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INNERVATION OF THE VENTRAL DIAPHRAGM OF THE LOCUST (*LOCUSTA MIGRATORIA*)

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SUMMARY

1. Innervation and some electrical properties of the locust ventral diaphragm were investigated with electrophysiological and histological methods.

2. Muscle fibres are coupled electrically. Electrical stimulation evokes a graded active membrane response.

3. Each segment is innervated by four motor neurones as follows. Two motor neurones are situated in each abdominal ganglion. Branches of their axons supply the ventral diaphragm in the respective and the next posterior segment.

4. This pattern of innervation was confirmed by axonal Co and Ni staining of the motor nerve endings.

5. Neuromuscular junctions are excitatory. EPSPs show summation but no facilitation.

6. Spontaneous electrical activity of the diaphragm is to a certain degree coupled to activity of the main inspiratory muscles.

INTRODUCTION

In many insect orders there is a thin muscular septum, the ventral diaphragm (Richards, 1963), dorsal to the abdominal ventral nerve cord. In dissected preparations the ventral diaphragm often shows rhythmical waves of contraction, running posteriorly, and it is therefore supposed to play a part in haemolymph circulation (Jones, 1954; Heinrich, 1971; Hessel, 1969). In *Locusta migratoria*, contractions can be observed coupled to contractions of inspiratory muscles (Hustert, 1975), so the diaphragm possibly has a ventilatory function also. More detailed physiological information is only available about *Schistocerca* (Guthrie, 1962) and *Periplaneta* (Miller & Adams, 1974). Contractions of the ventral diaphragm of *Schistocerca* are mainly myogenic in origin and influenced by inhibitory innervation. The hyperneural muscle of *Periplaneta*, on the other hand, is electrically inexcitable and innervated by excitatory fibres of the median nerves. The present study was undertaken to evaluate some functional properties of the ventral diaphragm of *Locusta*, and to study its innervation. The diaphragm consists of a single layer of muscle fibres, rendering it a relatively suitable object for histological studies as well as electrophysiological investigations under visual control.

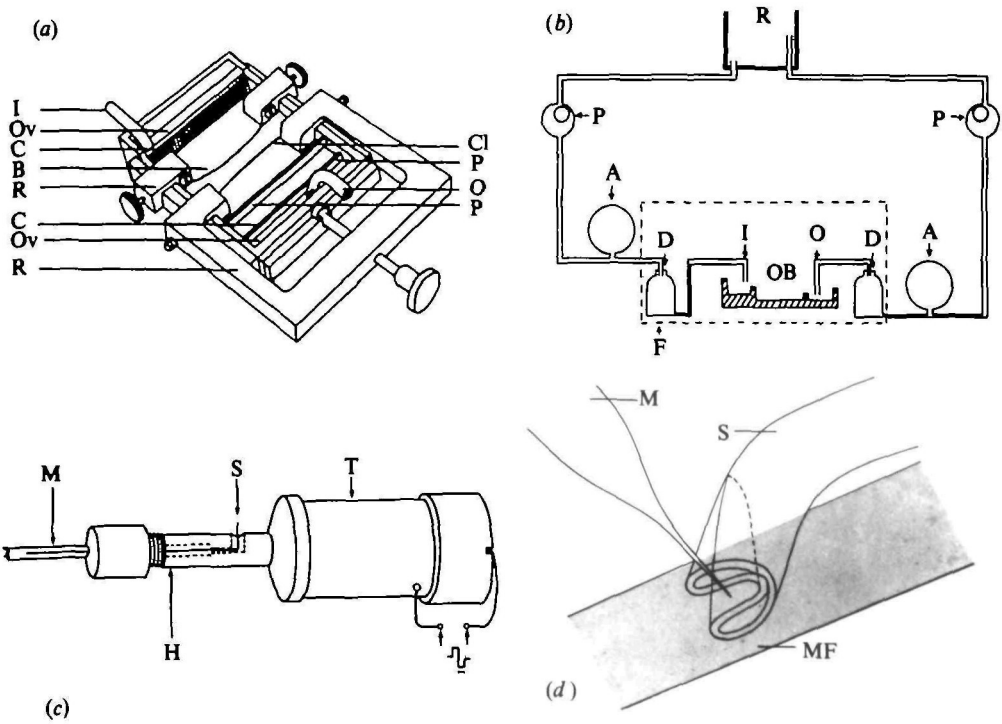


Fig. 1. Experimental arrangement. (*a*) Organ bath. The preparation (not shown) is clamped to the glass bottom (B) by clamps (Cl), the positions of which are adjusted by riders (R). Through the inlet (I) saline flows firstly into the overflow reservoir (Ov) and from there inside capillary tubes (C) down into the organ bath. It then passes below a partition (P) and finally through a slit formed by two capillary tubes arranged horizontally parallel to each other at a distance of 0·3 mm into the second overflow reservoir, which is formed by a further partition in the organ bath. From there saline is sucked off through the outlet (O). The capillaries serve to avoid discontinuities in saline flow at the borders of the overflow reservoirs and thus provide a smooth and constant saline level in the organ bath. (*b*) Perfusion system. Saline from a reservoir (R) is circulated over the preparation in the organ bath (OB) by means of two peristaltic pumps (P). Air vessels (A) damp pumping strokes. Disconnecting jars (D) in the inlet (I) and outlet (O) branch of the perfusion system prevent electrical interference from entering the Faraday cage (F). (*c*) Piezoelectric drive. The microelectrode (M) is inserted into a Perspex holder (H) which is fastened to a piezoelectric transducer (T) (Vernitron Ltd. Thornhill Southhampton; PZT-5A tube 12-8125-5A). The transducer changes length when a voltage step is applied to it. Connexion from microelectrode to preamplifier is made by a chlorided silver wire (S). (*d*) Electrode arrangement for intracellular recording. A muscle fibre (MF) is held by a U-shaped suction capillary (S) and impaled by a microelectrode (M).

