*J. Physiol.* (1981), **316**, *pp.* 279–291
*With 6 text-figures and 2 plates*
*Printed in Great Britain*



# THE NEUROMUSCULAR JUNCTION OF THE MOUSE AFTER BLACK WIDOW SPIDER VENOM

BY L. W. DUCHEN, S. GOMEZ AND L. S. QUEIROZ

*From the Department of Neuropathology, Institute of Neurology and the National Hospital, Queen Square, London WC1N 3BG*

(*Received 7 October 1980*)

## SUMMARY

1. A sublethal quantity of black widow spider venom was injected into the calf muscles of mice. After 30 min to 6 weeks soleus muscles were examined by light and electron microscopy and by electrophysiological techniques.

2. Within 30 min motor nerve terminals were swollen and depleted of synaptic vesicles and by 6 h were disrupted and engulfed by Schwann cells. By 24 h every end-plate examined was denervated. Some preterminal myelinated axons also showed degenerative changes.

3. Re-innervation was first seen at 2 days. By 3 days axon terminals were present at most end-plates and by 8 days their morphology was nearly normal. The normal pattern of innervation of the muscle was re-established in that axons re-innervated their original end-plates and very few ultraterminal axonal sprouts were found.

4. Physiological study showed complete failure of transmission and absence of miniature end-plate potentials (m.e.p.p.s) and end-plate potentials (e.p.p.s) until day 3, when muscles responded weakly to indirect stimulation and m.e.p.p.s were recorded at 30% and e.p.p.s at 40% of fibres. The mean quantal content of e.p.p.s was low and there was rapid fatigue on repetitive stimulation. Extrajunctional sensitivity to acetylcholine developed within 1 day, was maximal at 3 days and declined to normal at 12–14 days.

5. The proportion of fibres at which m.e.p.p.s and e.p.p.s were recorded returned to normal by day 6 and mean quantal content was normal by day 9.

6. These findings show that the re-innervation of original end-plates is of importance in facilitating the rapid return of transmission to normal levels and limiting the extent of axonal growth.

## INTRODUCTION

Black widow spider venom (BWSV) acts on a variety of nerve terminals to cause an initial increase in the spontaneous release of transmitter followed by the complete block of transmission (Frontali, Ceccarelli, Gorio, Mauro, Siekevitz, Tzeng & Hurlbut, 1976). In frog nerve–muscle preparations Longenecker, Hurlbut, Mauro & Clark (1970) recorded a massive increase in the frequency of miniature end-plate potentials (m.e.p.p.s) within a few minutes of adding BWSV to the bathing fluid. Electron microscopy showed that motor nerve terminals became markedly swollen and

depleted of synaptic vesicles and the mitochondria were disrupted (Clark, Mauro, Longenecker & Hurlbut, 1970). Similar effects have been seen in cat soleus (Okamoto, Longenecker, Riker & Song, 1971) and mouse hemidiaphragm (Kao, Drachman & Price, 1976). Most of the work on the effects of BWSV on motor end-plates has been limited to the acute changes and with the exception of the experiment described in the cat (Okamoto *et al.* 1971) has been done *in vitro*. We have studied the long-term effects of the venom on the neuromuscular junction of the mouse in order to determine the time course and the extent of the degeneration of the terminals. It was also of interest to study the regenerative capacity of the nerve endings and to determine whether re-innervation occurred at the original end-plates. A preliminary report of this work has been published (Duchen, Gomez & Queiroz, 1980).

## METHODS

Under ether anaesthesia the venom of *Latrodectus tredecimguttatus* was injected into the calf muscles of the right hind limbs of mice. The dose sufficient to produce local paralysis was found to be 0·01 of a gland pair dissolved in 0·1 ml physiological saline.

The mice were killed at times of survival ranging from 30 min to 6 weeks after injection of the venom. Saline was injected into the calf muscles of normal mice which were allowed to survive for 6 or 24 h. Muscles were also taken from normal untreated mice and from the left, non-injected leg of experimental animals. More than 100 mice were used in all.

*Histological methods.* Under anaesthesia mice were perfused with fixative through the left ventricle. For paraffin histology the fixative used was formol-calcium (10 % formalin in 1 % calcium acetate) followed by FAM (formaldehyde, 1 part; glacial acetic acid, 1 part; methanol, 8 parts). After decalcification in formic-citrate solution, the hind limbs were cut into longitudinal or serial transverse blocks. Paraffin sections were stained with haematoxylin–eosin, haematoxylin–van Gieson, Glees and Marsland's method of silver impregnation for axons and luxol fast blue combined with cresyl violet for myelin sheaths.

Other mice were perfused with cold (4 °C) formol-calcium. Serial longitudinal cryostat sections of the leg muscles were cut at 20 $\mu$m and stained by the method of Koelle & Friedenwald (1949) to demonstrate cholinesterase, followed by silver impregnation by the method of Namba, Nakamura & Grob (1967) to demonstrate axons. In some cases the cryostat sections were stained by the bromoindoxyl acetate and silver method (Pestronk & Drachman, 1978).

For electron microscopy, mice were perfused with 3 % glutaraldehyde–1 % paraformaldehyde in 0·07 M-cacodylate buffer, pH 7·3, at 4 °C. Blocks were taken from the middle third of soleus and from the superficial part of gastrocnemius, left overnight in fixative, post-fixed in 1 % osmium tetroxide, dehydrated in ethanol and embedded in Araldite. Longitudinal 1 $\mu$m sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate.

*Physiological methods.* At times ranging between 30 min and 14 days after injection of BWSV, mice were anaesthetized and soleus of the right leg dissected out together with a short length of its nerve. Muscles were placed in a chamber containing mammalian Ringer solution of the following composition, in mmol/l: NaCl, 115; KCl, 3·5; $MgSO_4$, 1; $CaCl_2$, 2; $NaHCO_3$, 25; $KH_2PO_4$, 1; glucose, 10. The solution was continuously gassed with 95 % $O_2$/5 % $CO_2$. Isometric tension was recorded by means of a Grass mechano-electrical transducer (Model FT.03 C). Muscles were stimulated indirectly using suction electrodes. For direct stimulation of the muscle two platinum wire electrodes were placed on the surface of the muscle. Intracellular recordings were made using conventional glass micro-electrodes filled with 3 M-potassium chloride. A train of end-plate potentials (e.p.p.s) was elicited on nerve stimulation at 50/s for 2 s. From 4 days survival, as well as in controls, D-tubocurarine chloride was added to the bathing fluid to give a final concentration of 0·3 $\mu$g/ml. Quantal contents of the e.p.p.s were calculated from the coefficient of variation of the amplitudes of sixty potentials (del Castillo & Katz, 1954; Martin, 1955). The first fifteen of each train were disregarded so as to avoid the phase of early rundown.

