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AXONAL ADAPTATIONS TO OSMOTIC AND IONIC STRESS IN AN INVERTEBRATE OSMOCONFORMER (*MERCIERELLA ENIGMATICA* FAUVEL)*

I. ULTRASTRUCTURAL AND ELECTROPHYSIOLOGICAL OBSERVATIONS ON AXONAL ACCESSIBILITY

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SUMMARY

The giant axons in *Mercierella* are overlaid by narrow glial processes which provide an incomplete covering of the axonal surface. Where more complete covering occurs the intercellular clefts are not sealed by tight junctional complexes. Ionic lanthanum penetrates to the surfaces of axons from sea-water-adapted animals (in normal saline and during initial exposure to hyposmotic saline) and, also, to the surface of hyposmotically adapted axons. A relatively free intercellular access to the axon surfaces is also indicated by the rapid electrical responses of sea-water-adapted axons to hyposmotic dilution and of hyposmotically adapted axons to sodium-deficient saline. The giant axon possesses an unusual ultrastructural specialization: hemidesmosome-like structures (associated with the axon membrane) which are connected to a network of neurofilaments within the axon. Theoretical considerations suggest that these structures could enable the axons to withstand appreciable excesses in intracellular hydrostatic pressure resulting from osmotic imbalance during hyposmotic stress.

INTRODUCTION

Our knowledge of the ionic basis of neuronal function has been almost exclusively derived from studies on the nerve cells of animals in which the body fluids are of relatively stable composition: notably the large axons and nerve cell bodies of certain marine annelids, molluscs and crustaceans and, to a lesser extent, the relatively small nerve cells of vertebrate animals. In these organisms the ionic homeostasis which is essential for normal nervous activity is provided by environmental stability (in the case of marine osmoconformers) or by effective ionic regulation of the body fluids (cf. Abbott & Treherne, 1977).

In contrast, the body fluids of some invertebrate animals are inconstant media. This is the case with estuarine osmoconformers, in which massive dilution of the

* The Editor reports, with deep regret, the apparent passing of *Mercierella enigmatica* which it is now proposed becomes *Ficopotamus enigmaticus* (Fauvel) (ten Hove & Weerdenburg, 1978, *Biol. Bull.* **154**, 96–120).

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body fluids can occur (cf. Potts & Parry, 1964) and terrestrial insects, in which the blood can undergo extremely large dietary, seasonal and daily fluctuations in ionic composition (Pichon & Boistel, 1963; Treherne, Buchan & Bennett, 1975; Lettau *et al.* 1977). In the latter case the nerve cells are, as in vertebrate animals, protected by a well-developed blood–brain barrier system (cf. Treherne & Pichon, 1972) and additional homeostatic mechanisms that maintain ionic microenvironments which can be markedly different from that of the blood (Treherne, 1974, 1976). Extreme estuarine osmoconformers, on the other hand, are representatives of invertebrate groups in which there is little evidence of homeostatic control of the composition of the neuronal microenvironment (cf. Treherne & Moreton, 1970; Abbott & Treherne, 1977).

The adverse effects of ionic and osmotic dilution on neuronal function can be judged from the irreversible damage caused to the spike-generating system in the axons of a stenohaline marine crustacean (*Maia squinado*) by relatively modest hyposmotic dilution (< 70%) of the bathing medium (Pichon & Treherne, 1976). In contrast, the nervous systems of estuarine osmoconformers can withstand varying degrees of change in the osmotic and ionic composition of the body fluids. The giant axon of the modest euryhaline osmoconformer, *Sabella penicillus*, has been shown to adapt to osmotic stress equivalent to 50% dilution of the body fluids (Carlson, Pichon & Treherne, 1978; Treherne & Pichon, 1978). However, the most extreme osmoconformer known is the polychaete annelid *Mercierella enigmatica* Fauvel, which occurs in natural waters of salinities between 1 and 55‰ (Seurat, 1927; Heldt, 1944; Tebble, 1953) and can withstand variations in the osmotic concentration of the blood of between 84 and 2304 m-Osmol (Skaer, 1974*a*; Benson & Treherne, 1978*a*). Previous studies have shown that axonal excitability is achieved by essentially 'conventional' ionic mechanisms: the resting membrane approximating to an ideal potassium electrode and the inward current of the action potential being largely carried by sodium ions (Carlson & Treherne, 1977). The axons of *Mercierella* can, nevertheless, adapt to tenfold dilution of the bathing medium (Treherne, Carlson & Skaer, 1977). This was shown in electrophysiological experiments on the axons of animals from a colony which was transferred from sea water and maintained in flowing distilled water. It was suggested that the ability of the axons to withstand rapid osmotic and ionic dilution of the bathing medium resulted from reduced accessibility of ions to the axon surfaces during the initial stages of hyposmotic adaptation, although no structural basis could be recognized for such a mechanism (Treherne, Carlson & Skaer, 1977). The present investigation was initiated to investigate the ultrastructural organization of the *Mercierella* axons and their associated structural elements and, in particular, to examine axonal accessibility using exogenous tracer substances and electrophysiological recording techniques. A preliminary account of some aspects of this research has been published elsewhere (Treherne, Benson & Skaer, 1977).

METHODS AND MATERIALS

Colonies of *Mercierella enigmatica* were collected from Weymouth Harbour, England, and kept in aerated sea water. Others were placed in distilled water, for at least 4 days, for experiments on hyposmotic adaptation.

Electron microscopy

(*a*) *Conventional fixation and embedding*

Ventral nerve cords of animals from the marine aquarium were fixed by the method described by Baskin (1971*a*). After postfixation specimens were stained *en bloc* for 1 h at R.T. with 2% uranyl acetate dissolved in sodium hydrogen maleate buffer, pH 6·0. Following dehydration, tissue was embedded in Araldite, sectioned using an LKB Ultrotome III, further stained with lead citrate and uranyl acetate and examined in a Philips EM 300.

