression of the two types of response coincided. Fig. 4 illustrates an experiment on a cat in which the dose of tubocurarine used caused an 85% block of the more frequently excited muscle and a 30% block of the more slowly stimulated contralateral muscle; the response to injected acetylcholine was completely abolished in both muscles and was still small 1 hr later even though the twitches fully recovered within 20 min. In order to produce, at the height of such a tubocurarine block, a response to injected acetylcholine comparable to that elicited before tubocurarine, it was necessary to increase the dose of acetylcholine about 20-fold.

Fig. 5 illustrates an experiment in which very large doses of triethylcholine were injected in an attempt to mimic the effects of tubocurarine shown in Fig. 4. A total of 0.5 g/kg of triethylcholine was necessary in order to produce a 40% depression of twitches elicited once every 10 sec, and this amount of triethylcholine completely abolished the response to acetylcholine. Despite the large dose given, the block was short-lasting, full recovery of the twitches occurring within about 15 min. A further dose of 0.25 g/kg of triethylcholine was injected to depress the twitches, and then a tetanic stimulus applied to the nerve produced a contraction which was

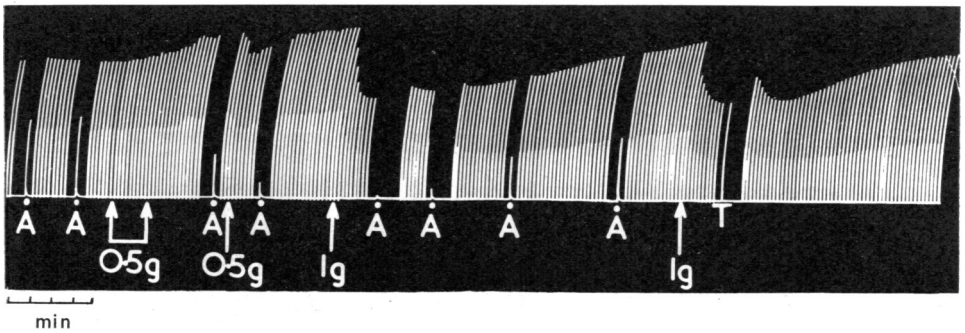


Fig. 5. Cat 4.0 kg. Maximal twitches of a tibialis anterior muscle elicited indirectly once every 10 sec. At *A*, 4 $\mu$g acetylcholine injected close-arterially. At the arrows, triethylcholine injected intravenously in the doses indicated. At *T*, a tetanus was elicited indirectly (100/sec for 10 sec), the kymograph speed being increased during the tetanus. Note the similarity to a twitch and compare with Fig. 7*c*.

unsustained to the extent that it resembled a twitch. The effect of a large dose of triethylcholine in depressing tetanic contractions of the muscle was also studied by displaying the muscle tension and action potentials on the oscilloscope (Fig. 7c). It can be seen that after triethylcholine the muscle responded only to the first 3 or 4 shocks of the tetanus.

Similar results were obtained using the rat diaphragm preparation. A concentration of 5 to 8 mg/ml. of triethylcholine was necessary to block twitches elicited once every 10 sec ; these concentrations of triethylcholine completely abolished the response to acetylcholine.

These results show that large doses of triethylcholine can produce a neuromuscular blockade which is a consequence of a depressed sensitivity of the motor end-plates, but the amounts of triethylcholine required, in both the cat and the rat preparations, were about 30 times greater than those which selectively depressed twitches elicited once every sec.

### *Potentiation of contractions produced by triethylcholine*

Bowman & Rand (1961b) noticed that triethylcholine produces a slight potentiation of the maximal twitch ; this effect precedes the block which develops in the more rapidly stimulated muscle and can be seen after intravenous injection in the cat in Figs. 1, 5, 6, 7b, 8 and 9. The effect was small and amounted only to 5 to 10% even

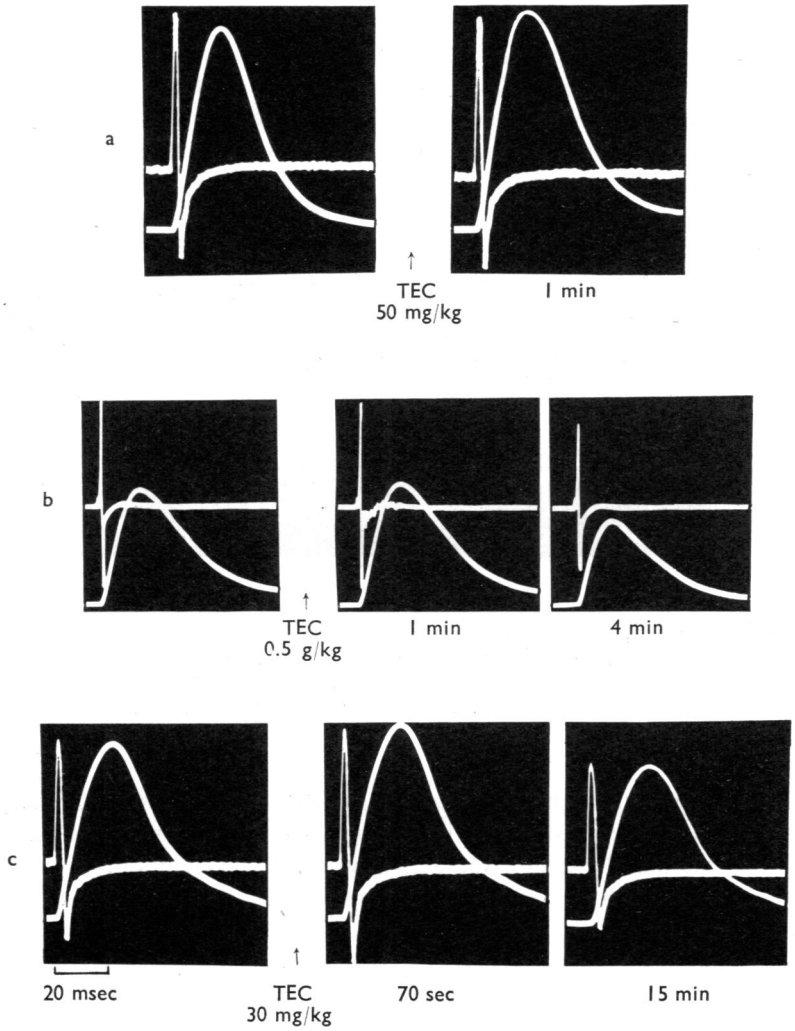


Fig. 6. (*a*) Cat 2.4 kg, (*b*) cat 2.7 kg, (*c*) cat 3.3 kg. Maximal isometric twitches and action potentials of tibialis anterior muscles elicited indirectly and recorded on a cathode-ray oscilloscope. The left-hand records were taken immediately before the intravenous injection of triethylcholine (TEC). The times below the remaining records indicate the times after injection. (*a*) Fresh preparation stimulated 1/10 sec. TEC caused a slight increase in twitch tension but no detectable change in the gross action potential recorded with belly-tendon electrodes. (*b*) Fresh preparation stimulated 1/10 sec. The increase in twitch tension produced by TEC was accompanied by action potentials showing slight repetitive firing (concentric needle electrodes). 4 min later the twitches were partially blocked. Compare Fig. 5. (*c*) Preparation previously stimulated for 4 hr at 1/10 sec but stimulated 1/sec during period illustrated. The increase in twitch tension produced by TEC was accompanied by a corresponding increase in the amplitude of the gross action potential (belly-tendon electrodes). 15 min later the twitches were partially blocked.

