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THE RELEASE OF ACETYLCHOLINE IN THE ISOLATED RAT DIAPHRAGM

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It is generally believed that acetylcholine (ACh) is responsible for the transmission of activity from nerve to muscle in vertebrates. This belief is based principally on a large amount of evidence that ACh has a powerful specific stimulating action on the muscle end-plate and that various agents which interfere with, or promote, its action have a corresponding effect on neuromuscular transmission (Riesser & Neuschlosz, 1921; Brown, 1937; Rosenblueth, 1950; Eccles, 1953; Fatt, 1954; Katz, 1958). There is very much less evidence that ACh is in fact released by the motor nerve endings. Since the classical experiments of Dale, Feldberg & Vogt (1936), comparatively little work has been published dealing with this problem. With few exceptions (e.g. Emmelin & MacIntosh, 1956) recent studies of the factors that influence release of ACh have been confined to experiments on sympathetic ganglia.

As a result, a large discrepancy has remained between the amounts of ACh known to be released in muscle, and the amounts needed for stimulation (cf. Acheson, 1948). There are, of course, many reasons why such a discrepancy might be expected (cf. Rosenblueth, 1950; Fatt, 1954; del Castillo & Katz, 1956), and the gap has tended to diminish in recent years, thanks to the more efficient application of ACh to end-plates from micropipettes (Nastuk, 1953; del Castillo & Katz, 1956; Krnjević & Miledi, 1958*c*). Nevertheless, even the smallest effective amount of ACh has been at least 15 times greater than the amount estimated to be released by a nerve ending.

In the experiments about to be described we have studied the release of ACh in the isolated diaphragm. This muscle seemed particularly suitable for such an investigation because much is already known about its behaviour during repetitive stimulation and about the amounts of ACh needed to produce end-plate activity (Krnjević & Miledi, 1958*b*, *c*). It is also convenient for various technical reasons: it has a long nerve, and only single innervation of its muscle fibres, and as it is flat and thin the inward diffusion of O_2 and the outward diffusion of ACh take place under relatively favourable conditions. We have shown in a previous paper (Krnjević &

Mitchell, 1960*b*) that one half of the extracellular ACh can diffuse out in $1\frac{1}{2}$ min.

Failure of neuromuscular transmission tends to occur rather quickly during sustained repetitive stimulation of the nerve, and this is accompanied by a marked reduction in the release of ACh (Dale *et al.* 1936). We have therefore endeavoured to keep the rate and duration of stimulation to a minimum determined by the sensitivity of the methods of bioassay. Our results show that ACh (or some very similar substance) can be collected in amounts which would be sufficient to produce appreciable depolarization if applied directly to the end-plate. A preliminary report of some of these results has already appeared (Krnjević & Mitchell 1960*a*). A comprehensive study of the release of ACh in the rat diaphragm during prolonged stimulation at various frequencies and temperatures has been published recently by Straughan (1960).

METHODS

Albino rats (Wistar strain) weighing between 200 and 400 g were used exclusively. They were anaesthetized with ether, and the left hemidiaphragm and phrenic nerve were removed and placed in cold (15° C) Ringer–Locke solution thoroughly aerated with 5 % CO_2 in O_2. The period during which the diaphragm was without an extra supply of oxygen (after opening the chest and before completing the dissection), varied between 3 and 5 min. The composition of the Ringer–Locke solution is given in the previous paper (Krnjević & Mitchell, 1960*b*).

Muscle holder. In most cases the hemidiaphragm was cut from its costal insertion and after trimming off excess connective tissue was tied to a rectangular Perspex frame at each corner. This operation was performed in Ringer–Locke solution at room temperature, with a fast stream of oxygen bubbles keeping the solution well stirred and aerated. The Perspex holder had a central turret with five platinum electrodes over which the phrenic nerve could be drawn when the muscle was to be stimulated (Fig. 1). The top of the turret was closed by a disk of Perspex, sealed with tap grease; under these conditions, the nerve remained moist for periods of at least $\frac{1}{2}$ hr, but, for safety, the nerve was left in the solution except during the periods of stimulation. Two types of frame were used, the second being somewhat smaller and lighter, and trapezoid in shape.

Collecting chamber. The muscle holder, with the muscle stretched out, fitted accurately into shallow rectangular (or trapezoid) chambers in a Perspex slab. When 5 ml. of solution was added the depth of fluid in the chamber was sufficient to keep the diaphragm well immersed. (For the lighter frames smaller chambers were used and only 3 ml. of solution.) There were several such chambers adjacent in the slab, and the holder, with the diaphragm attached, could conveniently be transferred from one to another without disconnecting the flexible leads of the electrodes. To maintain an adequate O_2 supply a fine polythene cannula was attached to the frame and a constant stream of O_2 (with 5 % CO_2) bubbles was directed at the muscle throughout (cf. Creese, 1954). Although most experiments were done at room temperature, the chamber could be kept at a higher temperature by placing the slab on a special warm water-bath.

Electrical stimulation and recording. With five electrodes it was possible to monitor continuously the spike potential of the phrenic nerve during stimulation. The second recording electrode was very close to the entrance of the nerve into the diaphragm; as long as the recorded spike was adequately diphasic, one could be sure that impulses were being transmitted to the intramuscular branches. The stimulating pulses had a duration of 10 μsec and their

intensity was usually kept at 1·5–3 times maximal for the fastest A fibres, except during a high-frequency tetanus when it was raised to 5 times maximal.

