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THE EFFECT OF ACETYLCHOLINE UPON MAMMALIAN MOTOR NERVE TERMINALS

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There is at present no conclusive evidence for any direct action of acetylcholine (ACh) upon mammalian motor nerve terminals. A clear demonstration of the presence or absence of such an action is obviously important, for recently hypotheses of neuromuscular transmission have been developed in which presynaptic actions of ACh are assigned an important role (Riker, 1960; Koelle, 1962). These hypotheses are based on the well-known fact that when mammalian muscle is exposed to ACh or anticholinesterases such as prostigmine bromide (PrBr) and eserine, applied either locally (Feng & Li, 1941) or via the blood stream (Masland & Wigton, 1940), action potentials are generated in the vicinity of motor nerve terminals and propagate antidromically towards ventral roots. However, the claim that these antidromic potentials provided evidence of a direct drug action upon nerve terminals (Masland & Wigton, 1940; Feng & Li, 1941) was not generally accepted, for it was soon shown that antidromic potentials could be generated by alternative post-synaptic mechanisms.

One alternative, the excitation of the terminals by muscle action potentials (Eccles, Katz & Kuffler, 1942; Lloyd, 1942) must be abandoned following the careful experiments of Werner (1960*a*, *b*, 1961) and Barstad (1962) who showed by different methods that anticholinesterases and ACh could initiate antidromic discharges without any contribution from muscle action potentials. Werner (1960*a*, 1961) was able to distinguish the short latency, ephaptic, back response of Lloyd (1942) from the drug conditioned back response by several criteria: the longer latency; the effects of paired and tetanic stimulation; the differential sensitivity to curare. Barstad (1962) demonstrated that the ACh-induced back discharges could still be detected

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when muscle fibres were so damaged (by cutting) that they could not develop action potentials.

These experiments were again not conclusive evidence for a direct presynaptic action of ACh, for it is known (Fatt & Katz, 1951; Takeuchi & Takeuchi, 1960) that in generating the end-plate potential ACh increases the extracellular potassium ion concentration in the synaptic cleft. Even in cut muscle fibres, this mechanism is still operative (Randić & Straughan, 1964). It has been suggested (Diamond, 1959; Katz, 1962) that in the presence of anticholinesterases this increase in extracellular potassium concentration might be large enough to depolarize the nerve terminals (Liley, 1956*b*; Takeuchi & Takeuchi, 1961) and thus generate the back discharge.

In the present investigation we have tested the hypothesis that ACh directly affects the excitability of nerve terminals (Masland & Wigton, 1940) and their ability to release transmitter (Riker, 1960; Koelle, 1962). Advantage has been taken of the techniques developed by Hubbard & Schmidt (1963) for stimulating motor nerve terminals in the rat diaphragm preparation *in vitro*, to explore the effect of ACh and related drugs on the excitability and after-potential sequence in the terminals. In addition we have examined the effects of ACh on the frequency of miniature end-plate potentials (m.e.p.p.s) and the quantal content of end-plate potentials (e.p.p.s). A brief report of some of the present experiments has been published (Hubbard & Schmidt, 1961; Hubbard & Yokota, 1964).

METHODS

The rat diaphragm–phrenic nerve preparation *in vitro* was used in all experiments. The dissection, the divided chamber in which the preparation was mounted and the composition of the bathing fluid have been previously described (Hubbard, 1961). There were two parallel inflow systems into the muscle bath, so that when solutions were changed, there was no time lag except for that involved in mixing in the bath, which had a fluid capacity of about 4 ml. when the preparation was mounted. At the rates of flow (4–6 ml./min) employed, thermal dilution methods showed that about 4 min were needed for 95 % exchange of solutions. The temperature of the bathing solution was monitored by a thermistor above the preparation. It was kept constant to within 0·5° C by a thermistor-controlled feedback circuit controlling the temperature of the water-bath in which the recording chamber was set.

All experiments fell into two groups which could be termed 'recording' and 'stimulating'. In 'recording' experiments, m.e.p.p.s and e.p.p.s were recorded with 3 M-KCl micro-electrodes of 5–15 MΩ resistance using conventional techniques. In 'stimulating' experiments, 4 M-NaCl-filled glass micro-electrodes with tip diameters of 1–3 μ and resistances of 1–3 MΩ were used. Nerve terminals were located (Hubbard & Schmidt, 1961, 1963) by the preliminary recording of extracellular m.e.p.p.s in magnesium-paralysed preparations and by the recording of focal extracellular e.p.p.s preceded by a presynaptic spike potential in curarized preparations. As previously reported (Hubbard & Schmidt, 1963) the positioning of the micro-electrode was very critical. A movement of a few microns upwards or sideways

greatly reduced or abolished the ability to record extracellular m.e.p.p.s It was thus necessary to prevent the three possible causes of muscle movement upon exposure to ACh and PrBr—muscle twitches, fibrillation and contracture. Twitches and fibrillation were prevented by blocking neuromuscular transmission, either by adding D-tubocuraine to the bathing fluid or by increasing its $MgCl_2$ concentration. In confirmation of Elmquist & Thesleff (1960) ACh and PrBr were found not to cause contracture in the innervated unblocked preparation when exhibited in the concentrations used in the present investigation. Indeed it was only when the ACh concentration was raised to $1{\cdot}1 \times 10^{-4}$ M that a small contracture could be detected (J. I. Hubbard and R. Close, unpublished experiments).

When a terminal had been located, the micro-electrode was connected to the isolation units of two Grass stimulators arranged in series and triggered from the oscilloscope time base at 1 sec or 2 sec intervals. The stimulating pulses were rectangular in form and 0·5 msec in duration. They were recorded across a 50 Ω resistance on the earth side of the stimulating circuit and monitored on the oscilloscope screen. The small, all-or-nothing, single antidromic impulses set up by stimulation (Hubbard & Schmidt, 1963) were recorded from the phrenic nerve (in oil in the adjacent chamber) with a pair of platinum electrodes connected to a high-gain amplifier. Another pair of electrodes on the phrenic nerve were used for stimulation. The threshold of the terminals was assessed by adjusting the pulse amplitude until two to four of six trials elicited an antidromic response. With practice this procedure took 10–20 sec. Drugs used were acetylcholine bromide (ACh—A. G. Fluka), prostigmin bromide (PrBr—Hoffman LaRoche) and D-tubocurarine chloride (dTC—Burroughs Wellcome). In all cases drugs were weighed dry and then dissolved in the control solution in amounts giving the appropriate concentration.

In experiments upon the quantal content of e.p.p.s the $MgCl_2$ content of the bathing medium was raised to 14–16 m-moles/l. At the temperature employed (35–37° C) the quantal content of e.p.p.s was small and there were intermittent failures to respond to stimulation. The number of failures of e.p.p. responses to each of 200–250 supramaximal nerve stimuli at 1/sec was then counted from filmed records and the quantal content (m) determined from the relationship:

$$m = \log_e \frac{\text{no. of stimuli}}{\text{no. of failures}} \quad \text{(del Castillo \& Katz, 1954).}$$

Under these conditions for significance at the $P = 0{\cdot}05$ level, m must increase by 33% or decrease by 25% (Edwards & Ikeda, 1962).

