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THE FORMATION OF SYNAPSES IN MAMMALIAN STRIATED MUSCLE REINNERVATED WITH AUTONOMIC PREGANGLIONIC NERVES

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SUMMARY

1. A study has been made of the formation of synapses during reinnervation of the hemidiaphragm of adult rabbits with preganglionic fibres of the thoracic vagus, using histological, ultrastructural and electrophysiological techniques.

2. Following reinnervation with preganglionic axons, silver-stained nerve terminals were found in association with cholinesterase-stained end-plates only in the region of the muscle corresponding to the original innervation band.

3. The fine preganglionic axons retained their normal structure in striated muscle, but were found to synapse over discrete areas of dimensions not larger than those of the original end-plates.

4. The regenerated varicose preganglionic nerve terminals were observed with the electronmicroscope in positions either overlying or in the vicinity of the old synaptic folds.

5. Spontaneous potentials and evoked synaptic potentials were recorded only in the middle of the muscle fibres after vagus reinnervation.

6. In a few cases, multiple synaptic potentials with similar time courses were recorded, suggesting that several axons had formed synapses at a single site on a muscle fibre.

7. It has been shown that, during reinnervation of adult mammalian striated muscle fibres with nerves other than those of the somatic system, synapses are formed preferentially in the region of the old end-plates.

INTRODUCTION

During regeneration of somatic nerves into amphibian (Miledi, 1960), avian (Bennett, Pettigrew & Taylor, 1973), and mammalian striated muscles (Bennett, McLachlan & Taylor, 1973), the pattern of innervation

is reconstituted, whether or not the original or a foreign somatic nerve supply is used. This result seems to hold for muscles with either a focal or a distributed innervation, even if the reinnervating somatic nerve does not normally form the same pattern of innervation. However, it is not known if the focal innervation will be reestablished if mammalian muscle is reinnervated by nerves other than those of the somatic system. The reinnervation of frog sartorius muscles by axons of the gastric vagus has recently been examined both structurally and functionally (Landmesser, 1971, 1972), and in this study it was found that junctions were formed mainly at the old end-plate regions on each muscle fibre. It might therefore be expected that the focal innervation of mammalian muscle fibres would be restored after reinnervation by autonomic preganglionic nerves, even though these axons normally form multiple 'en passant' terminals on the soma and dendrites of autonomic motoneurones (Elfvin, 1963, 1971*a*, *b*; Blackman, Crowcroft, Devine, Holman & Yonemura, 1969).

Functional reinnervation of striated muscle fibres by preganglionic axons has often been achieved after nerve cross-union (Langley & Anderson, 1904; Beattie, Duel & Ballance, 1932; Hillarp, 1946; Brown & Satinsky 1951; Dussardier, 1960), although other authors have claimed that function could be explained by the presence of aberrant somatic axons (Guth & Frank, 1959; Guth, Soutter, Frank, Campbell & Lloyd, 1960), and that such synapses do not occur (Zalewski, 1970). In the present study, an examination has been made of the formation of heterogeneous synapses by the regeneration of the preganglionic fibres of the thoracic vagus into the denervated hemidiaphragm of the rabbit. The location of these synapses and the events which occur during their formation have been investigated using combined silver-cholinesterase staining and electronmicroscopy, as well as an electrophysiological analysis of spontaneous and evoked potentials. The results show that the muscle is reinnervated primarily in the region of the denervated end-plates, although in some instances more than one preganglionic axon may be in contact with a single muscle fibre. The findings are consistent with the idea that the site at which synapses form during reinnervation of denervated muscle fibres is determined by some property of the muscle fibres at the end-plate region.

A preliminary report of this work has been presented (McLachlan, Taylor & Bennett, 1972).

METHODS

Rabbits (about 1 kg weight) were anaesthetized with sodium pentathione, and the left side of the thorax opened. After division of the thoracic vagus at the level of the diaphragm, about 15 mm of its central end was freed from the oesophagus. The left phrenic nerve was cut near its point of entry into the diaphragm, and a length of at least 20 mm resected from its cranial portion, which was allowed to spring back

high into the thoracic cavity. The central end of the cut vagus nerve was approximated to the distal stump of the phrenic nerve using an 8/0 silk ophthalmic suture (Ethicon V-767G), and the junction covered with about 3 mm of polyvinylchloride tubing. The animals were allowed to recover for periods of from 6 weeks to more than 4 months.

The animals were killed by cervical fracture and the chest opened. All possible nerves connected to the diaphragm were stimulated and contractions of the muscle were observed. In most experiments, the animals were first anaesthetized, and the left vagus nerve was stimulated in the neck before opening the chest, the diaphragm being inspected from its abdominal surface. The hemidiaphragm and about 15 mm of the vagus nerve connected to it were dissected free and mounted in a Perspex organ bath for electrophysiological examination of synaptic transmission. After this investigation was complete, the muscle was fixed for histological study. Electrophysiological, electronmicroscopic and light microscopic techniques were the same as those reported previously (Bennett, McLachlan & Taylor, 1973).

Some additional information on the properties of autonomic axons regenerating into striated muscles was obtained by applying the same techniques to study the sternohyoid muscles of guinea-pigs after reinnervation with the preganglionic fibres of the cervical sympathetic trunk. This muscle proved very satisfactory for electrophysiological and histological studies, but the irregular distribution of the innervation bands prevented an unambiguous description of the position of regenerated synapses.

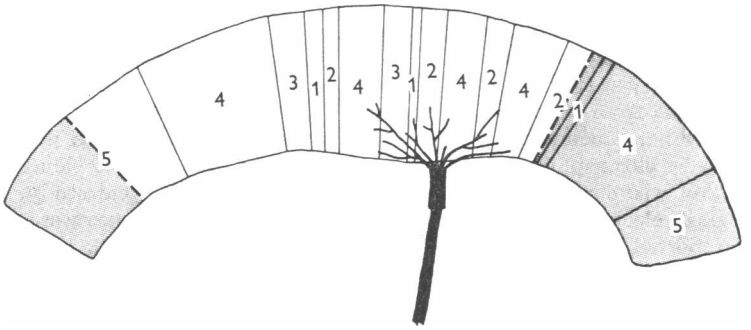
RESULTS

Successful functional reinnervation of the striated muscle fibres of the hemidiaphragm by the vagus nerve was achieved in six of eight operated rabbits after periods of more than 6 weeks. In one animal, the phrenic nerve had also regenerated into part of the diaphragm, and in two others, no response to vagal stimulation was seen, although nerve fibres were found in the muscle upon histological examination. Stimulation of the vagus nerve, either in the neck or in the thorax above the suture, resulted in contraction of parts only of the hemidiaphragm, even after more than 4 months of regeneration. Repetitive stimulation usually increased the size and extent of the contractions. However, extensive areas of the muscle remained denervated, showing fibrillation and atrophy.

Reinnervation of muscle fibres occurred in patches, not necessarily close to the point of entry of the nerve. There was no progressive reinnervation from the point of entry of the regenerating nerves as usually occurs during spontaneous reinnervation with the phrenic nerve (Bennett, McLachlan & Taylor, 1973), although the over-all extent of reinnervation was greater in the animals allowed to survive for longer periods. The example of Text-fig. 1 shows quite extensive reinnervation; only one muscle had greater distributions of Areas 1 and 2.

In three animals, the vagus nerve had sprouted above the PVC tubing and the regenerating axons grew over the outside of the tubing and then spread out diffusely into the diaphragm. Stimulation of the nerve above

the tubing led to contraction of muscle fibres. It may therefore be concluded that regeneration of preganglionic nerves into striated muscle proceeds even without growth down the old glial sheaths.