Procedure

The muscle was placed in one of the collecting chambers and stimulated for a given period. It was allowed to remain in the chamber for a further period of either 2 or 4 min, and then replaced in a large volume of aerated Ringer–Locke solution; as a rule there was no further stimulation for 30–60 min. When studying the release of ACh after a tetanus, the muscle was stimulated at a rate of 100–250/sec for 15–20 sec in a second chamber, and after 45–60 sec was shifted to a third chamber in which the standard period of stimulation at a lower frequency was repeated. The solutions were removed with a pipette and either assayed for ACh immediately, or kept on ice if the assay was delayed by an hour or so; they were in any case assayed within 2–3 hr of collection.

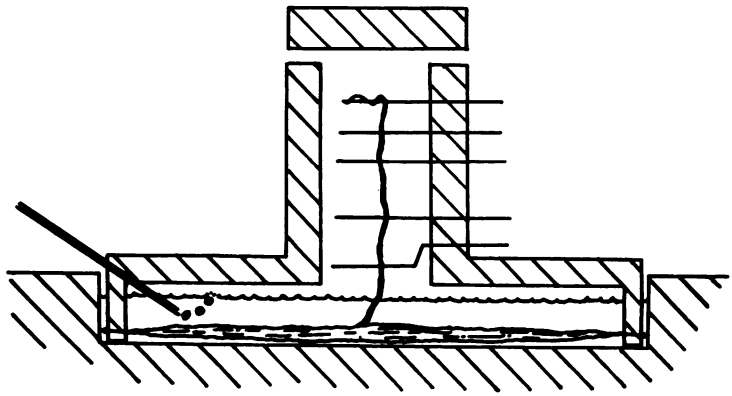

Fig. 1. Diagrammatic cross-section of rat diaphragm frame and chamber. Rat diaphragm is tied across the bottom of the frame, in Ringer–Locke solution bubbled continuously with $O_2 + CO_2$ mixture. Phrenic nerve passes over stimulating, earthing and recording electrodes. Top of frame is sealed with a greased Perspex disk.

Preservation of ACh. The hydrolysis of ACh was minimized by adding $1{\cdot}5 \times 10^{-5}$ M eserine sulphate to the solution in which the diaphragm was soaked for about 30 min before stimulating. In some experiments di-*iso*-phosphorofluoridate (DFP) was injected into the rats intraperitoneally in doses of 1–2 μmole/100 g body weight. This normally caused the animals to die after a period of some 3–5 min, when the diaphragm was removed as usual. As a further precaution, in a number of experiments, we used a solution rendered somewhat acid (pH 6·2–6·7 instead of 7·3–7·4) by reducing the bicarbonate content.

Assay of ACh

Since it was important to stimulate the diaphragm preparations as little as possible, only very small amounts of ACh were available for assay. It was therefore essential to use methods of assay which were highly sensitive, as well as reasonably specific. Since the frog rectus was too insensitive, a number of other preparations were tried in turn, with varying degrees of success. A few attempts at assaying with the frog lung (Corsten, 1940), were not very profitable, because of enormous variations in sensitivity. In four experiments longitudinal muscles from the locally available sea-cucumber (*Holothuria forscali*) were not found to be any more sensitive to ACh than the frog rectus, unlike longitudinal muscles of other sea-cucumbers which have a very low threshold to ACh (Bacq, 1939; Florey, 1956). The

following methods were more successful (Fig. 3) although each had its disadvantages. The accuracy of the assays varied with different preparations; it is estimated that the error of the best assays did not exceed $\pm 20\%$, and that of the poorest $\pm 50\%$.

Cat blood pressure. The samples were injected intravenously into eviscerated cats under chloralose anaesthesia (80 mg/kg), as in the method described by MacIntosh & Perry (1950). Although this was not a particularly sensitive method, it was little affected by moderate variations in the ionic concentration and pH of samples, and regularly gave predictable results.

Isolated rat duodenum. A short length of duodenum (2–2·5 cm) was taken from immediately below the stomach of a small rat (100–200 g), and after washing suspended in a 1 ml. Perspex bath through which oxygen was bubbled. The solution in the bath (De Jalon, Bayo &

