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ACETYLCHOLINE CONTENT OF THE RABBIT PLANTARIS MUSCLE AFTER DENERVATION

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Dale, Feldberg & Vogt (1936) found that skeletal muscle 10 days after it had been denervated no longer released ACh in response to direct electrical stimulation, a result which seemed to eliminate the possibility that any ACh normally released by nerve stimulation could be derived from the muscle fibres. The failure of the nerve endings to release transmitter agent has been attributed to failure of the degenerating axons to conduct (Lissák, Dempsey & Rosenblueth, 1939); but there is the additional possibility that at this stage of degeneration little or no ACh remains in the nerve terminals to be released. MacIntosh (1938) reported that denervation of the superior cervical ganglion is followed by a reduction of its ACh concentration to values averaging between 20 and 25 % of the concentration in normally innervated ganglia in 3 days. Feldberg (1943) found a somewhat steeper fall to 10 % in 3 days, and less than 5 % in 4–7 days in the same tissue; and he also found that the ACh in the degenerating part of the cervical sympathetic trunk falls at a similar rate. Lissák *et al.* (1939) and later MacIntosh (1941) observed that somatic nerves, the phrenic, the hypoglossal, the sciatic and its major branches also lose ACh fairly rapidly when they degenerate. According to MacIntosh the concentration falls in such nerves to between 3 and 25 % of the contralateral control tissue 5–8 days after their central connexions have been cut.

There do not seem to have been any systematic attempts to investigate ACh changes in nerves over longer periods of degeneration; but changes in choline acetylase, the enzyme which synthesizes ACh, associated with degeneration have been followed for as long as 4 weeks. In the denervated superior cervical ganglion and distal segment of the cervical sympathetic nerve trunk the enzyme concentration declines during the first week after section at about the same rate as ACh, and the reduction continues over the next 3 weeks at the end of which time the ganglion content is about 2 % of the control (Banister & Scrase, 1950) and the concentration (per gram of dry tissue) in both the ganglion and trunk are each less than 1·5 % of control tissue (Hebb & Waites, 1956). It has also been reported by Berry

& Rossiter (1958) that degenerating sciatic nerves lose their capacity to form ACh 2–3 weeks after section.

The conclusion to which all these observations seem to point is that most of the ACh in a degenerating motor nerve and the muscles it innervates will disappear if the period of degeneration is continued for much longer than a week. Other more recent evidence, however, indicates that this conclusion is wrong. The work of Bhatnagar & MacIntosh (1960) has shown that denervated skeletal muscle, which has about 30 % of the normal content of ACh 1 week after being deprived of its motor innervation still retains 10–15 % 5 weeks later. It also appears that after denervation voluntary muscle can still release ACh under certain circumstances. In the first place Birks, Katz & Miledi (1960) have found that the miniature end-plate potentials (m.e.p.p.) in frog muscle, which are at first silenced by section of the nerve, return at about seven days and may continue, although at a frequency lower than normal, for as long as 130 days. These m.e.p.p. have the characteristics of those observed in normally innervated muscle and on present evidence can only be attributed to the spontaneous release of small packets of ACh. In addition to this it has been observed by Straughan (1960) that there is a small resting release of ACh from the freshly-excised hemidiaphragm of the rat, amounting to 15–30 mμg/hr, and he has found about the same rate of resting release in tissue denervated, by section of the ipsilateral phrenic nerve, seven days before. Following up this observation, Mitchell & Silver (personal communication) have observed that even 4 weeks after denervation the resting release continues at about half the normal rate.

