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MEMBRANE POTENTIALS IN THE CENTRAL NERVOUS SYSTEM OF A PHYTOPHAGOUS INSECT (*CARAUSIUS MOROSUS*)

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INTRODUCTION

The extremely specialized ionic composition of the haemolymph in some phytophagous insect species presents certain difficulties in the interpretation of axonal conduction in terms of the classical membrane theory which has been found to be applicable in most animal groups. In particular the very low sodium level, together with the relatively high concentration of magnesium ions, represent conditions which are very different from the conventional ones encountered in the blood of most vertebrate and invertebrate species. Some recent experiments have indicated that there is some regulation of the sodium concentration in the extracellular fluid of the central nervous tissues of *Carausius morosus* (Treherne, 1965*a*). These preliminary studies were, however, carried out using extracellular electrodes. There have, in fact, been no studies carried out on the axons of phytophagous species using intracellular electrode techniques. The present investigation was, therefore, carried out to examine the membrane potentials in axons from the central nervous system of an insect in which the haemolymph is characterized by low sodium and high magnesium levels.

These experiments have been largely directed towards an investigation of the electrical events occurring in the intact nervous system as a necessary step prior to further work employing isolated preparations of the central nervous system of this species. Particular attention has been paid to the influence of the neural fat-body sheath, which has been recently described in this species (Maddrell & Treherne, 1966), on the resting and action potentials of the larger axons in the nerve cord.

METHODS

Most of the experiments in this investigation were carried out on intact nerve cords, exposed by cutting through the dorsal integument of insects which were held down with plasticine. The conduction processes were studied in axons in the connective between the pro- and mesothoracic ganglia. Platinum wire electrodes were placed at the distal end of the connective, adjacent to the prothoracic ganglion, and glass intracellular micro-electrodes were inserted in the region of the posterior third of the connective. This latter region of the connective was maintained under slight tension to facilitate penetration of the micro-electrode, by lifting with fine platinum-wire hooks

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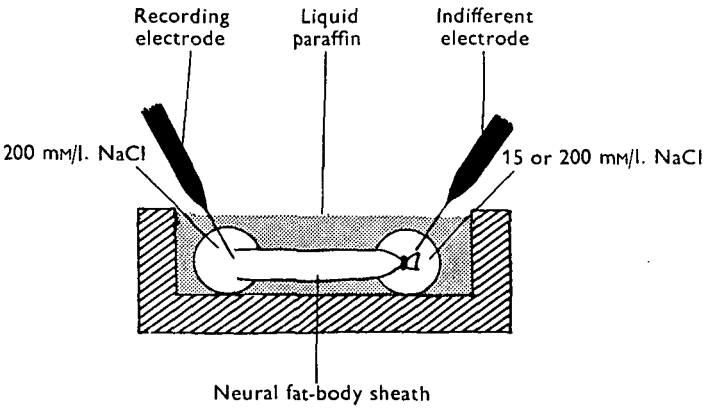
mounted on a micromanipulator. The nerve cord was irrigated with physiological saline, delivered from a glass hypodermic syringe, throughout the period of the experiment. The physiological solutions used in these experiments were based on those devised by Wood (1957). The normal solution, which approximated to the composition of *Carausius* haemolymph, had the following composition: Na 15 mM/l.; K 18 mM/l.; Ca 7·5 mM/l.; Mg 50 mM/l.; H_2PO_4 6·0 mM/l.; HPO_4 4·5 mM/l.; Cl 133 mM/l. The osmotic concentration of the fluid was maintained by the addition of 63·3 g./l. sucrose. The pH of the solution was approximately 6·6. Variations in the composition of this solution were effected with elevated concentrations of NaCl and also with additions of choline chloride and sodium sulphate. In these appropriate amounts of sucrose were omitted to maintain isosmotic conditions.



Text-fig. 1. Diagram of apparatus used to measure the potentials developed across the isolated neural fat-body sheath, when bathed with solutions of different ionic composition at the inner and outer surfaces. The tube, formed by the isolated sheath, was ligatured in the right-hand drop of fluid, which thus corresponds to the solution bathing the outer surface.

The nerve preparations were stimulated by uninterrupted series of rectangular pulses (1·0/sec.; 0·2 msec. duration) at low output impedance via an *RF* isolating unit. The glass micro-electrodes used in this investigation had resistances of between 20 and 60 MΩ when filled with 3·0 M KCl. Recording was effected using a high-impedance cathode follower, with unity gain, which was coupled to a Tetronix type 532 oscilloscope.