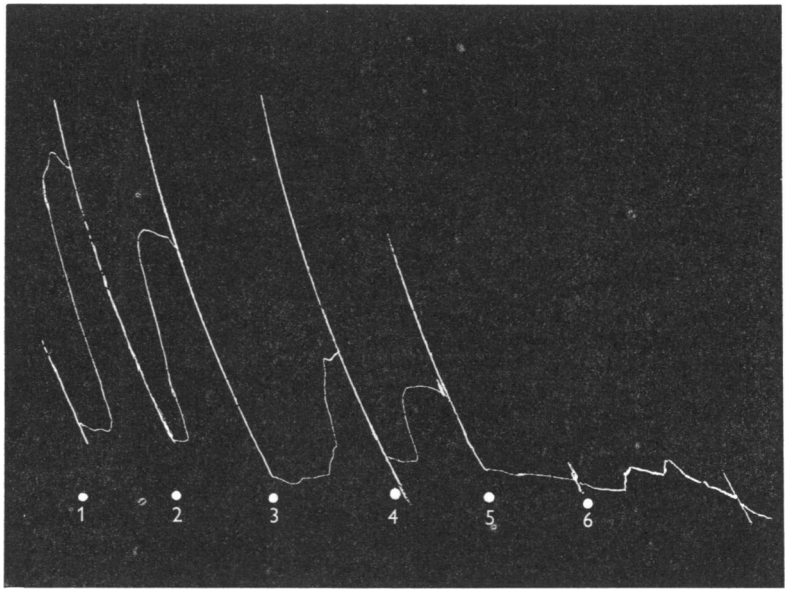

Fig. 2. Part of assay of ACh released from rat diaphragm on dorsal muscle of the leech with curare control. (1) 55 nM-ACh; (2) test solution (3 stim/sec for 5 min); (3) 2·8 nM-ACh; (4) 55 nM-ACh 7 min after tubocurarine ($1{\cdot}5 \times 10^{-5}$ M); (5) 55 nM-ACh 11 min after curare; (6) test solution 15 min after curare. After prolonged rinsing in curare-free solution, sensitivity to ACh returned.

De Jalon, 1945) was run through continuously (4–6 ml./min), except when test solutions were added. The volume of the fluid in the bath was kept constant at 1·0 ml. and the temperature maintained at 32° C. Movement of the gut was recorded by a balanced lever on a kymograph. ACh could be detected in concentrations as low as 5×10^{-11} M, although the usual threshold was at $1\text{–}3 \times 10^{-10}$ M. The test solutions were added to the bath (after equilibration at the correct temperature) in volumes of 0·1–0·4 ml.; larger volumes gave misleading results owing to insufficient dilution of the Ringer–Locke with De Jalon's solution. Although this preparation was very sensitive to ACh it deteriorated rather quickly, becoming more and more sensitive to slight changes in temperature, ionic composition (especially Ca^{2+}), pH or rate of flow of solution. It could only be used as a reliable method of assay for a rather small number of samples.

Dorsal muscle of the leech. The dorsal muscle was set up as described by MacIntosh &

Perry (1950), but in an 0·3 ml. Perspex bath and, on some occasions, with the addition of morphine sulphate ($1{\cdot}5 \times 10^{-5}$ M) to all solutions to facilitate relaxation (Murnaghan, 1958).

Heart of Mya arenaria (Hughes, 1955). The heart was suspended by the ventricles in 1 ml. artificial sea water (Tower & McEachern, 1948) at 16° C. The heart beat was recorded on a smoked drum. Although this preparation can be extremely sensitive, with threshold concentrations as low as 2×10^{-13} M, it is highly unpredictable, and the very sensitive hearts are not common. Furthermore, it has the great drawback that ACh often causes the beat to become not only weaker but slower; assaying can then be done only over a very small range of concentrations, since an amount 5–10 times threshold is likely to stop the beat altogether for a time. In this respect it is evidently unlike the heart of *Venus mercenaria* (Welsh & Taub, 1948). Its use would seem to be limited to cases in which very high sensitivity is absolutely essential and it can hardly be recommended for routine use.

ACh controls. The amounts of solution available were always too small for a really exhaustive series of control tests. However, whenever conditions were suitable, at least one control test was performed (this happened on the average once in every three assays). The most convenient procedure was to destroy the ACh in the sample by adding one-tenth volume of N/3-NaOH, letting the solution stand for 20 min at room temperature, and then neutralizing it with N/3-HCl. Specific antagonism by such drugs as curare (Fig. 2) or atropine was only used occasionally as a terminal experiment, the effects being too slowly reversible to allow useful assays to be performed again.

In several experiments the diaphragm was soaked initially in Ringer–Locke solution containing no anticholinesterase. No ACh could be detected under these conditions, even after prolonged stimulation.

Counts of fibres in the rat diaphragm. The counts were made in histological sections of diaphragms used in the experiments described above. The muscles were fixed in 10% formol saline and embedded in paraffin, and sections which included the entire width of the hemidiaphragm were cut at right angles to the fibres.

RESULTS

Most of the experiments were done at room temperature (19–23° C), because neuromuscular transmission in the isolated diaphragm persists for a longer period at lower temperatures (cf. Krnjević & Miledi, 1959). In fifteen experiments the temperature was kept at 37° C.

Only those experiments in which appreciable amounts of ACh were collected could be considered as yielding really useful information. The rejection of other experiments was based mostly on evidence that adequate excitation of the nerve endings was not taking place for various technical reasons, such as short-circuiting or displacement of stimulating electrodes, or because of obvious injury to the phrenic nerve or one of its main branches. Contraction of the diaphragm was not in itself very informative, since twitches of relatively few fibres may give the impression of widespread activity. However, in a substantial number of cases we failed to detect any ACh at all (this means less than about one tenth of the usual amount); sometimes this turned out to be because our anticholinesterases had lost their potency, but in other cases there was no obvious explanation.

