SUMMARY

1. *Mytilus edulis* could be adapted to salinities between 25% and 125% under laboratory conditions, with complete ionic and osmotic conformity of the body fluids.

2. Extracellular space, intracellular cation concentrations and water content were determined for the cerebro-visceral connectives of *Mytilus* adapted to either 100% or 25% salinity. These measurements suggested only a moderate degree of volume regulation (as indicated by relative cell hydration) and net losses of both potassium and sodium from the cells during acclimation to dilute sea water, although neither cation was reduced in proportion to the external concentrations.

3. Measurement of actual volume changes during acute hyposmotic stress indicated a greater capacity for volume control in the 25%-adapted connectives. However, these tissues also showed an increment of 11% in initial diameter, suggesting significant chronic swelling of the cells.

4. Fine-structural studies of *Mytilus* nerve indicated that the apparent swelling of the dilute-adapted tissues resulted from a roughly threefold thickening of the ensheathing neural lamella due to the deposition of extra collagen-like fibrils, with the axons in fact showing negligible volume increase. The connectives thus appear to exhibit almost perfect volume regulation.

5. To account for the conflicting estimates of volume regulating capacity, it is proposed that the cells are hyperosmotic to their environment at 25% salinity, the hydrostatic gradient thus created being countered by the restraint imposed by the thickened neural lamella. Physical stresses on the excitable membranes of the nerve would thus be minimized, and electrophysiological functioning in dilute media would be facilitated.

INTRODUCTION

The majority of marine invertebrates have body fluids approximately in osmotic and ionic equilibrium with normal sea water. Many such animals survive substantial changes in environmental osmotic concentrations, either by a moderate capacity for osmoregulation or by strict osmoconformity (Potts & Parry, 1964; Lockwood, 1976). In both cases, haemolymph concentration varies with salinity, yet the animal generally suffers no impairment of mobility or response, implying an inherent stability of the
excitable membrane systems in the face of both varied ionic gradients and changing cell volume.

Several recent studies have examined the quantitative processes of volume and solute regulation in the tissues of euryhaline invertebrates, but such work has so far been confined to muscles (Lange & Mostad, 1967; Lange, 1970; Freel, 1977) and gill tissues (Lange, 1968); though there are studies available on the nervous systems of crustaceans (Gilles & Schoffeniels, 1969; Gérard & Gilles, 1972) and of two annelid osmoconformers (Treherne & Pichon, 1978; Benson & Treherne, 1978) which have considered some aspects of this problem. As a preliminary to analysis of excitability under varying osmotic conditions, the present work concerns solute and water balance in the nervous system of a molluscan osmoconformer, aiming to determine the variations in ionic gradients and their effects on the active membranes. The consequences of both acute short-term stress and of long-term acclimation to dilute media will be considered.

The animal chosen for this analysis was the intertidal bivalve *Mytilus edulis*. Lamellibranchs have the advantage of commonly showing wider salinity ranges than most crustaceans or annelids without the need to osmoregulate, at least partly due to the tight apposition of the valves which can permit gradual exposure of the tissues to a new salinity. Furthermore, the bivalves have rather simply constructed nervous tissue (Nakajima, 1961; Gupta, Mellon & Treherne, 1968), so that flux studies for the determination of extracellular space and ion movements are more readily interpreted, while biochemical analysis and freeze-fracture examinations of axon surfaces also become feasible. Thus, although the choice of a bivalve limits the possibilities for electrophysiological dissection (Willmer, 1978b), such an animal provides nervous tissues ideally suited for an alternative physiological approach to the problem of neural adaptations to osmotic stress, and thereby gives results essentially complementary to those reported recently by Treherne & Pichon (1978) and by Benson & Treherne (1978) which rely primarily on intracellular recordings from annelid conformers.

*Mytilus edulis* also has the advantage of having been much studied with respect to many aspects of osmoconformity. However, there is considerable variation in the literature regarding its lower salinity tolerance (cf. Fox, 1941; Pilgrim, 1953a, b; Hegemann, 1964), so that a more rigorous study of the extent of ionic and osmotic conformity was required; the work of Gilles (1972) and of Baginski & Pierce (1975) has suggested that these earlier studies often allowed insufficient acclimation periods, at least 10 days being necessary for full biochemical adaptation. Based on this preliminary analysis, mussels have been compared at 100% and 25% salinities with respect to extracellular compartments, cellular ionic constituents and volume-regulating responses. These parameters are contrasted with the patterns of regulation displayed in other invertebrates, since the capacity for such regulation is widely assumed to play an important role in determining the degree of euryhalinity in marine osmoconformers (Schlieper, 1958). Finally, electron-microscopic comparisons have also been used in an attempt to locate structural correlates of the volume-regulating mechanisms.
Volume regulation and solute balance in nervous tissue of *M. edulis* 159

**MATERIALS AND METHODS**

(1) Storage and adaptation of mussels

Supplies of *Mytilus edulis* (each 4-8 cm in length) were obtained from the Marine Biological Laboratory at Plymouth and were maintained in tanks of recirculated natural sea water with an average osmotic concentration of 1044 m-osmol, at 7-10 °C. Food supplies were freely available.

Groups of 8-10 mussels were then adapted in 8 l vessels containing aerated and continuously filtered sea water of various dilutions, stored at 12 °C. Mussels were originally exposed to eight different salinities, the criteria for survival being the presence of a rapid shell-closure response when disturbed (Pierce, 1970). Unless otherwise indicated, an acclimation period of at least 14 days passed before the animals were tested further.