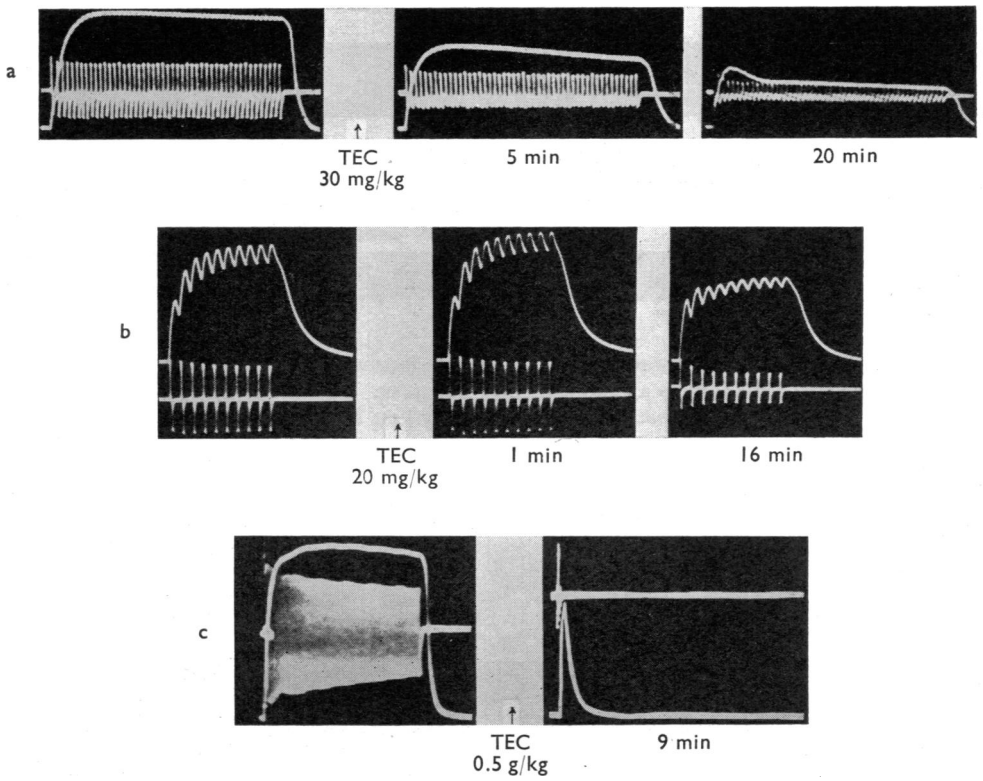


Fig. 7. (*a*) Cat 2.1 kg; (*b*) cat 3.4 kg; (*c*) cat 3.0 kg. Isometric tetanic tension and action potentials (belly-tendon electrodes) of tibialis anterior muscles elicited indirectly by stimulation with supramaximal shocks and recorded on a cathode-ray oscilloscope. Tetani were elicited every 5 min throughout each experiment. Between tetani, supramaximal single shocks were applied 1/sec in *a* and *b* and 1/10 sec in *c*. The left-hand records are examples of constant responses before the intravenous injection of triethylcholine (TEC). The times below the remaining records indicate the times after injection. (*a*) Fresh preparation. Tetanic stimulation: 50/sec for 1 sec. TEC depressed the contractions without initially augmenting them. (*b*) Preparation after stimulation at 1/sec for 1 hr. Tetanic stimulation: 20 sec for 0.5 sec. TEC initially augmented the contractions. (*c*) Fresh preparation. Tetanic stimulation: 100/sec for 1 sec. 9 min after TEC, tetanic tension was markedly unsustained. (Compare tetanus in Fig. 6.)

when very large doses of triethylcholine (0.5 g/kg) were given (Figs. 5 and 6b). No potentiation was observed in either rapidly or slowly stimulated muscles when triethylcholine was injected intramuscularly (Fig. 2).

In order to study more closely the factors responsible for the increase in twitch tension produced by triethylcholine experiments were carried out in which isometric tension and muscle action potentials were displayed simultaneously on an oscilloscope. Fig. 6 illustrates three experiments which show the largest degree of twitch potentiation obtained with triethylcholine in the cat. The subsequent degree of block which occurred in two of these experiments is also illustrated. In a fresh preparation, the twitch potentiation was usually unaccompanied by any detectable

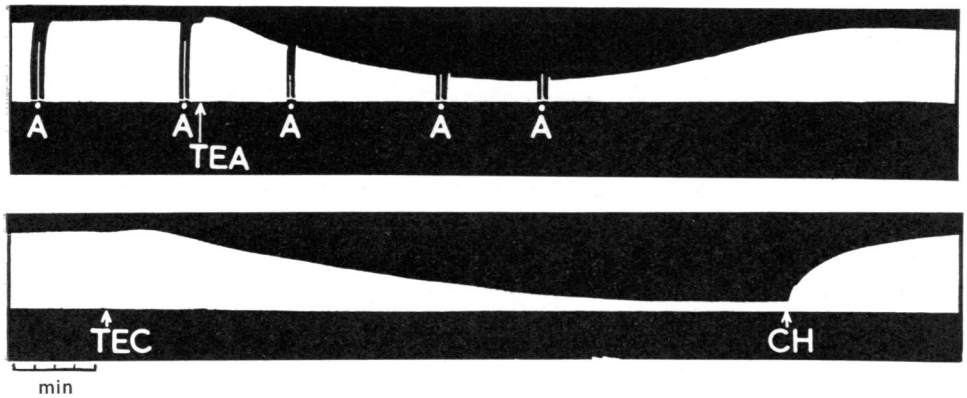


Fig. 8. Cat 3.2 kg. Maximal twitches of a tibialis anterior muscle elicited indirectly once every second. The lower record is a continuation of the upper. At *A*, 3 $\mu$g acetylcholine injected close-arterially. At TEA, tetraethylammonium (20 mg/kg); at TEC, triethylcholine (20 mg/kg); and at CH, choline (5 mg/kg) injected intravenously.

