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TWO FACTORS RESPONSIBLE FOR THE DEVELOPMENT OF DENERVATION HYPERSENSITIVITY

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SUMMARY

1. Innervated adult skeletal muscle is sensitive to acetylcholine at the end-plate region only. After denervation the entire muscle membrane becomes chemosensitive. The period of greatest increase in sensitivity in rat soleus muscles following section of the sciatic nerve in the thigh is between 48 and 72 hr post-operatively.

2. Direct electrical stimulation was found to prevent the onset of the development of denervation hypersensitivity during the first 2–3 days after nerve section. Thereafter, electrical stimulation only reduced the sensitivity of denervated muscles to acetylcholine (ACh).

3. The period of greatest increase in sensitivity follows loss of transmission and degeneration of the nerve terminals. Once this degeneration is under way, electrical stimulation is no longer as effective in preventing the development of denervation hypersensitivity.

4. Hypersensitivity is also seen in muscles on which a small piece of thread or degenerating nerve has been placed. Hypersensitivity following these procedures declines within a few days, unlike denervation hypersensitivity which persists until innervation is restored.

5. The present results suggest that activity alone cannot prevent the development of hypersensitivity in the presence of degenerating nerve fibres, or muscle damage. Activity does however counteract increased sensitivity. It is suggested that two factors interact to produce denervation hypersensitivity; the presence of degenerating nerve tissue and concomitant cellular changes bring about changes in the muscle fibre membrane causing it to become hypersensitive; and the loss of muscle activity, resulting in the persistence of hypersensitivity until innervation is restored.

INTRODUCTION

Following denervation skeletal muscles become hypersensitive to acetylcholine (ACh). This hypersensitivity was first seen when muscle contractures were observed in chronically denervated muscles in response to extremely small doses of ACh (Brown, 1937). Later it was noticed that whereas in a normal muscle only the end-plate region is sensitive to the transmitter (Kuffler, 1943; Nastuk, 1953; Miledi, 1960*a*) following denervation the whole surface of the muscle becomes sensitive (Ginetzinsky & Shamarina, 1942; Axelsson & Thesleff, 1959; Miledi, 1960*b*, *c*). The hypersensitivity of denervated muscles was explained by an increase of the chemosensitive area outside the end-plate region, and this led to several proposals being put forward to explain how the area outside the end-plate region is normally kept insensitive. Thesleff (1960) suggested that small amounts of ACh released from the nerve endings at the neuromuscular junction and seen as miniature end-plate potentials have a desensitizing effect on the area outside the end-plate region. Miledi (1960*b*, *c*) proposed that a special trophic substance independent of muscle activity is released from the nerve ending, and that this special material keeps the muscle fibre insensitive to the transmitter. Neither of these suggestions, however, explains the fact that the end-plate is always very sensitive to the transmitter although it must be in the closest contact with either the hypothetical trophic substance or the desensitizing effect of the miniature end-plate potentials.

The possibility that the absence of muscle activity is an important factor for the development of hypersensitivity has recently been reconsidered. When denervated muscles were directly stimulated during the first 2 days after denervation the onset of hypersensitivity was delayed (Jones & Vrbová, 1971). Lømo & Rosenthal (1972) reported that, when a plastic cuff containing local anaesthetic was placed round the sciatic nerve in rats, and the traffic of impulses was thus prevented from reaching the muscle, hypersensitivity developed which was comparable to that found after denervation. This finding differs from results obtained in rabbits where no changes in sensitivity or other muscle properties were found when the motor nerve was silenced (Robert & Oester, 1970). Other results also show discrepancies. Thomson (1952) reported that denervation hypersensitivity can be reduced by direct electrical stimulation of the denervated muscles, whereas Ginetzinsky (1956) stated that such treatment did not have any effect on the development of hypersensitivity to ACh. Thus contradictory results are reported by different authors concerning the contribution of inactivity to the development of denervation hypersensitivity.

It has been found that the time of onset of denervation changes in the muscle is related to the length of the peripheral nerve stump; denervation changes occur later when the peripheral stump is longer. The length of the peripheral stump also influences the onset of degenerative changes of nerve terminals in the end-plate (Miledi & Slater, 1969). In fact, Hájek, Gutmann & Syrový (1964) found that the biochemical changes that followed denervation can be correlated with the onset of degenerative changes at the end-plate. That factors other than inactivity may be involved is also suggested by findings obtained by Miledi (1960*b*) in the frog sartorius. Sartorius muscle fibres are innervated by two nerve fibres and it could be expected that if one is cut the other nerve fibre will activate the whole muscle fibre. Miledi (1960*b*) found, however, that denervation hypersensitivity in such partially denervated sartorius muscles developed on the part of the muscle that had been denervated, and concluded that activity did not prevent development of hypersensitivity of the denervated, but active, portion of the muscle fibre. Finally, inactivity cannot be the only factor responsible for hypersensitivity, for damaged innervated muscles, that presumably are active, become hypersensitive (Katz & Miledi, 1964). More recently, it has been reported that when a section of a degenerating peripheral nerve is placed on the surface of a normal muscle the area underlying the nerve becomes hypersensitive (Vrbová, 1967). Thus under these conditions activity fails to prevent the development of hypersensitivity. The present investigation was designed to examine the extent to which inactivity is important in the development of denervation hypersensitivity and to see what other factors might contribute to its development.

METHODS

1. *Electrical stimulation of denervated muscles*

In all experiments adult rats of 180–200 g body weight were used. The muscles of both hind legs were denervated by section of the sciatic nerve in the thigh, under ether anaesthesia with full aseptic precautions.

(i) *Stimulation in vitro*

Two to 6 days after denervation the soleus muscles of both legs were removed under ether anaesthesia and secured in a bath containing oxygenated Krebs–Henseleit solution. The muscles were attached to strain-gauges (Ether Ltd). Contractions were elicited by direct stimulation via bipolar silver wire electrodes, and recorded on a pen recorder (Devices Ltd). One muscle received trains of impulses at 40 pulses/sec for 500 msec once every 90 sec. The other muscle received single impulses at the same intervals. Stimulation was continued for between 1 and 4 hr, and contractions of both muscles were recorded throughout. After the period of stimulation, contractions of both muscles in response to ACh added to the bath at 10 min intervals were recorded and dose–response curves for each muscle obtained.

(ii) *Stimulation in vivo*

Two to 7 days after denervation rats were anaesthetized with pentobarbitone sodium (1 mg/kg). Small skin incisions were made at the knee and ankle joint and fine silver wires were inserted at the achilles tendon and at the knee joint. Leads from these electrodes were attached to a stimulator so that the muscles were stimulated directly with trains of impulses at 40 pulses/sec for 500 msec once every 90 sec. In most experiments, electrodes were implanted in the other leg, but there was no stimulation of these muscles. Stimulation was continued for 1–2 hr and at the end of the stimulation period soleus muscles were removed from both legs and their sensitivity to ACh tested using two different techniques.

In the first experiments muscles were secured in a bath containing Krebs–Henseleit solution, and contractions in response to diffusely applied ACh tested as previously described.