METHODS

Experiments were carried out at room-temperature on adult female locusts, bred at this institute. Usually the 5th abdominal segment was used because it can be regarded as the prototype of a praegenital segment. The saline that proved to be the most suitable had a pH of 6·8, and had the following composition (mM): NaCl, 120; KCl, 10; $CaCl_2$, 5; CH_3COONa, 10; $NaHCO_3$, 10; NaH_2PO_4, 6; $MgCl_2$, 5; Glucose, 10.

The abdomen was opened with a dorsal longitudinal cut. After removal of the ovary and intestines it was pinned, ventral side up, in a dish of saline. The sternites below the ventral diaphragm were then cut.

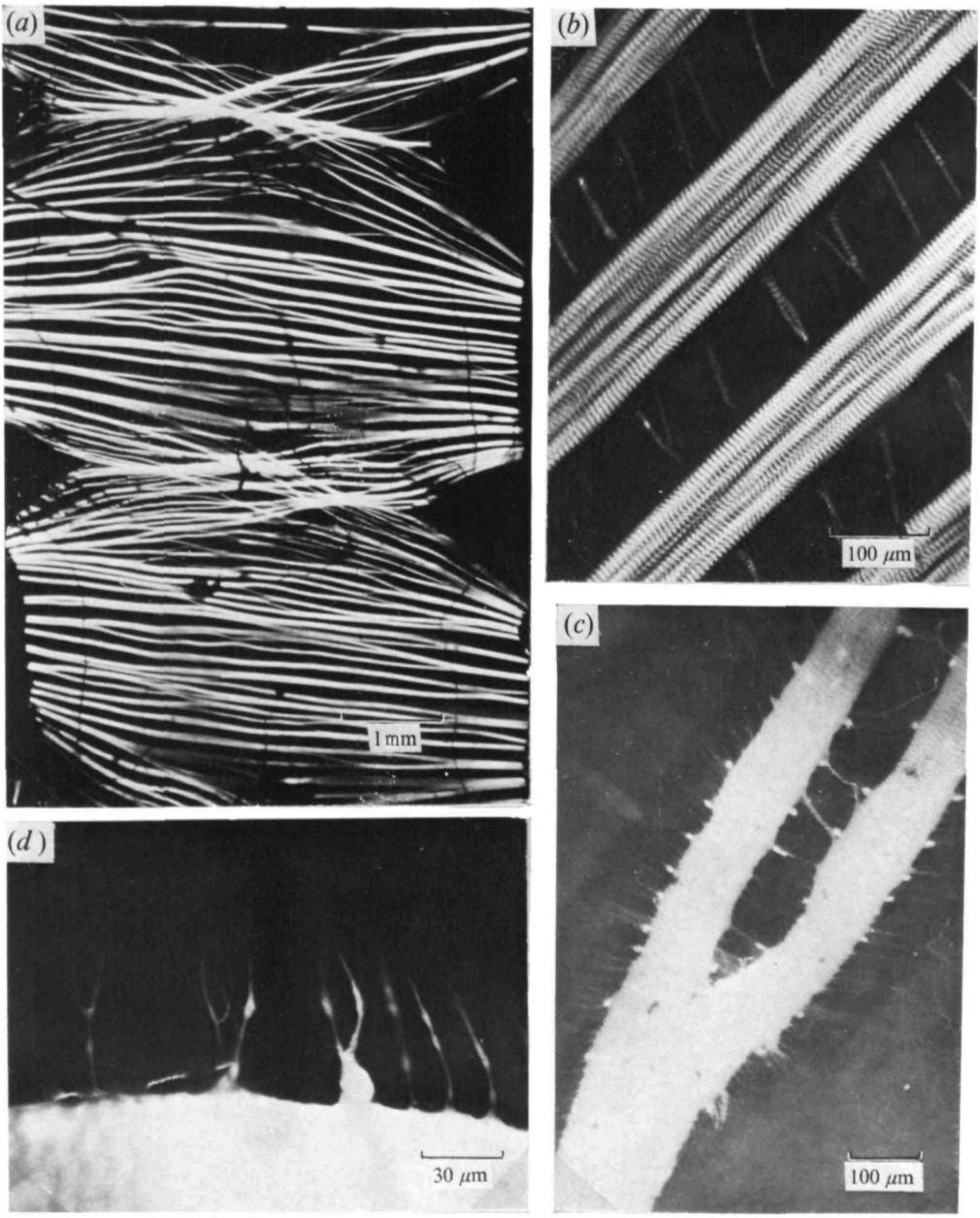


Fig. 2. Anatomy of ventral diaphragm muscle fibres. (*a*) Ventral diaphragm in organ-bath (polarized light)
(*b*) Muscle fibres and anastomoses more enlarged. The anastomoses show clear cross striations (arrow). (*c*) Procion yellow injected muscle cell. (*d*) Branches of a Procion yellow injected cell in the anastomoses.

