J. Anat. (1972), **112**, 2, pp. 173–184
With 5 figures
Printed in Great Britain

Some observations on the innervation of the striated muscle in the mouse oesophagus – an electron microscope study

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(*Accepted* 27 *April* 1972)

INTRODUCTION

The striated muscle cells of the oesophagus are not under volitional control and their response to acetylcholine is atypical (Bartlet, 1968*b*). A ganglionated plexus is present even where the oesophageal wall is formed exclusively of this cell type. The need for such a plexus and the reason for its formation seem to be unknown (Davenport, 1966).

The manner in which a peristaltic wave initiated in the upper striated muscular segment passes on smoothly into the lower segment composed of smooth muscle is not fully understood. Doty (1968) is of the view that the peristaltic wave is regulated by a spreading central excitatory pattern. Others have looked at the periphery for an explanation; Rohen (1955/56) has suggested connexions between striped muscle cells and smooth muscle cells while others have searched for peculiarities in the peripheral pattern of innervation. Light microscopic studies concerning these peripheral nerves have been reviewed by Stöhr (1957).

The present study was undertaken, firstly, to ascertain whether the oesophageal striated muscle cell has a distinctive morphology and if it has any special relationship with the smooth muscle cells in the muscularis externa, and secondly to see if there are any peculiarities in the pattern of innervation and the structure of the nerve ending on the striated muscle.

MATERIALS AND METHODS

Ten adult mice were killed by a blow on the head. In five of them, the oesophagus was exposed fully and preserved *in situ* by ice cold 2·5 % glutaraldehyde in Sorensen's buffer at pH 7·4. This material was dripped on the surface, and, after the pharynx and the cardiac end of the stomach had been tied with silk thread, was introduced into the lumen of the oesophagus to distend it slightly. The cervical, thoracic and abdominal segments were marked out with thread. In the remaining animals the oesophagus was dissected out, partially stretched, and secured to an applicator stick to maintain resting length. Preservation and marking out of segments were carried out as before. Each viscus was then immersed in fixative for 1 hour and washed thoroughly in the same buffer. The three segments were separated, samples of suitable size were obtained, and processed further in separate groups. The extreme ends

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and regions damaged by knots were discarded. Under a dissecting microscope the mucosa was stripped carefully off the muscularis externa in order to enhance the penetration of fixative into the externa. All samples were post-fixed in 1 % ice cold osmium tetroxide for 2 hours, dehydrated in ascending grades of ethanol, and embedded in Epon. Thick sections (1–1·5 μm), stained either in toluidine blue or methylene blue and basic fuchsin (Aparicio & Marsden, 1969) were used to assist in the selection of blocks containing nerves and neuromuscular junctions, and also in the search for muscle spindles. Thin sections cut on a Reichert OM U2 ultramicrotome, stained with uranyl acetate and lead citrate (Reynolds, 1963) were examined with a Phillips 200 electron microscope.

RESULTS

The muscularis externa of the entire cervical segment and the greater part of the thoracic segment is made up of striated muscle cells (Fig. 1) and is about six to eight cell layers thick. Smooth muscle cells begin to appear in the externa at the lower end of the thoracic segment just proximal to the diaphragm (Fig. 2). These cells increase in number and gradually replace the striated muscle cells, so that in the distal part of the abdominal segment the externa contains only smooth muscle cells.

Viewed under the dissecting microscope, the muscularis externa is seen as two layers disposed as opposing loosely wound helicals. Electron micrographs show these two layers to be separated by a plexus of nerves, ganglion cells, fibrocytes and their processes and collagen fibres (Fig. 2).

The striated muscle cells of the oesophagus exhibit a cross banding characteristic of other striated muscle cells. *Z*, *A* and *I* bands are clearly defined. Running across the *A* band is a definite *H* band within which is a lightly stained *M* band (Fig. 2). The muscle cell is rich in mitochondria, which are usually arranged in pairs, one on either side of the *Z* line. When single, they take up a variety of positions, and some may extend along the length of the sarcomere. On occasion, masses of mitochondria collect beneath the sarcolemma and may even give rise to protrusions (Fig. 1). The sarcoplasmic reticulum surrounds the myofibrils and, in keeping with the mammalian pattern, is interrupted in the junctional region between *A* and *I* bands by the *T*-tubule to form triads. Glycogen granules are numerous in the region of the *I* band and less frequent in between sarcomeres. Each cell is surrounded by a basement membrane and separated from neighbouring striated and smooth muscle cells by well defined intermuscular spaces containing collagen fibres, fibrocytes, blood vessels and bundles of nerve fibres. Connexions or points of intimate contact between smooth and striated muscle cells as suggested by Rohen (1955/56) were not observed.

