J. exp. Biol. (1978), 77, 181–205
With 18 *figures*
Printed in Great Britain

ELECTROPHYSIOLOGICAL CORRELATES OF IONIC AND OSMOTIC STRESS IN AN OSMOCONFORMING BIVALVE (*MYTILUS EDULIS*)

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

(*Received* 13 *March* 1978)

SUMMARY

1. *Mytilus edulis* suffered reductions in trans-membrane sodium concentration gradient during dilute salinity acclimation. Nevertheless the nerves of this osmoconformer had a conventional basis for their excitability, irrespective of the salinity to which they were adapted, and could produce full-sized spikes after acclimation to 25 % media.

2. The connectives showed rapid and predictable responses to sodium and to potassium ions, and the time-courses of these effects were unrelated to the presence or absence of the neural sheath at either of the acclimation salinities tested. Neural adaptation was therefore not a consequence of restricted access after acclimation to dilute media.

3. Classic pharmacological agents (tetrodotoxin, tetra-ethyl ammonium ions, 2,4-dinitrophenol and ouabain) elicited the expected responses, but invariably required rather high concentrations and long exposures for full effect.

4. Acute exposures of the connectives to hyposmotic or isosmotic dilutions resulted in changes in polarization accompanying the decline of the action potential. These were attributed to losses of potassium and of chloride from the axons, with a net loss of anion (and hence depolarization) during naturally occurring hyposmotic stress.

5. *Mytilus* connectives exhibited a critical salinity, with hyposmotic exposure below this level producing irreversible impairment of function. This salinity occurred at 20 % S.W. for 100 %-adapted tissues, and at 3·25 % S.W. (i.e. 15 % initial concentration) for 25 %-adapted nerves. Gross isosmotic dilutions produced no permanent decrements in axonal function.

6. The observed patterns of response to chronic and acute osmotic stress in *Mytilus* are contrasted with those reported in annelid and crustacean conformers, and possible adaptations which might underlie these responses are discussed.

INTRODUCTION

A previous paper (Willmer, 1978*a*) has indicated a significant reduction in the trans-membrane sodium concentration gradient in the axons of *Mytilus edulis* when adapted to dilute media. The present communication extends these observations and considers their effect on the resting membrane and on the spike-generating mechanisms

of this euryhaline bivalve, in which the ionic basis of excitability and the signalling capacity of the nerves are shown to be constant irrespective of blood concentrations over the salinity range tolerated by laboratory animals. The acute effects of altered osmotic concentrations on the electrophysiological performance of the nerves are also considered, since the axons of this osmoconformer clearly have considerably greater capacity for functioning in dilute media than those of a stenohaline marine animal such as *Maia squinado* (Pichon & Treherne, 1976) or the classic vertebrate nerve preparations (cf. Schmidt & Stämpfli, 1959).

Neurophysiological studies of lamellibranchs have been comparatively rare. Most of the early work using extracellular electrodes, with *Mytilus* (Woortmann, 1926), *Anodonta* (Nadort, 1943; Barnes, 1955) and *Mya* (Horridge, 1958, 1961), was concerned with establishing neural pathways and conduction velocities. There are only isolated analyses of the ionic basis of the spike; examples include both *Mytilus* (Twarog & Hidaka, 1971) and *Anodonta* (Treherne, Mellon & Carlson, 1969; Treherne, Carlson & Gupta, 1969; Sattelle & Howes, 1975), where conventional dependence on sodium has been demonstrated in spite of the very different blood concentrations of these two mussels. This relative lack of information is partially attributable to the uniformly small size of bivalve axons (cf. Willmer, 1978*a*), which precludes microelectrode penetration of the cells. All studies described here therefore involved a modified 'sucrose-gap' recording technique (Stämpfli, 1954). While this inevitably involves some attenuation of the signal, action potentials of considerable magnitude have been thus recorded in a variety of tissues; in particular, work on *Mytilus* myocardium has shown close agreement between intracellular and 'gap' records for both action potentials and d.c. resting potentials (Wilkens, 1972*a*, *b*). Similarly, Schofield & Treherne (1978) have shown a reasonable equivalence of Nernst slopes derived from intracellular and sucrose-gap recordings in the cockroach. However, since this paper describes experiments involving changes in total concentration (and hence conductance) of the bathing medium, attenuation of the signal cannot be assumed to be linear; consequently these results are primarily treated as non-quantitative.