The purpose of the present paper was to re-investigate the choline acetylase changes which occur in a somatic nerve during 4–6 weeks of degeneration induced by surgical section or crush injury; and to see how these are related to the changes in ACh content over a similar period in a skeletal muscle denervated by the same operation. In the experiments already quoted which were done earlier in this laboratory (Hebb & Waites, 1956) the choline acetylase activity of degenerating cholinergic nerves was usually less than 1 % of the control values, and because of limitations in the method of analysis was then too slight to be detected. It was therefore impossible to say in some experiments whether all the enzyme had disappeared or whether a very small fraction still remained. Since then, however, the method has been modified in a number of ways so that much lower concentrations of choline acetylase can be measured. It seemed, therefore, that it would be useful to re-investigate the reduction in choline acetylase which occurs in nerves during prolonged degeneration; the experiments also offered the advantage that the time courses for the loss of enzyme from the nerve trunk and for the changes in muscle ACh could be

directly compared. The muscle investigated was the rabbit plantaris. The rabbit was chosen in part because of availability but also because of an earlier report by Chenykaeva (1943) that denervation of the plantaris in this species increases the ACh content of the muscle. It seemed desirable to re-investigate this claim, particularly since, at the time Chenykaeva's experiments were done, it was not generally appreciated that the identification of ACh in tissue extracts can only be certain if the assay procedure incorporates certain necessary controls (see MacIntosh & Perry, 1950).

METHODS

Twenty rabbits, nine males and eleven females, 3 to 9 months in age, were used in the experiments. One to six weeks before experiments on twelve of the animals one plantaris muscle was denervated by crushing or cutting out 1 cm of the sciatic trunk at mid-thigh or knee level; in two animals the medial popliteal branch of the sciatic was cut instead, and 1 cm of nerve where it lies in the popliteal fossa was removed (see Fig. 1). In the remaining experiments both plantaris muscles had their normal innervation. Sodium pentobarbital (60 mg/ml. of 10 % ethyl alcohol) was used as anaesthetic (50–70 mg/kg given intravenously) in all the preliminary operations and in all save one experiment in which urethane was the sole anaesthetic (1·5 g/kg). In some experiments a small dose of urethane (500 mg/kg given intraperitoneally) was administered first and followed by sodium pentobarbital given intravenously (30–50 mg/kg). At the operation novocaine (Bayer Products) was injected subcutaneously at the site of the incision and occasionally around the sciatic nerve sheath before cutting or crushing it.

Preparation of extracts from plantaris muscle and assay of their ACh content. Each plantaris muscle was dissected out and dropped immediately into 10 ml. of ice-cold 10·5 % TCA (trichloroacetic acid) where it was chopped with scissors as rapidly as possible and reduced in this way to a fine mince. The dish containing the TCA was weighed before and after the tissue was put in, in order to obtain an estimate of the muscle weight. Control tests in which the two muscles from the same animal were compared showed that the ACh yield was about the same when instead of chopping with scissors the muscle was reduced to a fine mince in 2 min in a high speed homogenizer (M.S.E.) with blades rotating at 14,000 rev/min.

The subsequent procedure differed in some respects from that recommended by MacIntosh & Perry (1950). The TCA suspension was kept cold (1–4° C) for 2 hr, instead of at room temperature for 1½ hr, after which it was centrifuged for 3 min at 3300 rev/min (without temperature control), rewashed with 6 ml. 10 % TCA and centrifuged again at the same speed for 6 min. One ml. of frog Ringer's solution was then used to rinse the centrifuge tube and this too was added to the extract. Another difference in procedure was that the TCA extract was washed with considerably larger volumes of ether (20–25 ml. ether/ml. TCA extract) and was brought to pH 5–5·2 by four washes. This was important since the amount of TCA otherwise remaining in the extracts interfered seriously with the assay. The ether was not saturated with water, and the extracts were reduced in volume by the water taken up in the ether. In control tests of this procedure on muscle minced and left for some time at room temperature before being extracted with TCA (to remove the naturally occurring ester) to which a known amount of ACh was added after the TCA had been added, the value obtained by assay for the ACh present was equal to or slightly in excess (+15 %) of the amount added.

The next step was to heat the extracts (50–80° C) and blow the ether off with a stream of N_2 gas. The extracts were then partially neutralized (pH 6) by cautious addition of N/3 NaOH, made up to 9 ml. with frog Ringer's solution and divided into 2 equal portions.