For light microscopical examination of the motor innervation the diaphragm was pinned, ventral side up, on to the wax-floor of a Petri dish and continuously perfused with saline. One of the segmental nerves was then stained with $CoCl_2$ (Pitman, Tweedle & Cohen, 1972) or $NiCl_2$ via the cut axons (Iles & Mulloney, 1971), by means of an oil gap electrode (Peters, 1976). Although Co migrates much faster than Ni, with Co the glial cells surrounding the axons were also stained to some degree, whereas Ni remained more restricted in the axons and nerve endings. After axonal iontophoresis, Timm's sulphide-silver intensification was applied (Tyrer & Bell, 1974). For comparison the osmium-zinc iodide method was used (Jabonero, 1968). Microphotographs were made from whole mounts embedded in Caedax.

For electrical recordings, the preparation was fixed, ventral side up, under two P.T.F.E.-coated steel wire clamps in a chamber with a glass bottom (Fig. 1*a*). The chamber was fastened to the stage of an inverted microscope to allow intracellular penetration during observation under phase contrast. The perfusion system (Fig. 1*b*) produced no electrical interference, and provided a smooth flow of solution into the chamber, at a constant level.

Glass microelectrodes and fire-polished suction electrodes were used for recording and stimulation. Microelectrodes had short shanks and were filled with 2·5 M KCl or 4% Procion yellow solution. The resistance of the KCl electrodes was between 30 and 100 MΩ. To aid impalement, the microelectrodes were vibrated in a longitudinal direction with a piezoelectric transducer (Fig. 1*c*) (Chowdhury, 1969). In addition, the diaphragm was immobilized and stretched at the recording site by a suction electrode with a U-shaped cross section (Fig. 1*d*).

The nomenclature of Schmitt (1954) was used for nerves and that of Hustert (1974) for abdominal muscles.

RESULTS

The ventral diaphragm of *Locusta migratoria* consists of a single layer of flat muscle fibres up to 100 μm broad and 10 μm thick which run transversely across the abdomen. Muscle fibres give off side branches, which make connexions with fibres lying parallel (Fig. 2*a*). In addition, they are connected by many thin anastomoses, which clearly show birefringence and cross striation (Fig. 2*b*).

The ventral diaphragm lies dorsal to the ventral nerve cord, and is attached to it by connective tissue. The relative positions of ventral diaphragm and the main abdominal nerves are shown in Fig. 3.

Preparations of the ventral diaphragm lacking the ventral nerve cord are not spontaneously active. However, by electrical stimulation of a fibre it is possible to evoke in it an active graded membrane response which leads to its contraction (Fig. 4). If sufficient stimulation is applied to a fibre, all fibres in the same segment contract, indicating that there is coupling between cells. Since the excitation does not normally spread to neighbouring segments, it appears that coupling is weak at segmental boundaries.

To investigate electrical coupling between neighbouring fibres, one fibre was hyperpolarized through a suction electrode with a current pulse lasting several 100 ms while simultaneous intracellular recordings were made from the fibre or from adjacent ones (Fig. 5). (The resting potential of the fibres lay between 40 and 60 mV.)

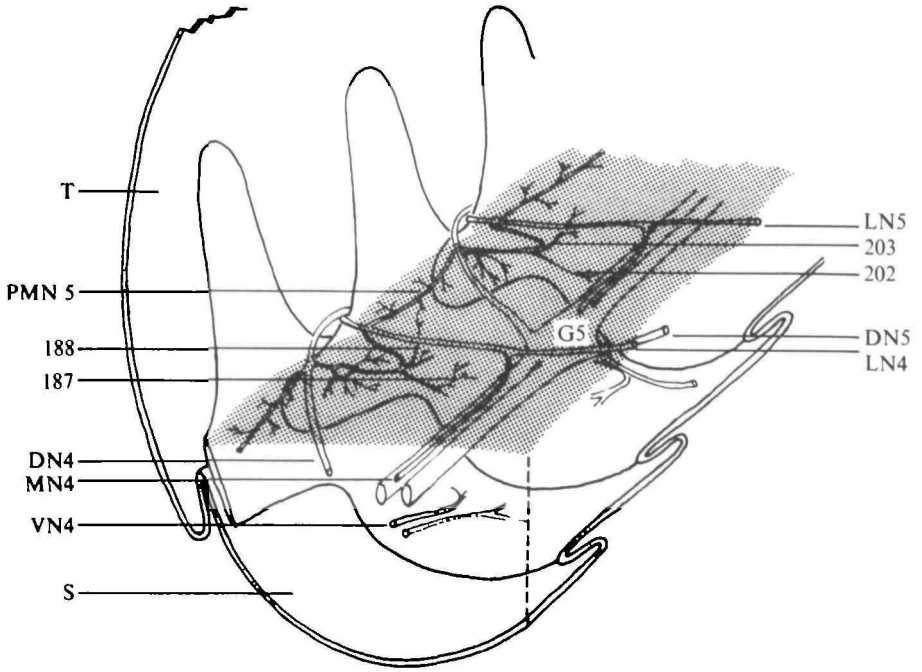


Fig. 3. Positions of ventral diaphragm and nervous system; semi-diagrammatic. Stippled, ventral diaphragm; G_5, 5th abdominal ganglion; PMN, paramedian nerve; DN, dorsal nerve; VN, ventral nerve; MN, median nerve; LN, lateral nerve; T, tergite; S, sternite. 187, 188, 202, 203: branches of dorsal nerves supplying muscles M 187, M188, M202, M203.