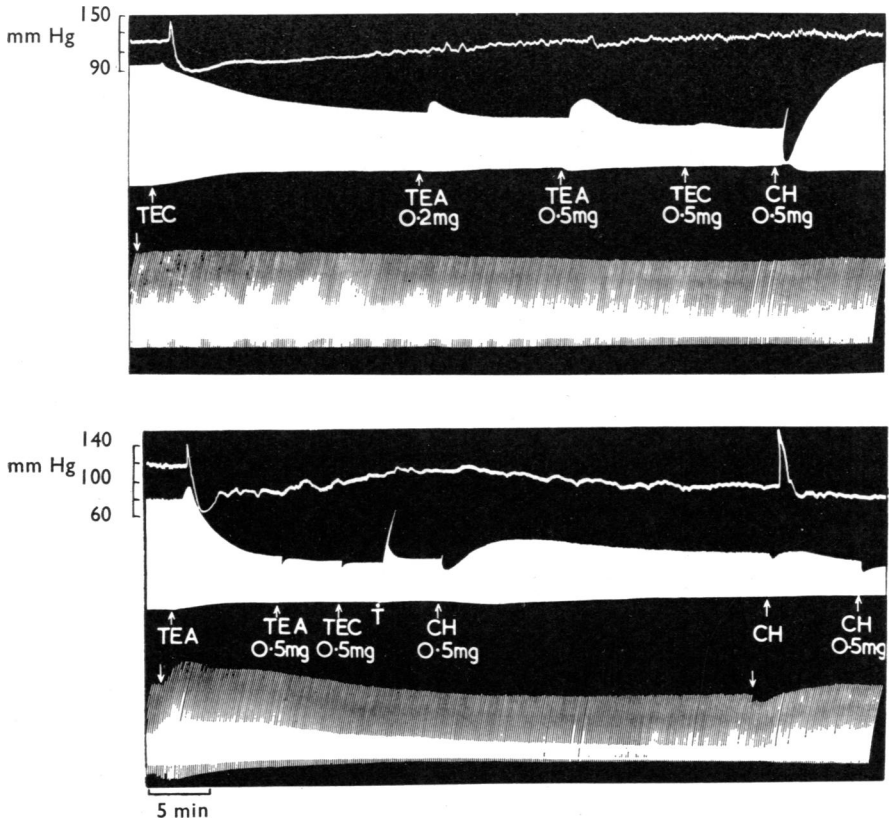


Fig. 9. Cat 2.7 kg. Records as in Fig. 1. The lower tracings are a continuation of the upper. At *TEC*, triethylcholine; at *TEA*, tetraethylammonium; and at *CH*, choline was injected. Single arrows indicate close-arterial injections of the drugs to the more frequently excited muscle. Double arrows indicate intravenous injections of 20 mg/kg TEC, 20 mg/kg TEA and 5 mg/kg CH. At *T*, a tetanus of the right tibialis anterior muscle was elicited indirectly (100/sec for 5 sec).

change in the gross muscle action potential as recorded with belly-tendon leads (Fig. 6a). However, when a concentric needle electrode was used to record the muscle action potentials, a small degree of repetitive firing was occasionally observed to accompany the increase in twitch tension (Fig. 6b). The appearance of repetitive firing in the record depended on the position of the needle electrode. When it was moved to other positions during the potentiation, it was usually possible to record action potentials not showing repetitive firing. These results indicate that in a fresh preparation, the small initial increase in twitch tension produced by triethylcholine is a consequence of repetitive firing occurring in a few motor units.

In preparations which had been stimulated for some time before studying the effects of drugs, comparison of the records with those at the beginning of the experiment often showed that the twitch tension and the amplitude of the gross action potential had become smaller by about 5 to 10% despite the fact that the nerve shocks remained supramaximal. This occurred particularly in rapidly stimulated preparations, but was also evident in infrequently excited muscle (1/10 sec) after long periods of stimulation. It appeared to be caused by a small degree of transmission failure possibly related to that described in rat muscle by Krnjević & Miledi (1958). In preparations showing slight transmission failure, the potentiating action of triethylcholine, though still small, was more pronounced than that in a fresh preparation. The records shown in Fig. 6c are from an experiment on a cat in which maximal twitches of the tibialis anterior had been elicited once every 10 sec for about 4 hr. The potentiation of the twitches produced by triethylcholine in this experiment was accompanied by a corresponding increase in the amplitude of the gross action potential, showing that recruitment of previously dormant fibres was an additional factor contributing to the effect in these circumstances.

Triethylcholine did not usually augment tetanic contractions of the muscles, either in fresh preparations or in those which had been stimulated for some time. The usual effect was a reduction in tension and in the ability of the muscle to sustain it (Fig. 7a); these effects were more pronounced the larger the dose of triethylcholine and the higher the frequency of tetanic stimulation (Fig. 7c). However, in preparations which showed a small degree of transmission failure, it was possible, providing the frequency of tetanic stimulation was low (10 to 20/sec), to produce a small initial potentiation of the contractions with small doses of triethylcholine (Fig. 7b).

The potentiating actions of triethylcholine described above could arise either through inhibition of cholinesterase or through an increased release of acetylcholine from the motor nerve endings. The possibility that triethylcholine exerts its effects through either or both of these mechanisms was therefore investigated.

### *Anticholinesterase activity of triethylcholine*

There is some superficial resemblance between the effects of anticholinesterases and of triethylcholine on skeletal muscle contractions. Thus when the frequency of nerve stimulation is low, anticholinesterases potentiate the maximal twitch, but at high rates of stimulation the maximal twitches are depressed (Bacq & Brown, 1937). Furthermore, in concentrations of 0.3 mg/ml., triethylcholine slightly potentiated the response of the frog rectus abdominis muscle to acetylcholine.

Experiments with the Warburg apparatus showed that the ability of triethylcholine to inhibit the acetylcholinesterase in homogenates of cat tibialis anterior muscles was very weak. The concentration of triethylcholine necessary to cause 50% inhibition of the enzyme was $2 \times 10^{-1}$ M, compared with $3 \times 10^{-7}$ M for neostigmine studied under the same conditions. A similar concentration of triethylcholine was necessary to cause 50% inhibition of the acetylcholinesterase present in homogenates of frog skeletal muscle. These results make it unlikely that any of the effects of triethylcholine on neuromuscular transmission are due to cholinesterase inhibition. Nevertheless, experiments were carried out in which the actions of triethylcholine were compared with those of neostigmine on the maximal twitches of the cat tibialis anterior muscle. The following differences were noted. The twitch potentiation caused by neostigmine was greatly in excess of that caused by triethylcholine. The subsequent block of transmission in the rapidly stimulated muscle was smaller and shorter in duration with neostigmine. When the dose of neostigmine was large enough to reduce the twitches of the muscle stimulated once every second, some reduction in the twitches of the contralateral muscle stimulated once every 10 seconds was also apparent. Only when the frequency of stimulation of the more rapidly excited muscle was increased to 3/sec was it possible to demonstrate a significant difference in the susceptibilities of the two muscles to the blocking action of neostigmine. During partial block produced by neostigmine, a tetanus applied through the nerve caused a subsequent decrease in the maximal twitches whereas post-tetanic potentiation was evident during triethylcholine block (Figs. 1 and 2). Contractions of the muscle produced by close-arterially injected acetylcholine were abolished at the height of a neostigmine block and the subsequent twitches were further reduced in tension; in contrast the response to injected acetylcholine was unaffected at the height of a triethylcholine block and the subsequent twitches were slightly increased in tension (Figs. 1 and 2). Finally, the injection of choline (5 mg/kg) increased the block produced by neostigmine but reversed that produced by triethylcholine (Figs. 1, 2, 3, 8).

