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STUDIES ON THE REPETITIVE DISCHARGES EVOKED IN MOTOR NERVE AND SKELETAL MUSCLE AFTER INJECTION OF ANTICHOLINESTERASE DRUGS

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Repetitive discharges recorded from the ventral root and from the gastrocnemius muscle in response to single motor nerve shocks applied close to the muscle after injection of edrophonium, neostigmine or ambenonium were studied in cats anaesthetized with chloralose. Two closely spaced volleys with an interval of 1 to 5 msec between them produced more repetitive firing than did a single shock. With longer intervals, the repetitive firing was not potentiated by the second volley. All frequencies of tetanic stimulation depressed the repetitive firing and, for successive stimuli to produce a degree of repetitive firing equivalent to the first, it was necessary to stimulate at frequencies below 2 shocks/sec. With stimulation frequencies higher than 100 shocks/sec, repetitive firing did not occur unless the duration of the tetanus was shorter than about 30 msec when slight repetition followed the last stimulus of the train. With stimulation frequencies of 100 down to 20 shocks/sec, repetitive firing was produced only by the first volley of the tetanus. Subsequent nerve action potentials of the tetanus occurring during the repetitive firing in the nerve following the first volley were partially extinguished by collision with the back discharge. This effect contributed to the waning tetanus, which is characteristic of treatment with an anticholinesterase, but the main depression of tetanic contractions appeared to be a consequence of depolarization block through accumulating acetylcholine. Tubocurarine and benzoquinonium reversed the initial "extinction" phase of the depressed tetani by abolishing the repetitive discharge in the nerve and in larger doses reversed the secondary depressant phase presumably by reducing the excessive end-plate depolarization. The results are discussed in relation to the hypothesis that anticholinesterases may effect transmission by acting at three sites at the neuromuscular junction—on acetylcholinesterase, at the motor nerve ending and at the motor end-plate—and that reaction at any one site may be augmented by the production of reverberating activity across the junction.

After injection of an anticholinesterase drug into a mammal, repetitive firing in response to single shocks applied to a somatic motor nerve may be recorded, not only in the stimulated muscle but also antidromically in the corresponding ventral root (Masland & Wigton, 1940; Feng & Li, 1941; Lloyd, 1942; Eccles, Katz & Kuffler, 1942; Riker, Roberts, Standaert & Fujimori, 1957; Riker, Werner, Roberts

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& Kuperman, 1959 ; Werner, 1960a and b ; 1961a and b). This paper describes a continuation of a previous series of experiments in which the influence of various pharmacological agents on the repetitive discharges was studied (Blaber & Bowman, 1963). Here, effects of multiple volleys made up of various numbers of stimuli delivered over a wide range of frequencies are reported. A particular object of these experiments was to determine to what extent, if any, the waning tetanic tension characteristic of treatment with an anticholinesterase (Briscoe, 1936) might be attributed to extinction of the orthodromic nerve impulses by collision with the high frequency back discharge.

METHODS

The experiments were carried out with twenty-two cats anaesthetized with intravenous chloralose (80 mg/kg). The preparation was similar to that previously described by Blaber & Bowman (1963). The cat was laid face downwards on the operating table and the right hind limb was clamped rigidly in position by means of steel drills inserted into the distal end of the tibia and into the distal and proximal ends of the femur. In addition the cat was immobilized by means of clamps on the spinous processes of the vertebrae. The gastrocnemius-plantaris muscle was separated from the soleus, and the nerve to the soleus was cut. The spinal cord was exposed from L6 to S1 and all visible dorsal and ventral roots were severed close to the cord. Bipolar platinum recording electrodes (4 mm apart) were placed on the peripheral end of the S1 ventral root so that the more central pole was in contact with the cut end of the root. The tendon of the gastrocnemius muscle was attached to an immovable support and a concentric needle electrode (26 S.W.G.) was inserted into the medial head. Occasionally the concentric needle electrode was replaced by two platinum wires inserted through the belly and tendon of the muscle. Closely-spaced bipolar platinum stimulating electrodes were usually placed on the nerve to the medial head of the gastrocnemius muscle after cutting all other nerve branches in the popliteal space. However, at the beginning of a few experiments the stimulating electrodes were applied to the whole sciatic nerve in order to observe the effect of restricting the peripheral innervation field later in the experiment. The nerve was excited by rectangular pulses of 10 to 100 μsec duration and of at least twice the voltage necessary to produce maximal nerve and muscle action potentials. Single shocks or brief trains of pulses were used. The trains consisted of various numbers of stimuli, the following stimulus intervals being used in different experiments: 0.5, 1, 2, 3.2, 5, 10, 13, 16, 20, 40, 50, 63, 100, 130, 200 and 400 msec. Intra-arterial injections into the gastrocnemius muscle were made retrogradely through a polyethylene cannula tied into the central end of the cut popliteal artery just below the muscle. At the moment of injection the popliteal artery was occluded by means of a loose ligature placed just above the gastrocnemius so that most of the injected fluid was forced into the muscle. Since all injections were given intra-arterially, no corrections for body weight were made. After differential amplification by Textronix (type 122) battery driven pre-amplifiers, the muscle and nerve action potentials were displayed simultaneously on a Tektronix (type 502) dual beam oscilloscope. In one experiment, isometric tension of the gastrocnemius muscle was recorded by means of an RCA 5734 mechano-electric transducer. In this experiment, muscle action potentials were displayed simultaneously with tension but ventral root recordings were not made. In all experiments the exposed cord and the muscle were immersed in warm medicinal liquid paraffin (B.P.) previously equilibrated with 95% O_2 and 5% CO_2. The preparation was kept warm by means of radiant heat lamps and the water heated operating table. Muscle temperature was occasionally checked by means of a copper-constantan thermocouple inserted into the belly of the muscle. It was not allowed to fall below 34° C.

The drugs used were neostigmine methylsulphate (Roche), edrophonium chloride (Roche), ambenonium chloride (Sterling-Winthrop), tubocurarine chloride (Burroughs Wellcome) and benzoquinonium chloride (Sterling-Winthrop). The doses quoted refer to these salts. The

drugs were dissolved in 0.9% (w/v) NaCl solution and the maximum volume injected into the gastrocnemius muscle was 0.4 ml.