Fig. 1. An electron micrograph of a large nerve bundle in the adventitia containing many myelinated axons (*My*) and fascicles of unmyelinated nerve fibres (*Um*). The bundle is surrounded by attenuated perineurial cell processes shown by arrows. Beneath the bundle is a striated muscle cell (*M*) showing a protrusion containing a mass of mitochondria (*m*). *F*, fibrocyte. × 5000.

Fig. 2. A part of the myenteric plexus represented by a bundle of unmyelinated fibres (*Um*). In the striated muscle cell (*M*) the *H* band has a distinct *M* band – shown by arrowheads. Mitochondria (*m*) are arranged in pairs around the *Z* line. *F*, fibrocyte cell process. *cf*, collagen fibres. *SM*, smooth muscle cell. × 5700.


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Um
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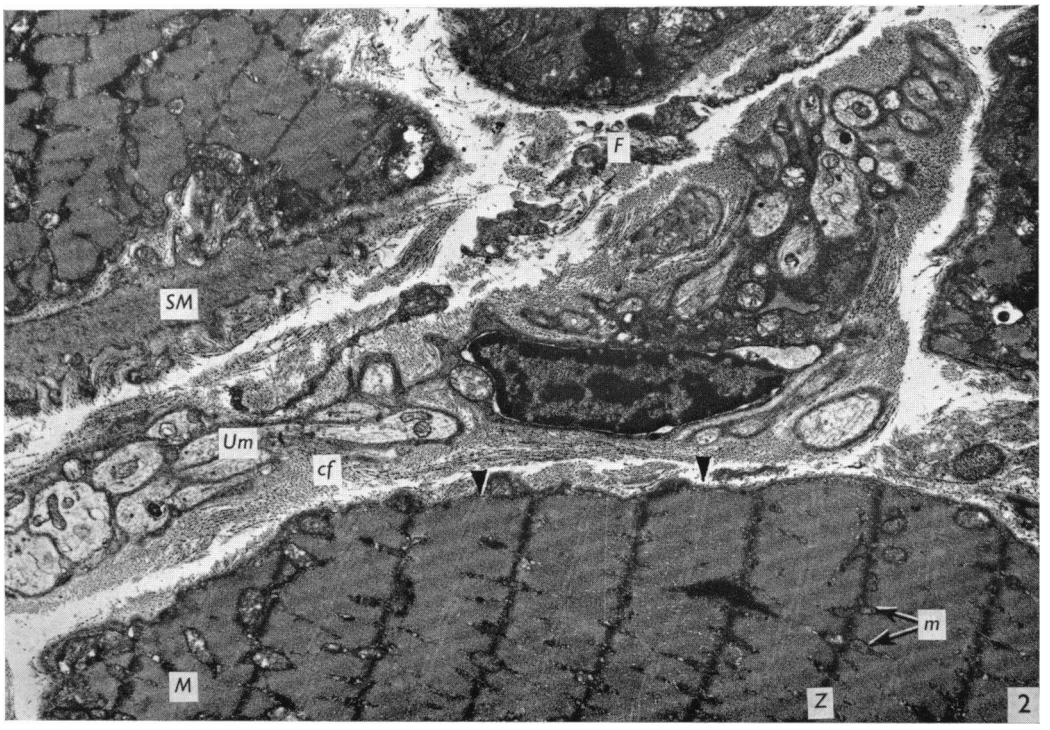
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The intramural distribution of nerves

Surrounding the oesophagus is a dense adventitial plexus, a nerve bundle from which is illustrated in Fig. 1. It contains many myelinated and non-myelinated nerve fibres, with each fibre type enwrapped by Schwann cells in the characteristic fashion. The non-myelinated fibre groups are separated from each other by collagen fibres, the majority of which are oriented along the length of the nerves. There is a peripheral covering one to two cell layers thick formed by interdigitating flattened processes of perineurial epithelium. External to the perineurial sheath is the adventitial coat of the oesophagus, formed of collagen fibres, fibrocytes, and their attenuated processes. These processes meet each other at special contact zones to form a sheath in portions of the oesophageal circumference. Smaller nerve groups composed of non-myelinated and myelinated neurites leave the adventitial plexus and enter the outer layer of the muscularis externa. They traverse the intermuscular spaces and, as the myenteric plexus is approached, shed their peripheral coverings, leaving only the Schwann cells in intimate relation to the nerve fibres.

