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THE SPONTANEOUS RELEASE OF ACETYLCHOLINE FROM THE DENERVATED HEMIDIAPHRAGM OF THE RAT

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Brooks (1954) has shown that acetylcholine (ACh) is released from the isolated guinea-pig diaphragm in the absence of nerve stimulation. A comparable resting release has been found in the rat diaphragm by Straughan (1960) and by Krnjević & Mitchell (1961). Furthermore, in two preliminary experiments Straughan (1960) could detect no great difference between the release from a rat hemidiaphragm denervated by phrenic nerve section 7 days previously, and that from the contralateral control hemidiaphragm acutely denervated at the time of the experiment; he suggested, therefore, that the ACh originated in some non-nervous tissue. Krnjević & Mitchell (1961), on the other hand, considered it possible that the resting release might represent the ACh responsible for the production of miniature end-plate potentials (m.e.p.p.s).

In the work to be described two types of experiment were done. In the first the ACh release from hemidiaphragms which had been denervated for different periods was compared with the release from the corresponding control hemidiaphragms, to determine whether or not the release was altered by chronic phrenic nerve section. In the second series of experiments measurements were made of the spontaneous release of ACh from rat hemidiaphragms subjected to alterations in the potassium concentration or in the temperature of the bathing fluid; these are procedures which are known to alter the m.e.p.p. frequency (Liley, 1956*a*, *c*).

No attempt was made to measure m.e.p.p.s in either type of experiment; but it should be mentioned that Liley (1956*a*) could record no m.e.p.p.s from the rat hemidiaphragm from about 18 hr after denervation until the time when the motor nerve terminals were re-established. On the other hand, Katz & Miledi (1959), working on the frog sartorius, found that while the m.e.p.p.s disappeared 3–4 days after denervation, activity was resumed at a much reduced frequency some days later.

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METHODS

One hundred and thirty-three rats (Wistar strain) of both sexes, weighing between 180 and 340 g, were used. In 69 of these, the phrenic nerve was divided on one side; 40 were used in experiments to investigate factors which influenced ACh release and the remainder served as unoperated controls.

Denervation experiments. In some early operations on rats anaesthetized with intraperitoneal sodium pentobarbitone (Nembutal, Abbott Laboratories; 3–4 mg/100 g) the phrenic nerve on one side was cut immediately below the level of the 5th cervical nerve; this procedure was abandoned because it was difficult to ensure complete section by this approach. In the majority of experiments the rats were anaesthetized with ether and one phrenic nerve was cut or crushed approximately 5 mm from the diaphragm through an incision between the 7th and 8th ribs. Usually denervation was performed on the left nerve because it was technically more convenient, but the right phrenic nerve was cut in a few animals. The rats were kept under normal conditions until the acute experiments; these were done at intervals after the initial operation, as shown in Figs. 4 and 5. In these experiments rats were anaesthetized with ether; the diaphragm was exposed and briefly observed for spontaneous movement and for movement in response to mechanical stimulation of the phrenic nerves. The whole diaphragm, still attached to the ribs and spinal column, was rapidly removed into cold (4–10° C) Ringer–Locke solution (Krnjević & Mitchell, 1960) aerated with 5 % CO_2 in O_2; the two halves of the diaphragm were then separated and the costal margins and the crura discarded to eliminate errors due to cross-innervation (Ogawa, 1959). Each half of the diaphragm was soaked for 30 min at 4–10° C in aerated Ringer–Locke solution containing 5×10^{-5} M eserine sulphate. The tissues were briefly rinsed with Ringer-Locke solution and then transferred to 2·0 ml. aerated non-eserinized Ringer–Locke solution at 37° C for the collection of ACh released spontaneously. After 30 min the bathing fluid was removed by suction and its volume measured before it was assayed for its ACh content.

In each rat the contralateral hemidiaphragm, acutely denervated during the dissection, served as a control for the chronically denervated tissue; to check that the ACh release from the right and left hemi-diaphragms was normally equal, 24 control rats were used in which both hemidiaphragms were acutely denervated at the time of the experiment.