Sensitivity of muscles to acetylcholine (ACh) was assessed by measuring the isometric tension developed on addition of ACh to the muscle chamber to produce a final concentration of $2 \times 10^{-5}$ M. Extrajunctional sensitivity to ACh was determined by the ionophoretic application of 3 M-ACh from

a micro-pipette. Sensitivity was estimated by the method of Miledi (1960*a*) as the membrane potential in millivolts per nanocoulomb of current applied through the pipette. The sensitivity to ACh was determined in five fibres about 0·5 mm proximal to the end-plate zone and then the process repeated at parallel 0·5 mm intervals passing distally through the end-plate region towards the tendon (Tonge, 1974*a*).

## RESULTS

Within a few minutes of the injection of BWSV there was weakness of the right hind limb and by 30 min the limb was completely paralysed. There were also signs of generalized toxicity, which lasted for about 2 days. On the third day the general condition of the mice improved, the right hind limb showed early signs of recovery from the paralysis and over the next few days the movements of the limb returned to normal.

### *Morphological observations*

In normal mice, the pattern of innervation of muscles supplied by spinal nerves conforms to that observed in other mammals (see Bowden & Duchen, 1976). Nerves penetrate the muscle at the neurovascular hilum and undergo repeated branching before reaching the region of the motor end-plates. The intramuscular nerve bundles eventually break up into a spray of myelinated preterminal axons, each of which extends for a short distance before ending at a single end-plate on a single extrafusal muscle fibre. Abundant axonal branching takes place in the intramuscular nerves but only about 10% of the axons divide after leaving the nerve bundles. The preterminal axon loses the myelin sheath shortly before entering the end-plate, where it forms a delicate terminal arborization. Cholinesterase activity at the end-plate is concentrated in the gutters and post-synaptic folds of the sarcolemmal membrane under the fine axonal endings. The axon does not extend beyond the end-plate region so that in the normal muscle there are no ultraterminal fibres.

Electron microscopy of normal neuromuscular junctions in mammalian extrafusal muscle fibres shows that the axon terminals lie in depressions in the surface of the muscle fibre and are separated from the sarcolemma by the basal lamina. Each motor nerve terminal contains filaments, mitochondria and abundant synaptic vesicles. The external surface of the terminal is covered by processes of Schwann cell cytoplasm. The post-synaptic sarcolemma is thrown into elaborate folds orientated approximately at right angles to the surface of the muscle fibre and the folds are also filled with basal lamina. The sarcoplasm at the end-plate region is abundant, particularly in slow muscles like soleus, and contains mitochondria, rough endoplasmic reticulum, stacks of Golgi membranes and sole plate nuclei.

*Changes after BWSV.* By *30 min* after the injection of BWSV it was not possible to demonstrate motor nerve terminals by silver impregnation techniques. The motor nerve fibres ended in stumps at variable distances from the end-plates, which could be recognized by the presence of cholinesterase activity. Occasional preterminal fibres and a few axons in small intramuscular nerve bundles had disintegrated into argyrophilic granules but the larger nerves appeared normal. The muscle fibres were largely unaffected but in some areas a few fibres were necrotic and a mild cellular reaction was present, which may have been due to trauma from the needle.

Electron microscopy showed that at 30 min the motor nerve terminals were

markedly swollen, electron-lucent and depleted of synaptic vesicles. Mitochondria in the terminals were swollen and rounded. Abundant whorls of membrane in some terminals were apparently continuous with the axolemma. Cisternae of irregular shape and size were common and could also occasionally be traced to axolemmal invaginations. In a few terminals the axolemma was disrupted. No abnormalities were seen in Schwann cells, in the post-synaptic membrane and folds or in the muscle fibre, except for a slight dilatation of the sarcoplasmic reticulum in some fibres. In one end-plate Schwann cell processes were interposed between the degenerating terminal and the sarcolemma. Many preterminal axons and a few axons in the intramuscular nerve bundles also showed degenerative changes. Some axons were shrunken, with dense axoplasm and clumped mitochondria, while others were swollen with electron-lucent axoplasm and mitochondria swollen and dispersed. Microtubules and neurofilaments could not be seen. Irregular membrane-bound cisternae similar to those in the terminals were also present. There were no abnormalities in the myelin sheaths, Schwann cells or perineurium.

By *6 h* all axonal endings examined electron microscopically in seventeen end-plates were severely degenerated. Masses of debris measuring up to 3·0 $\mu$m in diameter and consisting of electron-dense mitochondria and vesicles were seen within Schwann cell cytoplasm. The post-synaptic sarcolemma was covered by Schwann cell processes and shreds of basal lamina. The muscle fibres appeared unaffected.

*Twenty-four hours* after BWSV all end-plates seen with both light and electron miscroscopy were denervated. In silver-impregnated paraffin sections the appearance was similar to that at 30 min. In serial frozen sections of the calf muscles stained by the bromoindoxyl acetate technique, every end-plate was devoid of nerve terminals (Pl. 1 *A*). The preterminal axons ended blindly, sometimes in a small end-bulb. A gap of variable distance was seen between the axonal stump and the end-plate site shown by the cholinesterase activity. Occasionally the axon lay very near the end-plate but the fine terminal branches were absent. Two soleus muscles were examined by electron microscopy at 24 h and thirty-two end-plates identified. All were denervated (Pl. 1 *B*). The post-synaptic membrane was incompletely covered by Schwann cell processes, many of which contained electron-dense debris probably derived from the degenerated terminals. In many end-plates the Schwann cell nucleus was seen near the post-synaptic sarcolemma, an unusual finding in normal muscle. A number of preterminal myelinated axons and some axons in intramuscular nerve bundles had also degenerated. Schwann cells in those places had abundant cytoplasm and contained myelin figures.

*Two days* after BWSV a few motor nerve terminals could be seen in silver-stained paraffin sections but with light microscopy most end-plates appeared denervated. In one of two soleus muscles examined by electron microscopy a few end-plates were innervated by small axonal profiles containing synaptic vesicles and lying in contact with the post-synaptic sarcolemma (Pl. 2 *A*). Most of the sarcolemma of these end-plates was covered by Schwann cell processes. All other end-plates were denervated, with the post-synaptic sarcolemma covered by Schwann cell processes. No denervated end-plates were seen completely devoid of Schwann cell processes as was described by Miledi & Slater (1968) in the rat after 3 weeks or more of denervation.