In some experiments an attempt was made to measure the direction and the extent of the potential across the neural fat-body sheath in isolated preparations. To do this connectives and the encircling sheath were cut close to the pro- and mesothoracic ganglia. The connectives were pulled out with fine forceps so as to leave the fat-body sheath as a fluid filled tube. These isolated lengths of fat-body sheath were ligatured at one end and placed under liquid paraffin, as shown in Text-fig. 1. The potential was then recorded between an indifferent electrode, placed in the drop of saline at the ligatured end, and a recording electrode in the drop into which the open end of the fat-body tube was placed.

The ventral nerve cord and its surrounding fat-body sheath (Maddrell & Treherne, 1966) were prepared for examination with the light microscope using the following prodecure. Short lengths of the tissues were fixed in ice-cold, cacodylate-buffered

glutaraldehyde solution containing 175 mM/l. sucrose. After washing overnight in cacodylate buffer solution containing 350 mM/l. sucrose, the material was treated with ice-cold veronal/acetate buffered osmium tetroxide solution for 2 hr. Dehydration in a series of alcohols followed, when the tissues were embedded in Araldite. 1 μ sections were cut on glass knives using a Huxley ultramicrotome and were flattened by floating them on drops of water on a glass slide, kept on a hot plate. After the water had evaporated the sections were stained by flooding them with warm methylene blue solution (Mullinger, 1964) after which they were washed in distilled water and the slide was allowed to dry. The sections could then be covered with Euparol and a coverslip.

RESULTS

Light microscopical studies revealed that the nerve cord in this species is surrounded by a continuous sheath formed of fat-body cells. The accompanying photomicrograph (Pl. 1) shows that the fat-body sheath encloses an extraneural space and thus interposes an additional fluid compartment between the neural lamella and the haemolymph. The cells in the fat-body sheath overlying the ganglia tend to be thinner than

Table 1. *The resting and action potential, measured in intact preparations and in those from which the neural fat-body sheath was removed, with bathing solutions of different ionic composition*

Preparation	Bathing solution	Action potential (mV.) (Mean + S.E.)	Significance	Resting potential (mV.) (Mean + S.E.)	Significance
Intact	15·0 mM/l. NaCl	84·4 ± 3·4	$P > 0.2$	25·1 ± 1·5	$P < 0.001$
Fat-body sheath removed	15·0 mM/l. NaCl	78·8 ± 3·2		40·3 ± 2·3	
Intact	15·0 mM/l. NaCl + 185·0 mM/l. choline chloride	89·1 ± 4·5		43·8 ± 2·8	
Intact	200·0 mM/l. NaCl	92·5 ± 6·5	$P > 0.6$	39·9 ± 2·5	$P < 0.001$
Intact	100·0 mM/l. Na_2SO_4	86·6 ± 5·4		28·1 ± 1·5	

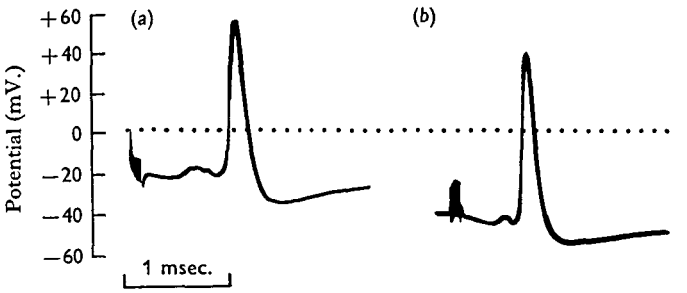
those associated with the connective (Pl. 1), the continuity of the sheath being maintained by the basement membrane. The structural organization of the cells in the neural fat-body sheath appears to be essentially similar to that of the cells in the fat-body deposits situated in other parts of the insect.

The axons within the thoracic connectives were found to be relatively small. It will be seen from Pl. 1 that only about sixty axons exceeded 7·0 μ in diameter, the maximum diameter being in the region of 11·0 μ.

The small size of the axons in the thoracic connectives made their impalement with micro-electrodes a matter of some difficulty. In these experiments the preparation was stimulated as the micro-electrode tip advanced into the tissues, so that penetration of an axon was signalled by the development of the resting potential and the appearance of a train of action potentials. Once an axon was impaled it was found that the amplitude of the action potential began to decline after a period which varied from 30 sec. to 20 min. This decline resulted from a reduction in the resting potential and would seem to indicate that the tip of the micro-electrode was causing appreciable damage to these small axons. The resting and action potentials were, therefore, only recorded

immediately following impalement of the axons so as to minimize the effects of axon damage.