In experiments with $4{\cdot}4 \times 10^{-5}$ M-ACh the quantal content was determined after 5 min exposure and again after 10 min washing. When $4{\cdot}4 \times 10^{-6}$ M-ACh was investigated, 5 or 10 min exposure was used, the results being the same for each interval. Ten minutes' washing was again allowed. With $4{\cdot}4 \times 10^{-7}$ M, the periods of exposure and of washing were both 10 min. With PrBr, 20 min of exposure and 20–30 min of washing were needed. In individual experiments the temperature was constant to within 0·5° C. During the experiments the resting potential, after d.c. amplification, was charted on a paper recorder (recti riter) which could be easily read to 0·5 mV.

RESULTS

Effect of ACh upon transmitter release

Of the four parameters of terminal activity explored, the quantal content of e.p.p.s provided the most decisive evidence of a presynaptic action of ACh.

Effect of ACh upon quantal content of e.p.p.s. In these experiments the quantal content of e.p.p.s was reduced to low levels by increasing the

$MgCl_2$ concentration of the bathing solutions. Quantal contents were then determined by the failure method of del Castillo & Katz (1954).

In all experiments with ACh, $3{\cdot}3 \times 10^{-6}$ M-PrBr was added to control and ACh solutions. In the absence of PrBr, ACh $4{\cdot}4 \times 10^{-5}$ M had no effect on quantal content, the mean ratio ACh/control (± 1 S.E.) being $1{\cdot}00 \pm 0{\cdot}04$ (8 trials). PrBr itself had a barely significant effect on quantal content, which proved difficult to demonstrate due to the long time (15–20 min) required for the onset of the effect and equally protracted period required to wash out the PrBr. Nine successful experiments were performed in three preparations, the mean ratio of quantal contents (PrBr/control) being $0{\cdot}73 \pm 0{\cdot}05$.

TABLE 1

A. Influence of $4{\cdot}4 \times 10^{-5}$ M-ACh on quantal content of e.p.p.s

No.	Quantal content			Ratio
	Control	ACh	Control	
1	0·89	0·55	—	0·62
2	1·93	0·77	0·31	0·69
3	0·90	0·74	1·34	0·66
4	1·43	0·25	0·69	0·24
5	1·17	0·10	1·02	0·09
6	0·54	0·17	0·48	0·34
7	1·94	1·04	1·64	0·58
			Mean ± 1 S.E. =	$0{\cdot}46 \pm 0{\cdot}08$

B. Influence of $4{\cdot}4 \times 10^{-6}$ M-ACh on quantal content of e.p.p.s

1	0·87	0·45	1·16	0·44
2	1·26	1·20	1·79	0·79*
3	0·58	0·56	0·59	0·96*
4	1·76	0·81	0·86	0·58
5	1·18	0·38	1·07	0·34
6	1·57	1·37	1·70	0·84*
7	1·10	0·59	0·89	0·59
8	1·67	0·92	1·08	0·68
9	0·68	0·34	0·57	0·54
10	1·57	0·99	1·35	0·68
11	1·05	0·88	1·65	0·65
12	2·59	0·61	1·83	0·28
13	2·45	1·97	1·03	1·13*
14	1·58	1·49	1·42	0·99*
			Mean ± 1 S.E. =	$0{\cdot}68 \pm 0{\cdot}07$

* Indicates no significant change in quantal content.

Table 1*A* shows that, in the presence of PrBr, ACh ($4{\cdot}4 \times 10^{-5}$ M) significantly depressed (see Methods) the quantal content of e.p.p.s in each of seven experiments, the quantal content in the ACh solution being on the average only 46% of the control value. When the ACh concentration was reduced to $4{\cdot}4 \times 10^{-6}$ M a significant reduction of quantal content was found at nine of fourteen junctions (64%) in which complete experiments were performed, the mean quantal content being only 68 ± 7% of the control (Table 1*B*). If the experiments in which there was only one run in

control are included, again at only 64% (14 of 22) of junctions was there significant depression. Acetylcholine, $4{\cdot}4 \times 10^{-7}$ M, was tried on nine junctions in three preparations but had no effect on quantal content, the mean ratio ACh/control being $1{\cdot}06 \pm 0{\cdot}09$. The reduction in quantal content produced by ACh is in sharp contrast to the effects of depolarization of terminals under the same circumstances. Depolarization by increasing the KCl concentration of the bathing medium increases quantal content (Liley, 1956*c*). Depolarization by applied currents in the presence of high concentrations of $MgCl_2$ either has no effect on or increases quantal content (J. I. Hubbard and W. D. Willis, unpublished observations).

It may be concluded, firstly, that the effect of ACh is most probably direct and not mediated by KCl release from a post-synaptic site and, secondly, that ACh does not depolarize the sites of transmitter release. This conclusion was tested by examining the effects of ACh upon m.e.p.p. frequency, which is a sensitive index of terminal depolarization (Liley, 1956*b*).

M.e.p.p. frequency. Despite a wide variety of experiments, no definite effect of ACh upon m.e.p.p. frequency was ever detected. Thus ACh in concentrations from 10^{-7} to 10^{-3} M had no effect on m.e.p.p. frequency in preparations paralysed with an excess of $MgCl_2$ or unparalysed and bathed in the normal Krebs Ringer. The presence of PrBr ($3{\cdot}3 \times 10^{-6}$ M) in either situation made no difference except that it was necessary in unparalysed preparations to await the subsidence of the fibrillation induced by the PrBr before testing the effect of ACh. In three experiments with Dr D. R. Curtis, one of us (J.I.H.) found that electrophoretic application of ACh from five-barrelled micropipettes close to nerve terminals did not accelerate m.e.p.p.s recorded by an intracellular electrode at the same junction. In these latter experiments there was no doubt that ACh and/or PrBr was reaching the vicinity of nerve terminals for the quantities released were varied over a range which at the lowest level did not affect m.e.p.p. amplitudes, and at the higher level decreased m.e.p.p. amplitudes. In the case of PrBr there was an intermediate level at which m.e.p.p. amplitudes were increased.

In these experiments and in experiments using ACh in the bathing solution m.e.p.p. frequency often appeared to decrease during the application of ACh. Close examination, however, always showed that this apparent decrease in frequency was due to a fall in m.e.p.p. amplitude, caused presumably by the densensitizing effects of the ACh. In further experiments the possibility that ACh stabilized m.e.p.p. frequency was tested by raising the KCl concentration of the bathing medium to 10 or 15 m-moles/l. ACh ($4{\cdot}4 \times 10^{-6}$ M) did not prevent the expected increase in frequency (Liley, 1956*b*), and when ACh was exhibited for 6 min during

the period of acceleration produced by 15 mM-KCl there was no significant change in frequency (eight complete trials, mean ratio of frequencies ± 1 S.E. ACh/control $0{\cdot}97 \pm 0{\cdot}14$).

Effects of ACh upon nerve terminal threshold

The results of the preceding section would suggest that ACh should not affect the threshold of nerve terminals. Indeed when, as judged by direct vision and/or the presence of a compound antidromic potential, we were stimulating intramuscular nerve branches, there was no threshold change upon exposure to ACh. However, when the stimulating electrode was precisely localized in the vicinity of nerve terminals (see Methods) ACh did lower the stimulus threshold in about 50% of trials. The effectiveness of ACh differed according to whether the preparations were paralysed with $MgCl_2$ or dTC and the results in each case will therefore be separately presented.