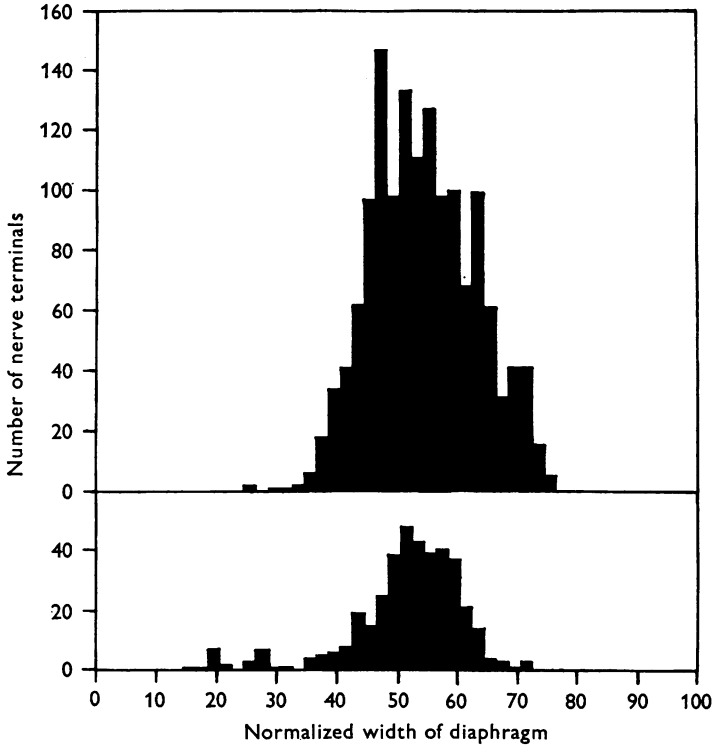
Text-fig. 1. Map of a rabbit hemidiaphragm reinnervated with preganglionic fibres of the thoracic vagus, three months after vago-phrenic anastomosis at the diaphragm. The continuous line indicates the visible branching of the regenerated axons. The stippled areas are the sections of the muscle removed for electronmicroscopy and therefore not examined electrophysiologically. The numbered regions indicate the extent of reinnervation of the different parts of the muscle, more than 80% of fibres in all regions being functionally and histologically denervated. '1' represents areas in which some fibres contracted in response to nerve stimulation, and in which nerves associated with ChE deposits were found on some fibres. '2' represents areas in which subthreshold e.p.p.s were recorded in some fibres, about the same proportion of fibres showing ChE deposits associated with nerves in histological sections. '3' represents areas in which no evoked electrical activity was observed, a few m.e.p.p.s of prolonged time course were noted, and in which a few ChE deposits were found together with nerves. '4' represents areas in which no electrophysiological evidence of synapses was seen, nerve trunks were present but there was no demonstrable ChE, and many action potentials typical of denervated fibres were recorded. '5' represents areas in which no nerves or ChE deposits were found histologically and many of the fibres were firing spontaneous action potentials typical of denervated muscle.

The location of synapses in vagus-reinnervated hemidiaphragms

Each striated muscle fibre has a single end-plate in the middle of the fibre, so that the normal hemidiaphragm has a single innervation band running around the middle of the muscle (Cöers, 1959; Bennett, McLachlan & Taylor, 1973). The position of the original synapses on the muscle fibres is therefore defined whether or not anatomical evidence of the old end-plates can be demonstrated.

Reinnervation of the hemidiaphragm with preganglionic fibres of the vagus nerve results in the formation of synapses only in the original end-plate region (Pl. 1). The position of silver-stained nerve endings associated

with cholinesterase (ChE) stain on muscle fibres were measured in sections from control (n = 10) and vagus-reinnervated (n = 6) hemidiaphragms. The position of each end-plate was measured as a percentage of the length of the muscle fibre between the central tendon and the rib insertion. The results show that the regenerated synapses were located in the region of the muscle fibres corresponding to the original innervation band (Text-fig. 2).



Text-fig. 2. The position of end-plates in control and vagus-reinnervated diaphragms. Histograms of numbers of end-plates in sections of control (upper) and vagus-reinnervated (lower) rabbit hemidiaphragms, measured at different positions along the length of the muscle fibres between the central tendon and the insertion on the ribs. Following reinnervation with preganglionic fibres of the thoracic vagus, end-plates are found only in the position on the muscle fibres corresponding to the original end-plate band.

In the most extensively reinnervated hemidiaphragm (more than 4 months post-operative), more than 80% of muscle fibres were still denervated. Eight ectopic end-plates (outside the normal extent of the innervation band) were found in this animal, located at distances of more

than 15 mm from the point of nerve entry. The formation of synapses at the region of the old end-plates would seem to be preferred, at least for the first few months after denervation.

Structure of the vagus-reinnervated end-plates

Distribution of nerves and end-plates. The gross appearance of the regenerating vagus nerve in the hemidiaphragm was quite similar to that of the regenerating phrenic nerve described in the preceding paper (Bennett, McLachlan & Taylor, 1973). The nerve trunks were generally fewer and smaller than those of the phrenic nerve, and were composed of thin unmyelinated axons, as described by Evans & Murray (1954). The nerve branched out through the muscle from the point of entry until it reached the end-plate region. The nerve trunks then coursed across the muscle at the level of the end-plates (Pls. 1, 2*a*). Small bundles of fine axons occasionally left the nerve trunks and ran either across or parallel to the muscle fibres and occasionally a synapse was formed with a muscle fibre (Pl. 2*a*). It was difficult to distinguish which axon (or axons) of the bundle was associated with the localization of ChE on the muscle fibre.

In one muscle which was examined at 6 weeks after nerve cross-union, and in which no functional innervation was detectable, isolated regenerating (preganglionic) axons were seen growing towards patches of ChE (Pl. 2*b*). These ChE deposits were also present at this stage in parts of the muscle which contained no nerve trunks (Area 5, Text-fig. 1) and so probably represented the ChE remaining at the old end-plates.

After more than 2 months post-operatively, in parts of the muscle which were functionally reinnervated (Area 1, Text-fig. 1), it was occasionally possible to trace fine axons terminating over ChE deposits which covered areas of muscle fibres of the same dimensions as the original end-plates (Pls. 2*c*, 3*a*).

Deposition of cholinesterase. At times longer than 2 months after nerve cross-union when all the remaining preparations were examined, ChE deposits were located only in areas of the muscle in which nerve bundles were demonstrated histologically in adjacent sections (Areas 1, 2, 3, in Text-fig. 1). ChE deposits at this time were found on fewer than 10% of fibres (Area 1, Text-fig. 1) in all but the most extensively reinnervated muscle, and usually appeared in isolated groups of five to fifteen muscle fibres. In areas which were functionally denervated (Areas 4 and 5, Text-fig. 1), no ChE deposits were found even with prolonged incubation times (greater than 1 hr).

In areas in which some electrical responses were detected (Areas 1, 2, 3, Text-fig. 1), ChE sometimes appeared in a strip along a muscle fibre associated with a regenerating axon which terminated a few μm further along

the fibre (Pl. 3*b*). Although appropriate enzyme inhibitors were not used, this strip deposition was probably pseudo-ChE, which is present in growing nerve sprouts (Nakai, 1969; Duchen, 1970). At the terminals, small spots of ChE occurred either singly (Pl. 3*c*), or in groups (Pl. 3*d*), or very occasionally at isolated points scattered over about 100 μm of the muscle fibres. After longer periods of reinnervation (Area 1), ChE was restricted to areas of dimensions similar to the normal end-plate (Pls. 3*a*, *e*). It appears as though the nerve induces ChE at the points where it contacts the muscle, and that this area becomes more extensive if the synapse matures.

Ultrastructure. The majority of the muscle fibres in twenty-two blocks sampled from the six vagus-reinnervated diaphragms (shaded areas, Text-fig. 1) showed typical signs of atrophy with disruption of the contractile elements and some fragmentation of the muscle fibres. Nerve trunks containing non-myelinated axons enclosed in Schwann cells could be found coursing between the muscle cells. Myelinated axons were never seen in the nerve trunks, even after more than 4 months of regeneration, which is consistent with the absence of somatic nerves in these preparations.

