THE ULTRASTRUCTURE OF THE PERINEURIUM IN TWO INSECT SPECIES, CARAUSIUS MOROSUS AND PERIPLANETA AMERICANA

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SUMMARY
The organization of the perineurium in two insect species (Carausius morosus and Periplaneta americana) has been examined with the electron microscope. In both species this cellular layer has been found to possess an extensive system of tortuous channels between the lateral cell walls. These channels are open at the outer margin adjacent to the fibrous connective-tissue sheath, but appear to be closed at the inner margin by regions of septate desmosomes and/or 'tight' junctions. There is an increased surface area at the inner margin of the perineurial cells produced by the presence of long inwardly directed flanges. An electron-dense coat has also been identified on the cytoplasmic side of the type II perineurial cell membranes at points of contact with the underlying extracellular system and at the outer surface adjacent to the connective-tissue sheath. This organization of the perineurium is strikingly similar to that observed in a variety of fluid-secreting epithelia and its possible function in fluid transport is discussed in relation to the available evidence on the physiology of the insect central nervous system. It is suggested, contrary to some earlier suppositions, that the perineurium may not be primarily involved in the control of the extracellular sodium level and that this regulation may be effected at a deeper level in the central nervous tissues.

INTRODUCTION
The chemical composition of the haemolymph is extremely specialized in some phytophagous insects. In particular the inorganic ion content is frequently unusual in the possession of very low concentrations of sodium ions together with relatively high magnesium levels (see Wyatt, 1961; Shaw & Stobart, 1963; Sutcliffe, 1963). Such low sodium concentrations in the haemolymph present obvious difficulties in the interpretation of nervous conduction in terms of the classical membrane theory, which has been shown to be applicable in all the other arthropod species which have been investigated (see Treherne, 1966). The ability of the axons of Carausius morosus to function when the nerve cord was bathed in low sodium solutions (Treherne, 1965a; Treherne & Maddrell, 1966b) did not appear to result from the presence of any impermeable peripheral diffusion barrier, for it was found that the exchanges of a variety of inorganic cations took place rapidly with the haemolymph (Treherne, 1965b). Axonal function in this phytophagous species was, in fact, found to depend upon a regulation of the extracellular sodium in the central nervous system. This regulation appeared to involve an active secretion of sodium ions from the haemolymph so as to

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produce a high sodium environment in the immediate vicinity of the axons. This effect does not appear to be achieved exclusively by the overlying neural fat-body sheath (Maddrell & Treherne, 1966), for the presence of this structure does not appear to be essential for prolonged nervous activity in low sodium solutions (Treherne & Maddrell, 1966a). It has been suggested that the most obvious site for the mechanism responsible for the regulation of the extracellular sodium would be the peripheral cellular layer or perineurium (Shaw & Stobbart, 1963; Treherne, 1965b). The present investigation was, therefore, undertaken in an attempt to throw some light on the possible function of the perineurium by relating the ultrastructural organization of this cellular layer to the available physiological information. Comparison has been made between the organization of the perineurium in the stick insect (Carausius morosus) and in the cockroach (Periplaneta americana), which possesses a normal haemolymph containing relatively high concentrations of sodium ions.

MATERIALS AND METHODS

Adult female specimens of Carausius morosus and Periplaneta americana, taken from laboratory cultures, were used in this investigation. Portions of their ventral nerve cords were fixed for 2 h in 2.5% glutaraldehyde kept at 0 °C and at about pH 7.4 in 50 mM/l cacodylate buffer containing 175 mM/l sucrose. After washing for 24 h in further cacodylate buffer solution containing 350 mM/l sucrose, the tissues were placed in cold 1% osmium tetroxide solution, buffered with veronal acetate, for 1 h. Dehydration in an ethanol series followed and, after treatment with propylene oxide, the material was embedded in Araldite. Thin sections were cut with a Huxley microtome and examined with a Philips EM 200 electron microscope. The sections were stained by successive immersions in 50% ethanol saturated with uranyl acetate (2 min) and Reynolds's lead citrate solution (4 min).

RESULTS

Carausius morosus

Perineurial cells of the interganglionic connectives. In the interganglionic connectives the perineurium consists of a rather thin layer of cells, rarely more than 1.4 μ in depth, immediately beneath the fibrous connective-tissue sheath. The cells show some structures which are thought to be characteristic of secretory tissues. Of these structural features perhaps the most striking is the organization of the lateral walls. These are very long and tortuous and occasionally they can become separated to form intercellular spaces (Figs. 1, 3 and 4). The lateral cell walls show no close adhesion towards the side facing the sheath. In all the cases examined, however, they are closely apposed at the inner ends where they are held together by septate desmosomes and there is usually a 'tight' junction (Figs. 1, 2).