ACh release at room temperature. On the basis of previous experiments with the phrenic-diaphragm preparation it was decided that there was

a reasonable chance of avoiding presynaptic failure of propagation if stimulation was effected at a rate not greater than 5/sec or did not last more than 5 min, arbitrarily limiting the number of impulses in one sequence of stimulation to 1500 or less. Results of experiments in which these limits were exceeded are considered separately. Clearly, even more moderate stimulation was preferable whenever the assay preparations were sufficiently sensitive; in several cases it was possible to obtain sufficient ACh while stimulating at 2/sec for 2–5 min. A few hearts of *Mya arenaria*

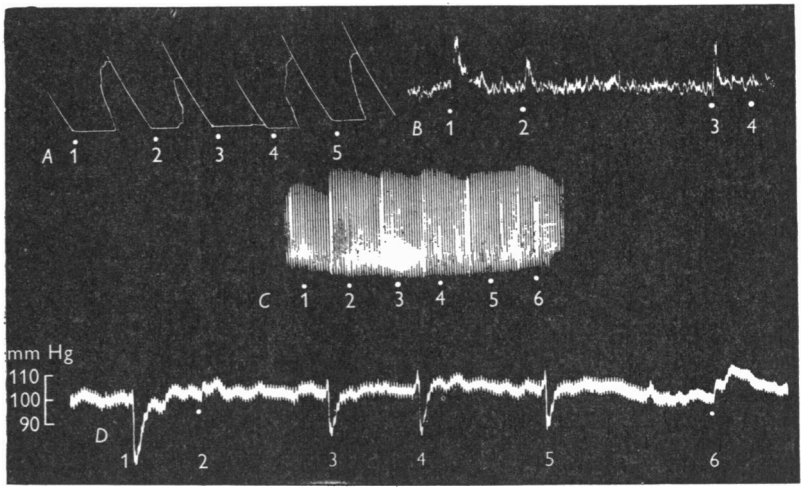

Fig. 3. Assay of ACh released from rat diaphragm during stimulation. Examples shown do not necessarily include complete assay. *A* Dorsal muscle of leech: (1) Test solution (5 stim/sec for 20 min); (2) 28 nM-ACh; (3) Test solution, alkali-treated; (4) Test solution; (5) 33 nM-ACh. *B* Rat duodenum: (1) Test solution (5 stim/sec for 5 min); (2) 11 nM-ACh; (3) 22 nM-ACh; (4) test solution, alkali-treated. Test solutions were diluted 5 times. *C* Heart of *Mya arenaria*: (1) 5·5 nM-ACh; (2) test solution (1·8 stim/sec for 15 min); (3) 4·3 nM-ACh; (4) test solution; (5) test solution, alkali-treated; (6) 5·5 nM-ACh. Test solutions were diluted 3 times. *D* Cat blood pressure: (1) 0·25 nmole ACh; (2) 1·0 ml. Ringer–Locke solution; (3) 0·1 nmole ACh; (4) 1·0 ml. test solution (5 stim/sec for 5 min); (5) 0·1 nmole ACh; (6) 1·0 ml. test solution, alkali-treated.

were so sensitive that we tried to stimulate even at 1/sec for only 2 min; the results were not very satisfactory, partly because such sensitive hearts tended to respond irregularly in the presence of solutions which had been in contact with the muscle, and partly because the yield seemed to be reduced, perhaps as a result of destruction by minimal amounts of cholinesterases or because of binding in the tissue (cf. Krnjević & Mitchell, 1960*b*).

The mean release of ACh from the hemidiaphragm, in twenty-four experiments at temperatures of 19–23° C, was 0·12 pmole (S.D. ± 0·079)

per impulse. These results were obtained by assaying the collected ACh either on the heart of *Mya arenaria*, the rat duodenum or the cat blood pressure (Fig. 3). Although these three methods of assay gave comparable answers, the mean result of assays on the rat duodenum was somewhat larger (0·19 pmole/impulse) than the mean value of assays on the heart of *Mya arenaria* (0·064 pmole), and the mean value of assays on the cat blood pressure which had an intermediate position (0·098 pmole). There was much overlap of individual results and the general scatter was such that no statistical significance can be attached to these deviations from the over-all mean (the greatest difference between mean values obtained with different preparations has a probability of just $> 0{\cdot}05$, and the other differences have probabilities $> 0{\cdot}25$ on the null-hypothesis, as determined by the t test).

ACh release at 37° *C.* No systematic study was made of the release of ACh from the same muscle at different temperatures. The mean release in six muscles excited at 2–3/sec for 5 min at 37° C was 0·15 pmole/impulse. The ACh was assayed on leeches and the rat duodenum. These results overlapped those at a lower temperature; it does not seem that there is a very pronounced change in the amount liberated at the higher temperature.

ACh release under other conditions

Release during faster and longer stimulation. The mean yield of ACh during activity at 37° C in a group of seven muscles stimulated at frequencies of 10–20/sec for 10–20 min was 0·025 pmole (range 0·011–0·033), i.e. one-sixth of the yield in a comparable series of six muscles during stimulation at 2–5/sec for 5 min. Similar differences were observed in experiments at room temperature. If the preparation was stimulated tetanically (at 100–200/sec) for 10–20 sec, the total number of impulses being greater than when stimulating at a slow rate for 5 min, the yield of ACh was as a rule below threshold amounts for assay.