(2) Determination of blood concentrations

For comparison with earlier bivalve studies, analyses of three different body fluids were performed. Osmotic concentrations of mantle fluid, pericardial fluid and blood were recorded with the Clifton cryostatic osmometer, independently calibrated with NaCl solutions; where possible, the readings were checked with the larger scale Knauer electronic instrument, and the two measurements were always within 2-3 m-osmol. Cationic concentrations were determined by photometry using the Pye-Unicam SP90A spectrophotometer; tests for interference between K and Ca yielded at least 95 % recovery of added aliquots of either ion. Chloride estimates were performed by microtitration against 0.01 m-AgNO₃, with electrical monitoring of the endpoint.

(3) Estimation of extracellular space

ECS was determined by an efflux procedure similar to that advocated by Nicholls & Wolfe (1967), to take into account possible cellular uptake of supposedly 'extracellular' markers. Isolated ligatured connectives were first either weighed on a torsion balance, or measured directly using a Wild binocular microscope fitted with a calibrated eyepiece graticule in conjunction with a fibre-optic light source from below the tissue. The latter technique permitted ready calculation of connective volume as these tissues have a very regular cylindrical form; and volumetric determinations in fact proved considerably more reliable than weighings. The mean tissue density was 1.08 mg μl⁻¹.

The tissues were then bathed for 1 h in salines containing 5 μCi of [¹⁴C]sucrose (S.A. 1.54 mCi mg⁻¹) or of [³H]dextran (mean M.W. 77500; S.A. 12 mCi g⁻¹), (both tracers being supplied by the Amersham Radiochemical Centre), before transfer to an efflux apparatus adapted from that described by Thomas (1976). This permits analysis of very fast effluxes, so that extracellular fractions are readily revealed. Aliquots of effluent were collected at intervals ranging from 1 s initially up to 15 min, over a total efflux period of 2-3 h. Each sample was corrected for quenching, and counted either in Bray's scintillant or in a Triton-X/toluene cocktail. The connective itself was removed from the apparatus, lysed in distilled water, sonicated and then counted as above in the Hewlett-Packard 3320 liquid scintillation counter.

Counts per vial were plotted conventionally as percent activity remaining in the
tissue with time, and the curves thus generated were subjected to compartmental analysis (cf. Solomon, 1960).

Some efflux experiments were conducted at reduced temperature. For these, the apparatus and solutions were equilibrated at 4 °C for 2 h, and the connectives were loaded at this temperature for 1 h, before commencement of efflux.

(4) Determination of intracellular cation and water concentrations

The total cationic concentrations of *Mytilus* nervous tissue were determined conventionally by photometry after heating the connectives in 100 μl conc. HDO₃ and redissolving the residue in 2.0 ml 0.1 M-HCl. But subtraction of the proportion predicted from known ECS and haemolymph ion levels as being due to extracellular cations left unexpectedly high figures for apparent intracellular concentrations. As an alternative, the method of Nicholls & Kuffler (1965) was adopted, whereby the ECS of the tissue is first filled with isotonic sucrose and the measured Na concentration is then related only to the cells. This technique should be reasonably accurate for *Mytilus* connectives as the characteristics of sucrose movements are known. Ligatured and measured connectives were therefore soaked for 3 min in 'sucrose Ringer' (1.00 M sucrose, 12.4 mM-KCl and 9.5 mM-CaCl₂), this time-period having been calculated from sodium efflux data described in a further paper (Willmer, 1978b) as sufficient to permit more than 99% efflux from extracellular spaces with only 6.5% loss of intracellular sodium. The connectives were then severed at the ligature and measured by photometry as above.

Water content of the tissue (and hence of the cells) was determined from wet and dry weight recordings obtained with a torsion balance to an accuracy of 2 μg.

(5) Volumetric determinations

Short dissected and ligated lengths of connective were mounted across the central channel of a Perspex recording chamber; each end was held in place with a Vaseline seal, with the central portion bathed by a relatively large volume of flowing saline which could be rapidly changed through a multi-way valve. At least 70% of the tissue volume was thus directly exposed to the test fluid. The diameter of the connective (and hence its volume) was recorded as above, with the Wild microscope mounted directly above the preparation to ensure viewing in a constant plane.

Volume changes were also recorded in connectives referred to hereafter as being desheathed. In these, the nerve sheath was split longitudinally for a considerable portion of its length, using sharpened tungsten needles (cf. Carlson & Treherne (1969) with *Anodonta*).

(6) Fine-structural studies

Mussels adapted either to full sea water or to 25% salinity were used for electron-microscopic analysis. Cerebro-visceral connectives from these animals were dissected after flushing the preparation with a primary fixative solution of 2% glutaraldehyde in 0.1 M-PO₄ buffer (pH 7.4) containing 1% CaCl₂ and 17.5% sucrose to give 1100 m-osmol (Baskin, 1971). After removal from the animal, the connectives were transferred to fresh fixative to give a total fixation period of 1 h at 18 °C. They were washed in
Volume regulation and solute balance in nervous tissue of M. edulis

0.1 M-PO₄ buffer + 22% sucrose, postfixed for 1 h at 18 °C in 1% OsO₄ in 0.1 M-PO₄ buffer (with 1% CaCl₂ and 22% sucrose), and rewashed as above. The tissues were then conventionally stained for 1–2 h in 2% uranyl acetate (in sodium hydrogen maleate buffer, pH 6.2), and treated with propylene oxide before embedding in Araldite resin. Sections were cut with the LKB Ultrotome III, double-stained with aqueous uranyl acetate and lead citrate, and examined in the Philips EM 300 microscope.