In the connective the perineurium consists of two cell types. Type I cells (Figs. 1, 3 and 4), are characterized by the presence of large mitochondria, the absence of microtubules and the presence of many free small granules, about 250 Å in diameter, which are somewhat similar to those found in the perineurium of the cockroach (Smith &
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Treherne, 1963) where they are thought to be deposits of glycogen. Groups of vesicles are occasionally seen which in some sections have some resemblances to Golgi secretion (Figs. 1, 4), but it is possible that they are swollen diverticula of the endoplasmic reticulum; no evidence was found to suggest that they might be pinocytotic vesicles. The type II cells typically contain very large numbers of microtubules and relatively few small mitochondria, but no ‘glycogen’ granules (Figs. 1–5). Type I cells do not appear to make contact with the Schwann cells which lie immediately under the perineurium, being always underlain by processes from type II cells (Figs. 1, 3 and 4). Thus, although both types of cell face on to the fibrous sheath, only one of them, the type II cells, makes direct contact with the deeper cells and extracellular spaces.

A noticeable feature of the type II perineurial cells is the presence of a layer of electron-dense material at various points immediately beneath the plasma membrane. Considerable areas of the outer surface of type II cells exhibit coating of such material immediately beneath the cell membrane. The type I cells, on the other hand, have only occasional patches of this material underlying the cell membrane at their outer surfaces (Figs. 3, 4). A similar electron-dense coat is also frequently found beneath the plasma membranes of the type II at the points of contact with underlying extracellular spaces (Figs. 1–5). In thin sections this layer often appears to be discontinuous (Fig. 5); it may be, therefore, that it consists of stalked particles similar to those associated with the cell membranes of the rectal papillae of the blowfly (Gupta & Berridge, 1966). The electron-dense layer averages about 250 Å in thickness, which approximates to the depth of the particulate layer seen in the rectal papillae cells of the blowfly.

The area of contact between the type II cells and the underlying extracellular spaces is increased by the presence of inward extensions of the cells (Figs. 3, 6). These may occasionally penetrate as deeply as 10 μ (Fig. 6). Longitudinal sections show these extensions to be flanges rather than microvillus-like processes.

Perineurial cells of the ganglia. The perineurial cell layer of ganglia is several times thicker than that of the interganglionic connectives and may be as thick as 8 μ. The cells are similar in structure to the type I cells of the perineurium of connectives (Fig. 7). In the ganglia, however, these cells make contact with the more deeply situated cells, but not, apparently, with the deeper extracellular spaces. These spaces, of which there are rather few in the ganglion, make contact only with glial cells which lie under the perineurium. In the thicker ganglionic perineurium the system of elongated lateral cell walls and intercellular spaces is considerably more extensive than in the connectives (Fig. 7). As in the connectives, the intercellular clefts appear to open only at the outer margin of the perineurium, the lateral cell walls being held together near their bases by septate desmosomes and tight junctions.

Periplaneta americana

The organization of the perineurium in this species appears to be essentially similar to that of the stick insect (typical sections are shown in Figs. 8–10). There are, however, some relatively minor differences. In the interganglionic connectives, the
flanges which project from the type II cells are rather less prominent than in the stick insect; they appear to be fewer in number and to penetrate less deeply (Figs. 8, 9). As in the stick insect there are numerous regions of contact between type II cells and the extracellular spaces below (Fig. 9). The cytoplasm of the type I cells is less dense than in the stick insect. In contrast to the condition in the stick insect, these cells appear to contain very few, if any, free darkly-staining 'glycogen' granules, despite the fact that the tissues were fixed and stained in an identical manner. In this connexion it may be relevant to point out that the interganglionic connectives in the cockroach are not surrounded by a fat-body sheath, as they are in the stick insect (Maddrell & Treherne, 1966). Moreover, in the ganglia of the cockroach, where there is close contact between the fat body and the nerve sheath, there is a profusion of glycogen granules in the perineurium (Fig. 10; see also Smith & Treherne, 1963).

