The Connective Tissue Sheath of the Locust Nervous System: A Histochemical Study

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SUMMARY

The connective tissue sheath surrounding the nervous system of *Locusta migratoria* has been studied histochemically. It consists of an outer non-cellular layer, the neural lamella, and an inner layer of cells, the sheath-cells.

The neural lamella has been identified as being composed of a collagen-type protein and neutral mucopolysaccharide on the evidence of its histochemical reactions and the identification of hydroxyproline by paper chromatography in a hydrolysate of the neural lamella.

The sheath-cells possess large numbers of lipochondria composed of phospholipids and cerebrosides, and small spherical mitochondria. The cytoplasm also contains lipids (some of which may be cerebrosides), glycogen, and RNA.

INTRODUCTION

SODIUM and potassium ions are always found in the body fluids of animals, a much higher concentration of sodium than potassium ions normally being present. Some insects, however, are peculiar in having very low concentrations of sodium ions, but high concentrations of potassium ions; in certain cases, less sodium than potassium may be present. Boné (1944) correlated the relative amounts of sodium and potassium ions in insect haemolymph with the feeding habits of the insect. He concluded that herbivorous insects have a very low sodium and a high potassium concentration, whereas omnivorous and carnivorous insects have the more usual higher sodium concentration.

These unusual sodium and potassium ratios might be expected to affect various physiological processes and especially those concerned with conduction in the nerves and muscles. The normal concentration of potassium ions in some insects is higher than that which would block conduction in crustacean or vertebrate nerves and this, together with the very low sodium concentration, led to the suggestion that conduction in insects involves a mechanism different from that in other animals (Hodgkin, 1951). Hoyle (1952, 1953) found that potassium concentrations of 70 mM had no effect on the action potential of the nerves of *Locusta migratoria*, but if saline containing 40 mM of potassium was injected under the sheath which surrounds the nervous system, conduction was blocked. From these observations it was concluded that the connective tissue sheath acts as a barrier, selectively permeable to ions, maintaining a constant ionic environment around the neurones and their axons; therefore, the nerves are not affected by the ionic concentrations in the haemolymph.

The sheath in the locust consists of two layers, an outer homogeneous, non-cellular layer, with an inner layer of flattened cells (Hoyle, 1952). Little is known of the chemical nature of the outer layer, nor of the underlying cells in the locust, but there is evidence that the outer layer of some other insects may contain collagen and some mucopolysaccharides (Baccetti, 1955, 1956, 1957; Richards and Schneider, 1958). This paper is an account of a histochemical study of this sheath in *L. migratoria*.

**Nomenclature**

Several different terms have been used for the two layers of the sheath. The outer, non-cellular layer has most commonly been called the neural lamella (Schneider, 1902; Scharrer, 1939; Wigglesworth, 1950; Hoyle, 1952; Imms, 1957; Hess, 1958), but it has also been referred to as the perilemma (Wigglesworth, 1956; Smith and Wigglesworth, 1959) and the ‘guaina neurale’ (Baccetti, 1955). A similar situation exists for the underlying cells, since they have been called either the perineurium (Schneider, 1902; Scharrer, 1939; Wigglesworth, 1950, 1956; Baccetti, 1955), the epineurium (Imms, 1957), or the perilemma (Hoyle, 1952; Hess, 1958). The two layers together have been called the perilemma (Scharrer, 1939; Baccetti, 1955) and the epineurium (Imms, 1925), while the developing sheath has been called the neurilemma (Eastham, 1930; Roonwal, 1937; Johannsen and Butt, 1941).

In this paper it is proposed to use the term ‘neural lamella’ for the outer, non-cellular layer. The underlying layer of cells will be referred to simply as the sheath-cells. It is suggested that the terms ‘perineurium’ and ‘epineurium’, which are used for different connective tissue layers in vertebrate nerves, should not be used, since the insect nerve-sheath is not homologous with that found in vertebrates.

**Methods**

The connective tissue sheath has been investigated in the metathoracic ganglion and the abdominal region of the nerve cord in *L. migratoria*. The locusts were kindly supplied by the Anti-Locust Research Centre, London.

The ganglia and nerve-cords were fixed in a variety of fixatives and then embedded in either paraffin wax, celloidin, or gelatin. The sectioned material was stained by a routine histological method or subjected to histochemical tests. The details of the procedures used appear in the appendix. Some living material was examined after staining with vital dyes.