The neuromuscular blocking action of neostigmine at high frequencies of stimulation can be ascribed largely to depolarization of the motor end-plates due to persistence of acetylcholine (Burns & Paton, 1951), but the blocking action of triethylcholine is clearly quite different.

### *Comparison with tetraethylammonium*

A second mechanism which might give rise to potentiation of the maximal twitch is an action on the motor nerve endings through which the amount of acetylcholine released by a nerve impulse is increased. Such an action has been ascribed to the closely related drug tetraethylammonium to account for its anticurare action in the mammal (Stovner, 1958), and Koketsu (1958) independently reached similar conclusions based on experiments in the frog. More recently Roberts (1962), who also worked with frog muscle, described actions of triethylcholine similar to those described by Koketsu (1958) for tetraethylammonium. Because of these findings, parallel studies of the effects of tetraethylammonium and of triethylcholine were made in the present experiments on mammals.

*Neuromuscular blocking action.* Tetraethylammonium showed a similar selectivity to triethylcholine in depressing the contractions of the more frequently excited muscle, both in the cat tibialis anterior muscle (Fig. 9) and in the rat diaphragm preparation. In the rat diaphragm preparation tetraethylammonium was almost twice as potent as triethylcholine in depressing the contractions of the more frequently excited muscle. In the cat, accurate comparison of the relative potencies was difficult since the previous administration of one potentiated the blocking action of the other. As far as could be estimated, the two drugs were about equipotent by intravenous injection. After intramuscular injection, however, triethylcholine was considerably more potent than tetraethylammonium.

During the depression of the maximal twitches of both the cat tibialis anterior and the rat diaphragm preparations produced by tetraethylammonium, contractions elicited by injected acetylcholine were depressed to a greater extent than they were during a comparable degree of block produced by triethylcholine. Fig. 8 illustrates the effect of tetraethylammonium on maximal twitches and on contractions produced by injected acetylcholine in the tibialis anterior muscle of the cat, and Fig. 3c illustrates a similar experiment on the rat diaphragm. These results show that the weak curare-like action of tetraethylammonium on the motor end-plates, which has been demonstrated by several workers (Atkinson, 1952 ; Jepson, Simeone & Lynn, 1953), plays a more important part than that of triethylcholine. However, the curare-like action of tetraethylammonium is too weak to account entirely for its selective effect in depressing the contractions of the rapidly stimulated muscle. This can be seen by comparing Fig. 8 with Fig. 4 ; when the block is entirely due to an action on the motor end-plates as with tubocurarine, the response to acetylcholine is completely abolished even though the maximal twitches are only partially depressed (Fig. 4). The depression of the twitches produced by tetraethylammonium in the cat was occasionally irreversible and resulted in complete failure of the more frequently excited muscle.

At the height of the depression of the twitches of the isolated rat diaphragm produced either by triethylcholine or by tetraethylammonium, changing the bath fluid for fresh McEwen's (1956) solution resulted in a restoration of the twitches to normal within 2 to 3 min, and this occurred despite continuous stimulation at a frequency of 1/sec. Fig. 3b illustrates this effect during block produced by triethylcholine.

A difference between the actions of tetraethylammonium and triethylcholine was evident in the cat when small doses of one of them were injected close-arterially during block produced by the other. Thus tetraethylammonium, in doses of 0.2 to 0.5 mg injected close-arterially into the tibialis anterior muscle, produced a small antagonism of triethylcholine block (Fig. 9a). However, the same doses of triethylcholine did not antagonize tetraethylammonium block (Fig. 9b) and larger doses increased the paralysis.

With doses of triethylcholine and tetraethylammonium which subsequently produced the same degree of depression of the twitches the initial potentiation of the twitches was more marked with tetraethylammonium (Figs. 3, 8 and 9).

*Anticurare actions.* Stovner (1957, 1958) demonstrated the ability of tetraethylammonium to restore the contractions of a muscle after transmission had been depressed by tubocurarine or by lack of calcium. These observations have been confirmed in the present study. According to Stovner, the introduction of a $\beta$-hydroxy group to form triethylcholine resulted in a complete loss of the properties