At *3 days* after BWSV many end-plates contained nerve terminals of normal

appearance and stainable with silver techniques. In sections stained for cholinesterase and nerve fibres some preterminal axons were very thin and appeared to reach an end-plate, suggesting that they were regenerating. However, those which did reach end-plates innervated only a single muscle fibre. Only exceptionally did axons grow beyond the end-plate, so that ultraterminal sprouts were rare. No collateral sprouts were seen. Fine granules of cholinesterase reaction product were found scattered along many of the regenerating axons. In all, twenty-eight end-plates were identified by electron microscopy in two muscles at this time. Most of these were examined in several sections although no attempt was made to cut serial sections of these blocks. In twenty-two (about 80%), axon terminals were in contact with the post-synaptic sarcolemma. The terminals were of variable size and contained filaments, mitochondria and synaptic vesicles. Many terminals were partly separated from the post-synaptic membrane by thin Schwann cell processes and the nucleus of the Schwann cell tended to lie over the end-plate. Normal-looking regenerating axons were seen in intramuscular nerve bundles. There were no abnormalities in the muscle fibres and in the post-synaptic sarcolemma.

From *4 days* onwards there was a rapid return to normal morphology. Preparations stained for cholinesterase and nerve fibres examined up to 6 weeks after BWSV showed a normal pattern of innervation with each preterminal axon innervating one end-plate and every end-plate containing terminals with a normal arborization (Pl. 1*C*). However, at 6 days and 2 weeks a few ultraterminal sprouts were seen, which appeared as thin tapering outgrowths usually with granules of cholinesterase reaction product along them. Most were short, less than half the diameter of an end-plate, while others extended for up to about 100 $\mu$m before ending blindly between the muscle fibres, sometimes forming a small end-bulb. Only occasional sprouts were seen to end in another normal end-plate or to form a small new end-plate. At 6 weeks only very rare ultraterminal sprouts could be identified. Electron microscopy of soleus at 5 days, 1, 2 and 6 weeks after BWSV (two mice at each time) showed a return of the motor nerve terminals to normal appearance (Pl. 2*B*). They were of normal size, contained filaments, mitochondria and abundant synaptic vesicles, and were in close contact with the post-synaptic sarcolemma. A total of 104 end-plates were examined ultrastructurally from 5 days to 6 weeks and all but one were found to be normally innervated. The only somewhat unusual finding was that the Schwann cells had an active-looking cytoplasm, with numerous ribosomes and stacks of Golgi membranes, and some Schwann cell nuclei were seen over the nerve terminals. Regenerated preterminal axons or those in nerve bundles were still unmyelinated or thinly myelinated at 8 days but by 2 weeks no thinly myelinated axons were seen.

### *Physiological results*

*Response to nerve stimulation.* Muscles of animals which had received an injection of venom were paralysed when examined between 30 min and 2 days. The first evidence of recovery from paralysis was seen at 3 days when two out of three muscles responded to nerve stimulation at 1/s with weak twitch tensions of 0·06 and 0·10 g/mg (Fig. 1*A*). Recovery continued rapidly so that by day 4 all muscles examined produced responses of normal tension and most tested between 5 and 14 days developed twitch contractions which were within normal limits. All paralysed muscles

responded normally to direct electrical stimulation. In control mice, injection of saline had no effect on the physiological responses of soleus.

*Fatigue.* In soleus of normal animals, tension developed in response to nerve stimulation at 100/s is well maintained while the stimulus is being applied, and the tension decreases to half-peak only after about 9 s. On switching off the stimulus the tension rapidly falls to the base line. Muscles recovering from paralysis due to BWSV

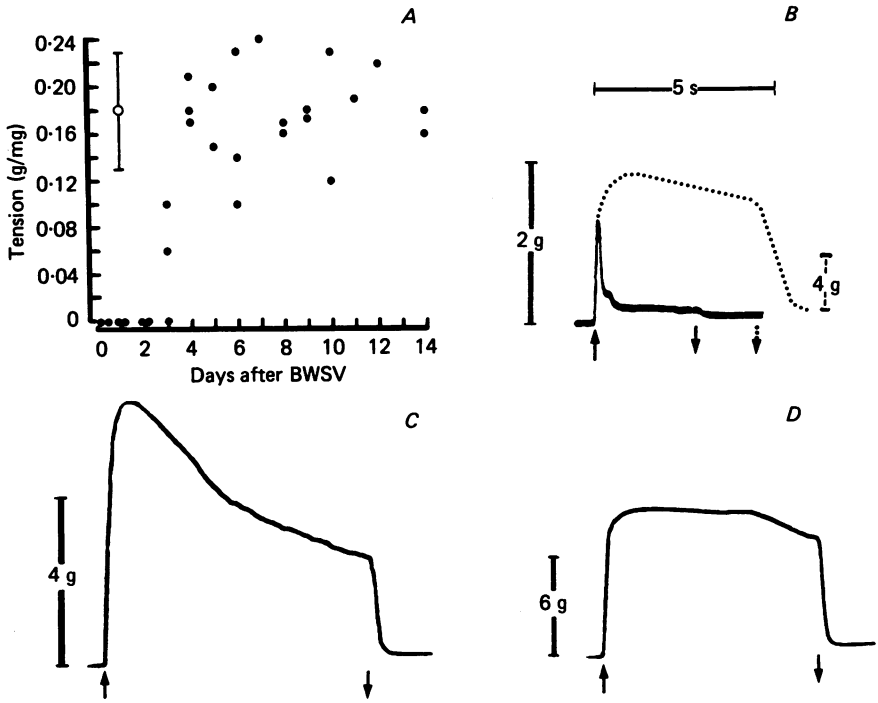



Fig. 1. *A*, isomeric twitch tension developed by soleus in response to nerve stimulation. Each point (●) represents a single muscle after BWSV. Mean tension of six normal muscles (○) was 0·18±0·05 (S.D. indicated by vertical line). Tension is restored to normal levels by day 4. *B–D*, isometric tetanic tension developed by soleus 3 days (*B*), 8 days (*C*) and 14 days (*D*) after BWSV. Response of a normal muscle is indicated by the dotted line and duration of stimulus by arrows.

showed fatigue within the first second of nerve stimulation at 100/s. At 3 and 4 days the rate of fatigue was very rapid as tetanic tension decreased to half-maximum in 0·15 and 0·62 s respectively, and after only 2 s the response had fallen to the baseline (Fig. 1*B*). The ability of muscles to maintain a contracture improved with increasing survival time, so that by 6 and 8 days the time to half-peak was 6·8 s (Fig. 1*C*). By 14 days no appreciable decrease in tension was seen within the first 5 s of stimulation (Fig. 1*D*). All muscles either paralysed by, or recovering from, BWSV were able to maintain tetanic contracture to direct stimulation at 100/s for 3 s.

*Fibrillation.* One out of three muscles examined at 3 days showed slight fibrillation *in situ*. In this muscle, spontaneous repetitive action potentials were recorded *in vitro* in three out of forty-seven muscle fibres tested. At no other time was fibrillation observed.

