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SODIUM FLUXES AND EXCHANGE PUMPS: FURTHER CORRELATES OF OSMOTIC CONFORMITY IN THE NERVES OF AN ESTUARINE BIVALVE (*MYTILUS EDULIS*)

By P. G. WILLMER
Department of Zoology, University of Cambridge

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SUMMARY

1. Analysis of sodium loss from *Mytilus* cerebro-visceral connectives indicated a slow efflux component attributable to movement across the cellular membranes. This component was significantly slowed at 4 °C, or by treatment with DNP, ouabain or K-free media; this implies that a conventional Na/K exchange pump contributes to cellular efflux.

2. Dose–response curves for the effects of ouabain on cellular Na efflux gave preliminary indications of a significant increase in the sodium pump frequency in connectives from mussels adapted to 25 % salinity relative to those from 100 % salinity.

3. Assays of ATPase activity in the connectives showed a 63 % increment in the Na/K-activated enzyme in 25 %-adapted tissues, with no significant change in the magnesium-activated enzyme. The Na/K-ATPase also exhibited a marked shift in its optimal sodium requirement, from 100 mM at 100 % salinity to 50 mM at 25 % salinity; these figures are close to the respective values of $[Na^+]_i$ determined for the axons under such conditions.

4. [^{3}H]ouabain binding studies confirmed that there was an increment in active sodium pumping sites (in this case of 76 %) in dilute-adapted nerves. Pump frequencies were in the range of 1000–4000 sites/μm^2, in agreement with previous studies of invertebrate nerve membranes.

5. The significance of extra pumping capacity for sodium (and potassium) ions in the dilute adapted tissues is considered in relation to the electrophysiological functioning, volume regulation and solute balance of the nerves of an osmoconformer.

INTRODUCTION

During the adaptation of invertebrate tissues to dilute media, the intracellular ionic environment of the cells undergoes considerable change, particularly with respect to sodium and potassium levels (Freel, 1977; Benson & Treherne, 1978; Willmer, 1978*a*). Such tissues must therefore have a capacity for long-term biochemical adaptation, since the total metabolic repertoire of the cells must be retained in spite of the profound effects of cytoplasmic cation levels on conventional enzyme functions. The nature and extent of such adaptations should be particularly evident in nerves and muscles, where the cell membranes are further critically dependent upon maintained ionic gradients for their characteristic responses. Hence it is of considerable interest to

discover any changes during acclimation to dilute conditions which might contribute to control of ion or solute balance or to cell volume regulation. An obvious candidate as a mediator of such control is the ubiquitous membrane-bound Na–K dependent exchange pump, whose functions in these respects have been widely reviewed (Skou, 1965; Whittam & Wheeler, 1970; Glynn & Karlish, 1975; Schwartz, Lindenmayer & Allen, 1975; Macknight & Leaf, 1977), and whose frequency is known to change in response to chronic alteration of internal sodium concentration during ouabain incubation (Boardman, Lamb & McCall, 1972) or to long-term potassium deprivation (Chan & Sanstone, 1969). The present study concerns several aspects of sodium movements and active pumping in the axons of *Mytilus edulis*, a bivalve which tolerates and conforms to extensive variation in its osmotic and ionic environment (Hegemann, 1964; Potts & Parry, 1964; Willmer, 1978*a*), and for which extracellular and axoplasmic concentrations have previously been determined (Willmer, 1978*a*).

METHODS

(1) *Sodium flux studies*

Cerebro-visceral connectives from *Mytilus edulis*, adapted to either full sea water or to 25% s.w. as described by Willmer (1978*a*), were dissected and ligatured. Each connective was incubated in 100 μl of an appropriate Ringer solution (see Willmer, 1978*a*) containing 10 μCi ^{22}Na (as NaCl, specific activity 25 μCi/mg, obtained from the Radiochemical Centre, Amersham). The increment of sodium thus introduced to the Ringer was always less than 1 mM. Where required, a potassium-deficient loading medium was obtained by replacing K with Na, giving an increase of less than 3% in total [Na]. Inhibitors were added as required at low concentrations without substitution.

Efflux analysis was performed by the modified fast-collection technique described in an earlier paper for studying ECS (Willmer, 1978*a*). The resultant effluent samples were each counted with 10 ml of Triton X-toluene emulsion fluid, with an efficiency of 55–58%.

Sodium uptake into *Mytilus* tissues was analysed by determining the volumes of individual connectives (Willmer, 1978*a*), loading them as above for varying time periods and estimating total activity taken up by counting after lysis and sonication of the tissue.

(2) *ATPase assay techniques*

Microsomal preparations of *Mytilus* membranes were obtained from pooled samples of 10 connectives, homogenized for 3 min in 100 μl of ice-cold medium containing 250 mM sucrose with 5 mM-EDTA and 0·2% sodium deoxycholate. The homogenate was made up to 600 μl with the same medium, and centrifuged in stages in an MSE Superspeed 65 ultracentrifuge to produce the heavy microsomal pellet. This was resuspended in 1–2 ml of a 250 mM sucrose solution and stored at 0 °C until required.

Reaction with ATP was performed at 20 °C, since preliminary tests indicated higher enzyme activity with this temperature regime than at 25 °C or 37 °C as used conventionally with vertebrate preparations. A series of reaction media was used, as explained in the Results section; each was adjusted to pH 7·0, and equilibrated with 2 mM sodium-ATP for 5 min before addition of the enzyme suspension. After 60 min

reaction was stopped with 30% trichloroacetic acid; proteins were precipitated by centrifugation and the supernatant was used to estimate released P_I by the method of Fiske & Subbarow (1925) using a Beckman 151 spectrocolorimeter at 650 nm. Protein concentrations of the original enzyme suspension were estimated by a micro-adaptation of the method of Lowry *et al.* (1951), using standards prepared from bovine serum albumin (fraction V, Sigma), with the Pye-Unicam SP 500 photometer.

(3) *Ouabain-binding studies*

Labelled ouabain may be used to analyse pump binding either in intact tissues and cells, or in microsomal preparations. The latter approach permits a considerable reduction of non-specific effects, but gives no real indication of *in situ* pumping activity because of possible inactivation or demasking effects inherent in the preparative procedures (see review by Jorgensen, 1975). Consequently the present study involved only intact connectives.