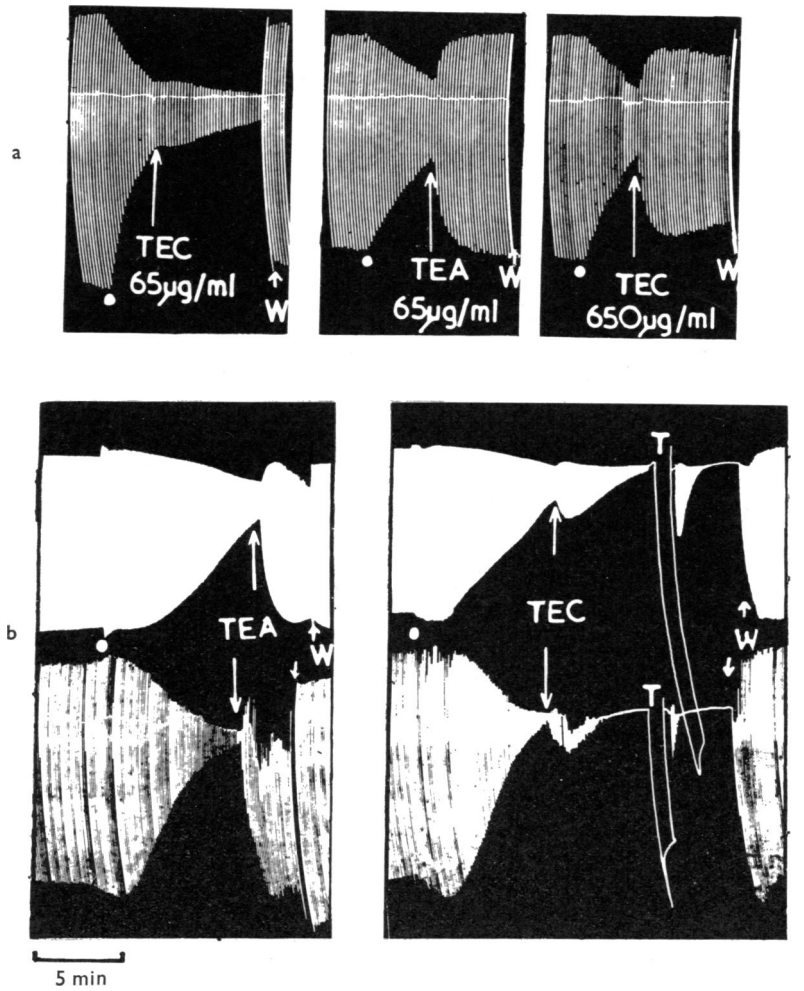


Fig. 10. Indirectly elicited maximal twitches of isolated diaphragm preparations of the rat. (*a*) Stimulation 1/10 sec. At the white dots, 1 µg/ml. tubocurarine added to the bath. At *TEC*, triethylcholine, and at *TEA*, tetraethylammonium was added to the bath in the concentrations shown. At W, the bath fluid was replaced with fresh McEwen's solution (temp. 32° C). (*b*) Both hemidiaphragms mounted in the same bath. Stimulation 1/sec (upper record) and 1/10 sec (lower record). At the white dots, the McEwen's solution was exchanged for a Ca-free solution and at *W* this was replaced by fresh McEwen's solution. At *TEA*, tetraethylammonium (1 mM), and at *TEC*, triethylcholine (2 mM) was added to the bath. At *T*, a tetanus (100/sec for 10 sec) was elicited on a fast kymograph (temp. 20° C). (Contractions downwards.)

which were characteristic of tetraethylammonium. In the present experiments, however, triethylcholine was shown to possess a weak anticurare action and a slight ability to restore the contractions of the diaphragm depressed by lack of calcium. Fig. 10 illustrates these effects of tetraethylammonium and of triethylcholine. When the maximal twitches were depressed by tubocurarine to about 10 or 20% of their original level, tetraethylammonium in concentrations of 50 to 150 $\mu$g/ml. added to the fluid bathing the isolated diaphragm, or in doses of 0.2 to 0.3 mg injected close-arterially to the cat tibialis anterior muscle, completely restored them to normal. Triethylcholine had a similar anticurare action, but doses about 10 times greater were required (Fig. 10a).

Stovner (1957) showed that in calcium-free solutions tetraethylammonium was most effective in restoring neuromuscular transmission in the isolated rat diaphragm when the temperature of the bath fluid was low (22° C). The optimal concentration of tetraethylammonium was 1 mM. The present experiments were therefore carried out at room temperature (20° C). When maximal twitches of the diaphragm were depressed through calcium lack to about 20% of their original tension, tetraethylammonium in 1 mM concentrations restored the twitches to normal (Fig. 10b). Triethylcholine, however, possessed only a very weak ability to restore the contractions of the diaphragm in concentrations up to 10 mM (Fig. 10b).

*Weakness after exercise.* In conscious animals triethylcholine produces a slowly developing muscular weakness which is accentuated by exercise (Bowman & Rand, 1961a, b). This action of triethylcholine is more pronounced than might be expected from results obtained in nerve-muscle preparations where maximal twitches are elicited once every second. Provided that the animal is exercised, doses of triethylcholine as low as 5 mg/kg given intravenously produce a definite weakening of muscular strength and the same dose given intramuscularly produces a more pronounced effect. Animals usually survive a dose of 75 mg/kg given intravenously if they are kept quiet, but if exercised they die from respiratory failure within 5 to 10 min, probably because the increased respiratory drive accompanying the exercise leads to transmission failure in the respiratory muscles. The more pronounced effects of triethylcholine in producing weakness in exercised conscious animals may be explained by the higher rates of nerve firing which occur in voluntary movements.

The effects of triethylcholine and of tetraethylammonium were compared after injection in conscious rabbits. At 5 min intervals after injection, the righting reflex was tested up to 20 times in rapid succession and the trial at which the rabbit failed to right itself was noted. The rabbits were left undisturbed between tests. Fifteen min after 10 mg/kg triethylcholine given intravenously the rabbits lost the strength to right themselves after 6 or 7 trials. This degree of weakness lasted during the next 15 to 20 min, then the rabbits gradually recovered so that by 60 to 80 min after the injection they responded vigorously to all 20 righting trials. The same dose of triethylcholine given intramuscularly produced more pronounced and longer-lasting effects. After tetraethylammonium, in doses up to 20 mg/kg intravenously, the rabbits continued to right themselves to all 20 trials. After intravenous doses of 30 mg/kg of tetraethylammonium, some rabbits showed a muscular weakness which developed 4 to 5 min after injection and which was not dependent on

exercise. The lethal dose of tetraethylammonium in rabbits was about 50 mg/kg. In further contrast to triethylcholine, tetraethylammonium was even less effective after intramuscular injection.

*Choline antagonism.* It was previously shown that choline restored the contractions of a muscle depressed by triethylcholine (Bowman & Rand, 1961a, b).

In the rat diaphragm preparation, contractions depressed by triethylcholine or by tetraethylammonium were equally well restored by choline (Fig. 3a and b). In the cat tibialis anterior muscle, however, triethylcholine block was more effectively antagonized by choline than was the block produced by tetraethylammonium. Reversal of tetraethylammonium block by choline was fully effective only when the degree of block was small. Figs. 1, 2 and 8b illustrate the marked antagonism of triethylcholine block by choline in the cat and Fig. 9b illustrates the much weaker antagonism of tetraethylammonium block.

In the cat, choline, whether injected intravenously (Figs. 1 and 8b), close-arterially (Fig. 9a) or intramuscularly (Fig. 2), antagonized triethylcholine. With close-arterial injections the antagonism was usually preceded by a transient reduction in the twitches (Fig. 9a). With intravenous injections of less than 5 mg/kg complete antagonism was produced without initial increase in the block (Figs. 1 and 8b), but with larger doses of choline an increase in the block preceded the antagonism. Doses of choline which produced this biphasic effect were sufficient to produce a transient block of the maximal twitches of normal muscle. This transient block had the characteristics of block by depolarization (Hutter, 1952; Bowman & Rand, 1962); it was less pronounced during the depression of the twitches produced by tetraethylammonium than during that produced by triethylcholine (Fig. 9), and this finding is in accord with the more powerful curare-like action of tetraethylammonium. The local concentration of choline resulting from intravenous injection, though sufficient to exert a powerful antagonistic action to triethylcholine, was presumably too small to cause persistent end-plate depolarization and consequent block. Choline was equally effective by intravenous and intramuscular routes in reversing triethylcholine block, although by the latter route the action was slower in onset (Fig. 2). Intramuscular injections of choline did not produce an initial depolarization block. Furthermore, intramuscular injections of choline in doses sufficient to cause maximal antagonism of triethylcholine block were completely without effect on the arterial blood pressure (Fig. 2). Choline injected intravenously or intramuscularly reversed the muscular weakness produced by triethylcholine in exercised rabbits, but was without any clear effect on the generalized weakness produced by large doses of tetraethylammonium.

