J. Physiol. (1963), **169**, *pp.* 641–662
With 9 text-figures
Printed in Great Britain

REPETITIVE STIMULATION AT THE MAMMALIAN NEUROMUSCULAR JUNCTION, AND THE MOBILIZATION OF TRANSMITTER

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(*Received* 7 *May* 1963)

The effects of nerve stimulation at a neuromuscular junction are both immediate and delayed. The immediate effect is the almost simultaneous release of many quanta of transmitter (Katz, 1958) which is detected most easily by its post-junctional result, the end-plate potential (e.p.p.). The delayed effects include an increase in the probability of the spontaneous release of transmitter detected as miniature end-plate potentials (m.e.p.p.s), and an increase in the amount of transmitter released by a testing nerve impulse following the first impulse or impulses at a suitable interval (Fatt & Katz, 1952; del Castillo & Katz, 1954). It is clear that changes in motor nerve terminals are largely responsible for these delayed effects of stimulation (Hutter, 1952; Liley & North, 1953; del Castillo & Katz, 1954) but the nature of these changes is still obscure.

Recently it has become technically feasible to study the properties of motor nerve terminals as they are revealed by extracellular recording and stimulation (Hubbard & Schmidt, 1963) and the application of polarizing currents (Hubbard & Willis, 1962). In the course of these investigations it was desirable to compare the changes in the nerve terminals with the concurrent alterations in transmitter release revealed by the recording of e.p.p.s and m.e.p.p.s. It was found that no systematic investigations had previously been made of three aspects of repetitive stimulation. First, there was no direct investigation of the effects of small numbers of stimuli, investigation having been confined to the effect of a single volley (Eccles, Katz & Kuffler, 1941; Lundberg & Quilisch, 1953) or a single volley and many hundreds of volleys (Liley & North, 1953). Secondly, the characteristics of repetitive stimulation at various frequencies had not been systematically studied. Finally, apart from the investigations of Feng (1941) and Liley (1956*b*), the effects of repetitive stimulation have been studied in curarized preparations rather than magnesium-paralysed preparations in which it is possible to record simultaneously changes in e.p.p. amplitude and m.e.p.p. frequency.

In the present investigation a comprehensive range of numbers and frequencies of stimulating volleys have been employed in the rat diaphragm *in vitro* paralysed with either D-tubocurarine or $MgCl_2$. A preliminary account of some parts of the present investigation has been published (Hubbard, 1959).

METHODS

The rat hemidiaphragm–phrenic-nerve preparation (Bulbring, 1946) was used in all experiments. The dissection, recording chamber, solutions and intracellular recording technique have been previously described (Hubbard, 1961). The amplifier was capacitively coupled and had a time constant of 30 msec. $MgCl_2$ or D-tubocurarine chloride (Burroughs Wellcome) was added to the bathing solution in sufficient amounts to paralyse neuromuscular transmission. The preparations were kept between 35 and 37° C. The nerve threshold was checked repeatedly during an experiment and stimuli were kept supramaximal. It was possible to make tetanic stimuli trigger the sweep of the cathode-ray oscilloscope tube, individual responses then appearing successively at the same point on the screen.

It is known that in Mg-paralysed preparations after 5–10 stimuli e.p.p. responses reach a new plateau level of amplitude, which only slowly increases with further stimuli (Feng, 1941; del Castillo & Katz, 1954; Liley, 1956*b*). Similarly in a curarized preparation, after 5–10 stimuli e.p.p. responses attain a plateau of amplitude (Liley & North, 1953), which was found in the present investigation to be maintained for 50–100 responses at frequencies below 100/sec. The amplitudes of e.p.p.s during the plateau phase of a tetanus were thus obtained by superposition of faint traces of the successive responses, the plateau height appearing as a sharply demarcated trace. At slow frequencies 5–10 responses were superimposed, but there were many more at higher frequencies. Measurements of m.e.p.p. frequency during tetanic stimulation (Fig. 3*A*) were made at junctions where the average e.p.p. amplitude was much larger than the average m.e.p.p. amplitude. The recognizable m.e.p.p.s were counted, but it is possible that some superposition of e.p.p.s and m.e.p.p.s might not have been detected at the sweep velocity employed in these experiments.

The post-stimulation effects were so short after a tetanus of fewer than 50 volleys that there was time to elicit only one test response per tetanus. The test response was therefore kept at a constant position on the cathode-ray oscilloscope screen and put on an extended sweep. After several control responses (5–10 in the curarized preparation, 10–15 in the Mg-treated preparation) had been obtained, the conditioning tetanus was applied at a known interval before the test stimulus. In curarized preparations at least three responses were obtained at each interval, but in Mg-treated preparations 10–15 were necessary because of the variability of response. The procedure was repeated for as many intervals as required, further control responses being obtained after the examination of every 3–5 intervals. The interval between two successive repetitions was adjusted in the light of preliminary experiments so that it always exceeded the duration of the post-tetanic effects. It was at least a second in Mg-paralysed preparations and 5 or more seconds in curarized preparations.

There was only a small acceleration of m.e.p.p. frequency after fewer than 20 stimuli, even at a frequency of 200/sec. This was assessed by placing the 1–20 conditioning volleys in the centre of a cathode-ray oscilloscope sweep lasting at least a second. M.e.p.p.s were counted in the 500 msec period before and after the stimulation. The 500 msec post-tetanic period was divided into 10 msec intervals for counting purposes, a photographic enlarger being used to magnify the film records. By comparing the number of m.e.p.p.s in pre- and post-tetanic periods after some 200 trials, it was possible to make estimates of the probability of m.e.p.p. release at varying intervals after stimulation.

After longer stimulation the acceleration of m.e.p.p. frequency was large enough for single trials to give significant information. The mean frequency was therefore determined for a

60 sec period before stimulation and this mean frequency was compared with the frequency found in successive 1 sec periods after stimulation. In many experiments, in Mg-treated preparations m.e.p.p. frequency and e.p.p. amplitude were measured at the same junction from records of the same cathode-ray oscilloscope sweep. Successive sweeps had a 1 sec duration with a 1 msec fly-back time. Stimuli fell in the centre of each sweep, and therefore arrived at 1 sec intervals. In these experiments the tetanic stimuli were applied without change of sweep speed and all responses appeared on the film record. Experiments in which there was any failure of response of the type described by Krnjević & Miledi (1959) were discarded.

RESULTS

After one impulse at a mammalian neuromuscular junction there is an increase in the number of quanta of acetylcholine (ACh) released, both spontaneously and in response to a testing nerve impulse (Brooks, 1956; Liley, 1956*a*). The time course of these effects (Fig. 1) is of interest in that it may provide a basis for the understanding of the effects of repetitive stimulation on transmitter release. Figure 1*A* illustrates the increased amplitude of the second of the e.p.p.s set up by paired stimuli in a Mg-paralysed preparation. The time course of this effect is shown in Fig. 1*C* (inset), in which the average amplitude of the second e.p.p. is plotted as a multiple of the amplitude of the first control e.p.p. The graph (Fig. 1*C*) and sample records (Fig. 1*A*) show that the potentiation of the second e.p.p. was largest at very short intervals after the conditioning stimulus, could be detected at intervals up to 100 msec and was not followed by any depression of e.p.p. response. A similar time course for this potentiation of e.p.p.s after a single impulse was found at the frog neuromuscular junction (Feng, 1941).