[^{3}H]ouabain was supplied by Amersham Radiochemical Centre (sp.act. 19 Ci/mM); it was dried to remove the ethanol–benzene solvent, redissolved in glass distilled water and stored at 0 °C. Connectives were incubated at 18–20 °C in 100 μl aliquots of either normal or K-free Ringers containing 2·5 μCi of the labelled source plus sufficient 'cold' ouabain to produce the desired concentration. Each nerve was loaded for 60 min; half-times for ouabain-binding vary from 2–4 min in HeLa cells to 15 min in heart cells (Baker & Willis, 1972*a*), so that connectives presumably approached saturation. The tissues were then washed for 5 min in two changes of unlabelled Ringer containing a matched drug concentration, to remove surface-adherent and extracellular tracer, and were then lysed in 300 μl of distilled water. Finally 5 ml of Bray's scintillation fluid were added, the vials were sonicated for 5 min, and then counted in a Hewlett Packard 3320 liquid-scintillation counter.

RESULTS

(1) *Sodium fluxes*

Control and low temperature patterns

Control efflux experiments were performed at 18–20 °C after a 1 h loading period, and an example of the effects is shown in Fig. 1. As with the efflux of saccharide molecules reported by Willmer (1978*a*), the loss of sodium from the connective could be described by three readily separable components which together accounted for all of the cation originally taken up. The mean half-times and loading proportions of these three fractions are summarized in Table 1, which also includes the effects of loading and effluxing the connectives in two low temperature regimes.

Half-times for all components were similar for sodium to those obtained with sucrose and dextran. It is therefore likely that the three phases represent the same compartments as were ascribed to them in the earlier paper; that is, the slow fraction is cellular efflux, fraction B describes losses from the conventional extracellular spaces, and fraction A represents an ill-defined superficial compartment probably related to the neural lamella. The putative cellular origins of fraction C are supported by the effects of reduced temperature on unadapted tissues, where a 15 °C drop caused an

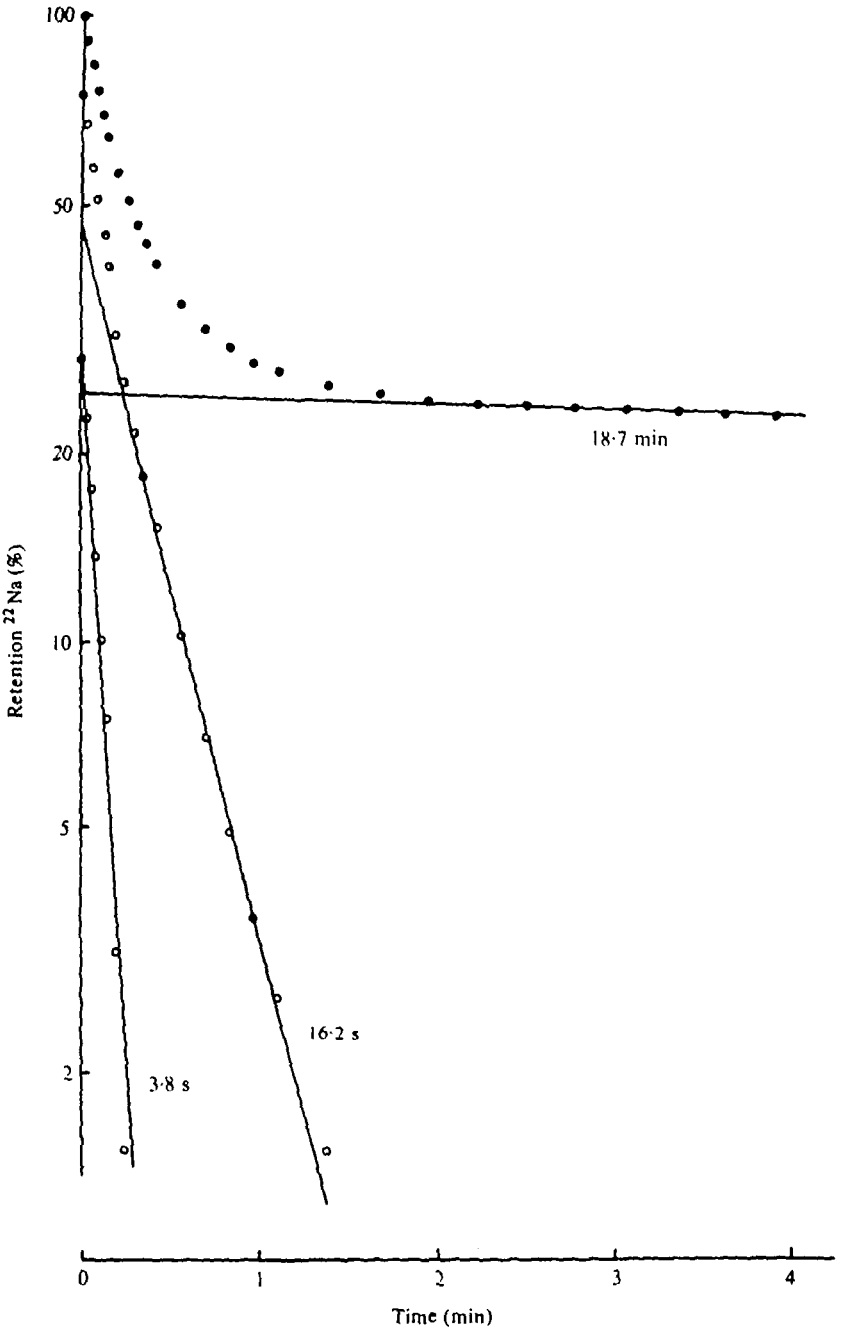

Fig. 1. An example of efflux of ^{22}Na from a 100 %-adapted *Mytilus* nerve at 18–20 °C. Efflux is shown up to 4 min, the slow phase having a constant $T_{0.5}$ thereafter for up to 2 h. Two further components may be extracted as shown, to account for all the remaining tracer in the tissue.

Table 1. *The percentage distributions and half-times for three components of sodium efflux identified in* Mytilus *nerves. It is proposed that C = cellular efflux, B = extracellular sodium and A = superficial (neural lamella?) sodium. In this and subsequent tables, results are given as means* ± 2 S.E.M.

		% Counts min^{-1}			$T_{0.5}$		
		C	B	A	C (min)	B (s)	A (s)
100 % s.w.	18–20 °C (n = 12)	28·75 ±3·41	38·27 ±3·93	32·98 ±3·48	29·67 ±4·95	17·49 ±0·79	3·12 ±0·26
	12 °C (n = 7)	34·56 ±9·87	21·18 ±6·13	44·26 ±7·18	207·00 ±53·90	22·40 ±3·33	3·52 ±0·21
	4 °C (n = 8)	35·50 ±10·43	19·62 ±6·20	44·88 ±7·37	263·00 ±59·90	36·81 ±2·41	4·80 ±0·88
25 % s.w.	18–20 °C (n = 10)	22·90 ±3·25	20·37 ±2·23	56·73 ±3·51	47·00 ±4·82	24·25 ±1·69	4·00 ±0·41

eightfold increment in $T_{0.5}$ for this component with comparatively limited effects on other efflux parameters. Furthermore, the ascription of fraction A sodium efflux to the surface/neural lamella is rendered more probable by the considerable increase in this compartment in dilute-adapted animals, since ultrastructural studies have indicated a marked thickening of the neural sheath in acclimated mussels (Willmer, 1978*a*). These two factors, together with the similarity of the half-time of extracellular (phase B) loss of sodium with that determined electrophysiologically (Willmer, 1978*b*), all support the given analysis of the three sodium compartments. In the present paper, efflux across the cell membranes is the focus of attention, and further tests to demonstrate the identity of fraction C with cellular loss were therefore pursued (with unadapted tissues only).