The toxicity of triethylcholine in mice is antagonized by choline (Keston & Wortis, 1946; Bowman & Rand, 1961a, b). Thus, one-quarter of the LD50 of choline caused a 7-fold increase in the LD50 of triethylcholine (Bowman & Rand, 1961b). In the present experiments this observation was confirmed, and in the same batch of mice the effect of choline on the toxicity of tetraethylammonium was tested. The maximal effect of choline was again obtained with one-quarter of its LD50, which increased the LD50 of tetraethylammonium by a factor of only 1.8 (from 125 mg/kg to 225 mg/kg).

During the antagonistic action of choline against triethylcholine in the cat tibialis anterior muscle, the response of the muscle to injected acetylcholine was usually slightly increased (Fig. 1). In the rat diaphragm, on the other hand, the response to acetylcholine was reduced even though choline caused a similar reversal of the failure in neuromuscular transmission. This action of choline in depressing the response of the diaphragm to acetylcholine was also observed in the absence of triethylcholine although the dose of choline used had no effect on the height of the maximal twitches (Fig. 3b). These differences might be explained by the different actions of choline on the motor end-plates in the two species. In the cat, choline blocks by depolarization (Hutter, 1952; Bowman & Rand, 1962); but in the rat diaphragm, choline produces dual block. The fact that choline reverses a triethylcholine block in both preparations suggests that the reversal is not related to the actions of choline at the motor end-plates.

### *Actions of acetyltriethylcholine*

Triethylcholine is acetylated by rat brain choline acetylase almost as effectively as is choline (Burgen, Burke & Desbarats-Schonbaum, 1956) and the possibility was considered (Bowman & Rand, 1961b) that triethylcholine might be acetylated in the nerve endings and the acetylated compound then released as a "false transmitter." However, whether or not this actually occurs, the transmission failure produced by triethylcholine is probably solely a consequence of a reduction in the acetylcholine output since acetyltriethylcholine was completely devoid of action at the neuromuscular junction in amounts greatly in excess of any ester that might be released from nerve endings. Thus doses of acetyltriethylcholine up to 0.3 mg neither caused contraction nor reduced the maximal indirectly elicited twitches when injected close-arterially into the tibialis anterior muscle of the cat. Furthermore, when acetyltriethylcholine (5 to 50 $\mu$g) was mixed with acetylcholine (2 to 10 $\mu$g) and the two injected together close-arterially, the resulting contraction did not differ from that produced by the same dose of acetylcholine injected alone in the same volume

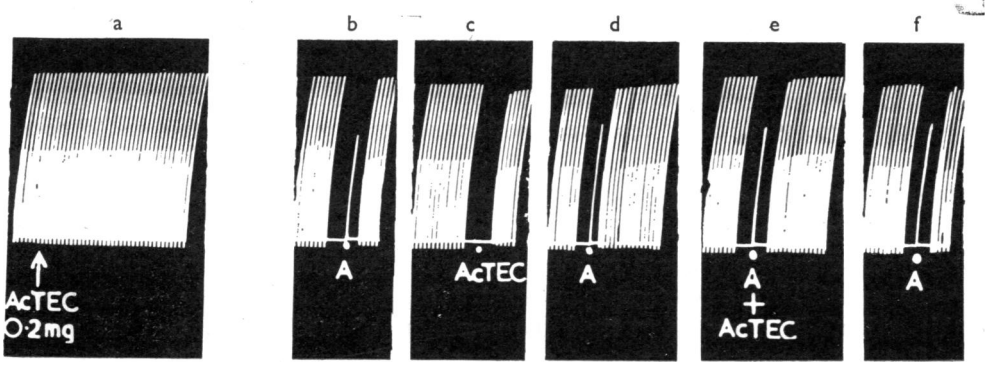


Fig. 11. Cat 3.0 kg. Maximal twitches of tibialis anterior muscle elicited once every 10 sec. All injections close-arterially in the same volume (0.2 ml.). (*a*) 0.2 mg. acetyltriethylcholine; (*b*), (*d*) and (*f*) 5 $\mu$g acetylcholine; (*c*) 50 $\mu$g acetyltriethylcholine; (*e*) 5 $\mu$g acetylcholine mixed with 50 $\mu$g acetyltriethylcholine. In *b* to *f*, electrical stimulation was stopped during the close-arterial injections.

(Fig. 11b). Holton & Ing (1949) showed that acetyltriethylcholine did not cause contracture of the frog rectus, nor did it depress the responses of the rectus to acetylcholine.

## DISCUSSION

One property expected of any ethonium ion is a curare-like action on the motor end-plates, and our results showed that in the mammal, as in the frog (Roberts, 1962), triethylcholine possesses such an action, but only to a very weak degree. In the cat, the curare-like action of triethylcholine was too weak and transient to contribute to the slowly developing and long-lasting transmission failure which occurred selectively in the more frequently excited muscle. As previously reported (Bowman & Rand, 1961a and b), the block produced by triethylcholine was clearly a consequence of a pre-junctional action. In the isolated diaphragm, the slight curare-like action of triethylcholine persisted throughout the time that the substance was present in the bath fluid. This difference between the results obtained in the whole animal and in the isolated preparation is probably due to the re-distribution or elimination of the drug which can occur *in vivo*. Thus the blood concentration is probably quickly lowered to a level which is too small to exert a curare-like action. Support for this conclusion lies in the finding that after intramuscular injections of triethylcholine, which would result in smaller peak blood concentrations than intravenous injections, the responses to acetylcholine were depressed to a lesser extent, yet there was a more powerful depressant action on the rapidly elicited twitches. Presumably the more prolonged persistence of the drug in the circulation favoured the development of the pre-junctional action.

Experiments, in which the effects of a drug in blocking maximal indirectly elicited twitches and in antagonizing the responses to injected acetylcholine are studied simultaneously, provide a sensitive method of determining to what extent a curare-like action of the drug contributes to its effects. Drugs which possess a powerful curare-like action, in doses which produce only a partial depression of the indirectly excited maximal twitches, completely abolish contractions of a muscle produced by injected acetylcholine. This is explained by the fact that the amount of acetylcholine released from the nerve endings is considerably greater than that necessary to produce the critical degree of end-plate potential necessary for a propagated muscle excitation wave (Brown, 1937 ; Kuffler, 1942). Therefore, some degree of end-plate block can occur without depression of the responses to nerve stimulation.