The myenteric plexus delimits the outer from the inner layer of the muscularis externa. It contains both non-myelinated and myelinated nerve fibres and ganglion cells. Surrounding these structures are fibrocytes and collagen fibres. Internal to the plane of the myenteric plexus, only non-myelinated nerve bundles are encountered, and myelinated fibres are conspicuous by their absence, none being seen despite an intensive search in over 200 sections. This seems to be the pattern for the entire oesophagus, irrespective of the type of muscle cell found in the externa of the cervical, thoracic and abdominal segments.

Nerve endings

Each striated muscle cell in the oesophagus appears to be innervated by a bundle of nerve fibres. These nerves always lack myelin, have an internal structure no different from the common unmyelinated neurite, and they are related to Schwann cells in the usual manner. Each bundle contains a variable number of fibres within the diameter range 0·1 μm to 1·8 μm, and is closely surrounded by a basement lamina.

At the point of neuromuscular contact the degree of specialization is comparable to that obtained in the classical somatic extrafusal motor end-plate. Yet the morphology, especially on the neuronal side, is greatly modified. One such end-plate, cut in the transverse plane, is illustrated in Fig. 3. Here the most striking feature is the retention of the form of the unmyelinated nerve bundle – a feature common to autonomic nerve endings A single Schwann cell is seen to contain nerve fibres as well as nerve terminals. The nerve fibres are usually located away from the side of the muscle cell and are either partly or completely surrounded by Schwann cell cyto-

