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THE RELEASE OF ACETYLCHOLINE FROM THE SPINAL CORD OF THE CAT BY ANTIDROMIC STIMULATION OF MOTOR NERVES

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SUMMARY

1. ACh was measured in the effluent from the perfused lumbosacral cord of the cat with or without stimulation of the central ends of the cut left sciatic and femoral nerves after section of the left dorsal roots.

2. In about 30% of the preparations ACh was obtained in the samples collected at rest (average 3·3 ng/min); the amount of ACh release was increased 1·3–9 times by stimulation of the peripheral nerves. The average amount of ACh collected during stimulation of the peripheral nerves at 5/sec was 6·9 ng/min. Antidromic motor nerve impulses responsible for the ACh release were likely to be only those in alpha motor fibres.

3. There was a depression in ACh release/stimulus as the stimulus frequency was increased more than 10/sec. Such changes in ACh release with various stimulus frequencies were correlated with depression in the response of Renshaw cells to excitation through motor-axon collaterals.

4. Amounts of ACh release during stimulation of the peripheral nerves at 5/sec were significantly increased for 1 or 2 min after a short tetanic stimulation of the nerves.

5. Intravenous injection of dihydro-β-erythroidine did not reduce the amount of ACh release produced by stimulation of the peripheral nerves.

6. It is concluded that antidromic impulses in alpha motor fibres liberate ACh from the presynaptic terminals at the central synapses on Renshaw cells.

INTRODUCTION

Antidromic impulses in motor nerves produce inhibition of motoneurones in the spinal cord of the cat (Renshaw, 1941). The pathway for this inhibition includes the motor-axon collaterals and a special group of interneurones, 'Renshaw cells' (Eccles, Fatt & Koketsu, 1954; cf. Renshaw,

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1946). It was suggested that transmission at the central synapses between these motor-axon collaterals and Renshaw cells is mediated by acetylcholine (Eccles *et al.* 1954; cf. also Dale, 1935) since acetylcholine (ACh) has been shown to be the transmitter substance at the neuromuscular junctions formed by the same motor-axons (see, however, Hayes & Riker, 1963). In fact, pharmacological investigations showed that Renshaw cells are readily excited by choline esters and that the response of Renshaw cells to synaptic excitation through motor-axon collaterals is prolonged by anti-cholinesterases and depressed by 'cholinergic-blocking agents' (Eccles *et al.* 1954; Eccles, Eccles & Fatt, 1956; Curtis, Eccles & Eccles, 1957; Curtis & Eccles, 1958*a*, *b*; Longo, Martin & Unna, 1960; Curtis, Phillis & Watkins, 1961). Further, the synaptic response of Renshaw cells was found to be diminished by hemicholinium which presumably prevents the synthesis of ACh (Quastel & Curtis, 1965).

Although there are several rigorous criteria for identification of a substance as a synaptic transmitter (Rosenblueth, 1950; Crossland, 1957; Paton, 1958; Florey, 1960; Toman, 1963), pharmacological confirmation of a 'receptor' to the substance on the post-synaptic membrane and evidence for its liberation from presynaptic terminals can be considered as two minimal requirements (Terzuolo & Edwards, 1962; Grundfest, 1964). While the former criterion for cholinergic synapses on Renshaw cells has for the most part been fulfilled (see above), no systematic approach has been made toward evaluating the latter criterion.

Bülbring & Burn (1941) have demonstrated that the rate of ACh release from the mammalian spinal cord is increased by stimulation of the central end of the cut sciatic nerve. It was thus envisaged that cholinergic synapses are involved in reflex transmission via *afferent fibres*. This surmise, however, was but weakly supported by recent pharmacological studies (Curtis *et al.* 1961; Curtis, 1963; cf. however, Fernandez de Molina, Gray & Palmer, 1958). It is possible that the liberation of ACh from the cord observed by Bülbring & Burn (1941) was mostly due to activation of recurrent collaterals by antidromic impulses in the motor nerve, since their preparations had both the dorsal and ventral roots intact. The present study was undertaken to test this possibility. For this purpose, the central ends of the cut peripheral nerves were stimulated after section of the appropriate dorsal roots, and ACh was measured in the effluent collected from the perfused spinal cord. Further, the output of ACh obtained under various conditions of stimulation was correlated with known electrophysiological properties of neuronal elements in the spinal cord. The results provide strong support for the postulated cholinergic nature of synaptic transmission from motor axon-collaterals to Renshaw cells. A preliminary report has been published (Kuno & Rudomin, 1965).

METHODS

Preparation and procedure. All experiments were performed on adult cats anaesthetized with sodium pentobarbital (Nembutal, Abbott laboratories, 40 mg/kg) given intraperitoneally. The lumbosacral cord was exposed by laminectomy, and the left dorsal roots L_5 to S_2 were cut. The cord was thereafter covered with modified Locke solution (see below) containing eserine (salicylate, Merck, or sulfate, National Biochemical Corporation), 0·1–0·5 mg/ml., and closed with the skin. The left femoral and sciatic nerves were dissected and cut distally. Their central ends were prepared for stimulation with buried electrodes. Stimulus strength used in the majority of experiments was supramaximal for all types of motor fibres. However, in some preparations only alpha motor fibres were selectively stimulated (see Results).

