J. Physiol. (1970), **207**, *pp.* 507–528
With 7 plates and 7 text-figures
Printed in Great Britain

ON THE DEGENERATION OF RAT NEUROMUSCULAR JUNCTIONS AFTER NERVE SECTION

BY R. MILEDI AND C. R. SLATER*

From the Department of Biophysics, University College London, Gower Street, London W.C. 1

(*Received 9 October 1969*)

SUMMARY

1. A study was made of functional and structural changes during degeneration of end-plates in the rat diaphragm after phrenic nerve section at two levels.

2. For 8–10 hr after cutting the nerve in the neck, all end-plates retain the ability to transmit impulses. During the following 8–10 hr, an increasing number of end-plates lose this ability so that after a total of about 20 hr, no end-plates can transmit.

3. Transmission failure occurs abruptly at most end-plates. This failure is usually accompanied by cessation of spontaneous miniature end-plate potentials (min.e.p.p.s), though in a few cases min.e.p.p.s persist after junctional transmission has failed. Several degenerating junctions were observed where the frequency of min.e.p.p.s was very low, suggesting an intermediate stage in min.e.p.p. failure.

4. The time of junctional failure depends on the length of the degenerating nerve stump. For each additional centimetre of nerve, failure is delayed about 45 min.

5. Changes in ultrastructure of nerve endings closely parallel those of function. For about 8–12 hr after cutting the nerve, nearly all end-plates appear normal. During the period when transmission is failing, some end-plates are clearly undergoing structural break-down. By the time functional failure is complete, all end-plates appear grossly abnormal.

6. During degeneration, the contents of the axoplasm undergo disruption and the nerve terminal breaks up into small fragments. In contrast, the Schwann cell appears to become very active and its processes extend into the synaptic cleft to surround fragments of the nerve terminal. Ultimately, the Schwann cell completely replaces the axon at the end-plate.

7. Increasing the length of the peripheral nerve stump delays the onset

* Present address: M.R.C. Laboratory of Molecular Biology, University Postgraduate Medical School, Hills Road, Cambridge.

of structural break-down. Disruption of end-plates near the site of nerve entry into the muscle occurs before those farther away.

8. It is suggested that end-plate degeneration is triggered by a signal which passes from the site of injury to the nerve terminal. The duration of the period after transection when end-plates appear to be normal would then reflect the time required for this signal to travel the length of the isolated nerve stump.

INTRODUCTION

This paper reports observations on the basic changes of structure and function occurring at mammalian neuromuscular junctions soon after motor nerve section. One aim of this study was to see how the failure of nerve impulse transmission during Wallerian degeneration is related to the disruption of the structural components of the junction. To do this, the time course of transmission failure has been determined and used as a guide for the selection of material to be observed in the electron microscope. We have compared our observations with those made in similar studies on the frog (Birks, Katz & Miledi, 1960; Hunt & Nelson, 1965).

An interesting feature of motor nerve degeneration is that degeneration is seen sooner at the neuromuscular junction than along the myelinated axon (Titeca, 1935; Birks, Katz & Miledi 1960). This suggests that the end-plate may respond to a signal which travels peripherally along the axon before degeneration of the nerve becomes apparent. One way of testing this hypothesis is to see if varying the length of the peripheral nerve stump has any influence on the time course of degeneration of the terminal. Previous experiments suggested that increasing the length of the stump does in fact delay the failure of neuromuscular transmission (Eyzaguirre, Espildora & Luco, 1952; Gutmann, Vodička & Zelená, 1955; Luco & Eyzaguirre, 1955; Birks, Katz & Miledi, 1960). It seemed worth while to investigate this point more fully and to examine its quantitative aspects. Some of these results have already appeared in a note (Slater, 1966).

METHODS

The experiments were made on the diaphragm of the rat. The end-plates of this muscle are distributed in a narrow band on one side of the intramuscular nerve trunk (Coers, 1953). In a normal preparation, the electrical signs of spontaneous transmitter release from motor nerve endings, the miniature end-plate potentials (min.e.p.p.s, Fatt & Katz, 1952) can be recorded from over 95 % of electrode penetrations made 1–3 mm lateral to the nerve trunk (cf. Miledi & Slater, 1968*a*). Thus, when most of these penetrations give negative results in an experimental muscle, it can be said with confidence that functional failure has occurred.

Female albino rats of Wistar origin (130–170 g) were used in all experiments. In an initial operation, performed under ether anaesthesia, the left phrenic nerve was

cut. This was done either in the neck, near the exit of the nerve from the spinal cord, or in the thorax, near its entrance into the muscle. For the operation in the neck, a ventromedial incision about 2 cm long running anteriorly from the clavicle was made in the skin, the sternohyoideus muscle retracted and the underlying musculature separated to reveal the brachial plexus. In order to ensure that all nerve fibres going to the diaphragm are cut, it was found necessary to transect the whole plexus, as well as the clearly visible branch of the phrenic nerve. To cut the nerve in the thorax an incision was made through the skin and superficial musculature between the 9th and 10th ribs. The intercostal muscles were then separated and the pleural cavity opened. A bent glass probe was inserted into the thoracic cavity and used to pull the phrenic nerve out of the thorax, where a piece about 5 mm long was excised.

In the terminal experiment the animal was anaesthetized with ether and the diaphragm was removed and placed in a modified Krebs solution which was equilibrated with 95 % O_2–5 % CO_2 and maintained at room temperature (17–23° C). The bathing solution had the following composition (concentrations in m-mole): Na^+ 140, K^+ 4·5, Ca^{2+} 2·0, Mg^{2+} 1·0, Cl^- 122·5, SO_4^{2-} 1·0, HCO_3^- 25, $H_2PO_4^-$ 1·0, glucose 11.

The portion of the muscle to be studied was mounted in a Perspex chamber (30 ml.) in which electrical recordings could be made. Fresh bathing solution which was equilibrated with the gas mixture flowed into the chamber at a rate of about 3 ml./min, a constant fluid level being maintained with a suction outlet. The nerve was led into a moist side-chamber, insulated from the main chamber by a 'Vaseline' seal, and rested on a pair of platinum electrodes which were connected to the output of a Grass S 4 stimulator.

To determine the state of neuromuscular transmission in the muscle intracellular recordings were made from the end-plate region using micro-electrodes filled with 3 M-KCl. The muscle was studied routinely by making penetrations in a track parallel to the clearly visible intramuscular nerve trunk, and just lateral to it. Penetrations were made at intervals of 0·5 mm along this track, measured by an eye-piece scale in the Zeiss dissecting microscope, and the distance of each penetration from the entry of the nerve into the muscle was noted, together with the observed electrical activity, both spontaneous and evoked.

For examination of end-plate structure, narrow strips of the diaphragm were fixed for observation in the electron microscope, the same or immediately adjacent regions being used to determine the extent of transmission failure. When end-plates were found in the tissue, several sections of the same ending were frequently examined, although no systematic attempt was made to study serial sections. The next 50–100 μm were then removed before examining further end-plates in the same block.

Tissues were prepared by fixation in a 6 % solution of glutaraldehyde in phosphate buffer (pH 7·2) for 2 hr at room temperature. Diaphragm strips were usually placed in the fixative within a minute or two after isolation from the animal. Following fixation, the specimens were washed in phosphate buffer for 1 hr, stained in a 1 % solution of OsO_4 in buffer for 0·5 hr and dehydrated in graded alcohols. Many sections were double stained with uranyl acetate and lead. Some muscles were fixed in OsO_4 and stained with phosphotungstic acid (cf. Miledi & Slater, 1968*a*). All samples were embedded in Araldite (CIBA Ltd.) and examined in a Siemens Elmiskop Ia.

RESULTS

The failure of neuromuscular transmission

Time course. Within less than 20 hr after cutting the nerve in the neck, electric stimulation of the isolated nerve trunk no longer evokes contraction of the muscle. To see if this rapid failure was not peculiar to the phrenic nerve, the sciatic nerve was transected in the thigh. The response of most leg muscles failed in 24 hr, and of all muscles within 36 hr. Thus degeneration is generally faster in the rat than in the cat, where complete failure of neuromuscular transmission takes 2–3 days (Lissák, Dempsey & Rosenblueth, 1939).

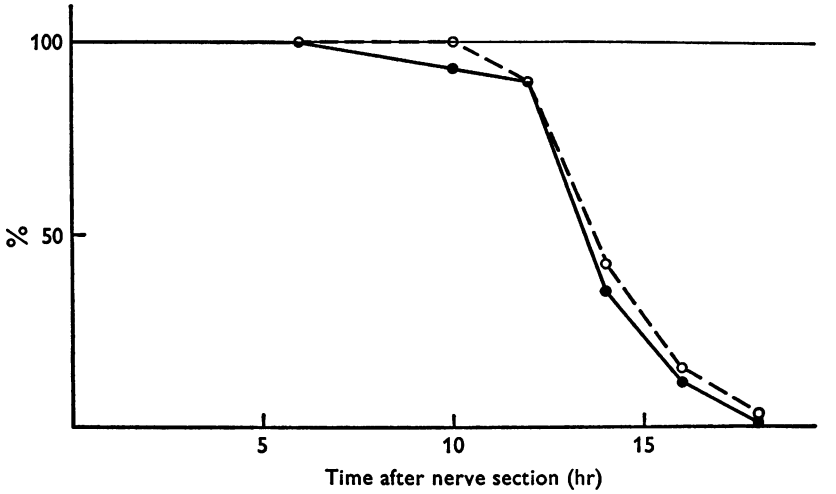
In other preparations undergoing Wallerian degeneration, it has been found that the nerve trunk is still electrically excitable when indirect excitability of the muscle fails (Titeca, 1935; Lissák *et al.* 1939; Birks, Katz & Miledi 1960). Similarly, when the peripheral stump of the phrenic nerve was removed from the rat up to 24 hr after section, it was possible to record normal extracellular compound action potentials. The first nerve fibres to fail, as indicated by a reduction in amplitude of the action potential, do so about 1 day after section; after the second day, all have failed. The earlier break-down of the indirectly evoked muscle response must therefore result from failure of the intramuscular nerve branches, or at the end plate itself.