RESULTS

Control responses

When a single shock was applied to the motor nerve every 10 sec in the absence of drugs, each antidromic nerve action potential recorded in the ventral root was followed by a brief asynchronous burst of small action potentials which started between 2.5 and 5 msec after the stimulus artifact. In most experiments, the peripheral innervation field was restricted almost entirely to the medial head of the gastrocnemius muscle and under these conditions the duration of the burst of small action potentials was about 2 msec. This back-response, which is evident as B in Figs. 1 and 2, has been previously described by others (Lloyd, 1942 ; Brown & Matthews, 1960 ; Werner, 1961a ; Blaber & Bowman, 1963) and has been shown

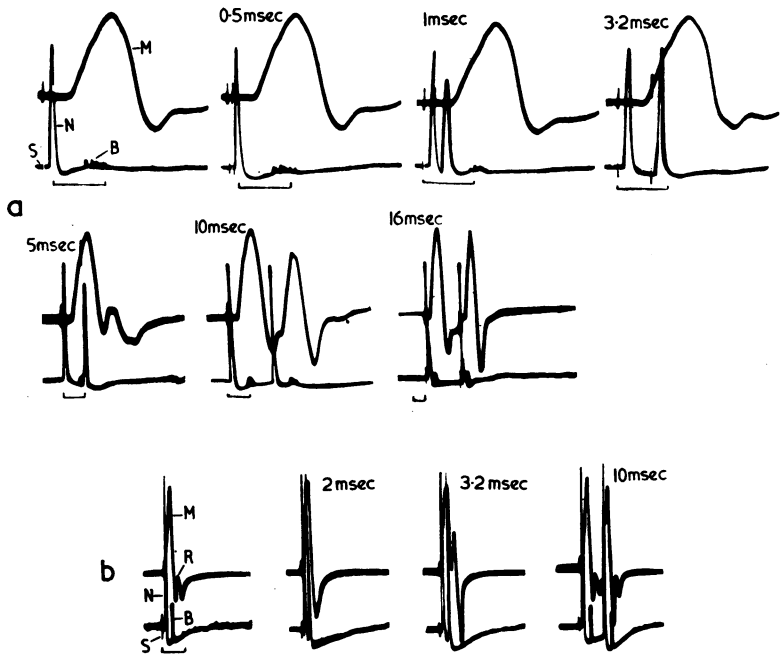

Fig. 1. Control records of responses to single and paired motor nerve volleys. Stimulus intervals are indicated above the responses to paired shocks. Upper beam, muscle action potentials from belly-tendon electrodes in a, and from concentric needle electrode in b; lower beam, ventral root potentials. S, stimulus artifact; N, antidromic nerve action potential; M, muscle action potential; B, back-response; R in b, small second muscle action potential caused by re-excitation of some muscle units by back-response in nerve. Note that R disappeared when the back-response in the nerve was abolished by delivering paired shocks at an interval of 2 msec. In b, and in the experiments of Figs. 2, 3 and 4, the gain on the ventral root recording was such that the amplitude of the main nerve action potential was greater than could be displayed on the oscilloscope screen and these records have been re-touched. Time calibrations, 5 msec in a, and 10 msec in b. In this, and in the experiments of all other Figs., peripheral innervation was restricted to medial head of gastrocnemius.

to depend upon gross muscle activity. It is believed to be caused by re-excitation of some of the α-motor fibres by electrotonic spread of the summed muscle action currents. The variation in the latency of the back-response in different experiments (2.5 to 5 msec) probably depended upon the threshold of the nerve to ephaptic re-excitation. When, as in the experiment of Fig. 1a, gross muscle action potentials were recorded from belly to tendon, it was seen that early back-responses in the nerve were initiated shortly after the onset of the muscle action potentials, indicating a low threshold, while later back-responses started nearer the crest of the muscle action potential, indicating a higher threshold to ephaptic re-excitation. No conclusions concerning the relationship between the back-response and the muscle action potential could be drawn from experiments in which the latter was recorded with concentric needle electrodes, since the axons carrying the back-response need not have been those innervating the muscle units in contact with the recording electrodes.

The back-response in the nerve propagates in both directions from the site of its initiation and, depending upon the position of the concentric needle electrode in the muscle, it could often be shown to re-excite some muscle units (Brown & Matthews, 1960 ; Blaber & Bowman, 1963). This effect, which is evident as R in Fig. 1b, was more prominent the later the onset of the back-response in the nerve, probably because fewer muscle units would then be in a refractory state. As shown by Lloyd (1942), the back-response in the nerve was longer lasting the less completely the peripheral innervation field had been restricted to the medial head of the gastrocnemius muscle. When the peripheral innervation field was left intact, back-responses in the nerve lasting as long as 5 to 8 msec were recorded. It appeared that such long-lasting back-responses in nerve were more effective than short-lasting back-responses in re-exciting muscle units in the medial head of the gastrocnemius muscle. This suggests that action currents generated in adjacent muscles (probably the lateral head of gastrocnemius) may re-excite motor fibres to the medial head of gastrocnemius. These results therefore emphasize the importance, in experiments on skeletal muscle, of stimulating only the muscle under study since repetitive firing of the muscle fibres in response to single nerve shocks may be more pronounced in the absence of this precaution.

Fig. 1 illustrates experiments in which single motor nerve shocks were replaced by paired volleys at various intervals delivered in the absence of drugs. Paired shocks with an interval of 0.5 msec between each gave responses identical with those elicited by single shocks, since the second fell within the absolutely refractory period of the nerve produced by the first volley (Fig. 1a). With a stimulus interval of 1 msec, the second nerve action potential of a pair had an amplitude of 40 to 75% of that of the first but only one muscle action potential was elicited (Fig. 1a). With a stimulus interval of 2 msec, both nerve action potentials were of the same size and some muscle units were invariably re-excited by the second. This is not evident in Fig. 1b but the presence or absence of the second muscle action potential in the record depended upon the position of the needle electrode. As the stimulus interval was lengthened, the amplitude of the second muscle action potential increased until it equalled that of the first at 6.4 to 12.8 msec. The amplitude of the second muscle action potential never exceeded that of the first as it does in avian muscle (Brown

& Harvey, 1938). When a second stimulus was delivered shortly before the onset of the back-response in the nerve, the occurrence of the latter was prevented, presumably because of the refractory period produced by the second nerve action potential. This is evident in Fig. 1a with a stimulus interval of 3.2 msec and in Fig. 1b with stimulus intervals of 2 and 3.2 msec. The small second muscle action potential (R), elicited by the back-response (B) in the experiment of Fig. 1b, also disappeared when paired shocks were delivered at an interval of 2 or 3.2 msec, but reappeared at an interval of 10 msec. In the experiment of Fig. 1a, a second stimulus delivered after 1 msec reduced but did not abolish the back-response in the nerve. Brown & Matthews (1960) were able to abolish completely the back-response in the nerve and its action in re-exciting the muscle fibres, by delivering a second stimulus 1 msec after the first. In the present study this occurred only in two out of eleven experiments in which this stimulus interval (1 msec) was used. In each of these experiments the back-response in the nerve was of short latency and duration. The more usual effect was as illustrated in Fig. 1 and, although it was always possible to abolish the back-response by delivering paired shocks separated by intervals of 2 or 3.2 msec, the second shock of such a pair itself always excited some muscle units. Consequently, it was usually impossible to produce a truly single muscle response by delivering paired nerve shocks. We are unable to account for the difference between these results and those of Brown & Matthews (1960) although it might be a consequence of the different anaesthetic agents used. In their experiments, the cats were anaesthetized with pentobarbitone sodium, while we always used chloralose.