RESULTS

(1) Osmotic and ionic adaptation

After 14 days' exposure to various salinities, the body fluids of Mytilus had the osmotic concentrations shown in Fig. 1. (Salinities of 15% and 10% were also used, but mussels rarely survived such dilutions and these tests were not pursued.) In all cases, the mantle fluid was essentially isosmotic with the sea water; pericardial fluid was hyperosmotic by an average of 10 m-osmol, and a further degree of hyperosmoticity occurred in the blood (mean 13 m-osmol, or 23 m-osmol above the environment). There was no evidence for any greater divergence from the isosmotic line at either very high or very low salinities.

The rate of adaptation in mussels is analysed in Fig. 2, showing the osmotic concentrations of mantle fluid and blood at intervals after a single stage drop of salinity from 100% to the lowest acceptable limit of 25% s.w. Acclimation was clearly complete within the 2 weeks normally allocated; most animals were apparently staying closed for at least the first 3–4 days of exposure.

The ionic concentrations in mussel fluids for each of the five ions studied are shown in Fig. 3(a–e). In every case, ionic levels closely followed those of the medium. For magnesium, calcium and chloride there were no significant differences; for sodium there was a just significant mean ionic excess of 4 mM, while for potassium the hyperionic condition of 5 mM was clearly very significant.

It is evident from these figures that Mytilus edulis is a true osmoconformer, all the ionic and osmotic measurements of the blood reflecting this property over a considerable salinity range. Internal and external concentrations are always approximately matched, so that the ratio Cₓ/Cₒ parallels the isosmotic line. The body fluids in fact show a small and roughly constant internal hyperosmoticity, but such an effect is common in invertebrates (Remmert, 1969) and is not inconsistent with osmoconformity. Protein in the haemolymph of Mytilidae has been recorded as 0.3–0.8 g l⁻¹ (Robertson, 1964), and this in conjunction with the elevated Na and K levels described above could adequately account for most of the recorded 23 m-osmol hyperosmoticity. A similar gradient of osmotic concentration from environment to blood has been reported in Modiolus spp. (Pierce, 1970), and in several related bivalves (Tiffany, 1972), though its existence is doubted by some authors on the grounds that excretory ultrafiltration would be impossible under these conditions (cf. Little, 1965; Fyhn & Costlow, 1975).

The osmoconforming range of Mytilus was found to be more limited in the present study than has been reported elsewhere, with little success below 25% salinity. While it is possible that physiological races of Mytilus exist (Wells, Ledingham & Gregory, 1940), it seems likely that the earlier studies underestimated the adaptation period
Fig. 1. Osmotic concentrations (m-osmol) of mantle fluid (○), pericardial fluid (△) and blood (○) of Mytilus after 14 days' acclimation to differing salinities. The solid line represents isoosmoticity of body fluids and medium. Values of 2 S.E.M. are in each case smaller than the symbol.
Volume regulation and solute balance in nervous tissue of M. edulis

![Fig. 2. Rate of adaptation of the mantle fluid (▼) and blood (○) of Mytilus when transferred from 100% sea water to 25% sea water at day 0. Each point represents samples from at least 5 animals, and solid bars indicate ± s.e.m.](image)

required, so that the animals still remained hyperosmotic to their environment. However, it is also known that laboratory salinity tolerances are not necessarily related to the ranges accepted by marine organisms in their natural environment (Kinne, 1971), and Mytilus has been observed in salinities as low as 8% (Freeman & Rigler, 1957). Nevertheless, in the remainder of this study, observations were confined to animals acclimated to 100% and 25% salinities only; adaptation to the latter was achieved by 3 days' exposure to 50% sea water followed by a minimum of 8 days in the 25% medium. Since the period of shell closure is roughly proportional to the magnitude of the imposed salinity change, the use of an intermediate stage allowed this slight decrease in total adaptation period.

Based on the present data, a Ringer solution for use in future experiments with Mytilus nerves was devised as follows: Na⁺ 442·1 mM; K⁺ 12·4 mM; Ca²⁺ 9·5 mM; Mg²⁺ 37·0 mM; Cl⁻ 500·4 mM; SO₄²⁻ 22·5 mM; HCO₃⁻ 2·1 mM; sucrose 18·0 mM (osmotic concentration 1044 m-osmol, pH 6·9). This is comparable with previous Ringers used with Mytilus (Potts, 1954; Twarog, 1967), though the overall osmolarity is somewhat lower. In accordance with the demonstrated lack of ionic regulation, Ringers required for use with 25%-adapted mussels were made by simple dilution of the above stock solution.
Volume regulation and solute balance in nervous tissue of M. edulis

Fig. 3. Ionic concentrations (in mM) in the blood of Mytilus at different salinities for (a) sodium, (b) potassium, (c) calcium, (d) magnesium and (e) chloride. The solid line in each case represents the isionic condition. Vertical bars indicate ± S.E.M. where this is larger than the symbol.

(2) Extracellular space

Examples of the efflux curves for [3H]dextran and [14C]sucrose from 100% adapted animals, conducted at 18-20 °C, appear in Figs. 4 and 5. In each case the curve has been analysed into three constituent phases, which invariably account for all the activity initially present in the tissue and give extremely good approximations to simple exponentials. Half-times and initial percentage activities of these three phases of efflux (referred to hereafter as A, B and C for fast, intermediate and slow components) are summarized in Table 1, together with the effects of reduced temperature. Precisely the same three phases could be identified in efflux from 25%-adapted nerves, and these results are also included in the table (though only sucrose efflux was attempted for such tissues).

