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SUPERSENSITIVITY OF SKELETAL MUSCLE PRODUCED BY BOTULINUM TOXIN

BY S. THESLEFF
From the Department of Pharmacology, University of Lund, Sweden

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In a chronically denervated mammalian skeletal muscle the entire membrane becomes sensitive to applied acetylcholine (ACh). About 4 days after denervation, the size of the ACh-sensitive area at the end-plate starts to increase and a few days later covers the larger part or all of the muscle membrane (Axelsson & Thesleff, 1959).

The conversion of the membrane, following denervation, into an ACh-sensitive surface might be due to the absence of some chemical influence exerted when the motor innervation is intact. The purpose of the present investigation was to see whether or not the release of the chemical transmitter agent might provide such an influence. Use was made of botulinum toxin, which is considered to prevent release of ACh from cholinergic nerve terminals (Burgen, Dickens & Zatman, 1949; Brooks, 1956). This mode of action of the toxin was confirmed, and it will be shown that when the transmitter output was reduced or abolished the size of the receptor area in muscle started to increase in a manner which was identical with that observed after denervation.

METHODS

Unless otherwise stated the experiments were made on the isolated tenuissimus muscle of the cat. The sensitivity of individual muscle fibres to ACh was determined by iontophoretic release of the drug from the tip of a micropipette as described by del Castillo & Katz (1955). When the tip of the pipette was close to the receptor structure, the ACh released by a current pulse of 10 msec duration produced a transient membrane depolarization of a few millivolts amplitude. This potential change was recorded with a conventional capillary micro-electrode inserted into the muscle fibre close to the point of drug application. For details of the experimental set-up, techniques for drug application and recording see Axelsson & Thesleff (1959).

The end-plate region of individual muscle fibres was located by pursuing fine superficial nerve twigs and by the appearance of miniature end-plate potentials (m.e.p.p.s) with a rapid time course. The mean frequency of m.e.p.p.s was calculated from recordings made over several minutes on moving film or with an ink-writer. The part of the muscle membrane at which ACh, when released from a micropipette, produced a depolarization with a latency of less than 10 msec was considered sensitive to the drug. Its length was measured with a binocular dissecting microscope and an eyepiece scale at 80 × magnification.

A powdered preparation of *Cl. botulinum* toxin type A with a mouse LD_{50} of 0·05 μg/kg was used. 1 mg of the toxin was dissolved in 1 ml. of sterile phosphate buffer as described by Ambache (1949). Further dilutions of the toxin were made from this stock solution immediately before use. A fresh stock solution was prepared for each day's experiment.

The toxin, in amounts ranging from 0·01 to 15 μg, was either applied to the exposed surface of the tenuissimus muscle or injected in divided amounts into the musculature of the hind leg. With these doses and modes of administration the action of the toxin was confined mainly to the site of application and generalized intoxications were usually avoided. Two to four weeks after the administration of the toxin the tenuissimus muscle was removed in pentobarbitone anaesthesia. In experiments on frog (*Rana temporaria*) the toxin was injected under the skin of the ventral surface of the thigh.

For examination of the ultrastructure of motor nerve terminals a number of botulinum-intoxicated and control muscles were removed and fixed at 4° C for 1 hr in 1% osmium tetroxide solution buffered with veronal-acetate to about pH 7·5, according to the procedure described by Palade (1952). After fixation the tissue was dehydrated in ethanol and small pieces of tissue containing end-plates were cut out. The specimens were stained in 1% phosphotungstic acid in absolute alcohol and embedded in 'Araldite' according to the method of Glauert & Glauert (1958).