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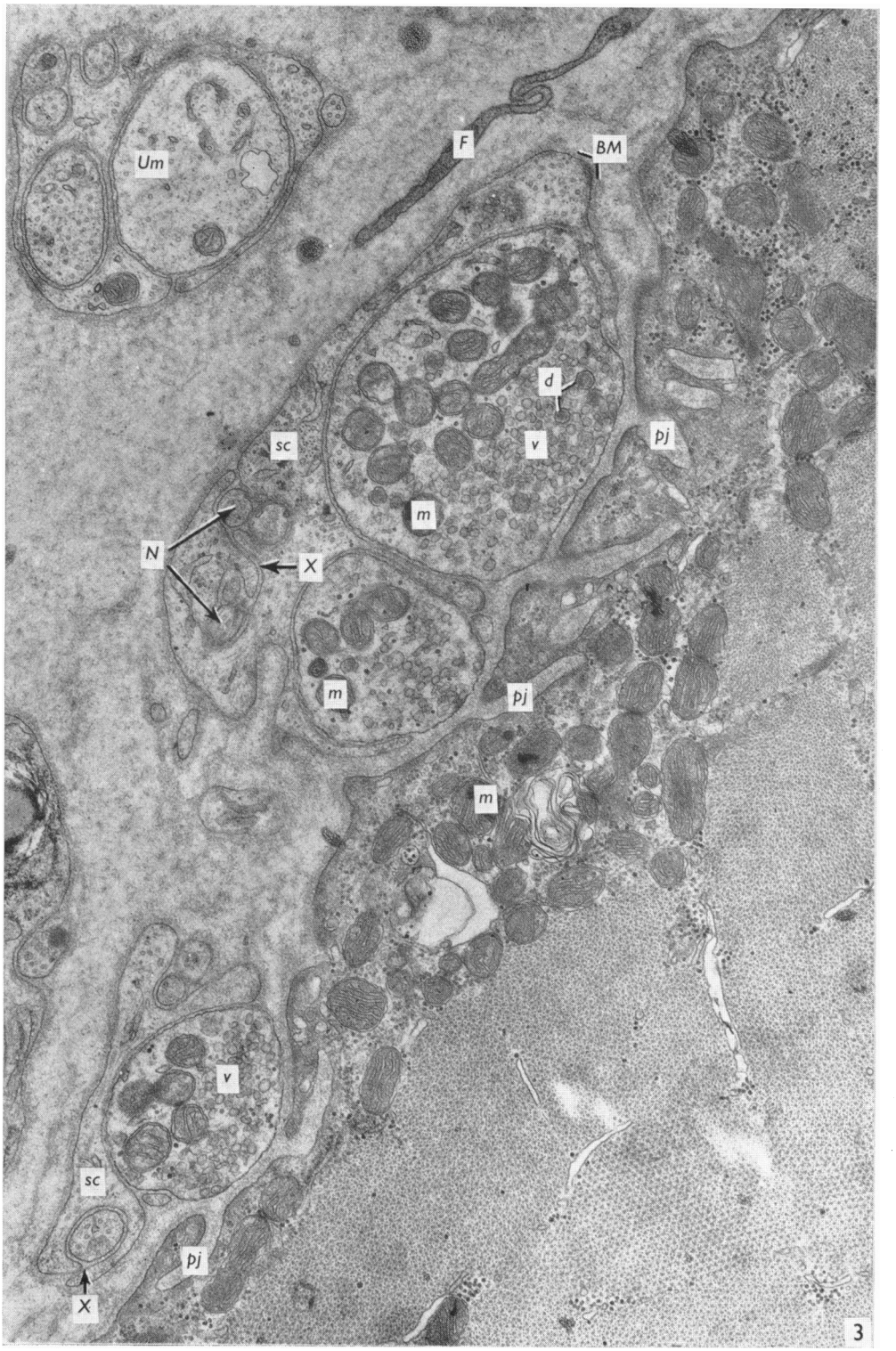
Fig. 3. A motor end-plate cut in the transverse plane illustrating the relationship between two bundles of unmyelinated nerves and a poorly developed synaptic gutter. Three nerve fibres on the side of the muscle are swollen and contain agranular vesicles (*v*) and mitochondria (*m*). The largest fibre contains a few large dense core vesicles (*d*). The nerves (*N*) away from the muscle fibre are related to the Schwann cell (*sc*) in the usual manner. In the proximity of the end-plate is a small bundle of unmyelinated nerves (*Um*). *F*, fibrocyte cell process. *X*, mesaxon. *BM*, basement membrane. *m*, mitochondria. *pj*, post-junctional fold. × 25800.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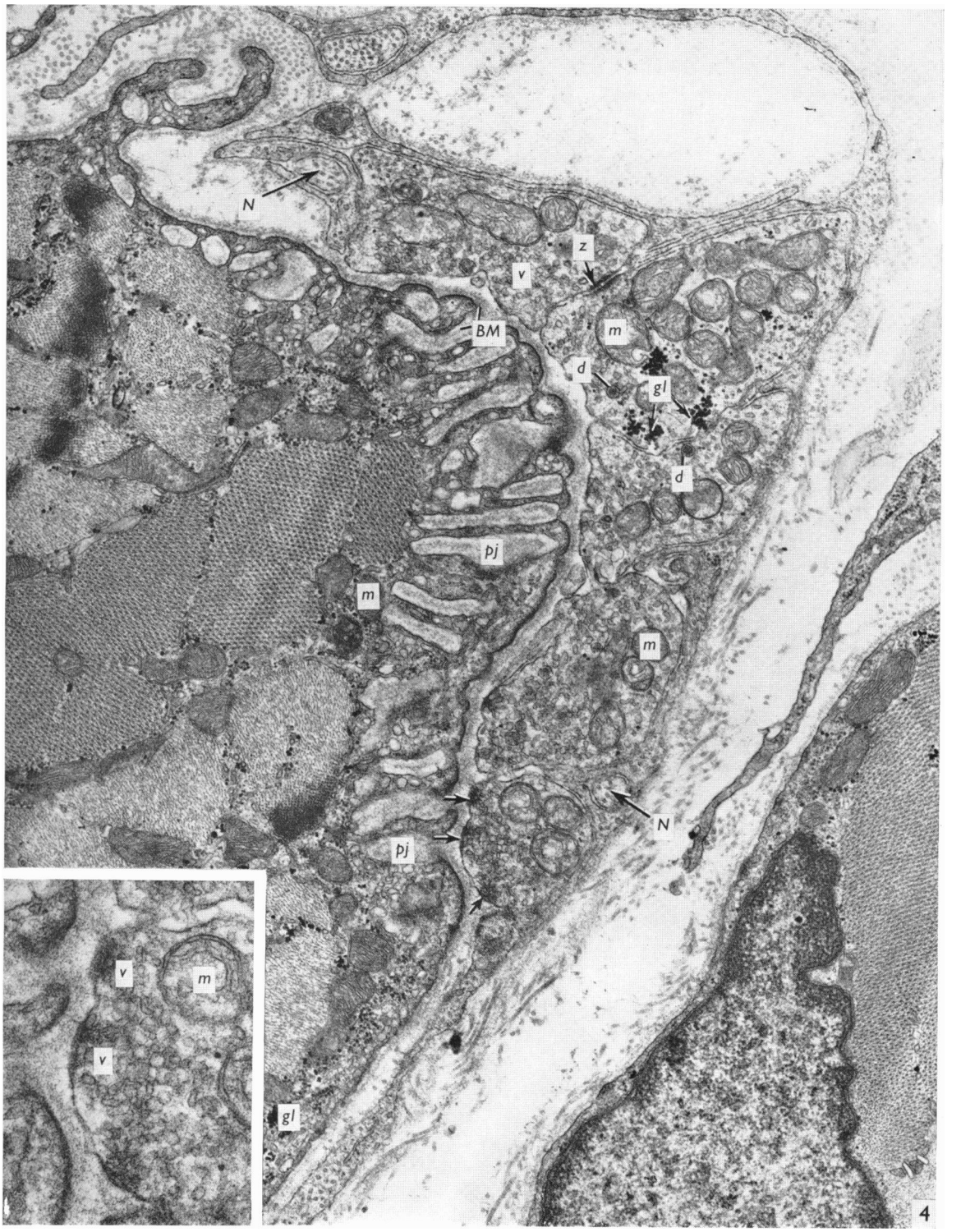
plasm. In the latter situation mesaxons are present. The nerve fibres are within the diameter range 0·1–1·8 μm, and exhibit the standard morphology of the unmyelinated nerve fibre. At this level they do not appear to have any special relationship with the muscle cells nearby. The nerve terminals, on the other hand, are always on the side of the muscle fibre. They are bulbous, and are partly exposed by a withdrawal of the Schwann cell cytoplasm away from the side of the muscle cell. The exposed part is usually lodged in a depression in the sarcolemma – the primary synaptic cleft (Figs. 3, 4). Infrequently, the nerve terminals may be located over flattened or even elevated zones of the muscle cell (Fig. 4). Unlike the somatic motor end-plate each primary synaptic cleft tends to contain more than one nerve terminal. In fact, there appears to be a rather strong tendency to a clustering of terminals in the motor end-plate as illustrated in Fig. 4. In this situation the adjacent terminals are not separated by a tongue of Schwann cell cytoplasm as in Fig. 3. Instead, there is a gap of about 20 nm between the adjacent neurilemmae with an occasional zonula adherens forming an intermediate junction (Fig. 4). On one occasion, an attachment zone between a neurilemma and a Schwann cell membrane was observed (Fig. 5).