Mg-paralysed preparation

ACh. ACh reversibly lowered the threshold to stimulation at nine of seventeen junctions (53%) exposed to concentrations of $4{\cdot}4 \times 10^{-6}$ M for at least 6 min. The number of units responding and the mean extent of the threshold change was the same with concentrations above $4{\cdot}4 \times 10^{-6}$ M (3/6, mean 28% with range 23–34%) as with $4{\cdot}4 \times 10^{-6}$ M (6/11, mean ± 1 S.E. 27 ± 5). With all concentrations the effect was rapid in onset (2 min 15 sec or less) but while with $4{\cdot}4 \times 10^{-6}$ M the threshold change was complete in 3–6 min and rapidly reversible, the effects of higher ACh concentrations were more prolonged.

Two types of experiment were employed to demonstrate the ACh action. In the first, illustrated in Fig. 1, the flow of the control solution was stopped and 1 ml. of various dilutions of ACh was added to the muscle bath. About 15 sec later the flow began again and the ACh was serially diluted by the continuously flowing bathing solution. Clearly the fall in threshold induced by $4{\cdot}4 \times 10^{-5}$ M-ACh was rapid in onset and reached its peak about 5 min after exposure to the drug. Acetylcholine, $4{\cdot}4 \times 10^{-4}$ M, produced a small additional effect but the addition of $4{\cdot}4 \times 10^{-3}$ M-ACh did not prevent the threshold returning to the control level after some overshoot. Evidently there was some desensitization (Katz & Thesleff, 1957) to further doses of ACh. In the second type of experiment ACh was applied by continuous infusion. Concentrations of $4{\cdot}4 \times 10^{-5}$ M and above were found to have a prolonged wash-out time while $4{\cdot}4 \times 10^{-6}$ M had a much briefer action and was therefore routinely used in experiments in which ACh was tested in the presence of other drugs.

ACh and PrBr. In these experiments $3{\cdot}3 \times 10^{-6}$ M-PrBr was added to

control and test solutions. The number of junctions at which a response to $4{\cdot}4 \times 10^{-6}$ M-ACh was obtained (9/17, 53%), and the mean fall ($\pm$ 1 S.E.) in threshold (27 ± 5%) were not significantly different from the corresponding values in the absence of PrBr. No threshold changes were detected during three trials of $4{\cdot}4 \times 10^{-7}$ M and $4{\cdot}4 \times 10^{-8}$ M-ACh at junctions responding to $4{\cdot}4 \times 10^{-6}$ M-ACh. At seven of the ten junctions at which there was a response to ACh the threshold fall resembled in both its onset (under 2 min 40 sec) and in its time to completion (5–6 min), the effect in the

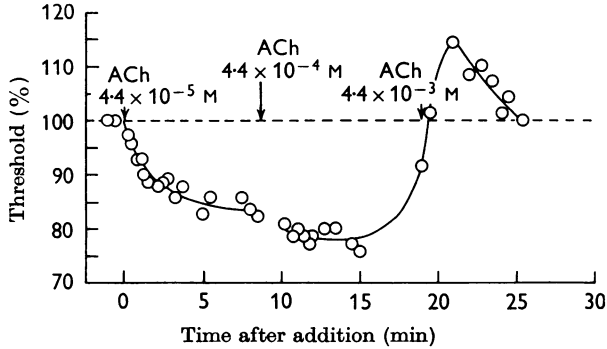

Fig. 1. Depolarization of nerve terminals by ACh. In this experiment 1 ml. of control solution, containing ACh at the stated concentration, was added to the muscle bath at the times marked by arrows. Threshold (ordinate) in this and in Figs. 2–5 is measured from the amplitude of a rectangular current pulse of constant duration (0·1 msec), required to set up an all-or-nothing antidromic potential in two to four of six trials. In each experiment illustrated, the average adequate pulse amplitude before the experiment is shown as 100% (horizontal interrupted line) and later pulse amplitude changes are expressed as percentage of the control pulse amplitude. Points are placed in the middle of the 10–20 sec period needed to assess the threshold pulse amplitude (ordinate). Time (abscissa) is shown as beginning from the first addition of ACh to the bath. All solutions in this experiment contained 14 mM-$MgCl_2$ and the temperature of the solution was 32° C.

absence of PrBr. At three junctions, however, a fall in threshold occurred $4\frac{1}{2}$–$5\frac{1}{2}$ min after exposure to ACh and took 7–11 min for completion. Reversal of the effect took a similar time to that required for its onset. In these cases then, it is possible that the presence of PrBr enabled the demonstration of an ACh effect.

ACh, PrBr and dTC. As Fig. 2 shows, the fall in threshold under the influence of ACh could be reversibly abolished by dTC. In this experiment in a preparation treated with $3{\cdot}3 \times 10^{-6}$ M-PrBr, after exposure to $4{\cdot}4 \times 10^{-6}$ M-ACh (vertical interrupted line), there was a fall in threshold to about 65% of the control value. The bathing fluid was then changed (Fig. 2, arrow and hatched bar) to a solution containing, in addition to ACh, $2{\cdot}5 \times 10^{-6}$ M-dTC. After 7–8 min delay the threshold began to increase

towards the control value, the complete process taking more than 20 min, which is comparable with the time needed to demonstrate the post-synaptic effects of curarization. The dTC was then washed out with the original ACh-containing solution (Fig. 2, arrow at end of hatched bar) and the threshold fell once more. Finally, the ACh was in turn washed out and the threshold returned approximately to the original control value. The same results were obtained in two similar experiments.

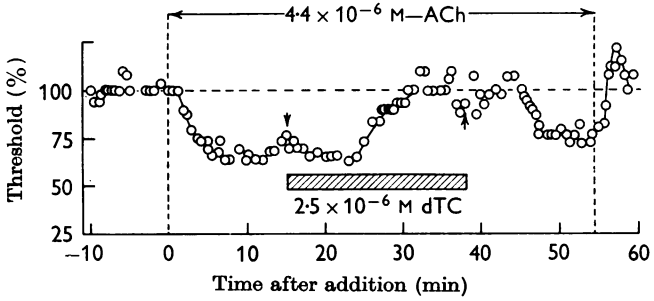

Fig. 2. The abolition of ACh action by dTC. The preparation was paralysed by 14 mM-$MgCl_2$. All solutions contained $3{\cdot}3 \times 10^{-6}$ M-PrBr. Threshold (ordinate) is measured as described in Fig. 1. At time 0 (vertical interrupted line) the bathing solution was changed to a solution containing $4{\cdot}4 \times 10^{-6}$ M-ACh. This concentration of ACh was maintained in the bath for the period between the vertical interrupted lines. Over the period marked by the arrows and the hatched bar, the solutions contained in addition $2{\cdot}5 \times 10^{-6}$ M-dTC. The temperature was 31° C.

PrBr. The threshold of nerve terminals was not affected by 3·3 or $6{\cdot}6 \times 10^{-6}$ M-PrBr in the course of a 30 min exposure. When the PrBr concentration was raised to $9{\cdot}9 \times 10^{-6}$ M a small reversible fall in threshold taking some 20 min to develop was detected.

dTC. In concentrations as low as $1{\cdot}27 \times 10^{-6}$ M-dTC was found to cause a reversible fall in nerve terminal threshold. This effect, like that of PrBr, was of long latency. Thus in the experiment illustrated (Fig. 3) using $5{\cdot}09 \times 10^{-6}$ M-dTC, the fall in threshold took 15 min to appear but was reversed with only 5–6 min washing. The long latency made it possible to explore the interaction of ACh and dTC (Fig. 2) before this complication occurred. In Fig. 2 there is, however, some indication of a threshold fall beginning about the period when the dTC was washed out (Fig. 2, second arrow).