In the middle of the muscle fibres (twenty-eight levels from fourteen blocks), small bundles of unmyelinated axons were observed enclosed in Schwann cell (from one to twenty-eight axons per Schwann cell) lying close to the muscle fibres. None were seen in other parts of the muscle (sixteen levels from eight blocks). Near the axon bundles, fine terminals could be found either singly or in small groups, containing agranular cholinergic vesicles (about 45 nm in diameter), and large granular vesicles (about 80 nm in diameter). The ratio of numbers of large granular vesicles to small agranular vesicles was much higher in regenerating vagal terminals than in regenerating somatic terminals, and resembled that normally found at ganglionic synapses (Pl. 4*a*).

Vagal nerve endings bare of Schwann sheath were found lying within 200 nm of the muscle membrane in areas close to the old end-plates (Pl. 4*b*). Serial sections revealed that the terminals were varicose (Pl. 5*a*, *b*) with intervaricosities ($< 0{\cdot}5$ μm in diameter) which contained neurotubules and occasionally large granular vesicles. The varicosities came into close contact with the muscle fibres, sometimes over the old synaptic folds (Pl. 5*c*, *d*).

Spontaneous electrical activity during reinnervation by the vagus nerve

Spontaneous action potentials, typical of denervated muscle fibres (Bennett, McLachlan & Taylor, 1973) were recorded in all areas of the vagus-reinnervated hemidiaphragms. This activity was particularly common (about 65 % of fibres) in the parts of the muscle in which no

synapses could be demonstrated histologically (Areas 4 and 5 in Text-fig. 1). No spontaneous potentials resembling miniature end-plate potentials (m.e.p.p.s) were seen in these denervated areas of the muscle.

The frequency of m.e.p.p.s recorded in autonomic motorneurons is normally low (Blackman *et al.* 1969; Blackman & Purves, 1969; Dennis, Harris & Kuffler, 1971). M.e.p.p.s were observed in only thirteen fibres among the many hundreds impaled in vagus-reinnervated hemidiaphragms and these include those muscle fibres from which synaptic potentials could be evoked by nerve stimulation. The frequency was always less than $0{\cdot}05\ \text{sec}^{-1}$. About half of the observed m.e.p.p.s had a prolonged time course similar to those seen at regenerating somatic synapses (Bennett, McLachlan & Taylor, 1973); both fast and slow time course m.e.p.p.s could not be detected in any fibre. Spontaneous miniature potentials of different time courses were observed in vagus-reinnervated frog sartorius muscles (Landmesser, 1971), and were attributed to a distributed innervation of the muscle fibres, although the rest of the data in those experiments was more compatible with the reinnervation of the muscle fibres only at the old end-plate regions.

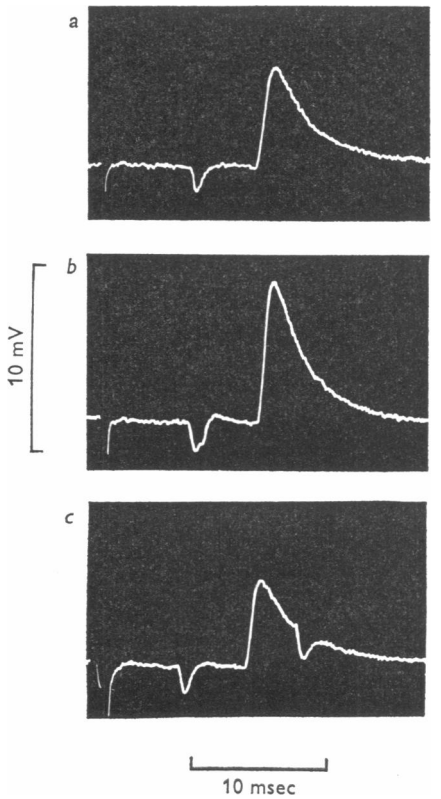
Evoked activity during reinnervation with the vagus nerve

Stimulation of the vagus nerve above the suture evoked subthreshold synaptic potentials (fifty-seven fibres) or sometimes action potentials (twenty-four fibres) (Areas 1 and 2 in Text-fig. 1) in many hundreds of impalements in the vagus-reinnervated diaphragms. Most of the adjacent muscle fibres were functionally denervated. These end-plate potentials (e.p.p.s) were only observed in the middle of the muscle fibres, in the region of the original end-plates, and even then only in areas of the muscle where ChE localizations associated with fine nerve terminals were later demonstrated histologically.

The latency of the responses was usually prolonged ($17{\cdot}48 \pm 1{\cdot}26$ msec, S.E. of mean, $n = 94$) compared with the response to phrenic nerve stimulation in the normal diaphragm ($1{\cdot}97 \pm 0{\cdot}07$ msec, S.E. of mean, $n = 63$) (Text-fig. 3), consistent with a low conduction velocity in unmyelinated preganglionic axons (Blackman & Purves, 1969; Perri, Sacchi & Casella, 1970). In 25 % of fibres with evoked activity, synaptic potentials occurred after a delay of more than 30 msec, which is considerably longer than occurs during reinnervation with somatic axons. In addition, the time course of these synaptic potentials was much longer (half decay time $10{\cdot}3 \pm 1{\cdot}6$ ms, S.E. of mean, $n = 13$) than that of the e.p.p. at junctions reinnervated with somatic axons (half decay time $3{\cdot}7 \pm 1{\cdot}02$ msec, S.E. of mean, $n = 32$) (Text-fig. 4). Action potentials were of normal amplitude and duration, although occasionally these exhibited a prolonged after-

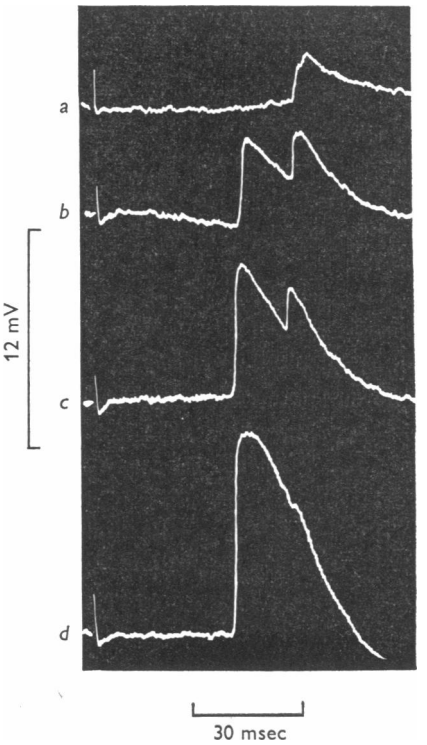
hyperpolarization similar to that observed in denervated muscle fibres, and attributed to a change in potassium conductance (Thesleff, 1963).

Increasing the strength of the stimulus to the vagus nerve did not usually change the amplitude of the synaptic potential (Text-fig. 3), indicating that only one axon was innervating each muscle fibre. However, in one preparation ($3\frac{1}{2}$ months after the operation), a restricted area of muscle was found in which seven polyneuronally innervated fibres were impaled. Progressive increases in stimulus strength produced multiple synaptic potentials (Text-fig. 4). In one fibre, at least five synaptic



Text-fig. 3. Evoked activity during reinnervation of mammalian muscle with preganglionic axons. End-plate potentials (e.p.p.s) in a muscle fibre of rabbit hemidiaphragm, evoked by single stimuli to the vagus nerve. Stimulus strength increased from *a* to *c* (no tubocurarine added). An action potential recorded from an adjacent muscle fibre can be seen between the stimulus artifact and the e.p.p. in *a* and *b*, and a second of these appears with a longer latency in *c*. Although there is considerable variation in the amplitude of the e.p.p., it is apparent that there is only one axon at this junction.