Each nerve terminal contains the usual complement of synaptic organelles. There is an abundance of mitochondria and vesicles, both exhibiting a degree of polarity. The mitochondria are located away from the area of nerve–muscle contact, whereas the vesicles are gathered nearer the sarcolemma. Dense zones in the axolemma seem to attract clusters of vesicles (Fig. 4). The majority of vesicles are clear, have a smooth surface and are about 40–60 nm in diameter. Scattered amongst them are a few large granular vesicles with a diameter of around 85–110 nm. Infrequently, a few of these granular vesicles are grouped together. The thick and thin types of terminals, each with a predominance of a specific type of vesicle (Gruber, 1968), were not observed. Isolated glycogen granules are scattered in the terminal axoplasm, and sometimes arranged in bunches (Fig. 4).

Following the pattern of the classical unmyelinated nerve bundle, the terminal Schwann cell and the related nerve fibre are closely bounded by a basement lamina. At the periphery of each neuromuscular junction this layer fuses with the basement lamina ensheathing the subjacent muscle cell, forming a single layer which insinuates itself between the plasma membranes of the nerve terminal and muscle cell. As in the classical end-plate this layer extends into the depths of the junctional folds closely following their contour. The folds tend to be shallow at the periphery and deep in the centre.

Besides an abundance of mitochondria, the sole-plasm contains some sarcoplasmic vesicles, glycogen granules, nuclei and profiles of endoplasmic reticulum.

Fig. 4. Another motor end-plate showing elevated and depressed portions of the sarcolemma. The postjunctional folds (*pj*) are well developed, but secondary folds are few. The basement membrane (*BM*) follows the contour of the foldings. The crests of the postjunctional folds are more electron-dense than the remainder of the sarcolemma. The terminal expansions are clustered together and at *z* is located a zonula adherens. There is a gathering of vesicles at dense zones shown by arrows, and a higher magnification of this region is shown in the insert. Two small nerves (*N*) are also present. *v*, agranular vesicles, *d*, large dense core vesicle. *m*, mitochondria. *gl*, glycogen granules. × 22600. Insert × 43300.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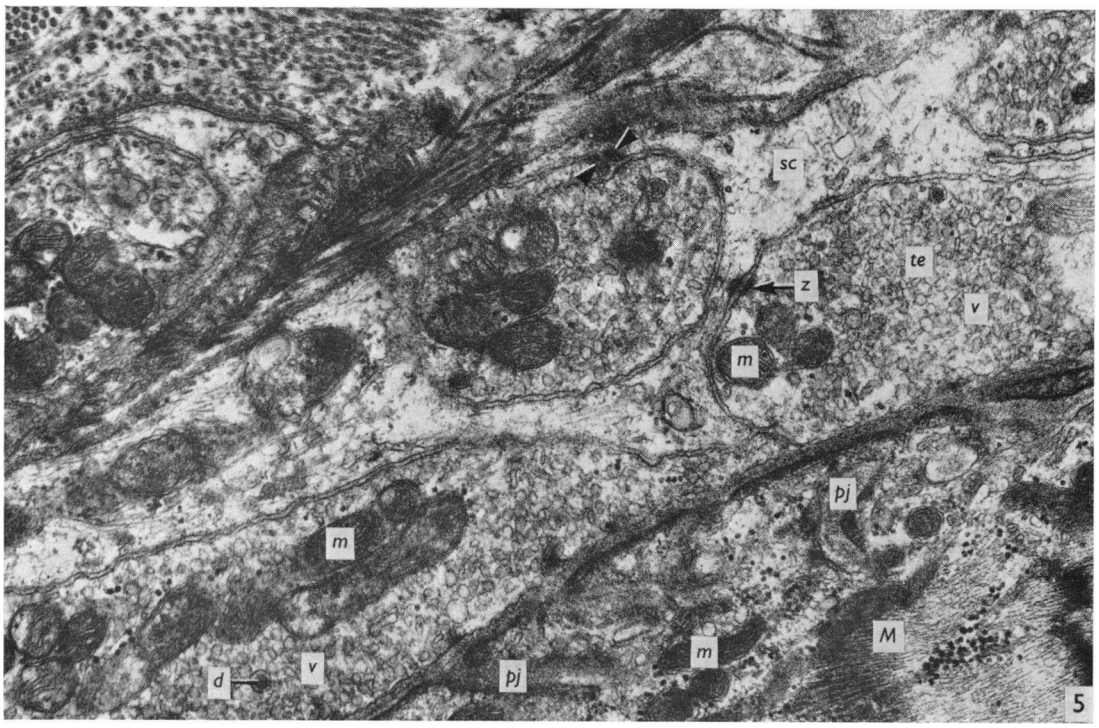

Fig. 5. A zonula adherens (*z*) between Schwann cell (*sc*) and terminal expansion (*te*). A comparable thickening at the nerve end of a mesaxon is shown by arrowheads. *M*, striated muscle. *pj*, postjunctional fold. *m*, mitochondria. *v*, agranular vesicles. *d*, large dense core vesicle. × 38 200.