The profound presynaptic depolarizing action of dTC (Fig. 3), while unexpected at the neuromuscular junction, is supported by the finding that cells in the thalamus (Andersen & Curtis, 1964), cerebellum (Crawford and Curtis—personal communication) and cerebral cortex (Krnjević & Phillis, 1963) are excited by dTC applied electrophoretically. Renshaw cells may

also be excited by relatively large electrophoretic applications of dTC (Curtis—personal communication).

Curarized preparation

ACh. It might be expected from the foregoing results that in curarized preparations ACh would have little or no effect. Trials showed, however, that curarized nerve terminals were surprisingly more sensitive to the effects of ACh than Mg-paralysed terminals. Thus of seven junctions exposed to $4{\cdot}4 \times 10^{-6}$ M-ACh the threshold fell within 4 min at three, the average change being 45% (range 27–68%). Of three junctions exposed to $4{\cdot}4 \times 10^{-5}$ M-ACh the threshold fell at two, the changes being 40 and 53% respectively. The wash-out time was prolonged as has been previously noted with the same concentrations in Mg-paralysed preparations.

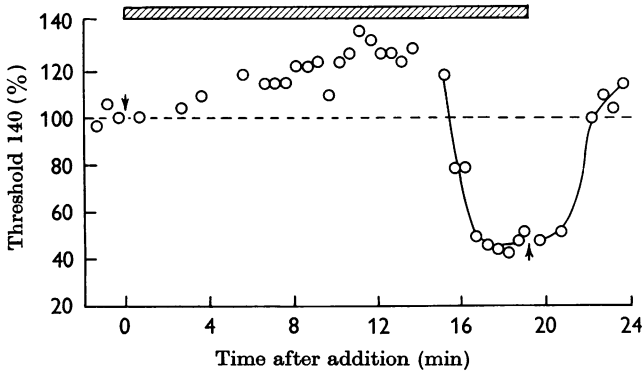

Fig. 3. The effect of dTC upon the threshold of nerve terminals. The preparation was paralysed with 13 mM-$MgCl_2$. Threshold (ordinate) was measured as described in Fig. 1. At time 0 for the period indicated by the arrows and hatched bar the bathing solution was exchanged for a solution containing in addition $5{\cdot}09 \times 10^{-6}$ M-dTC. The temperature was 32·5° C.

ACh and PrBr. As in Mg-paralysed preparations the presence of $3{\cdot}3 \times 10^{-6}$ M PrBr did not significantly affect the magnitude of the ACh action. Threshold falls were detected within 4 min at four of eight terminals exposed to $4{\cdot}4 \times 10^{-6}$ M-ACh, the average fall being 43% (range 17–62·5%), much the same as in the absence of PrBr. Under the same conditions $4{\cdot}4 \times 10^{-7}$ M ACh was equally effective. Four of six terminals exposed showed a fall in threshold, the average fall being 49% and the range 12–73%. A threshold fall was also detected at one of four junctions exposed to $4{\cdot}4 \times 10^{-8}$ M-ACh. Multiple concentrations were assessed at several junctions; one junction, for instance, responded to $4{\cdot}4 \times 10^{-7}$ M but not to $4{\cdot}4 \times 10^{-8}$ M while another was tested with a range of concentrations from $4{\cdot}4 \times 10^{-8}$ M. In this experiment the preparation was

exposed, first, to $4{\cdot}4 \times 10^{-8}$ M-ACh (Fig. 4*B*) the threshold falling by some 20% and returning to control values on washing out. Following washing out there was a slight rise in threshold to a new steady level, $4{\cdot}4 \times 10^{-7}$ M-ACh was then applied (Fig. 4*A*). The new threshold now fell by 60% and again returned to the control value upon washing out. Acetylcholine, $4{\cdot}4 \times 10^{-6}$ M, was also applied in the same experiment with much the same effect. This was a general finding (see above) perhaps brought about by the opposed ACh actions of depolarization and desensitization (Katz & Thesleff, 1957).

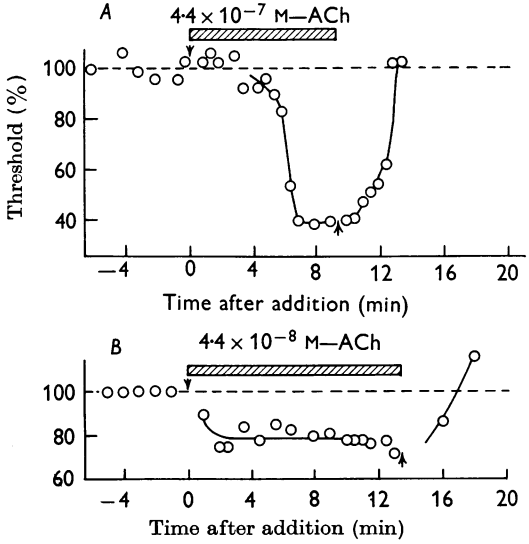

Fig. 4. The effect of ACh upon the threshold of nerve terminals in a curarized preparation. Threshold (ordinate) was assessed as in Fig. 1. In each graph the control solution was exchanged for a solution identical save for the addition of ACh, for the period marked by the arrows and hatched bar. In Fig. 4*A* the ACh concentration was $4{\cdot}4 \times 10^{-7}$ M and in *B* it was $4{\cdot}4 \times 10^{-8}$ M. All solutions contained PrBr $3{\cdot}3 \times 10^{-6}$ M and dTC $2{\cdot}29 \times 10^{-6}$ M. The temperature was 35° C.

PrBr. Prostigmine bromide, $3{\cdot}3$–$6{\cdot}6 \times 10^{-6}$ M, after 20 min exposure, brought about a reversible fall in threshold of some 60%. A similar time was required for reversal of the action. No effect was found with $3{\cdot}14 \times 10^{-7}$ M. The long latency of the action presumably reflects the known slow onset of cholinesterase inhibition with this drug (Wilson, 1955). These observations lead us to compare the mean threshold current required in the presence and absence of PrBr in all the terminals at which an effect of ACh was demonstrable. As expected, it was significantly ($P < 0{\cdot}1$) lowered in the presence of $3{\cdot}3 \times 10^{-6}$ M-PrBr (dTC, $8{\cdot}3 \pm 1{\cdot}8$ μA, $n = 5$; dTC + PrBr, $3{\cdot}0 \pm 0{\cdot}8$ μA, $n = 8$).

Effect of Mg. The presence of $MgCl_2$ in concentrations sufficient to block neuromuscular transmission increased the threshold of the terminals.

Evidence for this effect on threshold came from four experiments in which the threshold was followed during the substitution of dTC for $MgCl_2$ as the paralysing agent. There was invariably a fall in threshold of about 30% as the $MgCl_2$ was washed out, and a reversion to the control level as it was restored. This effect of Mg is of course similar to that observed in other situations, thus Mg ions increase the threshold to stimulation of muscle (del Castillo & Engback, 1954) and myelinated and non-myelinated nerve (Gordon & Welsh, 1948; Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser & Meves, 1958).