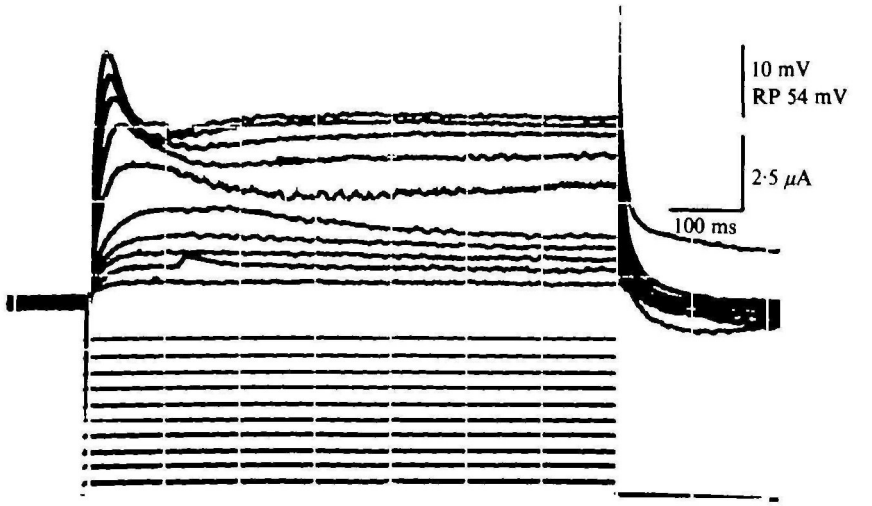
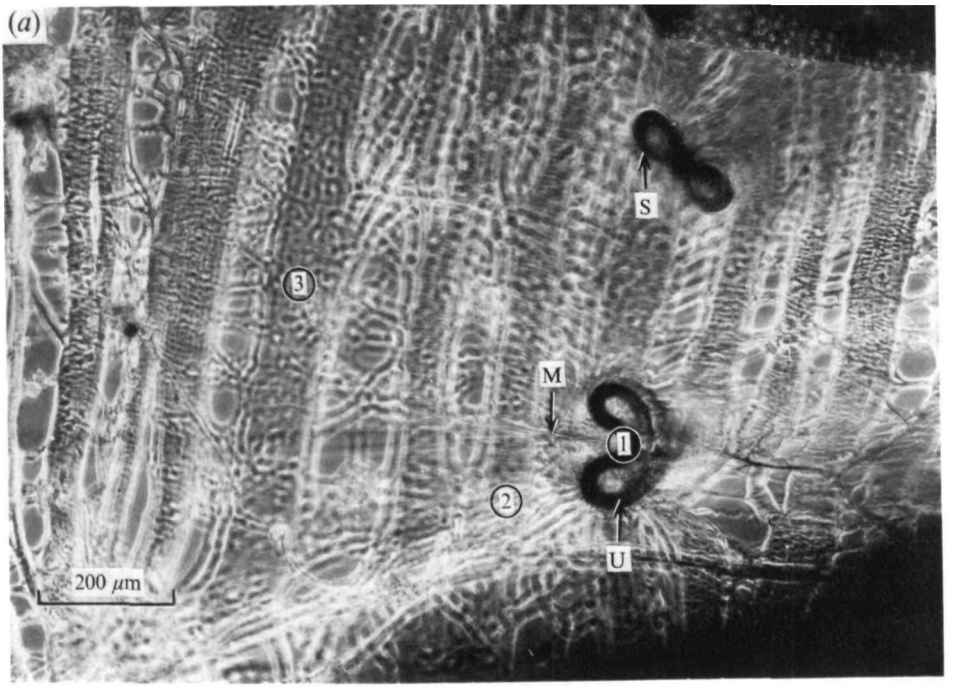


Fig. 4. Intracellular recording of muscle fibre electrical response to depolarizing stimulation (by suction electrode). Lower trace, current monitor.



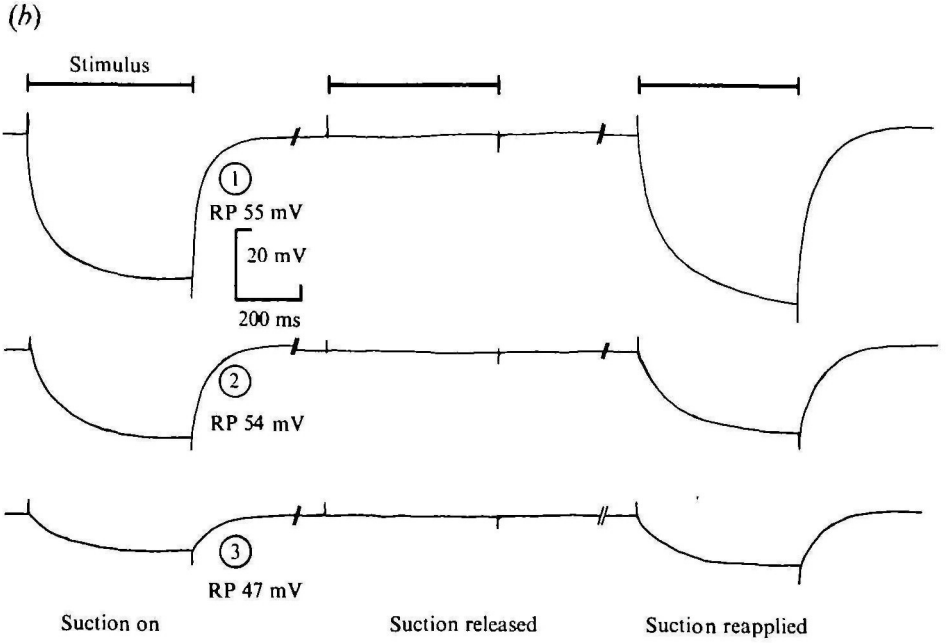


Fig. 5. Demonstration of electrical coupling between muscle cells. (*a*) Phase contrast micrograph of a portion of the ventral diaphragm, showing relative positions of stimulating and recording electrodes. M, glass microelectrode; S, stimulating suction electrode; U, U-shaped holding capillary (numbers depict recording sites). (*b*) Intracellular recording of electrical response from muscle fibres indicated in (*a*) to a hyperpolarizing stimulus. The stimulating electrode is stationary.