To determine the site of ACh release the left phrenic nerve was cut in three rats 7 weeks before the experiments. The two halves of the diaphragms from these rats were bathed in eserinized Ringer's solution for 30 min and then cut into strips containing either the line of nerve terminals or muscle tissue free of nerve terminals. The line of these strips was judged from diaphragms which had previously been stained for cholinesterase to show the nerve endings. The strips were each placed separately in 1·0 ml. of eserinized Ringer's solution which was oxygenated for 1 hr and then assayed for ACh.

Factors affecting release. Diaphragms from normal rats were dissected as above. As a routine, freshly prepared eserine sulphate (5×10^{-5} M) was added to the collecting fluid as well as to the preliminary soaking fluid whenever collections were continued for longer than 30 min. In a few experiments, however, the effect was observed of omitting the anticholinesterase, either entirely or from the collecting fluid; in some others di-*iso*-propyl phosphorofluoridate (DFP) 5×10^{-5} M, was used in place of eserine. To test the effect of the potassium concentration of the collecting fluid on release, the hemidiaphragm was kept at 37° C in 2·0 ml. Ringer–Locke solution containing 5, 15 or 30 mM-K^+ with equimolar reduction in Na^+; this was removed for assay and replaced by fluid of a different K^+ content every 30 min. To test the effect of temperature, the temperature of the hemidiaphragm in Ringer–Locke solution (of normal K^+ concentration) was altered every 30 min. The collecting fluid was removed at the end of each period. As the control in both cases, the contra-

lateral hemidiaphragm was maintained throughout at 37° C in 2·0 ml. Ringer–Locke solution changed every 30 min.

Assay and identification of ACh

The solutions taken from the diaphragm baths were diluted 1·8 times with distilled water to make them isotonic with leech Ringer's solution and assayed within 30 min against ACh chloride (Roche Products Ltd) on the sensitized dorsal muscle of the leech, set up as described by MacIntosh & Perry (1950), but in an 0·3 ml. Perspex bath. The volumes of the samples and their ACh concentration were necessarily small and this precluded the use of exhaustive controls, as described by Feldberg (1945); but in the course of each experiment it was usually possible to pool parts of several samples and to assay them again after alkali treatment (Krnjević & Mitchell, 1961). ACh samples treated in this way were inactive. In six of the experiments pooled samples were assayed against ACh on the rat blood-pressure preparation (Straughan, 1958) and in two experiments they were tested on the isolated guinea-pig ileum; on these occasions the assays agreed within $\pm 10\%$ in terms of ACh chloride.

In additional tests to identify the bio-active substance, active samples from 5 experiments were tested on the dorsal muscle of the leech after it had been treated with curare ($1{\cdot}5 \times 10^{-5}$ M) for 10 min, and their action was abolished. In other experiments, during which no anticholinesterase was used on the isolated diaphragm, the collected samples showed no activity when tested on the leech or rat blood-pressure preparation.

RESULTS

The spontaneous release of ACh from the isolated rat diaphragm

In the present experiment the amount of ACh spontaneously released was usually between 2·0 and 3·0 pmole/min/hemidiaphragm at 37° C. When cholinesterase activity had been initially blocked with eserine sulphate (5×10^{-5} M) the amount of ACh collected from the diaphragms began to fall after about 2 hr (Fig. 1), but it could be restored to near its former level by re-immersing the diaphragm in fresh eserine solution. When DFP (5×10^{-5} M) was used in place of eserine the ACh levels fluctuated considerably, but the over-all fall was slower than with eserine and further treatment of the tissue with DFP produced only a small increase in the amount recovered. Under these conditions, or in experiments when eserine was continuously present in the collecting solutions, detectable amounts of ACh were released from the hemidiaphragms for up to $14\frac{1}{2}$ hr. During the period of the experiment illustrated in Fig. 2 a total of 2040 pmole ACh was recovered from the fluid bathing the single hemidiaphragm; the maximum collected on any occasion was 3500 pmole in $14\frac{1}{2}$ hr.

The absolute amount of ACh collected during 30 min periods varied from rat to rat and also in relation to the time of day when the animals were killed. Figure 3 illustrates the release from hemidiaphragms of rats killed at intervals during a 24 hr period and shows that the highest values were obtained between 4 and 8 a.m. No correlation was found between the weight of rats and the ACh release from their diaphragms.