Bowman & Rand (1961a and b) concluded that the transmission failure produced by triethylcholine in rapidly excited mammalian nerve muscle preparations was a consequence of a reduced output of acetylcholine from the nerve endings, but Roberts (1962) in his experiments on isolated frog muscle was unable to demonstrate this action. However, apart from the possibility of species difference, the nature of Roberts's experiments was such as to exclude the occurrence of this effect. He used only low rates of nerve stimulation, but, in order to produce a pre-junctional transmission failure with triethylcholine, it is necessary to stimulate the nerve rapidly and continuously. Roberts used the technique of iontophoretic micro-application of acetylcholine to the motor end-plates and intracellular recording. With these

techniques the composition of the artificial bathing medium is deliberately adjusted to prevent muscle contractions, which would dislodge the microelectrode. This is achieved either by the addition of tubocurarine, which itself may influence the results obtained, or by altering the concentration of calcium and magnesium ions so that the quantity of transmitter released by a nerve impulse is too small to excite the muscle. In the latter case, any possibility of depleting the nerve endings of pre-formed transmitter, even with rapid stimulation, is probably prevented.

Hemicholinium is another substance which has been shown to cause transmission failure by a pre-junctional action (for a review, see Schueler, 1960) and the pattern of its effects resembles that of triethylcholine. This drug is a bisquaternary compound with an aromatic nucleus, and, as might be expected, it also possesses a curare-like action (Martin & Orkand, 1961 ; Bowman & Rand, 1961b ; Thies & Brooks, 1961 ; Hofmann, Feigen & Genther, 1962). Some authors have reported the curare-like action of hemicholinium to be the only blocking action produced. However, as in Roberts's experiments with triethylcholine, their choice of experimental methods precluded the demonstration of a pre-junctional blocking effect.

The species of animal used also appears to be an important factor. The pre-junctional blocking action of hemicholinium and of triethylcholine can be readily demonstrated in mammals, but experiments with hens and frogs have been unsuccessful. M. Totty, working in this laboratory (unpublished observation), found that intravenous injections of hemicholinium in the decerebrate frog produced only a curare-like depression of the rapidly elicited twitches of the gastrocnemius muscle, and triethylcholine, in doses up to 5 mg, caused only a potentiation of the twitches similar to that described by Roberts (1962). In other unpublished work from this laboratory, L. C. Blaber was unable to demonstrate that hemicholinium possessed a pre-junctional blocking action in the hen gastrocnemius muscle ; however, Reitzel & Long (1959) found that hemicholinium blocked indirectly excited contractions, but not acetylcholine contractures. One of us (B.A.H.) was unable to demonstrate a pre-junctional blocking action of triethylcholine in the isolated innervated biventor cervicis muscle of the chick although a myasthenic-like weakness was produced by exercise in conscious chicks (Bowman & Rand, 1961b).

In the present experiments, the effects of triethylcholine were compared with those of the closely related drug tetraethylammonium. There was evidence of differences in the type of block produced by the two drugs, particularly in the cat nerve-muscle preparation, and in conscious animals we were unable to demonstrate, with tetraethylammonium, the muscular weakness characteristic of the action of triethylcholine. Nevertheless, the similarities were such as to suggest that tetraethylammonium shares, to some extent, the pre-junctional blocking action of triethylcholine.

Roberts (1962) demonstrated the ability of triethylcholine to potentiate the maximal indirect twitches of frog muscle and this effect was accompanied by repetitive firing of the muscle fibres. The substance also possessed an anticurare action. Similar effects were observed in the present experiments on mammalian muscle, but they were slight and insignificant compared with those observed by Roberts in frog muscle. These experiments enabled Roberts to conclude that the facilitating effect of triethylcholine on neuromuscular transmission was a consequence

of its ability to increase the amount of acetylcholine released in response to a nerve volley. Triethylcholine is devoid of depolarizing action and of significant anti-cholinesterase action, and its only effect on the motor end-plates appears to be a weak depressant one. It seems likely, therefore, that an increase in transmitter output must also have been responsible for the weak facilitatory effects observed in the present experiments on mammals. However, the facilitatory effects of triethylcholine appear to be unimportant from the point of view of effects in conscious mammals. Voluntary movements do not resemble maximal twitches. A voluntary contraction is the algebraic sum of the asynchronous, intermittent and repeated contractions of the various motor-units. Even with powerful facilitatory agents, such as the anticholinesterases, repetitive firing of the muscle fibres is not produced during tetanic stimulation (Brown, 1937 ; Werner, 1960), and their only effect on tetanic contractions, as with triethylcholine, is to depress them (Briscoe, 1938) providing that there is no degree of transmission failure present to start with. In previous experiments (Bowman & Rand, 1961b) no potentiation of reflex contractions of the quadriceps muscle was evident after triethylcholine, and in conscious animals there was no evidence of an initial increase in muscular power before the onset of weakness. Similarly in the experiments of Laurence & Webster (1961) on rabbits treated with the toxin of *Clostridium tetani*, triethylcholine relieved the tetanic spasms without initially augmenting them. The only evidence of a facilitatory action of anticholinesterases in normal conscious animals is the occurrence of muscle fasciculations ; there was no sign of fasciculations after treatment with triethylcholine. In a previous paper (Bowman & Rand, 1961a) it was suggested that a drug like triethylcholine might be of use in the treatment of neurogenic spastic states since it would selectively relieve the spasm in the affected muscles. It seems possible, however, that in cases of prolonged spasticity some degree of transmission failure, corresponding to that described by Krnjević & Miledi (1958) as occurring in rat skeletal muscle after repeated stimulation, would be present in some muscle units. The use of a drug like triethylcholine in such cases might therefore be expected initially to increase the spasticity slightly, before relieving it.

Roberts suggested, from his experiments on the frog, that the explanation of the block previously recorded by Bowman & Rand (1961a and b) with triethylcholine might be that the synthesizing mechanism in the nerve endings was unable to supply acetylcholine fast enough to support the increased output, so that after prolonged stimulation the amount of transmitter released by each nerve impulse would decrease. The eventual neuromuscular block would therefore be due to depletion of transmitter resulting in an inadequate release by a nerve volley. While depletion of transmitter resulting from an initially increased output may contribute to the blocking action of triethylcholine in the mammal, there are reasons for believing that this is not the sole and probably not even the main mechanism underlying the reduced release.

Tetraethylammonium is much more powerful than triethylcholine in its ability to increase transmitter release in response to a nerve impulse, but triethylcholine is relatively more powerful in its blocking action than can be explained in this way. If an initially excessive release were the only factor accounting for depletion of acetylcholine, the striking antagonistic action of choline would imply, either that

choline can stimulate synthesis by some unknown mechanism, or that additional injected choline is necessary for incorporation into new acetylcholine to make good the initial loss. If this latter explanation were true, it would follow that the level of choline in the extracellular fluid is the factor limiting the rate of acetylcholine synthesis. This seems unlikely in the whole animal in view of the large amounts of free choline normally present in plasma (Bligh, 1952), although it might be an important factor in the isolated muscle. The strongest evidence that an initial excessive output was not the sole mechanism underlying the eventual reduced release of acetylcholine was obtained in experiments on the isolated diaphragm. In this preparation complete recovery from triethylcholine-block gradually occurred without stopping the rapid stimulation when the triethylcholine was removed from the organ bath. It was not necessary to allow the preparation a period of rest, which would have been essential if the transmission failure had been due solely to initial exhaustion of transmitter.