In a second group of experiments muscles were removed and placed in a bath perfused with Liley's modification of Ringer solution (Liley, 1956). The muscles were stretched over a Perspex dome set in a bed of paraffin wax on a glass slide which was then fixed in the bath. The muscles were secured by pinning the tendons at each end, and transilluminated via the Perspex dome by a light placed beneath the bath. Conventional 3 M-KCl filled micro-pipettes were inserted into superficial fibres using a Zeiss micromanipulator, and changes in membrane potential in response to ionophoretically applied ACh and, in some experiments, the input resistance of superficial fibres, were recorded using the methods described by Albuquerque & Thesleff, 1967.

(iii) *Chronic stimulation*

The sciatic nerves on both sides were cut. The operations were performed under ether anaesthesia with full aseptic precautions. On one side electrodes made of multicoiled stainless-steel pace-maker leads (Devices Ltd) covered with polyethylene tubing were implanted. A short length of bare pace-maker lead at the end served as the stimulating electrode. The bare ends of the leads were sutured on either side of the distal nerve stump in the popliteal fossa. The leads were then led under the skin of the back and externalized via an incision in the skin of the animals back. Here the leads were attached to press studs which in turn were attached to a small piece of 'Velcro'. The wound on the back was closed, and the Velcro was sutured to the skin of the back.

In another group of rats the implanted leads were of a soft, fine multicore flex (Filotex Ltd) with short silver wires attached at one end. The joints between the silver wire and the leads were covered with a short length of polyethylene tubing sealed at each end with Dow Corning medical adhesive. In these experiments one lead was sutured in the popliteal fossa as near to the proximal soleus tendon as possible, and the other lead was passed under the skin of the leg and attached to the achilles tendon. In this way the muscles of the back of the leg could be stimulated directly. The leads were passed under the skin of the back, and brought out via a skin incision on the back and secured as previously described. The animals were attached to a stimulator by means of a second set of press-studs attached to leads from a stimulator driven by a Digitimer via a gated pulse generator (Devices Ltd) to give trains of impulses (40 pulses/sec for 500 msec every 2 min). Stimulation continued for 7–8 hr each day for up to 3 days. At the end of stimulation period both soleus muscles were removed and placed in baths containing oxygenated Krebs–Henseleit solution. Contractions in response to different concentrations of diffusely applied ACh were measured. In two experiments the stimulated and unstimulated

soleus muscles of both legs were pinned in baths perfused with Krebs–Henseleit solution. Resting membrane potential and changes in potential in response to ACh added to the perfusing fluid were also measured, using conventional 3 M-KCl filled micropipettes. (See last section of Methods.)

II. *Induction of a chemosensitive area on the extrajunctional membrane of an innervated muscle*

In twelve rabbits and three cats 1 cm of the ulnar nerve was excised and placed on the surface of the lower third of the tibialis anterior muscle of one leg. Operations were carried out using pentobarbitone sodium (Martindale Samoore Ltd) anaesthesia (40 mg/kg) with full aseptic precautions. The final experiments were performed 2–12 days later. The rabbits were anaesthetized with 20% urethane, the tibialis anterior muscles of both legs were exposed, and their distal tendons cut and attached to hooks. The lateral popliteal nerve was exposed and cut at the knee joint. The rabbits were then placed on their backs on a heavy steel table. Both legs were held rigid at the knee and ankle joints by drills which in turn were attached to the heavy steel table. The distal tendons of the muscle were attached to strain-gauges (Statham Ltd). A pool was constructed by sewing the skin flaps to a semicircular bar in which liquid paraffin kept at 37° C was placed. The fascia was carefully removed from both muscles, and, after noting its position, the piece of degenerating nerve was removed from the operated muscle.

Cats were anaesthetized with chloralose (70 mg/kg) and Nembutal (0·1 mg/kg) but otherwise prepared in the same way as described for the rabbits.

In four rabbits and one cat Sherrington electrodes were placed on the belly and tendon of the experimental tibialis anterior muscle. The tendon of the muscle was attached to Statham strain gauges. Action potentials were amplified using a Devices FM 122 preamplifier and displayed together with changes of tension on the screen of a Tektronix 502 A oscilloscope. A glass pipette filled with solution of ACh and connected to a container by tubing was then mounted on a ratchet and moved along the longitudinal surface of the tibialis anterior muscle. Three drops of ACh were released from the pipette by opening a tap by a relay that also triggered the beam of the oscilloscope. The pipette was moved along the surface of the muscle and ACh was released at 10 min intervals.

In eight rabbits and two cats changes of membrane potential were recorded from the surface of the muscle using non-polarizable Ag-AgCl wick electrodes. The reference electrode was placed on the exposed distal head of the tibia. The recording electrode was mounted on a ratchet and could be moved along the whole length of the muscle. This recording was performed simultaneously from both legs, and the muscles lined up so that both recording electrodes were moved along comparable areas of the tibialis anterior muscles on both sides. The electrodes were connected to a high impedance input DC6 preamplifier and displayed on a pen recorder (Devices Ltd). In each leg the motor nerve was stimulated every 10 sec via bipolar silver wire electrodes. Muscle contractions were recorded on the pen recorder using Statham strain gauges connected to Devices DC2 subunit 1 preamplifiers. Decamethonium, suxamethonium and curare were administered I.V. via the jugular vein.

In twenty-two rats a small piece of ulnar nerve was excised and placed on the ventral surface of the soleus muscle of one leg. Operations were performed under ether anaesthesia with full aseptic precautions. Two to 7 days later both soleus muscles were removed under ether anaesthesia. In eight experiments the sensitivity to ACh of the operated and control muscles was compared by measuring contractions

in response to diffusely applied ACh using the method described previously for denervated muscles.

The remaining fourteen pairs of muscles were secured in a bath perfused with Krebs–Henseleit solution. The floor of the bath was coated with a layer of embedding wax and the muscles were secured by pins passed through each tendon into the wax floor. Control and operated muscles were placed in the same bath, and after noting the position of the degenerating nerve, it was carefully removed. Using an LPC micro-manipulator, 3 M-KCl filled micropipettes were inserted into superficial muscle fibres. Changes in membrane potential in response to doses of ACh added to the bath were recorded using an ELS negative capacitance amplifier connected to the amplifier of a Tektronix 502 A oscilloscope. The trace was filmed using a Langham Thompson camera.

In the present experiments the concentrations of the drugs quoted are for the salt of the drug. They are decamethonium diiodide (Allen & Hanbury Ltd.), acetylcholine chloride (Laboratiore Lematte et Boinot Ltd), suxamethonium chloride (B.D.H. Ltd). For *in vivo* experiments the drugs were diluted in 0·9 % NaCl (w/v) and for *in vitro* experiments in Krebs–Henseleit solution.

