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Fine Structure of a Lepidopteran Nervous System and Its Accessibility to Peroxidase and Lanthanum

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Summary. The avascular ventral nerve cord of the moth, *Manduca sexta*, possesses an extensive dorsal mass of connective tissue in which lie fibroblasts that produce a collagen-like protein. The lateral and ventral surfaces of the nerve cord are ensheathed by an acellular neural lamella. Beneath this lies a layer of microtubule-laden perineurial cells which are separated from one another at their peripheral borders by lacunae containing electron-opaque material to which the cells are attached by hemi-desmosomes. Beyond these spaces, narrow intercellular clefts occur between the interdigitating perineurial plasma membranes; these are then connected by both gap and tight junctions. The axons beneath are surrounded by glia which also contain many microtubules and which are linked to one another by desmosomes and tight junctions.

When intact nerve cords are incubated in horseradish peroxidase, reaction product is subsequently found within the neural lamella as well as in the lacunae and clefts between perineurial cells, but not beyond this level. Desheathed preparations, however, contain peroxidase within the cytoplasm of the exposed glial cells. Lanthanum penetrates the neural lamella and the lacunae, clefts and gap junctions between adjacent perineurial cells, but no further. It therefore appears that the tight junctions in the perineurium may be the site of restriction to the entry of ions and molecules, the existence of which has been suggested previously by electrophysiological investigations.

Key words: Moth — Nervous system — Gap and tight junctions — Peroxidase uptake — Penetration of lanthanum.

Introduction

The nervous system of lepidopterans, as in other insects, is avascular, so that substances attaining the surfaces of the nerve cells must do so by transfer processes through or between any structures interposed between the nerve membrane and the circulating body fluids. Electrophysiological investigations on the moth *Manduca sexta* suggest that there is restriction to the free diffusion of ions and molecules into and away from the immediate extraneuronal environment of axons in the ventral nerve cord (Pichon, Sattelle and Lane, 1972). Preliminary ultrastructural studies reveal an acellular neural lamellar sheath, a layer of perineurial cells, many glial cell processes and a system of narrow intercellular clefts separating the neuronal surfaces from the body fluids (Pichon, Sattelle, and Lane, 1972). The localization of a restriction to the diffusion of ions must therefore occur at one or more levels within this system. The presence of such a barrier to the free diffusion of ions is further suggested by the fact that although the haemolymph of *Manduca* possesses a sodium to potassium ratio as low as

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1.01 (S. E. $\pm$ 0.23), conventional ionic mechanisms for the generation of membrane potentials have been found in axons of the central nervous system (Pichon, Sattelle, and Lane, 1972). Electrophysiological experiments on nerve cord preparations that have undergone varying degrees of microsurgery indicate that in the intact system there is some peripherally located barrier to the movements of sodium and potassium into the central nervous system; peripheral extraneuronal potential changes are found to accompany variations in sodium and potassium concentrations in the bathing medium surrounding intact connectives, but no associated changes in the axonal action potential can be recorded (Pichon, Sattelle, and Lane, 1972). After desheathing the nerve cord, these peripheral changes of potential are abolished and exposure to sodium-free saline results in a rapid block of axonal conduction. It would therefore appear that a major restriction to ionic movements is destroyed by the act of desheathing. Electron microscopical observations have revealed that when the nerve cord is desheathed, the neural lamella is removed and the underlying perineurial cells are damaged to a certain extent (Lane, unpublished observations). These findings have led to the proposal that the most likely site of restriction to movements of ions and small water-soluble molecules might be the junctional complexes which occur between adjacent perineurial cells (Pichon, Sattelle, and Lane, 1972). Accordingly, the present investigation has been undertaken in an attempt to test this hypothesis by studying the uptake into the moth ventral nerve cord of the exogenous tracer molecules, peroxidase and lanthanum.

Materials and Methods

The abdominal nerve cord and peripheral nerves from adult specimens of *Manduca sexta* were dissected out and fixed at room temperature or 4° C either in 3% glutaraldehyde in 0.1 M cacodylate or phosphate buffer, pH 7.4, with or without added 0.2 M sucrose (Sabatini, Bensch and Barrnett, 1963) or in Karnovsky's fixative (1965). The tissues were subsequently washed in several rinses of the buffer in which they were fixed, post-treated in 1% osmium tetroxide in 0.1 M cacodylate or phosphate buffer, pH 7.4, dehydrated through an ascending series of ethanols to propylene oxide and embedded in Araldite. 1–2 micron sections were cut on an LKB Ultrotome III and treated with a solution of 1% toluidine blue in 1% borax. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a Philips EM 300 at 60 KV.

Block staining with uranyl acetate was carried out fixing portions of the ventral nerve cord in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with added 0.2 M sucrose, followed by treatment with 2% osmium tetroxide: 0.2 M collidine buffer, pH 7.4, 2:1 (Goodenough and Revel, 1970). Staining with 2% uranyl acetate in sodium hydrogen maleate (SHM) buffer, pH 6.0, then took place at 4° C in the dark for 2 hours with washes before and after in SHM buffer at pH 5.2 (Karnovsky, 1967). The tissues were then dehydrated and embedded as previously stated.

For the investigation on the uptake of exogenous peroxidase, a number of different preparations were studied. In some cases, the body cavity was opened up and the nerve cord exposed *in vivo* to the solution of enzymatic tracer. Alternatively, the nerve cords were isolated and incubated *in vitro* with the surrounding patches of fat body cells either left intact or removed. In the latter case the dorsal mass of connective tissue was either left in position or dissected off. Finally, prior to incubation in the peroxidase solution, some preparations were desheathed by careful removal of the neural lamella from a portion of the nerve cord. These various preparations were incubated at room temperature in a solution of horseradish peroxidase (Type II from the Sigma Chemical Co.) (6 mg/ml) in *Manduca* Ringer (KCl: 3 mM/l; NaCl: 150 mM/l; $CaCl_2$: 3 mM/l) for 4, 5 and 24 hours. They were then fixed in 3%