In view of the above considerations, the conclusion is reached that the reduced transmitter output produced by triethylcholine is largely a consequence of interference either with the synthesis or with the release of acetylcholine. The similarities between the actions of triethylcholine and hemicholinium (Bowman & Rand, 1961b) suggest that it is the synthesizing mechanism, and in particular choline transport, which is affected. Bowman & Rand (1961b) also considered the possibility that triethylcholine may be acetylated in place of choline during the re-synthesis of transmitter at the nerve endings. However, the possibility that some stage in the release mechanism is the site of the blocking action cannot be excluded at present. If this is so, the antagonistic ability of choline might be explained simply by competition for the same receptor by a substance which lacks the specific action necessary to inhibit the release mechanism.

We are indebted to Ward Blenkinsop & Co. for gifts of triethylcholine. W. C. B. is grateful to the Central Research Fund of the University of London for a grant towards apparatus, and B. A. H. to the Pharmaceutical Society of Great Britain for financial support.

## REFERENCES

ALDRIDGE, W. N. (1950). Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl-p-nitrophenyl thiophosphate (E605) and analogues. *Biochem. J.*, **46**, 451–460.

ATKINSON, W. J. (1952). Effect of tetraethylammonium on mammalian skeletal muscle action. *Amer. J. Physiol.*, **168**, 442–445.

BACQ, Z. M. & BROWN, G. L. (1937). Pharmacological experiments on mammalian voluntary muscle in relation to the theory of chemical transmission. *J. Physiol. (Lond.)*, **89**, 45–60.

BLIGH, J. (1952). The level of free choline in plasma. *J. Physiol. (Lond.)*, **117**, 234–240.

BOWMAN, W. C. & RAND, M. J. (1961a). The triethyl analogue of choline and neuromuscular transmission. *Lancet*, **i**, 480–481.

BOWMAN, W. C. & RAND, M. J. (1961b). Actions of triethylcholine on neuromuscular transmission. *Brit. J. Pharmacol.*, **17**, 176–195.

BOWMAN, W. C. & RAND, M. J. (1962). The neuromuscular blocking action of substances related to choline. *Int. J. Neuropharmacol.*, in the press.

BRISCOE, G. (1938). Changes in muscle contraction curves produced by drugs of the eserine and curarine groups. *J. Physiol. (Lond.)*, **93**, 194–205.

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

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

BÜLBRING, E. (1946). Observations on the isolated phrenic-nerve diaphragm preparation of the rat. *Brit. J. Pharmacol.*, **1**, 38–61.

BURGEN, A. S. V., BURKE, G. & DESBARATS-SCHONBAUM, M.-L. (1956). The specificity of brain choline-acetylase. *Brit. J. Pharmacol.*, **11**, 308–312.

BURGEN, A. S. V., DICKENS, F. & ZATMAN, L. J. (1949). The action of botulinum toxin on the neuromuscular junction. *J. Physiol. (Lond.)*, **109**, 10–24.

BURNS, B. D. & PATON, W. D. M. (1951). Depolarization of the motor end-plate by decamethonium and acetylcholine. *J. Physiol. (Lond.)*, **115**, 41–73.

HOFMANN, W. W., FEIGEN, G. A. & GENTHER, G. H. (1962). Hemicholinium No. 3 and mammalian neuromuscular transmission. *Nature (Lond.)*, **194**, 654–656.

HOLTON, P. & ING, H. R. (1949). The specificity of the trimethylammonium group in acetylcholine. *Brit. J. Pharmacol.*, **4**, 190–196.

HUTTER, O. F. (1952). Effect of choline on neuromuscular transmission in the cat. *J. Physiol. (Lond.)*, **117**, 241–250.

JEPSON, R. P., SIMEONE, F. A. & LYNN, R. B. (1953). Effects of certain autonomic blocking agents on striated muscle function. *Amer. J. Physiol.*, **173**, 70–74.

KESTON, A. S. & WORTIS, S. B. (1946). The antagonistic action of choline and its triethyl analogue. *Proc. Soc. exp. Biol. N.Y.*, **61**, 439–440.

KOKETSU, K. (1958). Action of tetraethylammonium chloride on neuromuscular transmission in the frog. *Amer. J. Physiol.*, **193**, 213–218.

KRNJEVIĆ, K. & MILEDI, R. (1958). Failure of neuromuscular propagation in rats. *J. Physiol. (Lond.)*, **140**, 440–461.

KUFFLER, S. W. (1942). Electric potential changes at an isolated nerve-muscle junction. *J. Neurophysiol.*, **5**, 18–26.

LAURENCE, D. R. & WEBSTER, R. A. (1961). The activity of the triethyl analogue of choline in suppressing experimental tetanus. *Lancet*, **i**, 481–482.

MCEWEN, L. M. (1956). The effect on the isolated rabbit heart of vagal stimulation and its modification by cocaine, hexamethonium and ouabain. *J. Physiol. (Lond.)*, **131**, 678–689.

MARTIN, A. R. & ORKAND, R. K. (1961). Postsynaptic effects of HC-3 at the neuromuscular junction of the frog. *Can. J. Biochem. Physiol.*, **39**, 343–349.

PRESTON, J. B. & VAN MAANEN, E. F. (1953). Effects of frequency of stimulation on the paralysing dose of neuromuscular blocking agents. *J. Pharmacol. exp. Ther.*, **107**, 165–171.

REITZEL, N. L. & LONG, J. P. (1959). The neuromuscular blocking properties of $\alpha$, $\alpha'$, dimethylethanolamino 4,4′ biacetophenone (hemicholinium). *Arch. int. Pharmacodyn.*, **119**, 20–30.

ROBERTS, D. V. (1962). Neuromuscular activity of the triethyl analogue of choline in the frog. *J. Physiol. (Lond.)*, **160**, 94–105.

SCHUELER, F. W. (1960). The mechanism of action of the hemicholiniums. *International Rev. of Neurobiol.*, **2**, 77–97.

STOVNER, J. (1957). The effect of low calcium and of tetraethylammonium (T.E.A.) on the rat diaphragm. *Acta physiol. scand.*, **40**, 285–296.

STOVNER, J. (1958). The anticurare activity of tetraethylammonium (TEA). *Acta pharmacol et toxicol.*, **14**, 317–332.

THIES, R. E. & BROOKS, V. B. (1961). Postsynaptic neuromuscular block produced by hemicholinium No. 3. *Fed. Proc.*, **20**, 569–578.

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

WISLICKI, L. (1958). Effects of rate of stimulation and of fatigue on the response to neuromuscular-blocking agents. *Brit. J. Pharmacol.*, **13**, 138–143.