The form of the action potential in axons from preparations in which the fat-body sheath was intact is shown in Text-fig. 2*a*. In these preparations the apparent resting potential was relatively small, only averaging 25·1 mV. (Table 1), while the measured overshoot amounted to about 59 mV. Removal of the fat-body sheath did not appear significantly to alter the form or the total amplitude of the action potential (Text-fig. 2*b* and Table 1). The measured resting potential in these latter preparations was, however, significantly increased, while the overshoot was correspondingly reduced, as compared with the action potentials obtained in preparations in which the fat-body sheath remained intact.



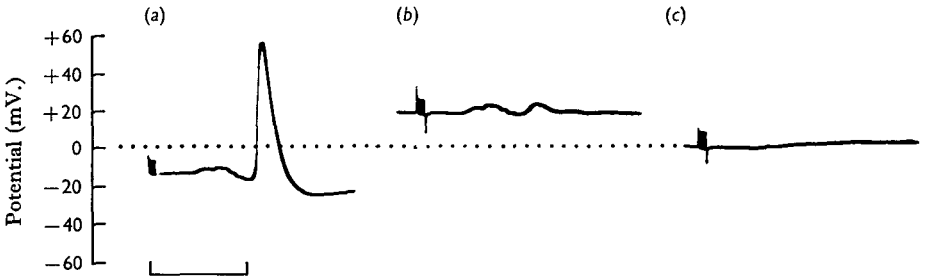
Text-fig. 2. Action potentials recorded from axons in thoracic connectives bathed with *Carausius* saline. (*a*) In preparations in which the neural fat-body sheath was intact. (*b*) In preparations from which the fat-body sheath had been removed.

Experiments were carried out in an attempt to determine the extent of any potential gradients between the extracellular fluid of the connectives and the bathing medium. Text-fig. 3 shows the action potential recorded in an axon from a connective in which the fat-body sheath was intact. In this experiment the tip of the micro-electrode was very slowly withdrawn from the axon and was then held in the position in which extracellular recording of the action potential could be seen (Text-fig. 3*b*). The micro-electrode tip was then withdrawn into the bathing fluid to give the position of zero potential between the recording and the indifferent electrode (Text-fig. 3*c*). These results indicate that there is a potential which can be measured between an electrode which is apparently in an extracellular situation in the connective, and the bathing medium. It is significant that this potential approximates in size and polarity to the differences in the apparent resting potentials measured in connectives in which the fat-body sheath was intact or absent.

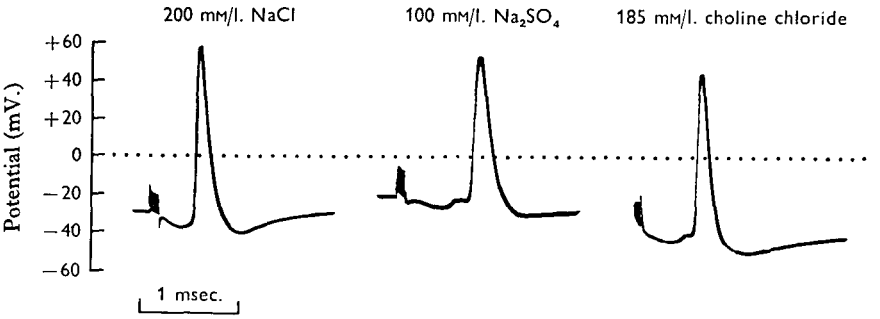
The apparent resting potential of axons was measured in connectives which were bathed with solutions of varying ionic composition. These experiments were performed in preparations in which the neural fat-body was intact. Text-fig. 4 shows action potentials obtained from such preparations which were bathed in the following solutions: one in which the NaCl content was elevated to 200·0 mM/l., a second in which 185·0 mM/l. choline chloride was added to the normal solution and a third in which 100 mM/l. Na_2SO_4 was substituted for the normal 15·0 mM/l. NaCl. The results show that the addition of elevated concentrations of NaCl and choline chloride resulted in increased resting potentials, corresponding to those obtained in connectives

in which the fat-body sheath was removed (Table 1). Substitution of 100 mM/l. Na_2SO_4 for the elevated chloride solutions, on the other hand, resulted in small apparent resting potentials which were not significantly different from those of intact connectives maintained in normal physiological solutions.