One (the test solution) was made up to 5 ml. by addition of 0·5 ml. frog Ringer's solution while to the other half (the control solution) 0·1 ml. N-NaOH was added; the alkali-treated solution was boiled briefly to destroy all ACh, neutralized with N-HCl and made up to 5 ml. In the subsequent assay on the eserinized frog rectus muscle, equal volumes of test and control solutions were compared with one another and the amount of ACh, when added with the control solution, which was required to produce a contraction equal to the test response was determined in the usual way (see MacIntosh & Perry, 1950). In one experiment the amount of ACh in the muscle extract was determined on both the frog rectus muscle and on the guinea-pig ileum. Both gave similar answers (within 5 %). In another experiment, the results obtained on the rectus muscle were checked by assay on the eserinized dorsal muscle of the leech. This too gave a similar result, but in general assays of the extracts on the leech muscle were not satisfactory in that the results were not always reproducible within the normal margin of error.

Some substance (or substances) was present in the extracts, both in the test and alkali-boiled fractions, which greatly sensitized the rectus muscle to ACh, and yet by itself had little stimulant action on the muscle. It seemed probable that the sensitizing action was due to adenosine triphosphate (ATP) and the break-down products of ATP, since extraction of rabbit skeletal muscle with TCA normally yields a solution containing relatively large amounts of ATP and this is known to sensitize the rectus muscle to ACh. Moreover, the sensitizing action of the extracts like the sensitizing action of added ATP was not altered by boiling with alkali (see Feldberg & Hebb, 1947, who also give earlier references). The increase in sensitivity observed with addition of 0·25 ml. of extract in a final volume of 1·0 ml. (the amount added to the muscle bath) was such that the rectus muscle, normally responding to 1 in 10^8 or higher concentration, usually responded to as little as 2 in 10^9 ACh. The same degree of sensitization could be obtained by adding 1 mg of the disodium salt of ATP in 1·0 ml.

Choline acetylase measurements. The choline acetylase activity of the tissues sampled was estimated according to the following procedure. The nerves were dissected as free as possible of connective tissue, weighed and minced with scissors; they were then immediately ground up in cold dry acetone as described earlier (Hebb, 1955) except that only two to three washes of acetone were used. The powdered tissue collected in a Buchner filter was dried over P_2O_5 at -500 mm Hg for 3–4 hr, suspended in cysteine-saline (3 mg of neutralized L-cysteine HCl/ml. 0·9 % NaCl) and frozen. The suspensions contained either 25 or 50 mg dried tissue ml. of cysteine-saline. They were usually tested 1–5 days after they were first frozen (at $-15°$ C) but could be stored without loss of activity for much longer periods.

TABLE 1. Composition of the acetyl-coenzyme A generating system

1. 0·06 ml. crude yeast extract containing 30 units of CoA
2. 0·04 ml. 4 % choline chloride
3. 0·04 ml. 30 % KCl
4. 0·14 ml. 10 % acetyl-phosphate
5. 0·08 ml. 1·2 % $MgCl_2.6H_2O$
6. 0·12 ml. 3 % L-cysteine HCl (brought to pH 6 with N-KOH; made up immediately before use)
7. 0·1 ml. 2·5 % phosphotransacetylase (extracted *Clostridium kluyverii*) in 0·02 M-$NaHCO_3$
8. 0·1 ml. 0·13 M Na phosphate buffer (pH 6·9)
9. 0·1 ml. 0·05 % eserine sulphate