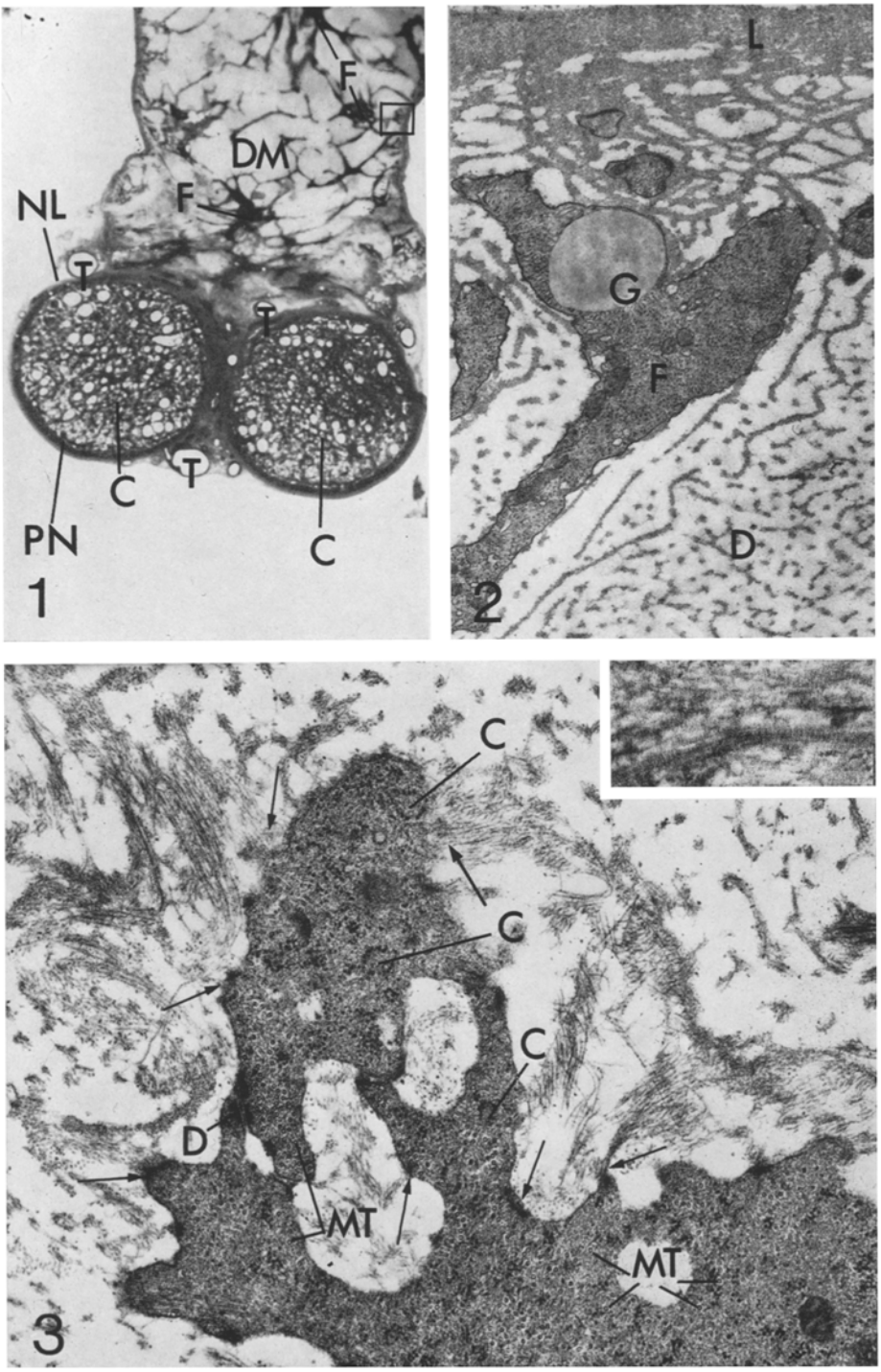
glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, plus 0.2 M sucrose, for one hour at room temperature, washed in buffer overnight at 4° C and incubated for 30 mins in a solution containing 0.05% diaminobenzidine (DAB), and 0.01% hydrogen peroxide in 0.05 M tris buffer, pH 7.6, as described by Cotran and Karnovsky (1968). The tissues were subsequently washed in buffer, treated with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, plus 0.2 M sucrose, for 60 mins at room temperature, dehydrated and embedded in Araldite in the usual fashion. Preparations were examined either unstained or stained briefly with uranyl acetate or lead citrate. Control preparations were incubated in Ringer without added peroxidase, or incubated in peroxidase and then treated with tris buffer and peroxide without added DAB.

Isolated lengths of connective from the ventral nerve cord were incubated at room temperature in a 1 millimolar solution of lanthanum chloride in *Manduca* Ringer for 15 minutes, 1 or 2 hours. This material was then fixed for 1 hour in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, plus 0.2 M sucrose, washed in phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer for 1 hr. at room temperature. The tissues were then briefly washed in SHM buffer, pH 5.2, before staining in 2% uranyl acetate in SHM buffer, pH 6.0, for 1 hr in the dark at 4° C. Rinsing with SHM buffer, pH 5.2, followed and the nerve cords were then dehydrated and embedded in the way described earlier. Control preparations were incubated in Ringer without added lanthanum, or in lanthanum without subsequent osmication nor staining with uranyl acetate.

Results

Ultrastructure

1. Connectives of the Abdominal Nerve Cord. A preliminary account of the fine structure of the paired connectives linking the ganglia of the ventral nerve of *Manduca sexta* has been published elsewhere (Pichon, Sattelle, and Lane, 1972). In adult moths the nerve cord is suspended in the body cavity by the attachment of its dorsal mass of connective tissue to muscles associated with the body wall; patches of fat body tissue surround it, but they do not form a continuous sheath. This dorsal mass is a striking U-shaped extension of the acellular neural lamella which ensheathes the nerve cord ventrally and laterally in the form of a thin layer about 2 to 8 μ thick (Fig. 1). The dorsal mass may measure up to 300 or 400 micra in length and, unlike the rest of the neural lamella proper, contains a cellular component; fibroblasts are scattered throughout its connective tissue matrix (Figs. 1 and 2). These fibroblasts have a number of attenuated processes which contain an occasional lipid globule (Fig. 2), many microtubules (Figs. 2 and 3) and much ribosome-studded endoplasmic reticulum, the cisternae of which may be swollen with a dense granular content (Fig. 3); at the cell borders there is an apparent extrusion of collagen-like fibres, indicating, as has been suggested for another lepidopteran (*Galleria mellonella*) (Ashhurst, 1964), that the fibroblasts synthesize the fibrous protein of the dorsal mass. The peripheral edges of the dorsal mass contain concentrated masses of the collagen-like protein, which elsewhere is more diffuse (Fig. 2). The periodic banding pattern of the fibres is not very distinct (Fig. 3), unlike the situation in other insects such as the cockroach (Smith and Treherne, 1963; Harper, Seifter and Scharrer, 1967). The neural lamella contains a fibrous component buried in an amorphous ground substance (Figs. 4 and 5); tracheoles appear scattered through its substance. The collagen-like fibres are oriented both parallel to and at right angles to the longitudinal axis of the nerve cord (Fig. 4).