The amino-acid composition of the neural lamella was analysed by paper chromatography. The neural lamella of the meso- and meta-thoracic ganglia was separated from the underlying cells; histological examination showed that the neural lamella could not be dissected entirely free from cells, but the cells dissected away were free from contamination by the neural lamella. As a control, therefore, the amino-acids of the cells were also analysed and the two analyses compared. After the cells and neural lamellae of about 45 ganglia had been separated, they were hydrolysed in 6 N hydrochloric acid...
at 100° C for approximately 16 h. The hydrolysates were evaporated to dryness under reduced pressure, redissolved in 10% iso-propyl alcohol, and then applied to No. 1 Whatman chromatography paper. The chromatogram was two-dimensional; the first solvent being a mixture of butanol, acetic acid and water (40:10:50) and the second phenol saturated with water. The chromatograms were sprayed with a mixture of isatin and ninhydrin in butanol (after Kolor and Roberts, 1957) to make the amino-acid spots visible.

RESULTS

Structure of the connective tissue sheath

Hoyle (1952) describes the sheath as consisting of two layers, the outer neural lamella and the inner sheath-cells, with a tracheolated membrane on the outer surface of the neural lamella. The two layers of the sheath are clearly visible in sections, but a tracheolated membrane has not been observed. There are, however, tracheae forming a network over the surface of the ganglia and nerves, but this network is not a continuous membrane surrounding the nervous system.

The neural lamella is about 7 μ thick around the metathoracic ganglion and 3 μ round the ventral nerve-cord. It is clearly differentiated from the cells by trichrome staining methods. It is non-cellular and homogeneous; no structure was visible in the light microscope with the methods used in this study.

The sheath-cells (figs. 1 and 2) form a continuous layer under the neural lamella, about 4 to 15 μ thick. The cell boundaries are not clear as the cells are irregularly shaped. The nuclei are approximately 8 μ in diameter. The cytoplasm contains many lipocondria of different sizes, ranging from 1 μ to 6 μ in diameter. They have a tendency to congregate in the region of the cell adjacent to the neural lamella. The mitochondria can be seen after vital staining with Janus green and in preparations made by Baker's (1957) HPO technique. They are all spherical bodies, about 0·5 μ in diameter. They are distributed in all regions of the cells, but are not very numerous.

Histochemistry of the neural lamella

Carbohydrates. Some sections were tested with the periodic acid / Schiff (PAS) test which indicates the presence of carbohydrate groupings. The neural lamella is strongly positive. The reaction is not reduced by previous incubation in saliva; this indicates that glycogen is not causing the reaction. Incubation with hyaluronidase does not affect the result; it seems, then, that hyaluronic acid is not the cause of the positive PAS reaction. The neural lamella is PAS negative if the periodic acid treatment is omitted; therefore the positive reaction is not due to free aldehydes present in the tissue. These results suggest that a mucopolysaccharide is present in the neural lamella.

To find out if any acid mucopolysaccharide is present, some sections were treated with toluidine blue, because acid mucopolysaccharides are
metachromasy does not occur in the neural lamella and so acid mucopolysaccharides are probably not present. After treatment with concentrated sulphuric acid (Lison, 1953), the neural lamella becomes intensely metachromatic and this suggests that most of the mucopolysaccharide present must be neutral.

This was further checked by estimating the basiphilia of the neural lamella by means of the methylene blue extinction test (Pearse, 1953); the ability to bind methylene blue at low pH, i.e. pH 2, indicates the presence of acid mucopolysaccharides or nucleic acids. In this case, the neural lamella does not stain with methylene blue below pH 5, and this again suggests that very little, if any, acid mucopolysaccharide is present.

*Lipids.* The neural lamella is not coloured by Sudan black B, nor does it give a positive result with the acid haematein test for phospholipids (Baker, 1946). Recent work has indicated that some lipids may not be in a detectable form after fixation in formaldehyde-calcium, since more lipids can be demonstrated in cells which have been fixed in a fixative containing chromic acid (Bradbury and Clayton, 1958). Several procedures for 'masked lipids', which
will be described in greater detail later, were used, but still no lipid could be detected in the neural lamella. Therefore, if the neural lamella does contain any lipid, it must be in amounts too small to be detected by these histochemical techniques.

