Sheaths of the motor axons of the crab *Carcinus*

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With 3 plates (figs. 1, 3, and 4)

Summary

In crab leg nerves, the largest axons, which are the motor axons usually isolated for physiological experiments, have a sheath structure which is different from that in medium sized and smaller axons of the same nerve or of any other described nerves. Axons with a diameter over 20 \( \mu \) have (a) an outer sheath, about 5 \( \mu \) thick, of well-spaced layers of alternating glial cell cytoplasm and extracellular fibrous material, formed from fewer cells than there are layers, and (b) an inner sheath of elongated cells which creep along the axon longitudinally and interdigitate where they meet, as seen 2 or 3 times round the outside of the membranes of axons in cross-section. Therefore, possible channels between inner glial cells are elongated and few. On these structural grounds, together with physiological evidence, they seem unlikely to be preferred pathways of diffusion of ions in crab axons. Smaller axons have simple sheaths; some occur in groups within a fibrous sheath; the thinnest axons frequently occur in bundles and have no glial cell membrane in contact with them.

Introduction

A sheath of considerable thickness surrounds the largest axons in crustacean leg nerves, as has been known for many years (Young, 1936). However, electron microscope observations are limited to short papers by Geren and Schmitt (1954) on axons in lobster leg nerve, McElear, Milburn, and Chapman (1958) on fibres in sections of crab brain, Uchizono (1960) on fibres in the crayfish ventral nerve cord, and Nunnemacher, Camougis, and McAlear (1962) on the size distribution of the axons in selected nerves. None of these works show detailed pictures of the relationships between the glial cells which immediately surround the axon membrane. For comparison, reports on other invertebrate axons having extensive sheaths have been made by Hama (1959) on earthworm giant axons, and by Villegas and Villegas (1960) on the squid giant axon.

Apart from the studies on squid axon, the above anatomical observations have not been related to the problem set by the movement of ions in the region just outside the axon membrane during and immediately following sub-threshold excitation or the passage of a single impulse. Recently one of us has shown that the membranes of crab motor axons show small but measurable changes during their response to direct current following a single impulse or even following subthreshold excitation (Chapman, 1963). These changes are reduced but not abolished by washing and are interpreted as arising from the accumulation of potassium ions outside the axon membrane.

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Therefore, it became imperative to determine the morphology of the spaces and membranes round the axon.

The thickness and structure of the sheaths described for invertebrate axons show a wide variation. Most axons in the size range 1 μ to 5 μ in diameter in Crustacea, Annelida and Mollusca are unilemnal, i.e. the sheath cell surrounds the axon once. Most axons in the range 0.05 μ to 1.0 μ are pressed directly against other small axons and the whole bundle is surrounded by a sheath cell common to all. Some may therefore be quite free from glial membrane, and axons totally lacking sheath cells are the rule in jellyfish, Hydra, anemones and ctenophores (Horridge and Mackay, 1962 and unpublished). Sheaths can be built up with a single plan through the whole thickness, as in earthworm giant fibres where a single loose spiral runs up to 30 times round the nerve, or made up of 2 or more distinct structures, as in the squid axon where there is an inner pavement of small cells round the axon within a fibrous non-cellular outer sheath. In general, investing cells wrap round invertebrate giant axons forming concentric layers, and have been compared to vertebrate myelin (McAlear and others, 1958). The inner glial cells creep along the axon surface. Where such cells meet each other on the outer surface of the axon there is the possibility that channels between the cells could convey ions between the axon membrane and the external bathing medium, as suggested by Villegas and Villegas (1960) for squid axons.