The inner and outer layers of the muscularis externa were examined to see if there was a difference in the morphology of the end-plates. All the end-plates examined were of the type described above, and no variations were seen.

In addition, there were two points of interest. The first was the presence of small unmyelinated nerve bundles in the proximity of the motor end-plate (Fig. 3). The second was the absence of muscle spindles in the sections examined.

DISCUSSION

The morphological characteristics by which the twitch type and the slow type of striated muscle fibre may be distinguished are listed by Hess (1970). On these criteria the oesophageal striated muscle of the mouse should be regarded as a twitch type of fibre. The presence of a 'terminaison en plaque' type of ending on the muscle cell further supports this conclusion.

There has been extensive research into the innervation of the twitch type of mammalian voluntary muscle (Tiegs, 1953; Cöers, 1967). The gastrocnemius (Reger, 1958; Birks, Huxley & Katz, 1960), the intercostal muscle (Reger, 1955; Andersson-Cedergren, 1959; Zacks & Blumberg, 1961; Saito & Zacks, 1969) and the diaphragm

(Padykula & Gauthier, 1970) are the popular sites selected for investigating the ultrastructure of the extrafusal motor end-plate. These studies have shown that both muscle and nerve are highly specialized at the point of neuromuscular contact. On the side of the muscle, the receptive zone is depressed to form a deep postsynaptic gutter or trough, which itself is thrown into a complex series of postjunctional folds, and in the subjacent sarcoplasm is an aggregate of mitochondria, postsynaptic vesicles and nuclei. In the oesophagus, this basic pattern is repeated with slight modifications. The trough is usually shallow. Sometimes it may be unduly flat, or, on occasion, the sarcolemma may protrude outwards. Postjunctional folds are always present but are not equally complex throughout the receptive zone. The relationship between the nerve fibre and subneural apparatus is most striking, with each synaptic gutter containing more than one terminal expansion. Previous workers (Robertson, 1956; Padykula & Gauthier, 1970) illustrating nerve–gutter relationships have shown that each trough contains a single nerve terminal. An infrequent variation of the established pattern is seen in pathological states where terminal sprouting is prevalent (Allen, Johnson & Woolf, 1969). Even so, the typical form of the unmyelinated nerve bundle seen in the oesophagus is lacking. The need for this special arrangement in the oesophagus is at present obscure. Perhaps it has some bearing on the quanta of acetylcholine or other trophic factor required for depolarizing what appears to be a slightly less specialized subneural apparatus.

On the side of the nerve fibres the differences are many. It is accepted that the end-plate on the extrafusal twitch fibre is supplied by a single myelinated nerve fibre. The fate of this nerve fibre is well illustrated by Cole (1957) using the light microscope and by Saito & Zacks (1969) using the electron microscope. The latter study has shown very convincingly that the myelin sheath ends in a structure resembling a node of Ranvier, and that the change occurs in the proximity of the end-plate. On the other hand, in the mouse oesophagus the myelinated nerve fibres have a peculiar pattern of distribution. They have been found in the adventitial and myenteric plexuses and in the neuronal links between the two, but none has been found internal to the plane of the myenteric plexus. Only unmyelinated nerve fibres are found within the intermuscular spaces of the inner layer. This is consistent with the observation made by Gruber (1968). Though myelinated nerve fibres are found in and around the outer layer of the muscularis externa they do not, as demonstrated in other muscle cells, terminate in the close proximity of the motor end-plate. The morphology of the neuromuscular junction in the outer layer has not been observed to be different from that in the inner layer. Thus, Irwin's (1931) view that the striped muscle of the oesophagus is innervated by myelinated fibres is not corroborated.

In the classical motor end-plate the axon beyond the terminal node of Ranvier is closely surrounded by multiple Schwann cell processes (Robertson, 1956; Cöers, 1967; Saito & Zacks, 1969). Once the terminal expansion enters the primary synaptic cleft only the border remote from the muscle is covered by Schwann cell cytoplasm. These processes of the Schwann cell are not known to enfold any nerve fibres other than that which is lodged in the synaptic gutter. In the present study it has been shown that only bundles of unmyelinated nerve fibres are available for the innervation of the striated muscle. Even at the ending, the classical organization of the unmyelinated nerve bundle is maintained. Unlike the situation in other end-plates, the terminal