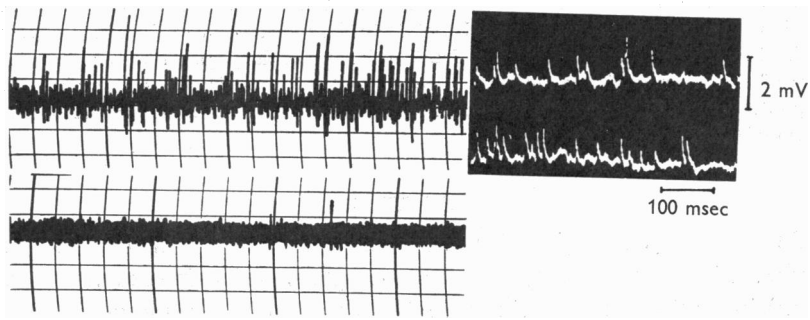
RESULTS

The ultrastructure of motor nerve terminals in botulinum-intoxicated muscles. It has previously been found by R. Thies (personal communication) in acute experiments on isolated serratus anterior muscles of the guinea-pig, that the paralysis produced by bath application of botulinum toxin was not accompanied by structural changes in the presynaptic vesicles. It seemed possible, nevertheless, that chronic intoxication might induce degenerative changes in the nerve endings similar to those described by Birks, Katz & Miledi (1960) during Wallerian degeneration. However, no structural abnormalities were found with the present technique in end-plates from paralysed frog and cat muscles, even after periods of 3–4 weeks. Examples are shown in Pls. 1–3, taken from a frog's sartorius 13 days and from a cat's tenuissimus 27 days after botulinum injection. During the experiments neither of these muscles showed any m.e.p.p.s or responses to nerve stimulation, and the cat muscle had become supersensitive to ACh all along its length. If one compares the micrographs with those from normal end-plates (for frog muscle, see Birks, Huxley & Katz, 1960; for mammalian muscle, see Reger, 1958, and Andersson-Cedergren, 1959), the internal structure of the nerve endings and the relations of their membranes to the muscle fibre and its junctional folds appear to be quite unchanged, nor is there any difference in the size and spatial distribution of the presynaptic vesicles.

The present method would not, of course, reveal changes of a much more minute nature. Thus to demonstrate the location of the toxin molecules or to show up slight changes in the structural detail of the terminal nerve membrane would require a more powerful technique.

Effects of botulinum toxin on transmitter release. As described by Guyton & MacDonald (1947) it was observed that the neuromuscular block produced by a single dose of botulinum toxin reached its maximum in about 5 days and thereafter remained at a constant level for a period of several months.

In amounts exceeding 1 μg the toxin completely abolished transmitter release from motor nerve terminals in the tenuissimus muscle of the cat. In such muscles spontaneous m.e.p.p.s were normally absent and end-plate potentials (e.p.p.s) were not recorded following nerve stimulation.



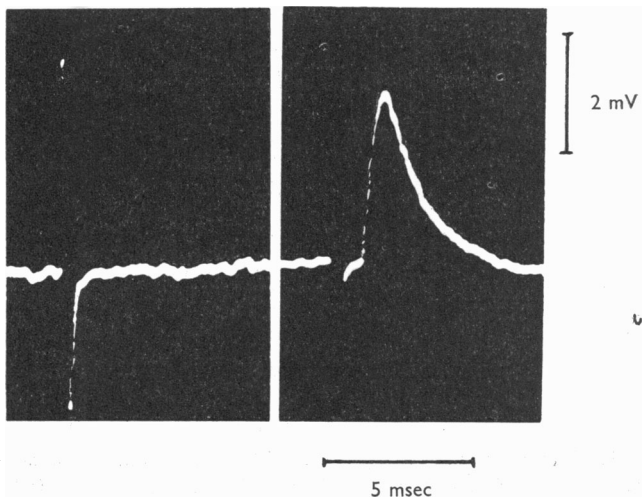
Text-fig. 1. The insertion of the tip of the micro-electrode into the end-plate region of a single muscle fibre gave rise to the burst of m.e.p.p.s shown in the upper ink and cathode-ray oscilloscope recordings. Two minutes later the electrical activity at the end-plate had almost subsided (lower record). The records are from an experiment made on the tenuissimus muscle of a cat which 3 weeks previously had received an injection of 2 μg of botulinum toxin into the hind leg. Neuromuscular transmission was completely blocked and transmitter release was not observed in any other end-plate. Calibration of ink recordings: upper record, 3 squares = 1 mV and 1·5 sec; lower record, 3 squares = 1 mV and 15 sec.

However, the insertion of the micro-electrode occasionally gave rise to a short-lasting burst of m.e.p.p.s at a high frequency of discharge (Text-fig. 1). Apparently this was due to mechanical injury to nerve terminals caused by the tip of the electrode when it penetrated the muscle membrane in the end-plate region.