In the experiment illustrated by Fig. 1a, two shocks delivered at an interval of 3.2 msec produced nerve action potentials equal in size but, when the stimulus interval was increased to 5 msec, the second nerve action potential was smaller than the first. In this experiment, the back-response in the nerve followed the stimulus artifact after 3.5 msec so that a second stimulus delivered 5 msec after the first fell during the back-response and the action potential was therefore probably extinguished in some nerve fibres by the refractory period produced by the back-response. When the interval between stimuli was wide enough for each to produce identical muscle action potentials, similar back-responses in the nerve followed both antidromic nerve action potentials (Fig. 1a and b).

Longer trains of stimuli with intervals of 2 to 40 msec between each (as produced by frequencies of 500 down to 25 shocks/sec) were also studied. The duration of these tetani did not exceed 1 sec and they were elicited at intervals of at least 10 sec. With stimulus intervals of 2, 3.2 and 5 msec the muscle showed the well-known effect of high-frequency (Wedensky) inhibition. With stimulus intervals of 2 msec, the electrical activity of the muscle rapidly waned and disappeared after about the fifth or sixth shock of the tetanus. With intervals of 3.2 and 5 msec the electrical activity of the muscle continued for 100 to 150 msec and 250 to 500 msec respectively. Eccles & Kuffler (1941a and b) showed that an end-plate potential elicited while the muscle was refractory delayed the recovery process. This effect was reflected by the finding that, at stimulus intervals of 2 and 3.2 msec, the response of the muscle to the nerve impulse became delayed as the tetanus continued and, at 2 msec intervals, there did not appear to be a one-to-one relation-

ship between the nerve action potentials and the deflections of the muscle record. With stimulus intervals of 10 msec and above, the muscle and nerve responses were constant, or showed only a slight and regular decline, as in record *i* of Fig. 8, and each nerve action potential was followed by a back-response.

Effects of anticholinesterase drugs

After intra-arterial injection of edrophonium (10 to 30 μg), neostigmine (3 to 10 μg) or ambenonium (1 to 5 μg), the muscle action potentials in response to motor nerve shocks delivered once every 10 sec became repetitive and each antidromic nerve action potential was followed by high frequency repetitive firing in the nerve. Fig. 2 illustrates this effect of ambenonium. At the time of maximal drug effect, the repetitive firing in the ventral root began between 1 and 4 msec after the ephaptic

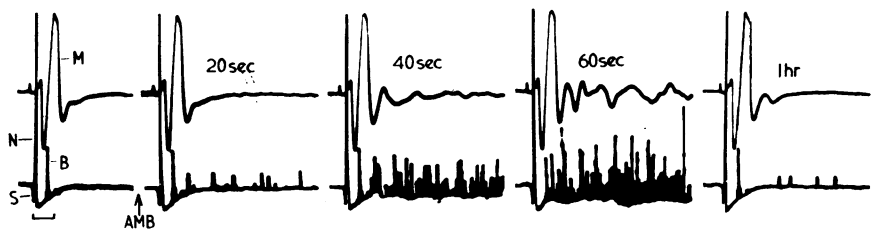

Fig. 2. Recording as in Fig. 1b except that negative deflection of the muscle action potential is downwards. The motor nerve was stimulated with single shocks every 10 sec. After 5 μg of ambenonium (AMB), repetitive firing in both nerve and muscle was evident up to 1 hr later. The times denote the times after injection of ambenonium. Time calibration, 5 msec.

back-response (B) and persisted for 30 to 80 msec after each stimulus in the case of edrophonium and neostigmine, and for 100 to 300 msec in the case of ambenonium. Repetitive firing in response to single shocks was observed during the following periods after drug injection: 5 to 10 min for edrophonium, 20 to 30 min for neostigmine and 60 to 90 min for ambenonium (Blaber & Bowman, 1963). The effects of multiple volleys delivered during these responses were similar for all three drugs but, because of the constancy and long duration of the effect of ambenonium, this drug was used in most of the experiments to be described.

At the beginning of each experiment, the responses of the preparation to the appropriate multiple volleys delivered every 10 sec were recorded in the absence of drugs. Single shocks were then delivered every 10 sec and the drug under study was injected intra-arterially. At the time of maximal drug effect, the single volleys were replaced by multiple volleys and their effect on the repetitive firing was observed.

Stimulus intervals of 0.5 to 10 msec

When the single shocks were replaced by paired shocks with an interval of 0.5 msec between them, the repetitive firing in both nerve and muscle appeared identical with that following a single stimulus, since the second shock fell within the refractory period of the nerve produced by the first. When a third shock was delivered 0.5 msec later, the repetitive firing remained unaltered. Double, triple or quadruple shocks, with an interval of 1 or 2 msec between each stimulus, potentiated the

repetitive firing, double shocks usually being more effective than triple or quadruple shocks. When the stimulus interval was 3.2 msec, double shocks were markedly effective in potentiating the repetitive firing. Fig. 3a illustrates the increase, both in magnitude and duration, of the repetitive firing produced by ambenonium when single shocks were replaced by paired shocks at an interval of 3.2 msec. Triple shocks given with this interval between each stimulus were no more effective than double shocks (Fig. 3a) and frequently produced no more repetitive firing than single shocks (Fig. 3b). Quadruple shocks at this frequency never potentiated the repetitive firing. When the stimulus interval was 5 msec, double volleys slightly potentiated the repetitive firing but triple volleys did not. The potentiation of repetitive firing usually appeared more pronounced in the nerve than in the muscle. With stimulus intervals of 10 msec and above, neither double nor triple volleys potentiated the repetitive firing. It therefore appeared that a second, third or fourth stimulus potentiated the repetitive firing only when it was delivered after the refrac-