It was also possible to examine the time course of the effect of single impulses upon the spontaneous release of transmitter. This was done by comparing, in a large number of trials, the incidence of m.e.p.p.s in equal periods before and after stimulation. In the graph (Fig. 1*C*) the incidence after stimulation is plotted as a multiple of the number to be expected in the absence of stimulation. In the 10 msec period immediately after the impulse the probability of spontaneous quantal release was raised some $2\frac{1}{2}$ times. At longer intervals after the stimulus the difference between the number of m.e.p.p.s found and the number expected was small. For instance, there were 64 m.e.p.p.s in the period 10–100 msec after the impulse, whereas 49 were expected, and in the period 100–200 msec after the impulse there were 63 m.e.p.p.s, the expected number being 54. In the period 200–300 msec after the impulse there was no significant difference between the number of m.e.p.p.s expected (54) and the number found (55).

In curarized mammalian preparations potentiation of the second e.p.p. response to paired stimuli has been described only as an occasional finding (Lundberg & Quilisch, 1953). In the present investigation such potentia-

tion was found at 27 of 30 junctions in which intervals of 1–15 msec were systematically explored. Figure 1*B* shows sample records of e.p.p.s in response to paired stimuli, the size of the second e.p.p. from these and other records being plotted in the graph (Fig. 1*D*, filled circles) as a

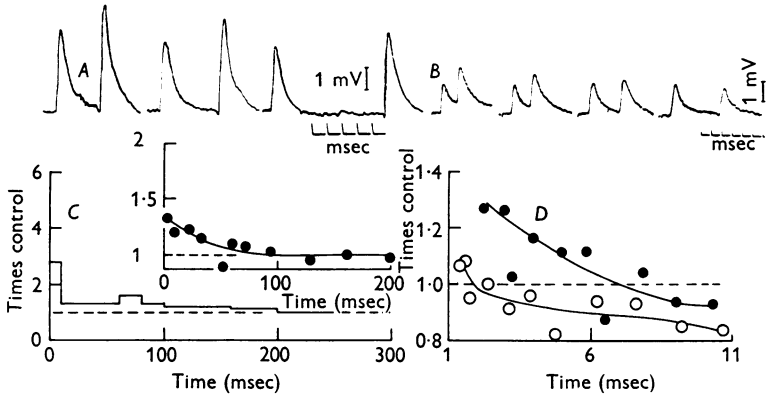

Fig. 1. The effect of one impulse on e.p.p. amplitude and m.e.p.p. frequency. *A* shows potentiation of an e.p.p. elicited by the second of paired stimuli in a Mg-paralysed preparation. In *C* (inset) the amplitude of this second potentiated e.p.p. is plotted as a multiple of the first e.p.p. for intervals up to 200 msec; each point represents the average of 10–15 trials at each interval. Also shown in *C* is the probability of m.e.p.p. occurrence at intervals up to 250 msec after an impulse. This graph was constructed from data obtained in over 200 trials in which m.e.p.p.s were counted in a 500 msec period before a single impulse and in 10 msec periods thereafter. From the total count before stimulation (270) the average number of m.e.p.p.s to be expected in any period after stimulation could be calculated (assuming stimulation had no effect). In fact there was always an excess of m.e.p.p.s in the first 200 msec period after stimulation, the greater part of this excess falling in periods immediately after stimulation. In the graph this has been shown by lines placed to indicate m.e.p.p. numbers as a multiple of the number expected in that interval. The intervals are shown by the line lengths, which represent 10 msec immediately after the stimulus, then 20 msec and at later periods 50 msec. In some places the lines are confluent, the numbers of m.e.p.p.s in adjacent periods being then the same.

B shows potentiation of an e.p.p. elicited by the second of paired stimuli in a curarized preparation. In the graph in *D* the amplitude of the second e.p.p. is plotted as a multiple of the first e.p.p. for intervals up to 11 msec. Each point is the average of 3–6 trials at each interval. Two examples are shown, one (○) showing potentiation only at intervals less than 2 msec, the other (●) showing potentiation at intervals up to 6 msec.

multiple of the first control e.p.p. As this graph shows, the interval between stimuli for which the second response was on the average larger than the first could be as long as 10–15 msec. More generally it was only 2–5 msec, as in Fig. 1*D*, open circles. At three junctions no absolute potentiation could be found. The increasing depression of e.p.p. amplitude which can

be detected for up to 200 msec after this potentiation and the recovery at longer intervals has been well documented (e.g. Eccles *et al.* 1941; Lundberg & Quilisch, 1953). It was noticed that the duration of the period required before a second impulse elicited an e.p.p. of control size varied from 1 to 10 sec at various junctions, as might be expected from the ability of some junctions to maintain e.p.p. amplitudes at a constant size when stimulated repetitively at such intervals (see below, Fig. 2).

It may be concluded that in both curarized and Mg-treated preparations a single impulse increases ACh release for a period of about 100 msec. This effect could be caused either by an increased ability of impulses to release transmitter, or to an increase in the available transmitter, a process which has been termed mobilization (Eccles, 1957). Recent investigations (Hubbard & Willis, 1962, 1963; Hubbard & Schmidt, 1963) support the hypothesis that there is a mobilization of transmitter. The present results suggest that this mobilization is largest immediately after an impulse, and wanes over a 100 msec period thereafter. Del Castillo & Katz (1954) have analysed this effect in solutions with a high Mg content, where the quantal content of e.p.p.s is small and often there is no transmitter release upon stimulation. In these circumstances they found that the potentiation of the second e.p.p. response to paired stimuli took place, whether or not there was any release of transmitter by the first impulse. In the rat diaphragm a similar clear separation of the mobilizing and transmitter-releasing functions of a nerve impulse is also possible. By increasing the Mg content of the bathing solution to 15–17 m-mole/l. at 37° C the individual e.p.p.s were so reduced in quantal content that in a third to a half of trials there was no ACh release on stimulation.

TABLE 1. Comparison of total and selected responses to the second of paired stimuli. The number of responses in each class is shown in brackets. In the 4th and 6th columns the size of response in each class is shown as a multiple of the average size of all responses to the first stimulus

Expt.	Interval (msec)	Mean of all responses to second stimulus		Mean of selected responses (no response to first impulse)	
		mV	2/1	mV	2/1
1	3·25	0·25 (317)	1·54	0·25 (181)	1·56
2	4·1	0·31 (315)	1·50	0·31 (143)	1·51
3	4·1	0·31 (342)	1·47	0·30 (179)	1·42
4	5·5	0·51 (373)	1·34	0·52 (125)	1·38
5	7·5	0·29 (218)	1·20	0·32 (106)	1·28
6	19·8	0·36 (386)	1·21	0·38 (142)	1·24
7	67·5	0·25 (433)	1·11	0·24 (188)	1·07

Table 1 shows that in these conditions when paired stimuli were applied at intervals up to 67·5 msec, the longest studied, the average response to the second stimulus was larger than the response to the first stimulus as in solutions of higher Mg content (Fig. 1*A*, *C*). For all these intervals also

the response to the second stimulus was of much the same size whether or not the first impulse released any ACh (Table 1, compare columns 3 and 5). It seems reasonable to assume therefore that the mobilizing function of the impulse may occur independently of its releasing function in mammalian nerve terminals as in amphibian (del Castillo & Katz, 1954) and crustacean nerve terminals (Dudel & Kuffler, 1961).