The enzyme activity was routinely measured by incubating 0·1 or 0·2 ml. of each preparation for 1 hr at 38° C with 0·78 ml. of the acetyl-coenzyme A (acetyl-CoA) generating system described in Table 1. When the expected activity was very small, as in the case of nerves that had degenerated for 4–6 weeks, 0·1 or 0·2 ml. was incubated with 0·4 ml. of the generating mixture. It should be mentioned that in earlier experiments the generation of

acetyl-CoA depended upon addition of CoA, ATP, liver enzyme extract and either citrate or acetate. With these systems, or with a system similar to the one now used except for the addition of pure CoA instead of crude yeast extract, the yield of ACh from incubation of a standard preparation of choline acetylase was found to be only about two-thirds of the yield obtained by using the generating system shown in Table 1. Crude CoA probably yielded higher values because it is more stable than pure CoA. The measurement of choline acetylase activity was also made effectively more sensitive by the system now described because in relation to the concentration of enzyme extract in the incubation medium as finally prepared for assay (see below) the amount of choline was only one tenth or one fifth of the amounts used earlier. This was important because it meant that incubation media in which very little ACh had formed could be tested in dilutions of 1 in 20 or 1 in 40 (in frog Ringer's solution) on the rectus muscle whereas in earlier experiments in which a larger amount of choline was used it was necessary to dilute the incubation media by as much as 1 in 100; otherwise the concentration of choline would be sufficient to mask the action of a small quantity of ACh, and so this might escape detection.

The generating mixture was usually prepared beforehand in bulk in amounts sufficient for ten to twenty separate incubation tests. After all ingredients had been added in the proportions shown in Table 1, the mixture was kept at 38° C for 10 min in order to obtain a high starting concentration of acetyl-CoA which with choline is a required substrate for the enzyme. It was then used immediately or stored at −15° C until required. At least two samples of each tissue preparation were incubated in order to determine its ACh-synthesizing activity. In the case of samples with very low enzyme activity, four incubation tests were done. The tissue concentration of enzyme, expressed as μg ACh synthesized per gram of nerve per hour was then calculated from the mean of these tests. The volume of each extract which was available for testing was usually less than 1·0 ml.

The amount of ACh formed during the hour's incubation was determined by assay on the eserinized rectus abdominis muscle of the frog. In most assays the control solution for addition with the standard ACh solution was prepared by incubating a heat-treated sample of a similar enzyme preparation with the generating mixture in parallel with the test preparations. At the end of 1 hr at 38° C a few drops of Universal Indicator (BDH) and 0·5 ml. N/3 HCl were added to both test and control which were then boiled briefly, cooled and made up, after the addition of 1 ml. 0·5% Na_2HPO_4 to 40 ml. with frog Ringer's solution. After partially neutralizing them with a few drops of N/3 NaOH they were ready for testing.

The procedure was somewhat different for preparations in which it was expected there would be very little enzyme activity. In these the incubation volume was smaller, since there was only 0·4 ml. of the generating mixture in each tube. The same amount was also put in a control tube containing heat-treated enzyme and both were prepared for testing as already described except that the amount of N/3 HCl used was only 0·25 ml. and the final volume was 20 ml. Another difference was that an alkali-boiled control solution was prepared from a sample of the test solution; and the results which were obtained by assaying the test against the incubated control solution were checked in a second assay using the alkali-boiled control. This double assay procedure was used in a number of experiments in which the concentration of ACh in the solutions under test was less than 20 mμg/ml. The two methods of control gave similar answers.

As a means of sensitizing the frog muscle in some experiments in which the ACh concentration was too low to produce more than a slight response from the muscle, sodium ATP (adenosine triphosphate, the disodium salt made by Pabst) was added to the muscle bath with the test and control solutions in concentrations of 1 or 2 mg/ml. This increased the sensitivity as mentioned earlier. The validity of the method was checked by doing duplicate assays on the eserinized leech muscle without addition of ATP. Although these gave slightly lower values (possibly an effect of the potassium present in the incubates) the differences did not exceed 10% and were consistent.