*Miniature end-plate potentials* (*m.e.p.p.s*). In an acute experiment, BWSV was added to a nerve–muscle preparation *in vitro* to give a final concentration of 0·025 gland pair/ml. The frequency of m.e.p.p.s before the addition of venom was 1·4 ± 0·8/s (mean ± S.D. of eighteen muscle fibres). About 2–4 min after adding the venom the frequency increased to 201 ± 101·7/s (five fibres). After 5 min m.e.p.p.s were no longer recorded. This result confirms the findings of Clark *et al.* (1970) and Okamoto *et al.* (1971) who showed a hundredfold increase in the frequency of m.e.p.p.s after adding BWSV to amphibian and mammalian nerve–muscle preparations.

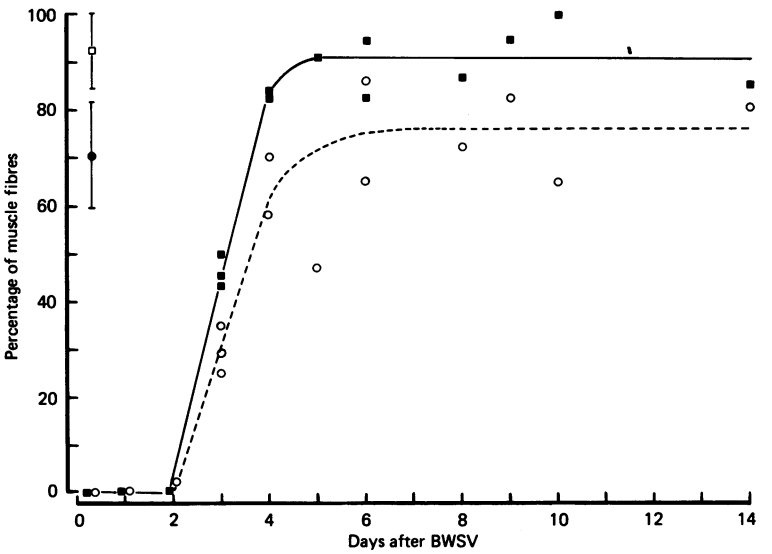



Fig. 2. Proportion of fibres tested from which m.e.p.p.s (○) and e.p.p.s. (■) were recorded. Each point represents a single muscle. In four normal muscles the range of the proportion of fibres showing m.e.p.p.s (●) was 60–82 % and e.p.p.s (□) was 84–100 % (as indicated by vertical lines).

Miniature end-plate potentials could not be detected in muscles examined between 30 min and 1 day after BWSV. They were first recorded at 2 days, but from only one out of forty-two fibres tested in one muscle (no e.p.p. was elicited in that fibre). At 3 days the proportion of fibres showing m.e.p.p.s had increased to about 30 %, i.e. seventeen out of fifty-seven fibres tested in three muscles (Fig. 2). Thirteen of these fibres had m.e.p.p.s of abnormally high frequency (Fig. 3). In most of the thirteen fibres the high frequency persisted for as long as the electrode was in place (this ranged between 30 s and 4 min). In four of the thirteen fibres the potentials occurred in bursts which had a duration varying from 0·25 to 1·0 s and a periodicity of 0·2–1·5 s. Between each burst there was either a period of silence or only an occasional m.e.p.p. The frequency during each burst was so high that many m.e.p.p.s summated forming complex potentials. It was also observed that in the muscle which did not respond to nerve stimulation at 3 days, frequencies of m.e.p.p.s ranged between 11 and 28/s in all seven fibres at which they were recorded. In the other two muscles which gave weak responses at 3 days both high and low frequencies were recorded.

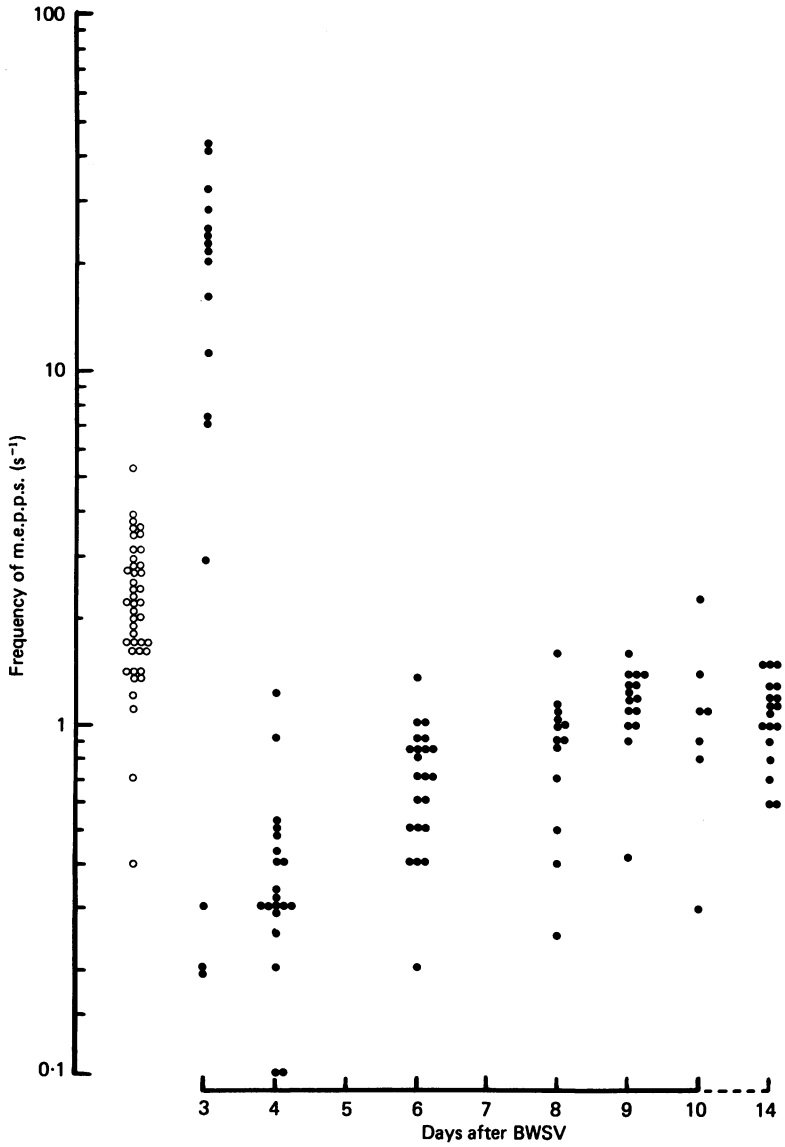



Fig. 3. Frequency of m.e.p.p.s (plotted on a log scale) in normal (○) and experimental (●) muscles. Each point represents a single muscle fibre. No m.e.p.p.s were recorded from 8 min after BWSV until the third day, when abnormally high frequencies were seen.

Between 4 and 14 days the percentage of fibres at which m.e.p.p.s could be recorded was within normal limits (Fig. 2). At 4 days the frequency was $0{\cdot}39 \pm 0{\cdot}25$/s (twenty fibres from two muscles), which was low compared with normal muscles. The mean frequency in treated muscles increased over the next 10 days, so that by 14 days it was $1{\cdot}09 \pm 0{\cdot}29$/s (eighteen fibres from one muscle), which was just within normal limits.