With smaller quantities of the toxin (0·05–1 μg) m.e.p.p.s with a frequency of discharge about 100 times less than in a normal muscle were observed (mean intervals were of the order of 10–100 sec instead of 0·1–1 sec). In confirmation of earlier investigations (Brooks, 1956) it was found that the amplitude and time course of these m.e.p.p.s were roughly the same as those recorded at a normal end-plate. In these muscles nerve stimulation was either ineffective or produced an e.p.p. of a few millivolts amplitude. At such a relatively light state of intoxication the addition of Ca^{2+} to the bathing fluid in a concentration of twice normal increased the trans-

mitter output by a nerve stimulus (Text-fig. 2). Tetraethylammonium in a concentration of 1 mM had a similar facilitatory action (cf. Koketsu, 1958).

Katz & Miledi (1959) made the interesting observation that in denervated frog muscle spontaneous subthreshold potential changes reappear at the end-plate region several days after cessation of activity. This renewed activity resembled the normal m.e.p.p.s except that the average frequency of discharge was much lower and that the amplitude distribution was wider. Probably end-plate potentials which occur in denervated frog muscle are produced by quanta of ACh released by the Schwann cell, as suggested by Birks, Katz & Miledi (1959).

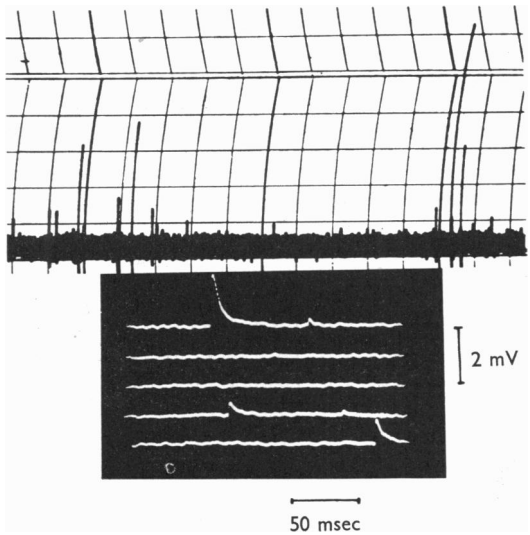


Text-fig. 2. The addition of a twice normal concentration of Ca^{2+} to the bathing fluid resulted in an e.p.p. in response to a nerve stimulus (right-hand record). Before the increase in the external $[Ca^{2+}]$, nerve stimulation had no effect (left-hand record).

Since the origin of ACh release in a chronically denervated junction presumably differs from that in an innervated one it was of interest to see whether botulinum toxin would also prevent this type of spontaneous activity. In frogs kept at room temperature the sartorius muscle was denervated on one side and 5 days later 2 μg of botulinum toxin was injected into each leg. This amount of toxin produced a complete paralysis of all leg muscles. Two weeks later the innervated as well as the denervated sartorius muscle was removed, and both were examined for the presence of spontaneous electrical activity at the end-plate region. As shown in Text-fig. 3, a slow rate of spontaneous discharge was observed at end-plate regions in a denervated and botulinum-treated muscle. In the muscle with an intact nerve, however, the same amount of toxin had completely

abolished m.e.p.p.s. Spontaneous ACh release in a chronically denervated junction appears consequently to be less affected by the action of botulinum toxin than the ACh release which in the innervated end-plate originates from motor nerve terminals.

ACh-sensitivity of botulinum-intoxicated muscles. When transmitter release is blocked by the use of botulinum toxin the fibres of the tenuissimus muscle become sensitive to applied ACh along their entire length.