**Proteins.** The cytochemical reaction for proteins described by Barnard and Danielli (1956), involving the coupling of proteins with a diazonium compound, gives a positive result with sections of the neural lamella. This coupling reaction may be prevented by prior treatment of the sections with benzoyl peroxide, except in the nuclei and in other special cases, for example, collagen (Barnard, personal communication), where it is not blocked by benzoylation. It was, therefore, interesting to find that the neural lamella is still positive to the coupling reaction after benzoylation. It may be mentioned here that the neural lamella is also positive to the PAS test after benzoylation, another characteristic of collagen (Barnard, personal communication).

In addition, the neural lamella gives a positive result with Baker’s (1947) modification of the Sakaguchi reaction for arginine and other guanidine derivatives, which indicates that arginine is present. The results with the Hg-nitrite test for phenols, especially tyrosine (Baker, 1956), were negative.
and suggest that there can only be a small amount of tyrosine in the neural lamella.

**Conclusions.** The results of the histochemical tests for proteins suggest that a collagen-type protein may be present in the neural lamella. This possibility is supported by the results with the coupling reaction and also by the presence of arginine and the absence of large amounts of tyrosine in the neural lamella. Baker (1956) states that collagen is negative with the Hg / nitrite test and, furthermore, amino-acid analyses of various collagens show that it is usual for collagenous proteins to contain much more arginine than tyrosine (Randall, 1953).

Collagen is also associated with mucopolysaccharide (Jackson, 1954), but it is not yet certain how the protein and polysaccharide groups are linked together. As a result, collagen is found to be PAS-positive (Pearse, 1953), and it is interesting that the neural lamella also is PAS-positive. Furthermore, collagen does not contain appreciable quantities of lipid; nor does the neural lamella. These results, therefore, indicate that the neural lamella may possess a collagen-type protein with associated neutral mucopolysaccharide.

**Histochemistry of the sheath-cells**

**Carbohydrates.** The cytoplasm is positive to the PAS reaction, the lipochondria appearing more strongly positive than the rest of the cytoplasm. The reaction is reduced if the sections are first treated with saliva; glycogen is therefore present. The reaction is negative if the periodic acid treatment is omitted.

**Lipids.** The lipids of the sheath-cells are rather complex in their distribution. In Sudan black preparations, there is a very dark coloration of the sheath-cells. It can, however, be seen that the lipochondria are very darkly coloured, whilst the cytoplasm is a much lighter colour. The same is true after coloration with Sudan IV.

The lipochondria are strongly positive to the acid haematein test (Baker, 1946), but the cytoplasm is negative and may still be coloured by Sudan IV. This suggests that the lipochondria contain phospholipids and that the cytoplasm must possess other lipids. That the positive reaction in the lipochondria is due to the presence of phospholipids is confirmed by the negative result after the pyridine extraction test. In the neurones, phospholipids in the lipochondria are associated with cerebrosides (Shafiq and Casselman, 1954), so it seemed possible that a similar combination may occur in the sheath-cells. To investigate this possibility some ganglia were fixed in either hot or cold acetone and then tested for the presence of lipids with Sudan black. After cold acetone fixation, both the cytoplasm and lipochondria are still coloured by Sudan black, but after hot acetone, the cytoplasm is negative and the lipochondria only very faintly coloured. This suggests that cerebrosides are present in both the lipochondria and cytoplasm, since they are soluble in hot, but not cold acetone (Casselman and Baker, 1955). These lipochondria may be called
'cerephos globules', a name suggested by Baker (1957) for lipochondria containing cerebrosides and phospholipids.

There is recent evidence (Bradbury and Clayton, 1958) that after fixatives containing chromic acid, e.g. Flemming's fluid, it is possible to detect more lipids in cells. This fixative was used and followed by the Sudan black and acid haematein tests, but no further lipid material could be detected.

**Proteins.** The cytoplasmic inclusions cannot be differentiated from the rest of the cytoplasm after the protein tests. The cytoplasm and nuclei are positive with the coupling reaction, but after benzoylation the reaction is positive only in the nuclei. The Sakaguchi test for arginine (Baker, 1947) and the Hg / nitrite test for tyrosine (Baker, 1956) are also positive in both the nuclei and the cytoplasm.