To obtain further information, intracellular recordings of electrical activity were made from end-plate regions of isolated diaphragms. At each end-plate studied, the response to single supramaximal shocks delivered to the phrenic nerve was tested. In normal muscles, such a stimulus evokes an end-plate potential (e.p.p.) large enough to trigger an action potential in practically all muscle fibres. After nerve section, stimulation of the nerve gave rise either to a normal e.p.p. and action potential or to no response at all. It was thus an easy matter to determine the number of end-plates at which transmission had failed at any time after nerve section.

Since the development of failure could not be followed in a single muscle, a sampling method was used to determine the time course of the process. Animals were killed at intervals during the first day after nerve section, and the diaphragms removed for electrophysiological examination at room temperature. The failure of transmission in the isolated muscles was then practically static, presumably because the degeneration process is greatly slowed by lowering the temperature (Birks, Katz & Miledi, 1960). The period from 10 to 18 hr after nerve section was studied in detail. Several muscles were examined at each 2-hourly interval during this period, and

about sixty end-plates were observed in each muscle. These results are shown in Text-fig. 1.

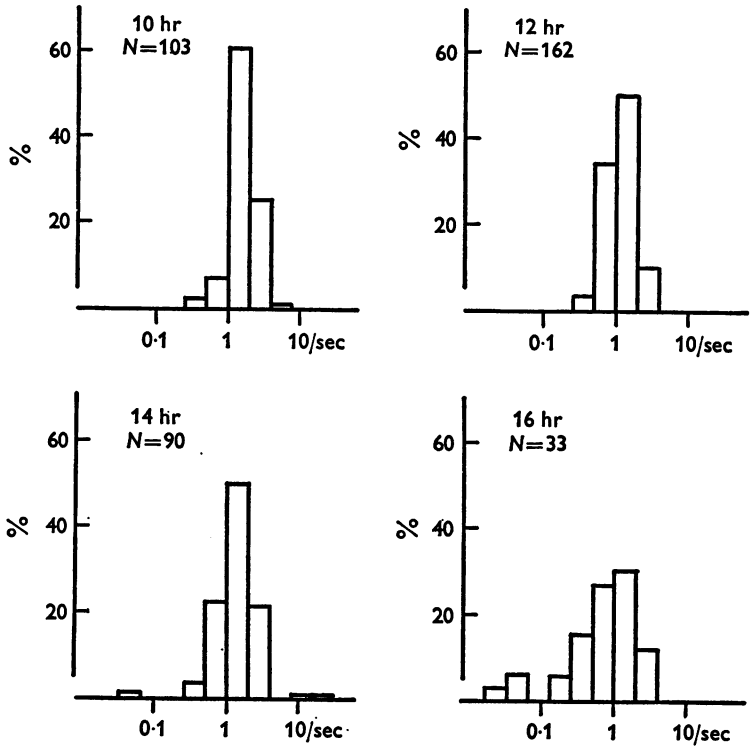
In muscles examined up to 10 hr after nerve section, practically all endings retained the ability to transmit impulses. This initial latent period was followed by a period of 8–10 hr in which progressively fewer end-plates would transmit. At the end of this second phase, some 18–20 hr after cutting the nerve, virtually all end-plates were electrically inactive, thus accounting for the loss of the indirect response of the whole muscle.



Text-fig. 1. Time course of end-plate failure after phrenic nerve section in the neck. Abscissa, time in hr; ordinate, fraction (per cent) of impaled fibres at which activity was observed. Filled circles, end-plates at which normal impulse transmission occurred in response to a single stimulus to the phrenic nerve; open circles, end-plates at which miniature end-plate potentials (min.e.p.p.s) were seen. Each point represents pooled results of about 150 impalements in several muscles.

Mode of transmission failure. As mentioned above, stimulation of degenerating nerves resulted either in normal transmission or in no response at all. This suggests that the failure of transmission occurs abruptly, in an all-or-none manner. Only one end-plate was ever encountered which gave an 'intermediate' response to phrenic nerve stimulation, in that the e.p.p. was much reduced in amplitude and gave rise to a delayed muscle action potential. Failure of the ability to transmit action potentials was usually accompanied by cessation of spontaneous min.e.p.p.s (Text-fig. 1). These findings confirm those made in the frog, where impulse transmission and spontaneous activity at myoneural junctions failed abruptly and at about the same time (Birks, Katz & Miledi 1960).

Some exceptions to the pattern just described were seen. At twenty-eight of the 795 end-plates examined, min.e.p.p.s were present, although response to nerve stimulation had already failed. (The reverse situation, transmission at an end-plate where min.e.p.p.s were *not* present, was never observed.) At these few endings transmission had probably failed as a result of the inability of the action potential either to invade the terminal (see Krnjević & Miledi, 1959), or to release transmitter.



Text-fig. 2. Distribution of min.e.p.p. frequencies at end-plates during nerve degeneration. Abscissa, min.e.p.p. frequency, note logarithmic scale; ordinate, fraction (per cent) of end-plates observed. Time after nerve section and number of end-plates observed is shown on each histogram.

In contrast to the abrupt failure of the e.p.p., there was some indication that the cessation of spontaneous min.e.p.p.s may be preceded by a gradual decrease in their frequency. In muscles whose end-plates were mostly still transmitting, the distribution of frequencies of min.e.p.p.s at different end-plates, presented in a logarithmic histogram, was symmetrical about the geometric mean. Sixteen hours after nerve section a few end-plates with unusually low frequencies were found, giving rise to a marked

assymmetry and a 'tail' on the low frequency side of the histogram (Text-fig. 2). Particularly noticeable was a small number of end-plates at which the frequency was of the order of several per minute instead of several per second. While normally fewer than 5% of end-plates had frequencies of 0·5/sec or less, 25–30% were in that class 16 hr after nerve section.

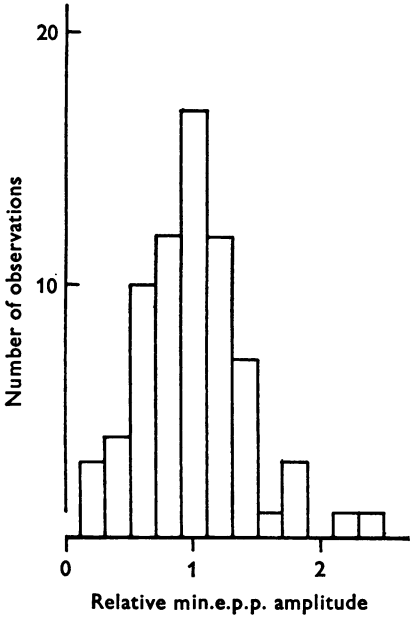
In the group of end-plates where transmission had failed before cessation of min.e.p.p.s, the frequency of the latter ranged from 17/sec to 1/50 sec. Low frequency is therefore not a necessary preliminary condition for transmission failure. On the other hand, of the five end-plates whose min.e.p.p. frequency was less than one tenth of the normal value, only one responded to nerve stimulation.

A further indication that the end-plates with a very low min.e.p.p. frequency were at an advanced stage of functional failure comes from a few experiments in which the stimulating effect of raising the external potassium concentration from 4·5 to 18·5 mM was tested. This procedure normally causes the min.e.p.p. frequency to increase at least tenfold (Liley, 1956). At degenerating end-plates, however, where the frequency was already low, increasing the potassium concentration had little or no effect. In these respects the low frequency activity resembles that occurring after much longer periods of denervation, and associated with the presence of the Schwann cell (Birks, Katz & Miledi 1960; Miledi & Slater, 1968*a*). It should be recalled, however, that in the diaphragm a period of about 2 weeks intervenes between the complete cessation of min.e.p.p. activity and its later resumption at a very small number of end-plates which have Schwann cells associated with them. We feel, therefore, that the low frequency of min.e.p.p.s observed at some degenerating end-plates probably reflects a decline in the transmitter release from the failing nerve terminals rather than an onset of new activity from a different source. A similar situation has been seen in degenerating neuromuscular junctions of the locust (Usherwood, 1963). There it is commonly found that min.e.p.p.s persist for some time after the failure of impulse transmission. When this occurs, increasing the external potassium concentration does not cause the normal increase in min.e.p.p. frequency.

While at some end-plates the frequency of min.e.p.p.s is greatly reduced during this stage of degeneration, there was no obvious change in the amplitude or time course of the individual potentials. Even at those few end-plates where the frequency had fallen to a few per minute, the mean amplitudes (0·5–0·8 mV) were well within the normal range, and the distribution of individual amplitudes about the mean was symmetrical, as is the case at normal junctions (Text-fig. 3). These observations indicate that the amount of transmitter in each packet is not greatly altered, even at an advanced stage of degeneration.

In summary, failure of evoked transmitter release at the end-plate occurs suddenly, after a delay of at least 8–10 hr. The cessation of min.e.p.p. discharge occurs at very nearly the same time as the failure of transmission and may be preceded by a gradual reduction in frequency, while the size of the unit potentials is unchanged.