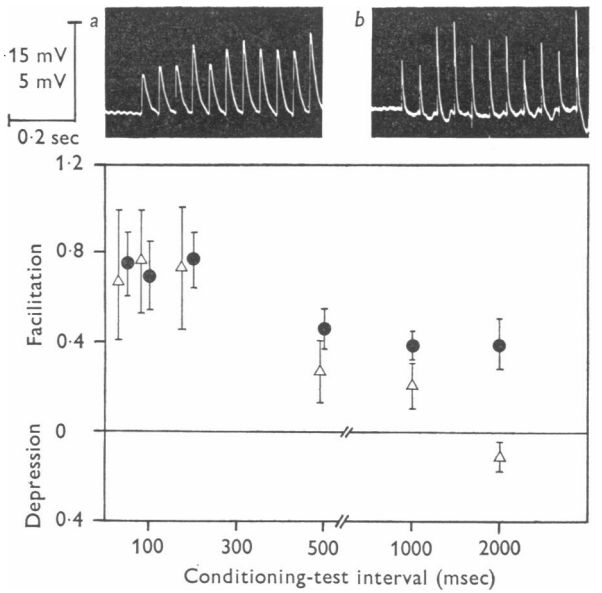
potentials could be distinguished before action potential firing was initiated. The time courses of these multiple synaptic potentials in a single muscle cell were similar, although the latencies were different. Insertion of the micro-electrode at different points along the length of these fibres established that the potentials could only be recorded from a restricted area in the middle of the muscle fibres, and therefore probably arose in this region. Histological studies of this part of the muscle showed



Text-fig. 4. Multiple innervation of a muscle fibre by preganglionic axons. E.p.p.s in a muscle fibre of rabbit hemidiaphragm evoked by single stimuli to the vagus nerve. Stimulus strength increased from *a* to *d* (no tubocurarine added). Note the extremely long latencies of the responses. At least four axons are being stimulated in *d*. Further increase in strength of stimulus led to action potential firing. The time courses of these e.p.p.s are very similar, and the responses are probably derived from the same part of the muscle fibres. It may be concluded that this muscle fibre was polyneuronally innervated at a single site.

fine bundles of axons close to single ChE depositions on each muscle fibre, similar to that seen in most vagus-reinnervated muscle, but it was not possible to identify any obviously multiply innervated end-plates.

Transmitter release is facilitated during short trains of impulses at frequencies greater than 0·1 Hz at control preganglionic nerve terminals (Blackman *et al.* 1969; Text-fig. 5*a*) and at preganglionic nerve terminals reinnervating striated muscle fibres (Text-fig. 5*b*). The variation in the amplitude of the responses suggests that the quantal content of the evoked potentials is low (del Castillo & Katz, 1954).



Text-fig. 5. Release of ACh during repetitive stimulation of preganglionic nerves. Synaptic potentials recorded in *a* ganglion cell of the guinea-pig superior cervical ganglion, and *b* muscle fibre in vagus-reinnervated rabbit hemidiaphragm (no tubocurarine added, cell in *a* hyperpolarized by 10 mV). Frequency of stimulation, 20 Hz. Upper calibration (15 mV) applies to *a*, lower value to *b*. In the graph the ratio $(v-v_0)/v_0$, where v_0 is the amplitude of a conditioning synaptic potential, and v the amplitude of a test synaptic potential, is plotted against the time interval between the stimuli. The filled circles represent the mean values obtained in ganglion cells of guinea-pig superior cervical ganglia, and the open triangles represent the mean values obtained in muscle fibres of rabbit hemidiaphragms reinnervated with thoracic vagus nerve fibres. The bars indicate the standard errors of the mean ($n \geqslant 9$). The release of transmitter from preganglionic nerve terminals is facilitated in both effectors.

A comparison has been made between the responses in autonomic motoneurones and in vagus-reinnervated muscle fibres to pairs of conditioning-test stimuli to their nerves (Text-fig. 5). The facilitation of transmitter release produced by a conditioning impulse on a subsequent test impulse at ganglionic synapses is maximal at short times, and declines

over a period of 30 sec (E. McLachlan, personal observation). Regenerated preganglionic terminals in the diaphragms were only tested at short conditioning-test intervals; the degree of facilitation was not very different from that seen at the ganglionic synapse, and followed a similar time course.

DISCUSSION

The possibility exists that the reinnervating axons were not of parasympathetic origin. No myelinated axons were observed in the vagus-reinnervated diaphragms, thus confirming the extensive study of the composition of the rabbit vagus of Evans & Murray (1954), who showed that it contains only unmyelinated axons at the level of the diaphragm. Most of these are probably sensory, but the peripheral extensions of sensory nerve fibres do not form functional synapses with striated muscle fibres (Gutmann, 1945). The failure of the regenerating axons to reinnervate the majority of muscle fibres over which they passed, together with their varicose nature and the occurrence of relatively large numbers of large granular vesicles, suggests that preganglionic terminals were responsible for the reinnervation. Furthermore, stimulation of the cervical vagus led to contraction of the reinnervated regions of the diaphragms. It therefore seems unlikely that the synapses formed by cross-union of the vagus and phrenic nerves in the thorax were a result of the presence of aberrant somatic nerve fibres.

The regeneration of the preganglionic axons along the degenerating phrenic nerve stump was probably the means by which the axons arrived at the muscle in most preparations, although growth in some cases over the plastic tubing, instead of inside it, suggests that any surface may serve to guide the axons (Weiss, 1945). There were fewer nerve bundles in the muscle than during reinnervation with somatic nerves (Bennett, McLachlan & Taylor, 1973), and their disposition in the muscle was similar. Dussardier (1960) briefly mentioned histological findings of preganglionic fibres running parallel to the muscle fibres before terminating in the vicinity of the old end-plates during the reinnervation of the diaphragm of sheep and goats, and vagal nerve fibres were shown to wander diffusely over frog sartorius muscles before reinnervating the old end-plates (Landmesser, 1971, 1972). It therefore seems unlikely that, in any of these experiments, the preganglionic axons were simply led to the old end-plate region along the old intramuscular nerve paths.

It is of interest that the preganglionic axons in mammals can induce ChE at synapses on mammalian muscle fibres, as there is a complete absence of histologically or functionally detectable ChE at vagal junctions in frog sartorius (Landmesser, 1971). This may represent a species differ-

ence in ChE activity, since the staining of ChE at amphibian end-plates normally requires more than ten times the duration of incubation needed in mammalian muscle. The synthesis of ChE at the end-plate is dependent on an intact innervation (Filogamo & Gabella, 1966), but preganglionic axons do not induce high concentrations of ChE at the synaptic region on the autonomic motoneurone (Couteaux, 1963; Koelle, Davis & Smyrl, 1971). It seems that the ChE may be produced at the end-plate by the muscle in response to the re-establishment of cholinergic synapses.

Some muscle fibres were reinnervated by several preganglionic axons, although the sites of origin of the synaptic potentials appeared to be very close to each other, and were located in the middle of the muscle fibres. It might be that the preganglionic axons are not capable of making their effector refractory to innervation by a nerve arriving subsequently (Harrison, 1910; Fort, 1938), perhaps because they normally synapse on a cell which is polyneuronally innervated. Alternatively, all the axons may have arrived at the end-plate within the period necessary for the development of this refractory property (about 6 weeks; Edds, 1955), as hyperneurotization occurs occasionally during reinnervation of muscle by somatic nerves (Hoffman, 1951*a*, *b*; Guth, 1962; Gutmann & Hanzlíková, 1967). In either case, the nerve terminals were all in the end-plate area, and were not dispersed over the length of the muscle fibres.

Some nerves show a preference during reinnervation for the muscles which they normally innervate (Hoh, 1971). The failure of autonomic axons to innervate all of the denervated muscle fibres they encounter, despite the fact that many nerves are present in the muscle, may indicate that selectivity of synapse formation is expressed as a preference of a nerve for an end-plate, rather than a preference for a nerve for a muscle. If this is so, then the reinnervation of muscle fibres by preganglionic nerves might result from the occasional failure of the selection mechanism.