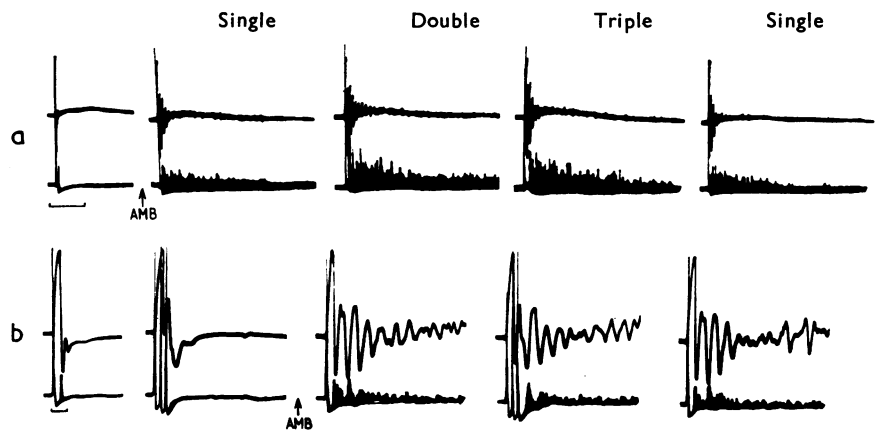

Fig. 3. Recording as in Fig. 1b. The preparations were stimulated every 10 sec with single, double or triple shocks, the intervals between stimuli in the double or triple volleys being 3.2 msec. In a, the repetitive firing produced by 5 μg of ambenonium (AMB) was augmented when the single shocks were replaced by double or triple shocks, but triple shocks were no more effective than double shocks. b was recorded with a faster time base than a and only single and triple shocks were used. Triple shocks produced no more repetitive firing than single shocks. Time calibrations, 100 msec in a and 10 msec in b.

tory period in the nerve produced by the first volley and before the time of onset of the repetitive discharge that would have been induced by a single shock. With the stimulus intervals used in these experiments, double shocks at 2 or 3.2 msec were most effective in potentiating the repetitive firing, and these results closely agree with those of Werner (1960a), who studied the responses of single ventral root fibres as well as those of whole rootlets. With short trains of closely spaced stimuli (1 to 5 msec between each), the onset of repetitive firing in the nerve was always delayed until after the last stimulus of the train (Fig. 3) presumably because of the refractory periods produced by the orthodromic volleys.

Longer lasting trains of closely spaced volleys inhibited the repetitive firing. Thus in an experiment with ambenonium, repetitive firing in the nerve in response to

single shocks persisted, at the time of maximal drug effect, for 175 msec after each single stimulus. When double or triple shocks were delivered with intervals of 3.2 msec between each, the repetitive firing was potentiated and persisted for 375 msec after each multiple volley. However, when the number of volleys was increased to five, repetitive firing following the last stimulus lasted for only about 100 msec, and with ten stimuli at the same frequency no repetitive firing was evident at all even though the duration of the tetanus was much shorter than the period during which repetitive firing followed single, double or triple shocks delivered subsequently. Thus with stimulus intervals of 5 msec or less, repetitive firing did not occur during tetanic stimulation and was completely absent even after the last stimulus when the duration of the tetanus was longer than 25 to 30 msec.

The high frequency inhibition of the muscle responses which occurred with tetanic stimulation at frequencies of 500 to 200 shocks/sec occurred more rapidly in the presence of the drugs. However, although the muscle response disappeared sooner, the amplitude of the muscle action potentials during the period when they were present was actually greater after injection of an anticholinesterase drug so that the declining curve was steeper. The inability of the muscle to respond to all of the nerve action potentials of a high-frequency train was exaggerated in the presence of the drugs and the frequency of stimulation at which this effect was evident was lowered. For example, in an experiment in which the motor nerve was stimulated with trains of shocks each separated by 3.2 msec, complete inhibition of the muscle had not occurred in the absence of drugs after 80 msec of continuous stimulation. A short train of twelve shocks produced twelve waning muscle action potentials. After the injection of 2 μg of ambenonium, complete failure of transmission occurred after 50 msec of continuous stimulation and a short train of twelve shocks now produced only seven potentials in the muscle record. This effect may be explained by the observation of Brown & Harvey (1941) and of Eccles *et al.* (1942) that the refractory period of the muscle membrane was prolonged by an anticholinesterase drug (physostigmine), and by the more recent finding by Hubbard & Schmidt (1961) that the refractory period of the nerve endings is also prolonged.

Stimulus intervals of 10 to 500 msec

With these stimulus intervals, repetitive firing in response to the first shock had already begun in both nerve and muscle at the time when the second shock was delivered. A second shock delivered during the period of repetitive firing produced by the first did not augment the firing in either nerve or muscle and it simply followed its normal time course. Fig 4a compares the repetitive firing produced by a single shock after injection of 5 μg of ambenonium, with that produced by a pair of shocks separated by an interval of 63 msec. Paired shocks produced very little change in the repetition, and the amplitude of the second muscle action potential was only about 20% of that of the first. In other experiments, stimuli delivered during the repetitive firing produced by the first shock did not elicit any response at all from the muscle. This is evident in records *iv* and *v* of Fig. 6. A second stimulus delivered soon after the period of repetitive firing produced by the first, gave rise to a full-sized muscle action potential and a normal back-response in the nerve but

Fig. 4. Recording as in Fig. 1b. First record in a shows repetitive firing in response to one of a series of single shocks delivered every 10 sec at the time of maximal effect of 5 μg of ambenonium. When a second shock was delivered 63 msec after the first (second record), there was little change in the repetitive firing and only a small muscle action potential was elicited. b illustrates a similar experiment in which a second shock was delivered shortly after the cessation of repetitive firing produced by the first. In this case the amplitude of the second muscle action potential was equal to that of the first but relatively little repetitive firing was produced.

very little or no repetitive firing in nerve or muscle. Fig. 4b illustrates the effect of a pair of shocks separated by an interval of 200 msec. Only very slight repetitive firing followed the second shock. In the same experiment a third shock, delivered 200 msec later, still produced a full-sized muscle response but no repetitive firing was evident at all. In order for a second shock to produce repetitive firing in nerve and muscle comparable with that produced by the first, a stimulus interval of 300 to 500 msec was necessary with edrophonium and neostigmine, and of at least 1 sec with ambenonium. These results are similar to those of Brown (1937), who studied the muscle action potentials in response to paired volleys in the presence of physostigmine, and to those of Werner (1960a), who studied both muscle and ventral root responses in the presence of physostigmine and hydroxyphenyltrialkyl ammonium salts.