The choline acetylase activity in corresponding lengths of the sciatic nerve and its branches was found to be equal for the two nerves within the limit of the assay method ($\pm 10\%$). Therefore in order to evaluate the effect of section on the choline acetylase of one sciatic nerve, and find what changes had occurred in the proximal and distal segments, two samples of the other nerve corresponding in level and length to these were used as controls with which the interrupted nerves were compared. Since there might be considerable changes in the weights of these, the concentration of enzyme, that is the activity expressed as μg ACh synthesized/g fresh tissue or/g dried tissue/hr was not used as the basis of comparison. Instead the activity in the whole of each sample was calculated and the values so obtained for the proximal and distal segments expressed as a percentage of the values for the corresponding controls.

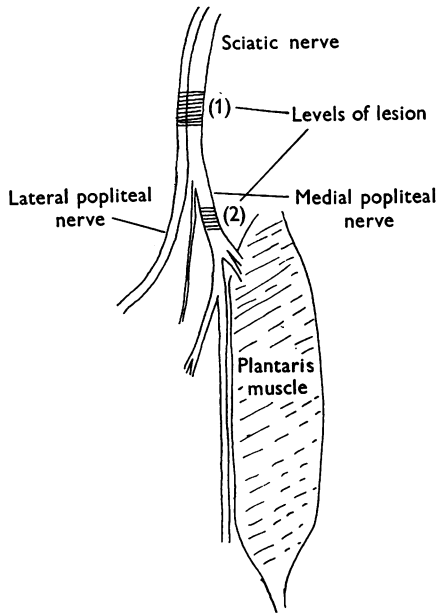

Fig. 1. Sketch of sciatic nerve and plantaris muscle to show level of section of (1) sciatic, or (2) medial popliteal nerve.

Figure 1 shows the branches of the distal segment of nerve which were taken for analysis. When the section or crush was low in the thigh in order to obtain enough material for analysis some or all of the larger branches in the leg were included in the sample; alternatively if only the medial popliteal had been interrupted then the branches of this nerve were dissected out to the limits indicated in the sketch. One to 2 cm of the distal segment nearest to the lesion was discarded in experiments carried out more than 2 weeks after the operation, since re-innervation might have proceeded this far especially when the lesion was a crush injury. As mentioned later in the text re-innervation probably did affect the results of one experiment.

RESULTS

ACh content of the plantaris muscles

Expressed as the chloride the mean amount of ACh estimated to be present in each of eighteen plantaris muscles with intact innervation

was 262 ± 79 (S.D.) while the mean concentration was 62 mμg/g muscle. This is very close to the concentration of ACh in rabbit skeletal muscles, 60 mμg/g, found by Chang & Gaddum (1933). Two of the lowest values in the series, 28 and 34 mμg/g, were obtained from experiments in which the fourth ether wash of the extracts was omitted and the assays were unsatisfactory because the alkali-boiled control by itself caused a large contraction of the rectus muscle (see Methods). Even if these values are not taken into account, however, there is still a rather wide scatter in the results, which can probably be attributed in part to differences in anatomical relations of the muscle, which was sometimes quite separate and sometimes joined to other muscles, and which varied considerably in size,

TABLE 2. ACh in denervated plantaris muscle and choline acetylase in the degenerating segment of interrupted sciatic nerves

Expt.	Time after operation (weeks)	Nature of lesion	ACh in denervated muscle as % of control: Concentration	ACh in denervated muscle as % of control: Content	Choline acetylase in distal segment (% of) control nerve)
4	1	L sciatic section	27·0	22·5	22
16	1	R sciatic crush	21·6	16·6	4·2
8	2	L med. popliteal section	27·0	19·0	5·2
15	2	L sciatic crush	24·3	14·3	3·8
14	3	R sciatic section	21·3	13·3	0·2
11	3	R sciatic crush	20·3	10·6	4·7
17	4	L sciatic section	22·2	10·6	0·2
7	4	L sciatic section	7·0	4·5	1·1
12	4	R sciatic crush	15·0	8·9	0·3
18	4	L sciatic crush	22·0	11·4	0·3
6	5	R med. popliteal section	24·1	12·1	0·2
9	6	L sciatic section	12·0	6·0	0·4
10	6	L sciatic section	27·0	11·1	0·6
13	6	R sciatic crush	25·0	13·2	1·0

In Expt. 13, re-innervation of the distal segment of the crushed sciatic nerve may have led to the inclusion of some new nerve tissue in the sample analysed for choline acetylase.

possibly because of differences in age and breed of rabbit; and in part to differences in the experimental conditions, such as the anaesthesia, under which the muscles were removed. No consistent differences were found in the ACh content of the muscles removed after, rather than before, death.