TABLE 2. Comparison of total and selected numbers of m.e.p.p.s found in a 200 msec period before and after a single impulse. The number of m.e.p.p.s found when there was no e.p.p. response is shown in brackets. Also in brackets (Col. 2) is the number of trials in which stimulation did not elicit an e.p.p.

Expt.	No. of trials	Number of m.e.p.p.s		B/A	
		Before (A)	After (B)	All	No e.p.p.
1	220 (78)	317 (108)	362 (122)	1·14	1·13
2	259 (138)	210 (121)	242 (139)	1·15	1·15
3	263 (163)	163 (98)	185 (111)	1·13	1·13

It might be expected that the increased probability of spontaneous release of ACh found after an impulse (Fig. 1C) would also be demonstrable, even if the impulse did not release any ACh. Table 2 shows the results of 3 experiments in which the numbers of m.e.p.p.s found in a 200 msec period before and after an impulse were compared in a large number of trials. In each experiment a 13–15% greater number of m.e.p.p.s was found after stimulation, whether or not there was an e.p.p. response to the stimulus. Similar results were obtained in 3 smaller experiments (not shown). Apparently therefore mobilization affects both spontaneous and nerve-impulse-induced quantal release in the absence of any activation of the transmitter release process.

During repetitive stimulation

During repetitive stimulation a summation of the mobilizing action of single nerve impulses would be expected to increase progressively the quantal content of responses. In the Mg-paralysed rat diaphragm this appears to be true especially for the first 5–6 impulses at high frequencies of stimulation (Liley, 1956*b*). Following upon this rapid increase in ACh release, there was a further more gradual increase.

The first two impulses of a tetanic train at any frequency above 10/sec correspond to a pair of impulses at intervals less than 100 msec, i.e. the situation illustrated in Fig. 1 and Table 1. If the mobilizing action demonstrated in this situation applies also to later responses in the tetanic train it should be possible to show that the potentiation of these responses can also be developed in the absence of any previous transmitter release.

The relation of e.p.p. amplitude during tetanic stimuli to previous ACh release was tested in 6 experiments, 4 stimuli being used at a frequency of

50/sec (1 experiment), 100/sec (2 experiments) and 200/sec (3 experiments). In each case the amplitude of the third e.p.p. in the train was measured and compared with a series of third e.p.p.s selected because there was no response to the first and second stimuli. In each experiment there was no significant difference between the average e.p.p. amplitudes in the two groups. The largest experiment, in which there were 463 trials of 4 stimuli at 200/sec, enabled a similar test to be made of the fourth e.p.p. response. In this experiment the average e.p.p. response, on the 39 occasions when there was no response to the first 3 stimuli, did not differ significantly in amplitude from the average amplitude of all 463 fourth responses. It seemed therefore that the increased quantal content of the third and fourth e.p.p. responses to tetanic stimuli was brought about by a process activated independently of the release of ACh.

Again, if mobilization is active during repetitive stimulation the frequency characteristics of the e.p.p. potentiation found during repetitive stimulation should be consonant with the time course of the process in its simplest state—after one impulse. Thus it would be expected, first, that there would be no potentiation if successive impulses fell at intervals of 100 msec or more, and secondly that potentiation would be especially marked at intervals shorter than 10–20 msec. The frequency characteristics of the e.p.p. potentiation during repetitive stimulation were tested in a large number of experiments. A typical experiment is illustrated in Fig. 2*B*, in which sample records of superimposed e.p.p.s obtained during frequencies of stimulation from 10 to 200/sec in a magnesium-paralysed preparation are displayed. The graph (Fig. 2*D*) of e.p.p. amplitudes obtained from these and other records shows that there was no increase in e.p.p. amplitudes during repetitive stimuli at frequencies less than 10/sec, that is, an interval between stimuli of 100 msec. At shorter intervals (higher frequencies) e.p.p. amplitudes progressively increased, this increase being greatest for frequencies of stimulation above 100/sec.

In the curarized preparation the sample records of Fig. 2*A* and the graph (Fig. 2*C*, open circles) show the decline of e.p.p. amplitude with increasing frequency of stimulation, that is, a decreasing interval between stimuli. Also plotted in the graph (Fig. 2*C*, filled circles) are results from another junction which showed a more profound depression of e.p.p. amplitudes as the interval between stimuli decreased. It will be noted that at one junction (Fig. 2*A* and *C*, open circles) the e.p.p. amplitudes remained constant as the interval between stimuli fell from 5 to 2 sec. At the other junction (Fig. 2*C*, filled circles) a fall in interval from 10 to 7·5 sec was sufficient to reduce e.p.p. amplitudes some 10%. At both junctions the e.p.p. amplitudes fell as the interval between stimuli decreased from 1 sec to 20 msec. At close intervals corresponding to those

at which absolute potentiation was found after one impulse (Fig. 1*B*, *D*) there was characteristically some recovery of e.p.p. amplitude (Fig. 2*C*, *A*, 80/sec). When the stimuli fell at intervals of 5–6 msec or less, however, a further decline in amplitude occurred.

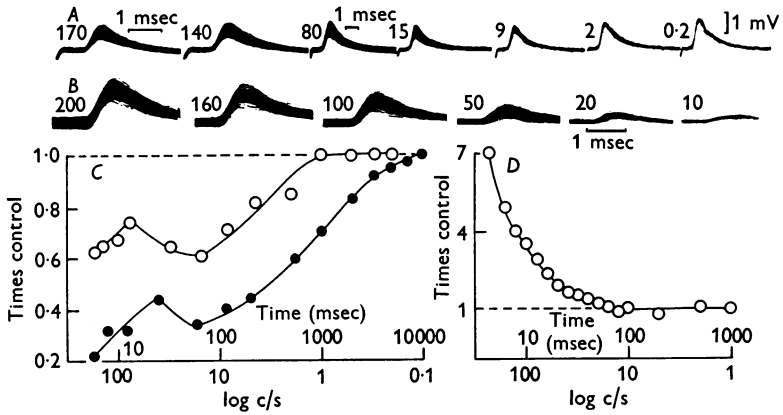

Fig. 2. The effect of stimulation frequency upon the amplitude of e.p.p.s during repetitive stimulation. *A* shows records of e.p.p. responses made by superposing faint traces at the indicated frequencies of stimulation (c/s) in a curarized preparation. In *C* (○) the average e.p.p. amplitude found in these and other records from the same experiment is plotted as a multiple of the e.p.p. amplitude during stimulation at a frequency of 0·1/sec (interrupted line). Also shown in *C* (●) are the results of another experiment similarly plotted, showing a much greater depression of e.p.p. amplitude as the frequency of stimulation increased. In these and all similar experiments there was some relief of the depression of e.p.p. amplitude at stimulation frequencies between 10 and 100/sec.