Waning tetanic tension

Longer-lasting trains of stimuli were studied to find whether repetitive firing reappeared during prolonged stimulation and also to determine whether the waning tetanic tension produced by anticholinesterase drugs (Briscoe, 1936 ; Fig. 5) could be attributed in part to extinction of the orthodromic nerve volleys by collision

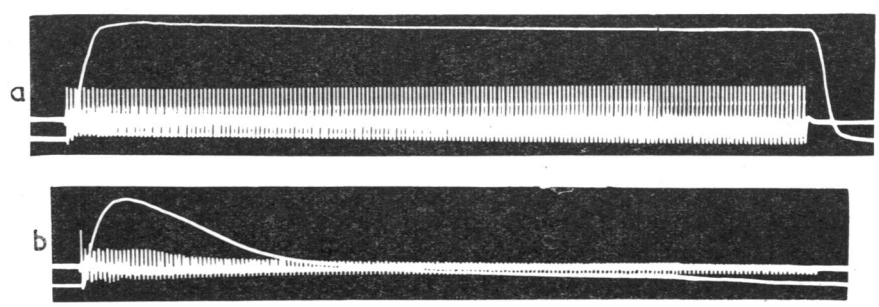

Fig. 5. Isometric tension and muscle action potential records from belly-tendon leads in the gastrocnemius muscle in response to motor nerve stimulation at 100 shocks/sec. a, before, and b, 5 min after intra-arterial injection of 10 μg of neostigmine. After neostigmine, the mechanical and electrical responses of the muscle waned characteristically.

with the back-discharge. Trains of stimuli with pulse intervals of 3.2 to 40 msec lasting up to 1 sec every 10 sec, or trains of stimuli with pulse intervals of 64 to 200 msec lasting for 5 sec every 30 sec, were delivered to the nerve. Lower frequencies of stimulation with stimulus intervals of 0.5 to 5 sec were applied continuously for periods up to 5 min.

Repetitive firing did not occur at all with stimulus intervals below 10 msec. With stimulus intervals of 10 to 64 msec, repetitive firing in nerve and muscle was produced only by the first shock of the train and did not reappear during the whole period of stimulation even when, as in records *v* and *vi* of Fig. 6, the muscle action potentials regained their control amplitude. When 100 to 200 msec elapsed between each stimulus, repetitive firing after injection of edrophonium or neostigmine followed the second and sometimes the third shocks of the train but was not evident after subsequent shocks. Similar results were obtained with ambenonium when stimulus intervals of 200 to 400 msec were used. When 0.5 sec elapsed between each stimulus, repetitive firing followed all shocks in the train but became markedly diminished in extent with continuous stimulation, particularly after injection of ambenonium. It was necessary to allow an interval between each stimulus of at least 1 sec with edrophonium and neostigmine and of at least 2 to 3 sec with ambenonium for each nerve volley to continue to produce equal degrees of repetitive firing. These results, therefore, support the conclusions of others (Brown, 1937; Feng & Li, 1941; Werner, 1960a) that repetitive firing plays little part in the action of anticholinesterase drugs at frequencies of stimulation above 2 shocks/sec and therefore cannot be important in the effects of these drugs on sustained voluntary muscular contractions.

In the experiments so far described, the gain for the ventral root recordings was high so that the repetitive discharges in the nerve could be clearly seen and the peaks of the main antidromic nerve action potentials were not visible on the oscilloscope screen. Consequently any effect of the drug on the amplitude of these nerve action potentials could not be observed. Experiments were therefore carried out with a lower amplification of the nerve action potentials.

With stimulus intervals of 1 to 5 msec, trains of volleys after drug administration produced a series of nerve action potentials similar to the control responses. The increased waning of the muscle responses produced by the drugs could not therefore have been due to an effect on nerve conduction as has been described for ethylpyrophosphate (Murtha, McNamara, Edberg, Bergner & Wills, 1955).

When the second and subsequent shocks were delivered during the period of repetitive firing in the nerve produced by the first (as with stimulus intervals from 10 up to 80 msec for neostigmine and edrophonium and from 10 up to 300 msec for ambenonium), the amplitudes of the second and subsequent antidromic nerve action potentials were reduced, as were the corresponding muscle action potentials. Fig. 6 illustrates an experiment in which repetitive firing in response to single shocks was produced by the intra-arterial injection of 3 μg of ambenonium. At the height of the repetitive firing, the single shocks were replaced in successive sweeps by 2, 3, 4, 10 and 20 shocks with intervals of 20 msec between each shock. The amplitudes of the 2nd to the 7th nerve and muscle action potentials were reduced, the maximal

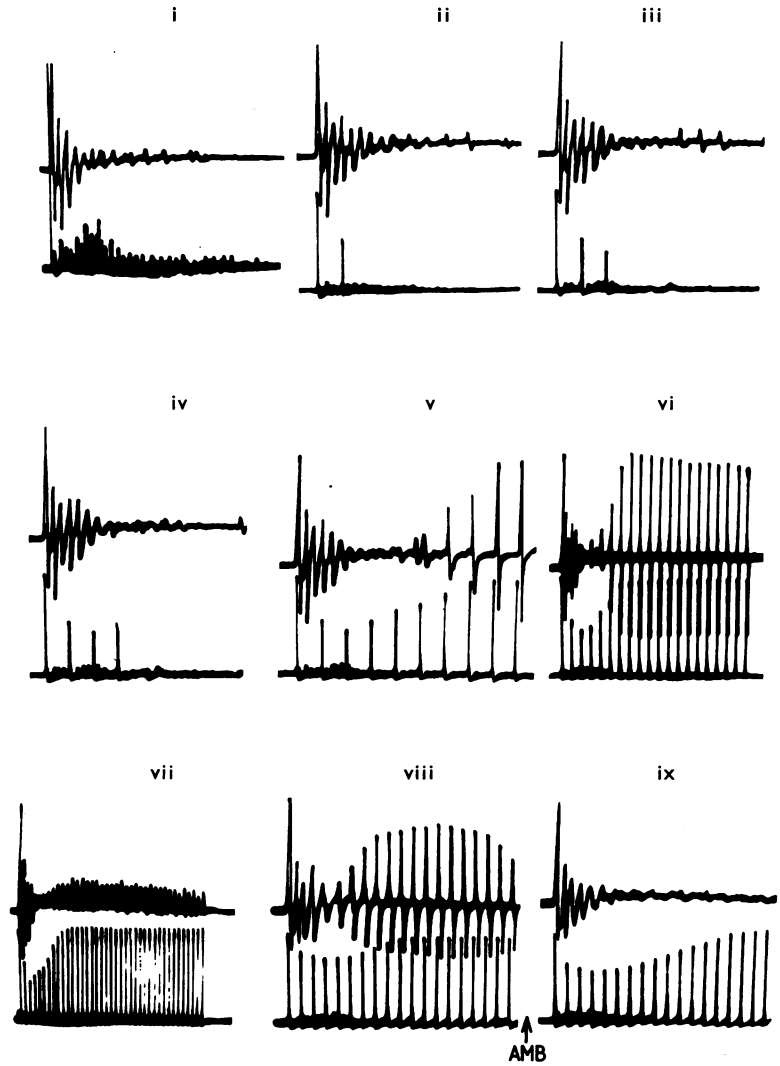

Fig. 6. Recording as in Fig. 1b. Record i shows repetitive firing in response to one of a series of single shocks delivered every 10 sec at the time of maximal effect of 3 μg of ambenonium. For records ii to ix the amplification of the ventral root potentials was reduced so that the whole of the antidromic nerve action potential was visible. Records ii to vi illustrate the effect of replacing the single shocks in successive sweeps by 2, 3, 4, 10 and 20 shocks, each shock being separated by 20 msec. Antidromic nerve action potentials occurring during the repetitive firing were reduced in amplitude and did not elicit muscle action potentials. Repetitive firing occurred only in response to the first stimulus of each train. Records vii to ix illustrate the effects of reducing the stimulus interval to 10 msec. At this frequency even full-sized nerve action potentials elicited only small muscle responses. Record viii was taken 20 min after record vii and illustrates partial recovery from the first dose of ambenonium. Before record ix, a further 5 μg of ambenonium (AMB) was injected. The muscle did not respond to the second and subsequent stimuli of the train even though the nerve action potentials were only temporarily depressed. Note that vi and vii were recorded with a slower time base than the others.