Influence of the length of the nerve stump. To test the influence of the length of nerve on the time course of degeneration, a series of experiments was made in which the extramuscular nerve stump was kept very short.

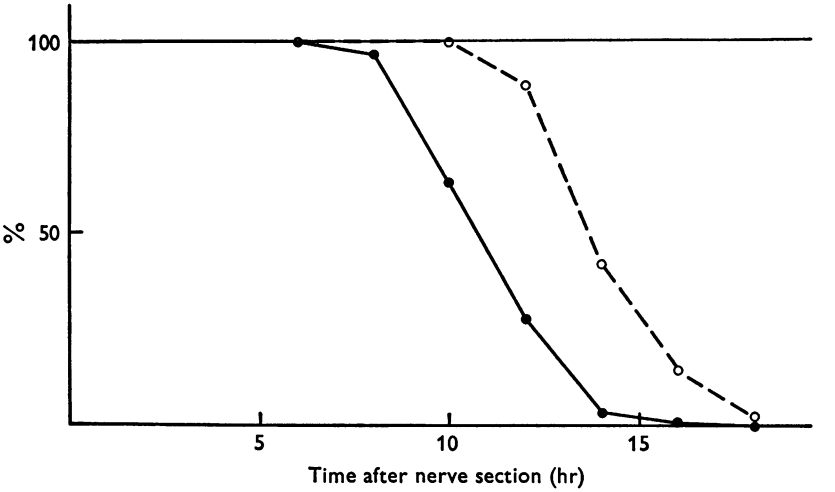


Text-fig. 3. Distribution of min.e.p.p. amplitude observed at four degenerating end-plates where the frequency was less than 5/min. Abscissa, min.e.p.p. amplitude, expressed as a fraction of the mean amplitude for the end-plate from which it was recorded; ordinate, number of observations.

The phrenic nerve was cut close to its entry into the diaphragm, thus reducing the total length of the peripheral stump from a total of 6 cm to the 2 cm left within the muscle. Since the remaining, extramuscular nerve was at most a few millimetres long, stimulation to test transmission was not always practicable, so the presence or absence of spontaneously occurring min.e.p.p.s was then used as the criterion of functional failure.

The effect of cutting the nerve close to the diaphragm was to cause the failure of transmitter release to occur about 3 hr earlier than when the operation was in the neck. This is seen in Text-fig. 4 which shows the clear difference in the initial latent period without substantial alteration of the shape of the curve (cf. Text-fig. 1).

This result shows that the onset of degeneration depends on the length of the *extramuscular* nerve. To see if the length of the *intramuscular* nerve branches had a similar influence we compared end-plates at different distances from the nerve entry. When the results from four successive 0·5 cm intervals along the course of the nerve are considered, a systematic variation in the extent of failure along the nerve is revealed. Regardless of whether the nerve was cut in the neck or at its entry into the muscle, failure occurred sooner at end-plates near the nerve entry than at those



Text-fig. 4. Time course of min.e.p.p. failure in the diaphragm after phrenic nerve section at two different levels. Abscissa, time after operation; ordinate, fraction (per cent) of impalements at which min.e.p.p.s were observed. Filled circles, phrenic nerve cut at its entry into the diaphragm; open circles, nerve cut in neck, 4 cm from diaphragm. Each point represents pooled results from 150 to 200 impalements from several muscles.

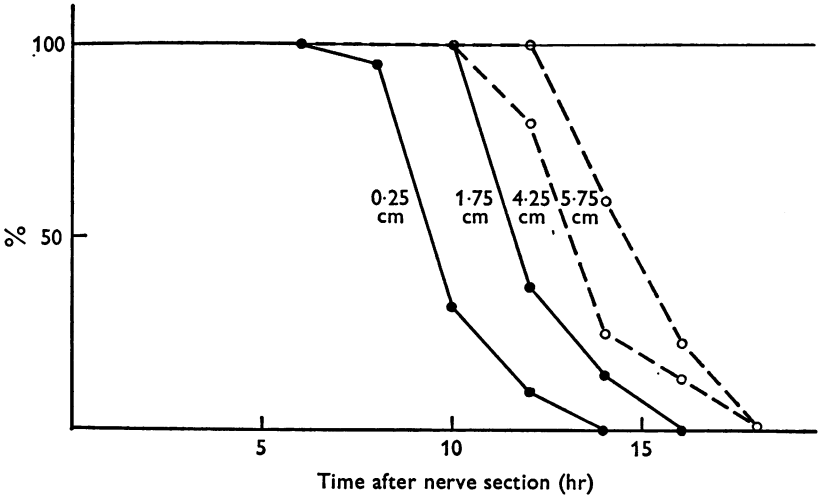
farther from it. The data from the regions of the muscle nearest and farthest from the nerve entry, for both short and long stumps, are shown in Text-fig. 5.

These results show that the duration of the period preceding end-plate failure depends on the length of the severed stump. To present this relation more clearly, the time when failure has occurred at 50 % of the end-plates in each of the four regions of the muscle, for both long and short extramuscular nerve lengths, was estimated from the eight curves such as those in Text-fig. 5. The relation between this time ($t_{50\,\%}$) and the length of the nerve is shown in Text-fig. 6. Thus, the delay in the onset of synaptic degeneration is determined by the nerve stump, and directly related to its length. The relation is such that the period preceding synaptic failure is increased by about 45 min for every additional centimetre of nerve.

End-plate structure during axonal degeneration

Normal endings. In an attempt to correlate the functional failure just described with the structural break-down of the end-plate, muscles were examined in the electron microscope at various times after nerve section.

The structure of normal end-plates in the rat diaphragm was similar to that described elsewhere (Reger, 1959; Andersson-Cedergren, 1959; Miledi & Slater, 1968*a*). The axon terminal contains various organelles: mitochondria, neurofilaments, neurotubules, lyosomes, glycogen, lamellar



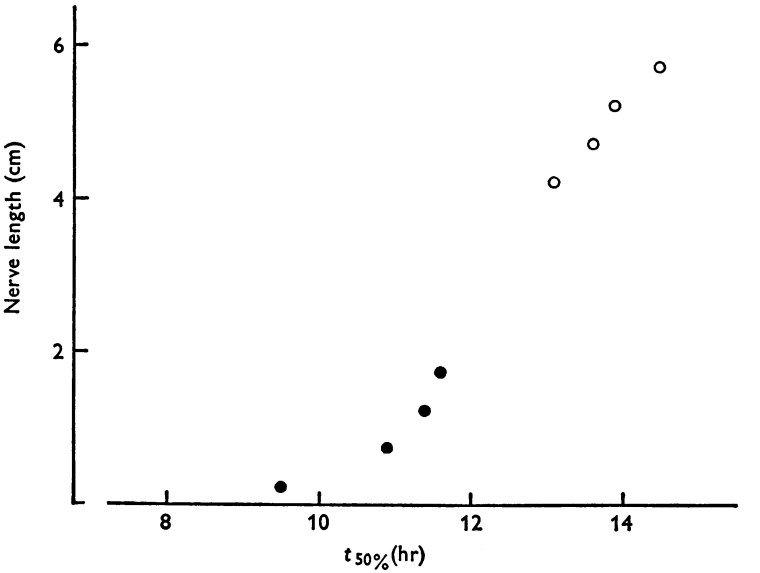
Text-fig. 5. Progressive failure of min.e.p.p.s in regions of muscle associated with different lengths of intramuscular nerve. Abscissa, time after operation; ordinate, fraction (per cent) of impalements at which min.e.p.p.s were observed. Each point represents the pooled observations from about sixty impalements. Points corresponding to the same nerve length have been joined for clarity. The appropriate nerve length is indicated for each curve. Filled circles and continuous lines, nerve cut at entry to diaphragm; open circles and dashed lines, nerve cut in the neck.

bodies, multivesicular bodies and synaptic vesicles (Pls. 1–3). The latter deserve further comment.

Several types of vesicles were found in the axon terminals. The most common were simple, round or oval, vesicles (*ca.* 40 nm diam.) whose interiors appear uniform, but slightly darker than the surrounding cytoplasm. Very early in our work we noticed that complex vesicles, like those originally found by Gray (1961) in the cerebellum of the rat are frequently observed at rat neuromuscular junctions (Pls. 2, 3*a*). These structures consist of a central vesicle, approximately 30–40 nm in diameter, covered by a layer of smaller 'vesicles' (*ca.* 15 nm diam.) the over-all diameter of a complex vesicle being about 70 nm, but some up to 130 nm are also seen

(cf. also Andres, 1964; Düring, 1967; Nickel, Vogel & Waser, 1967). Occasionally other vesicles are found which contain a central dense core. In addition, there are also virus-like clusters of small vesicles (about 10 nm diam.), the total size of the aggregate being only 30–40 nm.