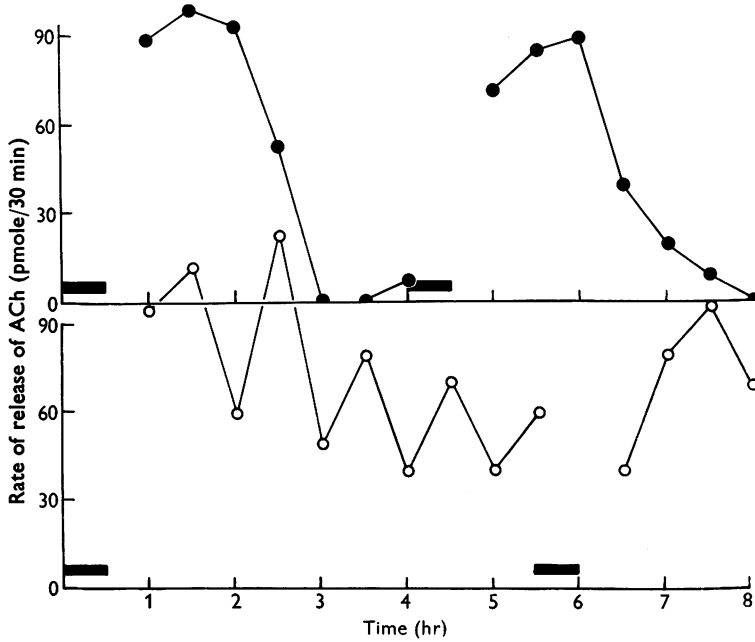

Fig. 1. The preservation in one hemidiaphragm by eserine (above) and in another by DFP (below) of spontaneously released ACh. The hemidiaphragms were soaked in eserine (5×10^{-5} M) or DFP (5×10^{-5} M) as indicated by solid horizontal lines. Collections were made over 30 min periods without anticholinesterase in the bathing solutions. Ordinate: rate of release of ACh (pmole-min) during 30 min collection periods.

The effect of denervation on the spontaneous release of ACh

It was first important to establish whether the quantities released from the two halves of the same diaphragm were equal. This was tested in 24 unoperated rats. In all these rats the amount of ACh released from the two halves of the same diaphragm was within 10 % over a 30 min-collection period (see Figs. 4 and 5).

Control experiments. Rosenblueth, Alanís & Pilar (1961) reported that in cats, rabbits, dogs and monkeys the diaphragm was innervated by intercostal and other nerves, and that dividing the phrenic nerve did not secure complete denervation. These observations were confirmed by us on three rabbits by recording the electromyograph (e.m.g.) from 12 points on the diaphragm before and after cutting the phrenic nerve in the chest. In the rat, however, no muscle activity could be recorded (3 rats) from any part of the diaphragm after denervation and it was concluded that in the rat this procedure completely denervates the diaphragm. A further observation which supports the claim that the diaphragm was completely

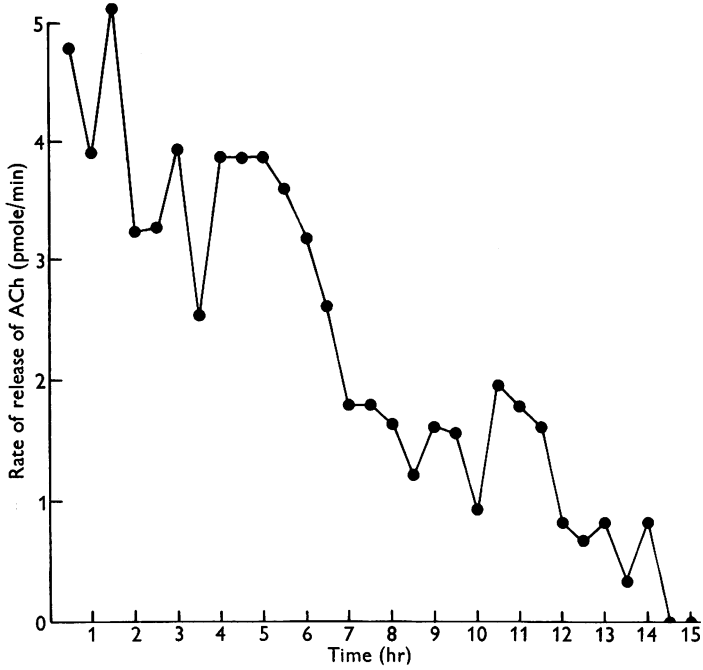

Fig. 2. The rate of spontaneous release of ACh from a single hemidiaphragm during 15 hr. Eserine (5×10^{-5} M) present throughout.