DISCUSSION

The organization of the perineurium in both Periplaneta and Carausius is characterized by the following structural arrangements: (i) an extensive system of long, tortuous channels between the lateral walls of the perineurial cells, apparently opening at the outer margin immediately adjacent to the fibrous connective tissue sheath; (ii) the presence of septate desmosomes and/or tight junctions between the lateral cell walls towards the inner margin of the perineurium; (iii) an increased surface area at the inner margin of the perineurial cells produced by the presence of long inwardly projecting flanges; and (iv) the presence of an electron-dense coat immediately beneath the plasma membrane of the type II perineurial cells at the points of contact with the underlying extracellular system and at considerable areas of the outer surface adjacent to the connective tissue sheath. It is now relevant to consider the organization outlined above in relation to the apparent ability of Carausius to regulate the extracellular sodium level of the central nervous tissues, for the perineurium has been regarded as the potential site for the uptake of sodium ions into the extracellular space in phytophagous insect species (Shaw & Stobbart, 1963; Treherne, 1965a, b).

The most obvious specialization in epithelia which are capable of active transport of sodium ions appears to be an enlargement of the surfaces at the outer and inner margins of the cells. The anal papillae of mosquito larvae may be used to illustrate this point. These structures, which are known actively to transport sodium ions in the absence of appreciable solvent drag (Ramsay, 1953; Treherne, 1954; Stobbart, 1959, 1960; Shaw & Stobbart, 1963), show a series of regular infoldings of the outer cell membrane, together with an extensive canalicular system in the cytoplasm which opens into the haemolymph (Copeland, 1964). The organization of the perineurium in Carausius does not obviously parallel that of an epithelium such as that of the anal papillae, although the increased surface area produced by the long intercellular spaces and inwardly directed flanges could be regarded as being equivalent to the apical foldings and canalici found in this sodium transporting system. The organization of the perineurium in these insect species is strikingly similar to that observed
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in a variety of fluid-secreting epithelia in both vertebrate (cf. Kaye, Lane, Wheeler & Whitlock, 1965; Kaye & Pappas, 1965; Diamond & Tormey, 1966) and invertebrate animals (Berridge & Gupta, 1967) in which the intercellular channel is regarded as the intermediate compartment in a three-compartment transport system (Curran & Macintosh, 1962). This similarity can be illustrated by comparison with the structure of the epithelial cells of the vertebrate gall bladder. As with the apical surface of the gall-bladder epithelial cells, the inwardly directed surfaces of the perineurial cells bear a series of projections, the flanges, which correspond to the microvilli of the vertebrate tissue. Similarly the occluded region of septate desmosomes and/or tight junctions between the lateral perineurial walls occur towards the surface bearing the latter projections. As with the gall-bladder epithelium, the intercellular channels are long and tortuous and appear to open at the outer surface of the epithelium. The cell membrane at this outer surface is, as in the vertebrate tissue, without any significant membrane infoldings. Finally in both tissues the surface at which the intercellular channels open is bordered by connective tissue. The possibility thus arises that the insect perineurium may be involved in fluid transport between the central nervous tissues and the haemolymph. By analogy with the vertebrate fluid-transporting epithelia and the rectal epithelium of an insect (Berridge & Gupta, 1967) it could be envisaged that solute would be actively transported into the closed end of the long intercellular channels and that osmotic equilibration would proceed continuously as the solute diffused along the channels. Such a mechanism could thus produce fluid movement directed from the inner surface of the perineurium into the haemolymph.

The available electrophysiological evidence is, however, difficult to explain in terms of the perineurium functioning as a system capable of regulating the extracellular sodium level in the central nervous system of Carausius. Recent experiments using intracellular microelectrodes have shown that the action potential is independent of the sodium concentration of the bathing medium in intact nerve cords, although a conduction block rapidly develops in low-sodium media in desheathed preparations (Treherne & Maddrell, 1966). With intact preparations, however, the axons continue conduction for extended periods when bathed in sodium-free solutions. It is difficult to explain this latter observation in terms of an inwardly directed sodium pump located at the periphery of the perineurium, for, under these circumstances, it would be expected that the extracellular sodium would fall in the absence of this ion in the bathing medium. In Periplaneta, also, the axons have been shown to function for prolonged periods in sodium-deficient saline (Twarog & Roeder, 1956), despite the fact that the exchanges of this ion with the haemolymph take place rapidly (Treherne, 1961a, b, 1962a). In this species the rapidly exchanging sodium fraction was, in addition, found to be related to the concentration of this cation in the bathing medium (Treherne, 1962a). From these considerations it would seem reasonable to suppose that the regulation of the sodium at the axon surface must involve tissues at a deeper level than the perineurium. It would appear possible, for example, that the regulation of the sodium surrounding the axons might involve the activity of the glial membranes. Under these circumstances some local recycling of the sodium could be envisaged which would be sufficient to allow axonal activity to continue in the event of a reduction
in the concentration of this ion in the remainder of the extracellular system. These processes might be expected to be affected by the desheathing used in the earlier experiments, for it is likely that this procedure has a variety of effects on the underlying tissues (Treherne, 1962a). In addition, the electron-dense material, which occurs in the extracellular spaces adjacent to the axon surfaces, may contribute to the maintenance of electrical excitability in conditions of low sodium in the external medium. This material has been identified as acid mucopolysaccharide (Ashhurst, 1961; Smith & Treherne, 1963; Rehberg, 1966) and could, as suggested by Treherne (1962b) and Rehberg (1966), function as a cation reservoir which might be involved in maintaining a relatively high sodium environment at the axon surface.