The cat was then eviscerated. The perfusion of the lumbosacral cord was made through a polyethylene cannula placed into the right external iliac artery. All the small blood vessels below the renal branches were ligated except the external iliac arteries, the common iliac veins and those maintaining circulation of the lumbosacral cord (Fig. 1 *A*; cf. also, Bülbring & Burn, 1941; Holmstedt & Skoglund, 1953; Curtis *et al.* 1957). Another polyethylene cannula was placed into the right common iliac vein to collect the venous effluent from the cord. Upon perfusion, the abdominal aorta and the inferior cava vein were temporarily occluded with clips at the level between the renal and internal spermatic arteries (Fig. 1 *Ab*) as well as the left external iliac artery (*c*) and the left common iliac vein (*d*). The perfusion could then be stopped at will, and the natural circulation restored, by removing these clips and closing the inlet and outlet cocks. With this arrangement, however, the effluent was often contaminated with a considerable amount of blood. This could be avoided by adding another occlusion clip to the aorta just below the diaphragm (Fig. 1 *Aa*). At the end of the experiments, methylene blue was injected through the inlet cannula. It was thus found that the segments of the spinal cord perfused were usually from L_4 down to the whole sacral cord.

The perfusion fluid was Locke solution modified by the omission of bicarbonate. The composition was (mM): NaCl 154, KCl 5·6, $CaCl_2$ 2·2, dextrose 10·4. In some experiments, the concentration of calcium ions was increased to approximately 3–4 times (7–9 mM) of the normal solution. In the sympathetic ganglion ACh release is potentiated by increasing external calcium concentration (Harvey & MacIntosh, 1940; Hutter & Kostial, 1954, 1955; Lipicky, Hertz & Shanes, 1963). In the present study, however, there was no significant effect of increased calcium levels on the amount of ACh release.

After addition of eserine, 10–20 mg/l., the perfusion fluid was saturated with pure oxygen under high pressure. The pressure produced by oxygen supply was adjusted by a valve (Fig. 1 *Bv*) so as to maintain the perfusion pressure constant at 120–180 mm Hg during the experiments. The rate of perfusion flow could be also adjusted by a cock attached to the venous outlet (Fig. 1 *A*). The flow rate so attained was usually 4–8 ml./min. The temperature of the perfusion fluid was kept at 36–40° C. The whole animal was also eserinized by intravenous injection of eserine, 0·1 mg/kg, 10–20 min before the perfusion.

Collection and bioassay of samples. Preliminary experiments showed that perfusion of the spinal cord produced an initial augmentation in monosynaptic reflex responses followed by a gradual depression. The perfusion time was thus subjected to a limitation since the reflex activity persisted only for 6–10 min after onset of the perfusion (Fig. 3, interrupted line; cf. also, Bülbring & Burn, 1941). The period of each perfusion, therefore, never exceeded 7 min, after which the natural circulation was restored. In spite of such a short period of perfusion, the preparation often deteriorated as evidenced by a fall in the blood pressure and oedema in the liver and the spinal cord. Occasionally the animal died 5–20 min after the cessation of perfusion. Only in relatively few preparations was ACh detected in the second perfusion performed 30 min after the first.

After the start of perfusion the venous effluent for the first 10–40 sec was discarded in order to wash the blood out of the system. The venous samples during 1 min periods were then successively collected, with or without stimulation of the central ends of the cut peripheral nerves. Each period of collection was separated by intervals of 5 sec. These samples were collected in separate chilled tubes and stored at 0° C until required for assay. When there was some mixture of blood in the effluent fluid, the blood was eliminated by centrifugation immediately after collection. The perfusates thus collected were assayed within 3 hr against ACh chloride (Roche Products) on the blood pressure of eviscerated cats as described by MacIntosh & Perry (1950). The detectable levels of activity varied in