Schwann cell cytoplasm contains a number of nerve fibres. Those adjacent to the sarcolemma are swollen and contain the usual complement of synaptic organelles, while those on the opposite side show no such specialization and appear to be in transit. The fate of these nerve fibres is at present unknown. It is possible that they contribute to the innervation of other striated muscle cells, and may thus become a part of the peripheral organization required for the segmental contraction of the striated muscle cells in peristalsis. Alternatively, they may be part of the same end-plate where contact between nerve and muscle is established beyond the plane of section, or they may give rise to accessory end-plates, as suggested by Cecio & Califano (1967). The small bundles of unmyelinated fibres in the immediate proximity of the end-plate may be branches leading on to other striated muscle cells. Only by a study of serial sections could any of these views be substantiated.

The terminal expansions contain vesicles which are predominantly agranular, and contrary to the description by Gruber (1968) only a single type of ending has been found. Large granular vesicles of the type he describes are scattered in the terminal axoplasm and their occasional gathering may have given the appearance of a second type of ending. Granulated vesicles of the same dimensions have been described in other sites (Grillo & Palay, 1962) and their role in the oesophagus could at best be speculative.

The evidence presented in this study suggests that the striated muscle cell in the oesophagus, though of the twitch variety, appears to be innervated by unmyelinated nerve fibres. The source of these fibres is as yet unknown. Gruber (1968) and Ham (1969) are of the opinion that the axons are postganglionic fibres from the myenteric plexus, a view not substantiated by pharmacological studies (Bartlet, 1968*a*). Further, Kantrowitz *et al.* (1970) have also shown that there is no difference between oesophageal striated muscle and striated muscle from other sites as far as responses to D-tubocurarine and atropine are concerned. Unanimity, however, has been reached as regards the course of these nerve fibres, and it is agreed that the vagus transmits the axons to the striated muscle cells. However, their nuclear source is in debate. Both the nucleus ambiguus and the dorsal motor nucleus of the vagus have been implicated, either singly or in combination, but more recent investigations point to the nucleus ambiguus as the only source (Lawn, 1964, 1966*a*; Lewis, Scott & Navaratnam, 1970). The motor neurons of this nucleus have been shown to have the same general form as motor neurons in the spinal cord (Lawn, 1966*b*) and it does not seem unreasonable to expect that the axons to which they give rise should be myelinated.

In this investigation it has been shown that the nerves innervating the striated muscle cells are of the unmyelinated type, at least in their pre-terminal and terminal parts. Myelinated nerve fibres are present, but their distribution is restricted to the region in and around the outer layer of the muscularis externa and at present there is no evidence to show their participation in the innervation of the striated muscle fibres.

SUMMARY

The termination of the nerves in the muscularis externa of the mouse oesophagus was investigated with the electron microscope.

The muscularis externa of the oesophagus was formed of striated muscle cells of the twitch variety, together with smooth muscle cells. The two types of muscle cells were not connected, nor were there any points of intimate contact.

Myelinated and unmyelinated nerve fibres were found within the myenteric plexus and external to it. Internal to this plexus, only unmyelinated nerve bundles were found.

The striated muscle cells were innervated by unmyelinated nerve fibres terminating in an end-plate. Although the receptive zone of the muscle conformed, by and large, to the classical pattern, the organization of the neural component appeared to be substantially modified. No terminal nodes of Ranvier were observed in the proximity of the end-plate. Instead unmyelinated nerve bundles, related to the Schwann cell in the usual manner, were present. The nerve fibres nearer the primary synaptic cleft were bulbous, and contained the standard synaptic organelles, whilst those further away showed no such specialization. Sometimes there was a tendency to a clustering of the terminal nerve fibres. This resulted in (*a*) a single primary synaptic cleft containing more than one terminal expansion; (*b*) the formation of zonulae adherentes between adjacent nerve terminals or between Schwann cell and terminal. In the proximity of the end-plate there were additional small bundles of unmyelinated nerves.

The author wishes to express his gratitude to Dr L. L. Ross for the advice and encouragement given during this investigation, and to Dr R. C. Swan for having sponsored him. Appreciation is also expressed to Mr Leo Cosio for photographic assistance. This work was done during the tenure of a U.S. Government Fulbright Research award.

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