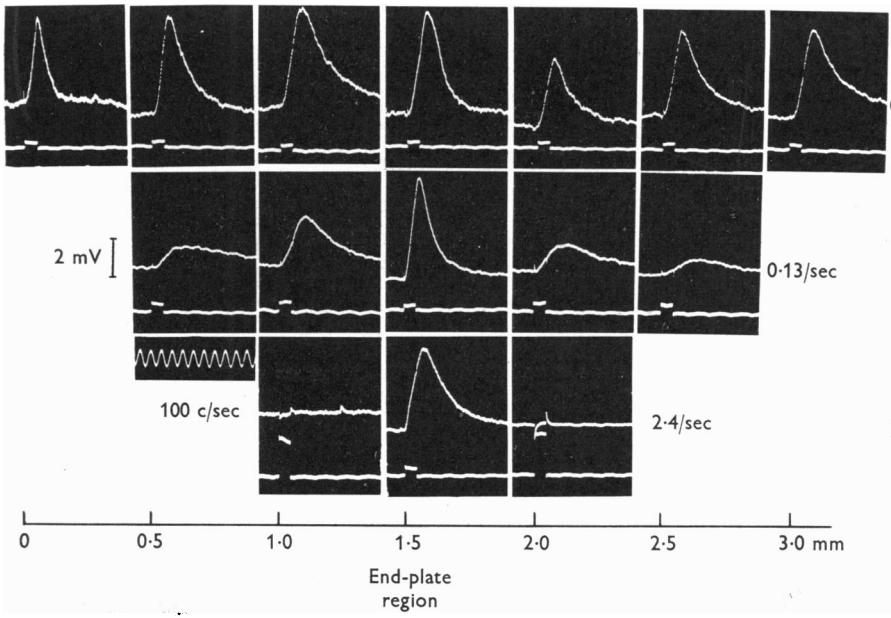
Text-fig. 3. Spontaneous subthreshold activity in a chronically denervated and botulinum-intoxicated frog end-plate (for explanation see text). Calibration of upper record: 3 squares = 1 mV and 15 sec.

One to two weeks after the administration of the toxin, ACh released from the tip of a micropipette produces depolarizations with a rapid time course wherever it is applied to the muscle membrane. The whole surface of the muscle fibre becomes as sensitive to ACh as the end-plate region, which maintains its original responsiveness to the drug (Text-fig. 4). The uniform sensitivity of the muscle membrane to ACh is similar to that which has previously been observed in chronically denervated mammalian muscles (Axelsson & Thesleff, 1959). As in a chronically denervated tenuissimus muscle, ACh produces in a botulinum-intoxicated one a graded and 'electrically silent' contracture.

With a small quantity of the toxin (0·01–0·05 μg) it is possible to obtain tenuissimus muscles in which the ACh-sensitive surface of individual muscle fibres varies in size. This is illustrated in Text-fig. 4. In some fibres the membrane was uniformly sensitive to ACh over at least 3 mm, as is shown in the upper records. In another muscle fibre (middle records) about

2 mm was sensitive to applied ACh, with the highest sensitivity at the end-plate. In a third fibre (lower records) only the end-plate region responded to ACh.

When spontaneous m.e.p.p.s were completely absent or when they appeared at a very low rate (< 0·1/sec) the whole fibre was invariably sensitive to ACh (Text-fig. 4, Table 1). With a higher rate of m.e.p.p. discharge (0·1–1·0/sec) the receptor surface was enlarged as compared to



Text-fig. 4. In a tenuissimus muscle, intoxicated 3 weeks earlier with a small amount of botulinum toxin, muscle fibres were observed in which the ACh-sensitive surface varied in size. The sensitivity of the membrane to ACh was tested by iontophoretic micro-application of the drug. The membrane potential of the fibre is recorded in the upper tracing of each record and the current passing through the pipette in the lower tracing. The fibre used for the upper records was uniformly sensitive to applied ACh over a distance of at least 1·5 mm at each side of the end-plate. The size of the ACh-sensitive surface in two other fibres was smaller (middle and lower records). The frequencies at which m.e.p.p.s occurred are shown by the figures to the right of the records.

normal, whereas at a frequency above 1·0/sec only the end-plate was sensitive to applied ACh. Table 1 shows the rate of m.e.p.p.'s and the mean length of the ACh-sensitive surface in fifty muscle fibres from seven tenuissimus muscles intoxicated 3–4 weeks previously with botulinum toxin. (For technical reasons distances longer than 2 mm could not be measured accurately in single fibres.) The results shown in the table

indicate that, despite individual variations, a relation exists between transmitter release and the extent to which a muscle fibre becomes sensitive to applied ACh beyond the end-plate region.

TABLE 1. The relationship between the frequency of spontaneous m.e.p.p.s and the length of the part of the muscle fibre which was sensitive to applied ACh. The experiments were made on seven tenuissimus muscles intoxicated 3–4 weeks previously with botulinum toxin. Figures within brackets denote the range of measurements

Frequency of m.e.p.p.s (sec^{-1})	No. of fibres	Mean length of ACh-sensitive area (mm)
0·01–0·1	16	2·0 (2·0)
0·1–1·0	8	1·6 (1·0–2·0)
> 1·0	26	0·8 (0·3–2·0)

DISCUSSION

Two observations are of particular interest in the present investigation: first, the finding that botulinum toxin prevents transmitter release from motor nerve terminals without altering their ultrastructure, and, secondly, that this action causes a change in the chemical sensitivity of the muscle similar to that produced by chronic denervation.