depression occurring with the third shock, that is 40 msec after the start of the tetanus. Subsequent responses then increased in size so that the 8th to the 20th nerve and muscle action potentials were equal in amplitude to the first although no further repetitive firing was evident. These results indicate that the 2nd to the 6th antidromic nerve action potentials had been partly extinguished in the refractory periods produced by the back-discharge. Since the muscle action potentials were correspondingly reduced the orthodromic nerve action potentials must also have been extinguished by collision with the back-discharge. The extent of the reduction in amplitude of the nerve and muscle action potentials showed that the back-discharge must have been travelling in a high proportion of nerve fibres. The period, at the start of a tetanus, during which the nerve action potentials were reduced was generally found to be shorter than the period during which repetitive antidromic discharges followed a single shock. This was probably due to the inhibitory action of tetanic stimulation on the repetitive firing already described.

That anticholinesterase drugs possess an additional and more powerful action in depressing tetanic contractions is also evident from the experiment illustrated by Fig. 6. When the 50 shocks/sec tetani were replaced by tetani of 100 shocks/sec, the muscle response only partially recovered from the effects of extinction of the nerve action potentials. A further gradual decline in the muscle action potentials then occurred during which the nerve action potentials remained constant at the control amplitude (Fig. 6, *vii*). The muscle began to recover from this secondary depressant stage while the initial "extinction" phase of the transmission failure was still present (Fig. 6, *viii*). A second dose of ambenonium (5 μg) injected at this stage markedly potentiated the second phase of the block. The muscle and nerve continued to respond repetitively to the first shock but for the remainder of the tetanus the muscle remained quiescent even though the nerve action potentials fully regained their control amplitude (Fig. 6, *ix*). This type of transmission failure produced by anticholinesterase drugs could occur at all frequencies of stimulation between 2 and 500 shocks/sec. It was more pronounced and quicker in onset the higher the frequency of stimulation and the greater the dose of anticholinesterase drug.

Effect of tubocurarine

Tubocurarine, and substances with a similar mechanism of action, have been shown to abolish the drug-induced repetitive discharges in nerve and muscle in doses below those required to produce neuromuscular block (Werner, 1961a ; Blaber & Bowman, 1963). Fig. 7 illustrates an experiment in which doses of tubocurarine were injected intra-arterially at regular intervals after marked failure of neuromuscular transmission had been produced by ambenonium. Stimulation in this experiment was applied to the motor nerve at a frequency of 100 shocks/sec for 170 msec every 10 sec. The first effect of tubocurarine was to reduce the repetitive firing following the first shock of the tetanus. As a result the depressed nerve action potentials increased in size and elicited larger muscle action potentials. With additional doses of tubocurarine the second phase of block was also antagonized until, after a total dose of 120 μg of tubocurarine, the transmission failure was almost overcome.

When the transmission failure produced by ambenonium was less complete than that illustrated in Fig. 7, it was occasionally observed that a small dose of tubocurarine (30 μg) partially reversed the initial " extinction " phase of the block but actually increased the second phase. This was interpreted as evidence that the second phase of the block was dependent on the nerve impulses reaching the nerve endings. Subsequent doses of tubocurarine then relieved the second phase of the block also. This effect was more prominent when benzoquinonium was used in place of tubocurarine. Benzoquinonium is relatively more powerful than tubocurarine in preventing the repetitive firing in the nerve (Blaber & Bowman, 1963) and the dose of this agent necessary to reverse the initial extinction phase, while at the same time potentiating the second phase, was therefore less critical. This effect of benzoquinonium is illustrated in Fig. 8. Similar results were obtained with edrophonium or neostigmine except that the doses of tubocurarine and benzoquinonium necessary to reverse their effects were about three times smaller.

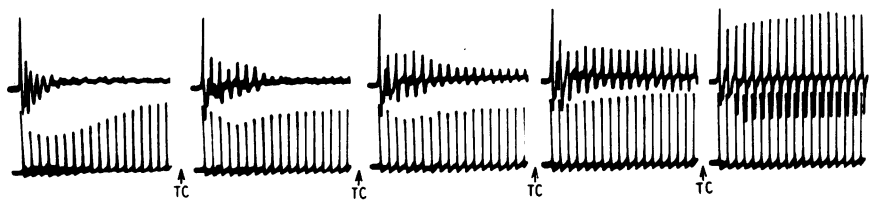

Fig. 7. Continuation of experiment illustrated by Fig. 6. The motor nerve was excited at a frequency of 100 shocks/sec for 170 msec every 10 sec. First record shows the maximum depression produced by ambenonium (total dose, 8 μg). Each of the subsequent records were taken 2 min after the intra-arterial injection of 30 μg of tubocurarine (TC) when the responses had become constant. Recovery from the effect of ambenonium was hastened by tubocurarine. Control experiments showed that, in the absence of tubocurarine, the transmission failure produced by 5 μg of ambenonium persisted for at least 90 min.