The vesicular organization of the terminal is further complicated by the presence of larger sacs and of tubes of various sizes, some of which appear to be continuous with vesicles. Some of the tubes have a cross-sectional



Text-fig. 6. Influence of the length of the degenerating phrenic nerve segment on the time course of end-plate failure. The time when 50 % of all end-plates in a given region of the diaphragm have failed ($t_{50\%}$) was estimated from the eight curves like those in Fig. 5, and is plotted against total nerve length. Abscissa, nerve length; ordinate, $t_{50\%}$. Filled circles, nerve cut within a few mm of entry into diaphragm; open circles, nerve cut in neck, 4 cm from diaphragm.

appearance similar to that of complex vesicles (Pl. 2). Furthermore, many of the simple vesicles seem to be interconnected by narrow necks, or joined by filaments. It has been suggested (Birks, 1966) that vesicles may arise from the break-down of tubules during fixation. It is difficult to rule out this possibility, but it seems more likely that vesicles and synaptic tubules are a normal constituent of axon terminals, since they are both seen after use of different fixatives (OsO_4, $KMnO_4$, glutaraldehyde, formaldehyde). After such fixatives, vesicles, tubules and microtubules of several types were still seen in the axon terminals, as well as in muscle and Schwann cells. Both complex and simple vesicles are frequently seen opening into the synaptic space, and less commonly into the axon-Schwann cell interspace.

In osmium-fixed tissue stained with phosphotungstic acid the synaptic axolemma shows some dark spots (cf. Gray, 1959; Birks, Huxley & Katz, 1960). These membrane thickenings have a particularly interesting relation to synaptic vesicles which sometimes appeared wedged between the dark spots (Pls. 1, 3*b–d*). Axolemmal thickenings are occasionally seen also on the Schwann side of the axon. In that case the corresponding portion of the Schwann cell membrane also appears darker.

The functions of all the intra-axonal organelles are not known. But with so many processes going on at the synapse, it would not be difficult to speculate on a possible morphological counterpart for some of them. For instance, plain vesicles, confluent or not, may contain the transmitter. Complex vesicles could be associated with the uptake of choline, and other substances, from the synaptic space, in much the same way that similar vesicles are responsible for yolk uptake in oocytes (Roth & Porter, (1964). A similar role for complex vesicles has already been suggested by Andres (1964) and Düring (1967). The small vesicles that form the clusters, and those making the rim of the complex vesicles, might contain the acetyltransferase required to synthesize the acetylcholine. These 'micro-vesicles' have the required dimensions to contain one molecule of the enzyme, which has a molecular weight of about 50,000 (Potter, Glover & Saelens, 1968). If that were so, an interesting possibility would be that micro-vesicles are detached from the complex vesicles when enough acetylcholine has been stored in the central vesicle. But of course, these are only speculations and many other interpretations are possible.

Degenerating endings. After denervation, the normal structure of the end-plate is greatly altered. Many features of end-plate degeneration in the rat are similar to those described for other peripheral and central synapses (Birks, Katz & Miledi 1960; Colonnier, 1964; Hunt & Nelson, 1965; McMahan, 1967; Nickel & Waser, 1968). Two of these will be described in some detail since they provide a convenient clue for the identification of end-plates early in the course of their disintegration.

Mitochondria. In normal nerve endings, the mitochondria are elongated sausage-like structures (Pls. 1–2) whose diameter, after glutaraldehyde fixation, is 0·1–0·2 μm. They sometimes branch or fold, and tend to lie parallel to the long axis of the terminal. The cristae usually lie parallel to the long axis of the mitochondrion. As mitochondria pass in and out of the plane of section, it is difficult to estimate their real length. In some sections they are as long as 1–2 μm, and it is possible that they may sometimes exceed this length.

At end-plates clearly undergoing degeneration, mitochondria had only large circular profiles, indicating that they had acquired a spherical shape (Pls. 4, 5*a*). The diameter of these profiles was typically about 0·4–0·5 μm, although some as large as 0·7 μm were seen. In addition to the increase in diameter, the cristae of these mitochondria sometimes appeared disorganized and broken up into small vesicular fragments. It may be that during degeneration some mitochondria break up into fragments, but it is

interesting that the usual area of these spherical mitochondria is the same as that of a cylinder with hemispherical ends 0·12 μm in diameter and 1·7 μm long. It may therefore be that these profiles represent mitochondria whose surface membranes have remained intact while the forces required to maintain the normal elongated shape have been lost. This situation may thus differ from that in denervated muscle of the rat where the normally long thread-like mitochondria break up into small spherical fragments, each with approximately the same calibre as the normal, and with apparently normal internal structure (Miledi & Slater, 1968*b*).

In some cases, clusters of mitochondria, surrounded by synaptic vesicles, form a compact mass enclosed within the Schwann cell (Pls. 5, 6); and fine dense grains are visible throughout. The nature of this precipitate is unknown. It could be related to the phosphate buffer used during, and after, fixation but whatever its nature it is interesting to note that the precipitate is deposited mainly in the degenerating axonic material (Pls. 5*a*, 6). In these conditions, mitochondria are not always easy to identify, and it is possible that some structures within the clusters represent a stage in the degeneration of other intracellular organelles. Indeed, a variety of other structures is seen in degenerating nerve terminals: bodies made up of concentric layers of membranes, clumps of vesicles reminiscent of the 'honeycomb' structures seen in degenerating frog synapses (Birks, Katz & Miledi 1960; Hunt & Nelson, 1965), bags of glycogen particles, and some more irregular structures enclosed by membranes (see Pls. 4–7).

Schwann cells. At normal mammalian end-plates the Schwann cell is folded into numerous thin layers (Andersson-Cedergren, 1959; Miledi & Slater, 1968*a*). The Schwann cell, and the nerve cell it covers, are surrounded by the ectolemma (basement membrane), which also extends into the junctional cleft between nerve and muscle, and even into the junctional folds of the muscle cell (Pls. 1–3). In the frog neuromuscular junction thin prolongations of the Schwann cell appear occasionally in the synaptic cleft, between the nerve and muscle cells (Birks, Huxley & Katz 1960; Birks, Katz & Miledi, 1960). These 'Schwann cell feet' are relatively rarely seen at normal rat end-plates.

An important feature of nerve terminal degeneration is the participation of the Schwann cell in disposing of the degenerating neural material. What is presumably an initial stage of this process was seen in the form of thin extensions of the Schwann cell (Pls. 4, 5*a*), sometimes little more than the two limiting membranes protruding into the synaptic cleft of degenerating junctions. In some sections these Schwann cell processes completely surround the axon, and it is clear that the area of synaptic contact between nerve and muscle must thereby be greatly reduced. In addition to becoming enveloped by the Schwann cell, and sometimes even before this

happens, the axon terminal undergoes a process of fragmentation. Several small axon profiles were frequently seen in a single synaptic groove, and usually the Schwann cell was interposed between them (Pls. 4, 5).

In what is probably a more advanced state of degeneration, the Schwann cell cytoplasm fills the synaptic groove more completely, and the axoplasm and its inclusions are seen as large, densely stained clumps (Pl. 6). In single sections these dense clumps are entirely surrounded by Schwann cell cytoplasm, but a membrane is sometimes seen enveloping them, and we are not certain that they ever become completely 'intracellular'.

The end-point of this process appears to be the replacement of the axon at the end-plate by the Schwann cell, as illustrated by Pl. 7*a*. However, it should be noted that this position of the Schwann cell is only temporary. A month or so after nerve section, while the characteristic synaptic folds remain in the muscle membrane no cells are seen in such close contact with the end-plate (Miledi & Slater, 1968*a*; Nickel & Waser, 1968).

Using mainly the change of the mitochondrial profiles and the intrusion of the Schwann cell cytoplasm into the synaptic cleft to identify degenerating end-plates, the time course of structural break-down could be compared with that of functional failure of the diaphragm.

End-plates in the latent period. We examined the ultrastructure of thirty-three end-plates in two muscles, 8 hr (short stump) and 11 hr (long stump), after cutting the phrenic nerve. At these times the electrical activity of both muscles was still normal in the sixty-five end-plates examined with micro-electrodes. But the physiological evidence indicates that failure would have occurred in about two thirds of them within the following 4 hr had they remained *in vivo* (see Text-fig. 5).

The structure of thirty-one of these end-plates was not obviously altered. The mitochondrial dimensions and cristae were normal, and Schwann cell processes were not observed in the synaptic cleft more frequently than in normal muscle. Neurofilaments, neurotubules and the various types of vesicles described above were still seen at this time. Lysosomes, membrane whorls and glycogen bags may be more prominent during this period than in normal endings, but more detailed studies are needed to confirm our initial impression. Similarly, in some cases the axoplasm was of unusually low density and appeared to contain relatively few synaptic vesicles, but no end-plates were clearly outside the rather wide range encountered in normal muscle.

In the remaining two end-plates, two clumps of vesicles were found in one region of the ending, while the remainder of the terminal was apparently normal. These two end-plates were near the nerve entry of the 8 hr (short nerve) muscle, which may be correlated with our electrophysiological findings (cf. Text-fig. 5).

End-plates during the period of functional failure. At the time when junctions cease to transmit, some end-plates appear normal while others are clearly undergoing gross disruption. In all we examined thirty-five different end-plates from muscles in intermediate or terminal stages of failure. From observations made on the same or adjoining regions of the diaphragm we estimate that about 40 % of these endings were electrically inactive. On the basis of structural appearance, about 50 % of the end-plates in this group appeared unambiguously to be in the course of degeneration (for example Pls. 4–6). Such end-plates usually had regions in diverse stages of degeneration; sometimes a contact appeared almost normal (Pl. 7*b*), while other regions of the same end-plate could already be completely surrounded by the Schwann cell.