METHODS AND APPARATUS

Lengths of at least 2·5 cm of cerebro-visceral connective were dissected free of the epithelial and renal tissues, and ligated at either end with fine cotton. In some instances the connectives were 'desheathed' as described in the previous paper.

The recording chamber consisted of five compartments drilled from Perspex, each bisected by the narrow central channel housing the nerve. Platinum wires in the two right-hand compartments provided stimulation from a Farnell unit, via an RF-coupled isolator. Chambers 3 and 5 were connected to Ag/AgCl indifferent and recording electrodes through agar bridges; the recording electrode passed signals to a cathode follower head and unity-gain impedance converter, and thence to a Tektronix 502 oscilloscope. Permanent records were obtained with a Nikon–Kohden camera and on a Smith-Servoscribe pen-recorder.

Compartment 3 contained the flowing test solutions, which could be rapidly changed via a multi-way valve, and compartment 4 was filled either with a pool of oil or with flowing isosmotic mannitol, these two non-electrolytes for the 'gap' having lower

ionic contents than sucrose (cf. Pichon & Treherne, 1970; Callec & Sattelle, 1973). All other chambers contained isosmotic salines and were isolated from each other by silicone grease seals (Blaustein & Goldman, 1966) to eliminate liquid-junction potentials.

The normal *Mytilus* Ringer was derived from measurements of blood concentration as described in an earlier paper (Willmer, 1978*a*). Osmotic dilutions of this basic Ringer were made with distilled water (hyposmotic) or with mannitol solutions (isosmotic) as appropriate. Increments in osmotic concentration were obtained by addition of aliquots of NaCl.

RESULTS

Compound action potentials recorded from *Mytilus* axons were up to 16 mV in amplitude, with a normal range of 6–10 mV. Although larger than those recorded from *Anodonta* nerves using similar techniques (Sattelle & Howes, 1975), these A.P.s are not comparable with intracellular recordings from molluscan cell bodies, and are presumed to be considerably attenuated. The spikes had a duration of 400–1000 ms, reflecting their origin from many slowly conducting small axons; their long time-course enabled direct pick-up with the pen-recorder, and in some cases these records alone are shown. Recorded A.P.s were identical in natural sea water and in the Ringer solution, and were unaffected by the non-electrolyte used. The ranges of amplitude, duration and shape were constant, whether the spikes were elicited from full-S.W. adapted animals or from 25%-adapted connectives, and were not affected by desheathing.

(*a*) *Ionic effects on* Mytilus *nerve*

(1) *Effects of sodium ions*

Variations of external sodium concentration produced considerable changes in amplitude of recorded A.P.s, whether the cation was replaced with Tris or with mannitol (Fig. 1). Using these data, a relationship equivalent to the Nernst slope may be plotted, if allowance for the variation in initial spike height is made by using a relative amplitude scale. The mean effects of sodium replacements on intact and desheathed connectives are shown in this way in Fig. 2, for both unadapted and dilute-adapted tissues. These plots indicate that the A.P. is always a simple logarithmic function of $[Na^+]_O$, confirming the primary role of this ion in axonal excitability. (Recorded slopes for individual preparations plotted on an absolute scale were in the range 11–18 mV per decade change in Na concentration, but the real sodium dependence clearly cannot be deduced from analysis of the Nernst gradient as the extracellular technique employed limits the significance of this parameter.) The results in Fig. 2 also suggest that the integrity of the neural lamella makes little difference to conduction processes in *Mytilus*, concurring with the earlier findings of Twarog & Hidaka (1971).

