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STIMULUS FREQUENCY AND NEUROMUSCULAR BLOCK

BY

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Muscle twitches of the rat isolated diaphragm and frog sartorius preparations were recorded. It was confirmed that, in the presence of tubocurarine, the degree of neuromuscular block was greater the higher the frequency of stimulation. The results suggest that the quantity of acetylcholine released by each nerve impulse was reduced by increasing the rate of stimulation so that, in a tubocurarine solution, muscle fibres which were only just being fired at a slow rate of stimulation failed to fire at a faster rate.

It has been observed repeatedly that neuromuscular block by tubocurarine and a number of other compounds is greater at faster than at slower rates of stimulation (for example, Rosenblueth & Morison, 1937 ; Chou, 1947 ; Guyton & Reeder, 1949 ; Preston & van Maanen, 1953 ; Wislicki, 1958). The most likely explanation of this is that the amount of acetylcholine released by each nerve volley is less at faster than at slower rates of stimulation. In the cat the second of two end-plate potentials is always about 20% smaller than the first, and only returns to a normal value after several seconds (Eccles, Katz & Kuffler, 1941). This finding, together with indirect evidence from both mammalian and amphibian muscle preparations, suggests that the depression is the result of a reduction in the amount of acetylcholine released (Lundberg & Quilisch, 1953a, b ; Takeuchi, 1958). Measurement of the acetylcholine released from rat and guinea-pig diaphragm preparations provides direct evidence that the amount released per nerve impulse falls off at higher frequencies of stimulation (Straughan, 1960 ; Krnjević & Mitchell, 1961).

This paper describes experiments consistent with this explanation. The possibility is considered that measurements of the " frequency dependence " of block at the neuromuscular junction may provide a useful method of studying the effect of drugs on acetylcholine release.

METHODS

Rat phrenic nerve-diaphragm preparation. Albino rats of either sex, weighing 100 to 250 g, were killed by a blow on the head. A portion of the left diaphragm with 3 to 4 cm of the attached phrenic nerve was removed quickly and placed in a 50 ml. organ bath at 37° C (Bülbring, 1946). For most experiments a Krebs-bicarbonate solution of the following ionic composition (mM/l.) was used: Na 144 ; K 5.9 ; Ca 2.54 ; Mg 1.2 ; Cl 129 ; H_2PO_4 1.2 ; SO_4 1.2 ; HCO_3 25 ; glucose, 2 g/l. In some experiments this solution was made up to contain

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1.27 mM of $CaCl_2$ and 35 mM of $NaHCO_3$; preparations in this solution survived as well as those in the former solution and gave similar results. In experiments requiring high concentrations of calcium, the following simplified solution containing less bicarbonate was used to prevent precipitation of calcium (mM/l.): Na 145; K 5.0; Ca 2.5; Mg 1.0; Cl 142; HCO_3 15; glucose, 2 g/l. All solutions were bubbled vigorously in both the reservoir and the organ bath with 95% oxygen and 5% carbon dioxide.

Frog sciatic nerve-sartorius muscle preparation. Preparations were made from *Rana temporaria* at all times of the year. The muscle with a length of its motor nerve was carefully dissected free and set up at room temperature (18 to 20° C) in a 50 ml. organ bath containing the following solution (mM/l.): Na 110; K 2.0; Ca 2.0; Cl 96; HCO_3 20; glucose, 2 g/l. This solution was bubbled with 95% oxygen and 5% carbon dioxide. Magnesium chloride (1 mM) was sometimes added.

A glass capillary electrode (Furshpan & Potter, 1959) was used for stimulation of the nerves of each preparation. Supramaximal rectangular pulses of about 125 to 250 μsec duration were applied through an isolating transformer.

A light-weight lever mounted transversely on a flat spring under tension recorded muscle twitches. At the beginning of an experiment, the resting tension of the muscle was adjusted to be between 1 and 2 g, and care was taken to keep this constant throughout the experiment. Within the range of the recorded twitches, the deflexion of the lever was directly proportional to an applied static load. Underswing of the lever was prevented by an adjustable stop close to the writing point.