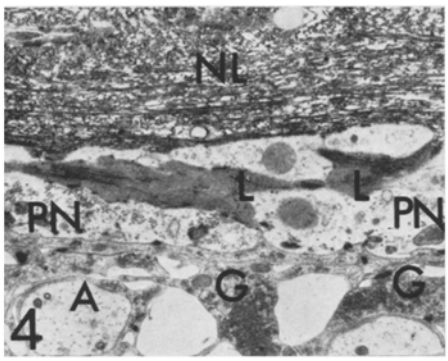
Figs. 1—3

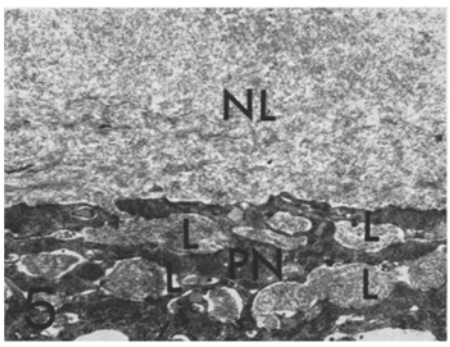
Beneath this dorsal mass and associated neural lamella lies a layer of modified glial cells, the perineurium, which, like the neural lamella, is continuous around the whole connective. The lateral borders of its component cells are often separated by lacunae which may contain electron dense material (Figs. 4 and 5). This possibly represents a mucosubstance since it resembles the material in those regions of the neural lamella which lie close to the perineurium and, in a related form, the moth *Gallaria mellonella*, the neural lamella and dorsal mass have been shown to contain neutral glycoproteins, chondroitin and dermatan sulphates (Ashhurst and Costin, 1971 b). The perineurial cells display hemi-desmosomes at their borders with the neural lamella as well as at their boundaries with the lateral lacunae (Figs. 8 and 9) and are joined to one another by both gap and tight junctions at their basal borders and to the underlying glial cells by desmosomes (Fig. 7). Block staining with uranyl acetate makes it possible to observe that the junctions formerly considered to be tight (Pichon, Sattelle, and Lane, 1972) are in fact gap junctions over a portion of their lengths (Figs. 6 and 7). These borders of the perineurial cells interdigitate in a complex fashion and the gap junctions run laterally for considerable lengths, showing a gap of 20 to 30 Å between their adjacent unit membranes thereby producing a septilaminar appearance in contrast to the pentalaminar structure seen in the regions of tight junctions (Fig. 6). The distribution of the gap and tight junctions appears to vary in different cells. In some oblique sections of gap junctions, a striated pattern appears, similar to that described in vertebrate junctions (Revel and Karnovsky, 1967). The perineurium may contain lipid globules and glycogen; numerous microtubules, which are present in profusion, appear spatially associated with the desmosome junctions (Fig. 8), lying parallel to the dense plaques on the membrane in a way similar to that in the wax moth (Ashhurst, 1970). These microtubules are usually of conventional size and appearance, but in some cases they contain an electron-opaque substance (Figs. 6 and 9) and in others a small dense central granule is present (Figs. 6 and 9). In addition, they may be surrounded by a clear zone and there are occasionally projections or cross bridges connecting

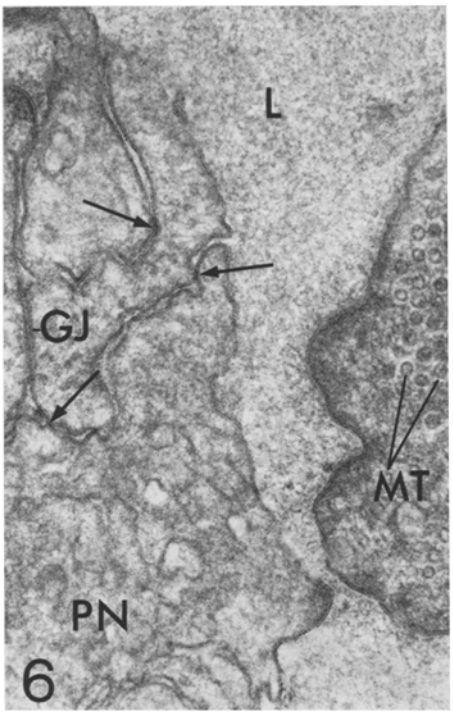
Fig. 1. Light micrograph of a transverse section through the paired connectives (*C*) of the ventral nerve cord of *Manduca sexta*. Note the extensive dorsal mass (*DM*), only part of which can be seen here; it contains tracheoles (*T*) and fibroblasts (*F*) with attenuated processes. The dorsal mass is continuous with the neural lamella (*NL*) which surrounds both connectives. Beneath this lies the perineurium (*PN*) encompassing the glial-ensheathed axons. The square outlined indicates the region illustrated in Fig. 2. ×330

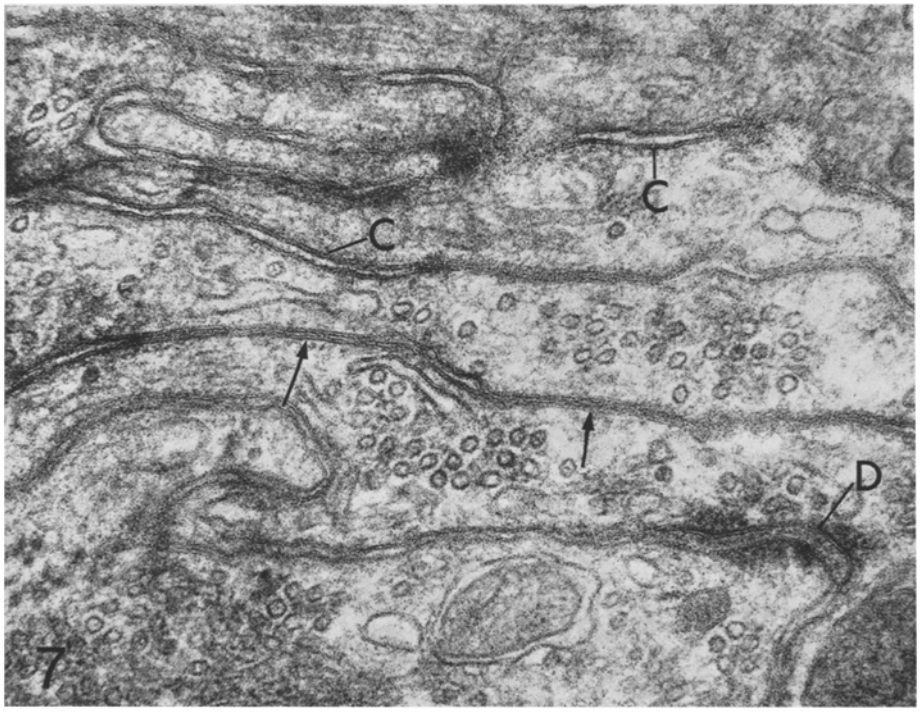
Fig. 2. Electron micrograph of the periphery of the dorsal mass showing a process (*F*) from a fibroblast lying near the border and producing the fibrous protein which forms the outer layer (*L*) and diffuse inner substance (*D*) of the mass. Note the lipid globule (*G*) in the fibroblast cytoplasm, as well as the numerous microtubules. ×16200

Fig. 3. Higher magnification of part of a fibroblast cell from the dorsal mass containing many microtubules (*MT*) and ribosome-studded cisternae of endoplasmic reticulum, sometimes swollen with a granular substance (*C*). Note that the fibrous protein seems to be arising from the fibroblast (arrows) and that desmosomal junctions (*D*) are present between adjacent membranes. ×26650. Insert shows a longitudinal section through fibres of the collagen-like protein showing its indistinct banding pattern. ×80000









Figs. 4—7

adjacent microtubules (Fig. 9). In some cases, the microtubules are seen to run into the periphery of large electron-dense bodies which are positioned within the perineurial cells where the microtubular bundles usually lie (Fig. 10); whether these represent a form of precipitated or disaggregated microtubular protein or some quite different structure, possibly lysosomal, is not clear.