Effect of KCl. When the effects of $4{\cdot}4 \times 10^{-6}$ M-ACh were compared at curarized and Mg-paralysed junctions (pooling the results in the presence and absence of PrBr) there was a significantly ($P < 0{\cdot}05$) greater effect in the curarized preparations, despite the demonstration of a dTC–ACh antagonism in Mg-paralysed preparations (Fig. 2). One explanation of this discrepancy would be that in the curarized preparation some additional factor was operating to lower the threshold. A strong possibility was, of course, that potassium ions released post-synaptically by ACh were reaching the site of stimulation (Katz, 1962). Indeed the threshold in curarized preparations was exquisitely sensitive to changes in the KCl concentration of the bathing medium. For example, at one junction (Fig. 5), when a solution containing 7·5 mM-KCl was exchanged for the control solution (5 mM-KCl), the threshold fell rapidly by 10% and remained at this lower level until the 7·5 mM-KCl was washed out (Fig. 5, arrow at end of hatched bar) whereupon the threshold returned to the control level. In other experiments we found that when higher concentrations (11–23 mM) were applied there was an initial fall in threshold, followed by a rise which with the highest concentrations ended in a loss of excitability, reversed only on washing. It remained to demonstrate that more potassium ions would be released in curarized preparations than in Mg-paralysed preparations.

A convenient, although indirect, index of potassium release was provided by the fall in resting potential at the junctional region of muscle fibres upon exposure for 6 min to $4{\cdot}4 \times 10^{-6}$ M-ACh, no more than five trials being made on any one preparation. Only fibres in which the resting potential remained constant (to within 0·5 mV) or showed completely reversible changes were considered. As expected there were no demonstrable resting potential changes in the absence of PrBr, in preparations paralysed with either 14 mM-$MgCl_2$ (eight fibres) or $2{\cdot}29 \times 10^{-6}$ M-dTC (nine fibres). When $3{\cdot}3 \times 10^{-6}$ M-PrBr was present together with 14 mM-$MgCl_2$ there was a reversible fall in resting potential at nine of ten junctions explored. All the falls were made comparable by adjusting the amplitude to that expected at a resting potential of 60 mV (the mean resting potential

for the group). The average fall (± 1 S.E.) for the ten fibres was then $5{\cdot}5 \pm 1{\cdot}5$ mV. With $2{\cdot}29 \times 10^{-6}$ M-dTC in the bathing medium together with $3{\cdot}3 \times 10^{-6}$ M-PrBr, the resting potential fell at only three of ten junctions (four preparations) upon the exhibition of ACh. The falls were 2, 9 and 11·5 mV respectively and were found in two different preparations. Clearly a large potassium effect could be expected only in the presence of 14 mM-MgC_2 and PrBr. Yet the effect of $4{\cdot}4 \times 10^{-6}$ M-ACh on terminal threshold in 14 mM-$MgCl_2$ was found to be the same whether or not PrBr was also present, and in the curarized preparation the effect of ACh was found to be greater than in the Mg-paralysed preparations. It thus seemed unlikely that the effects of ACh could be explained by the liberation of potassium from post-synaptic receptor sites.

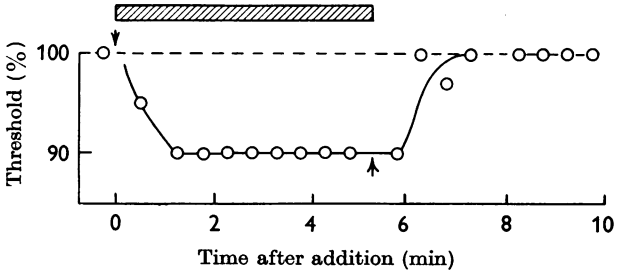

Fig. 5. The effect of KCl upon the threshold of motor nerve terminals. Threshold (ordinate) was calculated as described in Fig. 1. At time 0 (abscissa), the potassium concentration was raised from 5 to 7·5 mM for the period indicated by the arrows and hatched bar. The preparation was paralysed with $2{\cdot}54 \times 10^{-6}$ M-dTC and the temperature was 33° C.

Effect of PrBr and dTC upon after-potentials

As ACh in the bathing solution lowered the threshold for the initiation of antidromic impulses (Figs. 1, 2, 4) it seemed reasonable to look for a similar action produced by the ACh released by nerve impulses. The excitability cycle in the terminals after a conditioning impulse was therefore explored in the presence of PrBr and of dTC.

Effects of PrBr. The normal excitability cycle after one suprathreshold conditioning stimulus in a Mg-paralysed preparation is of the form shown in Fig. 6*A* (open circles). It consists of a relative refractory period lasting from 1·5 to 4 msec, followed by a supernormal period, which at 10–20 msec after the conditioning stimulus passes into a period of subnormal excitability. This becomes undetectable 50–90 msec after the conditioning stimulus (Hubbard & Schmidt, 1963). After exposure to $6{\cdot}6 \times 10^{-6}$ M-PrBr for 18 min the excitability cycle was re-explored (Fig. 6*A*, filled circles). The most marked change was a prolongation of both the super-

normal period and the refractory period. These changes were completely reversible and were found in three other experiments using this concentration and one experiment with $9{\cdot}9 \times 10^{-6}$ M-PrBr.

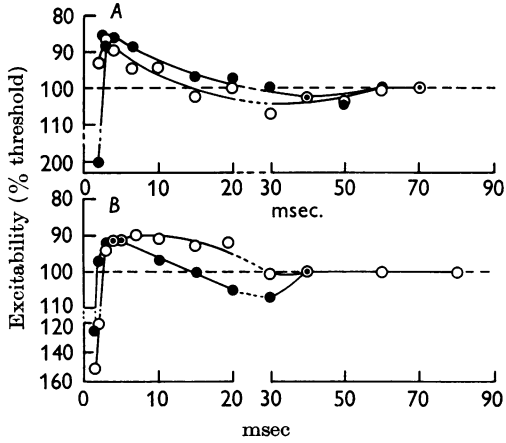

Fig. 6. Effect of PrBr and dTC upon the excitability cycle in motor nerve terminals. In this figure the excitability changes after a single conditioning stimulus are plotted at intervals (abscissa) from 2 to 70 msec (*A*) and 2–80 msec (*B*). In contra-distinction to Figs. 1–5 the percentage scale (ordinate) here denotes the ratio of the conditioned to the unconditioned threshold current at each interval. In the experiment illustrated in Fig. 6*A* these excitability changes were assessed first in the control solution which contained 11 mM-$MgCl_2$, then after 18 min exposure to $6{\cdot}6 \times 10^{-6}$ M-PrBr, and again 21 min after the PrBr had been washed out. The open circles indicate the averages of the pre- and post-exposure ratios and the closed circles indicate the ratios in the PrBr containing solution. The temperature was 36·5° C. In the experiment illustrated in Fig. 6*B* the control solution again contained 11 mM-$MgCl_2$ and the same procedure was followed, the test solution containing $1{\cdot}72 \times 10^{-5}$ M-dTC instead of PrBr. The conditioning curve was assessed 18–45 min after exposure to dTC and again 40 min after the dTC had been washed out. The temperature for this experiment was also 36·5° C. Note the breaks in the ordinate and abscissal scales and the changes of scale at those points.

Low concentrations of PrBr had similar effects in curarized and Mg-paralysed preparations. Figure 7 shows the effect of $3{\cdot}14 \times 10^{-7}$ M PrBr in a preparation paralysed with $5{\cdot}09 \times 10^{-6}$ M-dTC. Twenty minutes after the exhibition of the PrBr there was a marked but reversible increase in the duration and magnitude of the supernormal period (Fig. 7, closed circles) and in the duration of the refractory period as had been found in preparations paralysed with Mg (Fig. 6*A*, closed circles). Similar effects were found using $1{\cdot}27 \times 10^{-6}$ M-PrBr but were complicated by the marked fall in absolute threshold which occurred at the same time, and which made it difficult to retain the micro-electrode in close proximity to the terminal.