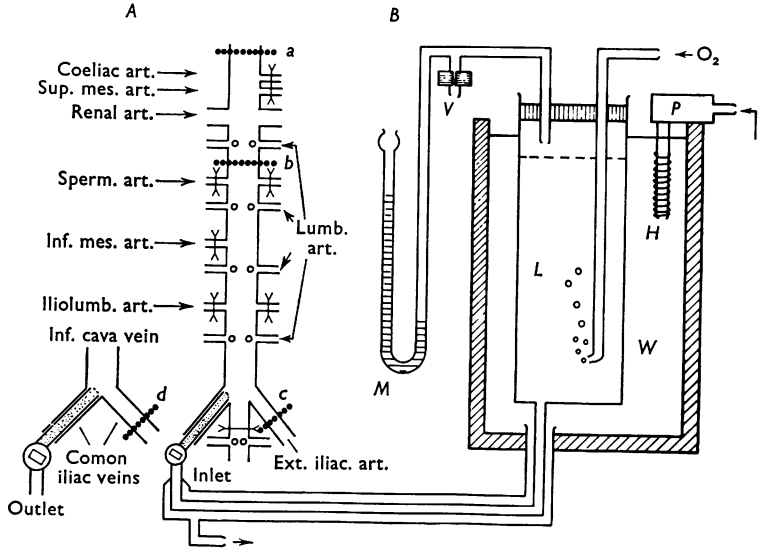

Fig. 1. *A*. Diagram of the abdominal aorta (right) and the inferior cava vein (left) viewed from the ventral side showing the branches tied and the positions of temporal occlusion (dotted lines). The cannulas for perfusion (inlet) and collection (outlet) are shown by stippled parts with their cocks. *B*. Eserinized Locke solution (*L*) is aerated by pure oxygen (O_2) under high pressure which is adjusted by a valve (*V*) connected to a mercury manometer (*M*). Temperature of a large water-bath (*W*) is controlled by a heater (*H*) attached with circulator pump (*P*) which induces flow of hot water outside the perfusion tube (arrows) preventing heat loss on the way to the preparation (*A*).

different preparations, the range being 0·5–2·0 ng of ACh. Less sensitive bioassay preparations were discarded. When the assay preparation deteriorated during testing, known amounts of ACh were added to the venous samples so that subthreshold activity of the samples was detected even with the relatively less sensitive preparation. The results observed by this procedure agreed, to 20 %, with those obtained by injection of the sample alone before deterioration of the preparation.

The identification of ACh, or a related choline ester, of the assayed bioactive substance was confirmed by alkaline hydrolysis and/or atropine tests in seven out of thirty-nine experiments. The action of the samples on the blood pressure was invariably abolished after the assay preparation had been atropinized, and alkali-treated samples were consistently inactive. All results in the text are expressed in terms of ACh chloride.

Electrophysiological studies. In a few experiments changes in the reflex activity during the perfusion were also studied (see above; cf. also Fig. 3). Monosynaptic reflex responses were recorded from ventral roots L_7 and S_1 following stimulation of the corresponding dorsal roots. Also, in several experiments the electrical activity of individual Renshaw cells was recorded extracellularly. Electrodes used for this purpose were glass micropipettes filled with 2·7 M-KCl solution with resistances between 5 and 15 MΩ. Renshaw cells were identified by their repetitive firing at high frequency in response to antidromic stimulation of the ventral root.

Additional technical details are given in the appropriate sections in the Results.

RESULTS

ACh release with and without nerve stimulation

The results were selected only from those preparations in which appreciable amounts of ACh were collected. In twenty-one out of sixty experiments no ACh release was detected. Some of these failures might have been due to destruction of ACh by the considerable amount of blood occasionally mixed in the venous effluent (cf. Emmelin & MacIntosh, 1956). However, in other cases there was no obvious explanation, as in some failures of collections from the rat diaphragm (Krnjević & Mitchell, 1961).

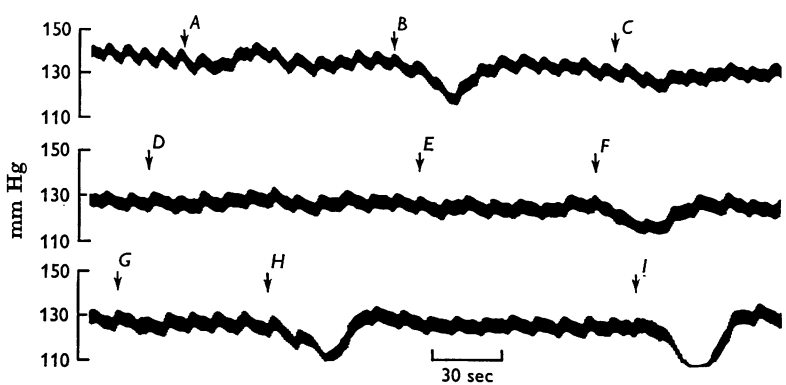

Fig. 2. Bioassay on the blood pressure of an eviscerated cat anaesthetized with chloralose, 80 mg/kg. *A*, *C*, *D* and *G* are control injections of 2 ml. of the eserinized solution used for the perfusion. At *E*, 2 ml. of the sample collected at rest was injected. *B* and *F* are the responses due to injections of 2 ml. of the sample collected during stimulation of the peripheral nerves at 5/sec. Responses due to injection of ACh are shown by *H* (2 ng) and *I* (4 ng).

Figure 2 illustrates representative assays of the venous samples collected from one animal. Injections of 2 ml. of perfusion solution produced no appreciable changes in the blood pressure (*A*, *C*, *D*, *G*). Similarly, 2 ml. of the sample collected at rest (without nerve stimulation) gave no detectable response (*E*). The responses at *B* and *F* were produced by injections of 2 ml. of the sample collected during stimulation of the peri-

pheral nerves at a frequency of 5/sec. These responses were similar in amplitude to that produced by injection of 2·0 ng of ACh (*H*) but were apparently smaller than that by 4·0 ng of ACh (I). Amounts of ACh in the samples were thus calculated from the ACh concentration/1 ml. and the total volume of samples collected during 1 min.