The three phases described by the figures given here are considered to represent true 'compartments' for the following reasons:

(1) Half-times are relatively constant for each component.
(2) Half-times of each fraction show no significant correlations with each other (cf. Tucker & Pichon, 1972), suggesting a dependence upon different variables.
Fig. 4. The efflux of $[^3H]$dextran from a 100 % s.w.-adapted *Mytilus* connective. Solid symbols indicate the original efflux curve, which beyond 2 min had a constant half-time; open symbols represent the two remaining components which may be extracted. The three components together account for all the tracer initially taken up.
Volume regulation and solute balance in nervous tissue of *M. edulis*

Fig. 5. Efflux of $[^{14}C]$sucrose from a 100% adapted connective of *Mytilus*. The original curve is shown only up to 2.5 min, since the half-time was constant thereafter (cf. Fig. 4); all effluxes in fact proceeded for at least 2 h. Again three compartments can fully account for the tracer loss.
Table 1. The percentage distributions and half-times of efflux of dextran and sucrose in 100%-adapted and 25%-adapted Mytilus connectives

The proposed identities of the three fractions are: C, cellular uptake of marker; B, conventional extracellular space distribution; A, fast-effluxing superficial tracer, probably associated with the neural lamella. All figures are given as means ± 2 S.E.M. in this and subsequent tables.

<table>
<thead>
<tr>
<th></th>
<th>C (min)</th>
<th>B (s)</th>
<th>A (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% 18-20 °C [1H]dextran (n = 7)</td>
<td>23.7</td>
<td>20.5</td>
<td>22.9</td>
</tr>
<tr>
<td></td>
<td>±3.7±3.7</td>
<td>±2.7±2.7</td>
<td>±2.7±2.7</td>
</tr>
<tr>
<td>100% 4 °C [14C]sucrose (n = 5)</td>
<td>57.2</td>
<td>57.2</td>
<td>57.2</td>
</tr>
<tr>
<td></td>
<td>±4.4±3.0</td>
<td>±5.4±3.0</td>
<td>±5.4±3.0</td>
</tr>
<tr>
<td>25% 18-20 °C [14C]sucrose (n = 7)</td>
<td>29.1</td>
<td>20.3</td>
<td>20.3</td>
</tr>
<tr>
<td></td>
<td>±3.6±3.6</td>
<td>±1.6±1.6</td>
<td>±1.6±1.6</td>
</tr>
</tbody>
</table>

(3) There was no residual activity unaccounted for by the three fractions: in most such analyses, a significant percentage remains due to the failure of conventional effluent collection techniques to detect very fast fractions.

Sucrose and dextran clearly behaved rather similarly, although dextran is normally considered to be confined to extracellular spaces while sucrose certainly enters cells. In both cases in this study, there were significant proportions of tracer having a slow efflux half-time (20-25 min); since the only physical barrier is likely to occur at the glial and axonal membranes, this fraction should represent cellular uptake and loss of markers. This view is supported by the effects of loading and effluxing at 4 °C, where fraction C efflux is considerably slowed and uptake to the compartment reduced, with other half-times incurring no greater increases than can be predicted from the effects of cooling on free diffusion ($Q_{10} \approx 1.3$; Ussing, 1960). Fraction C is therefore considered to represent the cellular distribution of both sucrose and dextran, dependent for both uptake and loss on energy-requiring processes; such fractions are conventional for sucrose and have been described for dextran in both leech CNS (Nicholls & Wolfe, 1967) and in mammalian CNS (Brown, Stumpf & Roth, 1969). The fast efflux fraction (A) is clearly extracellular in nature, and its rapid time-course indicates very easy access to the bathing medium. Since the tissue was not washed before effluxing, it is undoubtedly partly attributable to adherent tracer solution, but may also represent fluid associated with the neural lamella. Tracer activity of nerve sheaths has been estimated in other studies as about 10-15% of the total tissue uptake (Baker, Hodgkin & Shaw, 1962; Mellon & Treherne, 1969). Furthermore, this fraction is increased in 25%-adapted tissues (Table 1), which might correlate with the increased thickness of the neural lamella reported in this paper. Fraction A is excluded from considerations of the conventional ECS, as it clearly effuxes too rapidly to represent passage from clefts between the cells; thus, estimates of extracellular space described here are solely derived from fraction B, which has a half-time close to that determined for other invertebrate preparations, including Anodonta nerves (Mellon & Treherne, 1969) and Mytilus muscle (Potts, 1959).
Volume regulation and solute balance in nervous tissue of M. edulis

Table 2. Extracellular space determinations for connectives adapted to different salinities, using two alternative ECS markers

<table>
<thead>
<tr>
<th></th>
<th>Extracellular space (% tissue volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 % tissues</td>
<td>(n = 7) 19.97 ± 1.58</td>
</tr>
<tr>
<td>[3H]dextran</td>
<td>(n = 7) 19.97 ± 1.58</td>
</tr>
<tr>
<td>[14C]sucrose</td>
<td>(n = 13) 21.11 ± 1.37</td>
</tr>
<tr>
<td>25 % tissues</td>
<td>(n = 7) 20.60 ± 1.73</td>
</tr>
<tr>
<td>[14C]sucrose</td>
<td>(n = 7) 20.60 ± 1.73</td>
</tr>
</tbody>
</table>