Effects of ions and inhibitors

Experiments with metabolic poisons and with potassium-deficient media were initially attempted by applying test pulses to connectives during the steady phase of efflux (i.e. effectively during loss of fraction C only). This method has been of use with squid axons and similar preparations (Hodgkin & Keynes, 1955) but proved uniformly unsuccessful with *Mytilus*, since exposures required were too long and sample counts became unacceptably low. All further experiments were therefore performed by loading and effluxing continuously in the test medium; this has the advantage of revealing any effects on half-times for all three fractions and thus gives an extra test of the 'reality' of the proposed efflux compartments.

Experiments of this type were performed with K-free solutions, and with ouabain and 2,4-dinitrophenol (using a prepared calibration curve to correct for colour-quenching in the latter case). In every test there were no significant changes in most of the efflux parameters; the only recorded alterations were a very marked increase in the half-time of fraction C in all the situations tested, and sometimes a just significant increase in the relative loading of the fast fraction. Values of $T_{0.5}$ for the slow component are given in Table 2.

These results clearly indicated the cellular nature of fraction C efflux. Furthermore,

Table 2. *Effects of K-free media and of inhibitors on the half-time of efflux of fraction C (cellular) sodium from* 100 %- *and* 25 %-*adapted* Mytilus *connectives*

				Ouabain (M)			
$T_{0.5}$ (min)	Control	K-free	DNP 10^{-4} M	2×10^{-4}	5×10^{-4}	2×10^{-3}	5×10^{-3}
100 % s.w. nerves ($n \geq 6$)	29·67 ±4·95	172·25 ±43·46	140·00 ±17·50	41·00 ±1·52	98·33 ±12·34	130·60 ±15·36	121·33 ±18·66
25 % s.w. nerves ($n \geq 5$)	47·00 ±4·82			62·25 ±2·49	71·17 ±4·50	152·00 ±22·73	138·67 ±18·40

the effects of potassium-free media and of ouabain again suggest the involvement of a conventional Na–K exchange pump in controlling such efflux. The effects of 10^{-4} M DNP, of ouabain above 2×10^{-3} M, and of an absence of exchangeable potassium were all roughly similar with respect to the resultant half-time, suggesting that these conditions produce full inhibition of the pump such that residual sodium loss is entirely by a slow diffusive leak. Indeed, further slowing of efflux was only achieved by cooling to 4 °C, when complete pump inhibition would be accompanied by slower diffusional losses.

Comparison of the pumping contribution in unadapted and 25 %-adapted tissues may best be achieved by consideration of the concentration dependence of the ouabain effects; dose–response curves for this drug are shown in Fig. 2, using the slow-fraction

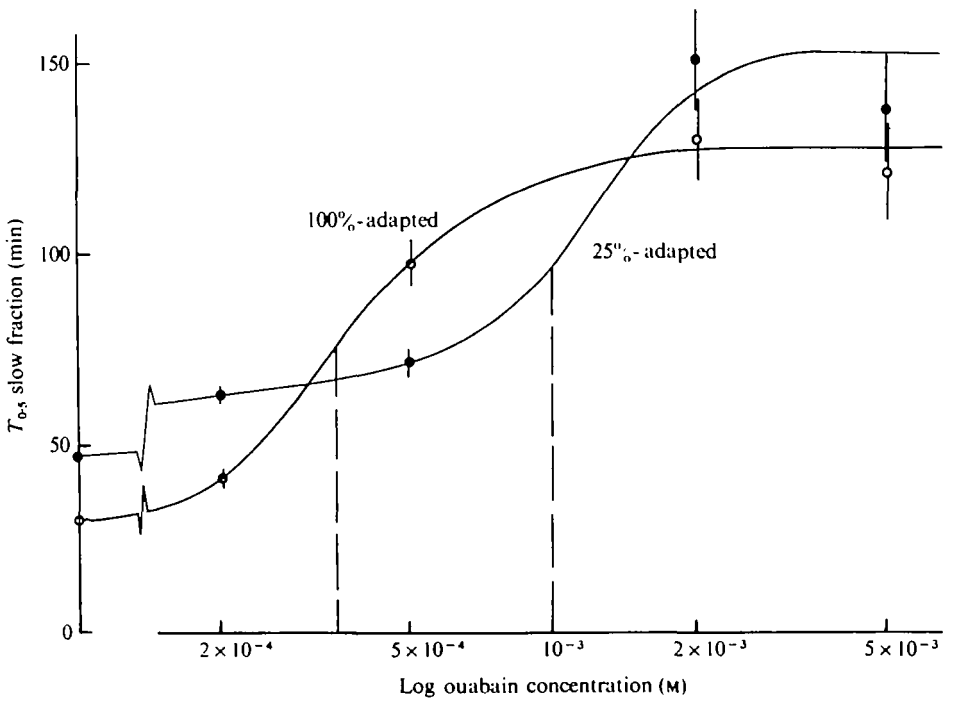

Fig. 2. Dose–response curves for the effects of ouabain on slow fraction efflux from the axons of *Mytilus*. ○, 100 %-adapted nerves; ●, 25 %-adapted nerves. Dotted lines indicate $C_{0.5}$ (the concentration required for half-maximal inhibition) in each case. Here and elsewhere the vertical bars represent 2 × S.E.M.