As shown in Fig. 2, in the majority of experiments ACh was collected only when the peripheral nerves were stimulated. However, in seven out of twenty preparations tested appreciable amounts of ACh were detected in the samples collected at rest. The average amount of ACh obtained at rest from such preparations was 3·3 ng/min with a range of 1·2–6·0 ng/min. However, in these preparations the amount of ACh release was further increased 1·3–9 times by stimulation of the peripheral nerves. Since invasion of sensory impulses into the spinal cord was prevented by section of the dorsal roots (see Methods), it is concluded that antidromic impulses in the motor nerve increase the amount of ACh liberated into the venous effluent from the spinal cord.

A considerable variation in amounts of recoverable ACh, including a substantial number of failures of collections, was the greatest obstacle to quantitative measurement of the ACh output. Even with the same preparation, amounts of ACh release produced by peripheral nerve stimulation varied, depending upon the time elapsed after the onset of perfusion. Figure 3 shows the average amounts of ACh obtained from six experiments in which 1 min samples were collected successively during continuous stimulation of the peripheral nerves at 5/sec (without measurement of the resting output). A tendency may be noticed for ACh release to fluctuate widely after 3 min of perfusion. Although this variation was not statistically significant, it seemed safe to take only the first 3 min samples for quantitative measurement, judging from some depression in the spinal reflex activity at the later stage of perfusion (Fig. 3, interrupted line). The average amount of ACh in the samples obtained from the first three collections was 6·9 ng/min with a range of 1·7–13·3 ng/min at a stimulus frequency of 5/sec.

In four experiments ACh release produced by antidromic motor nerve stimulation was compared with that due to sensory nerve stimulation. In these experiments the left dorsal roots L_5 to S_2 and the right ventral roots L_5 to S_2 were cut so that impulses elicited by stimulation of the peripheral nerves could reach the cord only through the motor nerve (antidromic) on the left side and through the sensory nerve (orthodromic) on the right side. In order to maintain the circulation of both hind-legs, the perfusion was made through the renal artery or the medial sacral artery, and the samples were collected from the renal vein. A 1 min sample was collected first during stimulation of the left peripheral nerves, and another 1 min sample was obtained during stimulation of the right peripheral nerves, or vice versa, both at a frequency of 10/sec. Amounts of ACh collected were always greater with motor nerve stimulation than with sensory nerve stimulation, the latter being approxi-

mately 77 % of the former on the average. However, this difference was not statistically significant while the increase of ACh release from the resting level was significant in both cases.

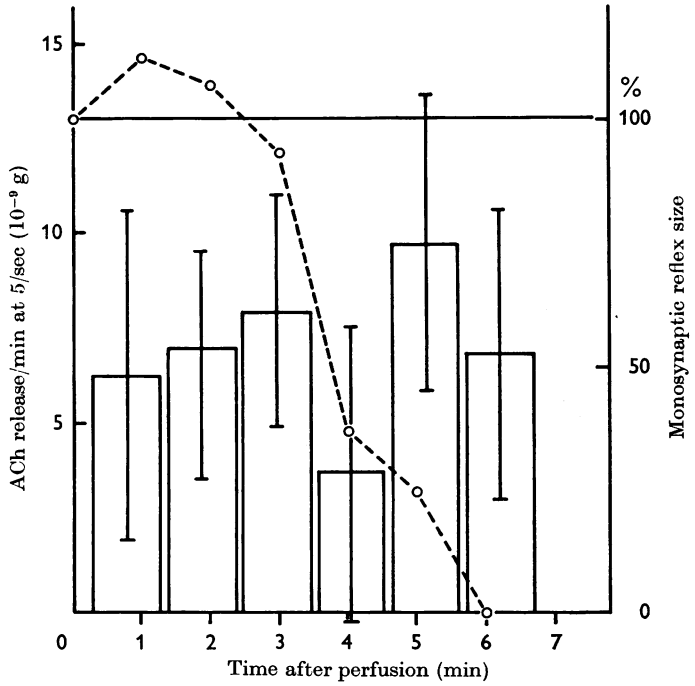

Fig. 3. Average amounts of ACh (ordinate)/min collected at different times after the onset of perfusion (abscissa) during continuous stimulation of the peripheral nerves at 5/sec from six different experiments with standard deviation (vertical lines). Open circles with interrupted lines show changes in monosynaptic reflex responses (right ordinate) during the perfusion obtained from one of preliminary experiments.