Methods

Pieces of leg nerve of Carcinus were partially teased, and stretched between hedgehog spines while they were fixed in 1% osmic acid in sea-water, buffered with phosphate to pH 7.5, to which a little sucrose had been added. The material was dehydrated in acetone, using calcium metal to extract the last traces of water, embedded in araldite and cut on a Porter Blum section cutter set to give silver sections. The grids bearing unsupported sections were stained by immersion for 30 to 60 min. in a saturated solution of lead acetate dissolved in a mixture of equal parts of absolute alcohol and ether, washed with absolute alcohol and dried. Staining of thick sections is carried out floating a 1 μ araldite section from the same block on to a slide, drying off the water, and finally immersing for a few minutes on the hot plate in an aqueous solution of toluidine blue made alkaline with borax. Material has been treated routinely in this way for monitoring the thin sections which are intended for electron microscopy but when viewed under oil immersion without a coverslip the method yields a light microscope image which makes

![Fig. 1 (plate). A typical region of a transverse section of the nerve-trunk in the meropodite of a crab walking leg, showing the wide range of axon diameters, that few of the axons are round, that small axons can be in contact only with other axons, that large axons have thick individual sheaths, that thick sheaths divide the number into bundles separated by partitions, and that occasional spaces between axons contain convoluted membranes of interlocking glial cells. b, boundary of a bundle which is defined by a sheath; c, coiled glial membrane; f, fusion of outer regions of 2 sheaths; m, mitochondrion; s, small axons; x, meeting point of mesaxon with axon surface.](image)
FIG. 1

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full use of the resolving power of the lens, unhampered by the section thickness, so that laminations of the sheath and mitochondria are readily seen.

**Results**

*The axon and its membrane*

There is a wide range of sizes of axons, from the largest of about 30 μ diameter to the smallest of about 0.5 μ (fig. 1). As pointed out by Nunnemacher and others (1962), the fine fibres far outnumber the few large sheathed ones, and the modal size is about 1 μ diameter. Contrary to expectations, the axons are rarely round in cross-section. The axon membrane of all is a smoothly continuous single membrane which always appears slightly darker and thicker than the neighbouring glial cell membrane. Mitochondria of the axoplasm are abundant in the region just within the membrane (fig. 1, m). In contrast to the findings of Geren and Schmitt (1954), we find no special relationship of the mitochondria with the axon membrane such as might suggest that they fused, one formed the other, or that the mitochondria had passed through. Mitochondria are rare in the adjacent glial cytoplasm.

Neurofibrils are sparsely scattered about the cytoplasm, never occurring in bunches or tufts and never related to the membrane or other structures as seen in longitudinal or transverse sections. A variety of small vesicles, usually smaller than mitochondria, abound in the axoplasm of some axons, but are rare in other more typical axons.

The axons show a wide variety of sheath structures, which are related in a broad sense to the axon diameter (fig. 2). The largest are surrounded by their own sheath of several different layers, each of which is complex in detail, while the smallest axons usually lack a special investing structure of any kind.

The sheath that surrounds a large typical crustacean motor axon of 20 to 30 μ diameter is substantially thicker than that described for other invertebrate axons of the same size, being generally between 4 and 5 μ thick (fig. 3, A). Under the low power of the electron microscope, this investing sheath is seen to be composed of 2 discrete structures (fig. 3, B). The outer three-quarters of the sheath consists of alternating flattened layers that form a loose concentric wrapping around the axon, called connective tissue cells by Geren and Schmitt (1954). Electron dense material, called connective tissue by Geren and Schmitt, fills the spaces between the concentric wrappings of cytoplasm. As interpreted diagrammatically in fig. 2 this connective tissue lies immediately outside the membranes of glial cells and is presumably secreted by them. The inner part of the sheath, about a quarter of the total, is made up of a layer of inner sheath or pavement cells called sheath cells by Geren and Schmitt; these closely adhere to the axon membrane and to each other, and form a mosaic over the axon membrane, as seen in transverse section, but run for long distances as seen in longitudinal section.

*The inner pavement sheath.* This is highly organized in the largest axons, progressively reduced in smaller axons, and is the sheath cell in those below 5 μ in diameter (figs. 1, 2). At any one place it is typically one or two cells
deep. The space between the axon and the membrane of the innermost flattened cell is fairly constant at 15 mμ, and is therefore larger in size than the corresponding space in squid axons (Villegas and Villegas, 1960). The interlocking of these sheath cells, where they meet on the surface of the axon, forms a pattern which is more meandering than in the squid axon, so that the channel between adjacent cells is often of considerable length, up to 20 μ (fig. 4, A to D). On the outer side of these cells is a band of electron dense material, 30 mμ across, lying between 2 cell membranes (bm in fig. 4, B). This layer is equivalent to that described as the basement membrane in lobster axons by Geren and Schmitt (1954), and is similar in fine structure to the extracellular material that occurs between the outer sheath cells, being in fact just the innermost of these layers.