Neighbouring fibres were also hyperpolarized, to a degree that was progressively smaller with increasing distance from the stimulation site. Hyperpolarizations could not be recorded when the suction electrode was released or when the recording electrode was withdrawn from the fibre. It is therefore probable that there is an electrotonic spread of the current from the stimulated fibre to adjacent fibres through low resistance pathways.

Some fibres are only connected by anastomoses so the electrotonic connexion may, in part, be mediated by these. To investigate whether there was cytoplasmic connexion between the fibres of a segment, Procion yellow was injected into a fibre by means of a microelectrode. Procion yellow does not normally cross cell membranes and thus depicts the geometry of an injected cell (Stretton & Kravitz, 1968). The injection revealed that branches of the fibres passed into the anastomoses (Fig. 2*c*), but there was apparently no cytoplasmic continuity between adjacent fibres, because these branches ended sharply (Fig. 2*d*). If there are electrical connexions between fibres in the anastomoses, they do not allow passage of Procion yellow and thus behave differently from some electrotonic synapses between nerve cells (e.g. Bennett, 1973).

Spontaneous activity

Diaphragm preparations to which the abdominal nerve cord and metathoracic ganglion together with all dorsal nerves was still attached showed rhythmic spontaneous electrical activity of the muscle fibres (Fig. 6*a*). Even in preparations to which only one abdominal ganglion was left attached, there was rhythmic electrical activity in the segments innervated by that ganglion.

In each segment, bursts of spikes in the paramedian nerves (PMN) were correlated with summating EPSPs in the diaphragm muscle fibres (Fig. 6*b*). Each EPSP was preceded by a motor nerve spike in the paramedian nerve (Fig. 6*c*). Spikes of the diaphragm motor nerve fibres could readily be recognized because of their relatively high amplitude. Excitation in neighbouring segments did not occur simultaneously. Electrical activity travelled posteriorly with an intersegmental delay of some 100 ms (Fig. 6*d*) between successive segments.

In both paramedian nerves of each segment, bursts appeared coupled to each other, but there was not a 1 : 1 relationship between the spikes in one nerve and those in the other (Fig. 6*e*).

Electrical activity of the diaphragm was coupled to inspiratory activity in other abdominal muscles (Fig. 6*f*) but not every inspiratory cycle was necessarily accompanied by excitation of the diaphragm.

Innervation pattern

To evaluate the innervation patterns of the muscle fibres of the ventral diaphragm, the dorsal, ventral and median nerves of one segment, as well as those of the next anterior and posterior segments, were stimulated while intracellular recordings were made from muscle fibres.

EPSPs were evoked in muscle fibres of a segment by stimulation of the dorsal nerves innervating that segment or the next anterior one (Fig. 7*a*, *b*). The EPSPs were preceded by spikes in the paramedian nerve. The amplitudes of the EPSPs generally ranged between 20 and 30 mV. They showed summation (Fig. 7*d*) but no