Release of ACh in the presence of curare. In ten experiments a comparison was made of the yield of ACh during ordinary activity and activity during paralysis by curare. After the preliminary control series the muscle was soaked for 30–60 min in 3×10^{-5} M tubocurarine chloride, and then stimulated as before in 3×10^{-6} M tubocurarine (with the usual amount of anti-cholinesterase also present). In two experiments there was no marked change in the yield of ACh per impulse. Figure 4 shows the assay on the heart of *Mya arenaria* of solutions obtained during indirect stimulation of a hemidiaphragm before and after soaking in tubocurarine. It can be seen that the sensitivity of the preparation to ACh was reduced by the presence of tubocurarine in the solution. However, the assay could be performed satisfactorily by adding the same amount of tubocurarine to

the standard ACh solution used for comparison. There was clearly no appreciable increase in the release of ACh in the presence of tubocurarine. In two other experiments the ACh release apparently rose by 50 and 100 %, respectively, and in one experiment it diminished by 15 %. In the five remaining experiments various technical difficulties in assaying prevented any accurate quantitative comparison, but it was evident that there was no marked change after curare: any difference could not have exceeded a factor of 2–3.

When simple solutions of ACh were compared with those in which the muscle had contracted, it was often found that the latter caused irregular

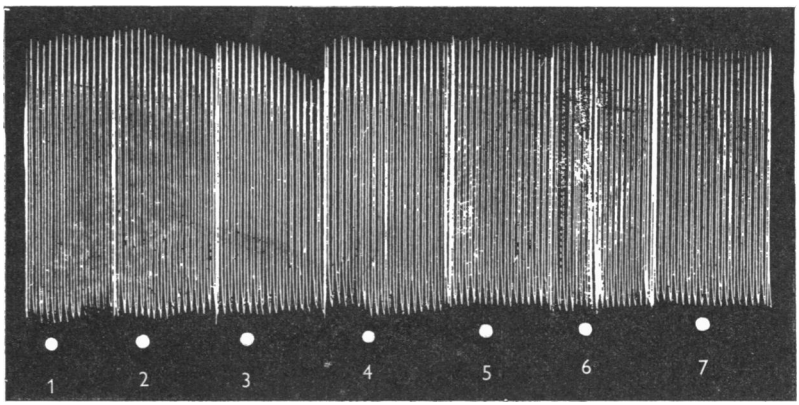

Fig. 4. Effect of tubocurarine ($1{\cdot}5 \times 10^{-5}$ M) on the *release* of ACh from the rat diaphragm. Assay on heart of *Mya arenaria*: (1) Test solution (2 stim/sec for 5 min) alkali-treated; (2) test solution; (3) 2·8 nM-ACh; (4) test solution, alkali-treated; (5) 2·8 nM-ACh + tubocurarine ($1{\cdot}5 \times 10^{-5}$ M); (6) test solution from curarized diaphragm; (7) 1·7 nM-ACh. Test solutions were diluted 3 times.

variations in base line, and also in ACh sensitivity. There was some evidence that solutions which had bathed muscles paralysed by curare were less likely to give rise to such effects, suggesting that disturbing factors are released mainly during contraction of the muscle fibres.

Spontaneous release of ACh. In some experiments the diaphragm was allowed to soak in the collecting chamber in the presence of anticholinesterase, but without stimulating the nerve. The period of soaking was usually 5–7 min, as in most experiments. In the majority of cases no ACh was found in the solution (i.e. less than 10^{-10}–10^{-9} M, depending upon the sensitivity of the assay). In several cases, however, there was an appreciable quantity of ACh in the solution, equivalent to a spontaneous release of about 1 pmole per hemidiaphragm per minute (at room temperature). The lower limit of the range of estimates was set, as always, by the threshold concentration for assay, and values of 0·5 pmole/min or less were

mainly seen when the period of soaking was extended to 30–60 min. The upper limit was reached in one experiment, in which the very high, apparently spontaneous, release of 9 pmole/min was seen twice out of four trials with one muscle; in the other two trials there was no detectable release. The high apparent rate of release in this case may conceivably have resulted from contamination with ACh.

Post-tetanic release of ACh. In seven experiments we were able to compare the yield of ACh before and after short tetani at a high frequency. Altogether fourteen such comparisons were made, as it was possible to repeat this procedure several times with some preparations.

It was clearly preferable to use the lowest possible rate of stimulation during the periods before and after tetanus. Unfortunately, it is important

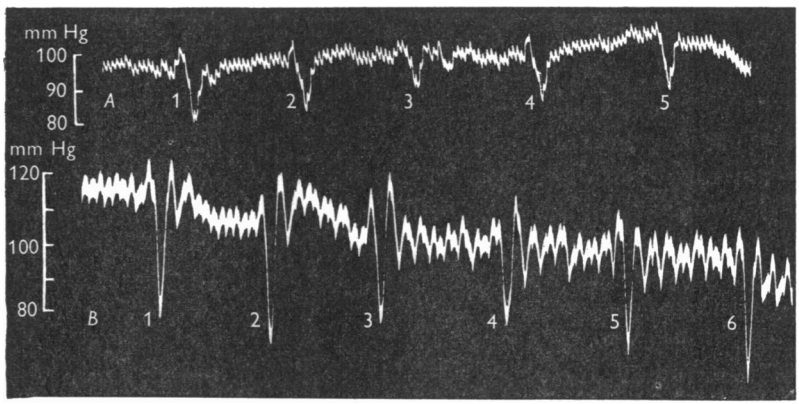

Fig. 5. Pre- and post-tetanic release of ACh from the rat diaphragm assayed on cat blood pressure. *A* (1) 0·023 nmole ACh; (2) 1·0 ml. test solution (5 stim/sec for 4 min); (3) 0·02 nmole ACh; (4) 1·0 ml. test solution (5 stim/sec for 4 min 50 sec after end of tetanus); (5) 0·023 nmole ACh. *B* (1) 1·0 ml. test solution (5 stim/sec for 5 min); (2) 0·03 nmole ACh; (3) 1·0 ml. test solution; (4) 0·025 nmole ACh; (5) 1·0 ml. test solution (5 stim/sec for 5 min, 60 sec after end of tetanus); (6) 0·03 nmole ACh.

to be able to analyse several samples quickly and reliably in this kind of experiment whereas the most sensitive methods of assay proved often very erratic, and seldom gave results at the highest level of sensitivity for very long. We were therefore only able to perform satisfactorily two experiments stimulating at 1 or 2/sec. The most stable conditions for assaying were available when using the cat blood pressure, which was relatively insensitive and required stimulation at 5/sec.