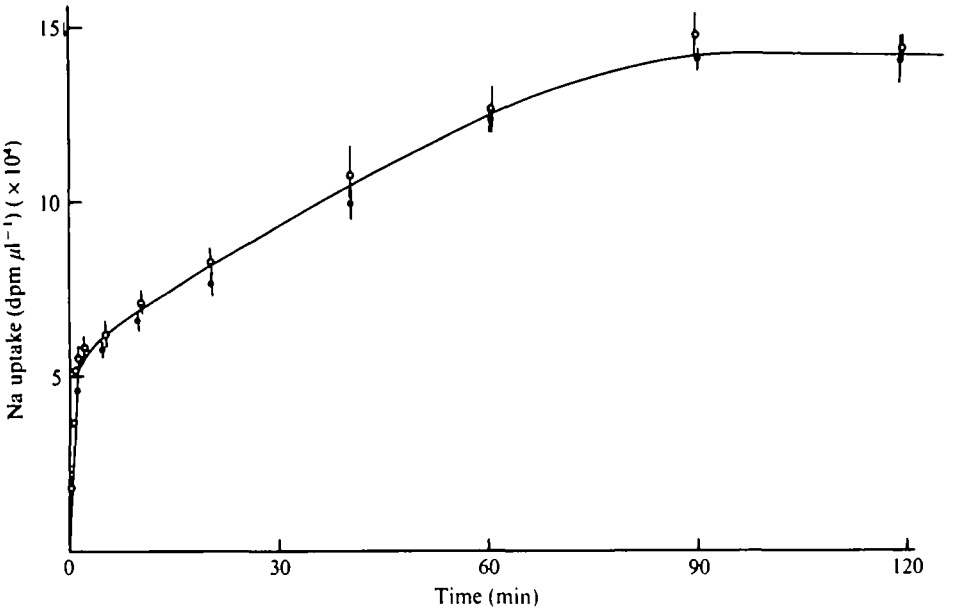

Fig. 3. Uptake of ^{22}Na with time in 100 %-adapted (○) and 25 %-adapted (●) *Mytilus* nerves. Data points represent at least triplicate determinations in this and the two following figures.

half-time as a measure of the inhibition produced. This plot exhibits a significant shift along the concentration axis in adapted nerves, which provides preliminary evidence that there may in fact be more sodium pumps present after acclimation; though the possibility of non-specific ouabain effects clearly cannot be excluded on the basis of these experiments.

Sodium uptake

The activity of ^{22}Na (as disintegrations min^{-1} μl^{-1}) with varying loading times is shown in Fig. 3 for each type of tissue. The rate of influx was clearly always high in the first 2 min, levelling off to a fairly steady inflow until a constant activity was reached at about 80–90 min; though the similarity of total uptake into 100 %- and 25 %-adapted nerves is probably coincidental, resulting from the unpredictable contributions of sheath uptake. The process of sodium uptake into cells would appear to be saturable in both tissues, implying regulation of $[Na^+]_I$ at a specific level appropriate to the acclimated state of the axons.

(2) *ATPase activity*

Enzyme activity in a basic salts medium

The great majority of assays recorded in the literature have used a salts medium for the ATP reaction containing 100 mM-Na, 20 mM-K and 5 mM-Mg (for total ATPase determination), or sucrose and 5 mM-Mg (for Mg-ATPase alone), the Na/K-ATPase being the difference between these two figures. The enzyme activities recorded in such media for unadapted *Mytilus* nerves were 20·03 ± 1·75 μmol P_I mg protein^{-1} h^{-1} for Mg-ATPase, and 15·24 ± 2·14 μmol P_I mg protein^{-1} h^{-1} for the Na/K-ATPase (means ± 2 S.E.M.).

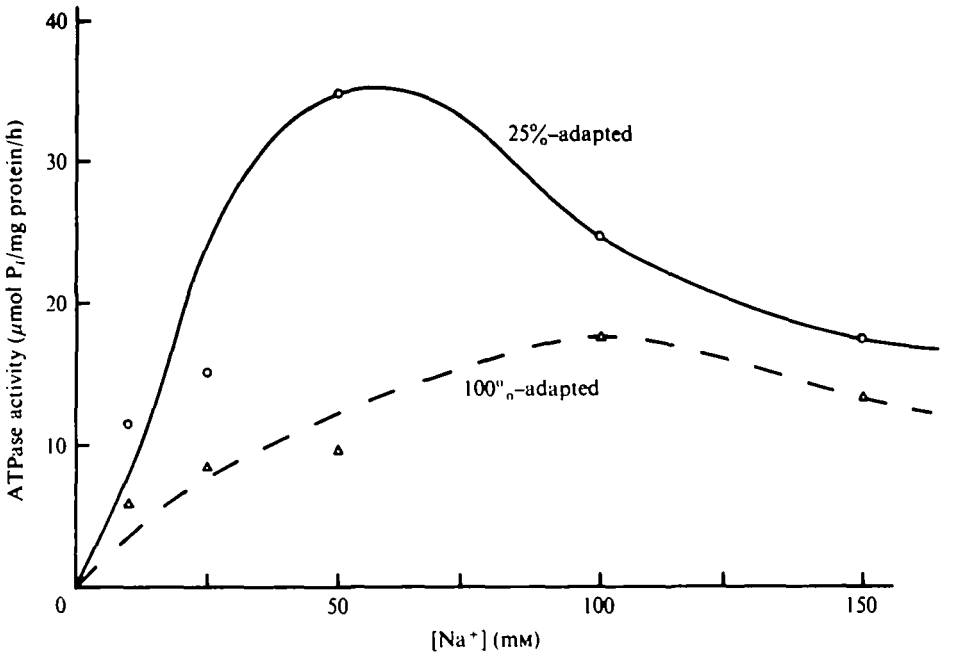

Fig. 4. Effects of Na^+ in the reaction medium on Na/K-ATPase activity in 100% (△) and 25% (○) adapted connectives.