Not only do preganglionic axons find it difficult to form synapses with the muscle fibres, but also they do not appear to exert a trophic influence on the muscle simply by their presence in it, as the muscle fibres which are not reinnervated continue to atrophy; many disappear and are eventually replaced by fat and connective tissue. The muscle fibres which are reinnervated regain their normal diameters (see also Landmesser, 1971). Trophic effects of somatic nerves on muscle can be seen early in regeneration, well before functional synapses are formed (Feinstein, Pattle & Weddell, 1945; Desmedt, 1959), and also after implantation of a nerve into an innervated muscle (Guth & Zalewski, 1963; Gutmann & Hanzlíková, 1967).

Histological, ultrastructural and electrophysiological evidence of synapses between preganglionic axons of the thoracic vagus and striated

muscle fibres of the rabbit hemidiaphragm has been found only in the area of the muscle corresponding to the old innervation band. This is in agreement with a previous histological demonstration of preganglionic axons of the cervical sympathetic trunk reinnervating some of the end-plates of the sternohyoid muscle in rats (Hillarp, 1946). Autonomic nerves in mammalian muscle were observed with the electron microscope to synapse at or near the old synaptic folds, as they do in amphibian muscle (Landmesser, 1972). The restoration of both the focal innervation of mammalian muscle and the multiple innervation of amphibian muscle by autonomic nerves is consistent with the idea that mature muscle fibres possess sites on their surface whch are preferentially innervated (Bennett, Pettigrew & Taylor, 1973; Bennett, McLachlan & Taylor, 1973).

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REFERENCES

BEATTIE, J., DUEL, A. B. & BALLANCE, C. (1932). The effects of stimulation of the hypothalamic pupillo-dilator centre after successful anastomoses between the cervical sympathetic and certain motor nerves. *J. Anat.* **66**, 283–299.

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

BENNETT, M. R., PETTIGREW, A. G. & TAYLOR, R. S. (1973). The formation of synapses in reinnervated and cross-reinnervated adult avian muscles. *J. Physiol.* **230**, 331–357.

BLACKMAN, J. G., CROWCROFT, P. J., DEVINE, C. E., HOLMAN, M. E. & YONEMURA, K. (1969). Transmission from preganglionic fibres in the hypogastric nerve to peripheral ganglia of male guinea-pigs. *J. Physiol.* **201**, 723–743.

BLACKMAN, J. G. & PURVES, R. D. (1969). Intracellular recordings from ganglia of the thoracic sympathetic chain of the guinea-pig. *J. Physiol.* **203**, 173–198.

BROWN, J. O. & SATINSKY, V. P. (1951). Functional restoration of the paralysed diaphragm following cross-union of the vagus and phrenic nerves. *Am. J. med. Sci.* **222**, 613–622.

COERS, C. (1959). Structural organization of the motor nerve endings in mammalian muscle spindles and other striated muscle fibres. *Am. J. phys. Med.* **38**, 166–175.

COUTEAUX, R. (1963). The differentiation of synaptic areas. *Proc. R. Soc.* B **158**, 457–480.

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

DENNIS, M. J., HARRIS, A. J. & KUFFLER, S. W. (1971). Synaptic transmission and its duplication by focally applied acetylcholine in parasympathetic neurons in the heart of the frog. *Proc. R. Soc.* B **177**, 509–539.

DESMEDT, J. (1959). The physio-pathology of neuromuscular transmission and the trophic influence of motor innervation. *Am. J. phys. Med.* **38**, 248–261.

DUCHEN, L. W. (1970). Changes in motor innervation and cholinesterase localization induced by botulinum toxin in skeletal muscle of the mouse: difference between fast and slow muscles. *J. Neurol. Neurosurg. Psychiat.* **33**, 40–54.

DUSSARDIER, M. (1960). Recherches sur le contrôle bulbaire de la motricité gastrique chez les ruminants. Thèses, Université de Paris.

EDDS, M. V. (1955). Collateral regeneration in partially reinnervated muscles of the rat. *J. exp. Zool.* **129**, 225–243.

ELFVIN, L. G. (1963). The ultrastructure of the superior cervical ganglion of the cat II. The structure of the preganglionic end fibres and the synapses as studied by serial sections. *J. Ultrastruct. Res.* **8**, 441–476.

ELFVIN, L. G. (1971*a*). Ultrastructural studies on the synaptology of the inferior mesenteric ganglion of the cat I. Observations on the cell surface of the post-ganglionic perikarya. *J. Ultrastruct. Res.* **37**, 411–425.

ELFVIN, L. G. (1971*b*). Ultrastructural studies on the synaptology of the inferior mesenteric ganglion of the cat III. The structure and distribution of the axo-dendritic and dendrodendritic contacts. *J. Ultrastruct. Res.* **37**, 432–448.

EVANS, D. H. L. & MURRAY, J. G. (1954). Regeneration of non-medullated nerve fibres. *J. Anat.* **88**, 465–480.

FEINSTEIN, B., PATTLE, R. E. & WEDDELL, G. (1945). Metabolic factors affecting fibrillation in denervated muscle. *J. Neurol. Neurosurg. Psychiat.* **8**, 1–11.

FILOGAMO, G. & GABELLA, G. (1966). Cholinesterase behaviour in the denervated and reinnervated muscles. *Acta anat.* **63**, 199–214.

FORT, W. B. (1938). An experimental study of the factors involved in the establishment of neuromuscular connections. Thesis, University of Chicago, U.S.A.

GUTH, L. (1962). Neuromuscular function after regeneration of interrupted nerve fibres into partially denervated muscle. *Expl Neurol.* **6**, 129–141.

GUTH, L. & FRANK, K. (1959). Restoration of diaphragmatic function following vago-phrenic anastomosis in the rat. *Expl Neurol.* **1**, 1–12.

GUTH, L., SOUTTER, L., FRANK, K. CAMPBELL, J. B. & LLOYD, J. B. (1960). Diaphragmatic function following the anastomosis of recurrent laryngeal and phrenic nerves. *Expl Neurol.* **2**, 251–260.

GUTH, L. & ZALEWSKI, A. A. (1963). Disposition of cholinesterase following implantation of nerve into innervated and denervated muscle. *Expl Neurol.* **7**, 316–326.

GUTMANN, E. (1945). The reinnervation of muscle by sensory nerve fibres. *J. Anat.* **79**, 1–8.

GUTMANN, E. & HANZLÍKOVÁ, V. (1967). Effects of accessory nerve supply to muscle achieved by implantation into muscle during regeneration of its nerve. *Physiologia bohemoslov.* **16**, 244–250.

HARRISON, R. G. (1910). The outgrowth of the nerve fibre as a mode of protoplasmic movement. *J. exp. Zool.* **9**, 787–848.

HILLARP, N. A. (1946). Structure of the synapse and the peripheral innervation apparatus in the autonomic nervous system. *Acta anat.* 2, suppl. **4**, 1–153.

HOFFMAN, H. (1951*a*). Fate of interrupted nerve fibres regenerating into partially denervated muscles. *Aust. J. exp. Biol. Med.* **29**, 211–219.

HOFFMAN, H. (1951*b*). A study of the factors influencing innervation of muscles by implanted nerves. *Aust. J. exp. Biol. Med.* **29**, 289–308.

HOH, J. F. Y. (1971). Selective reinnervation of fast-twitch and slow-graded muscle fibres in the toad. *Expl Neurol.* **30**, 263–276.

KOELLE, G. B., DAVIS, R. & SMYRL, E. G. (1971). New findings concerning the localization by electron microscopy of acetylcholinesterase in autonomic ganglia. In *Progress in Brain Research*, chap. 34, pp. 371–376. Amsterdam: Elsevier.

LANDMESSER, L. (1971). Contractile and electrical responses of vagus-innervated frog sartorius muscles. *J. Physiol.* **213**, 707–725.

LANDMESSER, L. (1972). Pharmacological properties, cholinesterase activity and anatomy of nerve-muscle junctions in vagus-innervated frog sartorius. *J. Physiol.* **220**, 243–256.

LANGLEY, J. N. & ANDERSON, H. K. (1904). The union of different kinds of nerve fibres. *J. Physiol.* **31**, 365–391.