Ventral nerve cords from animals adapted to dilute salinities were fixed in the normal physiological saline (Skaer, 1974*b*; Carlson & Treherne, 1977) diluted to one-tenth strength (1/10 Skaer) with 2·5% glutaraldehyde added and, in some instances, with one drop of 1% $CaCl_2$ added per 20 ml of solution. Specimens were then washed in 1/10 Skaer, followed by post-osmication and *en bloc* staining with uranyl acetate, using the same saline as the basis of the solutions. Subsequent treatment was as described above.

(*b*) *Lanthanum incubation*

Animals were opened along the dorsal midline, care being taken to leave the gut intact. The dissected animals were then flooded with a physiological saline containing 10 or 50 mM lanthanum chloride. This allows the uptake of lanthanum in near-physiological conditions. After periods from 30 min to 2 h the lanthanum solutions were pipetted out and replaced with a fixative solution containing phosphate ions to precipitate the lanthanum. The nervous system with surrounding muscle was then dissected out and placed in fresh fixative solution, after which it was treated in one of the ways outlined above.

Lanthanum incubation followed one of two pretreatments. (*a*) Animals were taken directly from normal aerated sea water in which case fixation was by the method described by Baskin (1971*a*). (*b*) Animals were taken from normal sea water, opened and then flooded with dilute saline (1/10 Skaer) for 5 min before incubation in lanthanum solutions made up with 1/10 Skaer saline. Fixation followed in this saline containing 2·5% glutaraldehyde and with 5 ml of 0·2 M phosphate buffer, pH 7·2, and one drop of 1% $CaCl_2$ added per 20 ml of solution.

Control preparations were incubated in solutions containing no lanthanum.

(*c*) *Freeze fracture*

A longitudinal section of the body wall containing the ventral nerve cords was fixed for 30 min either by Baskin's (1971*a*) method or in a dilute saline with added glutaraldehyde and calcium (as above). After several buffer washes the tissue was incubated for a further 30 min in 25% glycerol in a suitable buffer wash. The tissues were then mounted on copper holders in a yeast paste, quenched in melting Freon 22 and stored in liquid nitrogen. Freeze fracture was carried out in a Balzers BA 360 M device at 173 K, shadowing with a platinum–carbon mixture and backing with carbon taking place immediately after cleaving. Replicas were cleaned in solutions of sodium hypochlorite up to 50% in strength and were subsequently mounted on copper grids and examined in a Philips EM 300. The direction of

shadowing is indicated on the micrographs by an arrow in the lower right-hand corner.

Freeze fracture was carried out on animals from normal sea water and also after the tissues had been subjected to a brief (5 min) exposure to dilute saline (1/10 Skaer).

Electrophysiological techniques

The paired giant axons were exposed for intracellular recording by cutting the worms along the mid-dorsal line, pinning them flat (with fine entomological pins) under slight tension on to a layer of Sylgard and carefully removing the gut. The walls of the experimental chamber were formed by placing a greased Perspex ring (cut from 2·5 cm diameter tubing of 2·0 mm wall thickness) on to the Sylgard surface. The solutions in the experimental chamber were changed by vigorously syringing (at approximately 0·5 ml s^{-1}) on to the preparation a volume of at least 40 times (20 ml) the fluid capacity of the experimental chamber (0·5 ml). Excess fluid was removed by suction through a 19-gauge hypodermic needle connected to a suction pump. The preparation was illuminated from below using a fibre-glass light guide and viewed, from above, with a binocular microscope.

The axons were stimulated with insulated silver wire electrodes using rectangular pulses of 0·2 ms duration via an isolating unit from a Farnell stimulator. The glass microelectrodes were filled with 3·0 M-KCl and had resistances of between 20 and 40 MΩ. The intracellular recordings were made through a high impedance, negative capacitance, preamplifier (W.P. Instruments, Inc.) to a Tektronix oscilloscope. Continuous potentiometric recordings of both action and d.c. potentials were made on two Tekman chart recorders using a transient recorder and signal processer.

Experimental solutions

The normal physiological saline was as previously used (Carlson & Treherne, 1977). The composition of this saline is based on that of Skaer (1974*b*) and approximates in ionic composition to the blood of sea-water-adapted individuals (Skaer, 1974*a*): Na^+, 482·3; K^+, 30; Mg^{2+}, 77; Ca^{2+}, 31; SO_4^{2-}, 26; Cl^-, 663·8; OH^-, 12·5; Pipes 7·5 mM (pH 6·9; osmotic concentration, 1024 m-Osmol). Sodium-deficient saline was made by substitution with tris ions. Hyposmotic solutions were made by dilution with glass-distilled water.

RESULTS

Ultrastructural observations

The paired ventral nerve cords of *Mercierella* lie on either side of the ventral midline and mark the central boundary of the ventro-lateral blocks of longitudinal muscle. Each cord consists of a bundle of small axons (Figs. 1, 2) some of which contain dense core neurosecretory granules (Fig. 2). There is a single giant axon (Figs. 3, 4) associated with each cord but displaced laterally and separated from the smaller axons by a layer of connective tissue which also ensheaths the whole cord (Fig. 1). The glial investment of the small axons is sparse; glial cells are found more commonly at the surface of the cord but by no means give a complete peripheral covering and many axons are innocent of any glial covering at all (Fig. 1). Glial cells do ensheath the giant axon (Figs. 3, 4) and, although in some areas the successive