The total ACh content of all the muscles that had been denervated was much lower than that of normal muscles. The observed content varied from less than 5 mμg (one muscle denervated for 6 weeks after operation) to 66 mμg (one muscle denervated for 2 weeks). Table 2 shows the content and concentration of ACh in the denervated muscles expressed as a percentage of the values found for the innervated muscle of the other leg. This method of expressing the results is chosen in view of the large

individual variations in the ACh content of the muscle and because control tests showed that the values for the right and left muscles of the same animal agreed very closely (within 10 %). The largest change in content occurred during the first week when it was approximately 20 % of the ACh in the innervated muscle; over the remaining 5 weeks there was a further much slower reduction to a little less than half this level.

It will be noted from Table 2 that the concentration as compared with the content of ACh showed less change after the first week of denervation. The difference was related to shrinkage of the muscle which at 2 weeks after operation was only about two thirds of its fellow in weight; at 4 weeks it was about one half; while at 6 weeks it was a little less than one half (average of three experiments 48·3 %).

The changes in both content and concentration of ACh were similar to those reported by Bhatnagar & MacIntosh (1960) for denervated cat muscles.

Choline acetylase content of the sciatic nerves

Degenerating distal segment. Table 2 also shows the rate of change in choline acetylase in the distal segment of the interrupted nerves. The biggest decline occurred during the first week but the enzyme continued to fall, although more slowly, over the next 3–4 weeks. On average the amount of choline acetylase, expressed as a percentage of the activity in the contralateral control nerve, fell to 0·5 % at 4 weeks, and 0·2 % at 5 weeks (one experiment only) and was 0·6 % at 6 weeks. In one of the experiments at 6 weeks, no. 13, the value recorded for the choline acetylase concentration could well have been too high since re-innervation of the distal stump had begun and some new nerve tissue was probably included in the sample. This was an experiment in which the nerve had been crushed at operation. In all other experiments, however, the piece of the distal segment taken for analysis was sufficiently remote from the section to make it unlikely that re-innervating tissue could have affected the results. It may also be noted that in two experiments in which the analyses were done 4 weeks after operation, in one at 5 weeks and in another at 6 weeks, in all of which the nerves had been severed and 1 cm of the trunk removed, no connexion had been established between proximal and distal trunks, yet in all cases evidence of ACh synthesis was obtained on incubation. Although the amount of choline acetylase activity in the distal segment in these experiments was very small, repeated incubation tests of the preparation made from the degenerating nerves all yielded some ACh, the amounts found in each incubation varying in different experiments from 80 to 270 mμg per tube.

Figure 2 shows the curves for the logarithms of the averaged values given

in Table 2. This illustrates very clearly how the time courses for the changes in enzyme and ester differ. Since the level of activity of the enzyme, 0·2 %, shown at 5 weeks is based on only one experiment the fact that it is lower than the averaged values of 0·5 for 4 weeks (four experiments) and for 6 weeks (two experiments) may not be significant. In constructing this curve the experiment mentioned earlier, in which re-innervation of the distal stump may have affected the result, was not included.