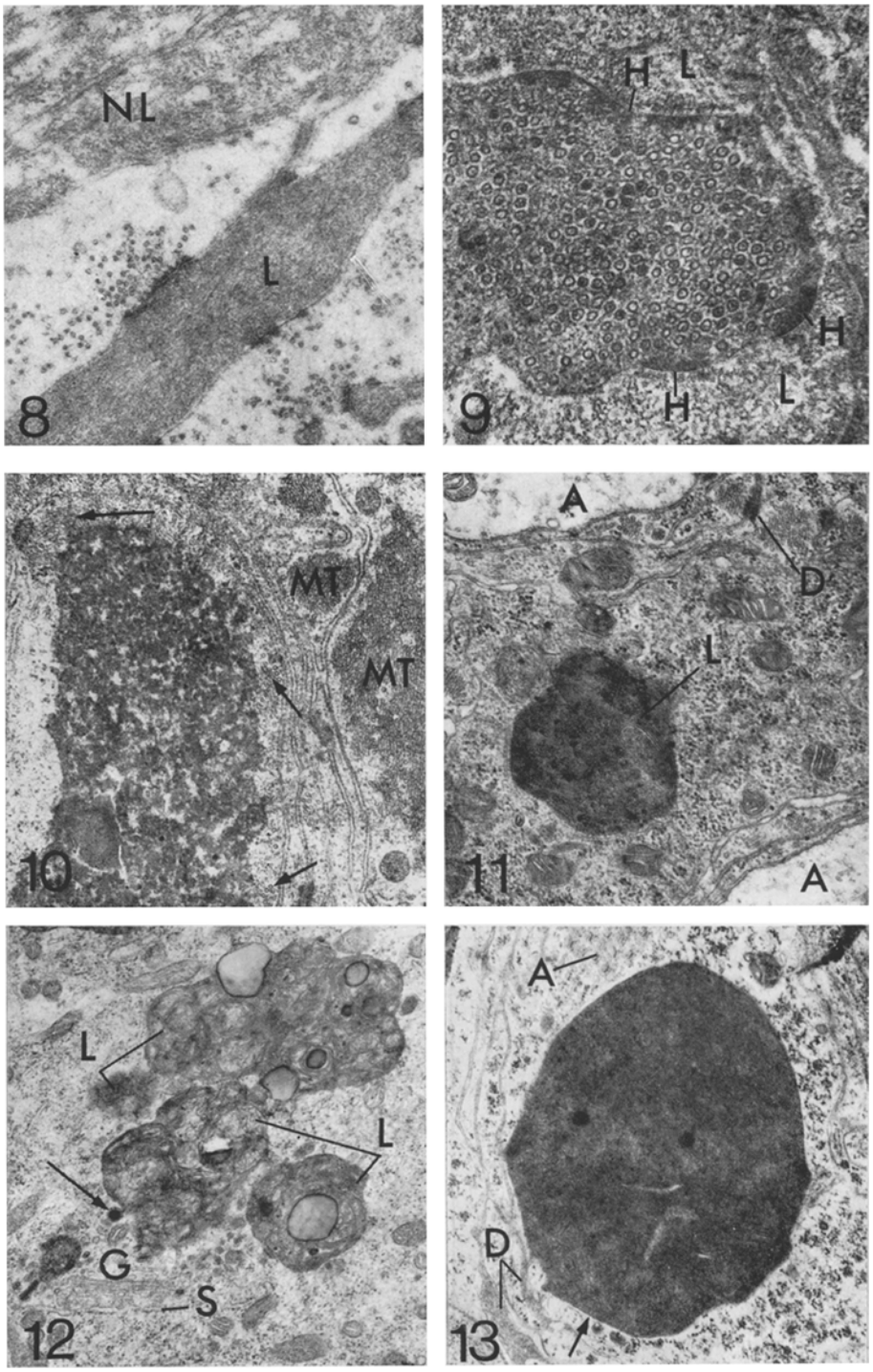
The glial cells in the connectives also contain large numbers of microtubules (Figs. 14 and 15), and, as in the perineurium, these are oriented for the most part parallel to the longitudinal axis of the nerve cord. Sometimes they too possess central granules or display an internal density, like the microtubules of the perineurium. Particularly in the perinuclear cytoplasm of the glia, large dense bodies, presumably lysosomes, are frequently encountered (Fig. 11). The extended glial processes are associated with one another by both desmosomes and tight junctions (Figs. 11, 14, 15 and 16). Like those in the perineurium, the glial desmosomes are typical of others reported in invertebrates, in that an electron-dense plaque of material lies in the extracellular space between two glial membranes which are highly electron-dense due to fibrous material adhering to their inner surface. The tight junctions appear very numerous after conventional fixation (Figs. 14 and 16), but after block staining with uranyl acetate, they are much more difficult to find, and when present, only run for very short distances. It may be that the extensive tight junctions observed are not present as such *in vivo* and may in fact be gap junctions, with a 20 to 30 Å space lying between their closely apposed unit membranes; preparations block stained with uranyl acetate show some areas of close membrane apposition which could be interpreted as such. The axons ensheathed by the glial cells contain neurotubules and mitochondria and in some cases, electron-dense granules, possibly a hormonal neurosecretory product. The spaces between adjoining glial cells may be quite considerable (Fig. 14) but are sometimes negligible (Figs. 11 and 15). Rapid preservation with glutaraldehyde in 0.1 M cacodylate buffer plus sucrose generally appears to give optimal results and after such fixation the intercellular spaces are not usually very large. These

Fig. 4. Cross section through the periphery of one of the connectives from the ventral nerve cord of *Manduca*. The collagen-like fibres of the neural lamella (*NL*) are arranged both parallel to and at right angles to the longitudinal axis of the nerve cord. Some fibres continue into the lacunae (*L*) which lie between adjacent perineurial cells (*PN*), beneath which lie glial (*G*) ensheathed axons (*A*). Fixed for several hours in glutaraldehyde in cacodylate buffer plus 0.2 M sucrose followed by post-osmication ×7700

Fig. 5. Similar region and fixation to that illustrated in Fig. 4 except that this preparation was fixed overnight. The perineurial cytoplasm (*PN*) appears more electron-opaque and the material in the lacunae (*L*) is less dense in comparison with the material shown in Fig. 4. The continuity of the lacunae with the neural lamella (*NL*) is evident. ×7500

Fig. 6. Section through the perineurium (*PN*) after block staining with uranyl acetate. Cells on either side of a lacunae (*L*) can be seen and both gap junctions (*GJ*) and tight junctions (arrows) are evident between adjacent cell borders. *MT* microtubules. ×79800

Fig. 7. Perineurial cells after block staining with uranyl acetate showing lengths of gap junctions (arrows) between adjacent plasma membranes. The narrowness of the gap in these junctions is emphasized by the dimensions of the normal clefts (*C*) between interdigitating perineurial cells. *D* desmosome between perineurium and underlying glial cell. ×95700



Figs. 8—13

extracellular spaces never contain electron-opaque material as do those of the cockroach and stick insect (Smith and Treherne, 1963) and this would appear to support the contention that they represent an artefact of fixation.