ACh release at various stimulus frequencies

The sensitivity of assay preparations used in the present study was not enough to demonstrate changes in the rate of ACh release by stimulation of the peripheral nerves at frequencies lower than 5/sec. When the stimulus frequency was increased from 5/sec to 10/sec, there was a parallel increase in the amount of ACh release/min. However, the relation between rate of ACh release and stimulus frequencies was no longer linear at frequencies higher than 10/sec. Consequently, there was a gradual depression in ACh release/stimulus as the stimulus frequency was increased. Figure 4*B* (open circles) shows percentage changes of amounts of ACh liberated by one stimulus at different stimulus frequencies relative to those at 5/sec. All the measurements were made from the samples collected at the 2nd and 3rd min after the onset of perfusion (see Fig. 3), and the average was

obtained from five experiments except that at 5/sec which was from twelve experiments. The difference in amounts of ACh release/stimulus was insignificant between 5/sec and 10/sec as well as between 50/sec and 100/sec. However, amounts of ACh release/stimulus at 20/sec were significantly ($P < 0{\cdot}10$ by t-test) smaller than those at 5/sec and greater than those at 50/sec. This suggests that the activity of the neuronal elements

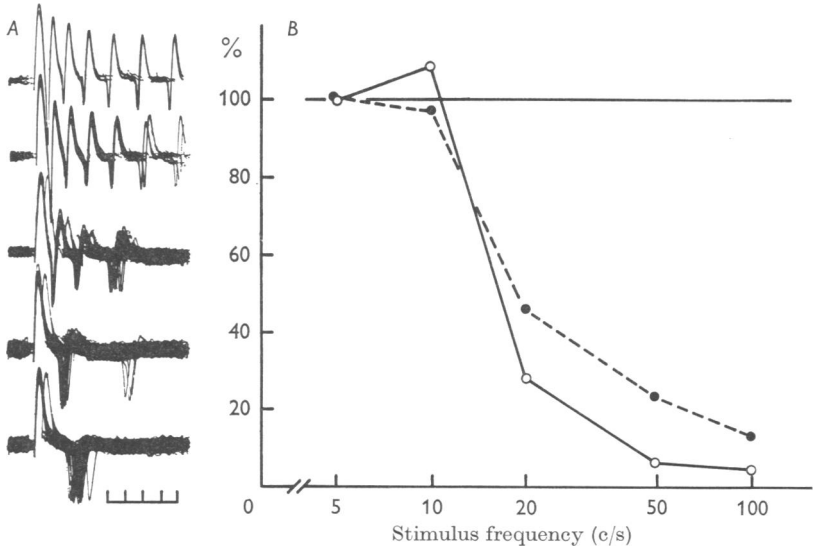

Fig. 4. *A*. Extracellular recording from a Renshaw cell in response to stimulation of the ventral root L_7 at various stimulus frequencies, 5/sec, 10/sec, 20/sec, 50/sec and 100/sec from the top downward. Time, 1 msec. *B*. Open circles, amounts of ACh release/stimulus at various stimulus frequencies expressed as percentage relative to those at 5/sec. Each point is the average from five experiments except at 5/sec which is from twelve experiments. The actual value of each point is $24{\cdot}1 \pm 10{\cdot}1$ (S.D.) pg at 5/sec, $26{\cdot}1 \pm 17{\cdot}4$ pg at 10/sec, $6{\cdot}7 \pm 3{\cdot}1$ pg at 20/sec, $1{\cdot}4 \pm 1{\cdot}0$ pg at 50/sec and $1{\cdot}1 \pm 0{\cdot}1$pg at 100/sec. Filled circles, the activity of Renshaw cells at various stimulus frequencies expressed as percentage relative to that at 5/sec. Each point is the average from seven Renshaw cells. Note, abscissa on logarithmic scale.

which may liberate ACh by antidromic motor nerve impulses is sensitive to relatively low frequency of stimulation. It is unlikely that the fibres in the ventral root itself are the major source of ACh obtained in the present study since these can follow high frequency stimulation up to 600/sec.

Figure 4*A* illustrates changes in the electrical activity of a Renshaw cell by increasing the frequency of stimulation applied to the ventral root. There was little difference in the response at stimulus frequencies between 5/sec and 10/sec (upper two records). A further increase in the stimulus

frequency produced a strong depression of the response of Renshaw cells (lower three records). Quantitative measurement of the responses at various stimulus frequencies could be made in terms of the number of repetitive discharge during the first 20 msec after stimulation. Filled circles in Fig. 4*B* show the percentage changes in the average activity of seven Renshaw cells at different stimulus frequencies relative to that at 5/sec. There was a good correlation between the depression of the response of Renshaw cells and the decrease in amounts of ACh release by increasing the stimulus frequency (Fig. 4*B*).