B shows records of e.p.p. responses made as in *A* by superposing faint traces during repetitive stimulation, but in a Mg-paralysed preparation. In *D* the average e.p.p. amplitude measured from these and other records in the same experiment is plotted as a multiple of the e.p.p. amplitude during stimulation at a frequency of 1/sec (interrupted line). In these experiments e.p.p. amplitudes during stimulation at frequencies above 10–12·5/sec were always greater than the control amplitude.

M.e.p.p. frequency has been considered an index of transmitter availability (Eccles, 1957). It would be expected therefore that the frequency changes during tetanic stimulation would parallel the changes in e.p.p. amplitude. In agreement with this hypothesis Fig. 3*A* shows that m.e.p.p. frequency increased rapidly during the first few seconds of tetanic stimulation and thereafter only slowly as stimulation continued. In this figure m.e.p.p. frequency is plotted over 5 sec periods before, during and after a tetanus of 1500 impulses at 50/sec. It will be noticed that the m.e.p.p. frequency more than doubled in the first 5 sec period and did not increase much more after a further 25 sec. In the first 5 sec after stimulation the

frequency did not drop much below the mean level during the tetanus. Indeed, in experiments of this type it was found that for the first 1–2 sec after prolonged tetanic stimulation m.e.p.p. frequency remained close to the level attained during the tetanus.

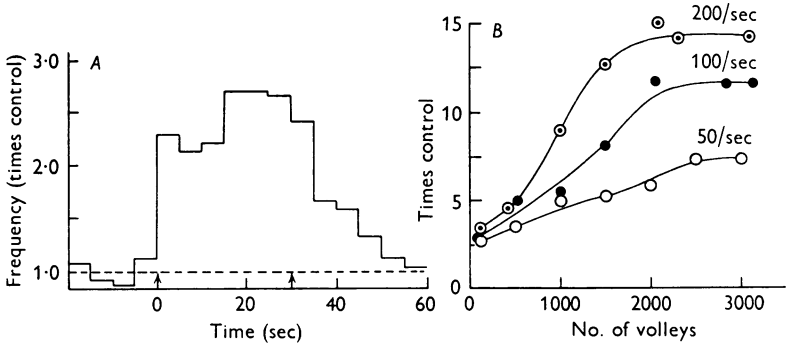

Fig. 3. M.e.p.p. frequency during repetitive stimulation of a Mg-paralysed preparation. *A* shows m.e.p.p. frequency over 5 sec periods before, during and after a tetanus of 1500 impulses at 50/sec. The frequency is shown as a multiple of the mean frequency for 20 sec before stimulation (interrupted line); arrows mark the beginning and end of the tetanus.

In *B* the mean m.e.p.p. frequency in the first 2 sec after stimulation is shown as a multiple of the mean frequency for 60 sec before stimulation. The stimulation was at frequencies of 50/sec (○), 100/sec (●) and 200/sec (⊙) and the numbers of stimulating volleys varied between 100 and 3000. Each series at the same frequency is from the same junction and all series are from the same preparation.

Figure 3*B* shows the frequency in this 2 sec post-tetanic interval after 100–3000 volleys at 50, 100 and 200/sec. After some 2000 volleys at 100 and 200/sec and 2500 at 50/sec m.e.p.p. frequency did not increase further. The final levels attained were approximately proportional to the frequency of stimulation, so that the effects of frequency and number of volleys were not interchangeable. During stimulation at frequencies of less than 10/sec the m.e.p.p. frequency changes were no greater than could be expected after single impulses. Investigations of both e.p.p. amplitudes and m.e.p.p. frequency suggest therefore that at frequencies above 10/sec a process which potentiates transmitter release is activated.

After repetitive stimulation

Mg-paralysed preparation. Figure 4*A* shows a short train of e.p.p.s elicited by 20 stimuli at a frequency of 200/sec and the e.p.p. set up by a testing volley (arrow) at various intervals after the tetanus. Clearly the process by which e.p.p. amplitudes were increased during the tetanus was progressively less potent as the interval between test volley and tetanus lengthened. The graphs inset in Fig. 4*B*, *C* and *D* show the average

amplitude of a testing e.p.p. as a multiple of the average amplitude in the absence of stimulation, at intervals up to 200 msec after 5 (*B*), 10 (*C*) and 20 (*D*) stimuli at 200/sec. All the graphs were made from records from the same junction. In each case, as after one volley (Fig. 1*A*, *C*), the testing e.p.p. was largest at the closest intervals and the potentiating process was short-lasting, persisting only about 200 msec at most after even 20 volleys (Fig. 4*D*, inset). Figure 4*B*, *C* and *D* shows the probability of m.e.p.p. occurrence after the same number of stimuli. It will be noticed that the period over which there was an increase in the probability of m.e.p.p. occurrence was longer than the period over which potentiation of an e.p.p. response to a testing stimulus could be detected. Similar observations of e.p.p. amplitude and m.e.p.p. occurrence were made after stimuli at 100/sec. The results were qualitatively similar to those at 200/sec, but the potentiation in each case was smaller in magnitude and shorter in duration for the same number of stimuli.

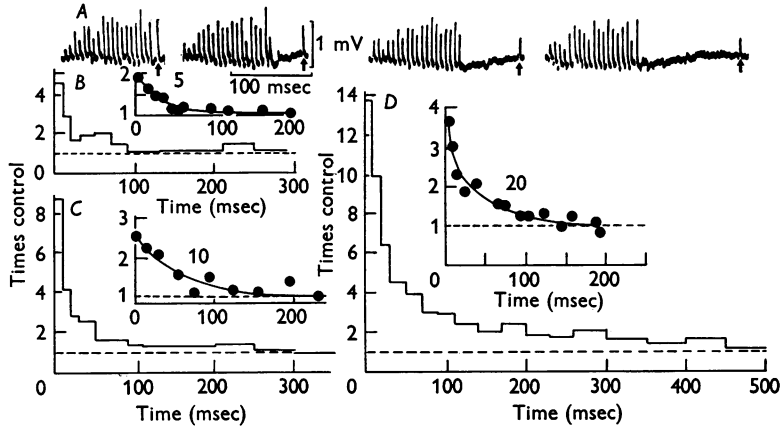

Fig. 4. Potentiation of e.p.p. amplitude and m.e.p.p. frequency after 5–20 nerve impulses in a Mg-poisoned preparation. *A* shows e.p.p.s. in response to 20 stimuli at 200/sec, and to a testing stimulus (arrow) at various intervals after the short tetanus. In the graphs inset in *B*, *C* and *D* the amplitude of the e.p.p. responses to a testing stimulus is plotted after 5 (*B*), 10 (*C*) and 20 (*D*) impulses at 200/sec, for intervals up to 200 msec. Each point represents the average e.p.p. amplitude determined from 10–15 trials at each interval and expressed as a multiple of the average e.p.p. amplitude in the absence of stimulation. The main graphs in *B*, *C* and *D* show the increased probability of m.e.p.p. occurrence after the same number and frequency of stimuli, calculated as in Fig. 1*C*. *B*, *C*, *D* were all obtained from the same junction. The temperature was 36° C and the Mg concentration 11 m-mole/l.