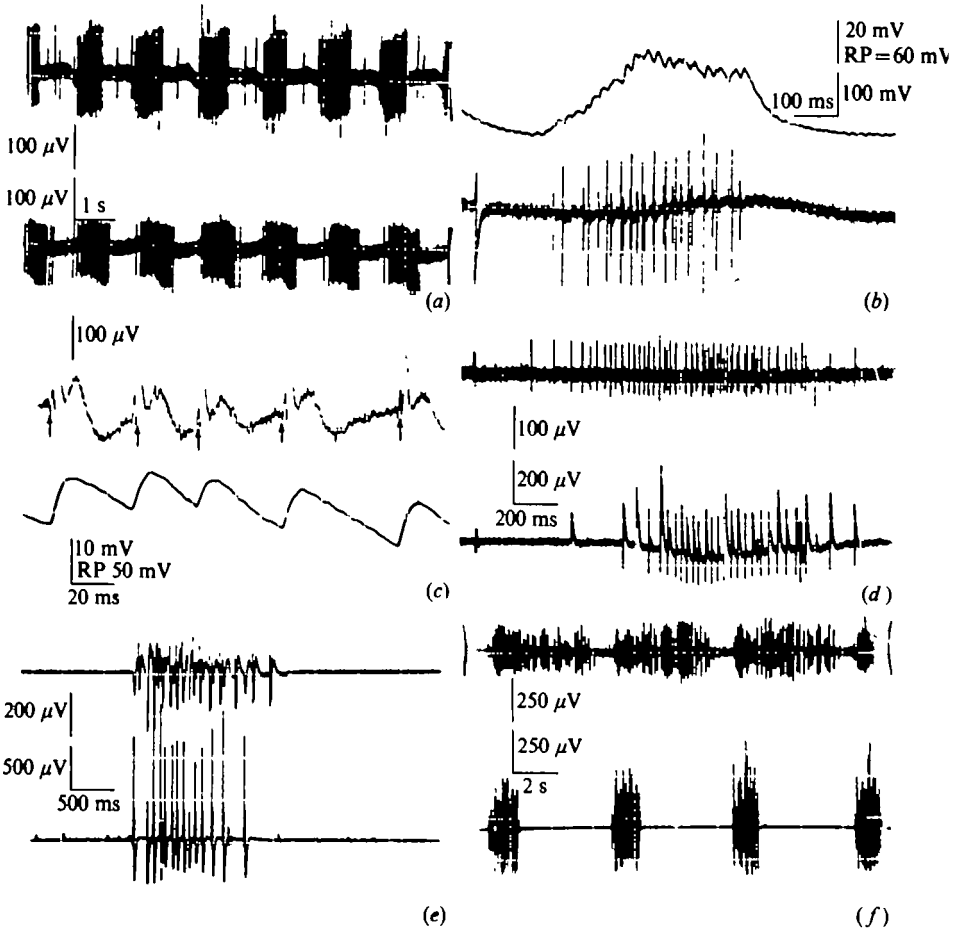


Fig. 6. Spontaneous electrical activity in diaphragm preparation with ganglion chain together with dorsal nerves up to metathoracic ganglion intact. (*a*) Recording from two adjacent segments; PMN segment 4 (upper trace) and PMN segment 5 (lower trace). (*b*) EPSPs in a diaphragm muscle fibre (upper trace) accompanied by a burst of spikes in the PMN of the same segment (lower trace). (*c*) Spontaneous activity at higher sweep speed. Each action potential in the paramedian nerve (arrows, upper trace) is followed by an EPSP in the ventral diaphragm (lower trace). (*d*) Activity in PMNs of two adjacent segments, segment 4 (upper trace) and segment 5 (lower trace), showing intersegmental delay. (*e*) Activity in both PMNs of one segment, segment 5, showing absence of 1:1 correlation of spikes. (*f*) Electromyograms recorded simultaneously from one main inspiratory muscle (M 207; upper trace), and the ventral diaphragm in segment 5 (lower trace).

facilitation (Fig. 7*c*) and because of these features may not be classified as either 'slow' or 'fast' EPSPs. Hyperpolarizing synaptic potentials were never observed. With stimulation above a particular threshold, stimulation of a dorsal nerve of a segment produced spikes in the PMN on one side of that segment simultaneously with the spikes in the PMN on the same side of the next posterior segment (Fig. 7*e*). This suggests that there is one motor axon in each dorsal nerve that has branches in the paramedian nerves of two successive segments.

The observation that spontaneously occurring spikes in a PMN did not show a 1:1 relationship with such spikes in the other PMN of the same segment suggests the presence of two motor neurones in each ganglion, each innervating one side

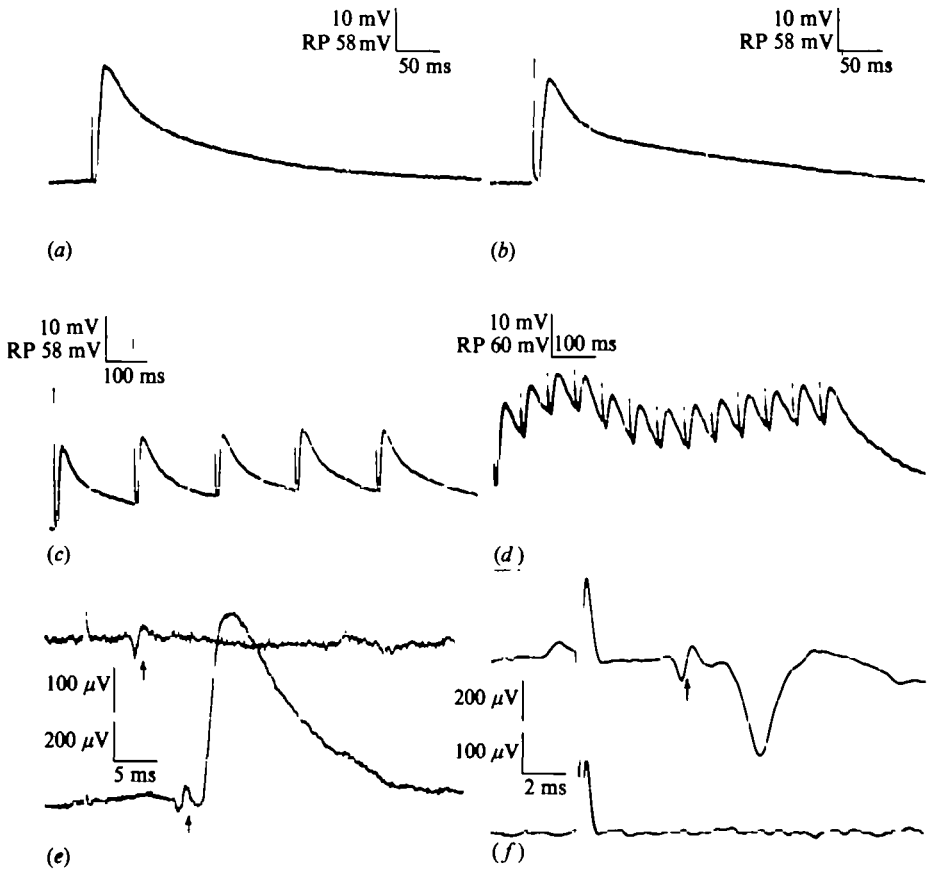


Fig. 7. Motor innervation. (*a–d*) Intracellular recordings from muscle fibres in segment 5. (*a*) Single EPSP following stimulation of DN 5. (*b*) Single EPSP after stimulation of DN 4. (*c*) Repetitive stimulation of DN 4 at 4 pulses/s, showing absence of facilitation. (*d*) Stimulation of DN_4 at 15 pulses/s, showing onset of summation. (*e*) Extracellular recording of motor-fibre spikes in two adjacent PMNs, in segments 4 (upper trace) and 5 (lower trace), with stimulation of DN_4. Action potentials in diaphragm motor fibres indicated by arrows. (*f*) Extracellular recordings from PMNs in segment 5 with *en passant* stimulation of the ipsilateral dorsal nerve (both dorsal nerves and G_5 intact). Upper trace, ipsilateral PMN; lower trace, contralateral PMN. Large deflexions following spikes on lower beam of 7 (*e*) and upper beam of 7 (*f*) stem from muscle fibres which have been sucked into the electrode together with the nerve.