*End-plate potentials* (*e.p.p.s*). End-plate potentials could not be elicited on nerve

stimulation at either 1 or 50/s in soleus between 30 min and 2 days after injection of venom. At 3 days, when neuromuscular transmission was recovering, e.p.p.s were elicited at 44–50 % of the fibres examined (a total of sixty-seven fibres were tested in three muscles). From 4 days onwards the percentage of fibres at which e.p.p.s could be elicited was within normal limits (Fig. 2).

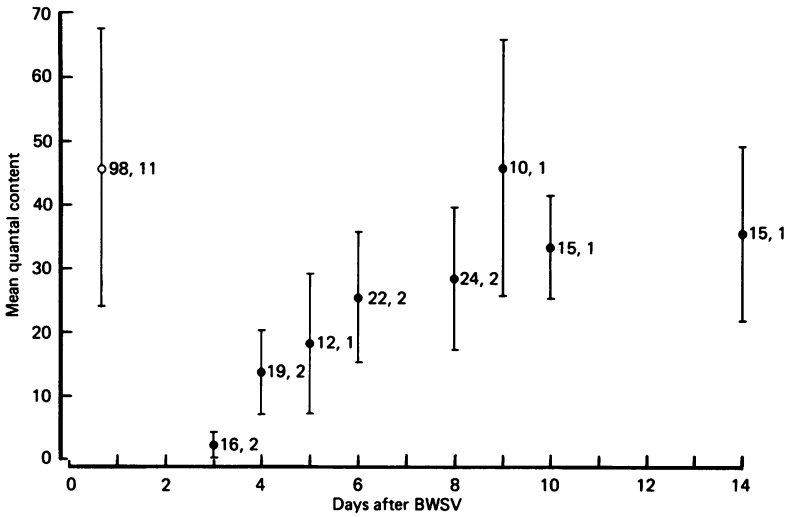



Fig. 4. Mean quantal content of normal (○) and experimental (●) soleus muscles. Each point represents the mean (S.D. indicated by vertical lines) of the number of fibres indicated by the first figure. The second figure refers to the number of muscles tested.

At 3 days failures were observed at about 50 % of the end-plates at which e.p.p.s were evoked. The mean quantal content in two muscles at 3 days was $2{\cdot}27 \pm 2{\cdot}20$ (S.D. of sixteen fibres; Fig. 4), at which stage no curare was required to prevent contraction. By 4 days no failures were observed but there was a large variation in the amplitudes of e.p.p.s, which indicated that the quantum content was still low. The mean quantal release of transmitter at 4 days was $13{\cdot}86 \pm 6{\cdot}72$ (nineteen fibres from two muscles). From 5 days onwards action potentials were produced on nerve stimulation and therefore recordings were made on partially curarized preparations. The mean quantal content steadily increased so that by 6 days the mean of two muscles was just within normal limits. Four muscles examined between 8 and 14 days also showed quantal contents within normal levels.

*Sensitivity to acetylcholine.* Muscles paralysed by BWSV developed supersensitivity to ACh and contracted strongly when ACh was added to the muscle chamber. As early as 24 h a response of 0·04 g/mg was obtained. Over the next 2 days the isometric tension to ACh increased rapidly and reached a peak at 3 days when a contracture of 0·31 g/mg was produced (Fig. 5). On return of neuromuscular transmission the response gradually decreased. Between 4 and 8 days muscles still contracted strongly to ACh, but by 9 days tension had fallen to a level similar to that at 1 day. At 12 and 14 days the tension response to ACh was negligible.

The ionophoretic application of ACh showed (Fig. 6) that in the normal muscle the

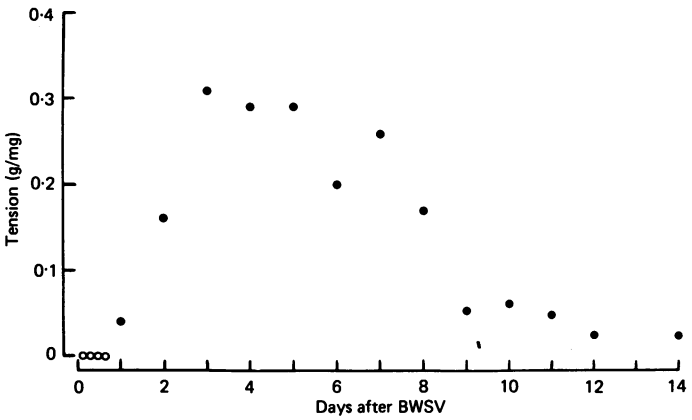



Fig. 5. Isometric tension developed in response to $2 \times 10^{-5}$ M-ACh by soleus after BWSV (●). Normal muscles (○) showed no response to ACh.

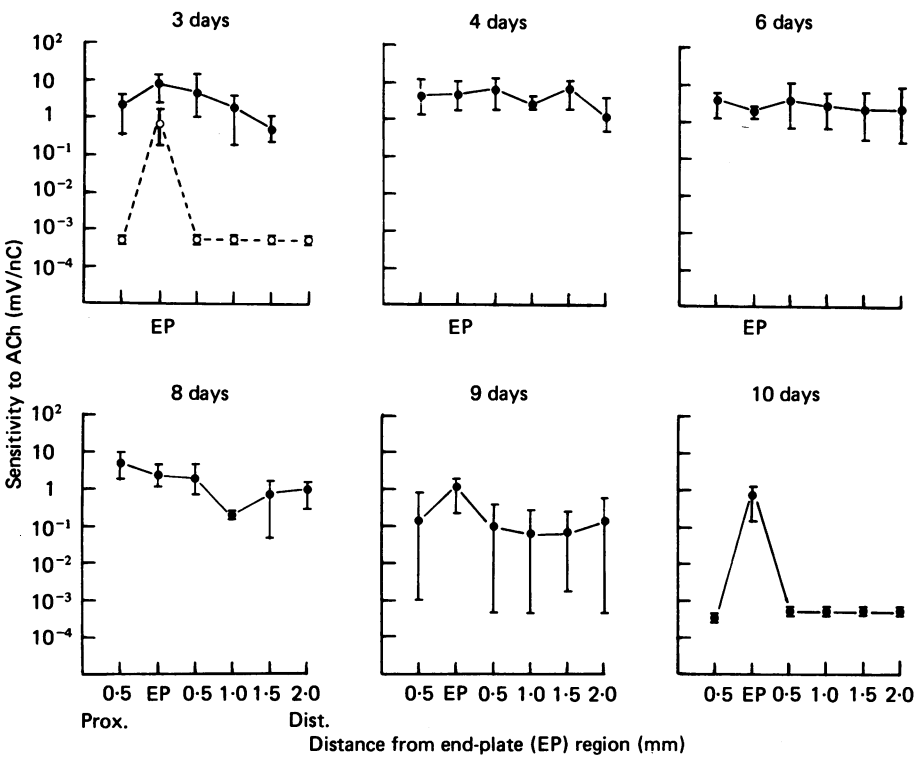



Fig. 6. The response to ionophoretic application of ACh (in mV/nC) determined at 0·5 mm intervals from the end-plate (EP). Each point represents the mean of five fibres, the range being indicated by the vertical lines. In normal soleus (dashed line) and soleus 10 days after BWSV the extrajunctional response to ACh was $< 10^{-3}$ mV/nC.

extrajunctional response was less than $10^{-3}$ mV/nC (but see Miledi & Zelena, 1966). After BWSV extrajunctional supersensitivity was observed from 3 to 9 days. At 4 and 6 days fibres were uniformly sensitive to ACh over the length tested. By 8 days extrajunctional sensitivity began to decrease and at 10 days was at normal levels.