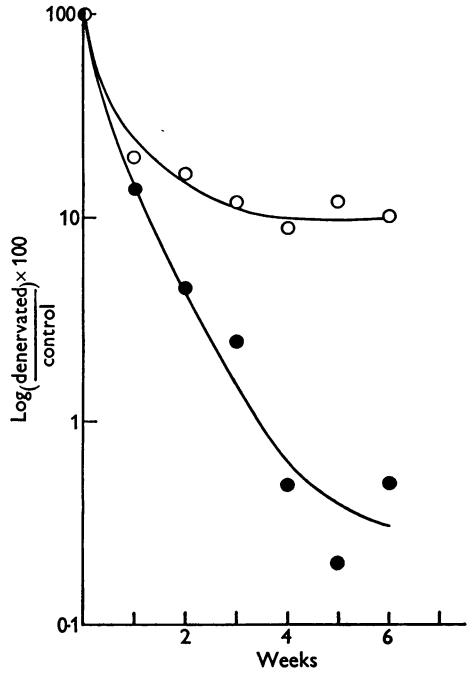

Fig. 2. Curves showing changes in (1) ACh content of denervated plantaris muscle, upper curve and (2) choline acetylase activity of degenerating segment of the interrupted sciatic or medial popliteal nerve, lower curve. The curves are constructed from the data of Table 2. *Ordinate:* log of averaged values expressed as a percentage of the contralateral control muscles (ACh) or nerves (choline acetylase). *Abscissa:* time in weeks.

Proximal segment. Three to 6 cm of the proximal segment, measured from the section or upper edge of the crushed piece of nerve, was analysed in each experiment and compared with an equal length, taken from the same level, of the opposite uninjured nerve. In confirmation of earlier work (Hebb & Waites, 1956; Hebb & Silver, 1961) the choline acetylase activity was observed to increase in the proximal segment of the operated nerve during the first week after section or crush injury. The increase was generally of the order of 100 %. That is, the output of ACh per centimetre

of nerve was about double the control level which was found to vary from 17 to 24 μg ACh/hr. Increased enzyme activity was observed in the proximal segment for as long as 4 weeks after section; but at this time the difference between the two nerves was generally less marked; and in some experiments in which the denervation period was 4 weeks or longer, there was no significant difference, so far as the proximal segment was concerned, between the operated and control nerves.

DISCUSSION

The ACh changes in the denervated plantaris muscle are similar to those observed by Bhatnagar & MacIntosh (1960) in the denervated leg muscles of the cat, except that the fall of ACh in the plantaris after section of its nerve is a little steeper in the first week and the level eventually reached at 6 weeks is somewhat lower, but this could be a species difference. In both cases the amount of ACh retained in the denervated muscle is proportionately larger than the amount of choline acetylase found in degenerating cholinergic nerves.

The question that these observations, and those which point to a continuing release of ACh from denervated muscle (Birks *et al.* 1960; Straughan, 1960), raise is whether there is any source of choline acetylase in the muscle other than the axons and endings of the degenerating nerve. The autonomic nerve supply of the muscle can probably be eliminated from consideration. In the first place Bhatnagar & MacIntosh (1960) showed in their experiments that sympathectomy did not affect the ACh content of the muscles which they investigated; secondly, it is now generally accepted that most of the autonomic fibres reach individual skeletal muscles through their main nerve supply. Some may run for short distances with the blood vessels to the muscle but these branches too are derived from the main nerve trunk (see Tiegs, 1953). Therefore by cutting the sciatic or the medial popliteal nerves the plantaris muscle would be deprived of its autonomic as well as of its somatic nerve supply.

Another possibility is that the muscle fibres are themselves the source of ACh. However, while this would account for the persistence of some ester in the muscle it would not account for the persistence of the m.e.p.p. activity after denervation. Moreover, other evidence indicates that the ACh concentration in parts of the skeletal muscle which are free of nerve tissue is much lower than the concentrations in the denervated plantaris and may in fact be less than is detectable. It therefore seems more likely that the ACh which persists in the muscle is associated with the degenerating axons and endings.