It is possible to perform linear extrapolations of the 'relative' Nernst plots of Fig. 2 to determine the point of abolition of the A.P., which would give an estimate of $[Na^+]_I$. Since Nernst relationships commonly cease to hold accurately at lower levels of external Na, this linear projection is strictly not justified, but should at least permit an estimate of maximum $[Na^+]_I$. The individual plots yielded a range of 86–119 mM, with a mean of 100 mM for unadapted nerves, and 34–47 mM, mean 40 mM, for 25%-

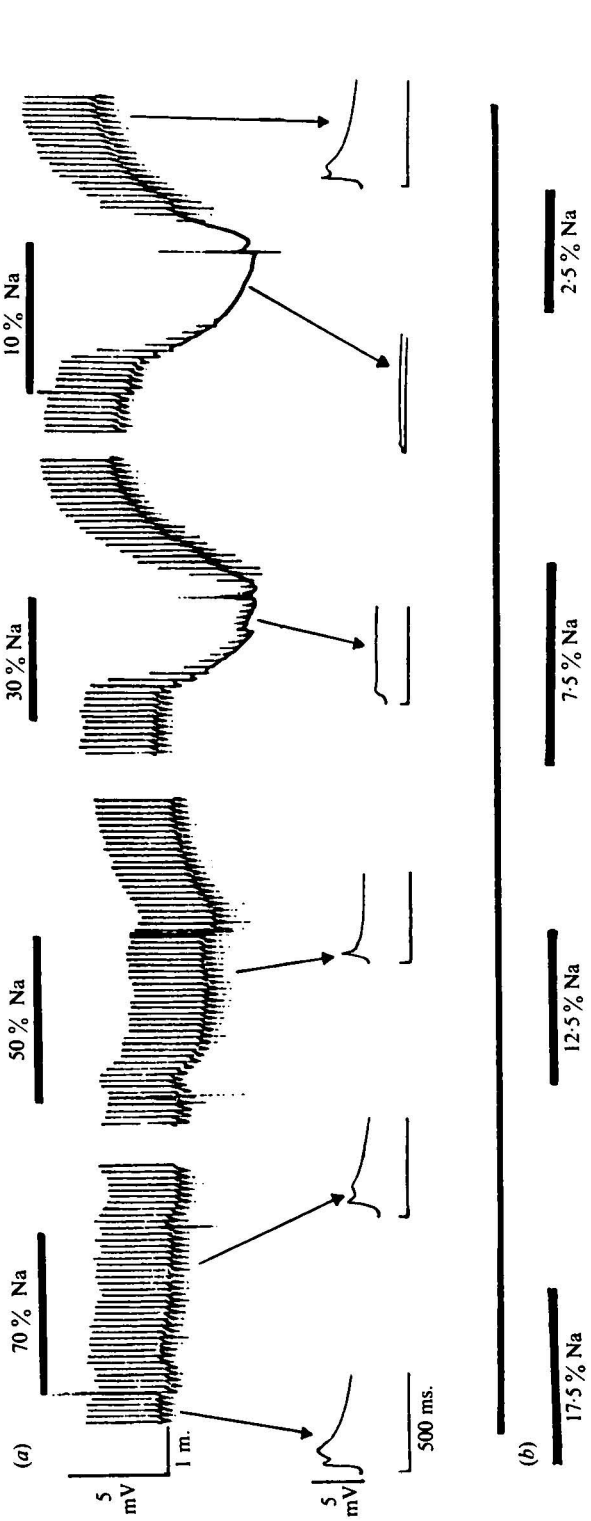

Fig. 1. Effects of reduced sodium concentration (substituted by Tris) on (*a*) 100 %-adapted and (*b*) 25 %-adapted *Mytilus* connectives. Chart records of the complete responses are shown together with individual A.P.s at each value of $[Na^+]_o$.