Table 3. Intracellular cation concentrations and percentage water content in the axons of mussels adapted to different salinities

<table>
<thead>
<tr>
<th></th>
<th>Unadapted (100 %)</th>
<th>Adapted (25 %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Na+], mM</td>
<td>104.5 ± 3.3</td>
<td>39.9 ± 1.9</td>
</tr>
<tr>
<td>[K+], mM</td>
<td>206.0 ± 5.1</td>
<td>109.2 ± 3.7</td>
</tr>
<tr>
<td>H2O, %</td>
<td>73.8 ± 3.0</td>
<td>82.5 ± 2.3</td>
</tr>
</tbody>
</table>

Calculations of ECS based on the above considerations yield the figures shown in Table 2, expressed as percentages of the (directly determined) tissue volume. These figures are satisfactorily constant with different tracers, so that the efflux method of ECS calculation seems to be an adequate technique so long as a full compartmental analysis is performed, and given that the tissue is sufficiently simple to make this possible. Values of ECS for *Mytilus* are within the range estimated for other comparable tissues; for example, Krogh (1939) found a thiosulphate space in *Mytilus* of 12 %, Potts (1958) found an ECS of 24.8 % in *Mytilus* muscle and 22 % in tissues of *Anodonta*, and Nicholls & Kuffler (1965) showed a fast-exchanging fraction of about 40 % in the leech, though this undoubtedly included the connective tissue space.

Extracellular space appears to be constant with salinity in *Mytilus*. Although Potts (1958) showed a decrease from 24.8 % at 100 % salinity to 20.3 % at 50 % salinity in *Mytilus* muscle, more recent studies by Bedford (1972) on *Melanopsis*, by Freel, Medler & Clark (1973) on *Nereis*, by Lockwood & Inman (1973) on *Gammarus*, by Siebers & Lucu (1973) on *Carcinus* and by Harris (1976) on *Carcinus* nerve have all found a similar apparent constancy of ECS at different salinities to that reported here.

(3) Intracellular water and cation concentrations

Determinations of cellular sodium and potassium levels, together with the percentages of cell water before and after acclimation to dilute sea water, are set out in Table 3. The figures given for [Na+] and [K+] are those obtained after filling the extracellular spaces of the connective with sucrose as described in the Methods section. Without this prior treatment, the figures calculated were at least twice as high. This result might obtain if the fast-effluxing component recorded for saccharides were also present for cations, so that a significant proportion of the total ion content of the tissue would be associated with the sheath or surface at an unknown concentration. Sodium efflux experiments to be described in a future paper indicate that this is in fact the case (Willmer, 1978b), so the figures shown in the table here are likely to be a more accurate reflection of actual [Na+] than would otherwise be available. Thus, although
the true activities of Na and K cannot be determined, it is likely that cellular adaptation involves less than complete dilution of internal cations.

(4) Volumetric changes

Examples of the effect of acute reductions in medium concentration to 25% of normal levels are shown in Fig. 6, representing intact and desheathed connectives from 100%-s.w. adapted mussels. The equivalent plots for nerves from 25%-adapted animals (exposed in this case to 6·25% salinity), are shown in Fig. 7. To summarize these experiments, the mean effects of all such treatments are given in Table 4, following the conventions marked in Fig. 6(a).

In every case, exposure to a 25% solution caused a rapid increase in volume, with a subsequent equilibration at a new slightly lower level. On return to full-strength solutions, the connectives always recovered to within a few percent of their initial diameter relatively quickly. This pattern of volume response is very similar to that recorded in a wide range of other tissues and cells from vertebrates and invertebrates (cf. Hoffmann, 1977). Major differences occurred between intact and desheathed connectives, however, the latter showing a great increase in the degree and speed of apparent swelling whether the connective was from full sea water exposed to 25% s.w. or was adapted to 25% salinity and exposed to 6·25% s.w. It could be argued that splitting the sheath has simply allowed a greater mechanical separation of the axons, with an increase in extracellular spaces, rather than actual cellular swelling, this process being assisted by the disturbance during solution changes. However, two considerations militate against this view: (1) No significant increase in diameter occurred when a desheathed connective was bathed in successive changes of its normal medium, implying that the axons adhere closely even when freed from the neural lamella. (2) The connectives clearly recovered to roughly normal proportions in both intact and desheathed nerves on return to normal Ringers (106% and 107% respectively for full-s.w. adapted tissues), suggesting no extra mechanical disruption in the latter after hyposmotic exposure. Therefore the recorded differences in diameter are deemed to be accurate reflexions of changes in tissue volume.

Comparison of the data in Table 4 leads to the further conclusion that swelling is reduced even more in 25%-adapted connectives if the neural lamella is intact: for while unadapted nerves show a diameter increment of about 20% in dilute media, the dilute-adapted tissues swell by only 10% in equivalent conditions. Measurements of initial connective diameter show that there is in any case a significant size increase after long-term exposure to 25% sea water (unadapted nerves = 0·123 ± 0·04 mm (mean ± 2 s.e.m.), adapted nerves = 0·136 ± 0·02 mm), so that both tissues will reach roughly the same maximum diameter of about 0·149 mm after acute hyposmotic exposures.