It was found that after 50 stimuli at 200/sec two distinct phases of e.p.p. potentiation could be distinguished (Fig. 5*A*, *C*). At intervals up to 400 msec there was potentiation of the response to a testing stimulus of a

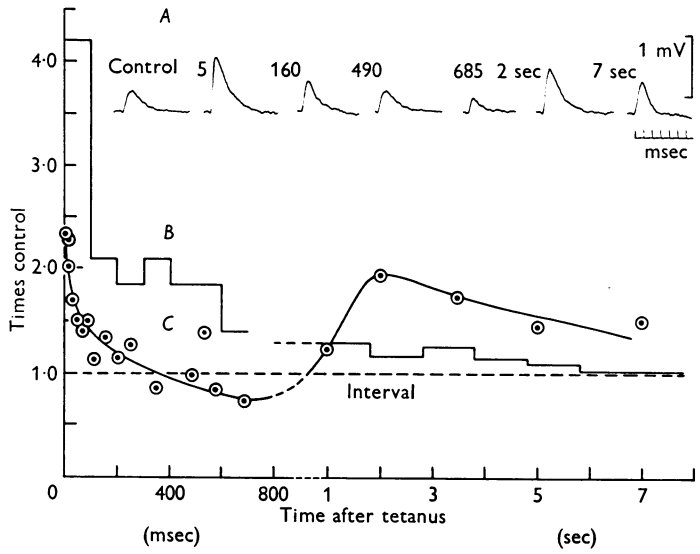

Fig. 5. The effect of 50 impulses at a frequency of 200/sec upon e.p.p. amplitude and m.e.p.p. frequency in a Mg-paralysed preparation. *A* shows records of testing e.p.p.s elicited at the indicated interval (msec or sec) after the tetanus. The records were selected to illustrate the average amplitude found in 4–6 trials at each interval. In the graph *C* this average amplitude is plotted as a multiple of the control e.p.p. amplitude found in the absence of tetanic stimulation. This control amplitude was measured repeatedly after 2–3 intervals had been assessed and was remarkably constant throughout the experiment. In this and other experiments the amplitude of testing e.p.p.s was potentiated at intervals up to 400 msec after the tetanus, was depressed at longer intervals up to 1 sec, and thereafter potentiated again. Also shown (*B*) is the probability of m.e.p.p. occurrence after 50 impulses at 200/sec, as a multiple of the control probability. This probability was calculated as in Fig. 1*C*, but for 100 msec periods for the first 700 msec after the tetanus and thereafter for 1 sec periods. The control m.e.p.p. probability and e.p.p. amplitude are shown by the same interrupted line. Note the break in the abscissal scale between 800 msec and 1 sec and the similar breaks in the graphs. The e.p.p. and m.e.p.p. results are not from the same junction, but are representative of 7 experiments in which e.p.p. amplitudes were successfully measured and 4 experiments in which m.e.p.p. frequency was assessed. The Mg concentration was 11 m-mole/l. and the temperature 37° C for all these experiments.

similar character to that detected after one (Fig. 1*A*, *C*), five, ten and twenty impulses (Fig. 4). At intervals between 4–500 msec and 1 sec the average amplitude of a testing e.p.p. was depressed below the control amplitude (Fig. 5*A*, 685 msec). At longer intervals (Fig. 5*A*, 2 and 7 sec) a second phase of potentiation was detected, the post-tetanic or post-activation potentiation described by Liley (1956*b*). There was an increase in the probability of m.e.p.p. occurrence paralleling the first phase of e.p.p. potentiation (Fig. 5*B*) but during the post-tetanic potentiation the

probability of m.e.p.p. occurrence declined to the control value. No depression of the probability of m.e.p.p. occurrence was found during the intervals after stimulation at which e.p.p. amplitudes were depressed.

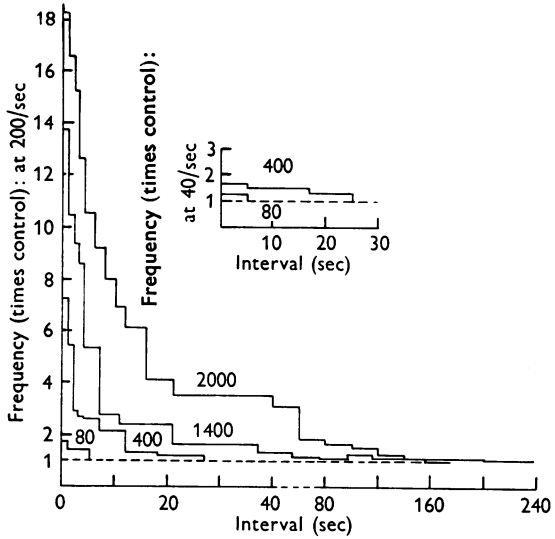

Fig. 6. M.e.p.p. frequency after 80, 400, 1400 and 2000 stimuli at 200/sec. Also shown is m.e.p.p. frequency after 80 and 400 stimuli at 40/sec (inset). The frequencies are shown as multiples of the mean frequency over a 60 sec period before stimulation (interrupted line). To show the rapid decline in frequency after stimulation, the intervals for which the frequency was calculated (represented by the line length), were short immediately after the tetanus and progressively lengthened as the frequency declined and became more irregular. Note the break in the abscissal scale between 40 and 80 sec.

With longer durations of stimulation m.e.p.p. frequency could be assessed directly from a single trial. The m.e.p.p. frequency declined from the level attained during stimulation (Fig. 3*A*) with a time course depending on the number and frequency of the conditioning stimuli. Figure 6 illustrates this decline after 80, 400, 1400 and 2000 stimuli at 200/sec. With increasing numbers of stimuli the frequency was initially higher and took longer to return to the control value. In Fig. 6 inset is shown the potentiation of frequency after 80 and 400 stimuli at 40/sec. Although the initial magnitude of the potentiation is much less than that found after the same numbers of volleys at 200/sec, the duration of the potentiation is about the same. This dependence of the duration of the potentiation of m.e.p.p. frequency upon the number of stimulating volleys rather than their frequency (provided it was above 40–50/sec) was a consistent finding. Frequencies of stimulation of 10/sec or below caused no persisting m.e.p.p. frequency changes.