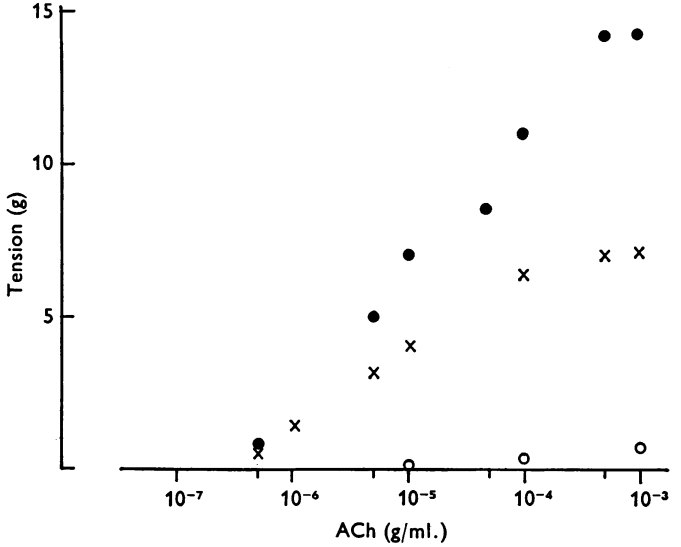
RESULTS

In some preliminary experiments the development of denervation hypersensitivity following sciatic nerve section was followed in rat soleus muscles. One to seven days after denervation muscles were mounted in a bath and the tension developed in response to diffusely applied ACh was recorded. It was found that a significant increase in sensitivity was already seen at 36 hr after denervation. The sensitivity continued to increase reaching maximum at 3–4 days after denervation. Text-fig. 1 shows dose–response curves of muscles denervated for 24, 48 and 72 hr respectively.

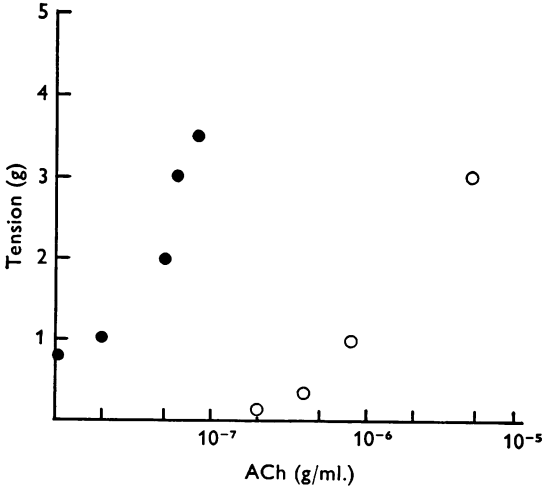
(*a*) *Stimulation in vitro*

The denervated soleus muscles of both hind legs were removed 2–6 days after the operation. Both muscles were placed in a bath and one muscle was stimulated at 40 pulses/sec for 500 msec every 90 sec while the other muscle was left unstimulated. After 1–4 hr of stimulation, contractions in response to different doses of ACh were recorded. It was found that the stimulated muscle was always less sensitive than the unstimulated muscle. The degree to which sensitivity was reduced varied depending on the length of time that the muscle had been denervated and the period of stimulation. Text-fig 2 illustrates the results obtained from a muscle denervated for 3 days. The dose–response curves show the response of the same muscle before and after a 4 hr period of stimulation. The twitch tension of the muscle before stimulation was 12 g and after 11·5 g. The difference in response to ACh was not therefore thought to be due to a change in the contractile properties. Table 1 shows the twitch tension and responses to a single dose of ACh before and after a period of stimulation lasting 1 hr. The muscles were denervated for 3–5 days. As the

Table shows there is often a small difference between the twitch tensions before and after stimulation but the direction of the change was not consistent. The response to ACh, however, was always smaller following stimulation. Thus the reduction of sensitivity was not related to a change in contractile characteristics.



Text-fig. 1. Tension developed in response to different doses of ACh by rat soleus muscles 1(○), 2(×), and 3(●) days after denervation.



Text-fig. 2. Tension developed by a 3 days denervated soleus muscle in response to different doses of ACh before (●) and after (○) 4 hr electrical stimulation.

(*b*) *Stimulation in vivo*

Two to 7 days after denervation the rats were anaesthetized and the soleus muscle on one side stimulated directly for 1–2 hr, using the same pattern of impulses as for the muscles stimulated *in vitro*. The muscles were attached to strain gauges so that changes in tension throughout the experiment could be recorded. Immediately after stimulation the muscles were excised and mounted in a bath for tension recording. In each experiment the twitch tension in response to direct electrical stimulation of each

TABLE 1. Response of muscles to ACh after a period of stimulation

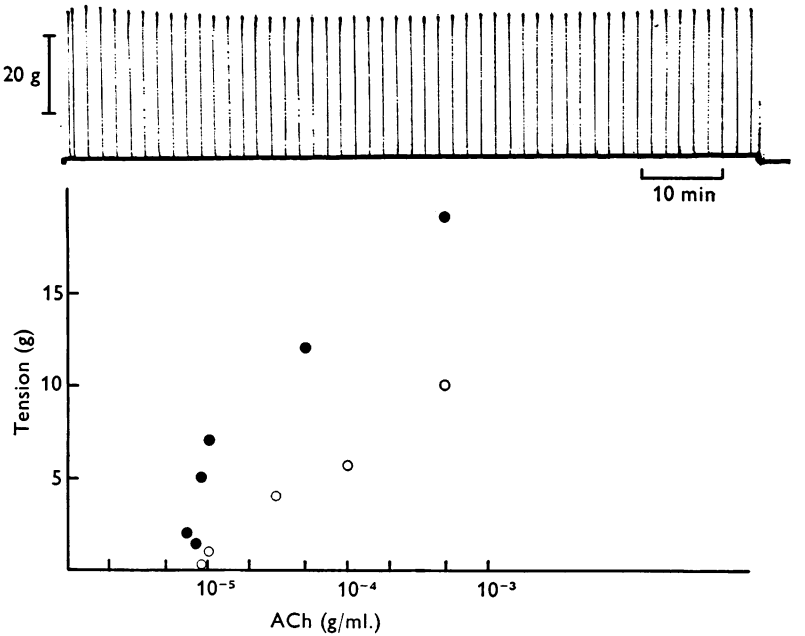
	Twitch tension in g		Response to ACh 5×10^{-7} g/ml. in g	
Days after denervation	Before stim.	After stim.	Before stim.	After stim.
3	14·5	13·0	2·9	1·1
3	13·0	14·5	1·5	0·4
4	13·0	12·2	1·9	0·9
4	11·0	14·0	1·4	0·6
5	12·0	11·0	4·0	1·2

muscle was recorded first. The twitch tension varied between 11 and 14 g, but for each pair of muscles studied the difference in tension was never greater than 20 %. In Text-fig. 3 tension developed in response to different doses of ACh by the stimulated muscle is compared to the responses of the control unstimulated soleus. It can be seen that the stimulated muscle was less sensitive to ACh. Text-fig. 3 (top trace) also shows that the tension elicited by direct electrical stimulation did not decrease throughout the experiment.

In addition, the resting membrane potential, input resistance and extrajunctional sensitivity to ACh of superficial fibres in ten stimulated and unstimulated muscles were measured. A second electrode containing KCl was used to pass current and the input resistance for stimulated and unstimulated fibres was recorded. Results are summarized in Table 2 below. The input resistance and resting membrane potentials were not significantly changed in the stimulated muscles. The sensitivity to iontophoretically applied ACh was also tested and the results expressed as mV membrane depolarization/nC passed through the ACh pipette are shown in Table 2. Although it can be seen from Table 2 that the mean sensitivity of the stimulated muscle was less than that of the unstimulated control, the difference between muscles was not statistically significant. This was thought to be due to the great variation in sensitivity between individual fibres from both stimulated and unstimulated muscles.