glial layers may be separated by connective tissue (Fig. 4), in other areas the glial folds are joined by macular gap junctions distinguishable both in thin sections (Fig. 3, inset) and after freeze-fracture (Fig. 4, inset). The gap junctions are characterized in thin section by the small (3–4 nm) intercellular space which may occasionally show a fine cross-banding suggestive of bridging structures between the two membranes. Freeze-fracture reveals plaques of particles (10–11 nm in diameter with a 14–15 nm centre-to-centre spacing) on the EF with corresponding pits on the PF (cf. Flower, 1977, who found similar fracturing characteristics but a tighter packing in unfixed, glycerinated tissue from the gut of the polychaete, *Perinereis novaehollandiae*). These arrays of particles and pits on *Mercierella* glial cell membranes are distinctive in their highly regular, almost paracrystalline, hexagonal packing. Desmosomes between glial cells such as have been found in nereids (Baskin, 1971*a*, *b*) and earthworms (Günther, 1976; Hama, 1959) have not been seen.

Although the giant axon is covered over most of its surface by one or more layers of glial folds, breaks in the investment are fairly frequently encountered (Fig. 5) and sometimes sizeable expanses of axonal membrane abut on to the ensheathing connective tissue. These naked areas of axonal membrane are typified by an increased density of neurofilaments inserted into the cell membrane, a characteristic feature of the giant axon, illustrated in Fig. 5 and its inset. The network of neurofilaments found in the axoplasm inserts into electron-dense plaques associated with the intracellular leaflet of the membrane. The extracellular space immediately overlying these areas is marked by an electron-dense band running parallel to the axonal membrane. These structures are reminiscent of hemi-desmosomes (Kelly, 1966; Campbell & Campbell, 1971). The average spacing between adjacent 'hemidesmosomes' is approximately 0·25 μm. Hemidesmosome-like structures have also been found in the nerve cell bodies and axons of the leech (Gray & Guillery, 1963; Coggeshall & Fawcett, 1964) but their distribution is irregular and infrequent contrasting with the situation in *Mercierella* giant axons. Insertion of filaments has been observed in the neuralemma of Dieters' neurones in rabbit brain where the filamentous network is thought to link the plasmalemma with the nuclear envelope and serve a transducing function (Metuzals & Mushynski, 1974; Lee & Metuzals, 1974). 'Insertion plaques' somewhat similar in appearance to those in *Mercierella* have been described in the gut of termites (Noirot-Timothée & Noirot, 1966). In these cells microtubules rather than microfilaments insert in the apical and basal plasma membranes of the gut epithelial cells and are thought to behave as strengthening structures.

The glial cells surrounding the giant axons sometimes contain numerous densely staining bodies decorated at their surface by granules resembling glycogen in appearance (Figs. 3, 6). Although dense granules and deposits of glycogen have been reported in the supportive glia of the central nervous system of *Nereis* species (Baskin, 1971*b*) this spatial association of the two structures was not found. Whether the prominent dense granules in some of the glial cells of *Mercierella* are gliosomes, as have been found in other nervous systems (for example, gastropods, Nolte, Breucker & Kuhlmann, 1965; arthropods, Pipa, Nishioka & Bern, 1962; polychaetes, Baskin, 1971*b*), serve a storage function (see, for example, Nicaise & Bilbaut, 1975; Treherne *et al.* 1969, and reviewed in Nicaise, 1973), or have some other role, awaits further elucidation. Some glial cells are rich in rough endoplasmic reticulum and

free ribosomes. In some instances the ER may become aligned with the attached ribosomes in register thus producing crystalline-like structures (Fig. 7).

Experiments involving *in vivo* incubation with lanthanum solutions under near physiological conditions indicate that in animals taken from normal sea water the ensheathing structures of the nervous system offer no resistance to the entry of lanthanum. Lanthanum penetrates both to the surfaces of axons in the bundle and to the giant axon (Figs. 8, 9). Lanthanum deposits can be seen outlining the intercellular spaces between glial cells ensheathing the giant axon (Fig. 8) and penetrating to the axonal surface (Figs. 8, 9). Even after a brief exposure to dilute saline, access to the axonal surfaces remains; lanthanum deposits are found surrounding the axons (Fig. 10).

Effects of hyposmotic dilution on axonal function

The preceding ultrastructural evidence indicates that the surfaces of sea-water-adapted axons appear to be accessible in normal saline and following abrupt hyposmotic dilution of the bathing medium. These observations are in apparent conflict with earlier electrophysiological observations which showed relatively slow axonal responses to the massive hyposmotic dilution of the bathing media (Treherne, Carlson & Skaer, 1977). This conflict between the present ultrastructural evidence and previous electrophysiological results prompted us to reinvestigate the electrical responses to hyposmotic dilution.

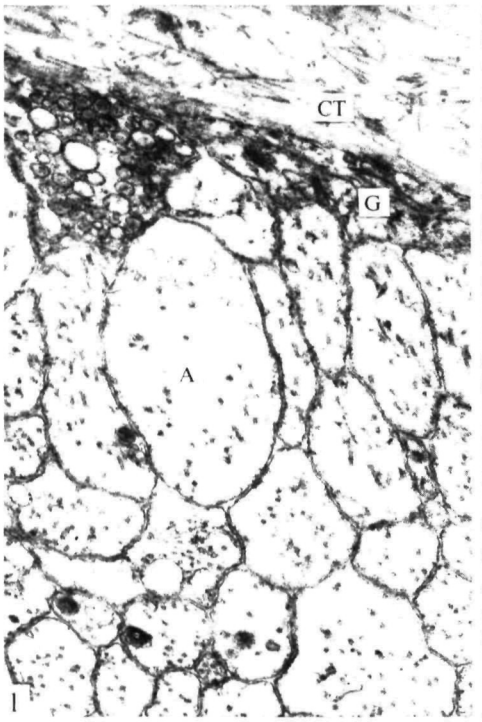
In the previous investigation a rectangular chamber with a baffle plate was used to create continuous and turbulent flow of the bathing solution. This system ensured a rapid exchange of dye solutions placed in the experimental chamber when the replacement solution was of approximately the same density as the dye solution. In the present investigation we replaced the rectangular chamber with a circular one and hyposmotic dilution was carried out by vigorously syringing the solutions directly on to the preparation. With this procedure, dilution of the bathing medium was found to produce relatively rapid axonal responses as reported here and in