The effect of stimulus frequency was usually observed over the range 0.125 to 2 shocks/sec. In all experiments the standard rate of stimulation was 0.125 shocks/sec, but at various times there was stimulation at faster rates. With the faster rates ten stimuli were usually applied, with rest periods of 30 sec (Fig. 2). In some experiments the preparation was stimulated each minute at three or four different rates in turn, and a typical cycle was 30 sec at 0.125 shocks/sec, 15 sec at 0.5 shocks/sec, and 15 sec at 2 shocks/sec (Fig. 1). Responses to such cycles of stimulation were well sustained by the rat diaphragm, but the frog sartorius muscle sometimes showed "fatigue" at higher rates.

The percentage block of neuromuscular transmission was expressed as the percentage by which the control twitch response at a given rate of stimulation was reduced by the drug. This was usually measured at a steady level of block at least 20 min after addition of the drug. On washing, the amplitude of the twitch usually returned to 95% or more of that recorded before addition of the drug. If the twitch amplitude did not recover to this degree on washing, the results were rejected.

In experiments in which a constant block was produced, the drug concentration could not be given accurately since it was reached by alternate dilution and drug addition. After treatment with calcium or increase in the tension, the former level of block was reached by making several small additions of the drug.

Drugs. Drugs were made up in 0.9% saline, and the solutions added directly to the bath in a volume normally not greater than 0.5 ml. Increases in the calcium and magnesium concentrations were made by adding some of a 10% solution of the appropriate chloride to the bath. No adjustment for the change in tonicity was made.

The drugs used were decamethonium bromide, 2-hydroxyethyltriethylammonium bromide (triethylcholine), neostigmine methylsulphate, tetraethylammonium bromide and (+)-tubocurarine chloride.

RESULTS

Stimulus frequency and neuromuscular block with tubocurarine

Normal responses. With the rat diaphragm, an increase in the rate of nerve stimulation was always accompanied by a small increase in the size of the muscle

twitch which declined again on returning to the slower rate of stimulation (Fig. 1). An increase in the twitch response was observed also when stimulation was resumed after a brief rest (Fig. 2, A). An increase in response might have been due to enhanced transmission but, in the present experiments, transmission was probably complete or nearly so, for doubling the concentration of calcium, which increases the release of acetylcholine (Castillo & Stark, 1952), did not increase the twitch response at slow rates of stimulation as might have been expected if some junctions were just failing to transmit (Muscholl, 1957). The facilitation was presumably muscular rather than junctional, due, perhaps, to prolongation of the active state of the muscle (Ritchie & Wilkie, 1955).

In the frog sartorius muscle, the facilitation of the twitch produced by increasing the stimulus frequency was sometimes preceded by a small decline in the twitch height (Takeuchi, 1958). Sometimes this decline was conspicuous, especially at faster rates of stimulation, and closely resembled the dependence on stimulus frequency observed with threshold blocking concentrations of tubocurarine.

Effect of tubocurarine. In several early experiments with the rat diaphragm, it was confirmed that the block produced by tubocurarine (2×10^{-6} M) was greater at faster stimulus frequencies (Chou, 1947). These experiments were complicated

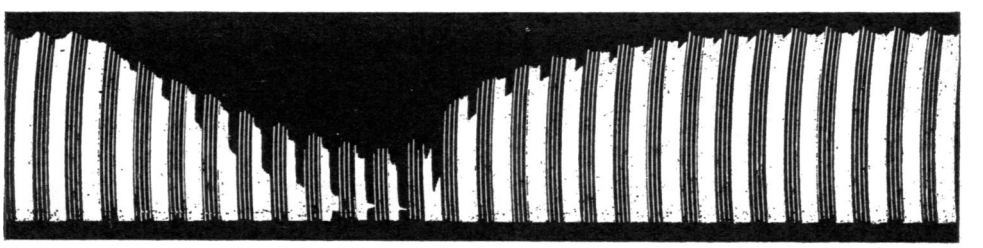

Fig. 1. Effect of stimulus frequency on neuromuscular block by tubocurarine in a rat diaphragm. Responses to stimulation at, successively, 0.125 shocks/sec for 30 sec, 0.5 shocks/sec for 15 sec, and 2 shocks/sec for 15 sec, for each cycle of 1 min. Tubocurarine added at TC to give a final concentration of 10^{-6} M. Bath fluid replaced by fresh Krebs solution at W.

by the tendency for successive additions of tubocurarine to produce greater block independently of the stimulus frequency, and it was more convenient to stimulate the preparation at different rates in turn throughout the experiment. In this way the dependence of the block on stimulus frequency could be observed at any stage of block or recovery (Fig. 1).