An attempt was made to measure the potential across the fat-body sheath in *in vitro* preparations (Text-fig. 1) in which a high concentration of NaCl was maintained at the inner surface of the sheath cells. It was found that with 200 mM/l. NaCl on the inside of the sheath and 15 mM/l. on the outside positive potentials were consistently recorded (Table 2). Replacement of the outer bathing solution with one of 200 mM/l. NaCl resulted in abolition of the potential difference.



Text-fig. 3. (*a*) Action potential recorded from an axon in a thoracic connective with the neural fat-body sheath intact. (*b*) The potential observed when the tip of the micro-electrode was withdrawn from the axon, but still retaining the extracellular recording of the action potential. (*c*) The potential obtained when the recording electrode was withdrawn into the bathing medium (normal *Carausius* saline).



Text-fig. 4. Action potential measured in thoracic connectives, with neural fat-body sheath intact, when bathed with physiological solutions containing 200 mM/l. NaCl, 100 mM/l. Na_2SO_4 and 185 mM/l. choline chloride.

Table 2. *The potentials measured across the isolated neural fat-body sheath, with* 200 mM/l. NaCl *at the inner surface, relative to bathing solutions of* 15 mM/l. *and* 200 mM/l. NaCl *at the outer surface*

Serial	Potential with 15 mM/l. NaCl in outer solution (mV.)	Potential with 200 mM/l. NaCl in outer solution (mV.)
1	+10	0
2	+ 5·0	0
3	+ 4·5	0
4	+12·0	0
5	+ 5·0	0

DISCUSSION

The above results demonstrate that the presence of the neural fat-body sheath did not alter the amplitude of the action potential, but reduced the apparent resting potential and correspondingly increased the measured overshoot as compared with action potentials recorded in connectives from which the fat-body sheath was removed. The reduced resting potential measured in the intact preparation appeared to result from the interpolation of a positive potential of some 15–20 mV between the recording and the indifferent electrodes.

The positive potential associated with the neural fat-body sheath was significantly reduced when the chloride concentration of the bathing medium was raised but appeared to be unaffected by the alterations in the cation concentration. Thus in 200 mM/l. NaCl or choline chloride the measured resting potentials of intact preparations approximated to those obtained in de-sheathed preparations maintained in normal *Carausius* saline. With 100 mMl. Na_2SO_4, on the other hand, the apparent resting potentials were relatively small, indicating that the positive potential had not been abolished by treatment with this solution. The dependence of the positive potential upon the level of chloride ions in the bathing solution suggests that it might result from a chloride diffusion potential across the neural fat-body sheath. This supposition derives some support from the results of the *in vitro* experiments in which it was shown that positive potentials could be obtained when a gradient of sodium chloride was maintained across the isolated fat-body sheath. These potentials were, however, smaller in magnitude than the postulated positive potentials in intact preparations. It is not clear whether this discrepancy results from damage caused to the fat-body sheath during the isolation procedure or to the selection of an inappropriate ionic gradient across the fat-body sheath in the latter experiments.

The presence of an apparent chloride diffusion potential across the neural fat-body sheath implies that there is probably a relatively high concentration of chloride ions in the fluid bathing the inner surface of the sheath. Now it has been emphasized that the cells of the neural fat-body sheath do not appear particularly specialized and do not, for example, exhibit any of the structural characteristics usually associated with secretory epithelia. It seems reasonable to suppose, therefore, that the maintenance of a relatively high ionic concentration at the inner surface of the fat-body sheath might result from the activity of conventional ion pumps situated in the plasma membranes of the fat-body cells. It could be envisaged, for example, that the extrusion of sodium in association with chloride ions might produce a relatively high concentration of these ions at the inner surface of the fat-body sheath merely as a consequence of the restricted space contained between the inner margin of the sheath and the neural lamella. The passive diffusion of these ions back into the haemolymph could thus produce a potential of the observed polarity if, as is observed in aqueous solution, the mobility of the chloride ions in the fat-body sheath is higher than that of sodium ions.

The results of some previous experiments have indicated that there is an appreciable rapidly exchanging sodium fraction in the nerve cord of *Carausius* (Treherne, 1965*b*). This fraction was identified as that contained in the extracellular compartment of the central nervous system. Unlike the state of affairs in nerve cord of *Periplaneta* (Treherne, 1961, 1962) this rapidly exchanging fraction did not result largely from a

Donnan equilibrium between the extracellular fluid and the haemolymph, but primarily from an active uptake of sodium ions into the intact nerve cord. This uptake appeared to be accompanied by a movement of chloride ions, for the influx of this anion into the nerve cord could also be reduced by the addition of metabolic inhibitors. It is conceivable, therefore, that the postulated chloride diffusion potential associated with the presence of the neural fat-body sheath could result from the efflux of this anion from a relatively high concentration at the inner surface of the sheath.