Fig. 1. Axonal field from a ventral nerve cord of *Mercierella enigmatica* fixed in dilute saline with added glutaraldehyde and calcium. The animal had been taken from sea water and exposed briefly to dilute saline before fixation. Note the absence of glial cells around the axons and also the incomplete glial layer at the periphery. A, Axon; G, glial cell; CT connective tissue. ×26000.

Fig. 2. Freeze-fracture preparation of an area similar to that shown in Fig. 1. The treatment of the tissue was also similar but fixation was carried out for only a short period before the tissue was glycerinated and frozen. Neurosecretory axons (NS) can be identified, the globular vesicles corresponding to the familiar dense core vesicles seen after conventional treatment (inset). A, Axon; NS, neurosecretory axon. ×30000; Inset, ×56000.

Fig. 3. Giant axon and a layer of ensheathing glial cells from an animal in sea water and fixed by Baskin's (1971*a*) method. The axonal cytoplasm (GA) contains a dense mat of neurofilaments and the glial cells (G) may contain large numbers of electron-dense granules as illustrated here. CT, Connective tissue. ×17000. Inset: gap junction associating neighbouring glial folds. ×91000.

Fig. 4. Freeze-fracture preparation showing the giant axon (GA) and two glial folds (G) separated from each other by a layer of connective tissue (CT). This animal had been hyposmotically adapted in flowing distilled water for more than 4 days and was fixed in glutaraldehyde made up in dilute saline enriched with calcium before being glycerinated and frozen. ×37000. Inset: gap junction associating two glial cells. The particles on the EF are overlaid by an area of adhering PF displaying the lattice of pits. ×49000.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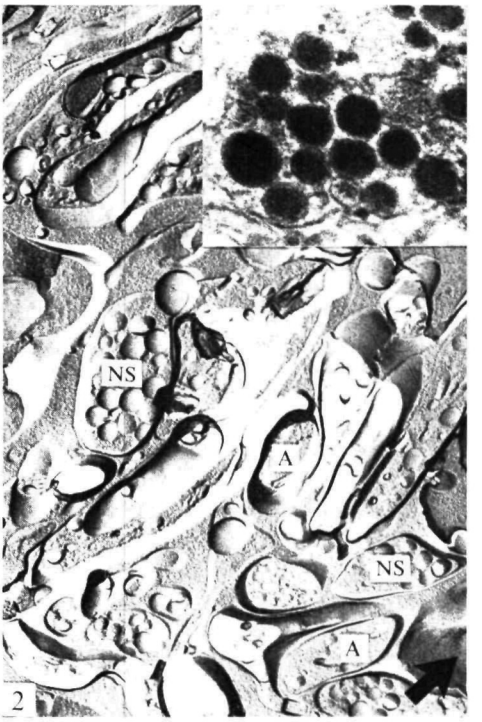
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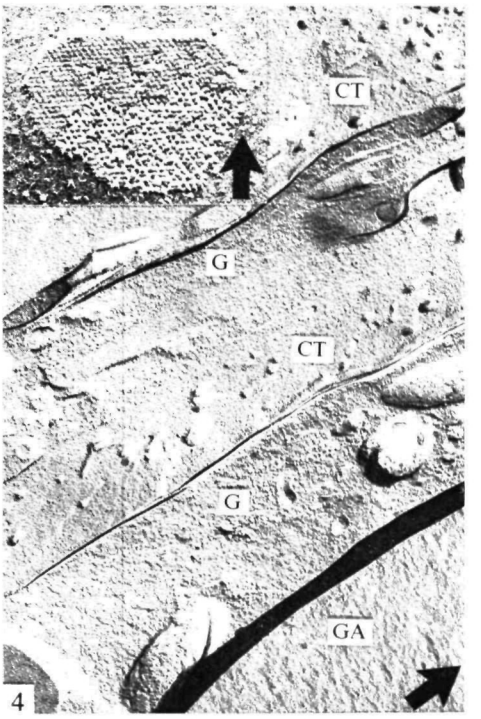
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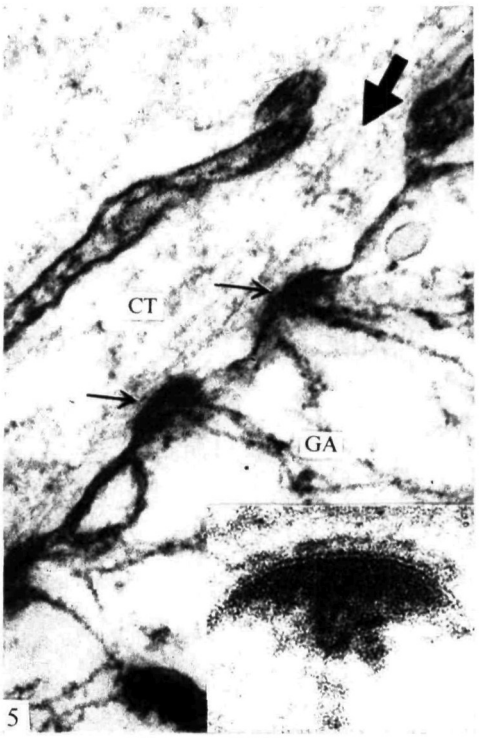
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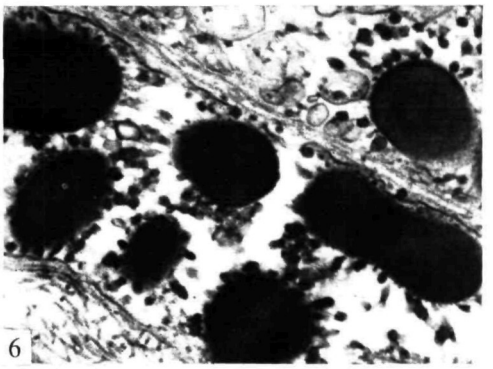
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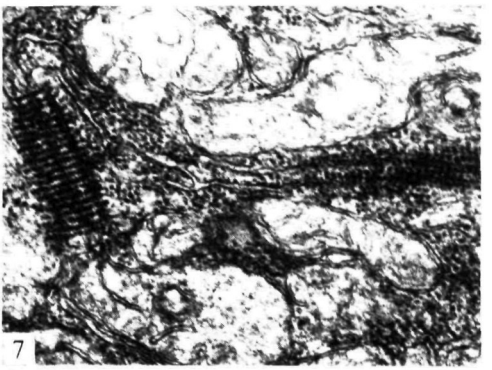
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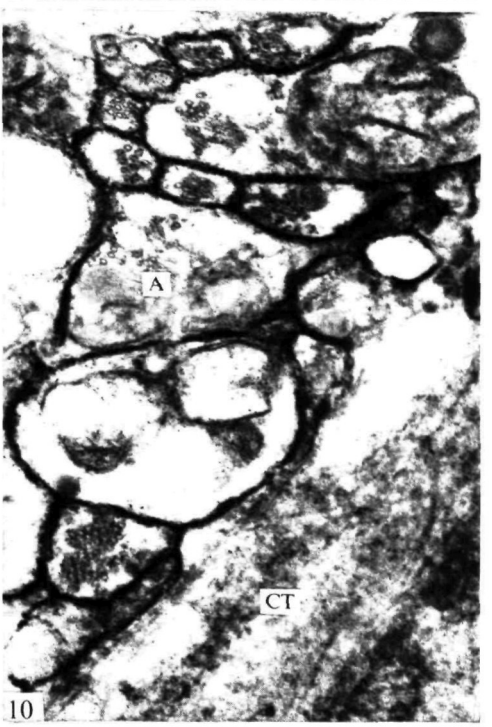
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subsequent papers (Benson & Treherne, 1978*a*; *b*). Fifty per cent dilution of the bathing medium, for example, produced an appreciable decline in the amplitude of the intracellularly recorded action potentials (Fig. 11). This was followed by a partial recovery in amplitude resulting from an increase in the extent of the overshoot of the action potentials. Exposure to 40% hyposmotically diluted saline caused a rapid decline in overshoot followed by conduction block and, later, rapid depolarization of the axonal membrane (Fig. 11). This depolarization appears to result from permanent axonal damage for it proved impossible to record a significant resting potential on subsequent attempts to re-impale the axon.