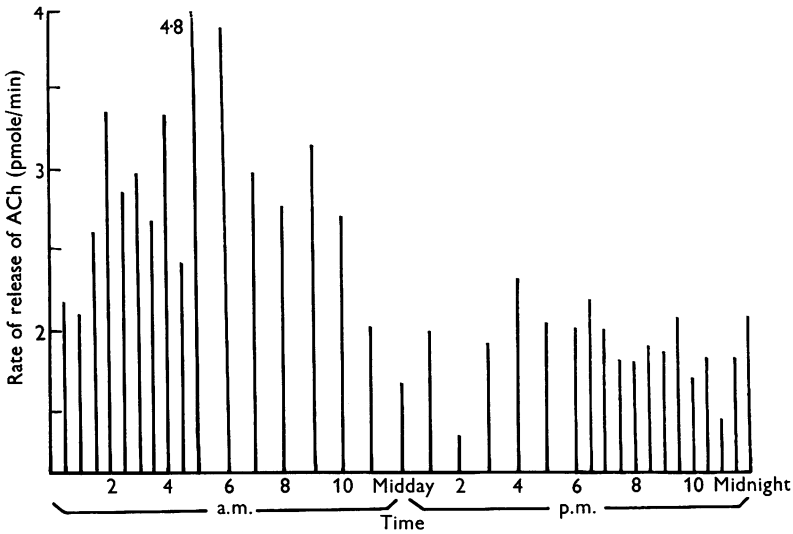

Fig. 3. The rate of release of ACh from single hemidiaphragms of rats killed at different times of the day or night. The results were obtained in three experiments covering different continuous periods: (*a*) 6 a.m.–12 noon; (*b*) 1 p.m.–5.30 p.m.; (*c*) 5.30 p.m.–5 a.m. Each vertical line represents the average rate of release of ACh from a single hemidiaphragm during 30 min.

denervated by division of the phrenic nerve was that in those rats in which re-innervation had not occurred after 8 or more weeks the diaphragms were very thin.

Short-term denervation. Observations were made on diaphragms removed from rats in which the phrenic nerve had been unilaterally divided 1–24 hr previously (Fig. 4). These showed that the output of ACh from the denervated hemi-diaphragm was quickly reduced, as compared with the contralateral control. One hour after denervation the resting release

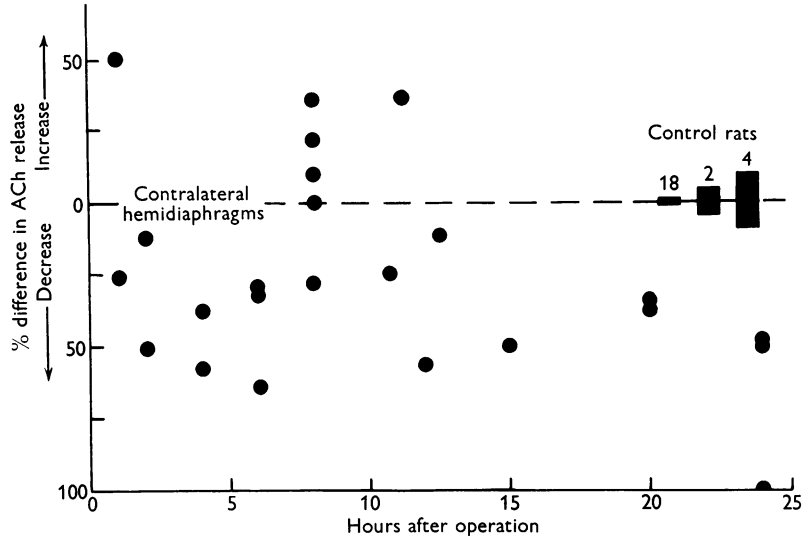

Fig. 4. The effect of short-term denervation on the spontaneous release of ACh from the hemidiaphragm. Ordinate: the percentage difference between the output from the denervated hemidiaphragm and that from the contralateral control; each point represents a single denervated hemidiaphragm. Abscissa: time in hours between denervation and collection. Solid blocks show the limits of difference in output between the two sides of the diaphragm in 24 control rats.