The convolute 'channels' between the inner sheath or pavement cells are

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**Fig. 3 (plate).** Sheaths of the fast 'closer' and fast 'opener' motor axons of a *Carcinus* walking leg.

A, light micrograph of a 1 μ araldite section stained with toluidine blue showing the multiple but loose sheath, the close association of the 2 sheaths, the intense staining of the inner pavement sheath and the distribution of mitochondria.

B, electron micrograph of the area shown by the rectangle in A, from a neighbouring thin section stained with lead acetate. d, dividing line between the axons, marked by a thicker membrane; i, interdigitating pavement cells of the inner glial sheath; m, mitochondrion; y, meeting point of 2 glial cells on the outermost noncellular layer.
Fig. 3

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Fig. 4
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characteristic only of large axons of 10 to 30 \( \mu \) diameter and are not found for small axons. Attention has not been directed to them previously, but a perusal of figures of other authors suggests that they will be found where sheaths are thick in other arthropods, for example in insect stretch receptors (Osborne, 1963). The form of the channels suggests that they function as a strong flexible mechanical attachment between the glial cells and not that they would function optimally as a passage for ions. The meeting points of glial cells are far apart, usually about 10 \( \mu \) in transverse sections and much further apart in longitudinal sections. The constant separation of 2 membranes 12 to 15 \( \mu \) apart suggests that the intervening gap is filled with a rigid substance which holds the 2 cell membranes apart, or, more likely, cements them together. The area between the membranes is more electron dense than background cytoplasm (fig. 4, c to d). Even if these 'channels' were passages it is clear that on their outer side they run into other layers which form the thick outer sheath; also, to function optimally as channels they should be short. In fact the electron microscope observations suggest that the idea of structurally observable channels must be abandoned.

The outer concentric sheath. This forms the bulk of the structure of the sheath and is quite different in pattern from a loose myelination, because the flattened glial cellular layers have non-cellular fibrous layers between them. There are broadly spaced alternating bands of extracellular material and clear cytoplasm of outer sheath cells, each generally between 0.2 and 0.5 \( \mu \) thick. The membranes round the glial cytoplasmic components do not form parallel sided channels of constant separation where the cytoplasm is occasionally squeezed out from between them on the inside, or where there happen to be no fibres between them on the outside, suggesting that they do not become cemented or 'zipped' together. The sheath is less uniform than that found around the earthworm giant axons, where there appears to be very little extracellular material although there is no regular myelin spacing (Hama, 1959). Squid giant axons also have a superficially quite different structure again, with a single sheath cell layer surrounded by extracellular material of high electron density. In fact, the multiple outer sheath around large crab axons is more complicated than the sheath of any large fibre previously described. Under higher magnification the extracellular material is seen to consist of alternating layers of circular and longitudinal fibrils 5 to 10 \( \mu \) in diameter, lying regularly separated from each other in a background matrix.

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**Fig. 4** (plate). Details of the meeting places of glial cells upon the axon membrane, showing the consistent separation of the glial cells from each other and from the axon membrane at a distance of 12 to 15 \( \mu \), and the interdigitation of the inner glial cells.

A, an axon with 4 interdigitating inner pavement cells 1 to 4.
B, enlargement of the area marked in A.
C, D, meeting points of glial cells on the axon membrane.
E, enlargement of the area marked in D. bm, the inner fibrous extracellular layer, called 'basement membrane' by Geren and Schmitt (1954); f, alternating layers of fibres; i, inner glial sheath; o, outer glial sheath; sp, space between layers of outer sheath; v, vesicles peculiar to a few axons; x, meeting point of glial cells on the axon membrane.
There is no cross-banding to suggest that they are collagen. These fibrils presumably account for the relatively great strength and elasticity of isolated crab axons. Such a sheath of alternating layers can surround a single large axon, or form a sheath around several small axons (fig. 1).