The most important argument against this view is the evidence already quoted in the introduction showing how rapidly ACh falls in degenerating

nerve trunks since it is probable that the fall in ACh in the intramuscular part of the nerve would follow a similar time course. However, the changes observed both by MacIntosh (1941) and by Feldberg (1943) were changes in concentration and not in total content of ACh and, since divided nerves increase very much in weight during the first stages of degeneration the rate of loss of ACh on the basis of their experiments would appear to be much greater than it really is. Furthermore, Saunders has recently observed that 7 days after crush injury degenerating somatic motor fibres (ventral roots) have about 30 % of the normal ACh content for such fibres and in one experiment a sciatic nerve which had degenerated for 6 days also contained about the same proportion of the normal (personal communication from Dr N. Saunders). Since the fraction of ACh which persists in muscle is also about 30 % or slightly less 1 week after denervation it could well be that it is in fact all derived from the intramuscular part of the nerve.

If ACh does persist in the nerve it could either be because preformed ester is protected from destruction during degeneration or because the amount of choline acetylase remaining, small though it is, is sufficient to maintain it at a reduced level. One possible change in the nerve fibre which would favour the continued maintenance of a store of ACh is the disappearance of a considerable part of the axonal true cholinesterase (Sawyer, 1946) following its section. The amount of ACh found in peripheral nerves is very much higher if they are removed after pre-treatment of the animal with an anticholinesterase (see MacIntosh & Perry, 1950) than if there is no premedication. This means that the level in normal nerves depends not only on the rate of production but also on the rate of destruction and so if the esterase is removed by degeneration more ACh will accumulate.

However, the amount of stored ACh remaining after 1 week of degeneration is probably only a small fraction of the total turnover necessary to account for the frequency and dimensions of the m.e.p.p. observed in denervated muscle. It is more pertinent therefore to inquire whether the amount of choline acetylase in the degenerating nerve, if it falls to the same extent, i.e. to about 0·5 % in 4 weeks, over the whole length of the distal segment of each cut fibre, would be enough to account for all the ACh involved in this activity.

It is not possible to make a precise estimate of the rate of ACh release from denervated muscle on the basis of the m.e.p.p. activity. It appears, however, that this is less than the total resting release and that has been directly measured by Mitchell & Silver (personal communication) who find that the rat hemidiaphragm 4 weeks after being denervated is able to release on average about 10 mμg/hr or approximately half the normal resting release. The question is how does this compare with the potential output of

ACh of the diaphragm if like the sciatic nerve its motor axons and endings only retain 0·5 % of their normal choline acetylase activity after nerve section. The rate of synthesis of ACh by an innervated hemidiaphragm is between 35 and 40 μg ACh per hemidiaphragm per hour (C. O. Hebb & K. Krnjević, unpublished experiments), and 0·5 % of this rate is between 175 and 200 mμg of ACh per hour. That is, the *in vitro* rate of synthesis would be about twenty times greater than the observed resting release. Therefore, in theory at least, sufficient enzyme remains in the nerve and endings to account for the continued release of ACh after denervation. It would clearly be of interest to test this directly by measuring the choline acetylase level in denervated muscle. That has not been possible in experiments on the plantaris because the enzyme is present in this muscle in too low a concentration to permit its accurate measurement even in the normally innervated organ.

SUMMARY

1. Following section or interruption of its nerve supply ACh in the rabbit plantaris muscle falls to about 20 % of the control content (contralateral innervated plantaris) in one week and to 10 % in 6 weeks.

2. Over a period of 4–6 weeks choline acetylase in the degenerating portion of the interrupted nerve (sciatic or its medial popliteal branch) falls to an average value of 0·5 % of the contralateral control.

3. In all experiments in which the degeneration period was 4 weeks or longer there was evidence that the distal segment could still synthesize ACh, although the amounts involved were extremely small (down to 0·2 % of the control).

4. It is suggested that the amount of choline acetylase remaining in the distal intramuscular portion of the degenerating nerves would be sufficient to account for the ACh released spontaneously in denervated voluntary muscle as observed by other workers.

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