(c) *Chronic stimulation*

In fifteen experiments the sciatic nerve on both sides was sectioned and stimulating electrodes were implanted. The peripheral stump of the sciatic was stimulated at 40 pulses/sec for 500 msec every 2 min for 7–8 hr daily.



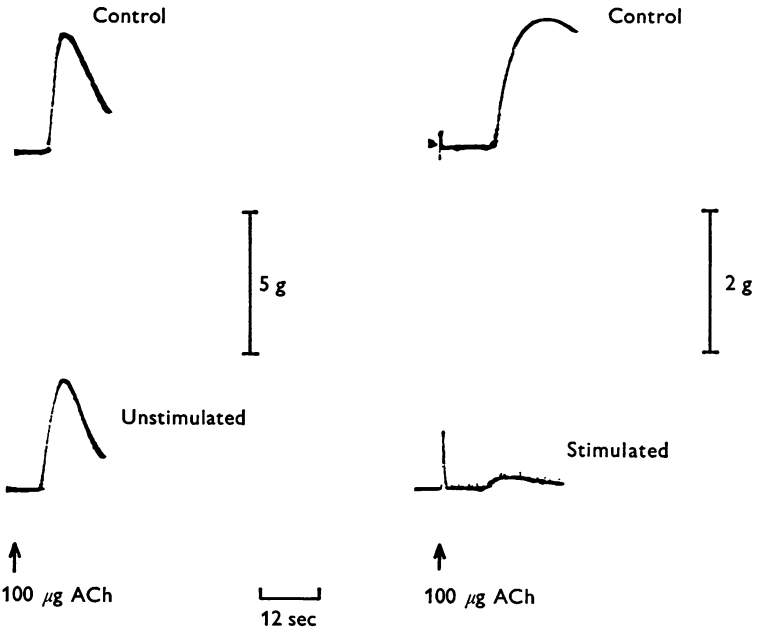
Text-fig. 3. Top tracing shows isometric contractions elicited by direct electrical stimulation in one of a pair of soleus muscles removed 3 days after denervation. The graphs shows the tension developed by the unstimulated (●) and stimulated (○) muscles plotted against concentration of ACh.

TABLE 2. Effects of muscle activity on some characteristics of the muscle fibre membrane

Days den.		Resting membrane potential (mV)	Input resistance (MΩ)	ACh response (mV/nC)
3	U	(30) 58 (S.D. ± 3·1)	(49) 0·43 (S.D. ± 0·14)	(15) 15·05 (S.D. ± 7·0)
	S	(30) 57 (S.D. ± 2·7)	(48) 0·38 (S.D. ± 0·13)	(10) 12·51 (S.D. ± 4·9)
5	U	(27) 62 (S.D. ± 4·0)	(17) 0·39 (S.D. ± 0·13)	(17) 14·9 (S.D. ± 6·4)
	S	(27) 61 (S.D. ± 3·6)	(21) 0·47 (S.D. ± 0·15)	(19) 10·25 (S.D. ± 4·6)
7	U	(15) 58 (S.D. ± 4·1)	(17) 0·69 (S.D. ± 0·23)	(6) 46·42 (S.D. ± 17·0)
	S	(17) 60 (S.D. ± 3·1)	(12) 0·63 (S.D. ± 0·21)	(6) 31·22 (S.D. ± 7·1)

Mean values for resting membrane potential, input resistance and ACh sensitivity in stimulated (S) and unstimulated muscles (U) at different times after denervation. Figures in brackets = *n*. ACh sensitivity was recorded at the edge of the muscle; approximately 3 mm from the distal tendon.

Transmission was seen to fail 32–36 hr after nerve section. The muscles were removed and tested for ACh sensitivity 48 hr after denervation. Text-fig. 4 shows the effects of such stimulation on the development of ACh sensitivity. Text-figure 1 shows contractions in response to ACh of denervated soleus muscles from two experiments from rats in which electrodes were implanted. The tracings on the left show contractions of the control (upper trace) and implanted (lower trace) muscles to the same dose of

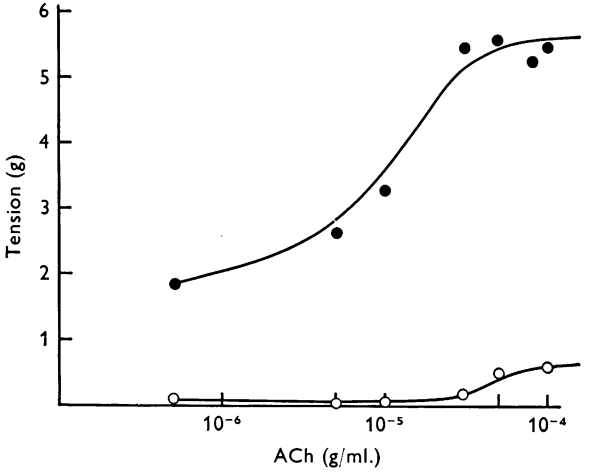


Text-fig. 4. Contractions of 48 hr denervated soleus muscles in response to ACh. Top tracings: denervated 'control' muscles, bottom left-hand trace – electrodes implanted, but muscle left unstimulated, bottom right-hand trace: electrodes implanted and muscles stimulated for 8 hr each day after operation.

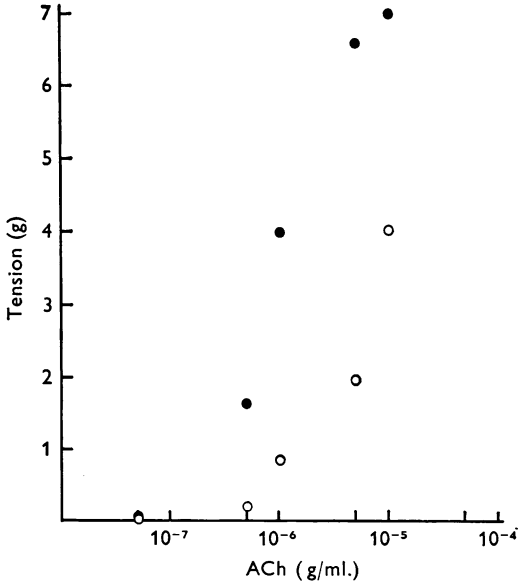
ACh. This rat received no stimulation, and it is clear that implantation alone does not affect the sensitivity of the muscle. On the right of the Text-figure are tracings from control (upper trace) and stimulated muscles (lower trace). In this experiment the development of denervation hypersensitivity was prevented altogether. The dose–response curves in Text-fig. 5 show that the stimulated muscle was much less sensitive to all doses of ACh tested. In all cases the tension developed by the denervated stimulated muscles in response to direct electrical stimulation was similar to that produced by the control muscle.