Post-tetanic potentiation of ACh release

It is well known that the synaptic response of a neurone may be enhanced for some minutes after tetanic synaptic activation (see review by Hughes, 1958). Such a post-tetanic potentiation was also observed in the response of Renshaw cells to synaptic excitation via motor-axon collaterals (Eccles *et al.* 1954). In observations on post-tetanic potentiation it is common experience that the lower the rate of test stimulation before and after tetanus, the greater is the potentiation. The test stimulus frequency in the present study had a limitation since when the peripheral nerves were stimulated at a frequency lower than 5/sec no ACh was detected in the venous samples (see above). In two experiments, however, the presence of a post-tetanic potentiation of ACh release was suggested by a recovery of appreciable amounts of ACh at a stimulus frequency of 1/sec when the nerves had previously been stimulated tetanically. This was further supported by two experiments shown in Fig. 5 in which the peripheral nerves were stimulated at 5/sec, and 1 min samples were successively collected before and after tetanization of 10 sec duration at 500/sec (hatched bar). Amounts of ACh release/min were increased significantly for 1 min ($P < 0{\cdot}02$ by t-test) and in the 2nd min ($P < 0{\cdot}10$ by t-test) after the tetanization (Fig. 5). In four other experiments, however, there was no appreciable difference in amounts of ACh release before and after tetanization. Such failures could, at least in part, be due to the high frequency used for the test stimulation (cf. Krnjević & Mitchell, 1961). In fact, with the electrical responses the post-tetanic potentiation was also observed in only two out of seven Renshaw cells examined at a test stimulus frequency of 5/sec while the potentiation was invariably present when tested at 0·5/sec before and after tetanization. However, the presence of a post-tetanic potentiation of ACh release, even though the number of observations was limited, excludes the possibility that the fibres in the ventral root itself and/or the motoneurone somata make significant contribution to the ACh recovered in the present study (see Discussion).

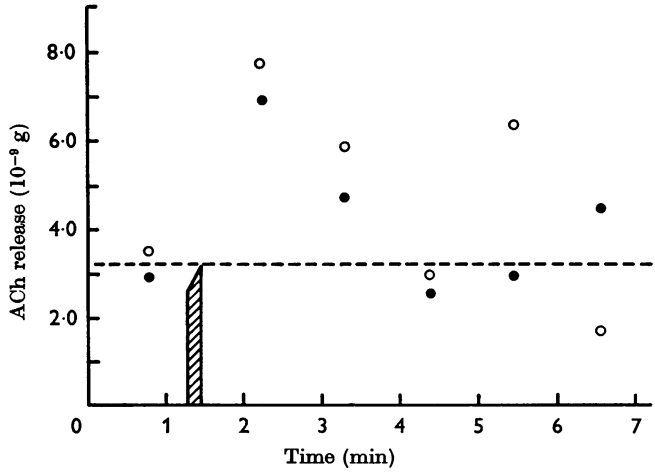

Fig. 5. Post-tetanic potentiation of ACh release in two experiments (open and filled circles). Each point represents amounts of ACh recovered from 1 min samples successively collected during stimulation of the peripheral nerves at 5/sec. The nerves were stimulated at 500/sec for 10 sec at the hatched bar. Abscissa, time after the start of the perfusion.

Exclusion of other possible sources of ACh

In the experiments described above, there was an uncertainty in assessing the origin of the resting output of ACh since the pathway of perfusion of the spinal cord also included the lumbar sympathetic chain and the paravertebral muscles. In addition, stimulus intensities used in these experiments were so strong that it was not possible to distinguish which group of motor nerve fibres was reponsible for the ACh release from the spinal cord (see Methods). Further, Renshaw cells themselves could be the source of the released ACh since there was a good correlation between the alteration in the activity of Renshaw cells and the change in amounts of ACh release during and after repetitive stimulation.

Observations to evaluate these possibilities were made in seven preparations in which bilateral sympathectomy was performed by removal of all the ganglia with the trunks below the renal arterial branches. Also, the responses produced by stimulation of the sciatic and femoral nerves were monitored from the peroneal nerve and the quadriceps nerve respectively. In order to activate only alpha motor nerve fibres, stimulus intensities twice the threshold for the most excitable nerve fibres were selected for the experiments. Further, before the perfusion a 'cholinergic-blocking agent', dihydro-β-erythroidine (DHE, Merck) 1·0 mg/kg, was intravenously injected. Previous observations that recurrent inhibition

through Renshaw cells on lumbar monosynaptic reflexes is significantly reduced by injection of DHE (Eccles *et al.* 1954; Brooks & Wilson, 1958; Kuno, 1959) were confirmed in one of the present preparations.

Table 1 (Expt. *A*) shows the results from the seven experiments. One minute samples were always collected first at rest and then during stimulation at rates of 10/sec and 50/sec (Expts. 1, 2, 5, 6) or vice versa (Expts. 3, 4, 7). Therefore, the significant difference ($P < 0{\cdot}01$ by χ^2-test) in rates of ACh release between 10/sec and 50/sec is not attributable to different times after the onset of perfusion. From Table 1 it is evident that amounts

TABLE 1. Rates of ACh release in 10^{-9} g/min at rest and during antidromic stimulation of motor nerves

Experiment *A*: preparations with bilateral lumbar sympathectomy and administration of DHE, 1·0 mg/kg. The peripheral nerves were stimulated at intensities twice the threshold for the most excitable nerve fibres. Experiment *B*: preparations with the sympathetic chain intact without administration of DHE. The peripheral nerves were stimulated at intensities supramaximal for all types of motor nerve fibres. Note, the dorsal roots, L_5 to S_2, were cut in all the preparations. Numbers of preparations investigated in parentheses.