MCLACHLAN, E. M., TAYLOR, R. S. & BENNETT, M. R. (1972). The site of synapse formation in reinnervated and cross-reinnervated mammalian muscle. *Proc. Aust. Physiol. Pharmacol. Soc.* **3**, 62.

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

NAKAI, J. (1969). The development of neuromuscular junctions in cultures of chick embryo tissues. *J. exp. Zool.* **170**, 85–106.

PERRI, V., SACCHI, O. & CASELLA, C. (1970). Electrical properties and synaptic connexions of the sympathetic neurons in the rat and guinea-pig superior cervical ganglion. *Pflügers Arch. ges. Physiol.* **314**, 40–54. Amsterdam: Elsevier.

THESLEFF, S. (1963). Spontaneous electrical activity in denervated rat skeletal muscle. In *The Effect of Use and Disuse on Neuromuscular Functions*, ed. GUTMANN, E. & HINK, P., pp. 41–54. Amsterdam: Elsevier.

WEISS, P. (1945). Experiments on cell and axon orientation in vitro: the role of colloidal exudates in tissue organization. *J. exp. Zool.* **100**, 353–386.

ZALEWSKI, A. A. (1970). Effects of reinnervation on denervated skeletal muscle by axons of motor, sensory and sympathetic neurons. *Am. J. Physiol.* **219**, 1675–1679.

EXPLANATION OF PLATES

PLATE 1

The location of synapses in diaphragms reinnervated with preganglionic axons. Longitudinal sections through (*a*) control and (*b*) vagus-reinnervated rabbit hemidiaphragms. The tendon insertion of the muscle fibres is on the left of each section. In the control muscle, a single band of end-plates can be seen running across the middle of the muscle fibres (arrows). After reinnervation with preganglionic fibres, synapses can only be found in this region. Calibration 2·5 mm, combined silver and ChE staining.

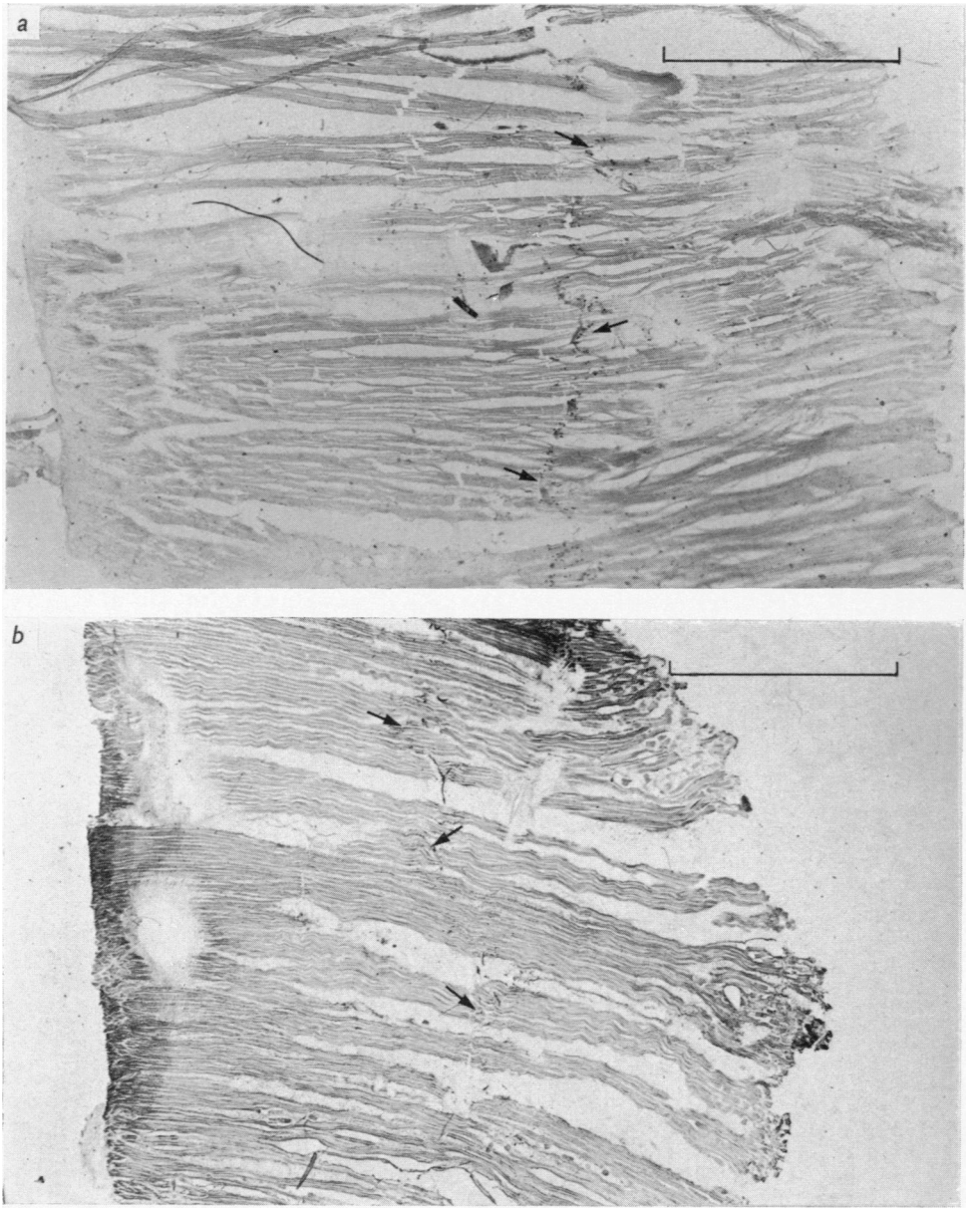
PLATE 2

The arrangement of preganglionic nerves in striated muscle. *a*, A small bundle of preganglionic axons leaving a large trunk and running across muscle fibres, on each of which ChE deposits are located. *b*, A preganglionic nerve growing towards the old ChE depositions (arrows) remaining after denervation of the muscle, about 6 weeks after nerve section. *c*, A fine autonomic axon apparently terminating on spots of ChE at the end-plate region of a muscle fibre. Calibrations: 100 μm (*a*) 30 μm (*b*) and (*c*). Combined silver and ChE staining of (*a*) and (*c*) vagus-reinnervated rabbit hemidiaphragm; (*b*), guinea-pig sternohyoid muscle reinnervated with cervical sympathetic trunk.

PLATE 3

a. Termination of preganglionic nerve at an end-plate. The fine autonomic terminal (arrow) branches over the end-plate region of a muscle fibre. Calibration 50 μm, combined silver and ChE staining of vagus-reinnervated rabbit hemidiaphragm.