## DISCUSSION

Early reports on the effects of BWSV indicated that muscle and peripheral nerve function were unaffected (D'Amour, Becker & van Riper, 1936; Sampayo, 1944). A paralysing action on the phrenic nerve–diaphragm preparation was observed by Cantore (1958) and subsequent work showed that the venom acted specifically at nerve terminals, leaving muscle fibres and Schwann cells intact (Longenecker *et al.* 1970; Clark *et al.* 1970; Kawai, Mauro & Grundfest, 1972; Frontali *et al.* 1976).

Destruction of motor nerve terminals after BWSV has previously been observed by Okamoto *et al.* (1971) in cat soleus *in situ*. Clark, Hurlbut & Mauro (1972) did not see such extensive damage to the nerve endings in frog nerve–muscle preparations and suggested that there are species differences. Ceccarelli, Grohovaz & Hurlbut (1979) noted that BWSV was neither lytic nor destructive. The disparity in reported effects of BWSV is therefore likely to be attributable to differences in technique, dose of venom used and survival time.

The venom denervates muscle fibres more rapidly than nerve section or crush, after which, depending on species and length of intact nerve, days may elapse before terminals degenerate and transmission finally fails. Rapid destruction of the nerve endings similar to that following BWSV has also been reported after the addition of beta-bungarotoxin in frog and rat nerve–muscle preparations (Abe, Limbrick & Miledi, 1976). The whorls of membrane and membrane-bound cisternae seen in some acutely degenerating terminals were similar to those observed by Clark *et al.* (1972) and may have been derived from the fusion of synaptic vesicles with the axolemma (Heuser & Reese, 1973).

The axonal terminals have a marked regenerative capacity, new terminals being present at end-plates within 2 days, and their size was almost normal on the eighth day. The morphological features of regenerating nerve terminals after BWSV closely resembled those after nerve section in that the new endings contained filaments, tubules, mitochondria and synaptic vesicles and established contact with the post-synaptic sarcolemma (Saito & Zacks, 1969*a*, *b*; Lüllmann-Rauch, 1971; Koenig & Pecot-Dechavassine, 1971).

At 3 days, when 80% of the end-plates contained small terminals, m.e.p.p.s of abnormally high frequency were recorded. High-frequency m.e.p.p.s were also found by Miledi (1960*b*) in newly re-innervated neuromuscular junctions of the frog. The abnormal spontaneous release of ACh may be related to the immature state of the terminals. The steady rise in quantal content can be correlated with the increase in size of the axonal terminals and the establishment of full synaptic contact (Bennett, McLachlan & Taylor, 1973). The abnormal fatigue of the tetanic response found in the early stages of recovery can also be related to the low quantal release of transmitter (Tonge, 1974*a*).

The rapid recovery of transmission following BWSV must be due, in part, to the

short length of axon to be regenerated. Another important factor determining the speed of recovery is that the regenerated axons re-innervate the old end-plate sites (Koenig & Pecot-Dechavassine, 1971). Tonge (1974*b*) found that when new end-plates were formed in soleus of the mouse, mean quantal content reached normal values in 7 weeks, but with re-innervation at original end-plates, mean quantal content returned to normal in 4 weeks. The results of the present experiments indicate that axonal growth is confined to the original end-plates when these sites can readily be reinnervated. The limitation of axonal growth can be correlated with the rapid return of neuromuscular transmission to normal levels of quantal release.

We should like to thank Dr N. Frontali for the venom; Mrs R. K. Bowden and Mrs J. Grainger for the microscopic preparations; Mr J. A. Mills for the photographs and Mr H. Goulding for the care of the animals. This work was supported by grants from the ALS Society of America, the Muscular Dystrophy Association and the Brain Research Trust. L. S. Q. is in receipt of a scholarship from FAPESP, Brazil.

## REFERENCES

ABE, T., LIMBRICK, A. R. & MILEDI, R. (1976). Acute muscle denervation induced by β-bungarotoxin. *Proc. R. Soc. B* **194**, 545–553.

BENNETT, M. R., MCLACHLAN, E. M. & TAYLOR, R. S. (1973). The formation of synapses in reinnervated mammalian striated muscle. *J. Physiol.* **233**, 481–500.

BOWDEN, R. E. M. & DUCHEN, L. W. (1976). The anatomy and pathology of the neuromuscular junction. In *The Neuromuscular Junction. Handbook of Experimental Pharmacology 42*, ed. ZAIMIS, E., pp. 23–97. Berlin, Heidelberg, New York: Springer-Verlag.

CANTORE, G. P. (1958). Contributo allo studio dell'azione farmacologica del veleno di *Latrodectus tredecimguttatus* Rossi. *Riv. Parassit.* **19**, 158–160.

CECCARELLI, B., GROHOVAZ, F. & HURLBUT, W. P. (1979). Freeze-fracture studies of frog neuromuscular junctions during intense release of neurotransmitter. *J. cell Biol.* **81**, 163–177.

CLARK, A. W., HURLBUT, W. P. & MAURO, A. (1972). Changes in the fine structure of the neuromuscular junction of the frog caused by black widow spider venom. *J. cell Biol.* **52**, 1–14.

CLARK, A. W., MAURO, A., LONGENECKER, H. E. & HURLBUT, W. P. (1970). Effects of black widow spider venom on the frog neuromuscular junction. *Nature, Lond.* **225**, 703–705.

D'AMOUR, F., BECKER, F. E. & VAN RIPER, W. (1936). The black widow spider. *Q. Rev. Biol.* **11**, 123–160.

DEL CASTILLO, J. & KATZ, B. (1954). Quantal components of the end-plate potential. *J. Physiol.* **124**, 560–573.

DUCHEN, L. W., GOMEZ, S. & QUEIROZ, L. S. (1980). Degeneration and regeneration at the neuromuscular junction in the mouse after black widow spider venom. *J. Physiol.* **300**, 20*P*.