of ACh had usually begun to fall below the control level. From about the second hour after operation onwards the output remained at about 50 % of the control, but during the 8th–10th hours it usually equalled or exceeded the control level. This effect was consistently observed, but could not be associated with any obvious recurring factor.

Long-term denervation. In these experiments the ACh output from hemidiaphragms was studied from 3 days to 20 weeks after denervation (Fig. 5). The lowest outputs were recorded in rats killed between 6 and 9 weeks after denervation, and the release was apparently rising again in those killed after 10 weeks. After 11 weeks the ACh levels had returned in most rats to near or above the control level, and in nearly all these it was possible

to obtain a muscle twitch on stimulating the operated phrenic nerve, indicating a functional re-innervation of the diaphragm.

Site of ACh release. No ACh was detected in the bathing solutions of muscle tissues free from nerve endings, whether they had been denervated for 7 weeks or not. The strips containing nerve terminals always released ACh; the nerve-terminal strips which had been denervated 7 weeks previously released less than half as much ACh as innervated nerve-terminal

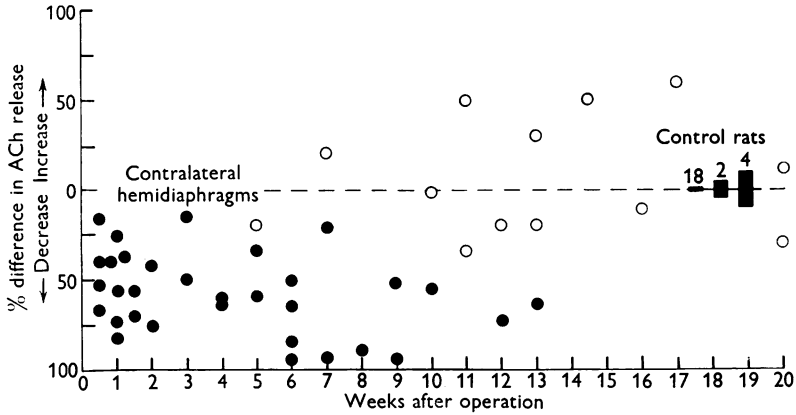

Fig. 5. The effect of long-term denervation on the spontaneous release of ACh from the hemidiaphragm. Ordinate: the percentage difference between the output from the denervated hemidiaphragm and that from the contralateral control. Abscissa: time in weeks for which the rats were allowed to survive after operation. Each point represents a single test hemidiaphragm. ○ indicates test hemidiaphragms from which a twitch was obtained on stimulation of the phrenic nerve; ● indicates a failure to twitch. Solid blocks as in Fig. 4.

strips. The actual amounts of ACh released lay within the range that would be predicted from the results of the experiments previously described, which suggests that all the ACh collected originated from nerve endings and not from the muscle of the diaphragm.

Effect of changes in potassium concentration

Liley (1956*c*) showed, on the isolated rat diaphragm at 37° C, that increasing the potassium concentration of the bathing fluid raises the frequency of m.e.p.p.s; to estt any association between these and the spontaneous ACh release from the diaphragm, a series of experiments was performed in which the bathing solution contained 5, 15 or 30 mM-K^+ with equimolar reduction in Na^+ concentrations. In the present experiments the maximum release of ACh in 15 and 30 mM-K^+ did not exceed 3 times the output in 5 mM-K^+ (Fig. 6).