b–e. Pattern of ChE depositions on muscle fibres reinnervated with preganglionic nerves. *b*, A dark band (arrow) near a few spot deposits probably represents pseudo-ChE associated with the growing nerve terminal; *c*, small single localization, the most commonly observed deposit; *d*, multiple spot-like deposit similar to that seen during reinnervation with somatic nerves; *e*, more extensive complex covering the same area as a normal end-plate. Calibration 50 μm, vagus-reinnervated rabbit hemidiaphragm.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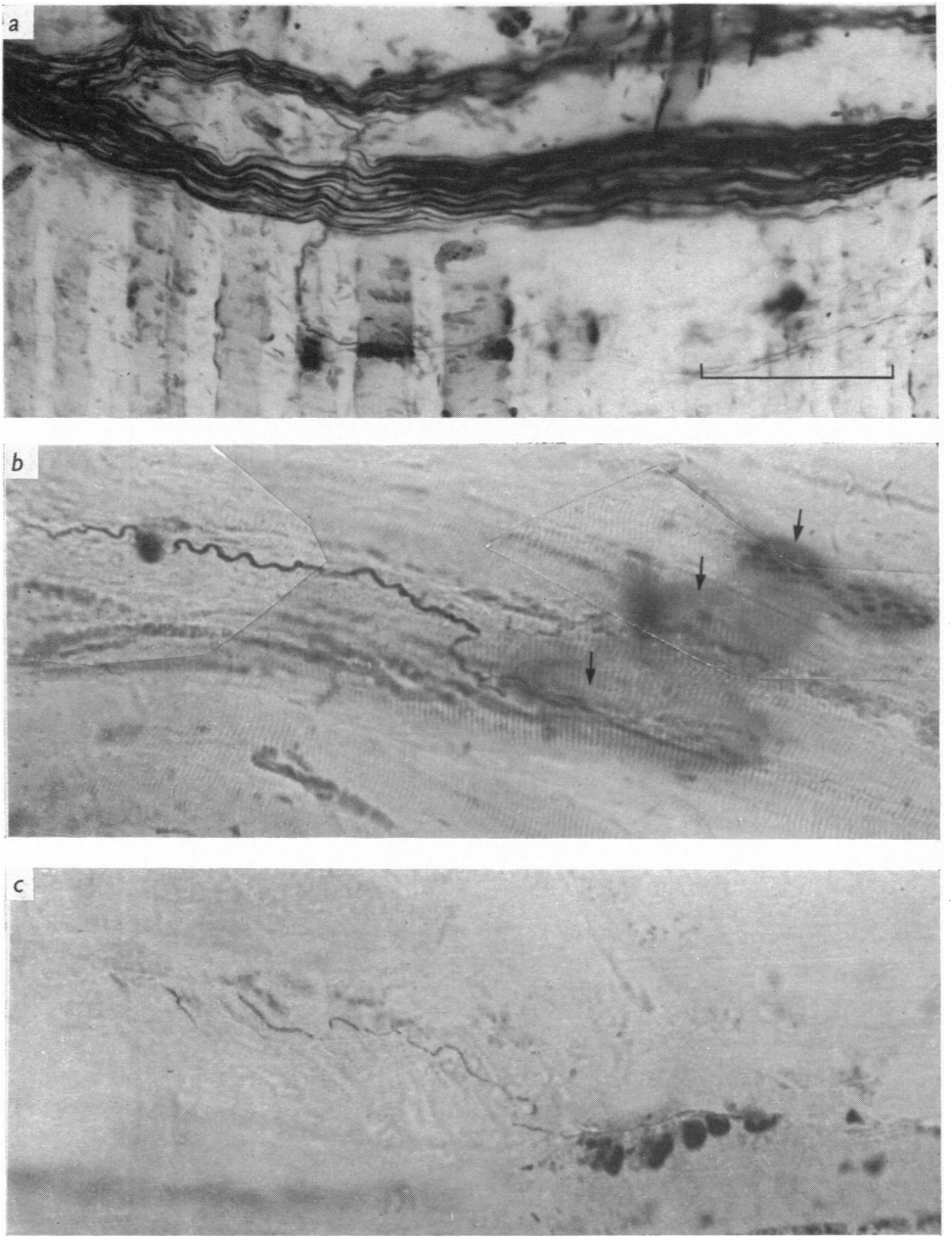
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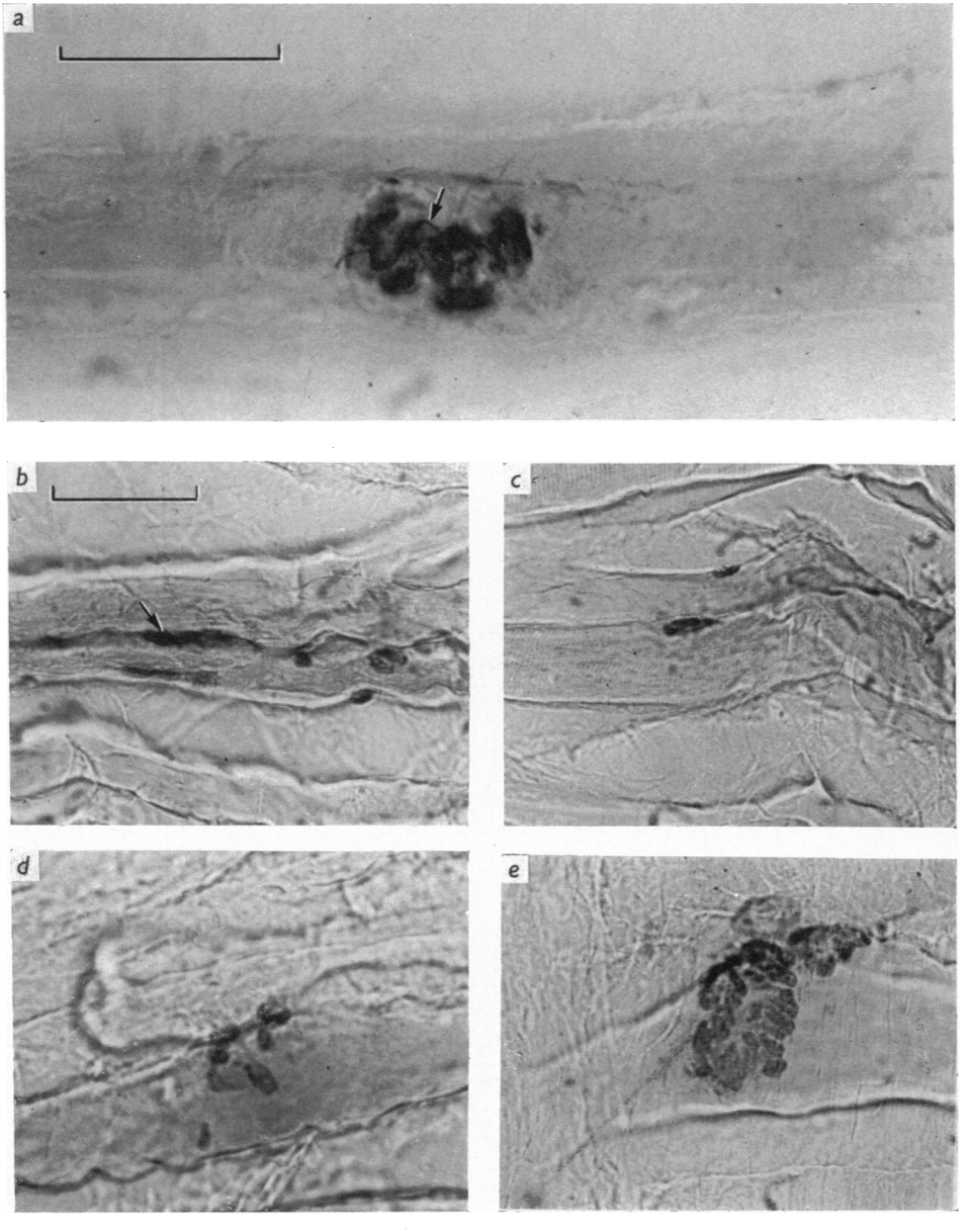
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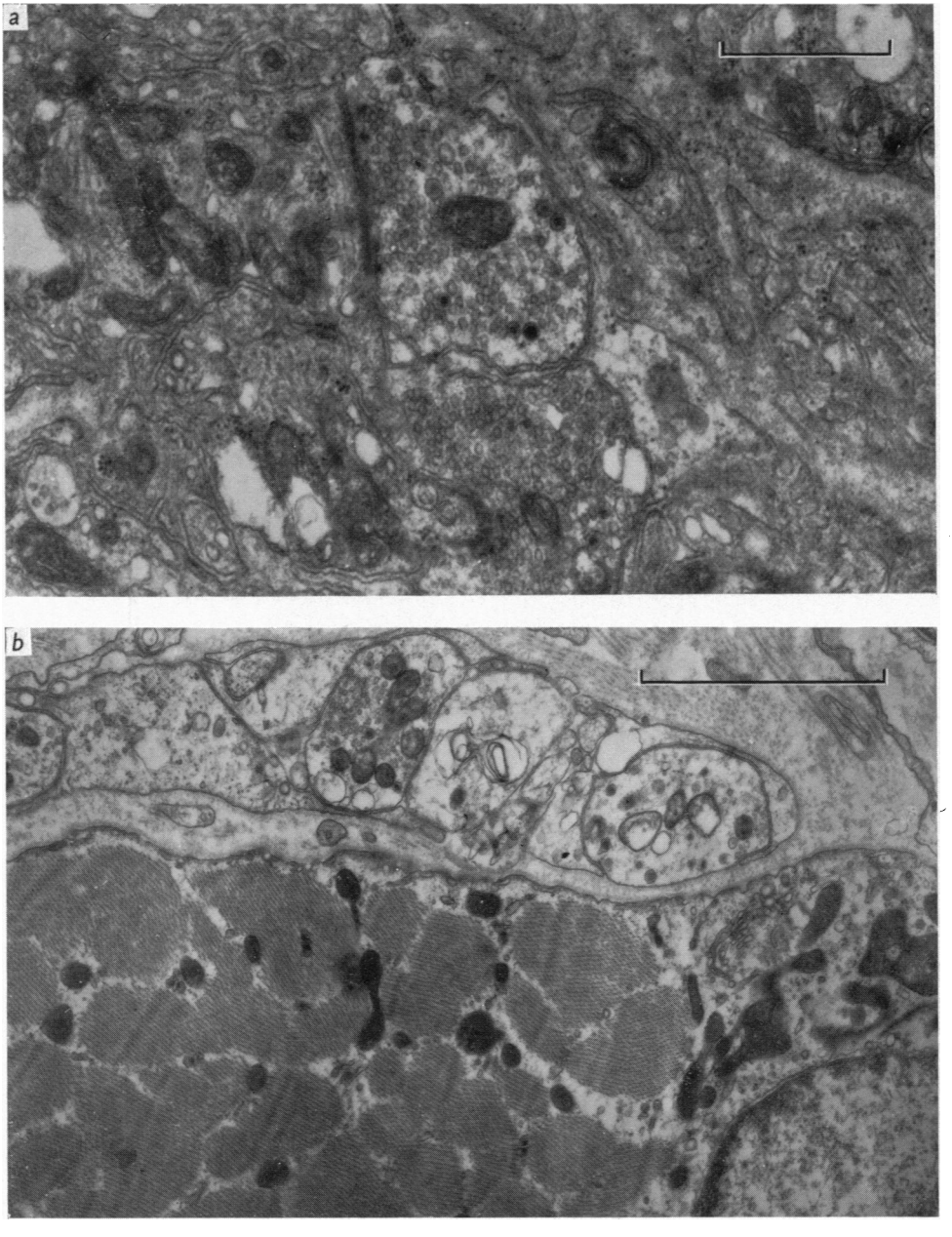
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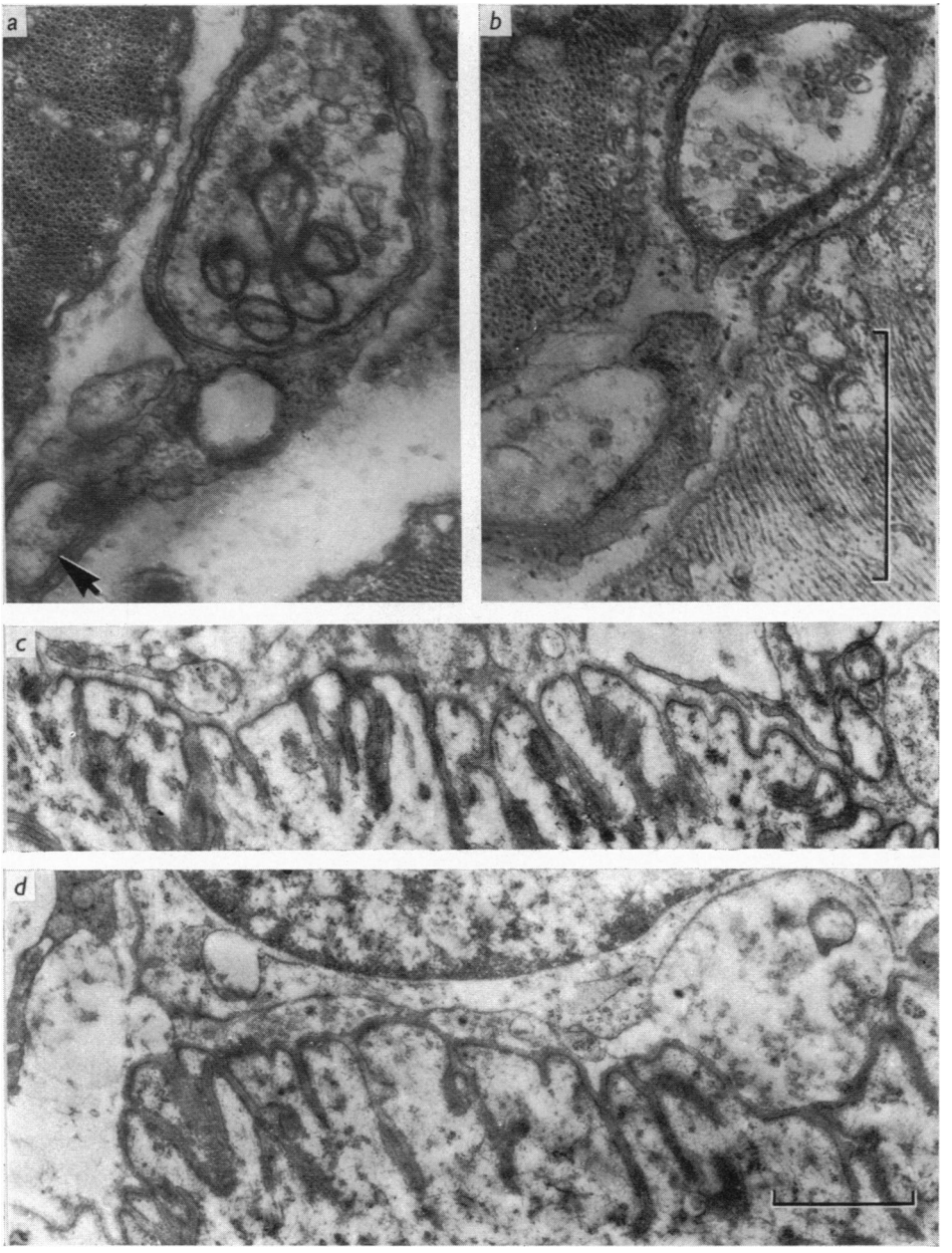
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PLATE 4