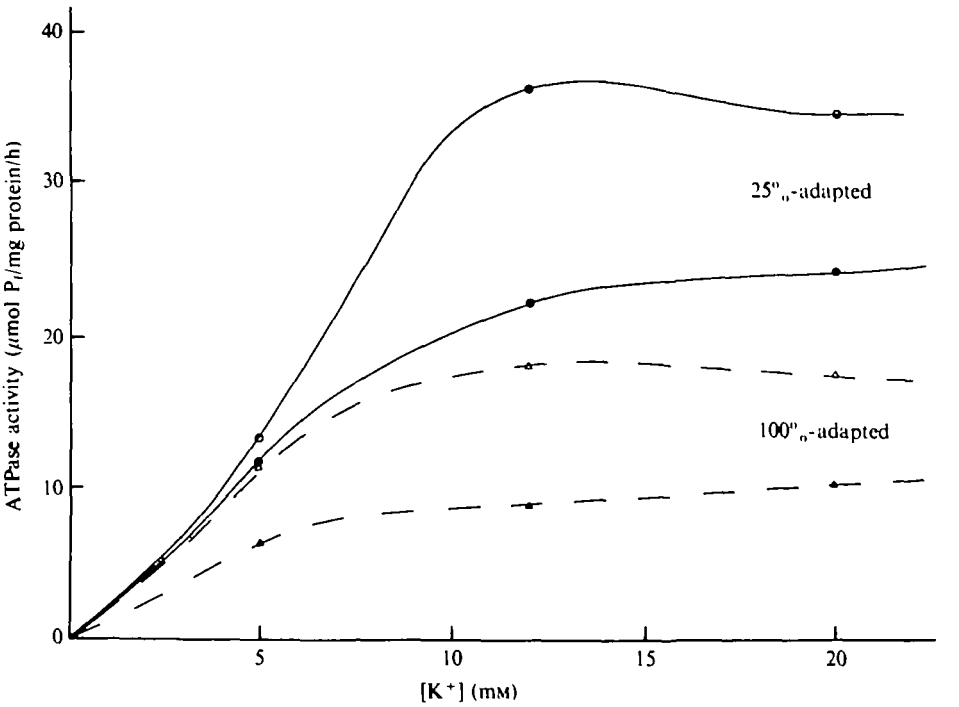

Fig. 5. Effects of potassium on Na/K-ATPase activity recorded in *Mytilus* nerves. △, 100%-adapted nerves with 100 mM-Na; ▲, 100%-adapted nerves with 50 mM-Na; ○, 25%-adapted nerves with 50 mM-Na; ●, 25%-adapted nerves with 100 mM-Na.

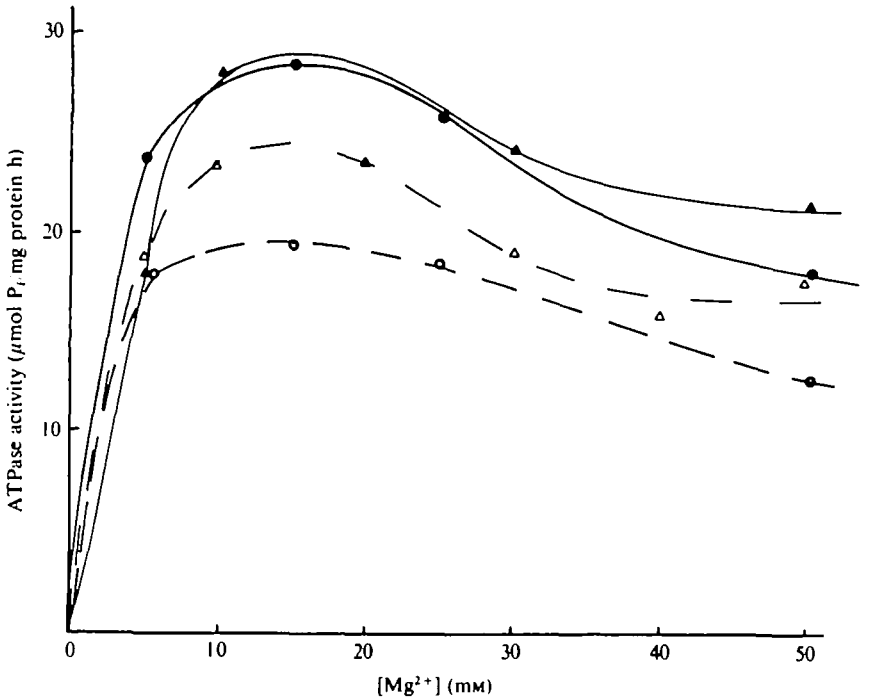

Fig. 6. The effects of magnesium concentration on Na/K-ATPase (solid symbols) and on Mg-ATPase (open symbols) from *Mytilus* connectives. △ ▲, 100%-adapted enzyme; ○ ●, 25%-adapted enzyme.

Ionic effects and peak enzyme activity

Since activities of the enzyme before and after adaptation to different ionic media were of particular interest, studies to determine the optimum salt incubation media were initiated before making direct comparisons between *Mytilus* tissues in different acclimated states. The effects of variation of Na, K and Mg in the reaction medium are summarized in Figs. 4–6, where each point represents at least triplicate determinations from the pooled connective samples. During variations of Na and K the activity of the Mg-ATPase remained roughly constant at a mean of 18·89 μmol P_I mg protein^{-1} h^{-1} for the unadapted enzyme, and 20·62 μmol P_I mg protein^{-1} h^{-1} in the adapted (25%) situation. Variations in the Mg-ATPase in different concentrations of magnesium are included in Fig. 6.

To summarize these effects, it may be said that the Na optimum for the enzyme undergoes a distinct and significant shift after acclimation, from 100 mM to around 50 mM, while the potassium and magnesium requirements remain roughly constant, with maximal activity always occurring above 12 mM-K and at about 15 mM-Mg. These figures confirm the view that simple comparisons of activity in a uniform reaction medium would be invalid, since the Na concentrations which produce maximal enzyme activation *in vitro* are roughly those to which the pump would be exposed from within the cell before and after acclimation (Willmer, 1978*a*, *b*). Peak enzyme activities were therefore recorded for a full comparison, under the optimal conditions set out in Table 3.

Table 3. *Reaction media ion concentrations and recorded enzyme activities (in μmol P_I mg protein^{-1} h^{-1}) for* Mytilus *connectives from two different salinity regimes*

	Total ATPase medium	Mg-ATPase medium	Mg-ATPase activity	Na/K-ATPase activity
100 %-adapted enzyme	100 mM-Na 12 mM-K 15 mM-Mg	224 mM sucrose 15 mM-Mg	21·30 ±1·36	18·34 2·36
25 %-adapted enzyme	50 mM-Na 12 mM-K 15 mM-Mg	124 mM sucrose 15 mM-Mg	21·97 ±1·71	29·64 ±3·13

Table 4. *Effects of varying ouabain concentrations on Na/K-ATPase activities recorded in* 100 %- *and* 25 %-*adapted mussel connectives*

	Control activity	Ouabain 10^{-3} M	Inhibition (%)	Ouabain 5×10^{-3} M	Inhibition (%)
100 % Na/K-ATPase	23·27	6·55	72·0	2·31	90·1
25 % Na/K-ATPase	41·20	28·63	30·4	2·81	93·2

These figures for enzyme activity include data from fourteen separate determinations and indicate a very significant increment of 63 % in maximal Na/K-ATPase activity in the adapted nerves, with no significant change in the Mg activated enzyme.

Effects of ouabain

To ensure that the measured Na/K activated enzyme was indeed related to the conventional sodium pump, the effects of ouabain on the *in vitro* enzyme system were checked. Preliminary tests indicated no significant inhibition of the Mg ATPase, and only limited effects on the Na/K enzyme, if the drug and microsomal suspension were not pre-incubated. However, the effects of ouabain on the latter enzyme were very pronounced if a 10 min pre-incubation was permitted; the magnitude of the inhibition produced is given in Table 4. Thus, while the sodium–potassium ATPase was clearly inhibited by the cardiac glycoside, confirming its specific relation to the pump which may be similarly inhibited, the adapted enzyme was significantly less sensitive to the drug at 10^{-3} M, with both enzymes requiring up to 5×10^{-3} M for nearly complete inhibition.