Nuclei and mitochondria of glial cells may lie anywhere in the glial cytoplasm. They turn up on the extreme outside of the axons, or in the cytoplasm between the fibrous layers of the outer sheath, or within the cells of the inner sheath. Round vesicles of 40 to 60 μm diameter occur in both inner and outer glial cells, but especially in the former.

**Discussion**

During experiments on the nature of the repetitive response, changes have been found suggesting that ions released during electrical activity accumulate around the axon membrane. These changes in excitability are prominent if the axon is surrounded by paraffin oil but still occur to a lesser extent when the axon is continually washed with normal sea-water. The organization of the sheath of these large motor axons shows that (a) there is only a small space, if any actual fluid-filled space, between the axolemma and the membrane of the inner sheath cells, and (b) there are plenty of layers of glial cytoplasm and other intervening layers and membranes which could counteract the effects of washing. We have also to bear in mind the fact that axons of differing size have differing relations to glial cells and possible channels between them, and yet we must suppose that this makes no fundamental difference to them.

The number of potassium ions released per impulse is $2.4 \times 10^{-12}$ moles/sq cm of membrane in crab axons (Keynes, 1951). If this potassium accumulates in the ‘space’ outside the axon membrane the available measured volume for it is $1.5 \times 10^{-6}$ ccm for each sq cm of membrane. To provide figures for comparison, the amount of potassium in this volume of the normal experimental bathing medium (sea-water) would be $1.5 \times 10^{-12}$ moles. Therefore, if the potassium concentration round the axon is initially similar to that of the bathing medium one impulse would more than double it. Comparison of the changes in membrane resistance with activity, with raised external potassium, show that this calculated change in concentration is much too large. We conclude from this that the concept of the space is not a useful approximation to reality. The signs are that, if the potassium ions behave in the same way as normal unbound ions, their rapid outward movement spreads beyond the ‘space’ and into the glial cell. In fact the ‘space’ is rather larger when determined electrically, being $2.46 \times 10^{-6}$ ccm (Hodgkin and Huxley, 1947).

Surrounding the isolated axon with paraffin oil as opposed to sea-water has an effect on excitability which is interpreted as due to a progressive accumulation of potassium at each impulse. It is not easy to see how the oil can cause this if the layers of the sheath are relatively impervious. Surrounding the isolated axon by running sea-water reduces the effects of impulses on the subsequent excitability. In fact, the nature of the medium surrounding the axon influences the features which can be attributed to the accumulation
of potassium ions round the axon deep within the sheath. We have ruled out channels to the exterior as being of especial importance and in addition have given a quantitative reason why the potassium released at each impulse must apparently spread into the inner glial cells. The action of the distant external medium can only be explained by supposing that an effect is passed on from the inner glial cells, progressively through the loose layers, to the outside. This effect is presumably related to the Donnan equilibrium set up by the basophil sheath structures, as described by Treherne (1962) for insect nerves.

The correlation of structure and ionic movements is further complicated by the reabsorption of the released potassium. The energy for transport of any ions presumably comes from mitochondria which in the axoplasm outnumber mitochondria in the glial cytoplasm by a factor of at least 10 to 1, allowing for the differing cross-sectional areas involved. In mammalian brain tissue the oxygen expenditure per unit weight of nerve-cells is about 10 times that of glial tissue (Korey and Orchen, 1959). The axoplasmic mitochondria are almost always close to the inside of the axon membrane. Assuming that the presence of mitochondria indicates a metabolically active region, it is evident that reactions supplying energy are situated on the inside of the axon membrane. This suggests that the membrane of the axon, not the membrane of the glial cell, is the principal partner of the pair of membranes in the processes of transport of ions back into the axon involving energy expenditure during recovery. The rate of reabsorption of liberated potassium in crab axons, surrounded by paraffin oil, has been estimated as $3 \times 10^{-10}$ moles/sec/cm² (Hodgkin and Huxley, 1947) and therefore, all the ions liberated by one impulse should be reabsorbed in 5 msec. The physiological effects last longer than this, and therefore if they are due to potassium, they suggest that estimates of the number of ions liberated are low, probably because during the measurements they were being reabsorbed so rapidly. In fact, consideration of the structural and physiological evidence together suggests that much remains to be worked out in relation to the amounts of potassium released and where it goes before it is reabsorbed.

References