The results clearly indicate that the axons of sea-water-adapted individuals show rapid electrical responses to abrupt hyposmotic dilution when the solutions are vigorously syringed directly on to the preparation. This suggests that with continuously flowing saline, as used before, the lack of effect of rapid hyposmotic dilution resulted from fluid stratification in the rectangular chamber which produced an unstirred layer of relatively high density and salinity covering the axon surface. Such an effect would not be expected, and was not seen, when external ion concentrations were altered at constant osmotic concentration (Carlson & Treherne, 1977) since fluid stratification did not occur during mixing of solutions of similar density.

Axonal accessibility was also tested in individuals which had been adapted to reduced salinity by placing the colony in flowing distilled water for at least one week. As previously reported (Treherne, Carlson & Skaer, 1977), the axons from such hyposmotically adapted individuals can conduct overshooting action potentials of relatively large amplitude in 10%, hyposmotic, saline (Fig. 12). As can be seen from Fig. 12, exposure to sodium-deficient saline caused a rapid decline in the amplitude of the intracellularly recorded action potentials, which recovered on return to normal 10% saline. Subsequent exposure to 1% hyposmotic saline appeared to cause irreversible axonal damage (Fig. 12). These results suggest that the surfaces of hyposmotically adapted axons are also accessible to water-soluble cations in the bathing medium.

Fig. 5. Giant axon (GA) from the ventral nerve cord of an animal hyposmotically adapted in flowing distilled water. The preparation was fixed in glutaraldehyde made up in dilute saline and shows the incomplete nature of the glial covering (large arrow). The neurofilaments can be seen inserting into specialized membrane areas (small arrows). GA, Giant axon; CT, connective tissue. × 58000. Inset: specialized membrane area at higher magnification showing the intracellular electron-dense mat and the extracellular bar of density lying parallel to the unit membrane. × 126000.

Figs. 6, 7. Glial cell inclusions. Fig. 6 shows large electron-dense granules peripherally associated with glycogen-like granules. × 28000. Fig. 7 illustrates the crystalline-like arrangements of ribosomes attached to the endoplasmic reticulum. × 42000.

Figs. 8, 9. Ventral nerve cord from an animal incubated for 30 min in normal sea water containing 10 mM lanthanum and subsequently fixed by Baskin's (1971*a*) method. Lanthanum deposits can be seen in the intercellular spaces of the glial sheath (Fig. 8) extending down to the axonal membrane (enlarged area in Fig. 9). GA, Giant axon. Fig. 8, × 9000; Fig. 9, × 23000.