(3) [^{3}H]*ouabain binding studies*

The uptake of ouabain over a considerable concentration range for normal and for 25 % adapted tissues is given in Figs. 7 and 8. Above 10^{-3} M glycoside concentrations, the uptake was linear in each case, but a small non-linear component approached saturation at about 10^{-3} M. This saturable uptake is represented by the dashed line. The presence of two uptake phases accords with the situation in other tissues (Landowne & Ritchie, 1970; Ellory & Keynes, 1969; Baker & Willis, 1969, 1972*a*), but in *Mytilus* the relative insensitivity of the tissue leads to the large non-specific drug uptake almost swamping the saturable phase, as predicted by Baker & Willis (1972*a*).

Evidence linking saturable uptake with binding to the sodium pump has been summarized elsewhere (Baker & Willis, 1972*b*), so that extrapolation can be performed

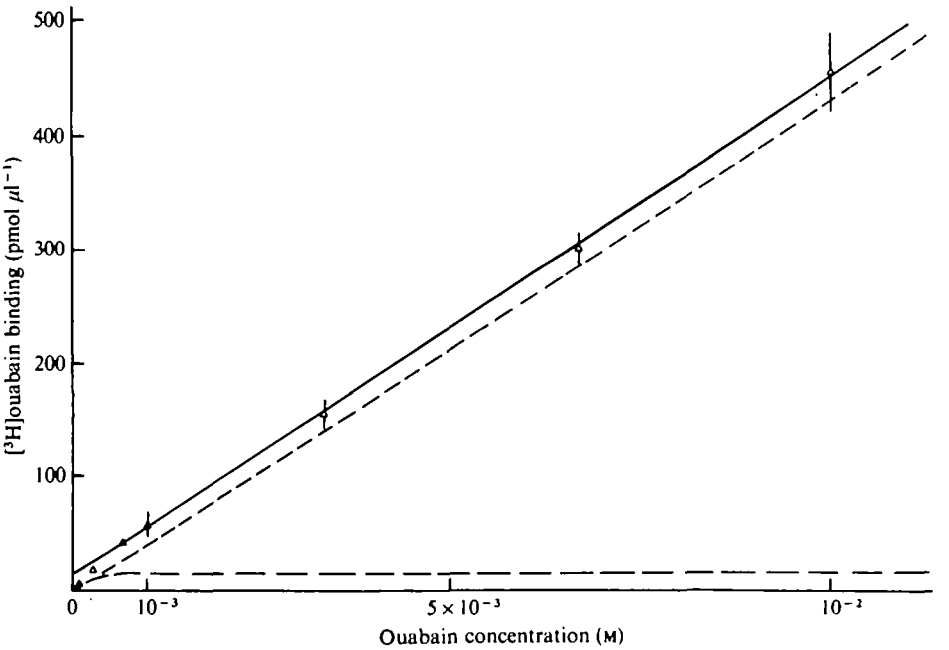

Fig. 7. [³H]ouabain binding to 100 %-adapted nerves, together with the linear and saturable components which comprise the total binding. Saturable uptake = 12·5 pmol μl^{-1}.

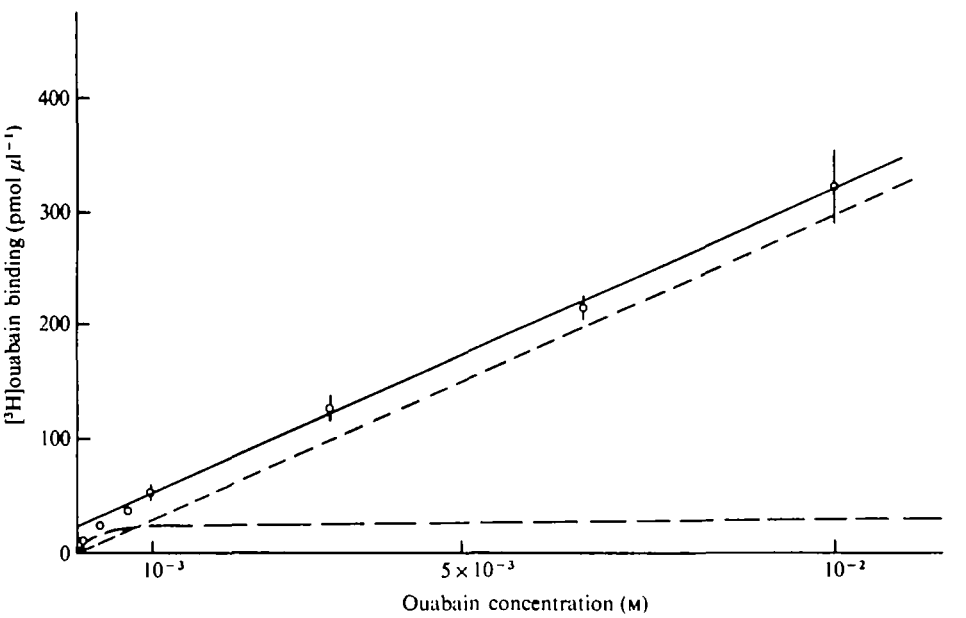

Fig. 8. [³H]ouabain binding to 25 %-adapted connectives. Saturable uptake = 22·0 pmol μl^{-1}.

to determine the actual concentration of specifically bound drug related to this pump. Values for *Mytilus* connectives thus found are 12·5 pmol μl^{-1} in unadapted nerves and 22·0 pmol μl^{-1} in dilute adapted tissues. These figures are clearly the result of a small difference between two much larger figures and are therefore subject to considerable error, but the relatively small standard errors obtained render the calculations reasonably justifiable, and the implied increment of 76% in binding sites in the adapted nerves is clearly in good agreement with the figures derived from ATPase assays.