2. Ganglia of the Ventral Nerve Cord. The ganglia of the abdominal nerve cord consist of glial-ensheathed nerve cells underlying the neural lamella and perineurium. Beneath the neurones a layer of attenuated glial processes lie in concentric array around the central neuropile; here the usual complex of axons and synapses are found, with very little extracellular space beyond the conventional cell to cell gap of 100 to 200 Å. The spaces between glia and nerve cell bodies also usually amount to no more than this although slightly larger spaces may be found. However, unlike the connectives, when present these gaps may contain an electron-dense material (Fig. 13). The glia display intercellular junctions, as in the connectives, and their perinuclear cytoplasm may contain prominent lysosomal bodies with a lamellar internum (as in Fig. 11). The nerve cell bodies themselves contain a good deal of ribosome-studded endoplasmic reticulum. In a few cases what appear to be annulate lamellae have been seen (Fig. 13). Golgi complexes, consisting of saccules apparently forming from the rough endoplasmic reticulum, and sometimes elaborating small, moderately electron-dense 'haloed' granules, are found (Fig. 12). Dense bodies of varying shapes and sizes, presumed to be lysosomes, occur (Figs. 12 and 13), sometimes lying in close association with mitochondria; these bodies contain a variety of inclusions including phospholipid-like configurations.

3. Peripheral Nerves. A fine structural examination of the peripheral nerves reveals that they too are bound by a neural lamella containing tracheoles. A perineurium is also present and it is similar to that of the connectives except that it

Fig. 8. Section through the perineurium of *Manduca* showing the microtubules associated with the dense plaques in the plasma membrane comprising the hemi-desmosomes. Note the continuity of the material composing the neural lamella (*NL*) with that in the lacunae (*L*) between perineurial cells. ×35900

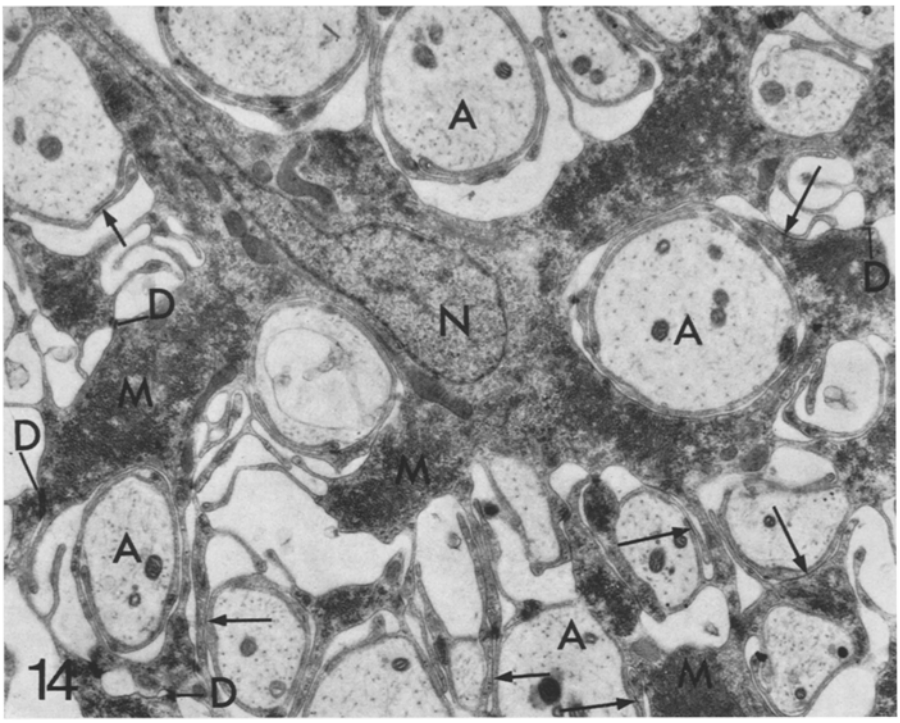
Fig. 9. Portion of the perineurium lying near lateral lacunae (*L*) and containing microtubules of varying density, some of which possess central granules or display side projections. *H* hemi-desmosomes. ×69000

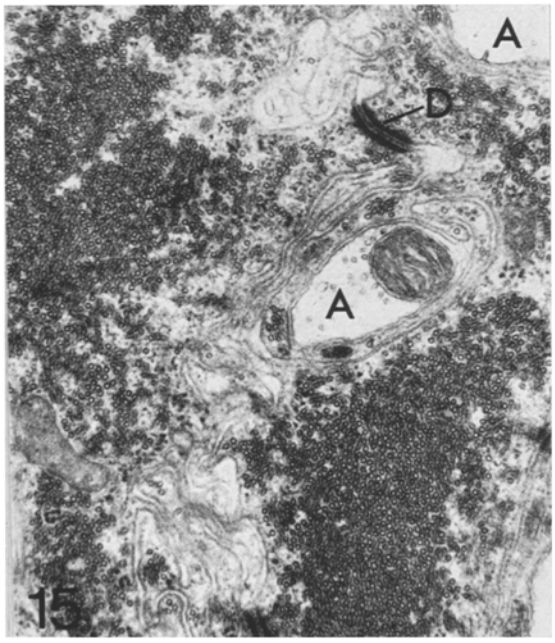
Fig. 10. Perineurial cell in which a mass of electron-dense material occurs; microtubules (*MT*) are in close spatial association with it in some areas (arrows). ×30750

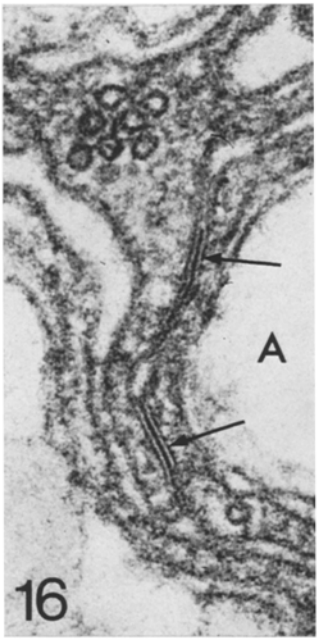
Fig. 11. Portion of a glial cell from a connective of a *Manduca* ventral nerve cord lying between two axons (*A*) and containing a dense lysosomal body (*L*). The intercellular clefts between glia in this preparation, fixed briefly in glutaraldehyde in cacodylate buffer with added sucrose, are not distended into large spaces. *D*, desmosomes. ×34150

Fig. 12. Nerve cell body from a ganglion of the ventral nerve cord of *Manduca* containing a number of dense lysosomal bodies (*L*) which have heterogeneous interna. A Golgi complex (*G*) lies nearby with the smooth border of a cisterna of endoplasmic reticulum (*S*) facing it; the ribosome-studded edge faces in the other direction. Dense, haloed, granules (arrow) are present; these appear from other micrographs to be elaborated by the Golgi saccules. ×20000

Fig. 13. Perikaryon of a ganglionic nerve cell body containing a large lysosome, delimited by a unit membrane, with a granular and fibrous internum. Note the cisternae of endoplasmic reticulum in continuity with what appear to be annulate lamellae (*A*). The extracellular space around the cell membrane contains an electron-dense material (*D*). ×25600







Figs. 14—16

is less deep; desmosomes and tight junctions are again found between adjacent glial cells (Fig. 16). As in the central nervous system, some of the axons in the nerve hough not 'giant' sized, are fairly large, although the majority are small to medium sized.