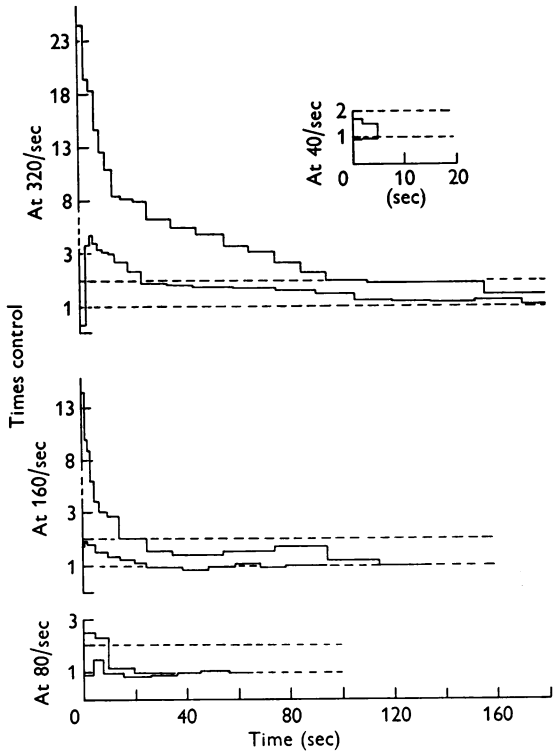

Fig. 7. The after effects of stimulation for 7 sec at frequencies of 320, 160, 80 and 40/sec (inset) in a Mg-paralysed preparation. In each graph the upper line represents m.e.p.p. frequency and the lower line e.p.p. amplitude expressed as a multiple of the control frequency and amplitude respectively. The control values shown by the lower of the pair of interrupted lines were respectively the mean m.e.p.p. frequency for the 60 sec before the tetanus and the mean e.p.p. amplitudes at 1/sec stimulation over the same period; the length of the lines represents the period over which m.e.p.p. frequency or e.p.p. amplitude was averaged; these periods as in Fig. 6 were progressively lengthened as they were remote from the tetanus. The upper of the pair of interrupted lines represents twice the control value of m.e.p.p. frequency. It will be noticed that when the post-tetanic average m.e.p.p. frequency was less than this value the corresponding e.p.p. amplitude approached the control level. Stimulation after the tetanus in each experiment was at 1/sec, the first stimulus falling 600–1000 msec after the end of the tetanus. Note the breaks in the ordinate scales. All results are from the same junction.

Parallel with the decline in m.e.p.p. frequency after tetanic stimulation there was a decline in e.p.p. amplitude. A large number of experiments were carried out to test this relation between m.e.p.p. frequency and e.p.p. amplitude, the numbers of stimulating volleys ranging from 100 to 3500 and their frequencies from 10 to 400/sec. Figure 7 illustrates a typical experiment, in which e.p.p. amplitudes and m.e.p.p. frequency were measured concurrently after tetanic stimulation for 7 sec at 40, 80,

160, and 320/sec. In these experiments the first testing stimulus fell 600–1000 msec after the tetanus. The type of e.p.p. potentiation found shortly after stimulation (Figs. 4, 5) was therefore not examined, but only the post-tetanic potentiation. M.e.p.p. frequency was measured from the end of the tetanic stimulation and thus covered both periods of potentiation of e.p.p. amplitude. With stimulation of 100 volleys or more at frequencies of 50/sec and above, the m.e.p.p. frequency changes outlasted the interval over which e.p.p. potentiation could be detected. In all experiments the acceleration of m.e.p.p. frequency was maximal immediately after the tetanus, thereafter declining at first rapidly and then more slowly. The e.p.p. potentiation, however, was not maximal immediately after stimulation ended. Indeed, after the longest stimulation (320/sec) the e.p.p. amplitudes were depressed below the control level for 2 sec. Further, the potentiation of e.p.p. amplitude was, as previously noted (Liley, 1956*b*), always smaller than the concurrent potentiation of m.e.p.p. frequency. As might be expected therefore it was possible to select frequencies and durations of stimulation which would accelerate m.e.p.p. frequency without any potentiation of e.p.p.s elicited within the same period (Fig. 7, inset). In general there was little or no e.p.p. potentiation unless the m.e.p.p. frequency was approximately doubled. Thus in Fig. 7 it can be seen that when m.e.p.p. frequency becomes less than twice the control value (upper interrupted line) e.p.p. potentiation was no longer detectable except after severe stimulation (320/sec).

Curarized preparation. The effect of 2–100 volleys at 200/sec was studied by examining the average e.p.p. response at various intervals after the conditioning stimuli. After 2–5 volleys, unlike 1 volley (Fig. 1*B*, *D*), there was no e.p.p. potentiation at close intervals, but rather depression, which increased for some 200 msec, and then recovery taking some 10 sec for completion (Hubbard, 1959). After 9–10 volleys (Fig. 8*B*, open circles) in about half the junctions explored there was still a profound depression of the e.p.p. response to a testing stimulus immediately after the tetanus, but at longer intervals a rapid recovery occurred, so that after some 100 msec a testing stimulus might elicit an e.p.p. within 5% of control size. At longer intervals there was again a small decline in amplitude before the final recovery to control size.

After 20 volleys a rapid recovery from depression, and a period of absolute potentiation was the rule. The sample records in Fig. 8*A* show the decline in e.p.p. amplitude during stimulation at a frequency of 200/sec and the potentiation of an e.p.p. elicited 105–170 msec after the tetanus. In the graph Fig. 8*B* (filled circles) the e.p.p. amplitudes obtained from strobed records are plotted as fractions of the control amplitude. The early phase of potentiation and the later decline and recovery to a second

smaller phase of potentiation are well shown. After 50 volleys (Fig. 8*C*) the early phase of potentiation was reduced in magnitude while the later phase was prominent. After 100 volleys (not illustrated) the early potentiation was detectable only as a hump on the recovery of e.p.p. amplitude, while the later phase of potentiation was still larger in amplitude. This late phase of potentiation as in the Mg-treated preparation (Fig. 5) was of course the well-known post-tetanic or post-activation potentiation (Liley & North, 1953; Liley, 1956*b*). It had the expected characteristics of increasing duration and magnitude with increasing duration and frequency of stimulation. In particular, the peak of potentiation was increasingly delayed as the duration of the repetitive stimulation increased.

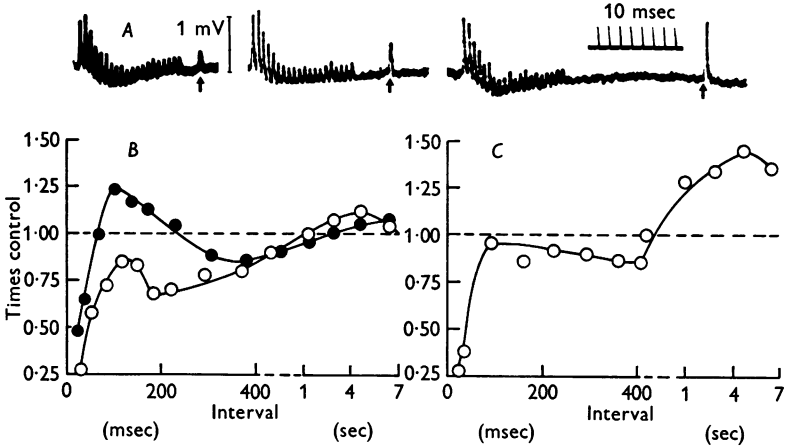

Fig. 8. E.p.p. potentiation after 10, 20 and 50 stimuli at 200/sec, recorded from a curarized preparation. *A* shows e.p.p. responses during and after (arrows) 20 stimuli at 200/sec; note the increased e.p.p. amplitude at the longest interval after the tetanus. In the graph *B* (●) the amplitude of this e.p.p. response to a testing stimulus is shown as a multiple of the e.p.p. amplitude in the absence of stimulation; all the points are averages obtained from 5–10 trials at each interval. Also plotted in *B* (○) are the e.p.p. amplitudes similarly obtained after 10 stimuli at 200/sec. In *C* the conditioning tetanus was 50 impulses at 200/sec. These results were all obtained from the same neuromuscular junction. It will be noted that after 20 impulses at 200/sec (*B*, ●) the e.p.p. amplitude was larger than the control amplitude at intervals from 100 to 200 msec after the tetanus. After 50 impulses this potentiation was lost, but a later phase (post-tetanic potentiation) was found beginning about 1 sec after the conditioning stimulation. The temperature was 36° C.