In the presence of tubocurarine, the decrease in the size of the twitch which followed each increase in the stimulus frequency was rapid; a steady level was usually reached by the fifth twitch after changing to the faster rate (Fig. 4). Similar observations have been made, for example, by Rosenblueth & Morison (1937) and by Maaske, Boyd & Brosnan (1938).

In most diaphragm preparations there was an approximately linear relation between the percentage block and the stimulus frequency (Fig. 6). The slope

of the line relating the percentage block to the stimulus frequency increased as the block developed and, for a given degree of block, was maximal when a steady partial block had been achieved. For this reason, comparisons of the dependence of block on stimulus frequency were made during steady block.

Similar results were obtained with tubocurarine and the frog sartorius preparation although, on changing from a slower to a faster rate of stimulation, a steady level of twitch was reached rather more slowly than with the rat diaphragm.

Factors influencing the dependence of tubocurarine block on stimulus frequency

A number of factors, believed to alter the release of acetylcholine from motor nerve terminals, affected the dependence of neuromuscular block on stimulus frequency in the presence of tubocurarine. Magnesium chloride was used to reduce the release of acetylcholine (Castillo & Engbaek, 1954). Calcium or an increase in the resting tension of the muscle was employed to increase the release of acetylcholine (Castillo & Stark, 1952 ; Kuffler, 1952 ; Ralston & Libet, 1953 ; Castillo & Engbaek, 1954 ; Hutter & Trautwein, 1956).

Magnesium chloride. An increase in the magnesium chloride concentration to 2 or 3 mM enhanced the partial block of the rat diaphragm or the frog sartorius caused by a steady level of tubocurarine (1 to 2×10^{-6} M). The dependence of block on stimulus frequency was always less than that associated with the same degree of block by tubocurarine at a normal magnesium concentration. Block by magnesium alone was little, if at all, dependent on stimulus frequency (Fig. 2).

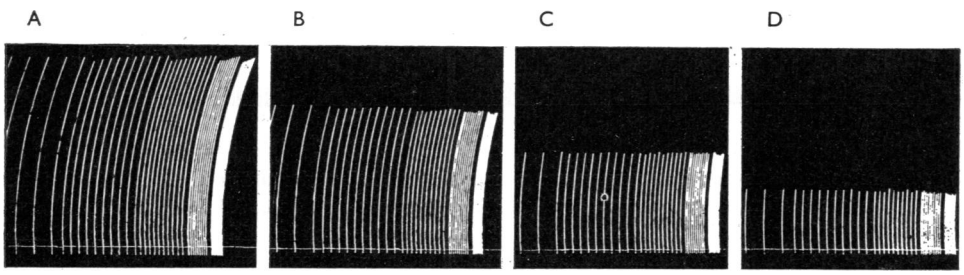

Fig. 2. Effect of stimulus frequency on block by magnesium in the rat diaphragm. Groups of ten stimuli were at 0.125, 0.25, 0.5, 1, and 2 shocks/sec successively. The drum was stopped for 30 sec between each group (first seven responses at 0.125 shocks/sec are omitted). Responses in (A) normal Krebs solution, 1.2 mM of Mg; (B) 6.6 mM of Mg; (C) 7.6 mM of Mg; and (D) 8.6 mM of Mg.

Calcium chloride. The effects of calcium were the opposite of those of magnesium. Thus, calcium rapidly reversed the partial block at a steady level due to tubocurarine (approximately 10^{-6} M). In the experiment illustrated in Fig. 3, doubling the calcium concentration (to 5.1 mM) restored transmission almost completely. However, a considerable degree of dependence on stimulus frequency remained. Addition of more tubocurarine restored the former degree of block, but the dependence on stimulus frequency was greater than when the calcium concentration was normal (Fig. 3, C).