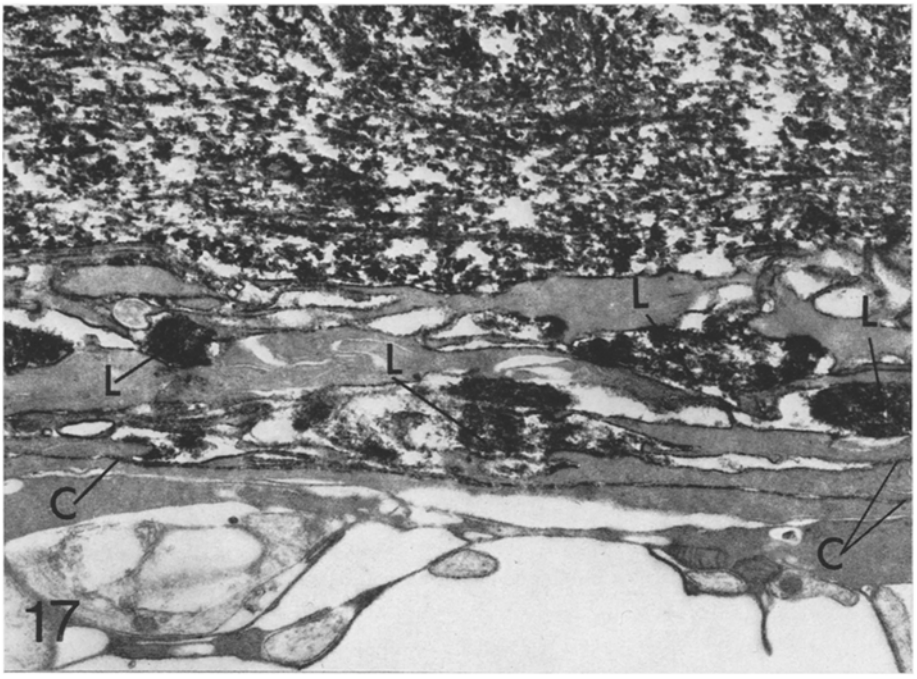
4. Sites of Uptake of Horseradish Peroxidase. In the intact connective, exogenous horseradish peroxidase is taken up by the neural lamella apparently without restriction (Figs. 17 and 18). This is found to be the case whether or not either the surrounding patches of fat body sheath are removed or the dorsal mass is cut off. When the dorsal mass is intact, the peroxidase penetrates less quickly. Reaction product for peroxidase is also found in the enlarged lateral lacunae and the narrow intercellular channels or clefts between adjacent perineurial cells (Figs. 17 and 18). However, reaction product has not been observed in the gap junctions nor is it found below the level of the basal borders of the perineurium; hence the tight junctions there seem to offer an effective barrier to entry of molecules of this size (M.W. 40000). In the desheathed connective, the neural lamella has been removed and the underlying perineurium often damaged. Incubation in solutions of peroxidase leads to uptake of this exogenous protein by the cytoplasmic substance of the glial cells (Fig. 19) as if the physical disruption had produced an alternate pathway to diffusion of molecules in addition to the normal extracellular route. Control preparations show no reaction product in either the intact or desheathed preparations.

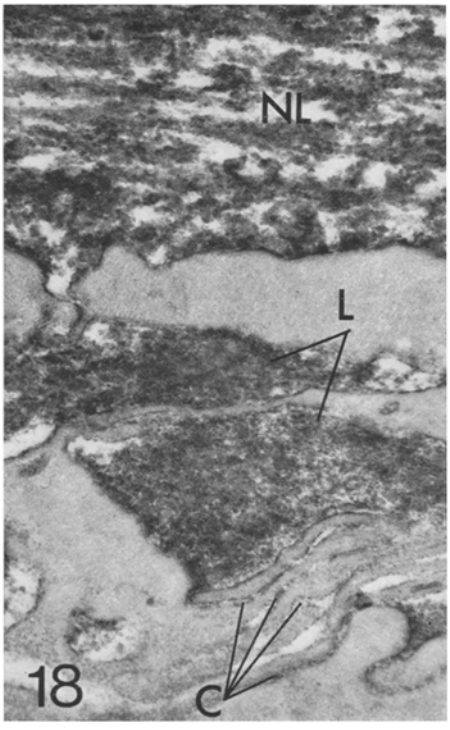
5. Penetration of Lanthanum. Intact tissues treated with lanthanum show scattered dense deposits in the neural lamella and in the peripheral lacunae between adjacent perineurial cells (Figs. 20 and 23). Even after only 15 minutes incubation, lanthanum is present in the inter-perineurial spaces. However, toward the basal portion of the perineurium, where the intercellular clefts are not dilated into lacunae, rather more dense accumulations of lanthanum crystals can be observed (Figs. 20 and 21). This suggests either that some lanthanum is removed by the treatment with the various fixative solutions after incubation, or that the concentration of lanthanum in the incubating medium was too low to permit the dense packing leading to the considerable electron opacity observed when lanthanum penetrates narrow extracellular channels in vertebrate tissues (Goodenough and Revel, 1970). A considerable density due to lanthanum can be seen in the space within the gap junctions that occur between the lateral borders of the perineurium close to the glial-ensheathed axons (Figs. 21 and 22). The unit membranes of the plasmalemma of the perineurium tend to stain very densely

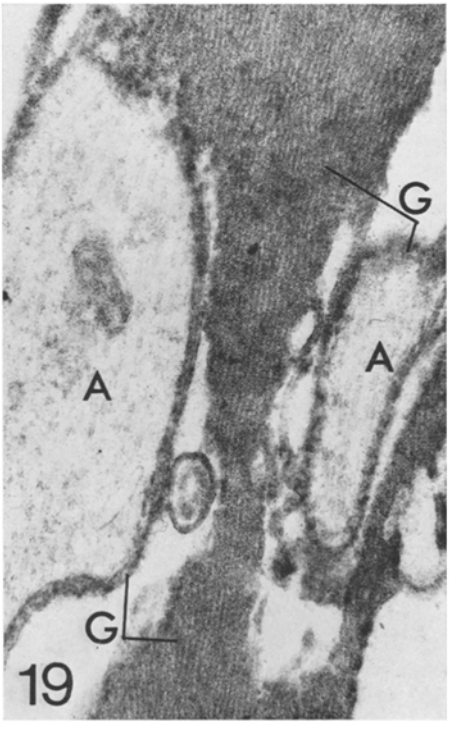
Fig. 14. Glial-ensheathed axons (*A*) within a connective of *Manduca*. Note the attenuated glial cell processes containing dense arrays of microtubules (*M*). Large extracellular spaces occur in this preparation and some tight junctions are present (arrows). *N* glial nucleus; *D* desmosome. ×12000

Fig. 15. Connective from *Manduca* fixed in glutaraldehyde in cacodylate buffer, plus sucrose, containing narrow intercellular spaces. Large arrays of microtubules, some with central granules, are shown. *A* axons; *D* desmosome. ×36000

Fig. 16. Tight junctions between adjacent glial cells in a peripheral nerve of *Manduca*. Similar complexes occur in the connectives. *A* axons. ×153750







Figs. 17—19

even in Ringer-incubated controls, as does the perineurial cytoplasm in some cases, so that it is at times difficult to distinguish this density from that due to lanthanum. However, control preparations incubated in Ringer without lanthanum show no crystalline precipitate as occurs in the experimental tissues.

In desheathed preparations lanthanum penetrates the central core of the perineurial microtubules. The preservation under these conditions is not sufficiently good to come to any conclusions as to whether lanthanum had penetrated the junctional complexes between the glia, thereby indicating whether they might be gap rather than tight junctions (see Goodenough and Revel, 1970).