(5) Fine structure

(a) 100%-adapted animals

The connectives of Mytilus edulis from full sea water proved to have a microscopic structure comparable with that demonstrated in related bivalves (cf. Nakajima, 1961; Fährmann, 1961; Gupta et al. 1968; Prior & Lipton, 1977). The tissue is completely
Volume regulation and solute balance in nervous tissue of M. edulis

Fig. 6. The diameter changes recorded in a 100%-adapted connective when exposed first to 25% sea water and then returned to conditions of 100% salinity. (a) Intact connective, (b) desheathed connective. Values of $d_1-d_4$ and $t_1-t_4$ are given in Table 4.
Fig. 7. Diameter changes recorded in a 25 %-adapted connective when exposed to a further dilution to 25 % (i.e. to 6·25 % s.w.), and during subsequent recovery on return to 25 % salinity. (a) Intact connective, (b) desheathed connective.
Volume regulation and solute balance in nervous tissue of *M. edulis* 173

Table 4. Changes in diameter recorded in isolated intact or desheathed connectives from mussels acclimated to 100% or to 25% salinity, when exposed to a further dilution to 25% of the original medium concentration

<table>
<thead>
<tr>
<th></th>
<th>d1%</th>
<th>t1m</th>
<th>d2%</th>
<th>t2m</th>
<th>d3%</th>
<th>t3m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact (n = 5)</td>
<td>27:5</td>
<td>17:0</td>
<td>20:6</td>
<td>30:2</td>
<td>6:0</td>
<td>5:5</td>
</tr>
<tr>
<td>Desheathed (n = 5)</td>
<td>±2:2</td>
<td>±1:9</td>
<td>±1:8</td>
<td>±3:5</td>
<td>±1:0</td>
<td>±0:5</td>
</tr>
<tr>
<td>100%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact (n = 6)</td>
<td>80:0</td>
<td>3:0</td>
<td>49:7</td>
<td>25:0</td>
<td>7:0</td>
<td>15:0</td>
</tr>
<tr>
<td>Desheathed (n = 5)</td>
<td>±8:2</td>
<td>±0:5</td>
<td>±3:2</td>
<td>±2:0</td>
<td>±1:0</td>
<td>±0:8</td>
</tr>
<tr>
<td>25%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact (n = 6)</td>
<td>13:2</td>
<td>7:0</td>
<td>10:0</td>
<td>15:5</td>
<td>0:0</td>
<td>10:0</td>
</tr>
<tr>
<td>Desheathed (n = 5)</td>
<td>±1:6</td>
<td>±0:8</td>
<td>±1:3</td>
<td>±1:5</td>
<td>±0:5</td>
<td>±1:3</td>
</tr>
<tr>
<td></td>
<td>75:2</td>
<td>3:0</td>
<td>45:4</td>
<td>10:0</td>
<td>6:0</td>
<td>6:0</td>
</tr>
<tr>
<td></td>
<td>±9:0</td>
<td>±0:8</td>
<td>±5:0</td>
<td>±1:0</td>
<td>±0:8</td>
<td>±0:4</td>
</tr>
</tbody>
</table>

bounded by an acellular sheath (Fig. 8), of fairly uniform thickness (2.5–5 μm). This is homologous with the ‘neural lamella’ of other invertebrate nervous systems, being composed of two structural elements: a fine fibrillar matrix forms concentric sheaths around the connective, alternating with lamellae of relatively coarse fibres mostly running parallel with the axis of the nerve. By analogy with other tissues, the matrix material may be a mucopolysaccharide (Ashhurst & Costin, 1971), while the fibres, each about 15 nm in diameter and with a characteristic banding in L.S., are probably collagenous. Within the neural lamella there are occasional groups of cells (Fig. 8), most of which are likely to be haemocytes loosely incorporated into the sheath material.

The central, cellular portion of the connective is mainly composed of numerous very small axons, ranging in diameter from 0.08–1.0 μm, with a mean of only 0.403 μm. These axons have irregular interlocking profiles, leaving extracellular clefts of only 15–25 nm. Morphological estimates of apparent total ECS from available micrographs gave a mean figure of 11.0%, a value in good agreement with that calculated by the method of Horstmann & Meves (1959) for fixed tissues, though predictably smaller than the more reliable figures obtained using tracers (cf. van Harreveld, Crowell & Malhotra, 1965).

The darker-staining profiles of glial cells have a fairly uniform distribution around the outer part of the connective (Fig. 8), with their nuclei commonly lying just below the cell/neural lamella interface. Such glial cells frequently send attenuated cytoplasmic projections towards the centre of the connective, as described in *Anodonta* (Gupta et al. 1968).

With regard to the accessibility of the connective from the external environment, it is apparent from the micrographs (as in Fig. 8) that most axons abut directly only on other axons, with no glial ‘wrapping’ around single or grouped excitable cells. Neither is there a continuous rind of glial cells at the periphery; in many places axons directly contact the neural lamella. Thus *Mytilus*, in common with other bivalves, does not show a ‘perineurial’ modification which could be construed as a physiological barrier. Furthermore, the only junctions to be found in this lamellibranch tissue are conventional hemidesmosomes, between glial cells and neural lamella (Fig. 9), at...
regular intervals around the periphery; no true junctional complexes which could structurally limit access to the interior of the connective have been located.

(b) 25%-sea-water adapted connectives

The basic structure of *Mytilus* nervous tissue described above is preserved after adaptation to dilute (25%) salinity, such that there are no differences in cell proportions or distribution (Fig. 10) (though the mean axon diameter is now 415 nm and the estimated ECS is 12.1%). There is no evidence for the appearance of structural barriers to the free passage of ions or molecules into the connective; hemidesmosomes occur with the same frequency as previously (Fig. 11), and no new types of junction appear.