DISCUSSION

This paper brings together several lines of evidence which combine to suggest a change in the sodium pumping capacity of *Mytilus* axons according to their state of acclimation. First indications of such changes came from sodium efflux studies designed to characterize Na movements across the cell membranes in this tissue, although these studies have also successfully highlighted certain other features of the distribution and flux of sodium within the connective. In particular, half-times for sodium loss from each of the three compartments proved to be only marginally shorter than those found for the saccharide molecules analysed in a previous communication (Willmer, 1978*a*), although dextran (120–160 Å) and inulin (32 Å) (Grotte, 1956) have much larger molecular sizes than a sodium ion (5·12 Å – Solomon, 1960). Application of the Hill (1928) equation to these data indicates values for the diffusion constant (D′) of about $1{\cdot}4 \times 10^{-6}$ cm^{-2} s^{-1} for fraction A, and of $2{\cdot}4 \times 10^{-7}$ cm^{-2} s^{-1} for fraction B, in 100%-adapted mussels, these figures being rather low for free diffusion (cf. Keynes, 1954). Sodium movements may therefore be somewhat restricted, partly by the tortuous intracellular paths which must be followed but perhaps also by an extracellular charged matrix. Similarly, the presence of a larger very fast fraction for Na (33%) relative to the saccharides (22%) may correlate with the occurrence of anionic charges in the neural lamella; for instance, collagen is likely to be present (Willmer, 1978*a*), and the free anionic equivalents associated with this material (Tristram, 1953) could produce such an effect. This view is supported further by using the flux data to calculate sodium concentrations within each compartment, as their relative volumes have already been determined (Willmer, 1978*a*). The intracellular concentrations for mussels were thus found to be 97·8 mM (unadapted) and 46·4 mM (25% adapted), in excellent agreement with previously published figures (Willmer, 1978*a*, *b*); while estimated concentrations in fraction A, assuming its volume to be that of the sheath, were 625 mM and 224 mM respectively, consistent with considerable ionic binding.

The movement of sodium across cell membranes in *Mytilus* is, on all the available evidence, related only to fraction C uptake and efflux, and the very similar degree of inhibition produced by DNP, ouabain and K-free media suggests that a conventional sodium pump is the main determinant both of the rate of efflux and of the equilibrium concentration reached in the cells. Calculation of the activation energy of sodium movements further strengthens this view; if the Arrhenius relation is definitely integrated between two temperatures (in this case 4 °C and 18 °C), the value of ΔH_A may be calculated as $\Delta H_A = (\log k_2/k_1\ 2{\cdot}303\ T_2T_1R)/(T_2 - T_1)$, where k_2 and k_1 are the rate constants at each temperature ($k = 0{\cdot}693/T_{0{\cdot}5}$). The value of ΔH_A for unadapted

Table 5. *Calculated values of influx* (m_I), *efflux* (m_O) *and permeability* (P_{Na}) *for sodium in* Mytilus *connectives adapted to either* 100% *or* 25% *salinity*

	m_I (pmol cm^{-2} s^{-1})	m_O (pmol cm^{-2} s^{-1})	P_{Na}(cm s^{-1})
100% axons	2·18	1·03	$1{\cdot}84 \times 10^{-6}$
25% axons	0·70	0·26	$2{\cdot}39 \times 10^{-6}$

Mytilus axons was 24·6 kcal mole^{-1}, a figure in good agreement with direct estimates of sodium pump activation energy in ox brain where a range of 18·5–29·1 kcal mole^{-1} was found (Charnock, Simonson & Almeida, 1977).

Given the assumption that the sodium pump is the primary determinant of outward sodium fluxes, consideration of actual fluxes across the membrane and of membrane permeability to sodium should provide useful information about the role of the pump before and after dilute acclimation. These figures, calculated from the uptake and efflux data using conventional equations (e.g. Keynes, 1954; Brinley & Mullins, 1965) are displayed in Table 5. The assumptions were made that uptake after 3 min and up to 90 min reflects net cellular gain of sodium, and that the membrane potential in *Mytilus* is −60 mV: available evidence suggests that the R.P. is unaltered after acclimation (Willmer, 1978*b*), so that comparisons based on such an assumption should be valid. Surface areas of axon membrane in the connective were calculated from micrographs.

Thus, while net influx and efflux of sodium in an adapted connective are reduced to around 25–30% of their pre-adaptation values, due to the dilution of sodium levels involved, the actual cell permeability to Na is approximately 30% greater after acclimation. All the values given in Table 5 are roughly one order of magnitude lower than for the squid axon (Brinley & Mullins, 1965), and for cockroach connectives (Treherne, 1966) (though they are more comparable with calculations for frog muscle – Mullins & Frumento, 1963). In comparison with other nerves, *Mytilus* connectives therefore appear to have a relatively low permeability, a finding which would accord with widespread reports that surface permeability of the tissues is reduced in euryhaline organisms (see review by Lockwood, 1976). In fact the half-time of efflux in *Mytilus* axons is slower than has been found in *Mytilus* muscle (Potts, 1959) and in *Anodonta* nerves (Mellon & Treherne, 1969), suggesting that the axons of osmoconformers may have a particularly low permeability. Furthermore, the tendency for inward permeability of the axon surfaces to increase in dilute media is the reverse of the effect found in other tissue types from invertebrate conformers, and may therefore reflect a specific response of the nerves to permit a maintained sodium-based spike; a comparable effect has been suggested earlier on the basis of an apparently increased Nernst gradient for active sodium entry in adapted nerves (Willmer, 1978*b*).

The remaining evidence presented in this paper seeks to establish the changes which occur in sodium pump activity during acclimation processes in *Mytilus* nerves. Firstly, from the dose–response curves for ouabain (Fig. 2), there is clearly a significant shift of sensitivity along the concentration axis after adaptation. This result could suggest a roughly threefold increase in ouabain-binding sites, providing there was no non-specific drug action, $C_{0.5}$ altering from $3{\cdot}5 \times 10^{-4}$ M to $1{\cdot}0 \times 10^{-3}$ M in dilute-adapted animals.

More convincing evidence for an increased activity of sodium pumps is obtained from the ATPase and ouabain-binding studies, which if taken together must suggest an actual increase in frequency rather than a change in individual pump molar activity. Each set of experiments indicated an increase of roughly 70% in the pump-sites. The ATPase assays also revealed a further effect on the pump, since the optimal requirement for Na showed a significant shift, being reset at a value closer to the acclimated level of $[Na^+]_I$ at which the pumps would be operating.