Expt. A	Resting	10/sec	50/sec
1	1·5	14·0	8·2
2	0	12·1	8·4
3	4·2	7·8	6·4
4	6·0	39·2	7·8
5	2·8	4·1	—
6	0	2·4	0
7	0	11·9	8·0
Average	2·1 (7)	13·1 (7)	6·5 (6)
Range	0–6·0	2·4–39·2	0–8·4
Expt. B			
Average	1·5 (20)	15·6 (5)	4·4 (5)
Range	0–5·4	1·9–26·7	1·7–7·1

of ACh release both at rest and during stimulation under the conditions described above (*A*) are still comparable with those obtained in the previous experiments (*B*) in their averages and ranges. It is thus unlikely that the paravertebral sympathetic ganglia are the origin of the resting output of ACh and that Renshaw cells are the major source of ACh released by stimulation of the peripheral nerves. Further, stimulus strengths used in the present series of experiments suggest that antidromic motor nerve impulses responsible for the ACh release are only those in alpha motor fibres since the threshold intensity for gamma motor fibres is 5–12 times the threshold for the most excitable fibres of the peripheral nerve (Eccles, Eccles, Iggo & Lundberg, 1960; cf. Leksell, 1945).

Validity of estimation of amounts of ACh release

In several experiments solution containing known amounts of ACh was perfused, and the venous effluent was collected. Figure 6 shows the relation between the concentration of ACh perfused and that of the samples collected from the venous outlet. Obviously, there was no appreciable loss of ACh in the pathway of the perfusion. The tendency, if any, was rather a slight increase in the ACh output which could presumably be due to some resting ACh release. However, in one experiment which was

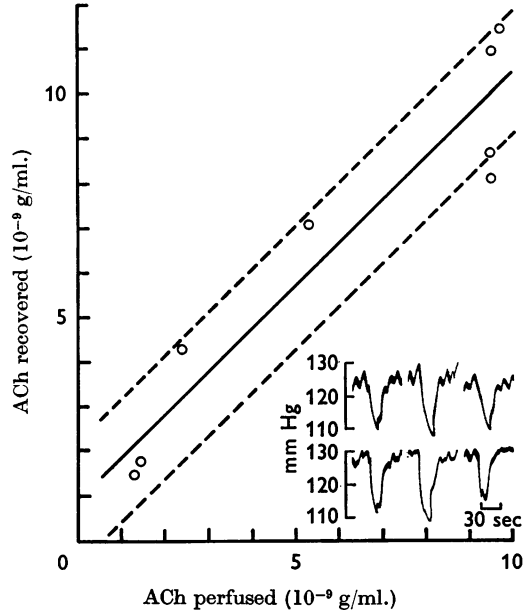

Fig. 6. Relation between ACh concentration in the perfusion fluid and that in the venous sample collected at rest. Interrupted lines show 2 S.E. of the correlation. Inset, assays of 1 ml. samples collected from one animal. Upper left, control response due to 1 ml. of the perfusion fluid containing about 9·5 ng/ml. Upper and lower middle, responses due to 1 ml. of the venous sample collected during peripheral nerve stimulation at 10/sec. Upper right, lower left and right, responses due to 1 ml. of the venous sample collected at rest.

not included in Fig. 6, no appreciable ACh was detected in the effluent from the cord perfused with solution containing approximately 6 ng/ml. of ACh. Since in this particular experiment there was a large amount of blood mixed with the effluent fluid, it was likely that the ACh perfused was destroyed by the blood (Emmelin & MacIntosh, 1956). Thus, as far as the venous effluent was free from the blood, loss of ACh in the pathway of the perfusion was negligible.

The inset of Fig. 6 shows assays of one experiment in which the spinal cord was perfused with solution containing about 9·5 ng/ml. of ACh, and 1 min samples were collected with and without stimulation of the peripheral nerves. The activity of the samples collected at rest (upper right, lower left and right records) was approximately the same as that of the solution used for perfusion (upper left record). Stimulation of the peripheral nerves at 10/sec apparently increased the rate of ACh release (upper and lower middle records). The rate of ACh release during the stimulation was 21·0 ng/min which was approximately the same as that obtained in the perfusion with solution containing no ACh at the same stimulus rate (15·6 with a range of 1·9–26·7 ng/min, see Table 1). Thus, the presence of relatively high concentration of ACh in the perfusion fluid had no significant influence upon the amounts of ACh collected. If the passage of ACh from the release site into the perfusate is simply determined by diffusion (Krnjević & Mitchell, 1960, 1961; Mitchell, 1963), the above experiment suggests that the concentration of ACh at the site of release is extremely high. Evidently, the amount of ACh collected in the present study was only a small fraction of the amount of ACh actually released within the cord.

DISCUSSION

Identification of ACh. The identification of ACh as the active substance collected in the present study was based entirely on the effects upon biological responses. Hence, it was not possible to identify ACh with absolute certainty. However, the assayed bioactive substance was similar in nature to ACh in that the activity of the substance disappeared when it was treated with alkali and/or when the assay preparation was atropinized. Since no other constituent of animal tissues is known to fulfil these criteria (MacIntosh & Perry, 1950), it is concluded that the bioactive substance collected in the present study is very likely to be ACh, or at least a similar choline ester.