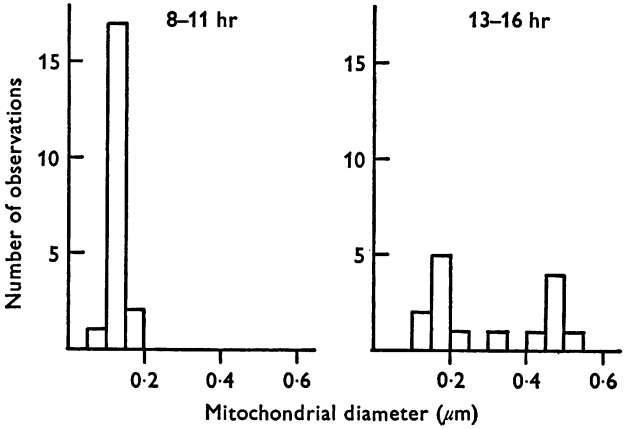
The diameter of the mitochondrial profiles was clearly increased in twelve of the twenty-one endings where measurements were made. While the average diameter of mitochondria of all twenty-one endings was about 0·3 μm, the distribution of these diameters suggests that two subgroups were present, one corresponding to the normal value (0·1–0·2 μm) and the other with an average of about 0·45 μm (Text-fig. 7). Of the end-plates whose mitochondria were enlarged, all but one showed conspicuous extension of the Schwann cell into the synaptic cleft. In only one case was such a Schwann cell extension seen in a section where mitochondria appeared normal. Thus, the alterations in mitochondria and Schwann cell probably occur very nearly simultaneously.

Our findings allow us to make a rough estimate of the time taken for structural break-down of an end-plate to occur. At 8–11 hr after nerve section, depending on the length of nerve attached to the muscle, almost all end-plates still seemed normal. Some 3–5 hr later, about half had swollen mitochondria and were largely surrounded by Schwann cell. In some sections the axon was already almost entirely replaced by the Schwann cell. Thus the break-down can occur within a few hours once the initial latent period is over.

Influence of nerve length. Just as with functional changes, structural changes are delayed if the length of degenerating nerve is increased. For instance, in one muscle with short nerve, denervated for 12$\frac{3}{4}$ hr, a large majority of end-plates showed clear signs of degeneration (Pls. 5, 7*a*); while in a muscle with long nerve attached, 13 hr after denervation, and 85 % of end-plates showing min.e.p.p.s, the majority of the end-plates appeared structurally normal (Pl. 7*b*). The physiological work indicates that these end-plates were very near their period of rapid disintegration, and even at this time most of the organelles within the terminal seemed normal. With a long nerve stump a few apparently normal end-plates were observed even 16 hr after denervation. Nevertheless, it is interesting to

note that the number of complex vesicles seemed to be reduced at these times.

In all the muscles examined in the electron microscope, the degeneration of nerve terminals was less advanced in the region farthest from the nerve entry. This indicates, in further correlation with the physiological findings, that both the extramuscular and intramuscular portions of the nerve stump contribute to prolonging the latency of terminal degeneration after nerve section.



Text-fig. 7. Distribution of the diameters of mitochondria in nerve terminals at different stages of end-plate degeneration. Time after nerve section indicated in each histogram. Abscissa, diameter of mitochondria; ordinate, number of observations.

To summarize, we find that the time course of structural changes of the end-plates closely parallels that of functional failure. For both there is a latent period of 8–12 hr during which no obvious changes are seen. This is followed by a period of similar length, during which all the end-plates in the muscle undergo a process of break-down. The actual time taken for an individual end-plate to disintegrate is only a matter of a few hours. The longer time taken for all the end-plates to degenerate is probably attributable to a lack of synchrony in the degeneration of end-plates; such asynchrony may be due, at least in part, to the intramuscular variations of the diameter and length of nerve fibre connected to each end-plate. The outcome of all this is that 18–20 hr after nerve section all end-plates are in an advanced stage of degeneration and nearly all have ceased to function.

DISCUSSION

It is clear from our study that changes in end-plate structure occur at very much the same time after nerve section as does the failure of neuromuscular transmission. Thus, there seems to be no difficulty in accounting

for the loss of function in terms of the observed structural changes. The alterations of the mitochondria, and the loss of the characteristic disposition of other organelles within the axon terminal would be expected to change its behaviour, and it would be surprising if the processes involved in the release of transmitter were not thereby impaired. In addition to the changes within the axon, the interposition of the Schwann cell between nerve and muscle would presumably prevent any acetylcholine which might be released from reaching the muscle membrane. This reduction in the extent of axonal synaptic contact may well account for those end-plates with very low min.e.p.p. frequencies, described on p. 512. It remains to see if some structural changes occur which can be specifically related to the cessation of the quantal release of transmitter. For this, correlated physiological and morphological observations of single end-plates will probably be necessary.

The changes observed during degeneration of rat end-plates are similar to those seen in the frog (Birks, Katz & Miledi 1960). In both cases, the failure of transmission is abrupt and is accompanied by a nearly simultaneous cessation of min.e.p.p.s. Nerve terminal disruption and proliferation of the Schwann cell are also seen in the two animals (cf. also Reger, 1959; Nickel & Waser, 1968), as well as in degenerating synapses of the frog sympathetic ganglion (Hunt & Nelson, 1965) and the vertebrate central nervous system (Colonnier, 1964; McMahan, 1967; see also Gray & Guillery, 1966). It seems that a common process of degeneration occurs in neurones of widely different functions and in a number of vertebrate species.

In some invertebrates, however, the response to nerve section can be quite different. Crayfish motor nerve terminals isolated from their cell bodies retain the ability to transmit action potentials for up to 3 months, during which time regeneration of the central stump may occur (Hoy, Bittner & Kennedy, 1967). In locust, while the failure of transmission occurs within 2 days at 30° C, min.e.p.p. discharge continues for some time, ultimately going through a complicated sequence of changes of amplitude and frequency before failing completely. At the time when transmission fails, the structure of the neuromuscular junction is grossly intact, although swelling of the mitochondria and clumping of the synaptic vesicles is seen (Usherwood, 1963; Usherwood, Cochrane & Rees, 1968). These observations suggest that one of the differences between the degeneration of vertebrate and invertebrate synapses may be in the role played by the Schwann cell.

The time course of the degenerative changes at end-plates in the diaphragm is of considerable interest. Our observations, while of a statistical nature, indicate that at end-plates in the diaphragm, nerve section is followed by a period of at least 8 hr when grossly normal structure and function

are maintained. After this initial delay, obvious changes occur, leading in a few hours to proliferation of the Schwann cell, complete disruption of the nerve terminal, and abrupt failure of neuromuscular transmission. While these changes are going on, normal electrical activity of the myelinated axons can still be recorded. Several closely related questions are raised by these findings: What initiates the internal disruption of the nerve terminal? What leads to the 'activation' of the Schwann cell? What controls the timing of these events?

The results of varying the length of the degenerating nerve stump are relevant to the last of these questions. These experiments show that at least a part of the survival time of an end-plate after nerve section is determined by its distance from the site of injury. Further, they define an apparent velocity with which the effect of cutting the axon reaches the presynaptic terminal. This is given by the relation in Text-fig. 6 which has a slope of about 1·5 cm/hr. This value is about ten times greater than that derived from similar experiments on the tenuissimus of the cat (Luco & Eyzaguirre, 1955). We have no adequate explanation for this difference.

The basis for the effect of nerve length is unlikely to be purely physical. A diffusion limited process would be expected to depend on the square of the distance, rather than on the first power. A similar argument probably applies to any change in hydraulic pressure which might occur. On the other hand, one might suppose that the process of end-plate degeneration is initiated whenever the concentration of some substance, originating in the cell body, falls below a critical level in the axoplasm of the nerve ending. If this substance were being used, or lost, at the end-plate, it would have to be continuously replenished from the soma. Transecting the nerve would limit the amount of substance available to the ending, to that present in the isolated nerve segment. The magnitude of the delay of degeneration resulting from the amount of 'trophic' substance present in the nerve trunk would depend on the balance between the rate at which the substance is used up, and the rate at which it is transported to the terminal. It is perhaps relevant that the apparent velocity we have observed is very similar to that indicated by several recent studies on the movement of substances in motor and sensory nerves (Miani, 1963; McEwen & Grafstein, 1968; Lasek, 1968; Ochs, Sabri & Johnson, 1969). Thus, it may well be that the duration of the period of end-plate survival after nerve section represents in part the time required for the translocation of material from the site of injury to the periphery by a normally occurring transport system.

One assumption of this scheme is that such a transport system, if it exists, continues to function for at least 8 hr after the loss of continuity with the cell body. Available evidence suggests that at least the gross integrity of the axon is maintained for this length of time. As noted above,

electrical activity of the nerve is essentially normal for up to 24 hr. Structural changes within the axoplasm may occur before this time, but probably not much before 12 hr after section (Terry & Harkin, 1959; R. Miledi, unpublished observation). It is further known that the movement of intracellular material in isolated chick neurites (Hughes, 1953) as well as in some protozoa (Allen, Cooledge & Hall, 1960; Thompson & Wolpert, 1963) persists for some hours in the absence of the nucleus. Clearly, a first step in obtaining direct evidence for the scheme we suggest would be the demonstration that a transport system does function, at the requisite rate, in the isolated phrenic nerve.

Our experiments do not indicate the chemical nature of the factors which cause the end-plate to degenerate. The nerve terminal could conceivably respond to a decrease either in the rate of appearance or of the concentration of some essential metabolite, or to the presence of a deleterious factor produced at the site of injury. We are equally ignorant of the factors responsible for the 'activation' of the Schwann cell. One possibility is that the Schwann cell responds to secondary changes in the neurones. Such an hypothesis would be supported if changes in the nerve terminal clearly preceded those in the Schwann cell. However, so far we have been unable to resolve such a difference in timing.