FRONTALI, N., CECCARELLI, B., GORIO, A., MAURO, A., SIEKEVITZ, P., TZENG, M. C. & HURLBUT, W. P. (1976). Purification from black widow spider venom of a protein factor causing the depletion of synaptic vesicles at neuromuscular junctions. *J. cell Biol.* **68**, 462–479.

HEUSER, J. E. & REESE, T. S. (1973). Evidence for recycling of synaptic vesicle membrane during transmitter release at the frog neuromuscular junction. *J. cell Biol.* **57**, 315–344.

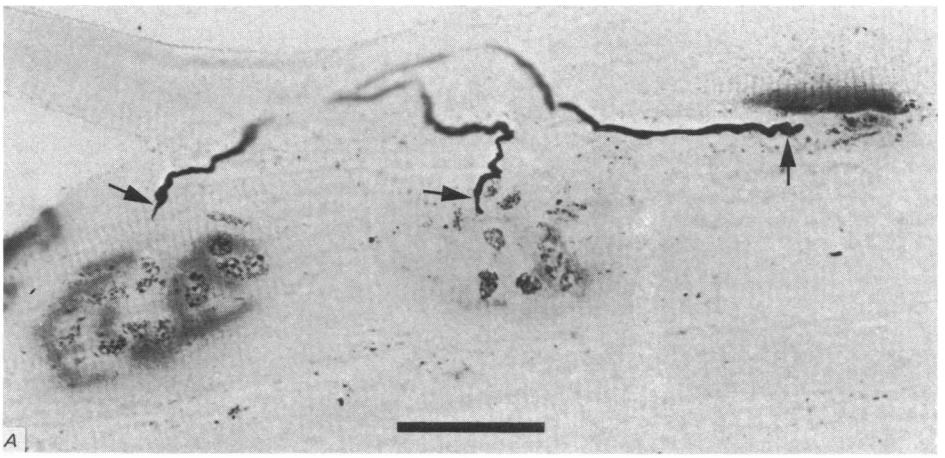
KAO, I., DRACHMAN, D. B. & PRICE, D. L. (1976). Botulinum toxin: mechanism of presynaptic blockade. *Science, N.Y.* **193**, 1256–1258.

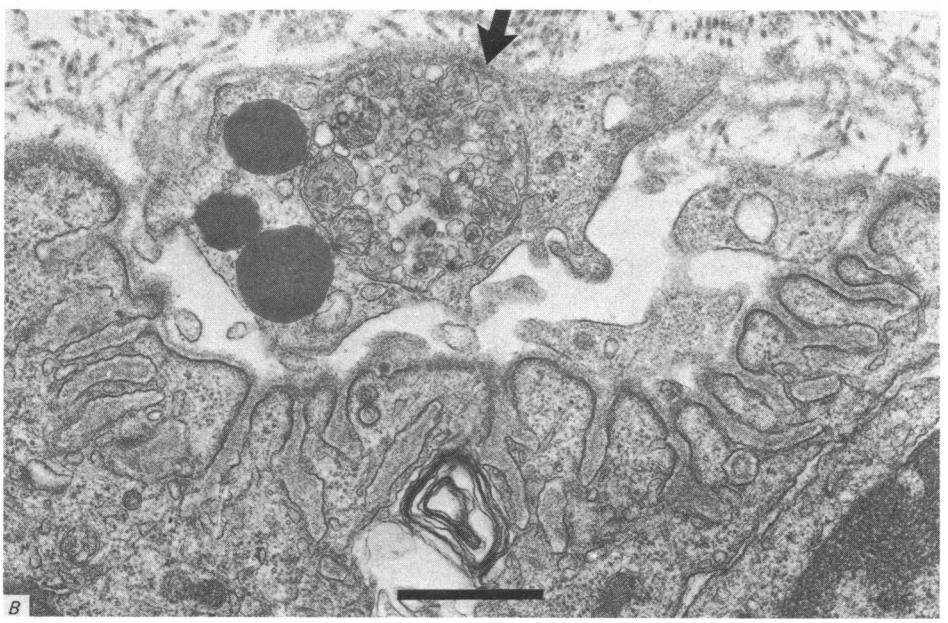
KAWAI, N., MAURO, A. & GRUNDFEST, H. (1972). Effect of black widow spider venom on lobster neuromuscular junctions. *J. gen. Physiol.* **60**, 650–664.

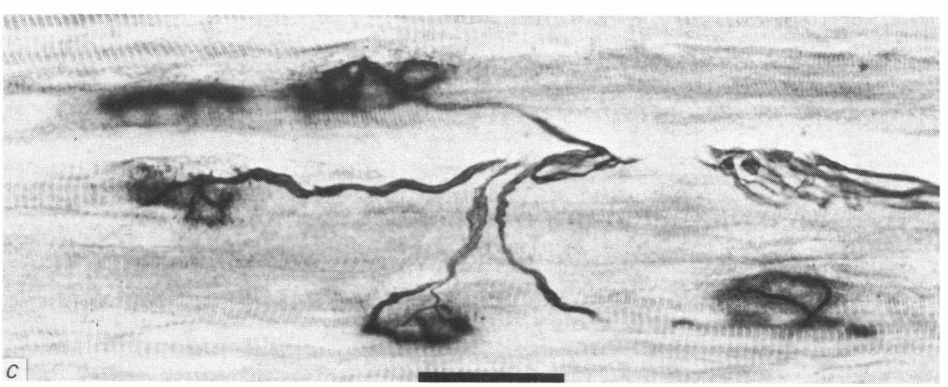
KOELLE, G. B. & FRIEDENWALD, J. S. (1949). A histological method for localizing cholinesterase activity. *Proc. Soc. exp. Biol. Med.* **70**, 617–622.