However, interesting changes may be discerned in the neural lamella. While retaining its form as a complete sheath around the axons, this structure was greatly thickened in each of the adapted animals examined (see Fig. 10), with a mean width of 10 μm (roughly three times its former thickness). Closer examination reveals an increased separation of successive lamellae of the matrix material (Fig. 11). However, the intervening areas of ‘collagenous’ fibrils retain a similar packing: counts of randomly selected regions indicated $415 \pm 77 \mu m^{-2}$ (mean ± 2 s.e.m.) before adaptation and $393 \pm 129 \mu m^{-2}$ after acclimation to 25% sea water. Therefore this thickening is not likely to be due simply to osmotic stress pulling the tissue apart; rather it is implied that synthesis and deposition of new material has occurred. This is supported by the appearance of particularly dense regions of fibrils near the periphery of the sheath in association with cellular margins (see Fig. 10), and by the presence of cells such as that shown in Fig. 12; this is a large, dense-bordered cell in the neural lamella, probably a haemocyte, apparently engaged in the deposition of the fibrillar component of the sheath. Many such active cells were found in adapted connectives; though presumably always present, their activity was not observed before dilute adaptation, so they may conceivably be quiescent (in the blood, or within the neural lamella), unless stimulated to increase production by stress such as an osmotic shock.

Two major points thus emerge from the fine-structural studies of *Mytilus* connectives. Firstly, there is an absence of potential structural barriers around the axons, suggesting ready accessibility for ions and small molecules. This freedom of access has also been demonstrated by tracer uptake (horseradish peroxidase) in *Anodonta* connectives (Lane & Treherne, 1972) and electrophysiologically by Sattelle & Howes (1975) in the same tissue; the suggestion of Twarog & Hidaka (1971) that the neural lamella has a regulatory function in *Anodonta* but not in marine bivalves thus cannot be supported on either structural or physiological grounds. There is clearly no evidence for a bivalve ‘neurone-haemolymph barrier’ of the type widely described in vertebrates and in insects (Smith & Treherne, 1963; Treherne & Pichon, 1972), nor is there any structure comparable with the thick encapsulating glial layer found in the gastropods (Sattelle & Lane, 1972; Reinecke, 1976) and in annelids (Kuffler & Potter, 1964; Baskin, 1971).

The second point of interest is the response of the connective to osmotic stress, the main potentially adaptive change occurring in the neural lamella. It has been suggested that this type of sheath has a degree of osmoregulatory function in both amphibian nerves (Lorente de No, 1952) and in insects (Twarog & Roeder, 1956), and such a
Fig. 8. Peripheral area of the cerebro-visceral connective from a 100% -adapted mussel. Glial cells (G) and their nuclei (GN) lie close to the neural lamella (NL). Their processes radiate between many very small, closely packed axons, some of which contain neurosecretory material (NS). A large haemocyte (H) is incorporated within the neural lamella. Scale bar represents 1 μm.

Fig. 9. Hemidesmosomes (Hd) at a glial cell/neural lamella interface in the periphery of a 100% -adapted Mytilus connective. Each junction is associated with dense filaments (GF) in the glial cell. The alternation of an amorphous matrix (Ma) and of bands of coarser collagen-like fibrils (Co) in the neural lamella is also shown. Scale bar represents 0.5 μm.
Fig. 10. Peripheral area of a 25%–adapted connective, showing the increased thickness of the neural lamella, with dense deposits of collagen-like fibrils near its outer margin. Glial cells, with associated hemidesmosomes, again occur at the cell/neural lamella interface. Scale bar represents 1 μ.

Fig. 11. A large hemidesmosome from a 25%–adapted connective, occurring where a glial cell abuts on to the neural lamella. Packed mitochondria (M), gliosomes (Gl) and glial filaments identify the glial elements. The packing of ‘collagen’ should be compared with that in the neural lamella of a 100%–adapted connective (Fig. 9). Scale bar represents 0.5 μ.

P. G. WILLMER
Fig. 12. A large cell (probably a haemocyte) within the neural lamella of 25% adapted connective, apparently involved in deposition of the collagen-like fibrils of the sheath. Scale bar represents 0.2 μ. 

P. G. WILLMER
property would clearly be of even greater importance to a euryhaline osmoconforming species. Therefore the observed thickening of the neural lamella by deposition of extra collagen-like material from haemocytes might be a specific response to control swelling in the nerve during low-salinity acclimation.

DISCUSSION

Living cells have a general tendency to behave as osmometers in anisosmotic media. However, this effect is partially controlled by the loss of cellular osmotic effectors (see review by Macknight & Leaf, 1977); in invertebrates, losses of amino-acids are particularly common (Florkin & Schoffeniels, 1965), whereas depletion of intracellular potassium and chloride is more frequently implicated in chordates (Macknight & Leaf, 1977). This balance between perfect osmotic swelling and regulation of cellular volume by intracellular losses can best be described by considering the relative cell hydration for a given tissue (Freel, 1977). Most of the available figures for this factor lie in the region intermediate between the theoretical extremes, as expected, though a few decapods apparently achieve complete volume regulation (see Fig. 1; Freel, 1977). Calculation of relative cell hydration for *Mytilus* (where \( R_{H_2O} = (g H_2O/g \text{ dry wt})_{25\%}/(g H_2O/g \text{ dry wt})_{100\%} \)) yields a figure of 1.67, again indicating a compromise between the two extreme conditions, with a moderate capacity for volume regulation. It is of interest that a different method of analysis has indicated a rather high degree of volume regulation in the muscles of *Mytilus edulis* (Lange & Mostad, 1967), and complete regulation in *Mytilus* gill tissue (Lange, 1968).