Fine structure of preganglionic terminals. *a*, Synapse between a preganglionic axon and the dendrite of an autonomic motoneurone; the terminal in the middle of the figure contains agranular cholinergic vesicles, and large granular vesicles; a thickening of the post-synaptic membrane is seen, towards which the synaptic vesicles cluster; the dendrite contains many mitochondria. Calibration 1 μm, guinea-pig superior cervical ganglion. *b*, Small bundle of unmyelinated preganglionic axons in striated muscle, in close contact with a muscle fibre near an old end-plate; accumulations of mitochondria and nuclei are seen in the sarcoplasm on the right of the Figure, and a naked varicosity lies close to the muscle in this region; the varicosities contain agranular cholinergic vesicles and large granular vesicles. Calibration 2 μm, rabbit hemidiaphragm.

PLATE 5

Preganglionic terminals reinnervating striated muscle fibres. *a* and *b*, Varicose nature of preganglionic axons in striated muscle; nearby sections from the same area of muscle, showing two axons in very close contact with muscle cells, although mostly surrounded by Schwann sheath in these sections; an intervaricosity appears in *a* (arrow). *c* and *d*, Vagal terminals overlying synaptic folds in striated muscle; nearby sections through an end-plate showing varicose axon terminals containing agranular cholinergic vesicles and large granular vesicles, partly overlying the old synaptic folds. Calibrations, 1 μm, vagus-reinnervated rabbit hemidiaphragm.