The early phase of potentiation was, as is shown in Fig. 9, barely detectable after 20 volleys at 50 or 100/sec, being similar in this respect to the early potentiation of excitatory post-synaptic potentials at motoneuronal synapses, which is likewise found only after brief high-frequency stimulation (Eccles & Rall, 1951; Lloyd, 1952; Curtis & Eccles, 1960).

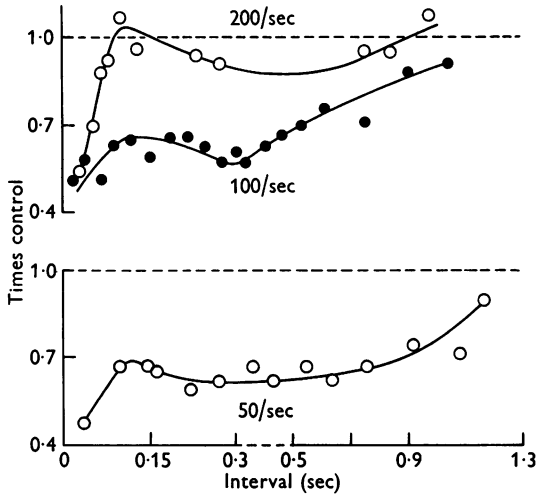

Fig. 9. E.p.p. amplitudes in a curarized preparation after 20 stimuli at 50/sec (○, lower graph), 100/sec (●, upper graph) and 200/sec (○, upper graph). The points show the average amplitude of e.p.p.s elicited in 3–5 trials at each interval, and expressed as a multiple of the e.p.p. amplitude found in the absence of tetanic stimulation. The whole experiment was carried out at the one junction. Note the break in the abscissal scale between 0·3 and 0·5 sec.

DISCUSSION

Undoubtedly the most interesting finding of the present investigation is the clear demonstration of two types of potentiation of e.p.p. amplitudes after repetitive stimulation (Figs. 5, 8). The first can be detected after one impulse (Fig. 1), is additive during repetitive stimulation (Fig. 2), and decays rapidly after stimulation (Figs. 4, 5). It must occur after all types of stimulation and has thus been named primary potentiation (Hubbard & Schmidt, 1963). The second type (Figs. 5, 7, 8) needs repetitive stimulation for its development and has therefore been called secondary post-tetanic potentiation (Lloyd, 1949; Liley & North, 1953). The separation of primary and post-tetanic potentiation renders untenable the view that all forms of potentiation are fundamentally similar (Feng, 1941; Liley, 1956*b*).

In both types of potentiation the increased e.p.p. amplitude is brought about by an increased release of ACh from nerve terminals. For primary potentiation the evidence is conclusive, for it has been shown at frog, rat and crayfish neuromuscular junctions that the potentiation of e.p.p. amplitude after one impulse and during a short tetanus, in Mg-paralysed preparations, is due to an increased quantal release of transmitter (del Castillo & Katz, 1954; Liley, 1956*b*; Dudel & Kuffler, 1961). For post-

tetanic potentiation the evidence is more indirect, resting as it does on the demonstration that the ACh-sensitivity of the muscle membrane is unchanged during the post-tetanic period (Hutter, 1952). Likewise the depression of e.p.p. amplitude in the curarized (Figs. 8, 9) and in the Mg-paralysed preparations (Fig. 5) is also of presynaptic origin, for the ACh sensitivity of the muscle membrane is unchanged after a short period of tetanic stimulation in both preparations (Otsuka, Endo & Nonomura, 1962).

Potentiation and depression of e.p.p. amplitude are thus reflexions of increased and decreased ACh release from nerve terminals respectively. Explanations for these changes in ACh release must therefore be sought in the little known properties of motor nerve terminals. For secondary post-tetanic potentiation a ready explanation is available in the observed increase in nerve terminal spike potentials after repetitive stimulation (Hubbard & Schmidt, 1963), which presumably is brought about by the concurrent hyperpolarization of the terminals. During repetitive stimulation in the Mg-paralysed preparation there is also an increase in pre-synaptic spike potential amplitude, logarithmically related to the simultaneously observed increase in e.p.p. amplitude (Hubbard & Schmidt, 1963). This increased spike amplitude may well be responsible for that part of the e.p.p. amplitude increase which occurs after the first few impulses and takes many impulses to reach a maximum (del Castillo & Katz, 1954; Liley, 1956*b*).

Primary potentiation cannot be attributed to the same mechanism as that operative in post-tetanic potentiation. Evidence for this distinction comes from two types of investigation. First, primary potentiation after one impulse is largest at intervals when the nerve terminals have an increased excitability, which presumably corresponds to a negative after-potential. Nerve spike potentials set up at this time, although eliciting potentiated e.p.p.s, are reduced in amplitude (Hubbard & Schmidt, 1963). At the squid giant synapses, on the other hand, spike potentials are increased at such close intervals (Takeuchi & Takeuchi, 1962). Nothing is known, however, of after-potential sequences in this preparation. Secondly, primary potentiation in both curarized and Mg-paralysed preparations can be increased during the great increase in e.p.p. amplitudes produced by a hyperpolarizing current applied to nerve terminals (Hubbard & Willis, 1962). Indeed, in Mg-paralysed preparations it is possible to increase selectively the second of paired e.p.p. responses by such means (Hubbard & Willis, 1963). The action of a hyperpolarizing current is apparently to increase the amount of ACh available for release from nerve terminals (Hubbard & Willis, 1962; Hubbard & Schmidt, 1963), and thus it would seem probable that primary potentiation is likewise due to an

increase in the amount of available ACh, as suggested by Eccles (1957).