In a further group of rats, electrodes were implanted so as to stimulate

the denervated muscles directly. In four rats soleus muscles of both hind limbs were denervated and electrodes implanted at the same time. On the fourth day the sensitivity of both muscles was compared. The dose–response curves shown in Fig. 6 illustrate the results of one of these

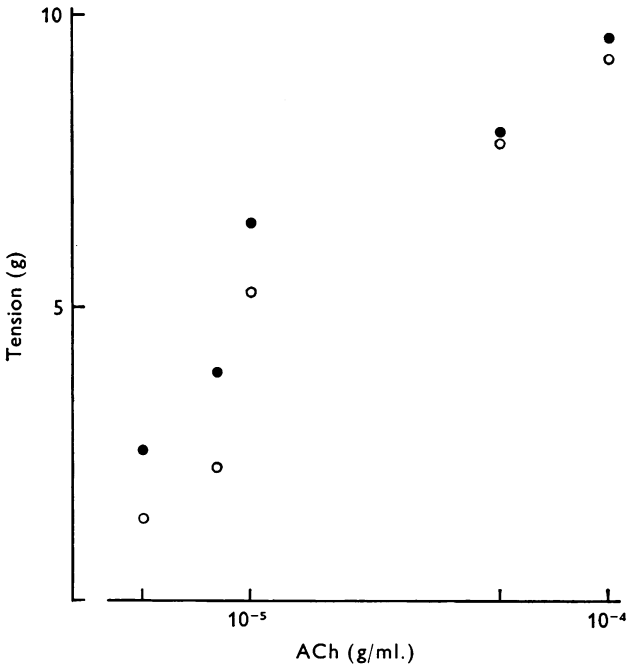


Text-fig. 5. Tension developed in response to ACh by a 48 hr denervated soleus muscles. (●) Unstimulated 'control' soleus; (○) stimulated soleus.



Text-fig. 6. Tension developed in response to ACh by 3 days denervated soleus muscles. (●) Unstimulated 'control' muscle; (○) stimulated muscle.

experiments. Although for each dose of ACh the response of the stimulated muscle was smaller, the difference between stimulated and unstimulated muscles was not as great as that seen in muscles examined at 2 days (Text-fig. 5). Further, when electrodes were implanted 3 days after denervation, and the muscles stimulated for 3 days, there is virtually no difference between the dose–response curves of stimulated and unstimulated muscles. This is illustrated in the dose–response curves shown in Text-fig. 7.



Text-fig. 7. Tension developed in response to ACh by 6-day denervated soleus muscles. (●) Unstimulated 'control' muscle; (○) stimulated muscle.

Thus the development of denervation hypersensitivity can be reduced during the early period of denervation by stimulation, but cannot be appreciably affected after the third day of denervation.

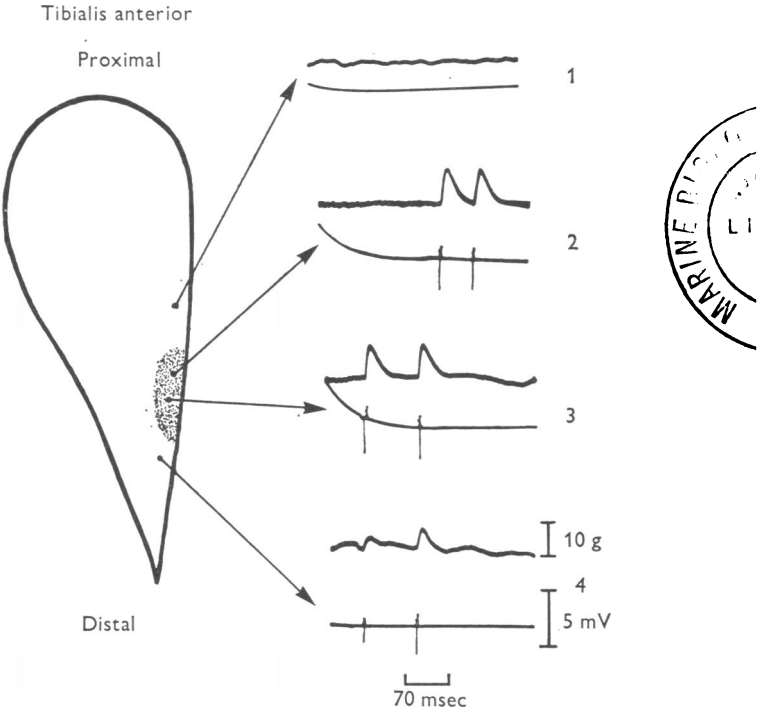
In order to see whether such rigorous stimulation of denervated muscle has any damaging effect on the muscle fibres, resting membrane potentials were recorded *in vitro* (Krebs–Henseleit solution) from two stimulated and two unstimulated soleus muscles which had been denervated 3 days previously. In the control muscles average resting membrane potentials were $69 \pm 1{\cdot}02$ (S.D.) in one and $70 \pm 0{\cdot}85$ (S.D.) in the other rat. The stimulated muscles had mean resting membrane potentials of $71 \pm 0{\cdot}81$ (S.D.) and $69 \pm 1{\cdot}3$ (S.D.). In each experiment recordings were made from between

ten and twenty superficial fibres. Stimulation, then, does not result in any change in the resting membrane potential, indicating that the muscle fibres are not damaged.

Effects of implantation of degenerating nerve on the chemosensitivity of the muscle fibre

(*a*) *In vivo experiments*

In twelve rabbits one cm of the ulnar nerve was implanted on to the surface of the lower third of the tibialis anterior muscle. Three to 15 days later the animals were anaesthetized and set up for recording action

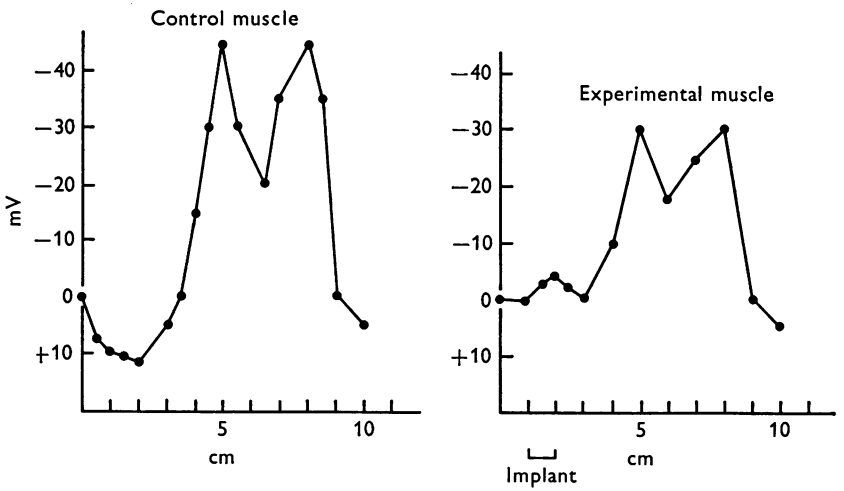


Text-fig. 8. Schematic representation of a rabbit tibialis anterior muscle. The shaded area represents the place where a section of the ulnar nerve was implanted 5 days before the final experiment. Traces 1–4 represent recordings of contractions (top) and action potentials (bottom) taken when ACh in concentrations 5×10^{-5} M was released on to the indicated place on the muscle.

potentials and contractions in response to ACh released from a glass pipette. When a solution of 5×10^{-5} M-ACh was released on to the area underlying the degenerating nerve, contractions and action potentials

could be recorded. When the pipette was moved to any other extra-junctional area no such effects were seen. Results from such an experiment are illustrated in Text-fig. 8. These responses were abolished by curare administered I.V.