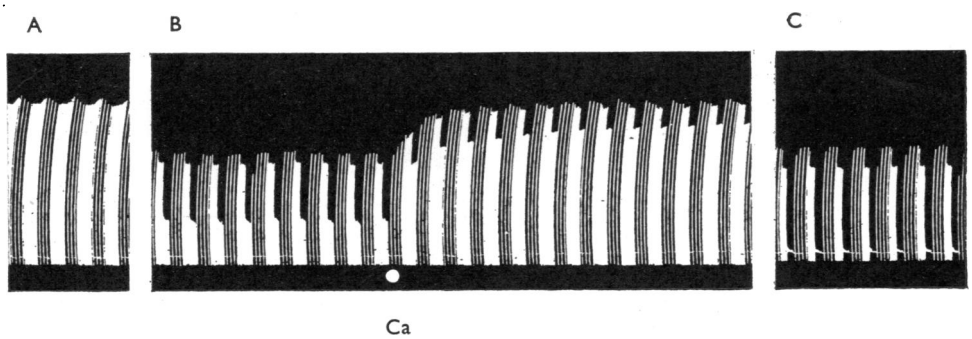

Fig. 3. Effect of calcium on the dependence of block by tubocurarine on stimulus frequency in a rat diaphragm. Stimulation at three rates as in Fig. 1. Responses (A) in normal Krebs solution; (B) during steady partial block by tubocurarine (approximately 0.5×10^{-6} mM) before and after doubling the calcium concentration (at Ca); and (C) following addition of more tubocurarine to restore former level of block.

Resting tension of muscle. Increasing the resting tension of muscle during steady partial block by tubocurarine (approximately 10^{-6} M) had a similar though less dramatic effect to that of adding calcium: the block was partly reversed, and addition of more tubocurarine to restore the former degree of block moderately increased the dependence on stimulus frequency.

Tubocurarine concentration

Although the changes in dependence on stimulus frequency were consistent with the view that such frequency dependence during block by tubocurarine was a normal presynaptic event, it was possible that the action of tubocurarine was itself dependent on stimulus frequency. This possibility arises because, in the above experiments, the effect of stimulus frequency was necessarily compared at different concentrations of tubocurarine. The following experiments were made to test this possibility.

Effect of neostigmine methylsulphate. The concentration of tubocurarine necessary to produce a given degree of block was greater when the drug was added in the presence of neostigmine. By using neostigmine it was therefore possible to compare the dependence on stimulus frequency of similar degrees of block produced by different amounts of tubocurarine. In the experiment illustrated in Fig. 4, the dependence on stimulus frequency of the steady block produced by tubocurarine (approximately 10^{-6} M) was first studied. Following the addition of 10^{-6} M of neostigmine, a similar degree of block was again reached by adding tubocurarine to give a final concentration of about 1.4×10^{-6} M. The dependence on stimulus frequency of the second block was similar to that of the first, and thus independent of the concentration of tubocurarine.

Calcium and magnesium together. Calcium and magnesium act together to depress the excitability of the muscle membrane (Engbaek, 1952; Castillo & Engbaek, 1954; Paul, 1960) but, at the same time, they are mutually antagonistic in their effect on acetylcholine release (Jenkinson, 1957). They were, therefore, added together to the frog sartorius or to the rat diaphragm preparation so that transmission

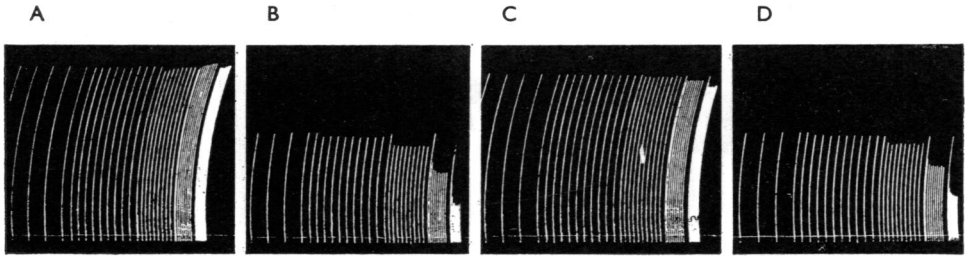

Fig. 4. Effect of neostigmine on the dependence of the block by tubocurarine on stimulus frequency in a rat diaphragm. Groups of stimuli were at 0.125, 0.25, 0.5, 1, and 2 shocks/sec successively, with 30 sec rests between groups (first seven responses at 0.125 shocks/sec omitted). Responses (A) in normal Krebs solution; (B) during steady block with tubocurarine alone (approximately 10^{-6} M); (C) after adding neostigmine (10^{-6} M); and (D) after adding more tubocurarine (final concentration of approximately 1.4×10^{-6} M) to give block similar to (B).