Fig. 10. Small axons from the nerve cord of an animal taken from normal sea water and exposed briefly (5 min) to dilute saline before incubation for 2 h in dilute saline with 50 mM lanthanum added. Fixation followed in 2·5% glutaraldehyde made up in phosphate-enriched dilute saline to which calcium was added. Lanthanum precipitates can be seen in the intercellular spaces between the axons. A, Axon; CT, connective tissue. × 44000.

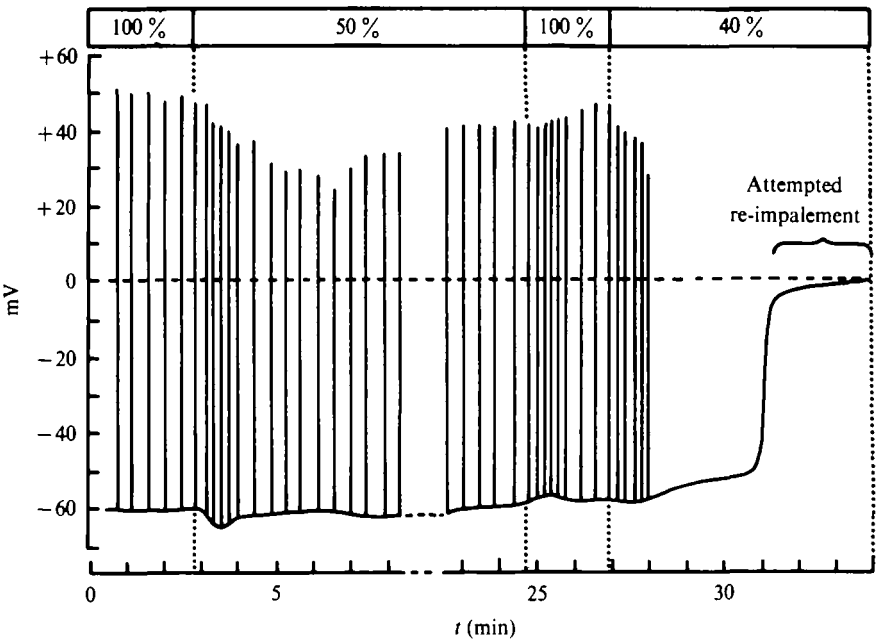

Fig. 11. Effects of successive exposures to 50% and 40% hyposmotic saline on the intracellularly recorded action and resting potentials in an axon from a sea-water-adapted individual. (Chart recording using a transient signal processor.)

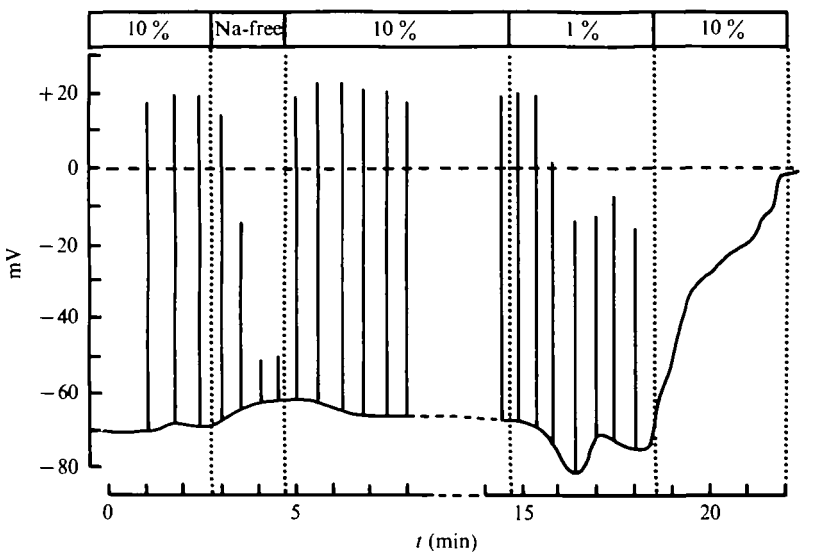

Fig. 12. Resting and action potentials recorded from the axon of an individual taken from a colony which had been maintained in flowing distilled water. The axons were impaled in 10%, hyposmotic saline before exposure to equivalent sodium-deficient saline. The effects of subsequent exposure to 1%, hyposmotic saline are shown.

DISCUSSION

Despite the external fluctuations in blood composition that can be experienced by the nervous system of *Mercierella* the ultrastructural evidence presented above indicates that the axon surfaces are unlikely to receive short-term protection by a drastic reduction in the intercellular access of water-soluble ions during the initial stages of osmotic stress, as had been supposed previously (Treherne, Carlson & Skaer, 1977). The giant axon is overlaid by narrow glial processes which provide an incomplete covering of the axonal surface. Where more complete covering occurs the intercellular clefts between adjacent glial processes are not sealed by tight junctional complexes. Furthermore, ionic lanthanum has been shown to penetrate from the bathing medium to the axon surfaces both in isosmotic conditions and during exposure to hyposmotic saline.

A relatively free intercellular access to the axon surfaces is also indicated by the rapid electrical responses of sea-water-adapted axons to hyposmotic dilution: a conclusion which contrasts with that of an earlier investigation in which a restricted access was postulated during the initial stages of hyposmotic adaptation (Treherne, Carlson & Skaer, 1977). The present evidence suggests that the lack of effect of hyposmotic dilution on the action and resting potentials in the previous study resulted from stratification of continuously flowing saline of different densities. On the basis of the data reported here and in a preliminary report elsewhere (Treherne, Benson & Skaer, 1977) it now appears that the axons of *Mercierella* are directly exposed to the rigours of osmotic stress resulting from the massive fluctuations in blood concentration experienced by this estuarine osmoconformer.