In four experiments changes of surface membrane potential in response to I.V. injection of suxamethonium were recorded. The recording electrode was placed on the area underlying the degenerating nerve and on a comparable area of the muscle on the other side. The reference electrode was

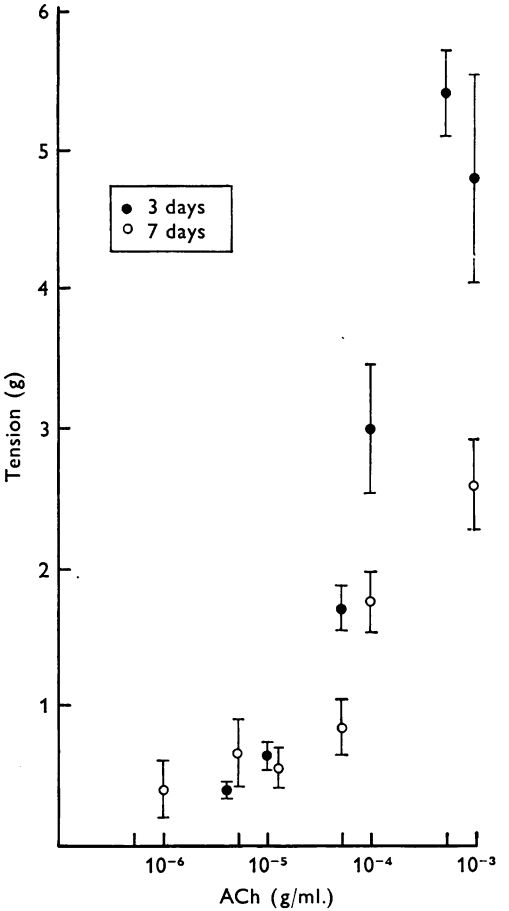


Text-fig. 9. Cat 2·5 kg. The graphs show the distribution of potential along the surface of a normal (upper section) tibialis anterior muscle, and a tibialis anterior muscle that had a nerve implant about 2 cm proximal from the distal tendon (lower section), 2 min after I.V. injection of decamethonium 20 μg/kg. The lower graph shows a depolarized area of muscle under the nerve implant.

situated on the cleaned bone in the ankle joint of the respective leg. Depolarization in response to I.V. injection of 40–80 mg/kg suxamethonium could be recorded from the area underlying the degenerating nerve whereas no change was seen in response to these drugs on the corresponding area of the control muscle.

In three cats similar experiments were performed, and the results obtained from these animals were comparable to those obtained from rabbits. In two experiments the membrane potential was recorded from the surface of the tibialis anterior muscle. The recording electrode was mounted on a ratchet and moved simultaneously along both of the muscles. Text-fig. 9 shows the changes of membrane potential after an I.V. injection of decamethonium. The experimental muscle had a nerve implant

about 2 cm proximal from the tendon and it can be seen that a small depolarization could be recorded from that area as well as from the two bands of end-plate regions typical for this muscle (see Maglagan & Vrbová, 1966). In some experiments a piece of catgut was placed on the surface of the muscle as a control. This procedure did not produce the development of a chemosensitive area on the tibialis anterior muscle.

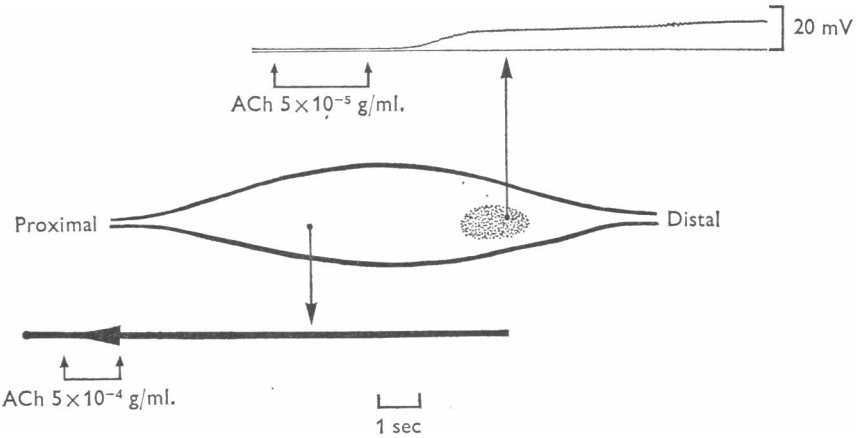


Text-fig. 10. Tension developed by soleus muscles in response to different doses of ACh. The muscles were tested at different intervals after the nerve implant, and each point represents a mean of eight experiments. Horizontal bars indicate S.D.

(*b*) *In vitro experiments*

The next series of experiments were performed on rats. A piece of nerve was implanted on to the surface of the soleus muscle. The sensitivity of these muscles was tested 2–12 days later. Initially, the muscles were placed

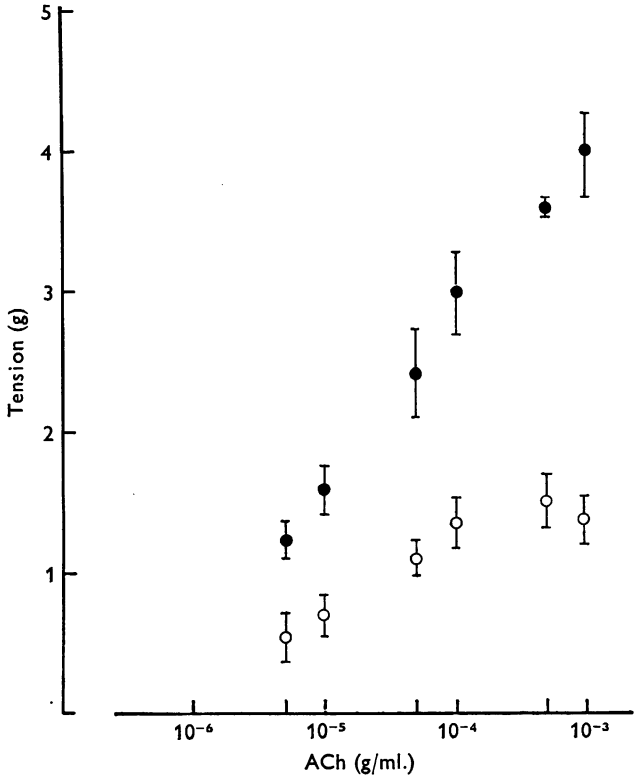
in baths containing oxygenated Krebs–Henseleit solution and contractions in response to ACh added to the bath were measured. Text-fig. 10 shows the dose–response curves obtained with muscles examined 3 and 7 days after nerve implant. Each point represents the mean responses of eight experiments and it can be seen from the graph that both groups become sensitive to ACh. Seven days after nerve implantation the sensitivity of the muscles started to decrease, and muscles examined at 12 days after nerve implant showed responses to ACh similar to those of normal muscles. The muscles were found to be most sensitive at 3–4 days after the nerve was implanted. Examination of sections of these muscles by electron microscopy showed that at 3 days there was an infiltration of macrophages in the area of the implant and much evidence of degenerating tissue (Pl. 1).