The presence of a fluid-transporting mechanism in the perineurium would obviously involve some system for the accumulation of ions into the cytoplasm for their subsequent secretion into the intercellular channels. It is, therefore, relevant to note the presence of the electron-dense coat on the cytoplasmic side of the type II cell membranes. The apparent resemblance of this system to the repeating particulate units described on the membranes of rectal papillae cells by Gupta & Berridge (1966) is of interest, for these authors suggest that the particulate units may be the sites of enzyme systems involved in an energy supply for ion transport mechanisms which are not mediated by ATP (cf. Siekevitz, 1965). It may be significant in this respect that in the type II perineurial cells, as in the apical area of the cytoplasm of rectal papillae cells (Berridge & Gupta, 1967), there are but few mitochondria. These considerations suggest that these possible sites for ion transport might be strategically placed for their involvement in a fluid-transporting mechanism.

The demonstration of a system in the perineurium which is closely similar to those found in fluid-transporting epithelia is, perhaps, somewhat unexpected in a cellular layer which has been primarily regarded as the potential site for the active uptake of sodium ions into the underlying extracellular fluid in those insect species which show exceedingly low levels of this cation in the haemolymph. It should be borne in mind, however, that the presence of a Donnan equilibrium between the haemolymph and the extracellular fluid in the central nervous system of *Periplaneta* (Treherne, 1962a) implies that these two compartments will not be in osmotic equilibrium. The situation in the central nervous system of *Carausius* is likely to be even more extreme, for the osmotic concentration of the haemolymph in this species is relatively low ($\Delta = 0.5$ °C (Ramsay, 1955)), whilst the inorganic ion content of central nervous tissues appears to be unusually high (Treherne, 1965b). It was suggested in an earlier paper that any excess of internal hydrostatic pressure resulting from an osmotic gradient across the perineurium might tend to be restrained by the relatively inextensible fibrous nerve sheath (Treherne, 1962a). However, in another arthropod species, *Libinia canaliculata*, some swelling of peripheral nerve occurs due to osmotic uptake of water at lowered external concentrations of the bathing fluid (Guttman, 1939), and it might reasonably be supposed, therefore, that a peripheral fluid-transporting mechanism would be of physiological advantage in a species such as *Carausius* which has a haemolymph of relatively low osmotic pressure. It is of some interest in this respect to note that a mechanism for the active removal of water through the
nerve sheath has been suggested from physiological evidence obtained in mammalian peripheral nerve (Krnjević, 1955). On the other hand, cyanide was found to have no appreciable effect on the efflux of tritiated water from lobster peripheral nerve (Nevis, 1958). In these latter experiments, however, the data for the measured effluxes were related to the intracellular water in these nerves and do not, therefore, exclude the possibility of active water movements from the extracellular fluid mediated by the perineurium. The possibility also exists that the long intercellular channels in the perineurium might serve an osmoregulatory function in the absence of a massive transport of water from the underlying central nervous tissues. The presence of a conventional solute transporting mechanism situated in the membranes of the intercellular channels could produce a solution of high concentration in these restricted regions which would function as an osmotic 'buffer' between the haemolymph and the underlying tissues. Under these circumstances a cycling of water could be envisaged between the haemolymph, the perineurial cells and the intercellular channels.