The results of recent investigations of ACh synthesis, storage and release in ganglia (Birks & McIntosh, 1961) and earlier investigations of ACh kinetics in ganglia (Perry, 1953) and motor nerve terminals (Liley & North, 1953; Thies, 1960; Brooks & Thies, 1962) suggest that the amount of ACh available for release is only a small part of the total ACh content of nerve terminals, perhaps consisting only of those synaptic vesicles immediately adjacent to the nerve membrane (Curtis & Eccles, 1960). Under normal conditions (as presumably in the curarized preparation) one impulse releases 33–45 % of this available transmitter (Thies, 1960; Liley & North, 1953), its replacement being very slow. During repetitive stimulation, however, this replacement is greatly speeded (Birks & McIntosh, 1961). The results of the present investigation suggest that even one impulse makes ACh available for later release either by nerve impulses or spontaneously (Tables 1 and 2). During repetitive stimulation, particularly with the first few impulses, this mobilizing action as it may be termed (Eccles, 1957) is additive (Figs. 2 and 3). After repetitive stimulation it rapidly decays (Figs. 4, 5). The different forms which primary potentiation takes in curarized and Mg-paralysed preparations may be simply explained by the effect upon the amount of available transmitter of the two opposing processes, depletion by release and mobilization, both brought about by the nerve impulse.

Thus in the Mg-paralysed preparation depletion of ACh by stimulation is small (del Castillo & Engbaek, 1954), mobilization more than compensating for depletion and the available ACh being increased. If the amount of ACh released by nerve impulses is greatly increased, e.g. by a hyperpolarizing current applied to the nerve terminals (Hubbard & Willis, 1962), the pattern of e.p.p. responses changes to the form found in the curarized preparation. Depletion of ACh by stimulation then exceeds mobilization, except under circumstances where mobilization is most effective, e.g. shortly after an impulse (Fig. 1*A*, *D*), or after short periods of high-frequency stimulation (Figs. 8, 9).

It will be noticed that primary potentiation apparently lasts longer at curarized junctions than at Mg-treated junctions. Thus after 20 impulses at 200/sec primary potentiation could be detected for 400 msec in a curarized preparation (Fig. 8) but only 200 msec in a Mg-treated preparation (Fig. 4). Possibly mobilization is increased by the greater depletion of ACh which occurs during stimulation of a curarized junction.

The increased probability of spontaneous release of ACh which occurred in parallel with primary potentiation of e.p.p.s (Figs. 1*C*, 4, 5) could also be well explained by an increase in available transmitter. This would

increase the probability of contact between quanta of transmitter and release sites in the nerve membranes as in the model suggested by Katz (1958). The longer duration of the m.e.p.p. frequency increase as compared with the e.p.p. amplitude increase (Figs. 1, 4, 5) may mean only that m.e.p.p. changes are a more sensitive indicator of small changes in available transmitter.

The post-tetanic potentiation of m.e.p.p. frequency (Figs. 6, 7) is not so easily explained. It had been thought that this potentiation and the post-tetanic potentiation of e.p.p. amplitude had a common origin (Liley, 1956*b*; Hubbard, 1959). The m.e.p.p. frequency changes cannot, however, be attributed to the hyperpolarization of nerve terminals in the post-tetanic period, for this would *reduce* m.e.p.p. frequency (Liley, 1956*c*). Further, the hypothesis of a common origin was based on a similarity in time course of the m.e.p.p. and e.p.p. potentiations, which later investigation has shown to be true only after conditioning with large numbers of impulses (Fig. 7). After smaller numbers of impulses the frequency changes can be detected without any parallel potentiation of e.p.p. amplitudes (Fig. 7, inset) and conversely e.p.p. potentiation may be detected without any corresponding increase in m.e.p.p. frequency (Fig. 5). It is possible that the frequency changes reflect an increase in available transmitter, but this cannot be large because on testing e.p.p. amplitudes in the post-tetanic period with short trains of stimuli rather than single shocks it was found that only the first 2–3 responses were potentiated, and the later members of the train were depressed (Liley & North, 1953; Hubbard & Willis, unpublished observations). Another possible mechanism is suggested by the close relation of the potentiation with the number of conditioning impulses. Thus at a frequency of 200/sec the number required to generate the potentiation lies between 50 and 80 (compare Figs. 5 and 6). Again, the duration of the potentiation increases with the number of conditioning impulses provided their frequency is 40/sec or greater (Fig. 6). These observations suggest that ionic changes in or around nerve terminals may be responsible for the m.e.p.p. frequency increases. The experiments of Takeuchi & Takeuchi (1961) rule out an increase of extracellular potassium in the synaptic cleft, but a change in the ionic composition of the nerve terminals themselves, perhaps increasing their volume (Eccles, 1953), remains an intriguing possibility.

It seems that other junctions may resemble the mammalian neuromuscular junction in responding to stimulation in a way which depends on the balance of available transmitter left by the opposing actions of mobilization and depletion. Thus, at neuromuscular junctions in amphibia and crustacea activity facilitates transmitter release in a fashion comparable to that found in the Mg-paralysed mammalian junction. A similar

dominance of mobilization over depletion may thus operate at these junctions. Similar considerations may apply to junctions in the central nervous system which show potentiation of synaptic potential amplitudes during repetitive stimulation. These include the synapses made by afferent fibres from Golgi tendon organs on the cells of origin of the ventral spinocerebellar tract (Eccles, Hubbard & Oscarsson, 1961), the synapses made by pyramidal cell axons with forearm motoneurones in the baboon (Landgren, Philips & Porter, 1962), the segmental monosynaptic connexions of the motoneurones of cat respiratory muscles (Sears, 1963), and also the lateral column fibre synapses on frog motoneurones (Fadiga & Brookhart, 1962). On the other hand, the monosynaptic connexions of muscle afferent fibres with lumbosacral motoneurones in the cat show responses to repetitive stimulation (Curtis & Eccles, 1960) which closely resemble the responses of the curarized neuromuscular junction, as also do the connexions of the same fibres with the cells of origin of the dorsal spinocerebellar tract (Curtis, Eccles & Lundberg, 1958; Eccles, Oscarsson & Willis, 1961).

SUMMARY

1. E.p.p.s and m.e.p.p.s were intracellularly recorded from curarized or Mg-poisoned rat diaphragm–phrenic-nerve preparations *in vitro*.

2. A single nerve impulse increased ACh release (measured as e.p.p.s and m.e.p.p.s) for a 100–200 msec period. This increased release was ascribed to a mobilization of ACh by the impulse. Mobilization was independent of the release of ACh by the conditioning impulse.

3. In Mg-paralysed preparations repetitive stimulation at frequencies above 10/sec increased e.p.p. amplitudes and m.e.p.p. frequency. In curarized preparations stimulation at frequencies above 0·1–1/sec depressed e.p.p. amplitudes, but there was some relief of this depression at frequencies between 20 and 80/sec.

4. After repetitive stimulation two types of potentiation of e.p.p. amplitude could be distinguished. The first, primary potentiation, could be detected after a single impulse, was additive during repetitive stimulation and decayed rapidly after stimulation. The second, post-tetanic potentiation, needed repetitive stimulation for its development and had a much longer time course.

5. There were also two types of potentiation of m.e.p.p. frequency after stimulation. One paralleled the primary potentiation of e.p.p. amplitude; the other required a small number of conditioning impulses for its development and had a time course which was similar to the time course of the post-tetanic potentiation of e.p.p. amplitude.

6. The effects of stimulation are discussed in relation to the properties of motor nerve terminals.

The author wishes to thank Sir John Eccles for his helpful comments during the preparation of the manuscript and Dr P. W. Gage for his assistance with three experiments.

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