It is characteristic of botulinum poisoning that there is a lack of transmitter release from cholinergic nerves, while in other respects nerve and muscle are unaffected by the toxin. The mechanism of this action is not understood, but it is conceivably localized to the nerve terminals (Guyton & MacDonald, 1947; Burgen *et al.* 1949; Brooks, 1954, 1956). The results of the present investigation do not explain the mode of action of the toxin but they suggest, by excluding the possibility of a morphological injury to presynaptic structures, that it is the ACh mechanism in nerve endings which is affected.

The number and size of presynaptic 'vesicles', supposed to contain the quanta of ACh released by the nerve (cf. del Castillo & Katz, 1956), appear to be normal in botulinum-treated muscles. That the terminals still contained packets of multimolecular quanta of ACh was disclosed when a mechanical injury to the end-plate produced a burst of m.e.p.p.s. ACh formation seems to be unaffected by the toxin. Burgen *et al.* (1949) and Stevenson & Girvin (1953) have shown that botulinum toxin does not interfere with choline acetylase systems, and in the present investigation it was observed that spontaneous ACh release from a chronically denervated amphibian junction was unimpaired by the toxin. The results of the present and previous investigations thus indicate that the toxin has a selective mode of action and that its point of attack is the mechanism responsible for the release of the chemical transmitter agent from cholinergic nerves.

In chronically denervated muscles the whole membrane becomes sensitive to applied ACh. This increase in size of the receptor surface can, as was shown by Axelsson & Thesleff (1959), account for the supersensitivity of denervated muscles to ACh and other chemical substances.

In botulinum-intoxicated muscles the spreading of the ACh-sensitive area of the end-plate to the entire membrane occurs in a similar manner and with about the same time course as in denervated muscles. Consequently, it is likely that the cause of the receptor change is the same in both instances. A reasonable assumption is that in denervated muscles, as in botulinum-treated ones, lack of transmitter release and not nerve degeneration is the cause which initiates the increase in size of the receptor surface. That supersensitivity following denervation is caused by the disappearance of transmitter release from the nerve has also been proposed by Burn & Rand (1959), who studied smooth muscles deprived of their sympathetic innervation.

Of interest was the observation that a diminished transmitter release, i.e. a low rate of m.e.p.p. discharge, produced an enlargement of the ACh-sensitive area at the end-plate. This suggests that the size of the chemosensitive region in a muscle fibre is variable and is regulated by the amount and frequency of transmitter released from the motor nerve.

There are no clues as to the nature of the mechanism by which transmitter release might affect the size of the receptor surface in a muscle fibre. A possibility is that the permeability increase produced in the end-plate membrane by the released ACh allows a chemical agent to enter the muscle fibre and that the presence of this substance prevents ACh receptors from being formed outside the end-plate region. Alternatively, the permeability increase may permit the efflux of a substance, formed in the myogenic part of the end-plate, whose presence inside the fibre induces the formation of ACh receptors. This latter possibility could explain the spatial sequence of the change in size of the receptor surface following denervation and reinnervation. This hypothesis is speculative but has at least the advantage that it can be experimentally tested. For example, it might be possible to determine whether long-lasting curarization produces changes in muscle similar to denervation.

SUMMARY

1. In mammalian skeletal muscle intoxicated by botulinum the entire muscle membrane becomes sensitive to applied acetylcholine. One to two weeks after the administration of the toxin the whole surface of the muscle is uniformly sensitive to acetylcholine. The spread of acetylcholine sensitivity from the end-plate to the whole membrane occurs in a similar manner and with about the same time course as in a chronically denervated muscle.

2. Botulinum toxin prevents transmitter release from motor nerve terminals without altering their ultrastructure. The present results indicate that the toxin acts by blocking the mechanism responsible for transmitter release from cholinergic nerve endings.

3. It is suggested that transmitter release from motor nerve terminals determines the size of the acetylcholine-sensitive area in the post-junctional membrane and that lack of transmitter agent and not nerve degeneration is responsible for initiating the process which leads to a high and uniform chemo-sensitivity in chronically denervated or botulinum-treated muscles.