C. R. Slater held a Marshall Scholarship during the early stages of this work, and was later supported by U.S. Public Health Service fellowship 1-F1-GM-15, 010-01A1.

REFERENCES

ALLEN, R. D., COOLEDGE, J. W. & HALL, P. J. (1960). Streaming in cytoplasm dissociated from the giant amoeba, *Chaos chaos*. *Nature, Lond.* **187**, 896–899.

ANDERSSON-CEDERGREN, E. (1959). Ultrastructure of motor end-plate and sarcoplasmic components of mouse skeletal muscle fiber. *J. Ultrastruct. Res.* suppl. **1**.

ANDRES, K. H. (1964). Mikropinozytose im Zentralnervensystem. *Z. Zellforsch. mikrosk. Anat.* **64**, 63–73.

BIRKS, R. I. (1966). The fine structure of motor nerve endings at frog myoneural junctions. *Ann. N.Y. Acad. Sci.* **135**, 8–19.

BIRKS, R., HUXLEY, H. E. & KATZ, B. (1960). The fine structure of the neuromuscular junction. *J. Physiol.* **150**, 134–144.

BIRKS, R., KATZ, B. & MILEDI, R. (1960). Physiological and structural changes at the amphibian myoneural junction, in the course of nerve degeneration. *J. Physiol.* **150**, 145–168.

COERS, C. (1953). Contribution à l'étude de la jonction neuromusculaire. II. Topographie zonale de l'innervation motrice terminale dans les muscles striés. *Archs Biol., Paris* **64**, 495–508.

COLONNIER, M. (1964). Experimental degeneration in the cerebral cortex. *J. Anat.* **98**, 47–53.

DÜRING, M. V. (1967). Uber die Feinstruktur der motorischen Endplatte von höherin Wirbeltieren. *Z. Zellforsch. mikrosk. Anat.* **81**, 74–90.

EYZAGUIRRE, C., ESPILDORA, J. & LUCO, J. V. (1952). Alterations of neuromuscular synapses during Wallerian degeneration. *Acta physiol. latinoam.* **2**, 213–227.

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

GRAY, E. G. (1959). Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.* **93**, 420–433.

GRAY, E. G. (1961). The granule cells, mossy synapses and Purkinje spine synapses of the cerebellum: light and electron microscope observations. *J. Anat.* **95**, 345–356.

GRAY, E. G. & GUILLERY, R. W. (1966). Synaptic morphology in the normal and degenerating nervous system. *Int. Rev. Cytol.* **19**, 111–182.

GUTMANN, E., VODIČKA, Z. & ZELENÁ, J. (1955). Veranderungen in quergestreiften Muskel bei Durchtrennung in Abhangigkeit von der Länge des peripheren Stumpfes. *Physiologia bohemoslov.* **4**, 200–204.

HOY, R. R., BITTNER, G. D. & KENNEDY, D. (1967). Regeneration in Crustacean motoneurons: evidence for axonal fusion. *Science, N.Y.* **156**, 251–252.

HUGHES, A. (1953). The growth of embryonic neurites. A study on cultures of chick neural tissues. *J. Anat.* **87**, 150–162.

HUNT, C. C. & NELSON, P. G. (1965). Structural and functional changes in the frog sympathetic ganglion following cutting of the presynaptic nerve fibres. *J. Physiol.* **177**, 1–20.

KRNJEVIĆ, K. & MILEDI, R. (1959). Presynaptic failure of neuromuscular propagation in rats. *J. Physiol.* **149**, 1–22.

LASEK, R. (1968). Axoplasmic transport in cat dorsal root ganglion cells: as studied with [^{3}H]leucine. *Brain Res.* **7**, 360–377.

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

LISSÁK, K., DEMPSEY, E. W. & ROSENBLUETH, A. (1939). The failure of transmission of motor nerve impulses in the course of Wallerian degeneration. *Am. J. Physiol.* **128**, 45–56.

LUCO, J. V. & EYZAGUIRRE, C. (1955). Fibrillation and hypersensitivity to ACh in denervated muscle: effect of degenerating nerve fibres. *J. Neurophysiol.* **18**, 65–73.

MCEWEN, B. S. & GRAFSTEIN, B. (1968). Fast and slow components in axonal transport of proteins. *J. cell Biol.* **38**, 494–508.

MCMAHAN, U. J. (1967). Fine structure of synapses in the dorsal nucleus of the lateral geniculate body of normal and blinded rats. *Z. Zellforsch. mikrosk. Anat.* **76**, 116–146.

MIANI, N. (1963). Analysis of the somato-axonal movement of phospholipids in the vagus and hypoglossal nerves. *J. Neurochem.* **10**, 859–874.

MILEDI, R. & SLATER, C. R. (1968*a*). Electrophysiology and electron-microscopy of rat neuromuscular junctions after nerve degeneration. *Proc. R. Soc.* B **169**, 289–306.

MILEDI, R. & SLATER, C. R. (1968*b*). Some mitochondrial changes in denervated muscle. *J. cell Sci.* **3**, 49–54.

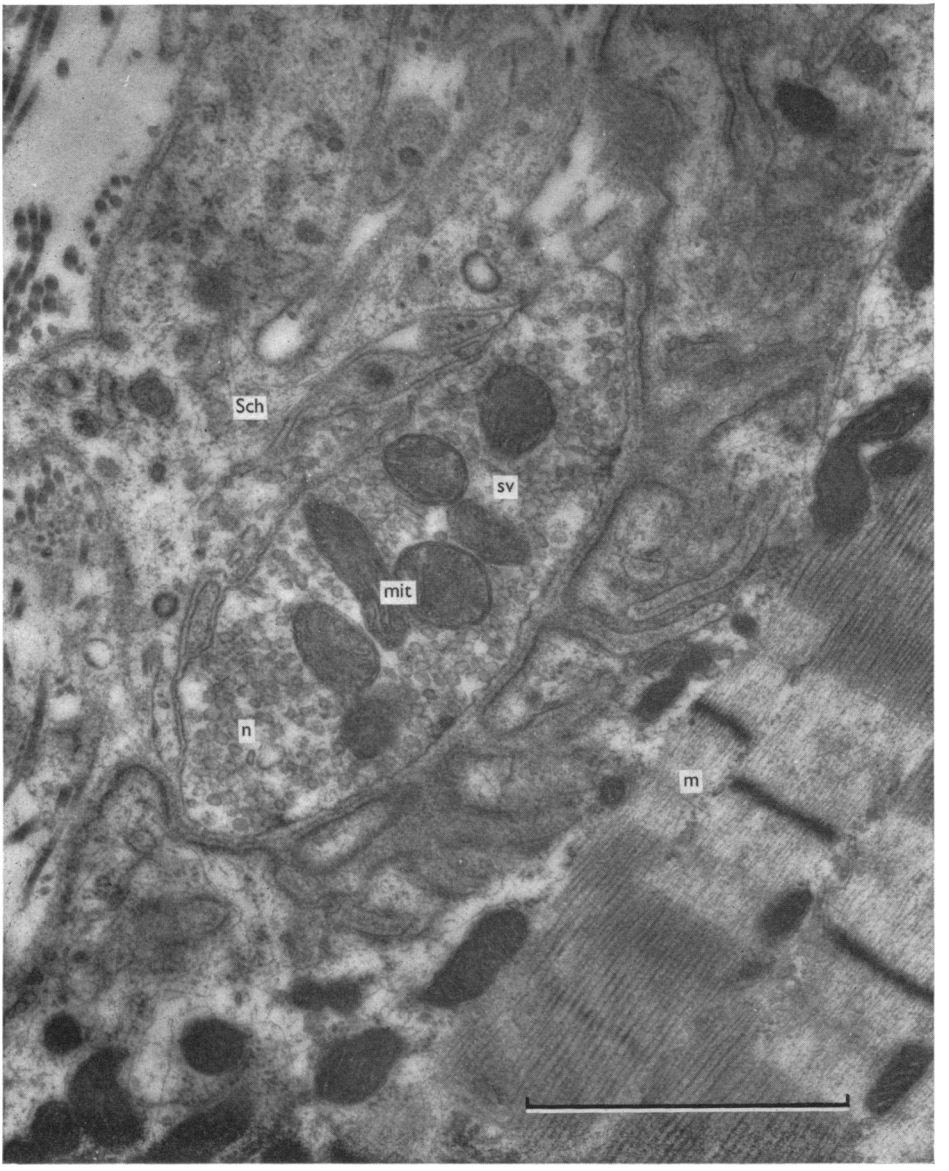
NICKEL, E., VOGEL, A. & WASER, P. G. (1967). Coated vesicles in der Umgebung der neuro-muskulären synapsen. *Z. Zellforsch. mikrosk. Anat.* **78**, 261–266.

NICKEL, E. & WASER, P. G. (1968). Elektronenmikroskopische untersuchungen am diaphragma der maus nach einseitiger phrenikotomie. I. Die degenerierende motorische endplatte. *Z. Zellforsch. mikrosk. Anat.* **88**, 278–296.