the diaphragm. This suggestion is strengthened by the following. If both sides were innervated by branches of a single neurone, stimulation of one dorsal nerve *en passant* should result in a spike in the ipsilateral PMN as well as an action potential conducted antidromically to the ganglion and from there to the contralateral PMN. In fact a spike could only be recorded from the PMN ipsilateral to the stimulated dorsal nerve (Fig. 7*f*).

The innervation pattern appears as presented in Fig. 8: in every abdominal ganglion 2 motor neurones are located whose axons run through the dorsal nerves to the periphery and branch in the paramedian nerves of two adjacent segments. In this way every segment of the diaphragm receives motor nerve endings from 4 motor neurones.

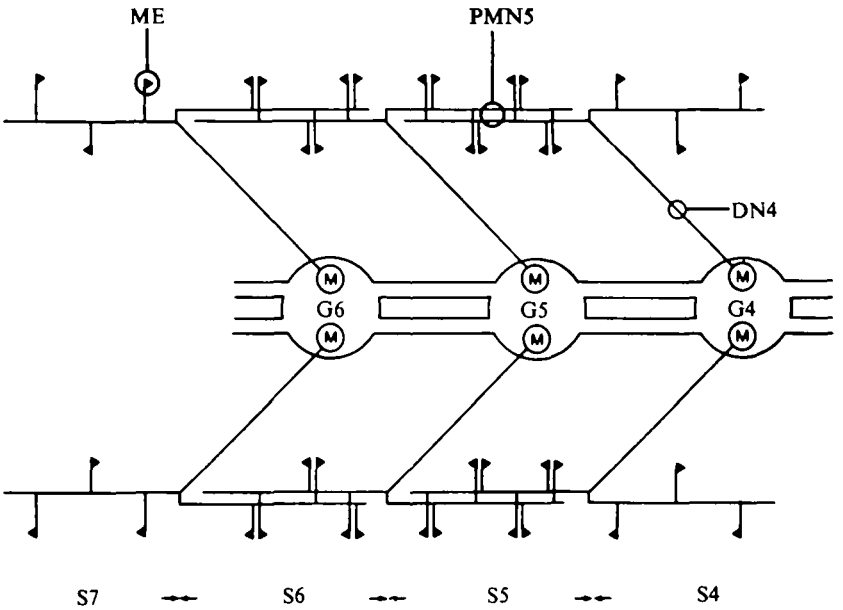


Fig. 8. Diagram representing motor innervation of the ventral diaphragm in overlapping fields. G, abdominal ganglion; DN, dorsal nerve; M, motor neurone; ME, motor nerve ending; PMN, Paramedian nerve; S, Abdominal segment.

The innervation pattern indicated by the electrophysiological results was confirmed by histological methods. By axonal iontophoresis of Co or Ni into the proximal cut end of one dorsal nerve, nerve-endings in the respective segment as well as in the next posterior segment are stained. The nerve-endings were to a large extent limited to the lateral regions of the diaphragm (Fig. 9*a*). The muscle fibres were innervated multiterminally (Fig. 9*b*) by brushtype nerve endings (Fig. 9*c*). Similar results were obtained after staining with osmium zinc iodide (Fig. 9*d*). This picture agrees with that obtained from *Schistocerca* by methylene blue staining (Guthrie, 1962).

DISCUSSION

In the ventral diaphragm of *Locusta*, individual muscle fibres are connected by branches and anastomoses, as for instance in various visceral muscles of the honey bee and in alary muscles of *Hyalophora cecropia* (Morison, 1928; Sanger & McCann, 1968), and neighbouring cells are electrically coupled, as has been described in the heart of *Periplaneta* (Miller & Usherwood, 1971). The anatomical mediator of this connexion in the diaphragm might be the intercalated discs which have been found (Dierichs, 1972).

Visceral muscles of insects typically show a spontaneous rhythmic myogenic activity (Miller, 1971; Cook & Reinecke, 1973; Nagai, 1973) but I could not observe such activity in the ventral diaphragm of *Locusta*. The muscle fibres of the ventral diaphragm could be excited electrically, but only responded with a graded active response and never with an all-or-nothing action potential.