**Nucleic acids.** The nuclei of the sheath-cells are Feulgen-positive after hydrolysis with dilute hydrochloric acid, so it may be concluded that they contain desoxyribose nucleic acid. The cytoplasm is strongly basiphil; this was shown by the pyronin / methyl green technique (Jordan and Baker, 1955). That the basiphilia is due mainly to the presence of ribonucleic acid in the cytoplasm was shown by treating the sections first with ribonuclease (Bradbury, 1956); the coloration with pyronin was then very much reduced.

**Phosphatases.** The tests for acid and alkaline phosphatases (Gomori, 1952) gave negative results in the sheath-cells.

**Chromatography**

Hydroxyproline is generally supposed to occur in large amounts in both invertebrate and vertebrate collagens, but only in small amounts elsewhere. Hence, if it is found that hydroxyproline is abundant in the neural lamella, it is reasonable to deduce that some collagen is present.

Chromatograms of both the neural lamella and the cell hydrolysates were developed as described earlier. The positions of the separated amino-acids were demonstrated by spraying the chromatograms with an isatin and ninhydrin mixture, which has a greater specificity for hydroxyproline than either reagent alone (Kolor and Roberts, 1957). The chromatogram of the neural lamella hydrolysate showed a distinct hydroxyproline spot, which was identified by running an authentic sample of hydroxyproline on the same paper. The cell hydrolysate gave a very faint hydroxyproline spot, but 150 applications of the hydrolysate were put on the paper, whereas only 30 applications of the neural lamella hydrolysate gave a very distinct spot. (Equal volumes of the hydrolysates in iso-propyl alcohol were obtained at the beginning of the experiment.) The much greater amount of hydroxyproline in the neural lamella hydrolysate must be due mainly to the neural lamella and not to the contaminating cells. The presence of small amounts of hydroxyproline in the cell hydrolysate suggests that small amounts of collagen may be present in the connective tissue within the ganglion.

The results of the amino-acid analyses, therefore, provide further evidence
for the presence in the neural lamella of collagen-type protein, since appreciable amounts of hydroxyproline are found only in the neural lamella hydrolysate.

**Discussion**

The possibility that the neural lamella may be composed of collagen fibres with associated mucopolysaccharide has been mentioned previously. This is inferred from the histochemical evidence for the presence of proteins and mucopolysaccharide in the neural lamella and also from chromatograms showing the presence of the amino-acid, hydroxyproline.

The mucopolysaccharide is thought to bind the collagen fibrils together, but it is not yet clear whether the protein and polysaccharide are chemically linked or merely in association with each other. In developing collagens, acid mucopolysaccharides are present; these have been identified as chondroitin sulphate and hyaluronic acid and it is thought that they serve to stabilize the collagen fibrils (Jackson, 1954). However, in the neural lamella there is no detectable acid mucopolysaccharide. This fact does not exclude the presence of collagen, since Williams (1957) found that the metachromatic properties of the ground substance of mammalian collagen are reduced as it matures, and Jackson (1957) found less sulphated mucopolysaccharides in mature collagen. Neutral mucopolysaccharides are found in other collagens, and Consden and Brown (quoted by Ward, 1958) suggest that since neutral mucopolysaccharides are not so readily removed as some other mucopolysaccharides, their association with the collagen must be very intimate. The neural lamella is then almost certainly composed of a collagen-type protein in association with an unknown mucopolysaccharide.

The polysaccharide content of collagens is variable, but it is thought that a high content of polysaccharides confers a greater degree of plasticity on connective tissue fibres (Bradfield, 1950). Although no estimations of polysaccharide content have been made in this case, the results suggest that a considerable amount is present. This would seem to agree with the mechanical functions of the neural lamella which are to hold together the cells and axons of the nervous system and yet be flexible enough not to resist or impede the movements of the body.

It has generally been assumed that the neural lamella is secreted by the sheath-cells (Scharrer, 1939). There appears to be no direct evidence for this. The origin of the sheath in the locust embryo is said to be from outlying ganglion cells which form a layer of cells around the ganglion (Roonwal, 1937). The formation of the neural lamella is not mentioned in this study, so it probably develops at a stage later than has been studied. If it is secreted by the sheath-cells, one might expect to find evidence for this in the enzyme content of the cells: Bradfield (1946) suggested that alkaline phosphatases are associated in insects with cells concerned with the synthesis of fibrous proteins. But both acid and alkaline phosphatases appear to be absent from these cells, at least in the adult locusts used in this study. It must be men-
tioned that Day (1949) found alkaline phosphatases in the cerebral ganglion, but not in the ventral nerve-cord of adult *L. migratoria*.