It is tempting, in the light of the above considerations, to suppose that the ability of *Carausius* axons to function in connectives bathed with low sodium solutions might result from the maintenance of a relatively high concentration of this cation by the activity of the neural fat-body sheath. However, the present study has demonstrated that the axons can propagate action potentials in connectives from which the fat-body sheath was removed. It is clear, therefore, that the postulated mechanism which regulates the concentration of sodium ions in the region of the axon surface must also result from the activity of cellular elements situated at a deeper level than the superficial connective tissue layer of the nerve sheath.

The present study has not revealed the existence of an appreciable potential between the extracellular system and the bathing medium in the absence of the neural fat-body sheath. It seems reasonable to suppose, therefore, that any mechanism responsible for the concentration of sodium of ions at the axon surface must also involve an uptake of anions in order to achieve electrical neutrality between the extracellular system and outside solution.

The apparent involvement of the neural fat-body sheath in the production of a positive potential, which tends to reduce the measured resting potentials in intact preparations, was deduced from the increased resting potentials obtained in de-sheathed preparations. The possibility exists, on the other hand, that these results might have been produced by some unidentified damage to the glial or perineural cells during the removal of the fat-body sheath. There is, however, little evidence for the involvement of the glial or perineural cells in producing the positive potential demonstrated in the connectives of *Carausius*. For example, action potentials were recorded in low-sodium solutions in the absence of the fat-body sheath, which suggests that some sort of glial or perineural regulation was still effective in these preparations. Furthermore, the *in vitro* experiments, which demonstrated a potential of appropriate polarity when a gradient of chloride ions was maintained across the isolated fat-body sheath, represent circumstantial evidence for the involvement of this structure in the production of the positive potential.

The resting potentials measured in connectives from which the neural fat-body sheath was removed was found to average 40·3 mV in preparations which were bathed in normal *Carausius* saline. This value approximates to the potassium equilibrium potential of 37 mV. which was calculated according to the Nernst equation in an earlier investigation (Treherne, 1965*b*). The latter value was based on estimates for intracellular and extracellular concentrations which were derived from the kinetics of ^{42}K exchanges in the nerve cord of this species. Similar methods were also used to calculate the sodium equilibrium potential of 22·3 mV. for the axons of the abdominal nerve cord. This estimated value does, however, fall short of the average overshoot of 38·5 mV. for action potentials measured in the present investigation. Such a dis-

crepancy between calculated sodium equilibrium potential and the measured overshoot is, perhaps, not surprising. The extracellular sodium concentration used in this calculation was obtained by relating the rapidly exchanging sodium fraction to the total estimated volume of the extracellular fluid in the intact nerve cord. This method, however, would not distinguish any localized regions of relatively high sodium concentration which might be maintained at the axon surface. Such factors would, of course, result in appreciable underestimates of the sodium equilibrium potential.

The results presented in this paper do little to increase our understanding of the precise mechanisms which are involved in the regulation of the sodium concentration in the region of the axon surface of this phytophagous species. Some efforts are currently being made in this laboratory to elucidate this problem using micro-electrode techniques. The results of this investigation will be discussed in a later communication.

SUMMARY

1. The nerve cord of the stick insect is surrounded by a fat-body sheath. This sheath encloses an extraneural space and thus interposes an additional fluid compartment between the neural lamella and the haemolymph. The axons in the thoracic connectives were found to be relatively small, the largest ones averaging 7–11 μ in diameter.

2. The apparent resting potentials of axons, impaled with glass capillary microelectrodes, were found to be relatively small, averaging only 25·1 mV., with an overshoot of 59·3 mV. in action potentials in intact preparations. In the absence of the neural fat-body sheath the resting potentials were increased to a mean value of 40·3 mV., there being no significant alteration in the total amplitude of the action potentials. This effect appears to result from the interpolation of a positive potential of some 15–20 mV. between the indifferent and recording electrodes.

3. The positive potential was abolished, in intact preparations, when the nerve cords were bathed with solutions of elevated chloride concentration. Positive potentials were also obtained when gradients of chloride ions were maintained across the isolated fat-body sheath. It is suggested that the positive potentials may result from a chloride diffusion potential across the neural fat-body sheath.