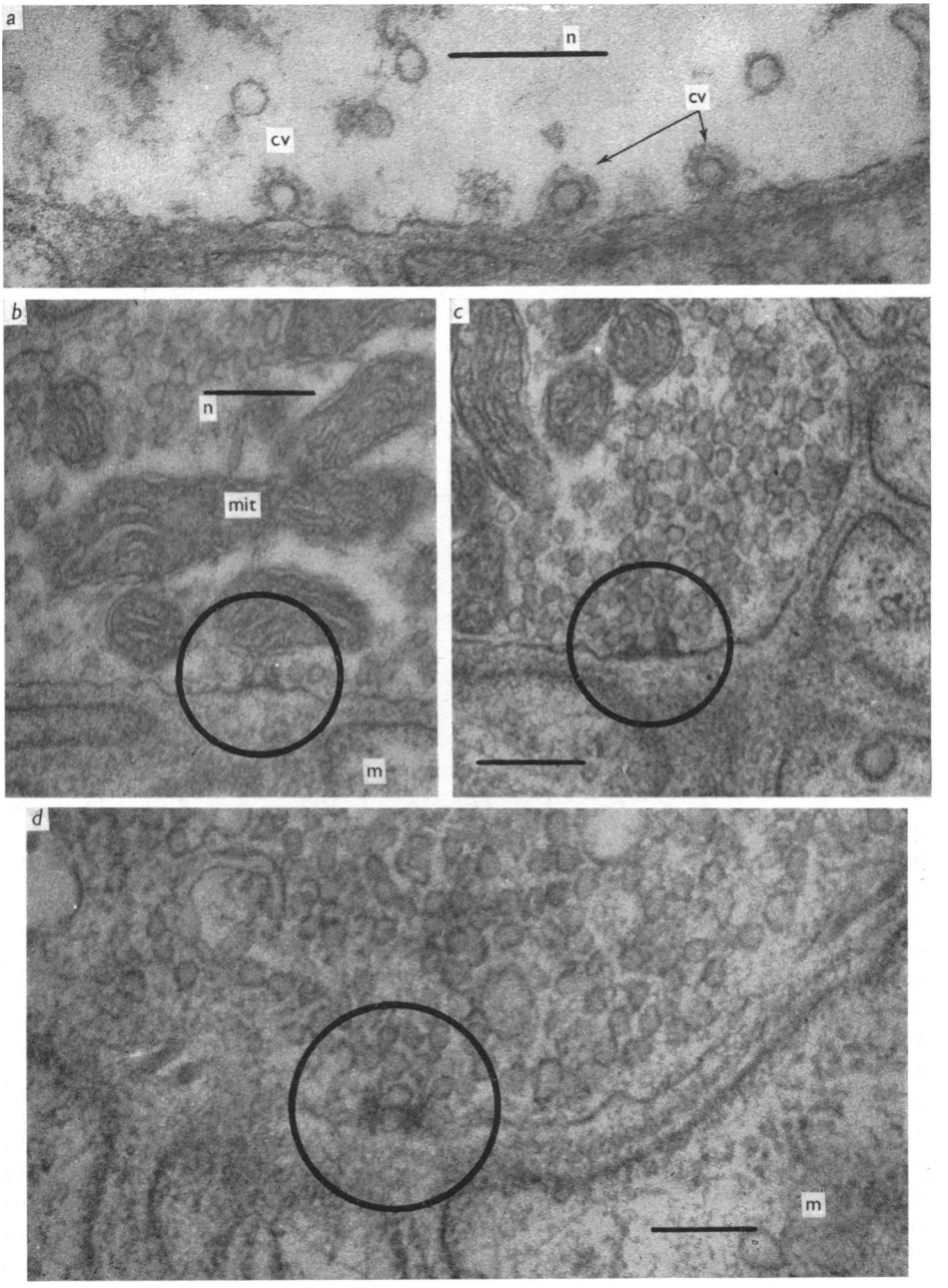
OCHS, S., SABRI, M. I. & JOHNSON, J. (1969). Fast transport system of materials in mammalian nerve fibres. *Science, N.Y.* **163**, 686–687.

POTTER, L. T., GLOVER, V. A. S. & SAELENS, J. K. (1968). Choline acetyltransferase from rat brain. *J. biol. chem.* **243**, 3864–3870.

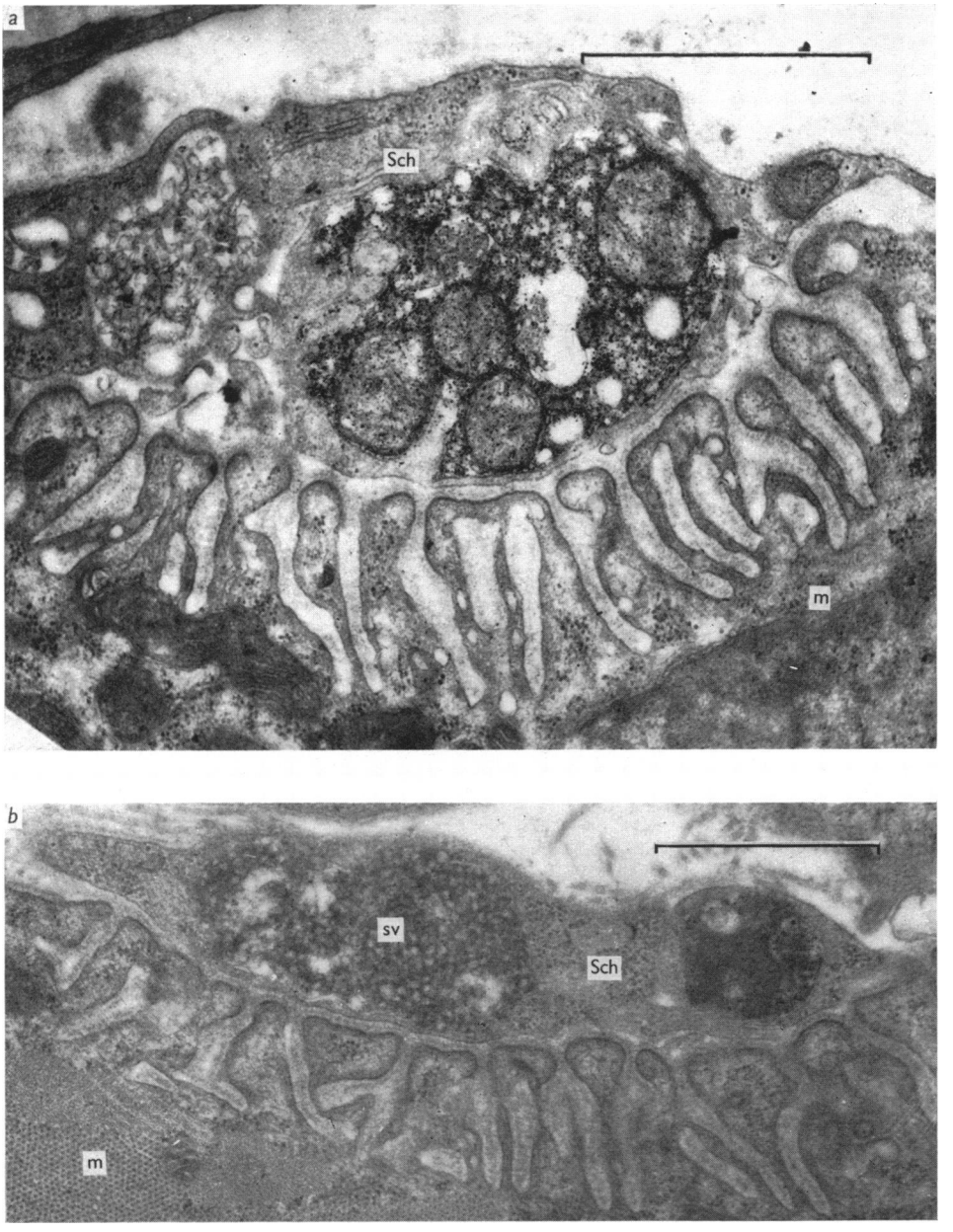
REGER, J. F. (1959). Studies on the fine structure of normal and denervated neuromuscular junctions from mouse gastrocnemius. *J. Ultrastruct. Res.* **2**, 269–282.