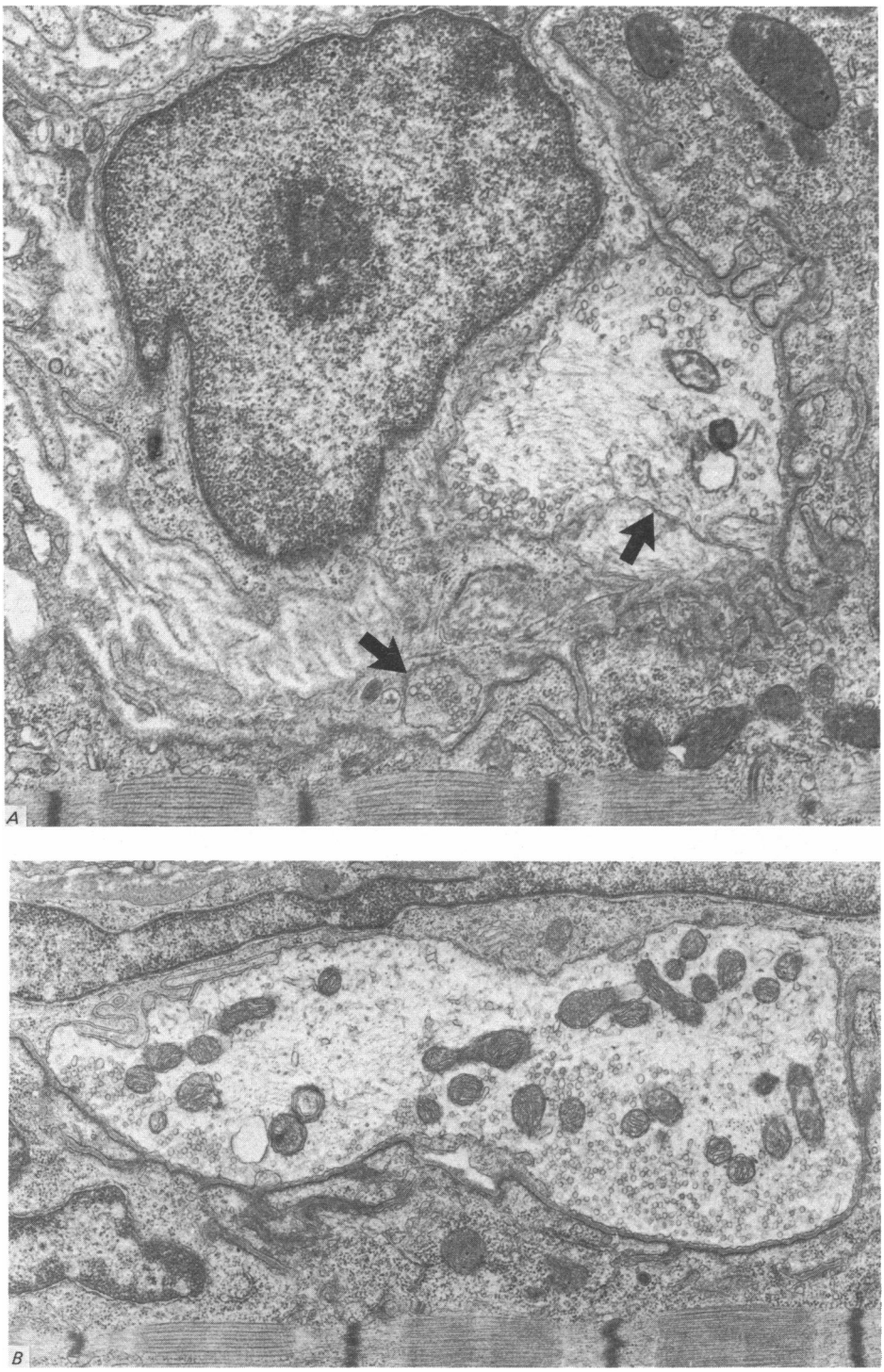
KOENIG, J. & PECOT-DECHAVASSINE, M. (1971). Relations entre l'apparition des potentiels et l'ultrastructure des plaques motrices en voie de reinnervation et de neoformation chez le rat. *Brain Res.* **27**, 43–57.

LONGENECKER, H. E., HURLBUT, W. P., MAURO, A. & CLARK, A. W. (1970). Effects of black widow


A


B


C


A
B

spider venom on the frog neuromuscular junction – effects on end-plate potential, miniature end-plate potential and nerve terminal spike. *Nature, Lond.* **225**, 701–703.

LÜLLMANN-RAUCH, R. (1971). The regeneration of neuromuscular junctions during spontaneous reinnervation of the rat diaphragm. *Z. Zellforsch. mikrosk. Anat.* **121**, 593–603.

MARTIN, A. R. (1955). A further study of the statistical composition of the end-plate potential. *J. Physiol.* **130**, 114–122.

MILEDI, R. (1960*a*). The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. *J. Physiol.* **151**, 1–23.

MILEDI, R. (1960*b*). Properties of regenerating neuromuscular synapses in the frog. *J. Physiol.* **154**, 190–205.

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

MILEDI, R. & ZELENA, J. (1966). Sensitivity to acetylcholine in rat slow muscle. *Nature, Lond.* **210**, 855–856.

NAMBA, T., NAKAMURA, T. & GROB, D. (1967). Staining for nerve fiber and cholinesterase activity in fresh frozen sections. *Am. J. clin. Path.* **47**, 74–77.

OKAMOTO, M., LONGENECKER, H. E., RIKER, W. F. & SONG, S. K. (1971). Destruction of mammalian motor nerve terminals by black widow spider venom. *Science, N.Y.* **172**, 733–736.

PESTRONK, A. & DRACHMAN, D. B. (1978). A new stain for quantitative measurement of sprouting at neuromuscular junctions. *Muscle Nerve* **1**, 70–74.

SAITO, A. & ZACKS, S. I. (1969*a*). Fine structure of neuromuscular junctions after nerve section and implantation of nerve in denervated muscle. *Exp. & mol. Pathol.* **10**, 256–273.

SAITO, A. & ZACKS, S. I. (1969*b*). Fine structure observations of denervation and reinnervation of neuromuscular junctions in mouse foot muscle. *J. Bone Joint Surg.* **51A**, 1163–1178.

SAMPAYO, R. R. L. (1944). Pharmacological action of the venom of *Latrodectus mactans* and other *Latrodectus* spiders. *J. Pharmac. exp. Ther.* **80**, 309–322.

TONGE, D. A. (1974*a*). Chronic effects of botulinum toxin on neuromuscular transmission and sensitivity to acetylcholine in slow and fast skeletal muscle of the mouse. *J. Physiol.* **241**, 127–139.

TONGE, D. A. (1974*b*). Physiological characteristics of re-innervation of skeletal muscle in the mouse. *J. Physiol.* **241**, 141–153.

## EXPLANATION OF PLATES

### PLATE 1

*A* and *C*, frozen sections stained by the method of Pestronk & Drachman (1978) showing cholinesterase activity and axons. Calibration bar, 50 $\mu$m. *A*, at 24 h after BWSV the preterminal axons (arrows) end blindly near the end-plates, which can be identified by the cholinesterase reaction product. Argyrophilic granules may represent remnants of axonal terminals. *B*, at 24 h after BWSV a denervated end-plate with intact post-synaptic sarcolemma which is still coated by basal lamina can be seen. Profiles of Schwann cell cytoplasm lie close to the end-plate and contain debris and a degenerated axon (arrow). Calibration bar, 1 $\mu$m. *C*, At 8 days after BWSV end-plates are innervated and arborizations of axonal terminals are demonstrated.

### PLATE 2

*A*, early regeneration 2 days after BWSV. Axonal profiles (arrows), containing vesicles and filaments and covered by a Schwann cell nucleus, lie in contact with the post-synaptic sarcolemma. *B*, end-plate 8 days after BWSV. The appearance is within normal limits. Calibration bar, 1 $\mu$m.