4. The results are discussed in relation to the ability of the axons of this species to function in ganglia and connectives bathed with solutions of low sodium concentration.

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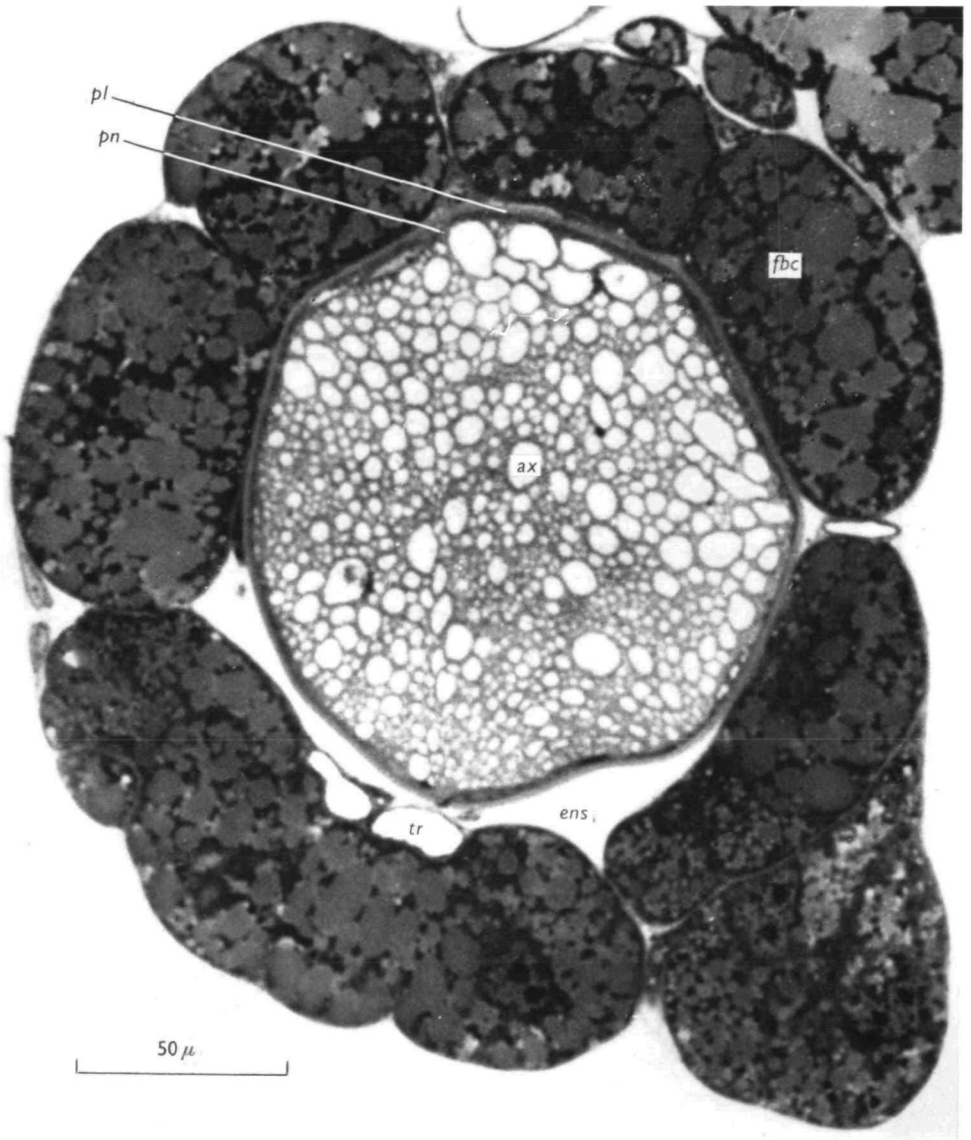

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EXPLANATION OF PLATE

A thin transverse section of one thoracic interganglionic connective and its associated fat-body sheath. The cylinder of fat-body cells (*fbc*) is continuous round the connective and is quite closely applied to it leaving only a narrow extraneural space (*ens*) in places. The acellular perilemma (*pl*) and the cellular perineurial layer (*Pn*) just beneath it can clearly be seen as concentric sheaths round the main central part of the connective, which contains the axons (*ax*). Also in the field are several branches of the tracheal system (*tr*).

The section was cut from material embedded in Araldite and stained with warm methylene blue solution (Mullinger, 1964). × 550.