might be blocked in the absence of tubocurarine without greatly affecting acetylcholine release (Castillo & Katz, 1954). With the sartorius preparation concentrations of calcium of 10 mM and of magnesium of 10 or 15 mM led to a dependence on stimulus frequency comparable to that with tubocurarine (Fig. 5). With the rat diaphragm, however, transmission was not blocked except with high ratios of the magnesium to calcium concentrations; there was little dependence on stimulus

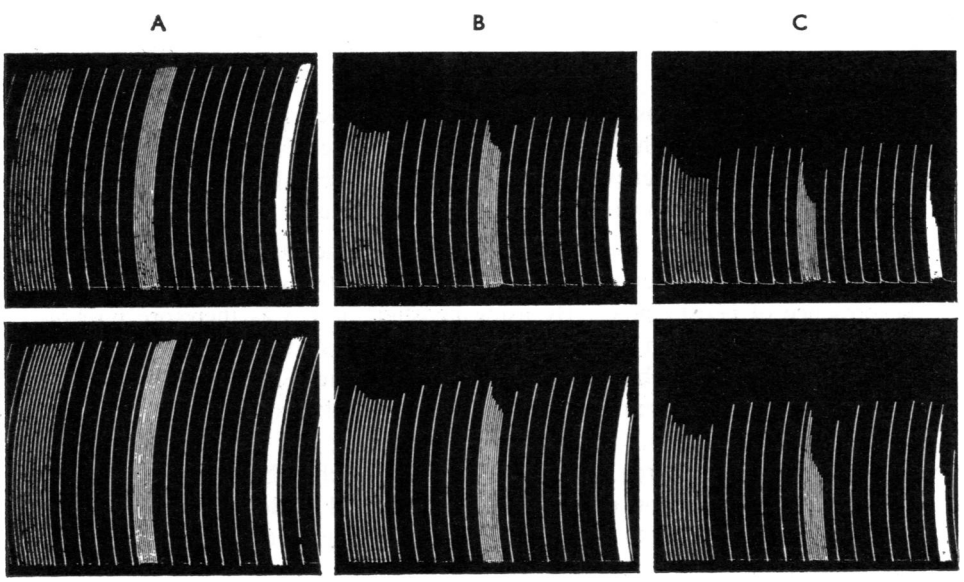

Fig. 5. Effect of stimulus frequency on block by tubocurarine (upper records) and excess calcium and magnesium (lower records) in a frog sartorius preparation. (A) Control responses to trains of 10 stimuli at 0.5, 1, and 2 shocks/sec successively, with basic rate of 0.125 shocks/sec; (B) during steady partial block by tubocurarine (approximately 2×10^{-6} M) (upper) and 10 mM of Ca+10 mM of Mg (lower); and (C) with more tubocurarine (upper) and 10 mM of Ca+15 mM of Mg (lower).

frequency, and presumably the block was due mainly to the presynaptic action of the excess magnesium.

Dependence on stimulus frequency with other drugs

Tetraethylammonium iodide. There is indirect evidence that, amongst its other actions, tetraethylammonium may increase the release of acetylcholine from motor nerve terminals (Koketsu, 1958 ; Stovner, 1958). The curare-like blocking action of tetraethylammonium should, therefore, be associated with a greater degree of dependence on stimulus frequency than the same degree of block by tubocurarine,