The refractory period changes appeared to be confined to the terminals because no detectable changes occurred during PrBr action in the latency of antidromic impulses set up by supramaximal stimuli, or in the latency of e.p.p.s set up by phrenic nerve stimulation.

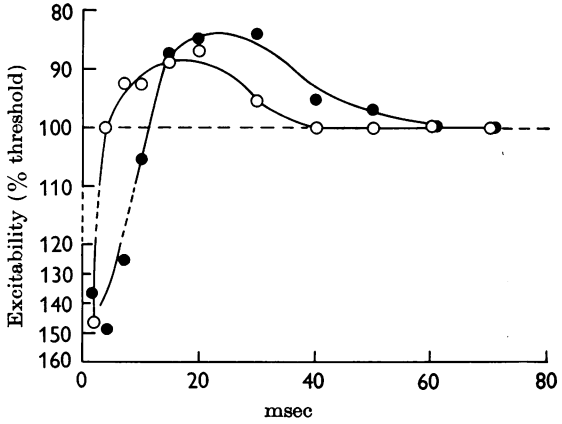

Fig. 7. The effect of PrBr upon the excitability cycle in a curarized preparation. The control solution contained $5{\cdot}09 \times 10^{-6}$ M-dTC (open circles) and the test solution contained in addition $3{\cdot}14 \times 10^{-7}$ M-PrBr (filled circles). The excitability changes were assessed as described in Fig. 6, first, in the control solution, then 22–41 min after exposure to PrBr and finally 30 min after washing out with the control solution. The temperature was 36·5° C. Note the break in the abscissal scale at 110 % and the subsequent change of scale.

Effect of dTC. This was explored successfully at six junctions, the typical finding (Fig. 6*B*) being a reversible reduction in the magnitude and duration of the supernormal period and a shortening of the refractory period. To demonstrate the effect of dTC it was found that the most useful preparations were those in which, due presumably to ageing (Hubbard & Schmidt, 1963), the supernormal period was prolonged and the subnormal period correspondingly shortened. Thus in Fig. 6*B* the control curve (open circles) exhibits a supernormal period lasting 30 msec after the impulse. Fifteen minutes after $1{\cdot}72 \times 10^{-5}$ M-dTC was added to the bathing solution the supernormal period was reduced to 15 msec, the refractory period was also shortened and a subnormal period could be detected from 15 to 40 msec after the conditioning impulse. Smaller concentrations had similar effects, the lowest tried being $1{\cdot}27 \times 10^{-6}$ M. As might be expected the effect was then smaller in magnitude and took longer to become manifest. In four experiments the $MgCl_2$ concentration was reduced to 1 mM at the same time as the dTC was added. The changes in the excitability cycle were of the same form as when the dTC was merely added to the solution (as in Fig. 6*B*).

The changes in the magnitude and duration of the supernormal period brought about by dTC and PrBr presumably reflect similar changes in the negative after-potential in the terminals (Gasser & Erlanger, 1930). There is no reason to believe that this effect is mediated by PrBr and dTC apart from their effects on ACh, presumably released by stimulation of the terminals. Nor has ACh previously been reported to affect negative after-potentials in nerve fibres, although effects on positive after-potentials have been reported (Armett & Ritchie, 1960). It seems reasonable therefore to explain the changes in the negative after-potential by an ACh initiated depolarization of the terminals superimposed upon the spike potential and true negative after-potential.

DISCUSSION

The depression of the quantal content of e.p.p.s by ACh (Table 1) supported by a similar finding at the frog neuromuscular junction (Ciani & Edwards, 1963) provides unequivocal evidence for a presynaptic action of ACh. This action is probably directly caused by ACh for, on the alternative hypothesis (Katz, 1962) of potassium release by post-synaptic action of ACh, an increase in quantal content would be expected (Takeuchi & Takeuchi, 1961; Liley, 1956*c*). Similarly depolarization by the local currents flowing between activated receptive areas on the muscle fibre surface and adjacent regions (del Castillo & Katz, 1956) must be ruled out for depolarization by applied currents increases or does not alter quantal content in high $MgCl_2$-containing solutions (J. I. Hubbard and W. D. Willis, unpublished experiments). The nature of the ACh action is obscure. The absence of any effect on m.e.p.p. frequency in the present and earlier investigation (Fatt & Katz, 1952; del Castillo & Katz, 1955) confirms the absence of terminal depolarization. In any case in high $MgCl_2$-containing solutions transmitter release is uncoupled from variations in spike amplitude (Hubbard & Schmidt, 1963; Katz & Miledi, 1965*b*). Thus ACh action must involve a more intimate part of the release mechanism.

A paradox now appears. The lowering of stimulus threshold (Figs. 1, 2, 4) in the presence of ACh is most plausibly interpreted as a depolarization (Wall, 1958) yet by the criteria of quantal content and m.e.p.p. release the terminals are not depolarized. The only possible solution is that the site of stimulation and the site of release are different. If, as is universally agreed, the non-myelinated terminals are the site of release, the site of stimulation must be above the site of release. Considering the location of the stimulating electrodes this can only be the adjacent node or nodes of Ranvier. It may be inferred that subthreshold depolarization here is not transmitted to the nerve terminals in a strength sufficient to increase the probability of transmitter release. Thus propagation of nerve impulses into

terminals (Hubbard & Schmidt, 1963; Katz & Miledi, 1965*a*) is apparently a necessity for transmitter release. Similar conclusions can be drawn from the presynaptic effects of PrBr described by Liley (1956*a*, 1957). PrBr did not alter m.e.p.p. frequency (Liley, 1956*a*) yet set up antidromic discharges, shown by simultaneous recording not to be associated with multiquantal potentials at innervated neuromuscular junctions (Liley, 1957). Standaert (1964) on other grounds, has concluded that antidromic discharges are initiated at the junction between the myelinated and non-myelinated segments of the nerve terminal. Electron micrographs of this junctional region (J. I. Hubbard, unpublished records) indicate that this must mean at the most distal node of Ranvier, for the non-myelinated nerve fibres adjacent to the terminal myelin appear similar in structure and vesicle distribution to those in more distal areas.

A more intractable problem is the mechanism of the ACh-initiated depolarization. The results in Mg-paralysed preparations (Figs. 1, 2) are compatible with a receptor system analogous to the post-synaptic system of ACh receptors. In the curarized preparation (Fig. 4) the ACh action appeared insensitive to dTC and was not affected in magnitude or duration by PrBr. The effect of ACh under these conditions is unlikely to be due to potassium release from post-synaptic receptors. If this occurred it should have been most prominent in Mg-paralysed preparations, especially in the presence of PrBr, for only under these conditions was it possible to detect any effect of ACh upon junctional resting potentials. Possibly the depolarization produced by dTC itself (Fig. 3) in some way facilitates the ACh action. The effects of PrBr and dTC on the excitability cycle (Figs. 6, 7) were demonstrable in the presence of high $MgCl_2$ concentrations and in the curarized preparations with low PrBr concentrations. There is thus a strong probability that these effects are mediated directly by the ACh released by nerve impulses. Certainly the observed alterations are suggestive of the superposition of a depolarizing potential on the normal excitability cycle. As both PrBr and dTC (Fig. 3) can depolarize, their opposing effects upon the supernormal period (Fig. 6) cannot be ascribed to any effect of the drugs upon the equilibrium potential for the negative after-potential.