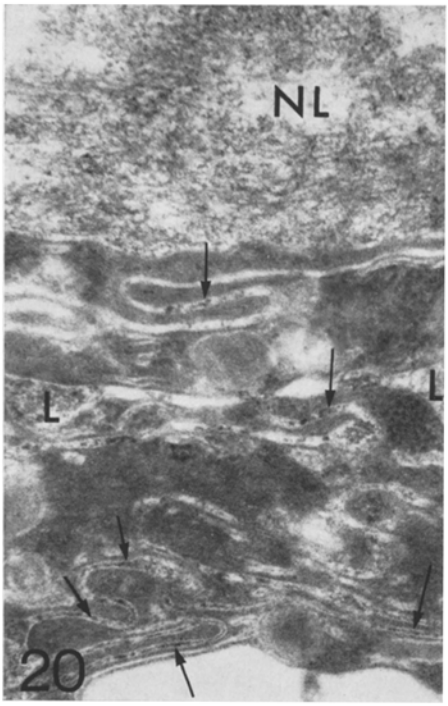
Discussion

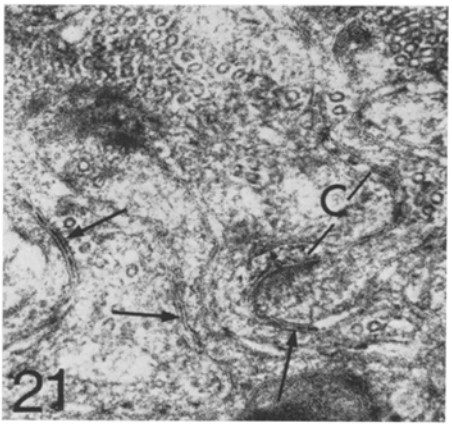
In the moth *Manduca sexta* the nerve cells of the avascular ventral nerve cord are separated from the surrounding body fluid by a series of sheaths and cell layers which substances dissolved in the haemolymph must negotiate in order to attain their surfaces. One of these layers, the perineurium, is composed of cells which are joined laterally by both gap and tight junctions. One or both of these junctions appear to act as sites of restriction to the entry of large molecular weight compounds such as horseradish peroxidase (M.W. 40000) which is unable to penetrate beyond this level. Reaction product for peroxidase has not been seen within the perineurial gap junctions or beyond the tight junctional complexes in *Manduca*; this corresponds with the results obtained in vertebrate system where peroxidase does not penetrate intact gap or tight junctions (Goodenough and Revel, 1971). Studies on vertebrate systems with a smaller molecular weight compound, lanthanum, on the other hand, show that it can penetrate gap junctions, but not tight junctions (Brightman and Reese, 1969; Goodenough and Revel, 1970). This has also been demonstrated in some invertebrate central nervous systems including the crustacean *Daphnia* (Hudspeth and Revel, 1971) and *Hydra* (Hand and Gobel, 1972). Similarly in *Manduca*, lanthanum appears to penetrate gap junctions but seems not to pass the tight junctions since no lanthanum can be seen beneath the perineurial borders in intact preparations. It would appear then that the tight junctions must be continuous rather than macular since they are causing a restriction to the entry of molecules such as peroxidase and lanthanum. If they were macular, ions and molecules could circumvent them (as peroxidase does in the case of macular gap junctions in

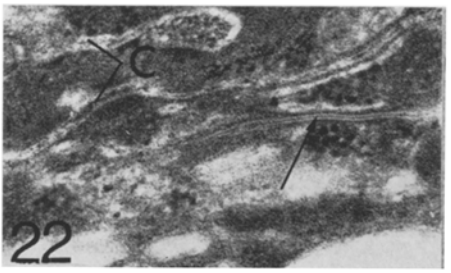
Fig. 17. Transverse section through an intact connective, from which the fat body has been removed, after incubation *in vitro* for 5 hours *in Manduca* Ringer containing horseradish peroxidase. Reaction product, indicated by its electron opacity, occurs in the neural lamella and in the lacunae (*L*) and clefts (*C*) between adjacent perineurial cells. Section unstained. The extensive spaces are probably artefacts resulting from the incubation. ×18700

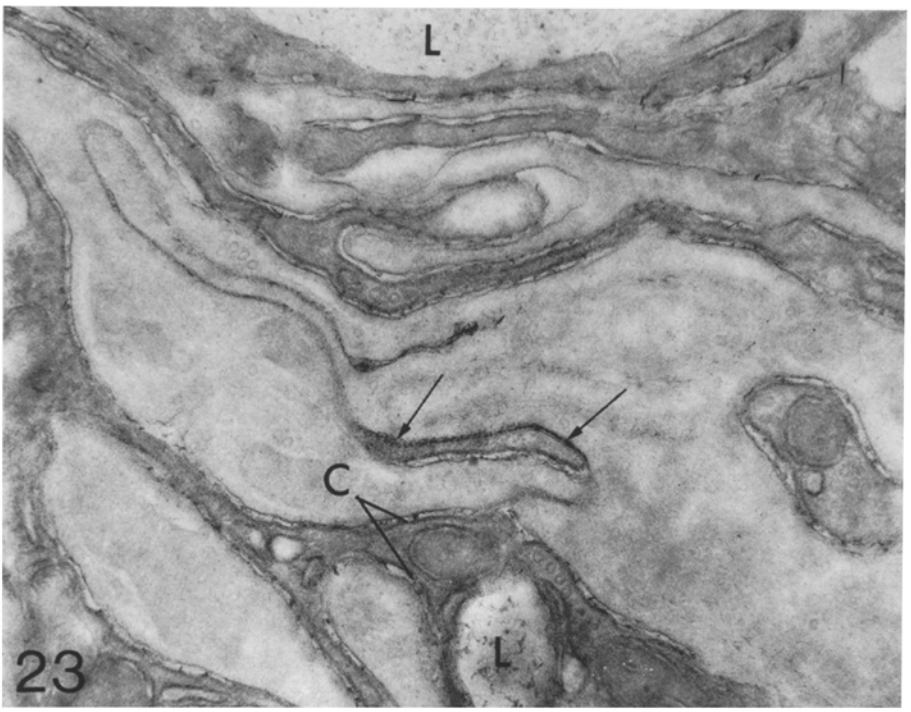
Fig. 18. Higher magnification of a portion of an intact connective incubated in a solution of exogenous peroxidase as in Fig. 17. Reaction product can be seen in the neural lamella (*NL*) and in the lacunae (*L*) and clefts (*C*) between adjacent perineurial cells which interdigitate in a complex fashion. ×47850

Fig. 19. Desheathed connective incubated *in vitro* in Ringer containing peroxidase for 4 hours. Reaction product occurs in the glial cytoplasm (*G*), (here showing their microtubules cut tangentially), attenuated processes of which envelope the axons (*A*). ×47850









Figs. 20—23

vertebrate systems, see Brightman and Reese, 1969, and Goodenough and Revel, 1971); if continuous, presumably only ions and very small molecules can penetrate and these possibly at reduced rates.