Site of ACh release. In some experiments appreciable amounts of ACh were detected from the samples collected in the absence of added nerve stimulation. Such a resting output of ACh could be due to background activity of neuronal elements in the spinal cord, the mechanism being similar to the spontaneous release of ACh from the cerebral cortex (MacIntosh & Oborin, 1953; Mitchell, 1963). The present study, however, failed to exclude a possible source of the spontaneous release of ACh from the paravertebral muscles. Further, the resting release of ACh could be due to continuing sensory and/or motor impulse activity from some injured nervous tissues. Therefore, no significance can be assessed on the resting output of ACh at present.

From the present results it seems clear that antidromic impulses in the motor nerve increase the rate of ACh release from the spinal cord. Amounts of the 'evoked' release of ACh were enhanced after a short tetanic stimulation of the motor nerve. This enhancement was similar in temporal course to post-tetanic potentiation of synaptic responses of central neurones. Since post-tetanic potentiation is a characteristic for synaptic transmission and is absent in antidromic responses of the ventral root fibres and motoneurone somata, it is unlikely that these neuronal elements are the major source of the ACh collected in the present study. It is generally agreed that the mechanism of post-tetanic potentiation is restricted to changes in properties of the presynaptic terminals (Lloyd, 1949; Wall & Johnson, 1958; Eccles & Krnjević 1959; Curtis & Eccles, 1960; Kuno, 1964). It is thus suggested that site of release of the ACh collected in the present study is the terminals of motor-axon collaterals which make synaptic contacts on Renshaw cells. In support of this suggestion, it was found that the response of Renshaw cells to synaptic excitation through motor-axon collaterals is depressed at high stimulus frequencies in the same rate as the accompanied reduction in amounts of ACh release (Fig. 4). Further, antidromic motor impulses responsible for the ACh release were likely to be only those in alpha motor nerve fibres. This agrees with electrophysiological observation that antidromic impulses responsible for activation of Renshaw cells are only those in alpha motor nerve fibres (Eccles *et al.* 1954; Longo *et al.* 1960). However, the ACh release was not directly related to the activity of Renshaw cells, since amounts of the ACh release were not influenced by administration of DHE which is known to depress the response of Renshaw cells to excitation of recurrent collaterals (Eccles *et al.* 1954; Brooks & Wilson, 1958). It is thus concluded that antidromic impulses in alpha motor nerve fibres liberate ACh from the presynaptic terminals at the central synapses on Renshaw cells.

In contrast with observations on the frog spinal cord (Mitchell & Phillis, 1962), the present study showed that sensory impulses also increased the rate of ACh release as much as did antidromic impulses in the motor nerve. It could be argued that such afferent volleys activated motoneurones reflexly, the associated ACh release then possibly being due to impulses in motor-axon collaterals. This is probably unjustified since the maximal stimulation of the dorsal roots usually fails to activate more than 25 % of the total motoneurone population (Hunt, 1955; Kuno, 1959). At the stimulus frequency used (10/sec), in particular the number of motoneurones reflexly excited must have been less than 10 % of the total (cf. Jefferson & Schlapp, 1953; Lloyd & Wilson, 1957). Since the above observations were made in preparations with the paravertebral sympathetic ganglia intact, the ACh output could be due to a reflex discharge of preganglionic neurones evoked by stimulation of the limb nerves (cf. Beacham & Perl, 1964). Alternatively, some other unidentified cholinergic synapses may exist in the mammalian lumbosacral cord (Fernandez de Molina *et al.* 1958)

Amounts of ACh release. Although loss of ACh in the pathway of perfusion was usually negligible, amounts of ACh collected seemed to be only

a small fraction of the amount of ACh actually released within the cord. There are several possible factors reducing the efficiency of collection of ACh. The blood–brain barrier may prevent the passage of ACh from the blood stream to the central nervous system (Eccles *et al.* 1956; Curtis *et al.* 1957; Curtis & Eccles, 1958*a*). If such a barrier exists also in the opposite direction, from the nervous system to the blood vessel, the released ACh cannot efficiently be collected, although the perfusion often produces oedema of the spinal cord (see Methods) which may break down the blood–brain barrier (Bakay, 1956). Similarly, another postulated barrier existing around the synaptic region at Renshaw cells (Eccles *et al.* 1956; Curtis & Eccles, 1958*b*) may also tend to diminish the amount of ACh collected. Further, the diffusion time of ACh from the site of release (cf. Krnjević & Mitchell, 1960, 1961; Mitchell, 1963) was ignored in the present study. Consequently, the value obtained from the venous samples gives only the minimum limit of amounts of actual release of ACh. The average amount of ACh collected during stimulation of the peripheral nerves at 5/sec was approximately 7×10^{-9} g/min or 21×10^{-12} g/stimulus. Stimulation of the peripheral nerves used in the present study probably activates nearly all alpha fibres of the ventral roots from L_5 to S_1. Since the number of large fibres of these ventral roots is about 8000 (Jefferson, 1954), the minimum amount of ACh release per impulse per fibre may be calculated as $2{\cdot}5 \times 10^{-15}$ g or about 10^{-17} mole.

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