Text-fig. 11. Schematic representation of a rat soleus muscle. Shaded area represents the place where 3 days previously a section of a peripheral nerve was placed. Records show changes of membrane potential in response to ACh added to the bath taken from the shaded area (top trace) and outside this area (lower trace). (Resting membrane potentials were 69 and 74 mV respectively.) In each instance electrodes were inserted in an area approximately 0·5 cm from the tendon. Min. end-plate potentials were not observed in any of the fibres impaled.

Next these muscles were examined to see whether, as in the rabbits and cats, the area most sensitive to ACh was the area underlying the piece of degenerating tissue. Muscles were secured in a bath perfused with Krebs–Henseleit solution and the depolarization in response to ACh added to the perfusion medium of superficial fibres in different parts of the muscle was measured using conventional 3 M-KCl filled micro-pipettes. Text-fig. 11 shows the response of two fibres, one underlying the degenerating piece of nerve, the other was situated on the other side of the muscle. The fibres underlying the nerve always responded to much smaller doses of ACh than

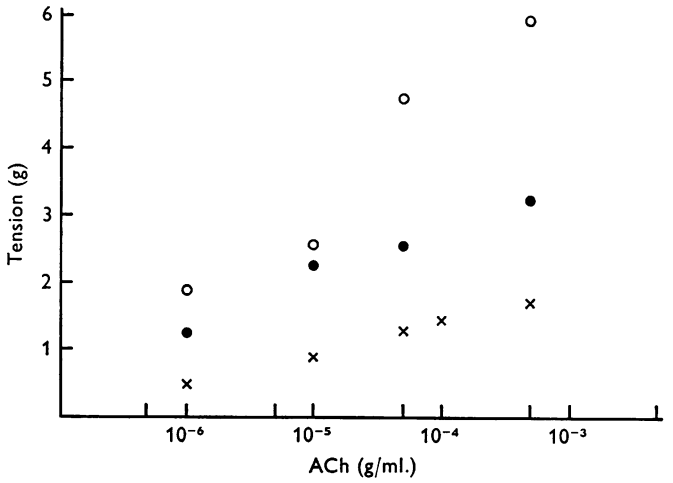
fibres elsewhere in the muscle. In both cases, the muscle fibres were known to be innervated, since on stimulation of the motor nerve an action potential was elicited in the muscle fibre. Again this response was seen to decline after about 7 days and at 12 days the sensitivity of all fibres examined was similar.



Text-fig. 12. Tension developed by soleus muscles in response to different doses of ACh. The muscles were tested at 3(●) and 7(○) days after implanting a thread on to the surface of the muscle. Horizontal bars indicate S.D.

Whether this development of hypersensitivity was a response to the specific stimulus, i.e. the degenerating nerve, or would occur in response to any foreign body implanted on to the surface of the muscle, was studied next. In a group of experiments a piece of silk thread was implanted on the surface of the muscle, and the sensitivity to ACh was tested at different intervals after the operation. An increase of sensitivity followed the implantation, but it was not as great as that seen in muscles with nerve implant. Text-fig. 12 shows the dose–response curves of muscles examined 3 and 7 days after the operation. Again, the increase in sensitivity declines with time.

It is possible that muscle activity somehow counteracts the effects of damage induced by the present procedures. If that was so then the effect of damage should be even greater in denervated muscles during the early period of denervation. In a group of rats, both soleus muscles were denervated and a piece of thread was placed on to the surface of one of the soleus muscles. Forty-eight hr later the sensitivities of both soleus muscles to ACh were compared. Text-fig. 13 shows that the muscle with the thread



Text-fig. 13. Tension developed by soleus muscles in response to different doses of ACh. Forty-eight hr denervated muscle (●), denervated muscle with thread implant (○), normal muscle 48 hr after thread implant (×).

was more sensitive than that without the thread. In another group of rats the thread was placed on to the surface of a normal soleus muscle and its sensitivity to ACh examined 48 hr after the operation. It can be seen in Text-fig. 13 that in this case the muscle was even less sensitive than with denervation alone. From Text-fig. 13 it appears as though the effects of denervation and damage in this experiment are additive.

The finding that even an active muscle, when damaged, can become hypersensitive suggests that inactivity alone is not responsible for the development of increased chemosensitivity of the muscle membrane following denervation. The active muscle, however, does not remain hypersensitive whereas denervated muscles remain hypersensitive until the motor innervation is restored.

DISCUSSION

The results of the present experiments show that relatively short periods of electrical stimulation can reduce the hypersensitivity of muscles denervated for periods of between 2 and 6 days. It was therefore surprising

to find that stimulation of denervated muscles *in vivo* for 8 hr a day reduced or prevented denervation hypersensitivity only in the first 2–3 days after nerve section. When muscles were stimulated 3–6 days after cutting the motor nerve, the effects of such activity were unexpectedly slight. It thus appears that when muscles are stimulated so that periods of rest are interposed between the periods of stimulation activity alone is not sufficient to prevent the development of denervation hypersensitivity.

The results of the present experiments seem to differ from those of Lømo & Rosenthal (1972) who claimed that denervation hypersensitivity could be reversed, using direct electrical stimulation even 11 days after nerve section. However, the period of time over which the muscles were stimulated in experiments of Lømo & Rosenthal were generally longer than here – for instance, a period of 11 days denervation was followed by 8 days stimulation. Nevertheless, they found that in five out of eight experiments there was considerable overlap between the sensitivity of fibres from stimulated and unstimulated muscles. They suggest that this overlap of sensitivity is due to inadequate stimulation. It is however possible that even adequate stimulation does not entirely restore the membrane sensitivity to normal while the muscle is denervated. The results reported in the present investigation favour the latter possibility.

The finding that chronic stimulation was effective in preventing the development of denervation hypersensitivity during the first 2 days, but would only reduce it by a little, if at all, thereafter, suggests that an additional factor to inactivity contributes to the development of denervation hypersensitivity. Findings in which denervation changes were seen to occur earlier in muscles attached to a short stump of sectioned motor nerve than in muscles connected to a longer nerve stump (Luco & Eyzaguire, 1955; Miledi & Slater, 1969; Harris & Thesleff, 1972) also indicate that inactivity alone cannot explain the development of denervation hypersensitivity.

It is interesting to note that the onset of denervation changes appears to be correlated in time with the onset of degeneration of the nerve terminals, and it is the time course of the onset of this process that depends on the length of the peripheral nerve stump (Gutmann, Vodlička & Zelená, 1955; Miledi & Slater, 1969). Thus nerve degeneration and onset of hypersensitivity seem to be closely correlated. It is of interest to note in this context that Miledi (1960*b*) reported that in a partially denervated muscle a localized area of high sensitivity was found in close proximity to a degenerating nerve fibre, a finding further suggesting that the presence of degenerating nerve terminals, or nerve fibres, may in some way be responsible for the development of denervation hypersensitivity.