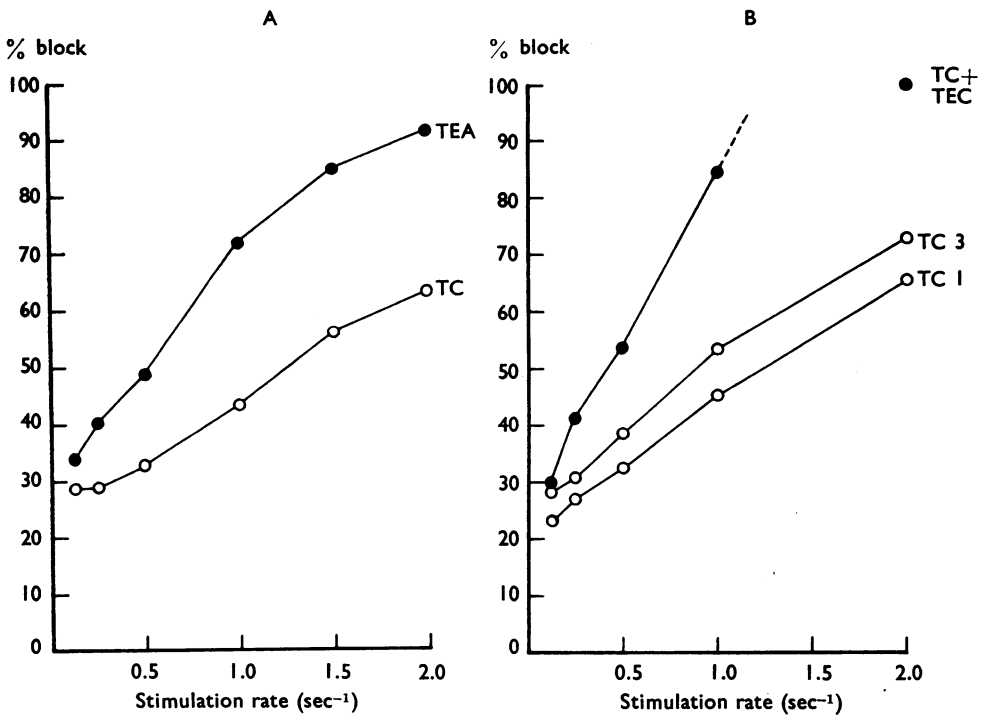

Fig. 6. (A) Effect of stimulus frequency during steady block by tetraethylammonium (TEA, 6×10^{-3} M) and tubocurarine (TC, approximately 10^{-6} M) in a rat diaphragm. (B) Effect of stimulus frequency in another rat diaphragm during steady block by tubocurarine (TC 1, TC 3, approximately 1.5×10^{-6} M) before and after block by tubocurarine (approximately 2×10^{-6} M) +triethylcholine (10^{-3} M; TC+TEC).

which presumably does not affect acetylcholine release. Greater dependence on stimulus frequency was, indeed, observed with tetraethylammonium and the rat diaphragm (Fig. 6, A) although, in the three experiments performed, the difference was not great.

Triethylcholine bromide. There is electro-pharmacological evidence that this compound enhances the release of acetylcholine from motor nerve terminals in the frog (Roberts, 1962) so that, like calcium or tetraethylammonium, it should enhance the dependence on stimulus frequency associated with a given degree of post-

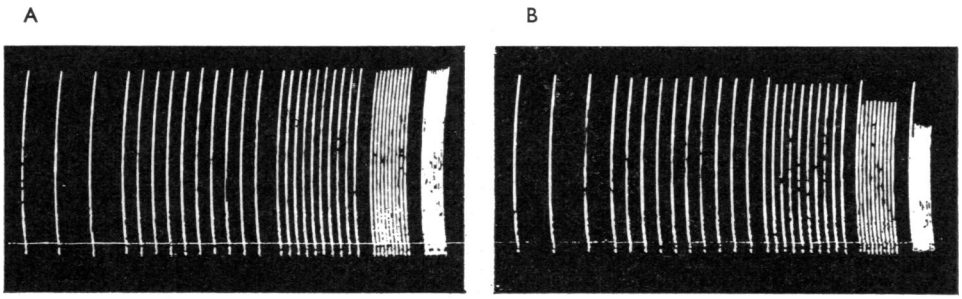

Fig. 7. Effect of stimulus frequency on a rat diaphragm in the presence of triethylcholine. Groups of stimuli were at 0.125, 0.25, 0.5, 1, and 2 shocks/sec successively with 30 sec rest between groups (first seven responses at 0.125 shocks/sec omitted). Responses (A) in normal Krebs solution; (B) 30 min after exposure to 10^{-2} M of triethylcholine (previous stimulation at 0.125 shocks/sec only).