The findings of the present investigation appear to explain the wealth of experiments in which ACh and related drugs have been shown to generate antidromic nerve impulses in motor nerves. These experiments have been of two kinds. First, there are experiments in which ACh and/or anticholinesterases generated antidromic impulses when exhibited in the vicinity of nerve terminals by intra-arterial injection (Masland & Wigton, 1940), or by local application in the whole animal (Feng & Li, 1941) or in the *in vitro* preparation (Van der Meer & Meeter, 1956; Barstad, 1962).

Initially the depolarization produced by ACh (Figs. 1, 2, 4) and PrBr and presumably other anticholinesterases would be sufficient to initiate impulses in the nodes of Ranvier adjacent to the terminals. These impulses, besides propagating antidromically, would propagate into other terminals of the same motor unit, releasing ACh to initiate the muscle twitches recognized grossly as fibrillation. The experiments of Barstad (1962) and of Randić & Straughan (1964) in which antidromic discharges were initiated in rat diaphragms incapable of twitching because the muscle fibres were cut, effectively rule out muscle action potentials as an essential component in the generation of antidromic discharges.

Secondly, there have been experiments in which after drug administration an orthodromic nerve volley was needed before there were any antidromic discharges (Riker, Roberts, Standaert & Fujimori, 1957; Riker, Werner, Roberts & Kuperman, 1959; Werner, 1960*a*, *b*, 1961; Blaber & Bowman, 1963*a*, *b*; Standaert, 1964). The present experiments indicate (Figs. 6, 7) that the terminal depolarization generating the discharge was set up by the combined effects of the drug acting as an anticholinesterase and/or depolarizing agent and the ACh released by the nerve impulses. It should be mentioned that the prostigmine analogues used by Riker *et al.* (1957, 1959) displayed anticholinesterase and depolarizing activity when applied electrophoretically at the mammalian neuromuscular junction (D. R. Curtis and J. I. Hubbard, unpublished experiments).

The abolition of antidromic discharges by dTC, reported by almost every worker in this field, is well explained by the ACh-dTC antagonism found in the present investigation (Fig. 2), while the inability of dTC to affect quantal release (Martin, 1955) is explained by the localization of its action at the nodes of Ranvier. The similar depressing effect of MgCl (Van der Meer & Meeter, 1956) may also be explained by a reduction in terminal sensitivity to ACh and the general increases in nerve threshold brought about by $MgCl_2$.

The presence of prejunctional ACh receptors may possibly have some developmental significance for it is considered that the number of ACh receptor sites in muscle is limited by a nervous mechanism (Miledi, 1962). If it could be shown that the sites in nerve terminals developed before ACh release it might support the hypothesis that the controlling nervous agent is not ACh as suggested by Thesleff (1960) but some other factor (Miledi, 1962). It seems more likely, however, that the presence of sites reacting with ACh is a universal property of non-myelinated nerve membranes, for such sites have been previously found in vagal and sympathetic C fibres (Armett & Ritchie, 1960, 1961), in lobster nerves (Dettbarn & Davis, 1963), in sensory receptors (Gray & Diamond, 1957; Gray, 1959) and in regenerating sensory nerve terminals (Diamond, 1959). At these

sites however, ACh depolarizes, whereas at the motor nerve terminal the depolarizing action is exerted at a locus above the site of action upon transmitter release. All these sites have in common the fact that ACh has no physiological role (Gray, 1959; Ritchie & Armett, 1963; Ehrenpreis, 1964). Presumably the proximity of the sites in motor nerve terminals to their natural excitant has contributed to make them a focus of pharmacological, if not physiological, interest.

SUMMARY

1. The effect of ACh upon the quantal content of e.p.p.s and upon m.e.p.p. frequency was measured at neuromuscular junctions in the rat diaphragm-phrenic nerve preparation *in vitro*. Acetylcholine in concentrations as low as $4{\cdot}4 \times 10^{-6}$ M (in the presence of PrBr $3{\cdot}3 \times 10^{-6}$ M) reduced the quantal content of e.p.p.s but did not affect m.e.p.p. frequency in the presence or absence of PrBr.

2. It was concluded that ACh did not depolarize the site of release of transmitter.

3. Motor nerve terminals in the rat diaphragm-phrenic nerve preparation *in vitro*, paralysed with dTC or an excess of $MgCl_2$, were stimulated with brief cathodal pulses delivered from a micro-electrode. The average pulse amplitude needed to set up antidromic potentials was used as a measure of the nerve terminal threshold.

4. ACh in concentrations as low as $4{\cdot}4 \times 10^{-8}$ M lowered the threshold of nerve terminals. The ACh action was antagonized by dTC and $MgCl_2$.

5. The after-potential sequence in nerve terminals was explored in the presence of dTC and PrBr. PrBr prolonged the refractory period and prolonged and increased the supernormal period. dTC shortened the refractory period and the supernormal period.

6. It was concluded that ACh depolarized the nerve terminals at a site more proximal than the site of release of transmitter, presumably the adjacent nodes of Ranvier. An explanation is offered for the antidromic action potentials set up in motor nerves following the exhibition of ACh, PrBr and related drugs in the vicinity of nerve terminals.

REFERENCES

ANDERSON, P. & CURTIS, D. R. (1964). The pharmacology of the synaptic and acetylcholine-induced excitation of ventrobasal thalamic neurones. *Acta physiol. scand.* **61**, 100–120.

ARMETT, C. J. & RITCHIE, J. M. (1960). The action of acetylcholine on conduction in mammalian non-myelinated fibres and its prevention by an anticholinesterase. *J. Physiol.* **152**, 141–158.

ARMETT, C. J. & RITCHIE, J. M. (1961). The action of acetylcholine and some related substances on conduction in mammalian non-myelinated nerve fibres. *J. Physiol.* **155**, 372–384.

BARSTAD, J. A. B. (1962). Presynaptic effect of the neuromusculal transmitter. *Experientia*, **18**, 579.

BLABER, L. C. & BOWMAN, W. C. (1963*a*). The effects of some drugs on the repetitive discharge produced in nerve and muscle by anticholinesterases. *Int. J. Neuropharmac.* **2**, 1–16.

BLABER, L. C. & BOWMAN, W. C. (1963*b*). Studies on the repetitive discharges evoked in motor nerve and skeletal muscle after injection of anticholinesterase drugs. *Brit. J. Pharmac. Chemother.* **20**, 326–344.

CIANI, S. & EDWARDS, C. (1963). The effect of acetylcholine on neuromuscular transmission in the frog. *J. Pharmacol.* **142**, 21–23.

DEL CASTILLO, J. & ENGBAEK, L. (1954). The nature of the neuromuscular block produced by magnesium. *J. Physiol.* **124**, 370–384.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the endplate potential. *J. Physiol.* **124**, 560–573.

DEL CASTILLO, J. & KATZ, B. (1955). On the localization of acetylcholine receptors. *J. Physiol.* **128**, 157–181.

DEL CASTILLO, J. & KATZ, B. (1956). Localization of active spots within the neuromuscular junction of the frog. *J. Physiol.* **132**, 630–649.