How is activity of the ventral diaphragm controlled by the nervous system? Innervation is of the polyneural and multiterminal type as is common in insect muscl

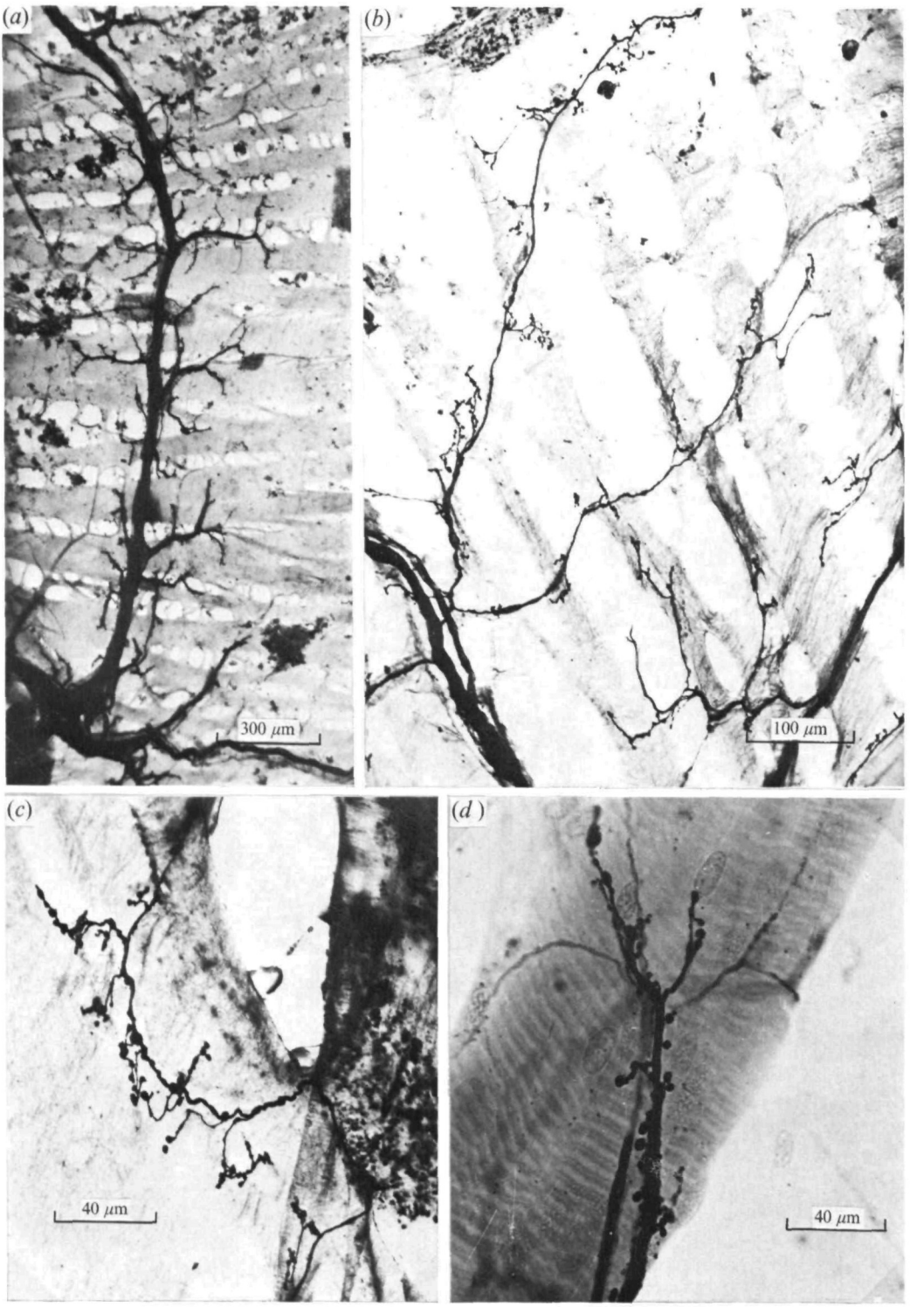


Fig. 9. Light microscopy of motor nerve endings. (*a*) Innervation of the left side of the diaphragm in segment 6. Staining via DN 6 ($CoCl_2$). Nerve branches in segment 7 (not shown) have a similar appearance.
(*b*) Branches of motor axon ($NiCl_2$). (*c*) Grape-shaped motor ending ($NiCl_2$). (*d*) Motor nerve ending (osmium zinc iodide).

Usherwood, 1967; Hoyle, 1975). Each muscle fibre is supplied by four axons originating in two adjacent ganglia. All synapses are characterized by EPSPs of similar amplitude and time course which do not show facilitation. Perhaps superposition upon the pure synaptic potential of an active membrane response renders facilitation (if present) unobservable, because in some preparations where EPSPs of a lower than normal amplitude were elicited (5–10 mV) strong facilitation was visible. The light microscopical picture gives no hint of different types of synapses, too. All nerve endings are of the brush type.

The limitation of the synapses to the lateral regions of the diaphragm contrast with the dense innervation of insect skeletal muscle fibres. The distance between synaptic endings in the retractor unguis muscle of *Schistocerca* amounts to 10–100 μm (Rees & Usherwood, 1972) whereas in the ventral diaphragm endings are spaced at intervals of several 100 μm. The relatively sparse nerve supply suggests that the capability of the muscle fibre membrane to elicit an active response is of major importance in triggering contraction of the whole fibre.

Innervation of muscle fibres by axons originating in different ganglia, as is the case in the diaphragm, seems to be quite common in locusts. Thoracic longitudinal muscles (Neville, 1963) as well as inspiratory muscles of the abdomen (Lewis, Miller & Mills, 1973) receive innervation from the next anterior ganglion as well as from the segmental ganglion. The present study strengthens the view that the ventral diaphragm plays a part in the ventilatory system (Hustert, 1975). In both the ventral diaphragm and ventilatory nerves or muscles rhythmical activity persists after isolation of a single abdominal ganglion (Lewis *et al.* 1973), and in dissected preparations activity can show a delay of up to several hundred ms between adjacent segments in *Locusta* (Hustert, 1975) and *Schistocerca* (Lewis *et al.* 1973). Strongest evidence for an involvement of the ventral diaphragm in the ventilatory system stems, however, from the fact that electrical activity is coupled to that of the main inspiratory muscles.

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