The demonstration of the occurrence of septate desmosomes and tight junctions in the intercellular channels of the perineurium raises the question of the precise route by which relatively rapid exchanges of ions and molecules occur between the haemolymph and the central nervous tissues (Treherne, 1961a, b, 1962, 1965a, b). The presence of such junctional complexes has previously been correlated with a degree of relative impermeability of various epithelia (Farquhar & Palade, 1963; Wiener, Spiro & Loewenstein, 1964). Similar occluded regions in intercellular channels have, however, been observed in the endothelium which surrounds the central nervous system of the leech (Coggeshall & Fawcett, 1964), although in this species, as in the insects studied, ion exchanges take place extremely rapidly between the extracellular system and the blood (Nicholls & Kuffler, 1964). In the latter investigation it was shown that substances exchanged rapidly with the nerve cord even in preparations cooled to 1.5 °C, which suggests that active processes are not involved in these exchanges. It is obviously not possible to resolve this question at this stage, but these considerations do suggest the possibility that such occluded regions might not seal off all the intercellular pathways between the haemolymph and the general extracellular system of the nervous tissues. Alternatively it might be necessary to postulate the existence of some low-resistance pathways across some position of the perineurial cells, perhaps with similarities to those demonstrated between adjacent gland cells (Loewenstein & Kanno, 1964).

REFERENCES


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Fig. 1. Perineurium of the interganglionic connectives of Carausius morosus. A transverse section to show the tortuous path of the lateral walls of the perineurial cells. The intercellular cleft (ic) can be followed from its opening beneath the fibrous sheath (arrow at upper right of picture) to the channel separating the perineurium from the underlying Schwann cells (arrow at lower left). The lateral walls are held together near the bases of the cells by septate desmosomes (sd) and by a 'tight' junction (tj). Note the presence of microtubules (mt) in the type II cells (II) and the darkly staining granules (g) together with the cluster of possible Golgi vesicles (ves) in the type I cell (I). Much of the extracellular space under the perineurium contains electron-dense material (asterisks) similar to that seen in the equivalent extracellular spaces of the cockroach (see Fig. 9). Other structures visible are axons (ax), Schwann cells (sc) and the fibrous nerve sheath (ns). $\times 34,000$.

Fig. 2. Micrograph at higher magnification showing the lateral cell walls of the perineurium held together at their bases by septate desmosomes and a tight junction. Carausius interganglionic connective. $\times 81,000$. 
Fig. 3. Perineurial cells of the interganglionic connectives of *Carausius*. Type II cells often have long inpushings (double arrows) into the deeper tissues. Note the electron-dense coat which lies just under the outer membrane of the perineurial cells at various points (single arrows), especially beneath the outer surface of the type II cells and under the areas of contact between the type II cells and the deeper-lying extracellular spaces (asterisks). At some points the lateral walls of the perineurial cells have become separated to produce intercellular spaces (tec). × 16000.

Fig. 4. Similar to Fig. 3 but at higher magnification. × 23000.

Fig. 5. Typical type II cell of the perineurium of the interganglionic connective of *Carausius*. A micrograph at higher power to show the electron-dense coat which is found underlying the plasma membrane. In the region marked by arrows, the coat appears to be regularly discontinuous. Note microtubules (mit) cut longitudinally and transversely. × 79000.
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Fig. 6. Perineurium of the interganglionic connective of *Carausius*. This field shows a very long inpushing or flange (double arrows) which runs deeply into the tissue underlying the type II cell from which it originates. Note that it makes contact with the extracellular space at several points along its length (single arrows). Stained with lead citrate only. × 21 500.
Fig. 7. Perineurium of a ganglion of *Carausius*. Note the extensive infolding of the lateral walls of the perineurial cells which results in a long intercellular cleft. The walls have become separated at many points to produce intercellular spaces (ics). The perineurial cells contain many granules (g), several large mitochondria (mit), a series of small electron-transparent vesicles (ves), some of which may be swollen diverticula of the endoplasmic reticulum (er) and a few much larger vesicles with more electron-dense contents (asterisks). × 22,500.
Fig. 8. Perineurium of the interganglionic connectives of *Periplaneta*. A transverse section through the connective to show the tortuous channel formed by the lateral cell walls. As in the stick insect, the lateral cell walls are not held together towards the outer surface and can become separated so that the intercellular cleft (*ic*) becomes a series of intercellular spaces (*ics*). More centrally the walls are held together by septate desmosomes (*sd*). Compare this micrograph with Fig. 1. ×47450.
Fig. 9. Perineurium of the interganglionic connectives of *Periplaneta*. Compare this micrograph with Figs. 3, 4. × 15 500.

Fig. 10. Perineurium of the ganglion of *Periplaneta*. Compare this micrograph with Fig. 7. The cytoplasm contains many granules (g) and occasional microtubules (mt). × 35 000.