Although the electrophysiological evidence (Benson & Treherne, 1978*b*) suggests that adaptation to hyposmotic media involves considerable loss of internal ions from the giant axon, the maximum loss recorded, on adaptation to a 25% medium, was equivalent to a drop in internal osmotic pressure of only 416 m-Osmol, as compared with the drop of 768 m-Osmol required for osmotic equilibrium. Since the volume of the axon does not change appreciably, it is clear that either a further reduction in internal osmotic pressure must occur, as for example by a reduction in free amino acid content (cf. Schoffeniels, 1976), or the internal hydrostatic pressure must rise to balance the osmotic gradient. Some increase in internal pressure would in any case be expected during the initial stages of hyposmotic adaptation, since it is difficult to see how the internal osmolarity could be adjusted fast enough. The electrophysiological evidence (Benson & Treherne, 1978*b*) indicates that, for the internal cations at least, adjustment lags behind the change in external osmotic pressure.

It is, therefore, relevant to consider how the *Mercierella* axon may be structurally adapted to withstand increases in the internal pressure during hyposmotic stress. Spyropoulos (1977) has investigated the osmotic relations of the squid giant axon, and concludes that the principal resistance to swelling in diluted external media lies in the axolemma and Schwann cell sheath. The axoplasmic gel actually makes a positive contribution to the internal osmotic pressure, and swells if extruded into the 'normal' bathing medium, so that an intact axon in such a medium will be

under slight pressure internally. There was little evidence of any resistance to swelling, resulting from internal restraints.

The *Mercierella* axon, however, does not possess the continuous, multilayered glial investiture which is provided by the Schwann cells in the squid. Its glial system consists only of small, isolated processes, with areas of bare axolemma between. Clearly, therefore, resistance to osmotic swelling imparted by the 'sheath' will be restricted to the modest tension which can be sustained by a single cell membrane. For convenience, we consider the effect of a notional excess internal osmotic concentration of 100 m-Osmol, corresponding to an equilibrium hydrostatic pressure (P) of $2{\cdot}42 \times 10^5$ Pa ($2{\cdot}42 \times 10^6$ dyn cm^{-2}) as calculated from the ideal gas equation (cf. Dick, 1966). Representing the *Mercierella* axon as an infinitely long cylinder of radius $R = 15\ \mu$m, the membrane tension (T) is given by Laplace's law as

$$T = PR = 3{\cdot}63 \text{ N m}^{-1} (3{\cdot}63 \times 10^3 \text{ dyn cm}^{-1}). \quad (1)$$

While this value is some 300 times smaller than the equivalent figure for the much larger squid axon, it is nevertheless about two orders of magnitude greater than the estimated values generally given for cellular membrane tension (cf. MacKnight & Leaf, 1977). For example, the membrane of the human erythrocyte can withstand only 20 dyn cm^{-1} (0·02 N m^{-1}) before haemolysis occurs (Rand, 1964).

It is clear, therefore, that in the absence of additional internal structural organization the membrane of the *Mercierella* axon cannot withstand the hydrostatic pressure resulting from any appreciable excess in intracellular osmotic concentration. Electron microscopy has shown, however, that the *Mercierella* axon is unusual in possessing numerous 'hemidesmosomes' which link the axonal membrane to a system of intracellular filaments. It is difficult to estimate reliably the overall spatial frequency of these structures, as their identification depends on a favourable plane of section of the membrane area in question. In many areas, however, they were found to occur quite regularly, with an average spacing of about 0·25 μm (cf. Fig. 5). It seems likely that this unusual ultrastructural specialization may be connected with resistance to osmotic swelling, as a firm internal attachment of the membrane at regular intervals will, by reducing the radius of curvature of the unrestrained portions, also reduce the membrane tension as given by equation (1).

Fig. 13 allows a quantitative assessment of the effectiveness of such a system. Here it has been assumed that the membrane consists of a mosaic of circular patches, each of radius a, and restrained around its edge by attachment to the internal filaments. Internal pressure causes each patch to be distended into a 'blister', forming part of a sphere of radius r, and subtending a semi-vertical angle θ at its centre (Fig. 13*a*). (Since the radius of the patch is much less than that of the axon, the cylindrical curvature of the axon surface may be ignored for all except very small values of θ). The value of θ may vary from zero (flat membrane) to $\frac{1}{2}\pi$ (hemisphere). r, θ and a are related through

$$a = r \sin \theta. \quad (2)$$

If the volume of the blister is v, and the area of its surface is A, then pressure and membrane tension are related by