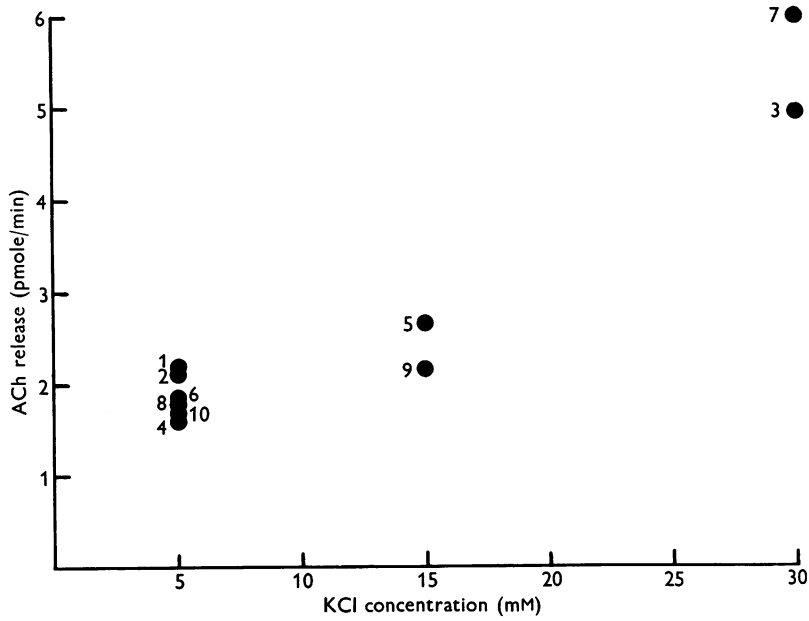

Fig. 6. The effect of the KCl concentration of the collecting fluid on the resting release of ACh from a single hemidiaphragm. The figure beside each point represents the order in which the collections were made.

Effect of temperature on the spontaneous release of ACh

The frequency of m.e.p.p.s is known to be affected by temperature changes (Liley, 1956*a*). At 16° C the frequency is about 70/sec, but at 25° C the frequency is less than 1/sec and at 37° C it increases again to about 1–2/ sec. In the present experiments, however, the amount of ACh released from the hemidiaphragm was simply related to the temperature over the complete range investigated (16–37° C) and showed no tendency to rise, as did the m.e.p.p. frequency, at temperatures below 25° C (Fig. 7). It was also found that a cooled diaphragm which had been releasing less ACh than its contralateral control for a 30 min collection period often produced more than the control during the subsequent 30 min collection period at 37° C.

DISCUSSION

We were satisfied by the results of cross-assays on the guinea-pig ileum and rat blood-pressure preparations, and from the use of assay controls, that the active principle collected from the diaphragm treated with an anticholinesterase was acetylcholine, since no other known naturally occurring choline ester fitted all the tests. The amount of ACh collected from

normal diaphragms varied considerably from rat to rat, but the amounts released from the two halves of a whole diaphragm did not differ by more than $\pm 10\%$ in the 24 control rats studied.

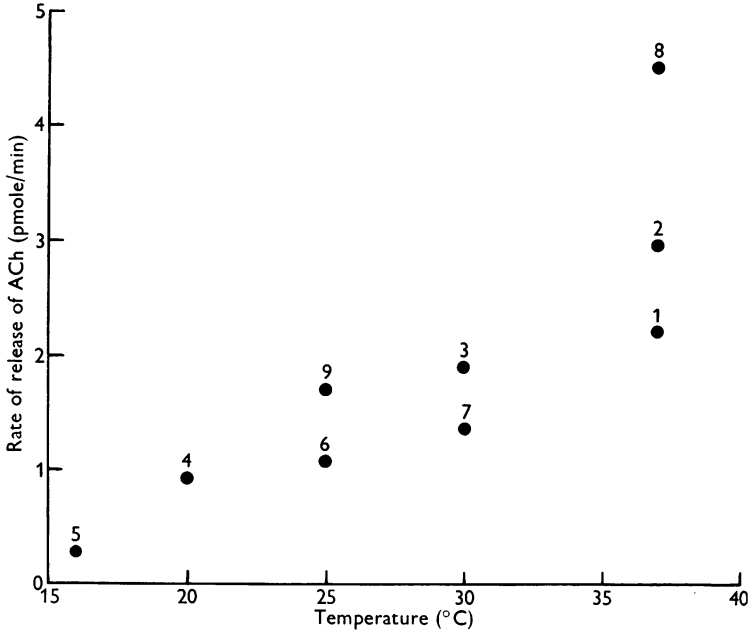

Fig. 7. The effect of the temperature of the collecting fluid on the resting release of ACh. Each point represents the rate of release from the hemidiaphragm during one 30 min collection period. The figure above each point shows the order in which the collections were made.