The ability of tubocurarine to reverse the transmission failure was most pronounced when it was injected after only one or two small doses of an anticholinesterase drug. When it was injected late in an experiment after the animal had received five or more doses of neostigmine or ambenonium, the reversal was weak or non-existent and further doses of tubocurarine increased the neuromuscular block. Two or three hours after an animal had received several doses of ambenonium, the muscle action potentials in response to single maximal motor nerve shocks often did not differ from the control responses and no repetitive firing was evident in either nerve or muscle. Single shock stimulation therefore gave the appearance of complete recovery from the effects of the anticholinesterase. However, the use of closely-spaced paired volleys showed that the refractory period was prolonged and the high frequency inhibition of the muscle response was more pronounced and occurred at lower frequencies of stimulation than it had done earlier in the experiment at a time when pronounced repetitive firing was produced by single shocks. In one such experiment during stimulation at 1 shock every 10 sec an intravenous injection of tubocurarine (100 μg/kg) caused an immediate and long-lasting block of the muscle action potentials. This dose is at least three times

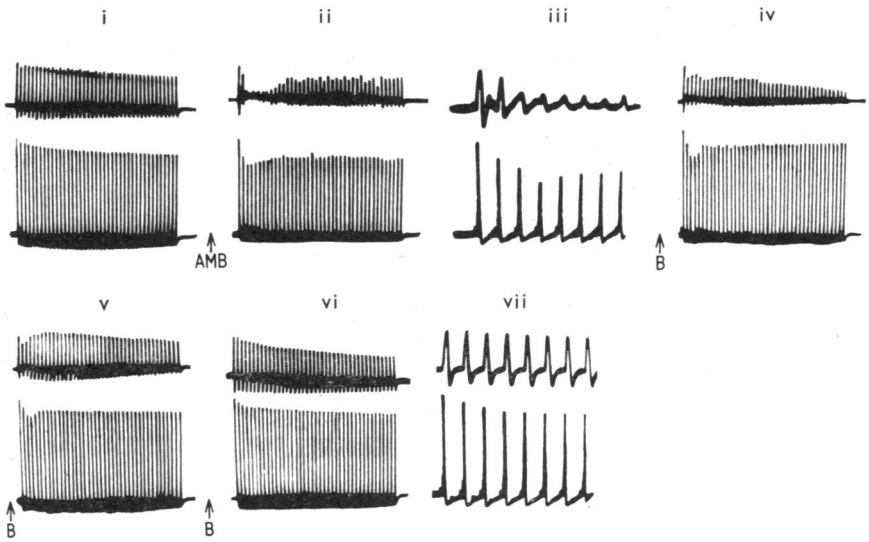

Fig. 8. Muscle (upper beam) and ventral root (lower beam) action potentials in response to stimulation of the motor nerve at a frequency of 100 shocks/sec for 0.4 sec every 10 sec. Records iii and vii were taken 10 sec after records ii and vi respectively, and show the first part of the tetanus recorded with a faster time base. Record i is one of a series of identical responses before drug treatment. Record ii illustrates the maximum depression produced by 2.5 μg of ambenonium (AMB). Records iv, v and vi were each taken 90 sec after injection of 5 μg of benzoquinonium (B). The first dose of benzoquinonium caused a partial recovery of the depressed nerve action potentials which then excited the muscle. However, the muscle action potentials at the end of the train were further depressed (compare iv with ii). Subsequent doses of benzoquinonium completely reversed the effect of ambenonium (vi).

smaller than that required to produce a similar block in an animal which has not previously been treated with anticholinesterases.

DISCUSSION

A consideration of some of the reported actions of anticholinesterase drugs on neuromuscular transmission will help to assess the significance of the results obtained in the present study. In earlier experiments, Blaber & Bowman (1963) studied factors affecting the repetitive discharges induced in nerve and muscle by single shocks delivered in the presence of anticholinesterase drugs. Their results, together with those of other workers (Masland & Wigton, 1940 ; Eccles *et al.*, 1942 ; Riker *et al.*, 1957, 1959 ; Werner, 1960a and b, 1961a and b), led to the suggestion that anticholinesterase drugs may affect transmission by acting at three sites at the neuromuscular junction—on acetylcholinesterase, at the motor nerve ending and at the motor end-plate—and that effects due to reaction of the drug at any one site may be augmented by the production of reverberating activity across the junction.

Hubbard & Schmidt (1961) have shown that the non-myelinated motor nerve terminals in the phrenic nerve-diaphragm preparation of the rat exhibit marked negative and positive after-potentials and that both are enhanced in extent and duration by neostigmine. The potentiated negative after-potential long outlasts the

refractory period of the adjacent myelinated axon. In the presence of an anticholinesterase agent, therefore, the nerve terminal will act as a current sink for the portion of myelinated axon immediately central to it and may give rise to repetitive antidromic discharges recordable in the ventral root (Werner, 1960a; Hubbard & Schmidt, 1961). Hubbard & Schmidt (1961) also showed that neostigmine caused a reversible increase in the duration of the refractory period of the nerve terminals, which implies that the duration of the action potential in the terminals was also increased. Since the quantity of transmitter released is a function of the extent and duration of the depolarization of the terminal membrane (Liley, 1956), Hubbard & Schmidt (1961) concluded that the period of transmitter release in response to a nerve impulse was prolonged by neostigmine, supporting the contentions of Bowman (1958) and Blaber & Bowman (1959, 1962a and b) based on more indirect evidence. These pre-junctional actions might themselves be sufficient to explain some of the facilitatory effects of anticholinesterase drugs on neuromuscular transmission but clearly they would be augmented by additional effects such as: (1) Liley (1956) has shown that cathodal currents applied to the nerve endings greatly increase the frequency of the miniature end-plate potentials. Thus the spontaneous release of transmitter is probably augmented during the potentiated negative after-potentials in the terminals and this would occur immediately after the excessive release produced by the nerve impulse. Excessive release of acetylcholine, coupled with a reduced rate of destruction due to cholinesterase inhibition, would lead to the greatly augmented and prolonged end-plate potentials and repetitive firing of the muscle fibres first described by Eccles *et al.* (1942) and by Brown, Dale & Feldberg (1936) respectively. (2) A repetitive discharge initiated in the nerve by the potentiated negative after-potential could not be transmitted orthodromically into the associated muscle fibre. However, even if initiated at only one neuromuscular junction, such a repetitive discharge would propagate by axon reflex into the remaining fibres of the motor unit and so augment the post-junctional repetition of the muscle as a whole. Such an effect would account for the finding of Riker *et al.* (1959) that, while recording from a single ventral root fibre and from the muscle unit innervated by it, occasionally there was a one-to-one relationship between nerve and muscle spikes in a repetitive discharge, and that the nerve spike preceded that of the muscle. Spreading activity of this type might partly explain the long duration of the repetitive discharge (up to 300 msec) when, as in the present experiments, the recording electrodes were in contact with many motor units. This period is greatly in excess of the duration of the potentiated negative after-potential in the individual nerve terminal which, from the results of Hubbard & Schmidt (1961), is unlikely to exceed 50 msec. (3) In the absence of drugs, gross electrical activity in the muscle can ephaptically re-excite the motor nerve (Lloyd, 1942; Brown & Matthews, 1960). During the potentiated negative after-potential produced by an anticholinesterase drug the threshold of the nerve terminal to electrical excitation must be lowered and under these conditions it is possible that the augmented end-plate potentials can ephaptically re-excite their associated nerve terminals. Eccles *et al.* (1942) showed that in the eserinized muscle which had been conditioned by a preceding nerve volley, a muscle spike, initiated by direct stimulation of the muscle at some distance from the end-plate, gave rise to a dis-

charge of impulses in the motor nerve. However, this retrograde transmission never occurred in the absence of eserine. (4) Although there is, as yet, no convincing evidence that acetylcholine can depolarize somatic motor nerve endings, there is strong evidence that it does possess this action at the nerve endings of sensory C fibres (Douglas & Ritchie, 1960). If the threshold of the motor nerve terminals to chemical excitation is lowered during the potentiated negative after-potential, the excess acetylcholine in the junctional region might then be capable of exciting the terminals and further augmenting the back-discharge. Masland & Wigton (1940) found that, after the administration of neostigmine, injected acetylcholine caused repetitive firing in motor nerves even in the absence of electrical stimulation.