$$T\frac{dA}{d\theta} = P\frac{dv}{d\theta}, \quad (3)$$

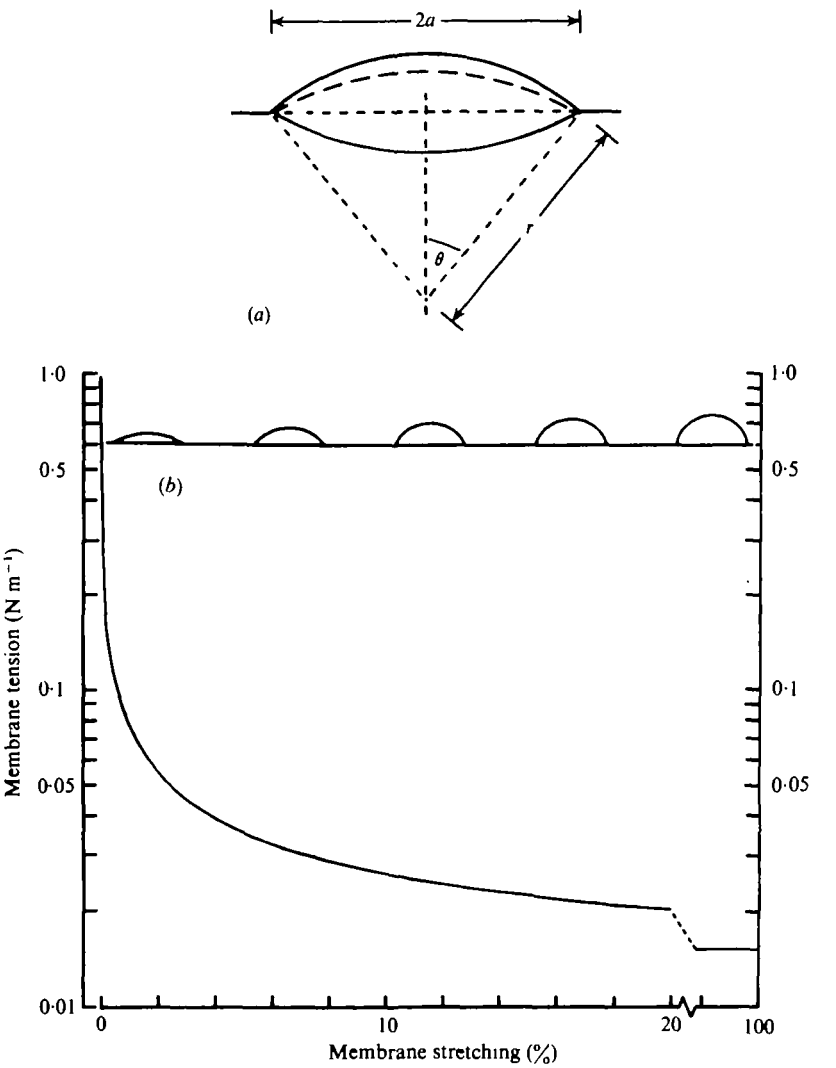

Fig. 13. (*a*) Diagram of a localized membrane swelling, or 'blister'. A circular area of membrane, of radius *a*, originally flat, is distended to form part of a sphere, or radius *r*, subtending a semi-angle θ at its centre of curvature. (*b*) Membrane tension plotted against membrane stretching, expressed as the percentage increase in area, as given by equations (4) and (6), for an internal pressure of $2{\cdot}42 \times 10^5$ Pa. Above the curve are sketched profiles of the 'blister' for various degrees of stretching.

which leads by a geometrical calculation to

$$T = \frac{\mathrm{Pa}}{2 \sin \theta} = \tfrac{1}{2} P r, \tag{4}$$

showing that the small 'blister' of membrane behaves exactly like a spherical bubble. As θ approaches $\frac{1}{2}\pi$, so that the blister approaches a hemisphere, T falls to a limiting value of $\frac{1}{2}$ Pa. If θ becomes very small (i.e. the blister is almost flat), T increases without limit – but of course the original approximation that the radius of curvature,

r, is much less than that of the axon will then break down, and in practice T must approach a limit given by the cylindrical curvature of the undisturbed axon (equation 1).

Clearly, a decreased membrane tension is not achieved without some cost in stretching of the membrane. The area of the blister is in fact given by

$$A = \frac{2\pi a^2}{1+\cos\theta}, \tag{5}$$

whence the percentage increase above the original area (πa^2) is

$$\Delta A = 100\frac{1-\cos\theta}{1+\cos\theta}. \tag{6}$$

By eliminating θ between (4) and (6), it is possible to plot membrane tension against extension for a given pressure. Fig. 13(*b*) shows a semi-logarithmic plot, starting somewhat below the limiting tension given by (1), and covering increasing values of θ up to $\frac{1}{2}\pi$, which represents a 100% increase in membrane area. The value chosen for P was $2{\cdot}42\times10^5$ Pa, as calculated above for a notional excess internal osmotic concentration of 100 m-Osmol, and the radius of the blister (a) was taken as 0·125 μm, being half of the smallest regularly observed spacing of the 'hemidesmosomes'.

It will be seen that a small membrane extension can allow a very large decrease in the tension, an increase in area of only 10% being sufficient to reduce the tension to about 0·03 N m^{-1}, less than one hundredth of the value for the unstretched axon. Further extensions up to 100% produce only a marginal fall in tension, the limiting value being close to 0·015 N m^{-1}. Clearly, the provision of regular supports for the cell membrane is a highly effective way of reducing the tension during osmotic stress, so that quite small amounts of stretching can reduce the tension to values close to those which can be withstood by other cell membranes (see above).

The pronounced flattening of the curve in Fig. 13(*b*) for extensions greater than about 20% implies conversely that, for pressures exceeding a certain value, the membrane will stretch very rapidly for only a small rise in pressure. Such local stretching could thus be responsible for the transient, drastic reduction in the size of the action potentials recorded at critical stages of hyposmotic adaptation, despite the absence of any visible overall swelling of the axon (Benson & Treherne, 1978*b*). As the internal pressure returns to below the critical value, the membrane stretching will rapidly subside, which could lead to the observed rapid recovery of the action potential.

It appears then that the axons in this extreme osmoconformer are accessible to water-soluble ions and molecules both during the initial stages of hyposmotic stress, in sea-water adapted and hyposmotically adapted animals. This implies that the *Mercierella* axon is directly exposed to and is able to withstand and adapt to massive dilution of the fluid immediately bathing the axonal membrane. This ability is associated with an unusual structural specialization which, it is proposed, enables the axon to withstand appreciable excesses in intracellular hydrostatic pressure during hyposmotic stress.

The responses and adaptations to osmotic stress of the ionic mechanisms associated

with excitation and conduction in the *Mercierella* axon are described in the two succeeding papers (Benson & Treherne, 1978*a*, *b*).

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