The periods of stimulation before and after the tetanus lasted 4 or 5 min and the second one usually began 45–60 sec after the end of the tetanus (120–250/sec for 10–20 sec), to allow at least some of the ACh which might have accumulated during the tetanus to diffuse out. In fact only very

small amounts of ACh were usually found after such tetanization, and three control experiments, in which the muscle was returned to the original chamber 45–60 sec after the end of the usual short tetanus and left there without further stimulation for 7 min, did not give a higher final concentration of ACh. These controls are in any case only of somewhat academic interest, since the twelve experiments performed when stimulating at 5/sec did not show a significant change in the release of ACh. The mean value of the twelve ratios of post-tetanic to pre-tetanic release was 1·08, with a standard error of $\pm 0{\cdot}05$. Examples of assays of solutions obtained in these experiments are shown in Fig. 5. On the other hand, two experiments performed at a low rate of stimulation (1 and 2/sec) suggested that more ACh was released after the tetanus, the yields being greater by a factor of 1·8 and 2·0, respectively (these values are unlikely to be incorrect by more than $\pm 0{\cdot}3$ and $\pm 0{\cdot}5$).

Counts of fibres in the rat diaphragm

The numbers of muscle fibres counted by one observer in histological sections of 3 hemidiaphragms came to 7896, 10,384 and 11,407, the mean count being 9896. As there is always some degree of arbitrary selection in deciding what is and what is not a 'fibre' in such sections, three other observers also counted independently the fibres in the first specimen; their estimates were 7094, 8016 and 8294. All four counts differed from the mean value by less than 10%. These results confirm an estimate of 10,000 ($\pm 20\%$) made by Krnjević & Miledi (1958*a*) on the basis of fibre counts in a small number of representative sections.

DISCUSSION

Identification of ACh. The amounts of substance released from the diaphragms were always so small that it was not possible to identify ACh with an extremely high degree of certainty by performing a series of parallel assays on different preparations (cf. Chang & Gaddum, 1933). However, if we consider that the active substance was only obtained in the presence of anticholinesterase, that it was destroyed in alkaline solutions, that different methods of bioassays gave approximately the same estimate in terms of ACh, and that such specific antagonists as curare and atropine had the expected action, notwithstanding the fact that these observations were made at different times, we can conclude that the substance in question is very likely to be ACh, or at least a similar choline ester.

Site of release. We have not excluded the possibility that ACh is released by antidromic activation of sensory nerve fibres in the diaphragm, but it is very unlikely that so much could be released by sensory fibres in

view of the observations of Dale *et al.* (1936) and Brecht & Corsten (1941). It is clear that autonomic nerve fibres were not involved, since stimuli just maximal for the fast A fibres were adequate for the release of ACh.

Amounts collected

As the muscles were bathed in 3 or 5 ml. of solution, the external concentration of ACh could never be greater than 1 or 2 % of that in the extracellular space of the muscle (the free ACh space in the diaphragm is 25 ml./100 g (Krnjević & Mitchell, 1960*b*); i.e. 0·05 ml. in a typical hemidiaphragm weighing 200 mg). If we assume that ACh released during stimulation is free to diffuse out, and to simplify matters, that the rate of outward movement is simply proportional to the amount already present, we can calculate approximately what fraction (f) of the total ACh released can be expected to remain in the muscle at a given time.

$$f = \frac{T}{ts}\left\{\exp -\frac{(t-ts)}{T} - \exp -\frac{t}{T}\right\},$$

where T is the time constant of outward movement, ts the period of stimulation, and t the total period in the bath from the beginning of stimulation. It was found in previous experiments (Krnjević & Mitchell, 1960*b*) that one half of the 'extracellular' ACh leaves the diaphragm in 1·5 min, and that the remainder moves out with a time constant of 3·6 min. Taking therefore a value of 2·9 min for T, the amount of ACh collected during a 7 min period, which includes a 5 min period of stimulation (our usual procedure), would come to 76 % of the total ACh released. It seems reasonable to conclude that our method would enable us to collect some 3/4 of the ACh in the muscle which was free to diffuse out.