Actual levels of ATPase activity recorded here are in good agreement with those obtained by similar preparative techniques in a wide range of tissues, including other invertebrate nerves (Skou, 1957; Bonting & Carravaggio, 1962; Camejo *et al.* 1969). The proportions of Mg-activated enzyme to Na/K-activated enzyme are also in accordance with these earlier studies. Calculation of the number of ATPase (pump) sites is possible for comparative purposes. If the molar activity of the enzyme is taken as 10000 molecules of ATP hydrolysed per minute (see Clausen & Hansen, 1974; Jorgensen, 1975), the site densities in 100% and 25% nerves are roughly 1200 and 1950 sites/μm^2 respectively. From the ouabain-binding data, values are somewhat higher, in the range 3000–5270 sites/μm^2 (incidentally implying that the pump molar activity may be low in *Mytilus*). Any of these values of pump frequency are comparable with the range found in other studies, including those on squid axon (Baker & Willis, 1972*b*) and non-myelinated mammalian nerves (Landowne & Ritchie, 1970), so *Mytilus* nerves are clearly not abnormal in their sodium pumping apparatus. However, these figures taken together with the sodium efflux data indicate a pump turnover rate of only 75–400 ions per site per minute, rather low in comparison to other studies (e.g. Baker & Willis, 1972*a*; Clausen & Hansen, 1974); the pump's cation transporting efficiency may therefore be limited. Some differences also occur in *Mytilus* with respect to ionic requirements of the pump when compared with other tissues; for instance crab nerve ATPase shows a 20 mM potassium optimum, with maximum activity at any Na concentration above 100 mM (Skou, 1957), whereas gills of *Procambarus* (Horiuchi, 1977) and of the eel (Kamiya & Utida, 1968) both show optima for each cation. Nevertheless it is apparent that *Mytilus* sodium pumps are essentially conventional in their action and requirements, and may be assumed to have conventional functions.

What then are the functions of the sodium pump ? The active extrusion of sodium from within the axons must have at least three major roles in any nerve operating in constant conditions: (1) long-term maintenance of sodium (and potassium) gradients which determine action potential and resting potential amplitudes; (2) short-term restoration of the ionic balance after electrical activity, and (3) volume regulation of the cells, since net cation transport can entrain osmotic water fluxes. Clearly each of these roles will be affected by acclimating the nerves to dilute media. Since the net passive influx of sodium is reduced in such conditions, and the active influx with each action potential is not likely to differ much in 100%- and 25%-adapted nerves (Willmer, 1978*b*), there is no obvious reason why more pumps should be an advantage in the acclimated axons with respect to electrical functioning, unless, on purely kinetic grounds, more pump sites must be available to achieve roughly the same net flux from a dilute solution.

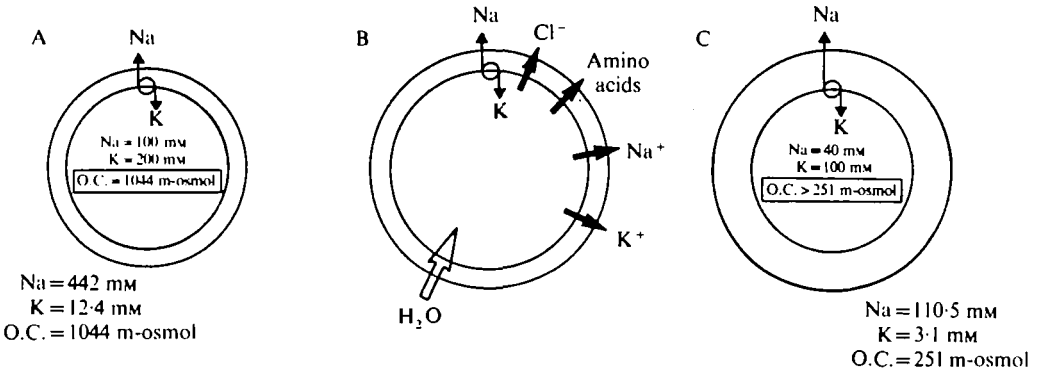

Fig. 9. A model of the likely ionic and osmotic concentrations in the extracellular and intracellular environment of *Mytilus* axons: (A) whilst acclimated to 100% salinity, (B) during acute exposure to 25% salinity, showing the probable fluxes which occur, and (C) after prolonged exposure and acclimation to 25% salinity. Swelling occurs during phase B, but after dilute acclimation the axons return to their original volume due to the control exerted by the thickened neural lamella (C).

The 'extra' sodium pumps may therefore be related to the osmotic and ionic concentrations across the nerve membrane. An earlier paper (Willmer, 1978*a*) has considered the solute balance in *Mytilus* nerves, from which the picture summarized in Fig. 9 emerges. It is considered that in dilute media the cells have a net hyperosmotic balance, losing certain amounts of intracellular ions and large proprtions of the amino-acids, but retaining non-proportional amounts of Na and K (about 40% normal $[Na^+]_I$ and 50% normal $[K^+]_I$ in a 25% dilute medium). This hyperosmoticity is maintained by an extra degree of volume control due to the thickened neural lamella, which balances the hydrostatic pressure difference; the cells show no significant long-term swelling. From this model it may be that the sodium pump has two important functions in the acclimated nerve which could explain its increased frequency.

(1) The axons clearly require vigorous volume control, presumably to prevent any tension increase being imposed on the excitable membranes; this factor may be central to the success of any acclimated nerve. To assist the structural constraints on volume, an active sodium extrusion system would be of considerable value, especially as the inward sodium permeability is somewhat increased; though since net Na flux is reduced, the kinetic argument would still have to be applied.

(2) The activity of the linked Na/K pumps will clearly partially determine $[K^+]_I$, and in the adapted axons there may be an absolute requirement to maintain $[K^+]_I$ at an acceptable value; levels above 100 mM are almost universally found in animal cells and particularly in axoplasm. The reasons underlying such a requirement have been considered by Bygrave (1967) and reviewed by Burton (1973); it is likely that cellular enzymes are dependent upon adequate potassium concentrations (of 100–250 mM) for efficient functioning.

It is not at present clear which, if either, of these postulates in fact explains the need for more sodium pumps in a dilute adapted nerve. However, the finding that such an increase occurs is of particular interest in view of a number of earlier studies which have shown comparable or even more pronounced increases (in ATPase activity,

though not necessarily in the number of pump sites) in tissues adapted in the reverse direction, from dilute media to more hyperosmotic sea water. For instance, Bonting *et al.* (1964) reported an increase in the enzyme activity in salt glands of marine herring gulls relative to those fed only on fresh water; Karlsson, Samuelsson & Steen (1971) found similar effects on duck salt glands; Epstein, Katz & Pickford (1967) and Jampol & Epstein (1970) have recorded increases in teleost gill Na/K-ATPase when transferred from fresh water to sea water; and Kamiya & Utida (1968) reported a fivefold increase in the branchial enzyme activity of eels treated similarly. Amongst the invertebrates, Horiuchi (1977) described an increment in the ATPase of freshwater crayfish gills when acclimated to 50% sea water. Each of these studies has concerned specific salt-absorbing epithelial tissues, so that together they form a significant contrast to the present work on nerves, where the biochemical and physiological priorities differ. The enzyme adaptations required in these two different types of tissue in an invertebrate must also differ accordingly, and it is to be hoped that the field of axonal osmotic adaptation will provide further informative comparisons with the considerable body of evidence already available on the adaptation of other individual tissues and of whole animals.

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