It has been reported by Gutmann & Young (1944) that 3 days after

denervation, cell division is seen in the muscle. The number of Schwann cells increases and other phagocytic elements appear, presumably to remove the degenerating nerve fibres. This may account for the increase in proteolytic activity in the muscle; indeed Hájek, Gutmann & Syrový (1964) noted that there was a good correlation between the percentage of degenerating end-plates and increased proteolytic activity in denervated muscle. The relative ineffectiveness of electrical stimulation after 3 days of denervation seen in the present experiments may be due to the appearance and activation of such phagocytic elements.

The mechanism by which hypersensitivity could be brought about by the activity of these cells is purely speculative. Results of Lunt, Stephani & De Robertis (1971) suggest that receptor protein is always present in the muscle fibre membrane. It is possible that the increase in phagocytic elements and proteolytic activity could result in the exposure of receptors normally inactive or protected in innervated muscles, resulting in general sensitivity to ACh over the entire muscle membrane after denervation. In support of this hypothesis it has been shown that, if the cellular proliferation which occurs after denervation (Gutmann & Young, 1944) is prevented, denervation hypersensitivity fails to develop (Blunt & Jones, 1972). Other workers (Fambrough, 1970; Grampp, Harris & Thesleff, 1972) have found that the administration of actinomycin D and other inhibitors of protein synthesis prevents the development of denervation hypersensitivity. Grampp, Harris & Thesleff (1972) suggested that such drugs prevent the synthesis of ACh receptors by blocking the expression of the genome, thus inhibiting the manufacture of new receptors of the extrajunctional membrane. However, these compounds can also arrest cell division (see Gottlieb & Shaw, 1967). If, as is suggested in the present discussion, the receptors are always present in the muscle membrane, but are exposed by the action of phagocytic cells which multiply within the muscle after denervation, then the action of drugs such as actinomycin D may be explained by their action on cell division.

Conversely, the role of activity in reducing hypersensitivity may be to cause receptors which have been exposed to become protected again and therefore 'unavailable'. Such a mechanism could account for the fact that the muscles of new-born animals are sensitive to ACh over their entire length (Ginetzinsky & Shamarina, 1942; Diamond & Miledi, 1962). These muscles become insensitive only gradually, and this may be related to the increasing activity of new-born animals.

Once denervation hypersensitivity has developed, it takes a long time for denervated muscles to become desensitized, whether activated by electrical stimulation, or by the motor nerve when the muscles become re-innervated. When, in the present experiments, hypersensitivity was

induced in innervated muscle by placing a degenerating nerve on the surface of the muscle, this hypersensitivity was seen to subside within a few days. These findings further suggest that activity counteracts hypersensitivity. Further, when an increase in sensitivity was brought about by placing a piece of thread on the surface of both active and inactive (denervated) muscles it was found that, within the same period of time, the inactive muscle became more hypersensitive than the active muscle. The results of Katz & Miledi (1964) in which the sartorius muscle of the frog was cut so that one portion of the muscle was free from end-plate and the other innervated, and therefore active, also suggest that activity may reduce hypersensitivity produced by muscle damage, for in these experiments the innervated section of the muscle was less hypersensitive than the nerve free segment.

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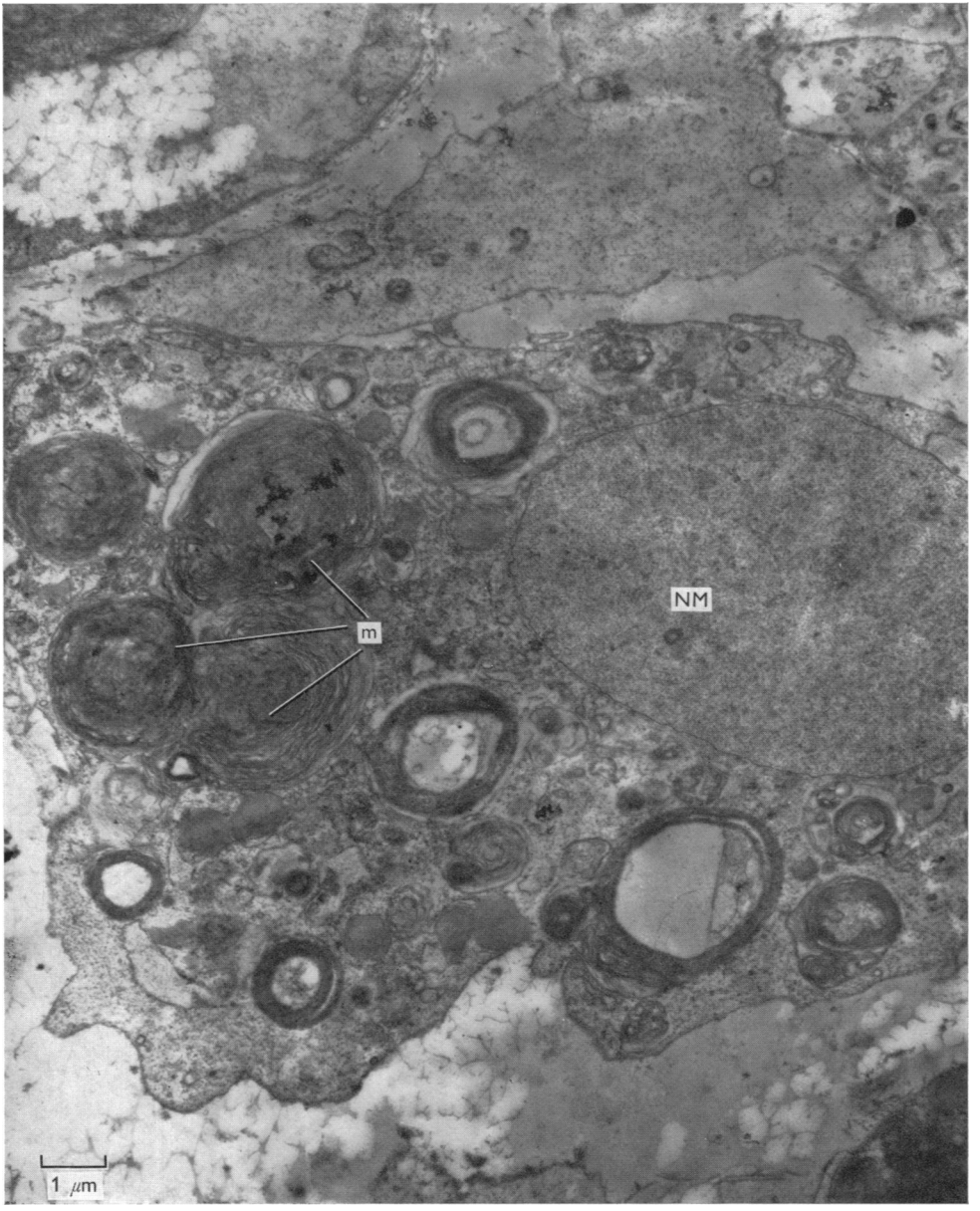
EXPLANATION OF PLATE

Electron micrograph of rat soleus muscle in the region of nerve implant, 3 days after operation. The nucleus of a macrophage (NM) is shown; included within the cytoplasm of the macrophage are several pieces of degenerating myelin (m) as well as other axonal debris.


NM
m
1 μm