The study of the ultrastructure of motor nerve terminals was made by Professor B. Katz, Department of Biophysics, University College London, who has kindly permitted me to publish his results. The expenses of this investigation were aided by grants from The Muscular Dystrophy Associations of America, Inc. and the Air Research and Development Command, United States Air Force, through its European Office. I am indebted to Dr J. Keppie, of the Microbiological Research Station, Porton, for a generous supply of botulinum toxin and toxoid. Unfailing technical assistance was provided by Miss E. Adler.

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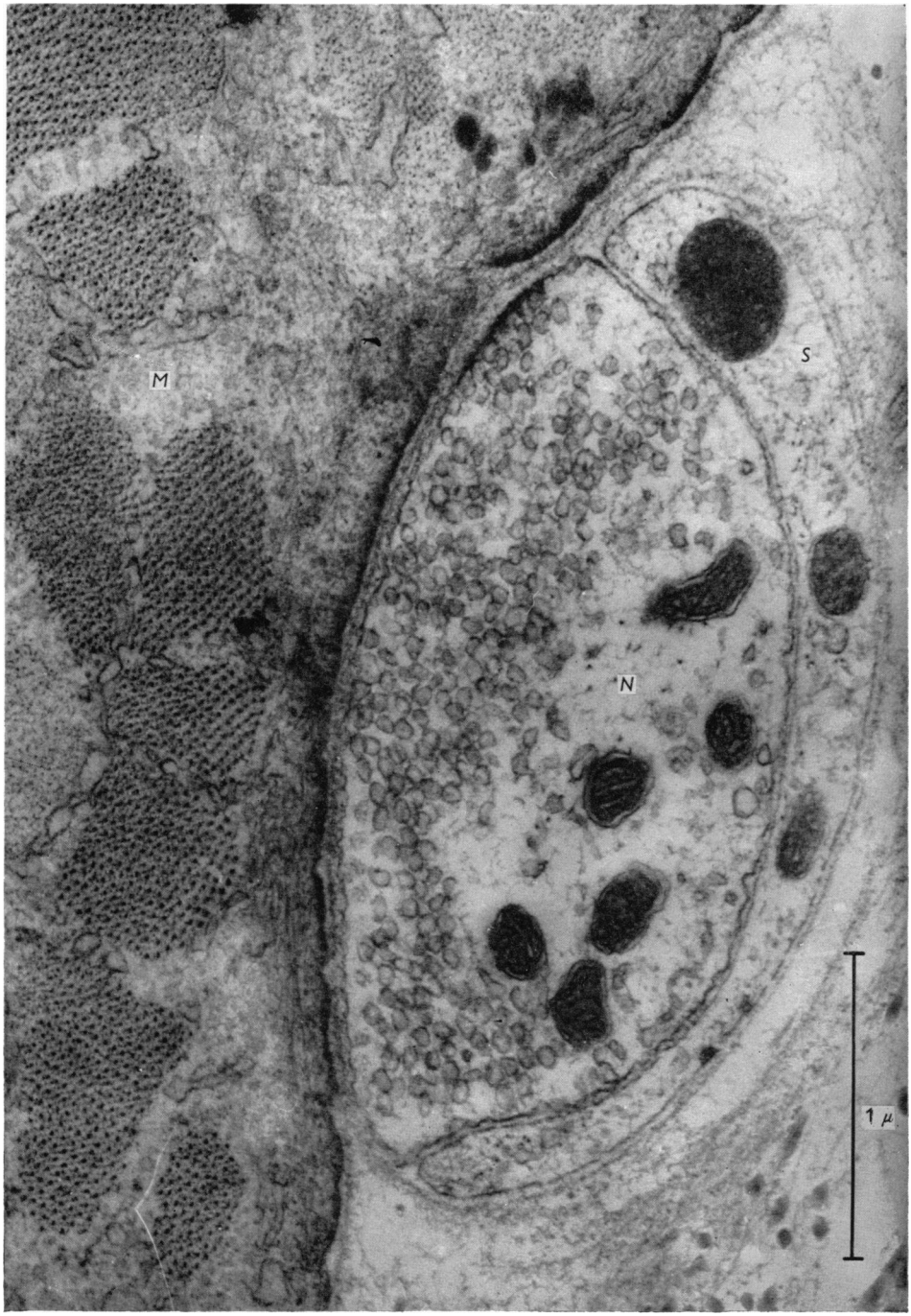