Release of ACh per impulse

Previous authors who have made observations on the release of ACh in the rat diaphragm have tended to use a standard technique in which the diaphragm is stimulated at 50/sec for 20 min (e.g. Burgen, Dickens & Zatman, 1949; Brownlee, 1957). It is very unlikely that the nerve endings would be capable of releasing ACh in anything like the normal amount at such a frequency and for such a long time. In any case it is certain that most phrenic nerve fibres would very soon begin to fail to transmit impulses at such a frequency, so that the over-all effective contribution of the nerve endings would be at a much lower rate (Krnjević & Miledi, 1958*b*, 1959). Hence it is not surprising that the yield of ACh per impulse in such experiments (about 0·006 pmole per hemidiaphragm) is considerably less than we have found when stimulating at 2–5/sec for periods of 2–5 min (0·12 pmole). In our own experiments faster and longer periods of stimulation yielded substantially less ACh per impulse. Even more significant

are the observations made recently by Straughan (1960), who has analysed systematically the release of ACh from the rat diaphragm during 20 min periods of stimulation at frequencies varying between 6/sec and 100/sec. He has found that increasing the rate of stimulation causes only a relatively small increase in total ACh release, which reaches a maximum at about 25/sec with little further change up to 100/sec.

The highest relative yield obtained by Straughan (1960) during stimulation at 6/sec for 20 min was about one quarter of the average amounts which we collected. Most of this difference can be ascribed to the shorter periods and lower rates of stimulation in our experiments, although other experimental factors such as temperature and methods of assay may have tended to exaggerate it.

It should not be thought surprising that comparatively small variations in the frequency of stimulation may lead to marked changes in the release of ACh at the nerve endings. In the curarized rat diaphragm the amplitude of the end-plate potential is very sensitive to the frequency of stimulation: Lundberg & Quilish (1953) have shown that an impulse is followed by a period of presynaptic depression lasting at least 3 sec, during which a second end-plate potential is appreciably reduced, presumably because of a smaller release of ACh. According to our own observations the end-plate potential in the curarized diaphragm is almost immediately reduced to about one third when the frequency of nerve stimulation is changed from 1/10 sec to 2–5/sec. It is highly probable that much lower rates of stimulation than we were able to use would be required to demonstrate the maximal release of ACh.

Spontaneous release of ACh. The finding of a spontaneous release of ACh in a number of unstimulated diaphragms confirms the observations of Straughan (1960), although the typical amounts were smaller by about one half, perhaps because of the lower temperature (cf. Liley, 1956). Such a small release is equivalent to 1/20–1/30 of the usual rate of release during stimulation, and would not as a rule be detected after soaking the muscle for only 5–7 min. It is most unlikely that injury discharge of the phrenic nerve could contribute appreciably to the spontaneous release, since no spontaneous twitching could be observed. To liberate this amount of ACh, one quarter of all the fibres would have to twitch at a mean rate of 1/sec.

The release of about 1 pmole/min can probably be correlated with the spontaneous miniature electrical activity recorded at the end-plate. The relatively small size of the end-plate potential during stimulation at 2–5/sec suggests that its quantum content may not be much more than 20. Taking our figure of 0·12 pmole for the release of ACh per impulse, a quantum of ACh would correspond to about 0·006 pmole (per

hemidiaphragm). It follows that a spontaneous quantal release at 3/sec (well within the common frequency range of miniature end-plate potentials in the diapragm; Liley, 1956) would be sufficient to give 1 pmole/min. However, it is difficult to associate single presynaptic vesicles (cf. del Castillo & Katz, 1956) with this release, which is equivalent to $3{\cdot}6 \times 10^5$ molecules per quantum at a single nerve ending and would require an improbably high concentration of ACh (10 M) within the 500 Å vesicles. Furthermore, the total bound ACh in the rat phrenic nerve endings (about 300 pmole according to preliminary observations by C. O. Hebb and K. Krnjević) would be accounted for by only 50,000 vesicles per nerve ending; if the density of vesicles is $1000/\mu^3$ of axon terminal, as in the frog (Birks, Huxley & Katz, 1960), the total volume of terminal would be only 50 μ^3, compared with 3000 μ^3 in frog muscle. Such a small volume seems unlikely if one considers that the motor ending spreads over an area with a diameter of some 20 μ on the diaphragm fibres (Cole, 1957).

Yield of ACh in the presence of tubocurarine. Our results confirm previous evidence that curare does not prevent the release of ACh in muscle (Dale *et al.* 1936; Emmelin & MacIntosh, 1956); they also show that under its influence there is no marked increase in the amounts of ACh which can be collected, presumably because the reaction between ACh and the receptor is so rapid (cf. del Castillo & Katz, 1957) that formation of a relatively stable receptor–curare compound does not displace any appreciable amount of fixed ACh. This is in contrast with the action of dibenamine on sympathetic transmission in the spleen (Brown & Gillespie, 1957).

ACh release during post-tetanic potentiation (PTP). It is unfortunate that it was not possible to perform most of the experiments on PTP under conditions more likely to give a conclusive answer. In most cases stimulation had to be at 5/sec, at which frequency PTP is not nearly so marked as, for instance, at 0·5/sec. We have found in the curarized rat diaphragm that PTP does clearly occur at 5/sec (Fig. 6), although its duration was shorter than during stimulation at lower frequencies, when appreciable PTP may be detected for over 6 min. The enormous apparent potentiation of the end-plate immediately after the end of the tetanus was probably largely caused by a short gap of 2–5 sec between the end of the tetanus and the resumption of stimulation at 5/sec.

It can be seen from the second curve in Fig. 6 that if one waited some 45–60 sec before resuming stimulation after the tetanus there was a much more rapid decline of PTP when testing is resumed, so that the curve soon joined and then followed that obtained by the usual procedure. This behaviour, which has been seen at other frequencies of testing stimuli,

suggests that the rate of decay of PTP depends upon time rather than on the discharge of impulses.