junctional block. Bowman & Rand (1961) showed that triethylcholine only blocked transmission in mammalian muscle when stimulation of the motor nerve was maintained and rapid. In the present experiments, it was confirmed that concentrations of triethylcholine up to 10^{-2} M were insufficient to block the rat diaphragm preparation at a slow rate of stimulation (8 shocks/min) although brief trains of fast stimuli caused a rapid but small block (Fig. 7). The effect of triethylcholine on the dependence on stimulus frequency of tubocurarine block was estimated in

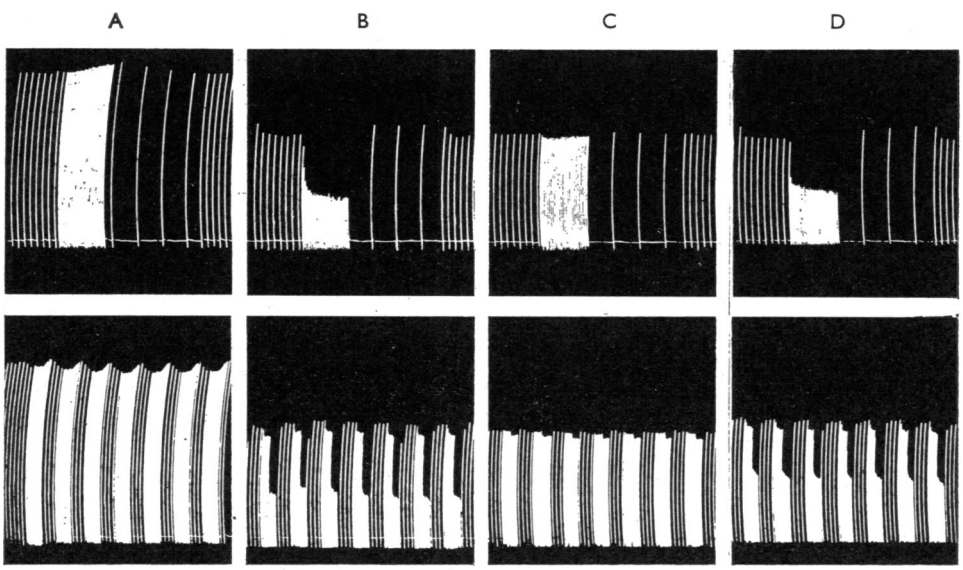

Fig. 8. Effect of stimulus frequency during steady partial block by tubocurarine and decamethonium in a rat diaphragm. Maintained stimulation at three rates as in Fig. 1. Responses recorded on fast drum (upper records) and slow drum (lower records). (A) In normal Krebs solution; (B) during block by tubocurarine (approximately 10^{-6} M); (C) during block by decamethonium (3×10^{-5} M); and (D) in the same solution after doubling the calcium concentration.

two experiments, one of which is illustrated in Fig. 6, B. Block by tubocurarine (approximately 2×10^{-6} M) in the presence of 10^{-3} M of triethylcholine was accompanied by greater dependence on stimulus frequency than was a similar degree of block by tubocurarine (approximately 1.5×10^{-6} M) alone.

Decamethonium bromide. With the rat diaphragm, block by decamethonium was often associated with considerably less dependence on stimulus frequency (Fig. 8, C) than was block by tubocurarine (Fig. 8, B). This was noticeable especially during the first 30 min of block. With time, the dependence on stimulus frequency usually increased slowly. Doubling the calcium concentration during steady block by decamethonium usually antagonized the block to a small extent only, but it effectively increased the dependence on stimulus frequency (Fig. 8, D). The dependence on stimulus frequency with decamethonium in the frog sartorius muscle, although less than that with tubocurarine, did not differ as much as that found with each drug in the rat diaphragm.