It would therefore seem that a form of 'blood-brain barrier', represented by the junctional complexes between adjacent perineurial cells, is present in lepidopterans as it is in other insects such as dictyopterans (Treherne and Pichon, 1972). In the cockroach for example, neither reaction product for exogenous macro- or micro-peroxidase nor lanthanum is found beyond the interperineurial cell junctions in the ventral nerve cord (Lane and Treherne, 1969, 1970a, 1972b). An exception seems to occur in peripheral nerves of the cockroach, however, where apparently no occluding junctional complexes occur in the outer sheath so that intercellular pathways between glial cells are accessible to the circulating body fluids. This is demonstrated by the ready penetration of lanthanum to the axonal membrane surfaces (Lane and Treherne, 1972b). Such a situation is not incompatible with axonal conduction by conventional ionic mechanisms since there exists a high sodium:potassium ratio in the haemolymph of this insect (cf. Treherne and Pichon, 1972). The situation in lepidopterans such as *Manduca* where the sodium:potassium ratio is approximately unit may account for the complete ensheathing of their peripheral nerves by the perineurium.

The presence of a blood-brain barrier in insects contrasts with the situation in the various molluscs which have been thus far examined, the nervous systems of which are avascular, as no effective structural barrier appears to be interposed between the circulating body fluids and their axon surfaces. For example, macro-peroxidase (Lane and Treherne, 1972a) and ferritin (Pentreath and Cottrell, 1970) are found to penetrate the connective tissue sheath and move beyond into the gaps between adjacent glial cells in the lamellibranch *Anodonta cygnea* and the pulmonate *Helix pomatia* respectively. Such results are in accord with electrophysiological observations in *Anodonta*, (Treherne, Mellon, and Carlson, 1969) and other pulmonate species such as *Limnaea stagnalis* (Sattelle and Lane, 1972; Sattelle, 1972) and *Helix aspersa* (Moreton, 1972); these experiments reveal rates of change of resting potentials in both intact and desheathed preparations that are consistent with potassium exchange by a first order diffusion process.

Fig. 20. Cross section through an intact connective of *Manduca* incubated in lanthanum for 2 hours. Unstained. Although the cytoplasm of the perineurium is very dense in this preparation, the lanthanum can nevertheless be seen in the perineurial lacunae (*L*) and clefts (arrows). *NL* neural lamella. ×79800

Fig. 21. Perineurium from an intact connective incubated in lanthanum for 2 hours. Stained with uranyl acetate and lead citrate. The dense lanthanum can be seen in clefts (*C*) and in gap junctions (arrows). ×79800

Fig. 22. Basal region of the perineurium from an intact connective incubated for 2 hours in lanthanum which can be seen in intercellular clefts (*C*) and a gap junction (arrow). Unstained. ×79800

Fig. 23. Region of the perineurium from an intact connective of *Manduca* incubated in lanthanum for 2 hours. The lacunae (*L*) between adjacent cells contain dense crystals of lanthanum as do clefts (*C*) and gap junctions (arrows). Unstained. ×109700

Similarly in the annelids, for example the leech, it has also been found that the avascular central nervous system presents no peripheral barrier to ion movements into and away from the nerve cell surfaces (Nicholls and Kuffler, 1964).

In desheathed preparations of *Manduca* connectives, reaction product for peroxidase has been found in the glial cell cytoplasm, as occurred when similar experiments were carried out with the cockroach ventral nerve cord (Lane and Treherne, 1969, 1970a). This suggests that in insects, the act of desheathing renders the glial system accessible to large molecules leading to the production of an alternative diffusion pathway from the bathing medium to the axonal surfaces in addition to the extracellular route via the narrow intercellular clefts between cells. This could help account for the rapid rate of potassium depolarization in desheathed connectives in comparison with intact ones following elevation of the concentration of this ion in the bathing medium (Pichon, Sattelle, and Lane, 1972).

The reaction product in the glial cells after desheathing and incubation in peroxidase could be due to trauma but alternatively might suggest that the exogenous protein may be moving from within one glial cell to an adjacent glial cell. If this is so, the observed tight junctions between glial cells may in reality be gap junctions which have been considered to provide intercellular cytoplasmic channels of high permeability to ions and molecules (Peyton, Bennett, and Pappas, 1969; Johnson and Sheridan, 1971). Moreover, it has been shown that when isolated gap junctions of vertebrate tissue are treated with horseradish peroxidase, unlike the case in whole tissue, reaction product is found within the 20 Å cleft; it has therefore been suggested that a cytoplasmic approach may have been used by the peroxidase to gain access to the gap. This could indicate that gap junctions play a role in the cell-to-cell passage of larger molecules such as peroxidase as well as ions (Goodenough and Revel, 1971). The fine structural investigations on *Manduca* have not clarified the nature of the interglial junctions since many glial tight junctions occur in all preparations except those block stained with uranyl acetate. These show only a very few tight junctions and seemingly few clearly distinguishable gap junctions, so it is not obvious to what extent the observed tight junctions may be an artefact of fixation. Both gap and tight junctions have been observed to occur between glia within the same system, however, for in another invertebrate group, the Nereid polychaetes, glia of the central nervous system exhibit desmosomes, gap and tight junctions (Baskin, 1971).

The desmosomes between adjacent glial cells and the hemi-desmosomes between the perineurial cells and their lateral lacunae or neural lamella are probably instrumental in maintaining the structural integrity of the nervous system. This would be especially important in lepidopterans where the nerve cord is in a state of agitation caused by the association of the dorsal mass with the body wall via muscle attachments. Microtubules found so prominently in the glial cytoplasm could also be functional in a skeletal capacity, in addition to any role they may play in such processes as transport. The same may be true of those in the perineurium; the significance of the variation in structural detail, in tubular density and central granule arrangement, is obscure. Central granules have been observed elsewhere in the microtubules of nervous systems (see references in Lane and Treherne, 1970b). The lanthanum staining of the microtubular

cores in desheathed preparations is similar to that observed previously in neurotubules from cockroach connectives (Lane and Treherne, 1970b).

The spaces which occur between adjacent glial cells in some preparations are very probably not present in the living state. This is suggested by the fact that those connectives which have been rapidly fixed during dissection of the nerve cord appear best preserved and usually display no spaces between glia other than the normal intercellular 100 to 200 Å gaps. Preparations incubated in lanthanum, peroxidase or Ringer for varying periods of time prior to fixation, usually show large spaces between glial cells ,these spaces seemingly increasing in size as incubation time is prolonged. Moreover, no electron-opaque material is observed in these spaces as has been found in the gaps between glial cells in the connectives of other insects (Smith and Treherne, 1963), which has been shown cytochemically to be a mucosubstance (Ashhurst and Costin, 1971a) and hence possibly to represent a cation reservoir (Treherne and Moreton, 1970). Electron-dense material is found in the extracellular spaces between neurones and ensheathing glia in the periphery of the ganglia of *Manduca*, but no cytochemical or electrophysiological experiments have so far been performed upon these areas to elucidate its function.

In conclusion it seems clear that these fine structural studies utilizing tracer substances substantiate the electrophysiological findings which indicated a peripheral barrier to inward movement of ions and small water-soluble molecules to the avascular ventral nerve cord of *Manduca sexta*. It would therefore appear that, unlike the situation in such invertebrates as annelids and molluscs, a 'blood brain barrier' is operative in this system as it is in other insect nervous systems thus far investigated (Treherne and Pichon, 1972).

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