Inspection of such PTP curves obtained during stimulation at 5/sec shows that if there is an increased post-tetanic release of ACh (Hutter, 1952) the mean increment over a period of 4–5 min is unlikely to exceed 10 % of the pre-tetanic release (assuming a simple relation between height of end-plate potential and ACh release). Our results are consistent with

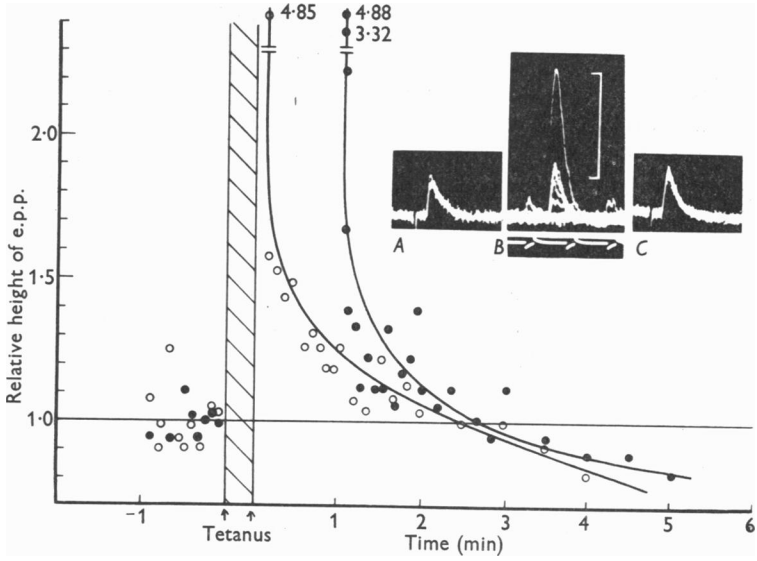

Fig. 6. Post-tetanic potentiation of end-plate potentials (at 5/sec) in curarized rat diaphragm, recorded beginning 5 sec (○—○) and 60 sec (●—●) after the end of tetanic stimulation of the phrenic nerve (250/sec for 20 sec). Inset, examples of intracellular potentials actually recorded: *A*, before tetanus; *B*, 10 sec after tetanus; *C*, 30 sec after tetanus; vertical scale 1 mV, horizontal scale 10 msec.

such a change, but they are too variable to be conclusive evidence one way or the other. On the other hand, the two results obtained during stimulation at a lower frequency can only be considered as suggestive evidence that appreciably more ACh may be released after a tetanus. The ephemeral nature of PTP during activity at 5/sec supports previous evidence that PTP may not be of great significance for normal function (Ström, 1951).

Release of ACh per nerve ending

With a mean number of 10,000 muscle fibres in the hemidiaphragm, we can take the release of ACh by an average ending to be about 10^{-17} mole per impulse (fibres in the diaphragm only extremely rarely show evidence of multiple innervation (Lundberg & Quilish, 1953; Liley, 1957; and

personal observations)). This amount of ACh is 10–20 times greater than previous estimates, calculated rather indirectly (e.g. Acheson, 1948; R. I. Birks, quoted by MacIntosh, 1959). It has been shown previously (Krnjević & Miledi, 1958*c*) that substantial end-plate potentials can be produced iontophoretically in the diaphragm with as little as 10^{-17}–10^{-16} mole of ACh; very short pulses of current were used, and under these conditions the ACh potentials were practically indistinguishable from normal end-plate potentials. We have already mentioned reasons for believing that the number of quanta released by impulses is likely to be reduced in our experiments, so that the amounts of ACh released can be compared directly with the amounts needed to produce 5–10 mV ACh potentials without gross error. It is clear that, although there is overlap of extreme values, a small gap remains between typical values; this is not surprising, since the liberation of ACh from a point source at the tip of a micropipette is inherently less efficient than the release of a similar amount spread over the whole area of the end-plate; the amounts which we have collected are likely to be substantially short of the maximal release for various reasons already mentioned. One can conclude that for the rat diaphragm the quantitative agreement between the effectiveness of applied ACh and its release during stimulation is about as satisfactory as could be expected.

SUMMARY

1. Estimations have been made by bioassay methods of the release of ACh in the isolated rat hemidiaphragm caused by not more than 1500 maximal impulses in the phrenic nerve (at frequencies of 2–5/sec). The mean release in twenty-four experiments was 0·12 pmole per impulse at room temperature.

2. The rate of release was substantially less during stimulation at a higher frequency and for longer periods.

3. The yield of ACh during low-frequency stimulation was not increased to any pronounced extent in experiments performed at 37° C, or in the presence of tubocurarine.

4. Post-tetanic potentiation during stimulation at 1–2/sec (in two experiments) was associated with a greater release of ACh; at 5/sec, although the mean increase was of the order expected, there was too much variation for the change to be significant.

5. Spontaneous release of ACh was seen in some cases, in amounts which are not incompatible with those expected from the spontaneous activity at the end-plate.

6. The calculated mean release of ACh in the rat diaphragm from a single nerve ending (10^{-17} mole) during low-frequency stimulation is of

the same order of magnitude as the smallest amounts of ACh which have previously been found to be effective when applied directly to the end-plate in the rat diaphragm.

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