DISCUSSION

The dependence on stimulus frequency of the block by tubocurarine at the neuromuscular junction might be explained in two ways. The first, that tubocurarine itself affects dependence on stimulus frequency by an action at the end-plate (Brown & Harvey, 1941 ; Guyton & Reeder, 1949), is unlikely for, by using neostigmine to adjust the block by different concentrations of tubocurarine to the same degree, the dependence on stimulus frequency was independent of the tubocurarine concentration. Further, treatment of the frog sartorius preparation with excess calcium and magnesium resulted in a block of transmission which was dependent on stimulus frequency in the absence of tubocurarine. That a similar block dependent on stimulus frequency could not be produced in the rat diaphragm with excess calcium and magnesium is due presumably to the comparatively high safety factor for transmission in mammalian muscle (Boyd & Martin, 1956), coupled with the fact that calcium may not depress the excitability of the muscle membrane in the rat diaphragm as much as in the frog sartorius (Paul, 1960).

A second, and more likely, explanation of the dependence on stimulus frequency which accompanies block by tubocurarine is that the acetylcholine normally released by each nerve impulse may be reduced by increasing the rate of stimulation, so that muscle fibres only just activated at a slow rate of stimulation in a tubocurarine solution may fail to fire at a faster rate. Dependence on stimulus frequency is not usually observed in the normal preparation even at comparatively fast stimulus frequencies such as 2 shocks/sec, presumably because few, if any, fibres in the muscle fail to fire in spite of a reduction in the amount of acetylcholine released. The block of transmission will be dependent on stimulus frequency if the effectiveness of the released acetylcholine is impaired, as for example in the present experiments when the sensitivity of the end-plate to acetylcholine was reduced by tubocurarine or (as in the frog sartorius) when the excitability of the muscle membrane was depressed by excess calcium and magnesium.

If the observed dependence on stimulus frequency is the result of a normal presynaptic event, alterations in the amount of acetylcholine released by a nerve impulse

might be expected to affect the degree of dependence on stimulus frequency for a given degree of block. Such changes were brought about not only by increasing the calcium or the magnesium concentration, or the muscle tension, three factors which normally determine the extent of acetylcholine release, but also by the quaternary ammonium compounds tetraethylammonium and triethylcholine, both of which are believed to enhance the release of acetylcholine from motor nerve terminals (Koketsu, 1958 ; Stovner, 1958 ; Roberts, 1962).

These findings suggest that the dependence on stimulus frequency is a presynaptic phenomenon. They also indicate that the extent of the depression of acetylcholine release which occurred during a train of stimuli is determined by the amount of acetylcholine released initially: the greater this amount, the greater the dependence on stimulus frequency associated with a given degree of transmission. It also follows that comparisons of the dependence on stimulus frequency associated with neuromuscular block by different drugs might give useful information about their effects on acetylcholine release. However, the results with decamethonium suggest that such comparisons should be interpreted with caution. Block by decamethonium in the rat diaphragm was less dependent on stimulus frequency than was block by tubocurarine, which might suggest that decamethonium, like magnesium, might inhibit the release of acetylcholine from the nerve terminals. However, a preferable explanation is based on the known ability of decamethonium both to depolarize and to " desensitize " the end-plate (Zaimis, 1951 ; Burns & Paton, 1951 ; Thesleff, 1955a and b, 1958). For example, during steady partial block by decamethonium, many end-plates at the junctions still permitting transmission may be depolarized to such an extent that even when the acetylcholine released by each impulse is considerably diminished, as occurs during stimulation at faster rates (1 to 2 shocks/sec), the muscle fibres will be activated. Under steady partial blocking conditions, the proportion of such junctions which will continue to transmit at all rates of stimulation may be high. The dependence on stimulus frequency that was actually observed would then be due to those remaining junctions at which there was less depolarization of the end-plate and perhaps more " desensitization," such junctions failing to transmit at faster rates of stimulation. In the early part of an experiment when the dependence on stimulus frequency with decamethonium was small, the number of such junctions was also presumably small, but later the extent of the depolarization by decamethonium diminished (Thesleff, 1955a and b, 1958) and the number of junctions which failed to transmit on increasing the stimulation rate presumably became greater ; that is, the dependence on stimulus frequency would increase with time, as indeed it did. Clearly, the ability of a drug to depolarize the end-plate will make it difficult to interpret studies of dependence on stimulus frequency. In the absence of depolarizing activity, however, such studies may indicate an action on acetylcholine release.

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