Because of individual variation the absolute values from different rats are probably of no great significance; what is important are the changes observed in any one rat. Nevertheless, it should be mentioned that the present results, though on an average slightly higher, are of the same order as those obtained by Straughan (1960). The release at temperatures below 25° C agrees well with that reported by Krnjević & Mitchell (1961) who worked at room temperature. The absolute values from different rats showed a diurnal variation. While we have no evidence about the cause of the fluctuation it is possible that it is associated with nocturnal activity and feeding. This should perhaps be borne in mind in experiments where absolute values of ACh levels are important.

ACh released from acutely denervated diaphragms

Krnjević & Mitchell (1961) tentatively suggested that the resting release from the diaphragm might represent that amount of ACh thought to come

from the nerve endings, which is responsible for the production of m.e.p.p.s. The present results indicate that while this ACh may contribute to the total amount collected, a large proportion is derived from some other source. Krnjević & Mitchell (1961) calculated that a quantum of ACh might be of the order of 0·006 pmole. If the discharge of each quantum results in a m.e.p.p. then the total ACh required to produce m.e.p.p.s at the rate of 2·5/sec (mean frequency found by Liley, 1956*a*, at 37° C) would be only 0·9 pmole/min (27 pmole in 30 min). This is only one third of our value of about 2·7 pmole/min (82 pmole/30 min). Moreover, when the potassium concentration in the collecting fluid is increased from 5 to 30 mM the m.e.p.p. frequency is raised to about 700/sec and is stable near this frequency for at least 20 min (Liley, 1956*c*). This means that the number of quanta released has increased 200–300 times, and were the quantal release the only source of the resting output, the resting output should increase by the same factor. In our experiments it was increased by a factor of only 3. Furthermore, when the temperature of the diaphragm is decreased below 25° C, the m.e.p.p. frequency increases and may reach 70/sec at 16° C (Liley, 1956*a*); the ACh release, however, shows no such increase but falls steadily with temperature. A further finding which suggests that the resting release is not solely concerned with m.e.p.p. production is that it continued in the chronically denervated diaphragms; Liley (1956*a*) could detect no m.e.p.p.s in such preparations.

The lack of correlation between the changes in m.e.p.p. frequency and spontaneous ACh release suggests that an important contribution to the release is made by a component which has nothing to do with m.e.p.p. production. Nevertheless, it is to be expected that the ACh associated with m.e.p.p.s will be included in the total collected and it is surprising that procedures which should alter this contribution have so small an effect on the total release. There seem to be two possible explanations. The first is that the 'basic' release of ACh (i.e. ACh release not associated with m.e.p.p.s) is also affected by changes in K^+ concentration and temperature but in the opposite direction, so that its fluctuation would mask the other changes. If, for example, ACh is merely diffusing from the 'basic' source it is to be expected that its release will be slowed by a lowered temperature.

The other explanation we suggest is that the amount of ACh involved in m.e.p.p. production was over-estimated by Krnjević & Mitchell (1961). The number of quanta of ACh involved in the production of a normal end-plate potential varies with the frequency of stimulation; Krnjević & Mitchell (1961) based their figure of 20 quanta on the size of the potentials produced by stimulation at 2–5/sec. Liley (1956*b*) suggested that the figure might be 80–100 in 'a normal e.p.p.' in rat diaphragm while Boyd & Martin (1956) quote a value of 200–300 in cat tenuissimus muscle. If the

quantal content in Krnjević & Mitchell's experiments was in fact more than 20, their derived value of 0·006 pmole for a quantum of ACh would be too large, as would be the amount of ACh they calculated to be necessary for the production of m.e.p.p.s at 3·0/sec. Thus, if the ACh involved in m.e.p.p. production represented only 1·0–3·0 %, as compared with 25–30 %, of the total release, this could explain, to some extent, the present findings that the total ACh release is little affected by conditions which alter m.e.p.p. frequency.