DETTBARN, W. D. & DAVIS, F. A. (1963). Effect of acetylcholine on axonal conduction of lobster nerve. *Biochim. biophys. Acta*, **66**, 397–405.

DIAMOND, J. (1959). The effects of injecting acetylcholine into normal and regenerating nerves. *J. Physiol.* **145**, 611–629.

ECCLES, J. C., KATZ, B. & KUFFLER, S. W. (1942). Effect of eserine on neuromuscular transmission. *J. Neurophysiol.* **5**, 211–230.

EDWARDS, C. & IKEDA, K. (1962). Effect of 2-PAM and succinylcholine on neuromuscular transmission in the frog. *J. Pharmac. exp. Ther.* **138**, 322–327.

EHRENPREIS, S. (1964). Acetylcholine and nerve activity. *Nature, Lond.*, **201**, 887–893.

ELMQUIST, D. & THESLEFF, S. (1960). A study of acetylcholine induced contractures in denervated mammalian muscle. *Acta pharm. tox., Kbh.*, **17**, 84–93.

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

FATT, P. & KATZ, B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* **117**, 109–128.

FENG, T. P. & LI, T. H. (1941). Studies on the neuromuscular junction XXIII. A new aspect of the phenomena of eserine potentiation and posttetanic facilitation in mammalian muscle. *Chin. J. Physiol.* **16**, 37–50.

FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**, 218–244.

FRANKENHAEUSER, B. & MEVES, H. (1958). The effect of magnesium and calcium on the frog myelinated nerve fibre. *J. Physiol.* **142**, 360–365.

GASSER, H. S. & ERLANGER, J. (1930). The ending of the axon action potential and its relation to other events in nerve activity. *Amer. J. Physiol.* **94**, 247–277.

GORDON, H. T. & WELSH, J. H. (1948). The role of ions in axon surface reactions to toxic organic compounds. *J. cell. comp. Physiol.* **31**, 398–419.

GRAY, J. A. B. (1959). In *Handbook of Physiology*, Section on Neurophysiology. Ed. FIELD, J., MAGOUN, H. W. & HALL, V. E., vol. 1, pp. 123–145. Washington, D.C.: American Physiological Society.

GRAY, J. A. B. & DIAMOND, J. (1957). Pharmacological properties of sensory receptors and their relation to those of the autonomic nervous system. *Brit. med. Bull.* **13**, 185–188.

HUBBARD, J. I. (1961). The effect of calcium and magnesium on the spontaneous release of transmitter from mammalian motor nerve endings. *J. Physiol.* **159**, 507–517.

HUBBARD, J. I. & SCHMIDT, R. F. (1961). Stimulation of motor nerve terminals. *Nature, Lond.*, **191**, 1003–1004.

HUBBARD, J. I. & SCHMIDT, R. F. (1963). An electrophysiological investigation of mammalian motor nerve terminals. *J. Physiol.* **166**, 145–167.

HUBBARD, J. I. & YOKOTA, T. (1964). Direct evidence for an action of acetylcholine upon motor nerve terminals. *Nature, Lond.*, **203**, 1072.

KATZ, B. (1962). The transmission of impulses from nerve to muscle, and the subcellular unit of synaptic action. *Proc. Roy. Soc.* B, **155**, 455–477.

KATZ, B. & MILEDI, R. (1965*a*). Propagation of electric activity in motor nerve terminals. *Proc. Roy. Soc.* B, **161**, 453–482.

KATZ, B. & MILEDI, R. (1965*b*). The effect of calcium on acetylcholine release from motor nerve terminals. *Proc. Roy. Soc.* B, **161**, 496–503.

KATZ, B. & THESLEFF, S. (1957). A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol.* **138**, 63–80.

KOELLE, G. B. (1962). A new general concept of the neurohumoral functions of acetylcholine and acetylcholinesterase. *J. Pharm. Pharmac.*, **14**, 65–90.

KRNJEVIĆ, K. & PHILLIS, J. W. (1963). Pharmacological properties of acetylcholine-sensitive cells in the cerebral cortex. *J. Physiol.* **166**, 328–350.

LILEY, A. W. (1956*a*). An investigation of spontaneous activity at the neuromuscular junction of the rat. *J. Physiol.* **132**, 650–666.

LILEY, A. W. (1956*b*). The effects of presynaptic polarization on the spontaneous activity at the mammalian neuromuscular junction. *J. Physiol.* **134**, 427–443.

LILEY, A. W. (1956*c*). Transmission at the mammalian neuromuscular junction. Ph.D. Thesis, Australian National University, Canberra.

LILEY, A. W. (1957). Spontaneous release of transmitter substance in multiquantal units. *J. Physiol.* **136**, 595–605.

LLOYD, D. P. C. (1942). Stimulation of peripheral nerve terminations by active muscle. *J. Neurophysiol.* **5**, 153–165.

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol.* **130**, 114–122.

MASLAND, R. L. & WIGTON, R. S. (1940). Nerve activity accompanying fasciculation produced by prostigmin. *J. Neurophysiol.* **3**, 269–275.

MILEDI, R. (1962). Induction of receptors. In *Ciba Foundation Symposium on Enzymes and Drug Action*. Ed. MONGAR, J. L. & DE REACK, A. V. S. London: Churchill.

RANDIĆ, M. & STRAUGHAN, D. W. (1964). Antidromic activity in the rat phrenic nerve-diaphragm preparation. *J. Physiol.* **174**, 130–148.

RIKER, W. F. JR (1960). Pharmacologic considerations in a re-evaluation of the neuromuscular synapse. *Arch. Neurol.* **3**, 488–499.

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

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

RITCHIE, J. M. & ARMETT, C. J. (1963). On the role of acetylcholine in conduction in mammalian nonmyelinated nerve fibres. *J. Pharmac. exp. Ther.* **139**, 201–207.

STANDAERT, F. G. (1964). The action of d-tubocurarine on the motor nerve terminals. *J. Pharmac. exp. Ther.* **143**, 181–186.

TAKEUCHI, A. & TAKEUCHI, N. (1960). On the permeability of end-plate membrane during the action of the transmitter. *J. Physiol.* **154**, 52–67.

TAKEUCHI, A. & TAKEUCHI, N. (1961). Changes in potassium concentration around motor nerve terminals, produced by current flow, and their effects on neuromuscular transmission. *J. Physiol.* **155**, 46–58.

THESLEFF, S. (1960). Effects of motor innervation on the chemical sensitivity of skeletal muscle. *Physiol. Rev.* **40**, 734–752.

VAN DER MEER, C. & MEETER, E. (1956). The mechanism of action of anticholinesterases. II. The effect of diisopropylfluorophosphonate (DFP) on the isolated rat phrenic nerve-diaphragm preparation. A. Irreversible effects. *Acta physiol. pharm. neerl.* **4**, 454–471.

WALL, P. D. (1958). Excitability changes in afferent fibre termination and their relation to slow potentials. *J. Physiol.* **142**, 1–21.

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

WERNER, G. (1960*b*). Generation of antidromic activity in motor nerves. *J. Neurophysiol.* **23**, 453–461.

WERNER, G. (1961). Antidromic activity in motor nerves and its relation to a generator event in nerve terminals. *J. Neurophysiol.* **24**, 401–413.

WILSON, I. B. (1955). The interaction of tensilon and neostigmine with acetylcholinesterase. *Arch. Int. pharmacodyn.* **104**, 204–213.