Two stimuli separated by short intervals of 2 to 5 msec augmented the repetitive discharge in both nerve and muscle. Werner (1960a) concluded that this effect was due to summation of the negative after-potentials at the nerve endings. In addition, however, closely spaced volleys must release more acetylcholine than a single volley and, since hydrolysis of the transmitter is prevented, this will lead to enlarged post-junctional end-plate potentials (Eccles *et al.*, 1942). As well as increasing the muscle repetition directly, these factors might produce additional ephaptic and chemical re-excitation of the sensitized terminals which would further augment the repetitive discharge. A second stimulus delivered 10 msec or more after the first did not increase the repetitive firing, and often produced no response from the muscle at all, probably because it was extinguished in many nerve fibres by collision with the back-discharge. As the repetitive firing began to wane, the second orthodromic volley reached the terminals in a greater number of fibres and gave rise to a larger muscle action potential. However, those nerve fibres which had ceased to conduct the repetitive discharge would have entered the period of reduced excitability due to the enhanced positive after-potential and this would temporarily depress the production of further repetition in the nerve no matter what the source of its initiation. Werner (1960a) also concluded that the positive after-potential exerts a limiting effect on the repetitive firing.

Riker *et al.* (1959a and b) showed that the frequency of the antidromic repetitive discharge in single ventral root fibres reached 100 to 250 impulses/sec at the time of maximal drug effect. Thus, although only single shocks may be delivered to the motor nerve, many muscle units, through axon reflexes, will be subjected to a high frequency tetanus. The nerve terminal appears quickly to regain the ability to release sufficient transmitter to produce maximal non-repetitive muscle action potentials. However, extra time will be required for replenishment of the excess acetylcholine necessary to produce a full-sized repetitive response and, since the hydrolysis of transmitter is prevented, this time may be longer than it would otherwise be owing to the nerve endings having been robbed of a source of choline for re-synthesis. This may be the explanation of the long interval which was found necessary between shocks in order for each to continue to produce repetitive responses equivalent to the first. As would be expected, the more prolonged the repetitive discharge following the first shock, the longer the necessary interval between stimuli.

Although closely-spaced double volleys augmented the repetitive firing, a few additional stimuli depressed it despite the finding of Eccles *et al.* (1942) that such stimulation, in the presence of an anticholinesterase drug, produces greatly augmented end-plate potentials. However, Hubbard & Schmidt (1961) showed that brief trains of volleys reduced the negative after-potential in the nerve terminals and increased the secondary subnormal phase. This latter effect, therefore, must contribute to the depressant effect of tetanic stimulation on the repetitive firing.

The main effect of anticholinesterase drugs on tetanic contractions is depressant, unless the sensitivity of the motor end-plates to acetylcholine has been previously reduced, for example by tubocurarine. Several factors may contribute to this depressant effect, and which of these is important will depend upon the frequency of stimulation and upon the dose of anticholinesterase drug used.

At very high frequencies of motor nerve stimulation (300 shocks/sec and above), the muscle response rapidly wanes even in the absence of anticholinesterase drugs. This may be largely explained by the inability of the nerve endings to mobilize transmitter sufficiently rapidly, since the acetylcholine released per nerve volley is known to diminish as stimulation frequency is increased (Straughan, 1960 ; Krnjević & Mitchell, 1961). In addition, closely-spaced volleys prolong the refractory period of the muscle fibre membrane (Eccles & Kuffler, 1941a and b) and this effect probably contributes to the rapid fall-off of the muscle response. An initial excessive release of acetylcholine produced by the anticholinesterase drugs would hasten the subsequent diminished release by each nerve impulse and this action, together with the further prolongation of the refractory period produced by these drugs, might partly account for the enhanced transmission failure occurring at very high stimulation frequencies. In addition, the preserved acetylcholine, accumulating at the neuromuscular junction, will lead to depolarization block of the motor end-plates (Eccles *et al.*, 1942 ; Burns & Paton, 1951 ; Douglas & Paton, 1954). A transmission failure, unaccompanied by depressed nerve action potentials, may be assumed to be due to depolarization block if it is reversed by tubocurarine which reduces the excessive end-plate depolarization. Depolarization block appeared to play a large part in the depressant action of the anticholinesterase drugs over a wide range of frequencies (down to 3 shocks/sec depending on the dose). However, after repeated administration of anticholinesterase drugs, even at slow rates of stimulation, tubocurarine may enhance the transmission failure. This may be explained if, at this stage, the block is due more to a reduced transmitter output than to excessive end-plate depolarization.

Tubocurarine and benzoquinonium, by abolishing the repetitive discharge in the nerve, also reversed the initial " extinction " phase of transmission failure which occurred during tetanic stimulation at frequencies of 100 shocks/sec and below. This action of the blocking drugs is probably mainly explained by Hubbard & Schmidt's (1961) observation that tubocurarine reduces the extent and duration of the negative after-potential in the motor nerve terminals. Since tubocurarine also causes the enlarged end-plate potentials to return to normal values (Eccles *et al.*, 1942), reduced ephaptic re-excitation of the terminals may also contribute to the depressed back-discharge.

In voluntary movements, the motoneurones rarely discharge at frequencies greater than 100 impulses/sec. Repetitive firing in the presence of an anticholinesterase drug may therefore occur in response to the first nerve impulse initiating the voluntary movement. The longest duration of the repetitive discharge in these experiments was found to be 300 msec and extinction of the nerve action potentials can therefore play only a very small part in the depressant effect of anticholinesterase drugs on sustained voluntary muscle activity. This effect is probably largely due to depolarization block caused by accumulating acetylcholine. Nevertheless, extinction of the nerve action potentials might be important in the depressant effect on muscles, such as those concerned in respiration, which are activated by short-lasting intermittent bursts of nerve volleys. In intact animals, however, central actions and effects on the γ-motor system, where transmission to the muscle spindles is also known to be cholinergic, may complicate the picture.

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