ACh released from the chronically denervated diaphragm

In experiments such as these the time course of changes cannot be followed in an individual rat, but the pattern of results for the whole series suggests that the release falls fairly sharply immediately after operation, and that there is only a gradual decline thereafter. This raises the question of where the ACh is coming from in the diaphragm. The present experiments suggest that in both the acutely and chronically denervated diaphragms it originates from nerve terminals and not from muscle fibres. There is evidence to suggest that the choline acetylase (ChAc) present at the nerve terminals has been manufactured in the nerve cell body (Hebb & Waites, 1956; Berry & Rossiter, 1958; Hebb & Silver, 1961) and that it is transported down the axon to the terminals, the site of ACh synthesis. If this theory is correct it is surprising that the release of ACh continues for some weeks after denervation. A possible explanation is that the ACh may be derived from a pre-formed store or it may be synthesized by ChAc persisting in the nerve terminals.

Hebb & Krnjević (personal communication) have found, in some preliminary experiments on control rats, that the total bound ACh in rat phrenic nerve endings is only 150 pmole/hemidiaphragm. Such a store could not support a continuous release of ACh of the order observed in these experiments (approximately 82 pmole/30 min) for even 1 hr.

We are then left with the alternative that ChAc is synthesizing the ester in the degenerating terminals. It is not easy to get direct evidence of this because, at present, ChAc activity has not been measured in the denervated diaphragm. On the other hand, certain indirect evidence discussed by Hebb (1962) indicates that there may be more than enough ChAc in degenerating terminals to synthesize the ACh found. Hebb (1962), using a specially sensitive assay system, found that 0·5 % of the normal ChAc activity was retained in the distal part of the divided popliteal nerve in rabbits 6 weeks after operation. She pointed out that the end-plate region of a rat hemidiaphragm is capable of ACh synthesis *in vitro* at the rate of 35–40 μg/hr (0·22 μmole/hr) (Hebb & Krnjević, personal communication), hence a chronically denervated diaphragm retaining only 0·5 % of normal

activity should, in theory, be capable of a synthesis *in vitro* of 175–200 ng/hr (1000 pmole), which would easily account for the amount found in the present experiments to be released from denervated diaphragms.

If the idea of continued synthesis is accepted the next question concerns the site of this activity. Birks, Katz & Miledi (1959, 1960) examined denervated motor end-plates in frog sartorius muscle with the electron microscope; they observed that 3–4 days after operation, Schwann cells (or what appeared to be Schwann cells) engulfed the products of end-plate degeneration. They suggested that these Schwann cells might acquire the power to produce ACh after absorbing the enzymic apparatus of the nerve ending.

Re-innervation. The majority of diaphragm removed later than 10 weeks after operation twitched when the regenerating phrenic nerve was cut during dissection. The release from these re-innervated diaphragms was in many cases greater than normal. Miledi (1960) found evidence that in the newly re-innervated sartorius muscle of frog the ACh responsible for producing m.e.p.p.s is liberated from both Schwann cells and from the nerve axon terminals. It may well be that both sources also contribute to the 'basic' release.

A completely different idea is that the intact nerve exerts some triggering effect on the basic release; the loss of this effect might account for the fall in output almost immediately after nerve section, while over-activity in recently regenerated fibres might result in excess release of ACh.

SUMMARY

1. The spontaneous release of ACh from the isolated diaphragm of rats has been studied after the inhibition of cholinesterase. The amount released usually lay between 2·0 and 3·0 pmole/min/hemidiaphragm at 37° C and was similar from the two halves of a single diaphragm.

2. ACh could be recovered for up to 14½ hr after isolating the diaphragm, during which time a total of 3500 pmole was collected from a single hemidiaphragm.

3. The ACh release from chronically denervated hemidiaphragms usually fell within 2 hr of operation to 50 % of the control level and returned to near or above the control level from about the 11th week onwards.

4. The spontaneous output of ACh was studied at various temperatures and in diaphragms soaked in Ringer's solutions containing high K^+ concentrations. These experiments suggest that only a very small proportion of the ACh spontaneously liberated can be associated with m.e.p.p.s.

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