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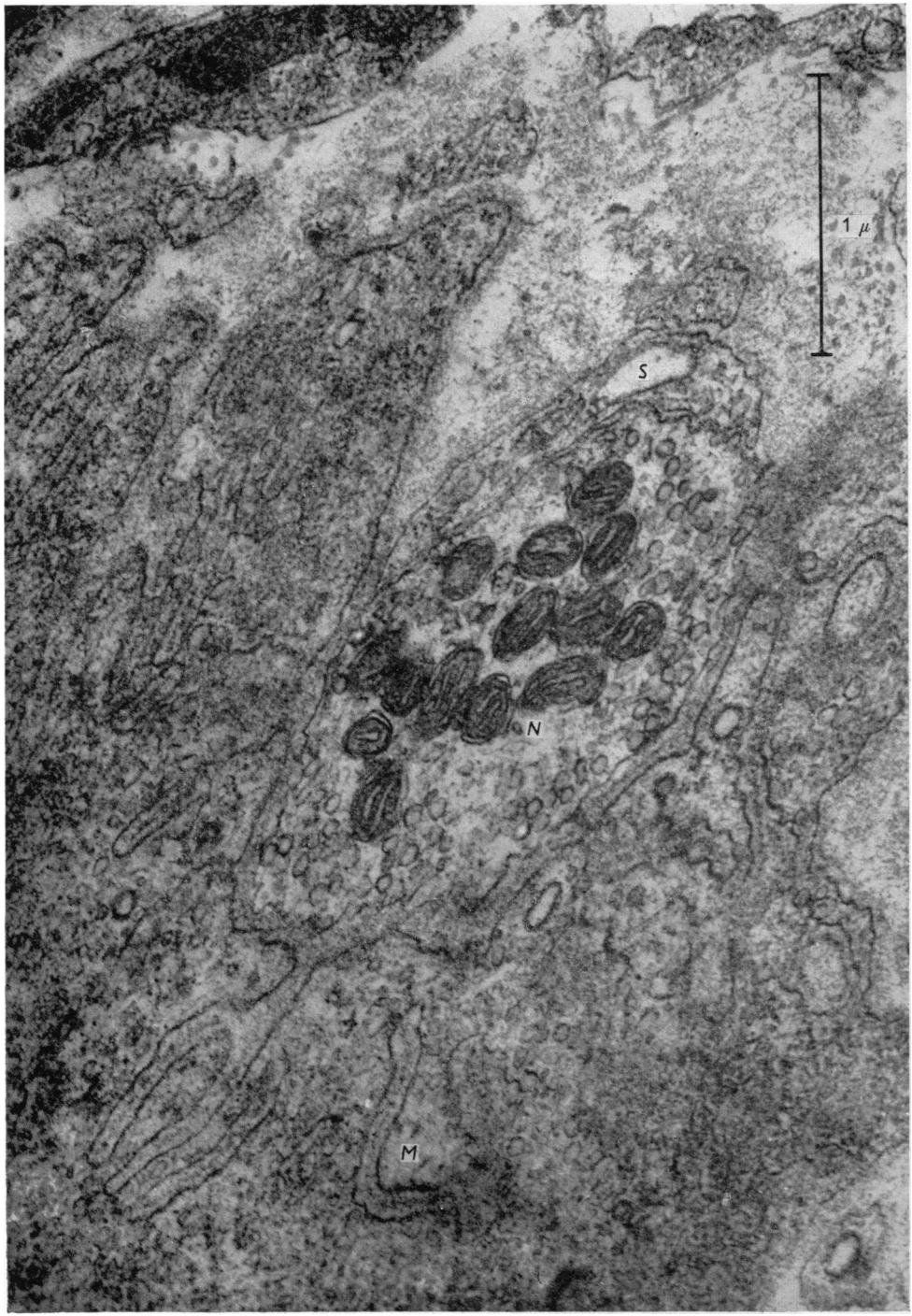
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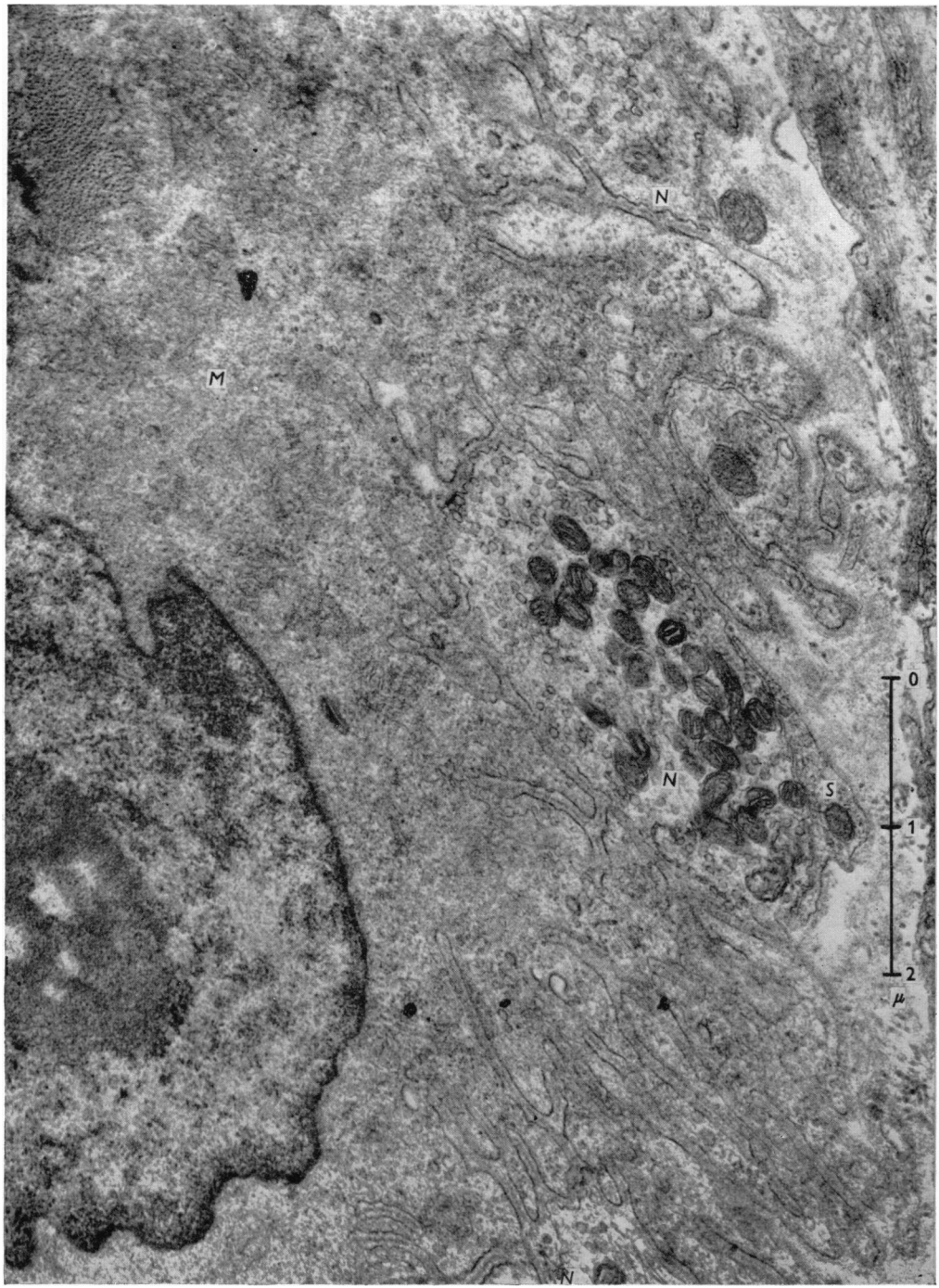
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EXPLANATION OF PLATES

Transverse sections of end-plate regions of botulinum-paralysed muscles. Preparations were fixed with osmic acid and stained with phosphotungstic acid in alcohol.

PLATE 1

Frog's sartorius, 13 days after toxin injection. *S*, Schwann cell; *N*, nerve ending; *M*, muscle fibre.

PLATE 2

Cat's tenuissimus, 27 days after toxin injection. *S*, *N* and *M*, as in Pl. 1.

PLATE 3

End-plate from same cat muscle, at lower magnification.