Apart from this ability to regulate cell water content, other features of intracellular solute variation are evident from the present data. Using the known values for intracellular and extracellular concentrations of sodium and potassium it is possible to calculate values of \( R_{\text{solute}} \), the relative cell solute concentrations on a dry weight basis (Freel, 1977), where \( R_{\text{solute}} = (\text{mm} / \text{kg dry wt})_{25\%}/(\text{mm} / \text{kg dry wt})_{100\%} \). For *Mytilus* the figures are \( R_Na = 0.57 \) and \( R_K = 0.79 \). Thus for both the major cations there is a net loss from the cells during low salinity acclimation. This finding is of particular interest in view of Freel's results which indicated net losses of Na and of chloride, but a passive re-equilibration of potassium \( (R_K = 1.0) \), in a variety of decapod muscle tissues. He postulated a conventional Gibbs-Donnan equilibrium as being sufficient to account for the observed distribution of K and Cl in these muscle fibres. However, he also predicted on the basis of his model that in marine nerves, where the major intracellular amino-acids glutamate and aspartate form both the bulk of the anion fraction required to balance \([K^+]_i\) and the major source of volume-regulating solute, that intracellular potassium would have to be more variable. Thus the present results, showing a significant variation in potassium content per g dry cell weight, provide a useful confirmatory test of Freel's model. Furthermore, in the nerves of *Sabella* (Treherne & Pichon, 1978) it appears that the only significant net losses in 60% salinity are of potassium, whereas *Mercierella* (Benson & Treherne, 1978) at 25% appears to lose moderate amounts of both Na and K; but the \( R_S \) values cannot be computed accurately in these cases as the cell hydration has not been determined.

*Mytilus* nerve cells, like other invertebrate nerves, therefore appear to show net losses of both major cations during very dilute acclimation, but the actual concentra-
tions of these ions are clearly not fully reduced in proportion to external levels. In fact, losses of sodium and potassium together with equimolar chloride could only account for a maximum of 314 m-osmol reduction in the cells, whereas osmotic equilibrium would require a total intracellular loss of 780 m-osmol and therefore considerable reductions in the cellular activity of amino-acids are likely to be occurring to produce the observed volume regulation (cf. Lange, 1963; Bricteaux-Gregoire et al. 1964).

The volume changes observed in hyposmotic media suggest that acute swelling is somehow reduced in the nerves of dilute-adapted animals, although such tissues show a net long-term increase in diameter relative to the 100 % s.w. adapted connectives. However, the fine-structural studies show that this diameter increment of 13 μm can be almost entirely accounted for by an increased thickness of the neural lamella, so that the cellular areas of the connective in fact show no long-term swelling: since the ECS is known to be approximately constant, the cells must also show no volume change. (Actual micrographic measurements, though unreliable for fixed tissue, also indicated only 6 % volume increase.) This finding seems to conflict with the calculation of cell hydration which indicated only a moderate capacity for cell volume regulation. However, the two results may perhaps be explained by the possibility of a non-isosmotic condition existing in the cells after adaptation; if the resultant osmotic gradient were countered by a hydrostatic force imposed physically by the neural lamella, the relative hydration of the tissue could be increased without changes in cell volume. Such a system, while unusual, is not without precedent, since a similar phenomenon has recently been postulated in the nervous system of the annelid *Mercierella enigmatica* (Skaer et al. 1978), where linked hemidesmosomes around the axons have been implicated in volume control. The tension imposed on the axon membrane of *Mercierella* (for a nominal 100 m-osmol osmotic gradient) was calculated as 3.6 × 10³ dyn cm⁻¹; since this parameter is dependent upon the radius of the cell, the equivalent tension in *Mytilus* axons would be roughly two orders of magnitude less, giving a membrane tension only marginally greater than that withstood by the erythrocyte membrane (Rand, 1964). Thus the problem of membrane stress is clearly reduced in a tissue containing many small axons; nevertheless, a hyperosmoticity of the adapted axons is likely to underlie the necessity for the thickened neural lamella, which would provide a further safeguard against physical damage, and which would otherwise appear irrelevant in a tissue where cell volume remains constant. Goldman (1964) has proposed an identical role for the lamellae which surround the earthworm nerve cord, calculating that each such lamella would have to withstand only 0.05 atmospheres to counter the osmotic gradient in the fully distended tissue.

The crucial factor in determining the existence of such mechanisms for volume control in nervous systems could be the necessity to reduce the stresses imposed on the excitable membranes; *Mytilus* axons, and those of the annelid conformers so far examined, do not appear to have any infoldings of the membrane which could act as a safety mechanism in the event of stretch, as are found for instance in the earthworm (Goldman, 1964). At the same time, and perhaps equally crucial (cf. Willmer, 1978A), undue losses of the cations on which metabolic function depends can be avoided. Such factors are likely to be of considerable importance in preserving the physiological performance of the nerves, which, as the following paper will demonstrate, can maintain full excitability after adaptation to 25 % salinity.
This work represents part of a Ph.D. thesis submitted to the University of Cambridge. The author would like to express sincere thanks to Dr J. E. Treherne for his help and advice throughout, and to Dr N. J. Lane, Dr H. leB. Skaer, Mr J. B. Harrison and Mrs L. Morris for their generous help with structural studies. This project was supported by an SRC grant, and by New Hall, Cambridge.

REFERENCES


Volume regulation and solute balance in nervous tissue of *M. edulis* 179