The identification of collagen in the locust is of special interest since there is very little reliable evidence for its presence in insect connective tissues. Rudall (1955) has identified collagen in the ventral nerve-cord of mantids. Baccetti (1955) in a histochemical study of the sheath round the nervous system of *Anacridium aegyptium*, rejected the possibility that collagen is present in the neural lamella on his data, but later (Baccetti, 1956, 1957), from studies of the birefringent properties, he identified a collagen-type protein in the neural lamella of this insect. He found that the neural lamella of *A. aegyptium* could be divided into three regions; the narrow inner and outer regions being differentiated from the middle region by possessing only neutral mucopolysaccharide, while the middle region has acid mucopolysaccharide. No such zonation of the neural lamella can be seen in the locust, and no acid mucopolysaccharide is present. The cockroach, also, has a sheath similar to that of the locust (Twarog and Roeder, 1956); the neural lamella possesses occasional nuclei which are thought to represent fibroblasts. This neural lamella was shown to be collagenous by Richards and Schneider (1958). Wigglesworth (1956) found that the neural lamella was PAS-positive in *Rhodnius prolixus*, which suggests a possible similarity with the locust's neural lamella. Moreover, electron micrographs of the neural lamellae of both the cockroach (Hess, 1958) and *R. prolixus* (Smith and Wigglesworth, 1959) show fibres with a periodicity similar to that of vertebrate collagen. A similar type of connective tissue sheath, the perineurium, consisting of an outer layer with collagen fibres and an inner epithelial layer, is present round vertebrate nerves.

In addition to the structural similarity between the sheath in insects and the perineurium in vertebrates, there appears to be also a similar function. There is evidence that the perineurium regulates the passage of ions into amphibian nerves (Feng and Liu, 1949; Huxley and Stämpfli, 1951; Krnjević, 1954). But as Twarog and Roeder (1956) point out, the locust sheath is far more efficient than the vertebrate or cockroach sheath, since nerves in these animals are rapidly blocked by saline containing 50 mM of potassium (Roeder, 1948), while 140 mM of potassium takes many hours to block a locust nerve. The ionic regulation is in both directions across the sheath and this explains why a block takes so long to occur in a sodium-free medium. The layer of cells and not the neural lamella seems to be responsible for the ionic regulation, since Krnjević (1954) and Twarog and Roeder (1956) found that silver nitrate penetrating the sheath of frogs or cockroaches was accumulated in the sheath-cells and did not go farther into the ganglion. Hoyle (1953) discovered that there was no ionic regulation in the locust if the tracheal supply to the nervous system was severed; an observation which suggests that regulation is an active process and hence would be more likely to occur in the sheath-cells than in the neural lamella. It has also been suggested by these authors that the sheath has an osmo-regulatory function.
It may be mentioned that Edwards, Ruska, and de Harven (1958), in an electron microscope study of wasp peripheral nerves, in which they identify the neural lamella as the basement membrane of the sheath-cells, or lemmoblast, suggest that this basement membrane may serve to maintain a constant ionic concentration at the plasma membrane of the cells, whilst the plasma membrane is the selective ion barrier. If this is the correct sequence of events, the mucopolysaccharides of the neural lamella may be responsible for controlling the ionic concentration; there is recent evidence that mucopolysaccharides might be concerned in the control of the passage of ions across tissues (Bradbury, personal communication; Hess, 1955; Kantor and Schubert, 1957).

The connective tissue sheath seems to have, therefore, two functions: the neural lamella encloses the nervous system and restricts it mechanically, and possibly controls the flow of ions across it, whilst the cells form a selective barrier to ions entering the nervous system. There is no apparent structural difference to account for the greater efficiency of the locust's sheath compared to that of other animals. Perhaps the difference is due to the fact that the locust has under normal conditions to tolerate fluctuations in potassium ion concentration not found in animals other than herbivorous insects (Hoyle, 1954).

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APPENDIX

Table of methods and results

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Key: +++ = strong reaction. ++ = medium reaction. + = weak reaction. — = no reaction.
### Carbohydrates

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### Lipids

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