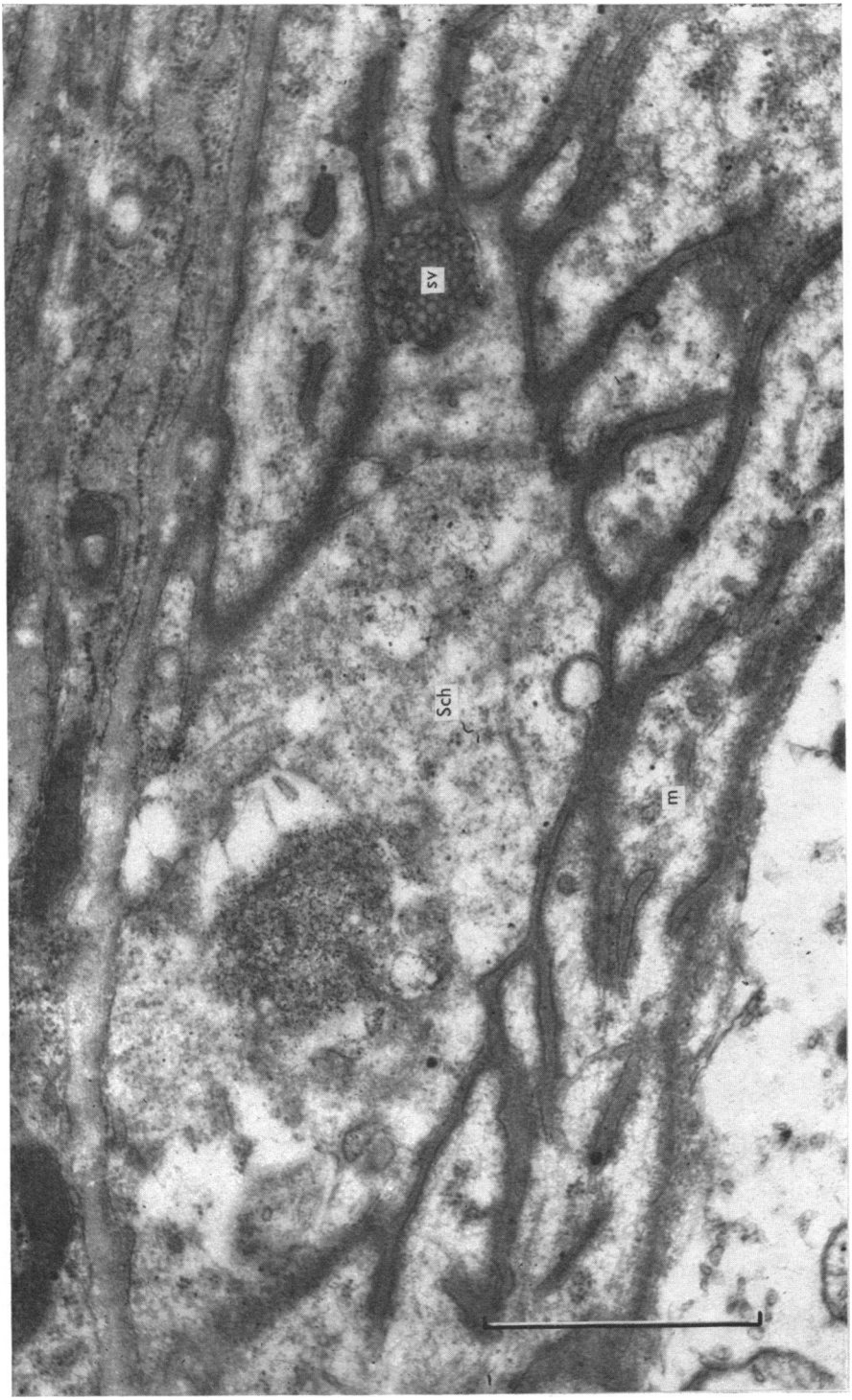

Sch
sv
mit
n
m


Sch
n
m
cv

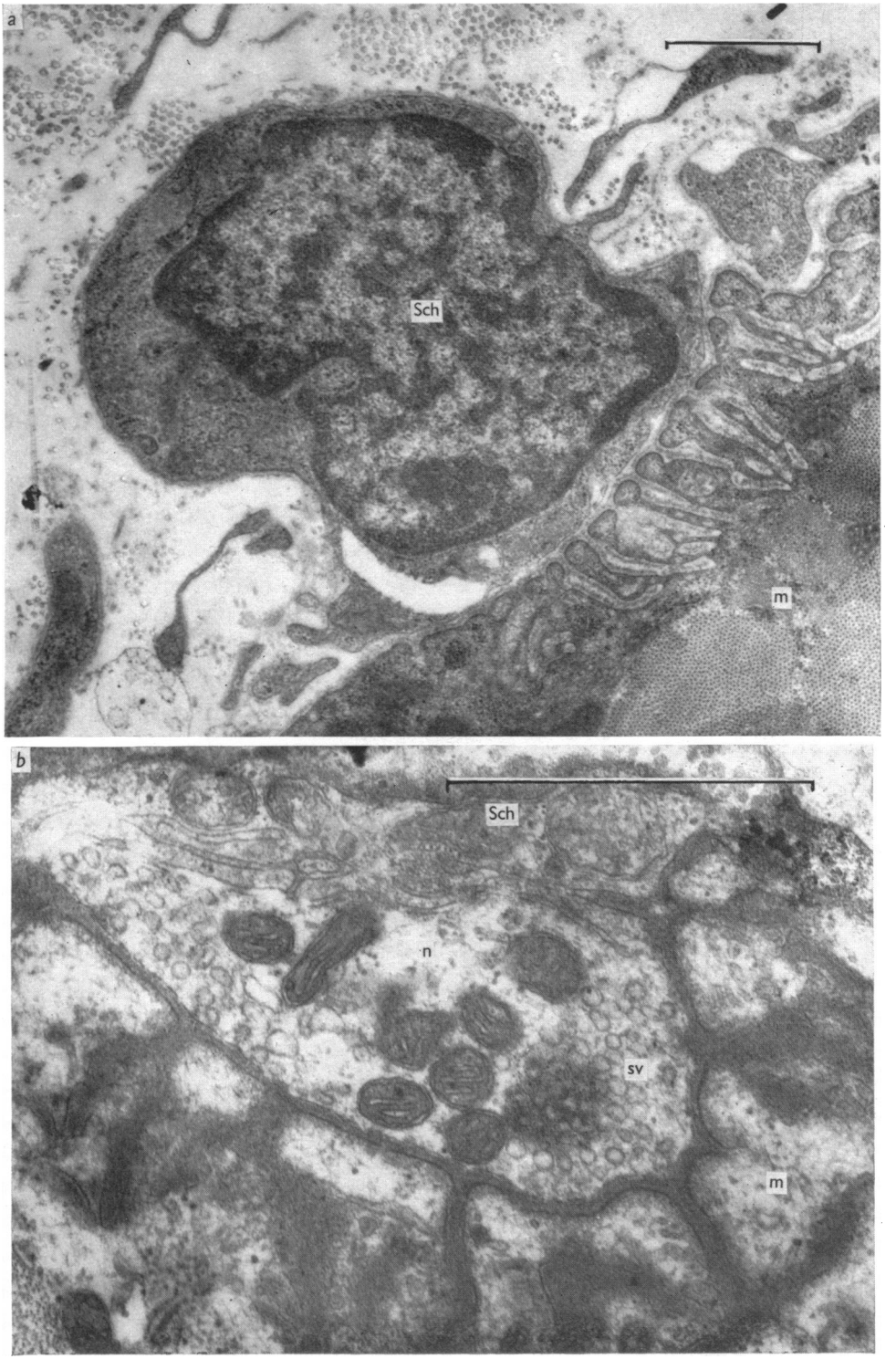

a
n
cv
cv
b
n
mit
m
c
d
m


n
Sch
n
mit
m
n
n


a
Sch
m
b
sv
Sch
m



R. MILEDI AND C. R. SLATER


a
Sch
m
b
Sch
n
sv
m

ROTH, T. F. & PORTER, K. R. (1964). Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti* L. *J. cell Biol.* **20**, 313–332.

SLATER, C. R. (1966). Time course of failure of neuromuscular transmission after motor nerve section. *Nature, Lond.* **209**, 305–306.

TERRY, R. D. & HARKIN, J. C. (1959). Wallerian degeneration and regeneration of peripheral nerves. In *The Biology of Myelin*, ed. KOREY, S. A., pp. 303–320. New York: Paul B. Hoeber.

THOMPSON, C. M. & WOLPERT, L. (1963). The isolation of motile cytoplasm from *Amoeba proteus*. *Expl Cell Res.* **32**, 156–160.

TITECA, J. (1935). Etude des modifications fonctionelles du nerf au cours de la dégénérescence Wallerienne. *Archs int. Physiol.* **41**, 2–56.

USHERWOOD, P. N. R. (1963). Response of insect muscle to denervation II. Changes in neuromuscular transmission. *J. Insect Physiol.* **9**, 811–825.

USHERWOOD, P. N. R., COCHRANE, D. G. & REES, D. (1968). Changes in structural, physiological and pharmacological properties of insect excitatory nerve-muscle synapses after motor nerve section. *Nature, Lond.* **218**, 589–591.

EXPLANATION OF PLATES

All electron micrographs from rat diaphragm. Pls. 1, 3*b–d* from muscles fixed in OsO_4 and stained with phosphotungstic acid. All other plates from muscles fixed in glutaraldehyde and stained with uranyl acetate and lead. All calibration bars indicate 1 μm, except Pl. 3, where they indicate 0·2 μm.

The following symbols are used on the Plates: n: nerve; m: muscle; Sch: Schwann cell; mit: mitochondrion; sv: synaptic vesicles; cv: complex vesicle.

PLATE 1

Normal end-plate showing complex folding of Schwann cell, nerve terminal with synaptic vesicles and mitochondria, and muscle fibre. Note junctional folds in surface of muscle fibre, and three regions of high density in synaptic surface of nerve terminal.

PLATE 2

Normal end-plate showing same structures as in Plate 1. Note complex vesicles in axoplasm.

PLATE 3

Normal end-plate at higher magnification. *a*, complex vesicles in nerve terminal. *b–d*, high density region of presynaptic vesicles surrounded by palisade of electron-dense material.

PLATE 4

End-plate denervated 16 hr, long nerve (see Methods). Note swollen mitochondria, thin extensions of Schwann cell into synaptic cleft, and fragmentation of nerve terminal.

PLATE 5

Two end-plates denervated 12¾ hr, short nerve. *a*, shows accumulation of high density material consisting in part of mitochondria. Dense granular material of unknown origin or composition is associated with degenerating axonal material. *b*, shows dense clump of synaptic vesicles in left nerve fragment.

PLATE 6

End-plate denervated 16 hr, long nerve. The Schwann cell has almost completely replaced the axon in the synaptic cleft leaving two clumps of degenerating axonal material.

PLATE 7

a, end-plate denervated 12¾ hr, short nerve. The large Schwann cell completely replaces the nerve in this section. *b*, end-plate denervated 13 hr, long nerve. The effect of the long nerve, in delaying structural break-down, is seen in the nearly normal structure of this end-plate, as compared with those in Pls. 5 and 7*a*. Note area of dense granular material which may indicate an early stage of degeneration.