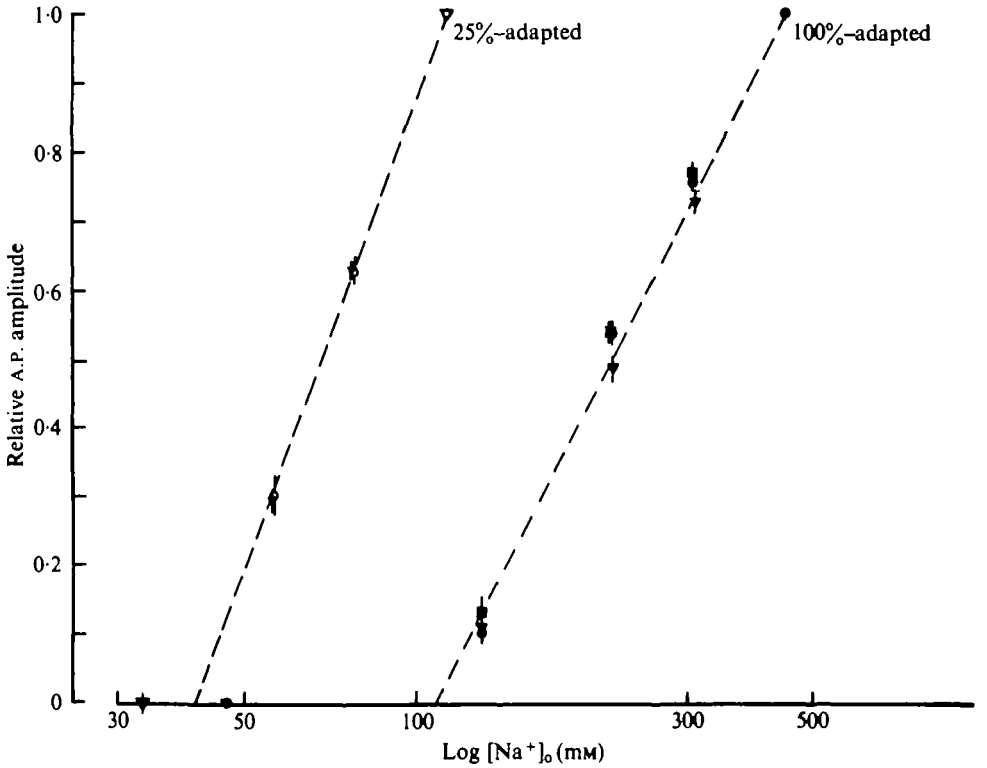

Fig. 2. Nernst plots for the relative A.P. height at varying $[Na^+]_O$ for connectives adapted to different salinities. Solid symbols indicate 100%-adapted nerves: ●, intact, Tris substitution; ■, intact, mannitol substitution; ▼, desheathed Tris substitution. Open symbols indicate 25%-adapted nerves: ○, intact; ▽, desheathed, both using Tris substitution. Vertical bars represent 2 S.E.M. in this and subsequent figures.

adapted nerves. The fact that adapted connectives have apparently reduced their internal Na by less than the predicted 75% is also reflected in the slight increase in gradient of the Nernst slope after acclimation, and accords very well with determinations of $[Na^+]_I$ described in the previous paper (Willmer, 1978*a*).

Calculation of the half-times of sodium responses was of interest, both for analysis of the 'symmetry' of the system with respect to this ion, and for comparison with tracer studies to be reported separately (Willmer, 1978*b*). Using directly plotted Nernst slopes for each preparation, momentary values of A.P. amplitude were converted to the equivalent Na concentration at the axon surfaces and a standard plot of $C_\infty - C_t/C_\infty - C_O$ was constructed against time. An example of such a relation is given in Fig. 3, showing initially complex movement followed by simple first-order diffusion. The results of all such calculations are summarized in Table 1 (for entry (I) and exit (O) of sodium at varying concentrations) as half-times of this linear phase.

Movement of Na in and out of the tissue thus appears to be essentially independent of concentration in intact nerves, and there are no significant differences between the intact connectives from the two salinities. At very low external sodium concentrations, the desheathed preparations tended to recover rather more slowly, but otherwise behaved similarly to their intact counterparts. There is therefore no significant asymmetry of ionic fluxes, and no evidence for a peripheral barrier to sodium.

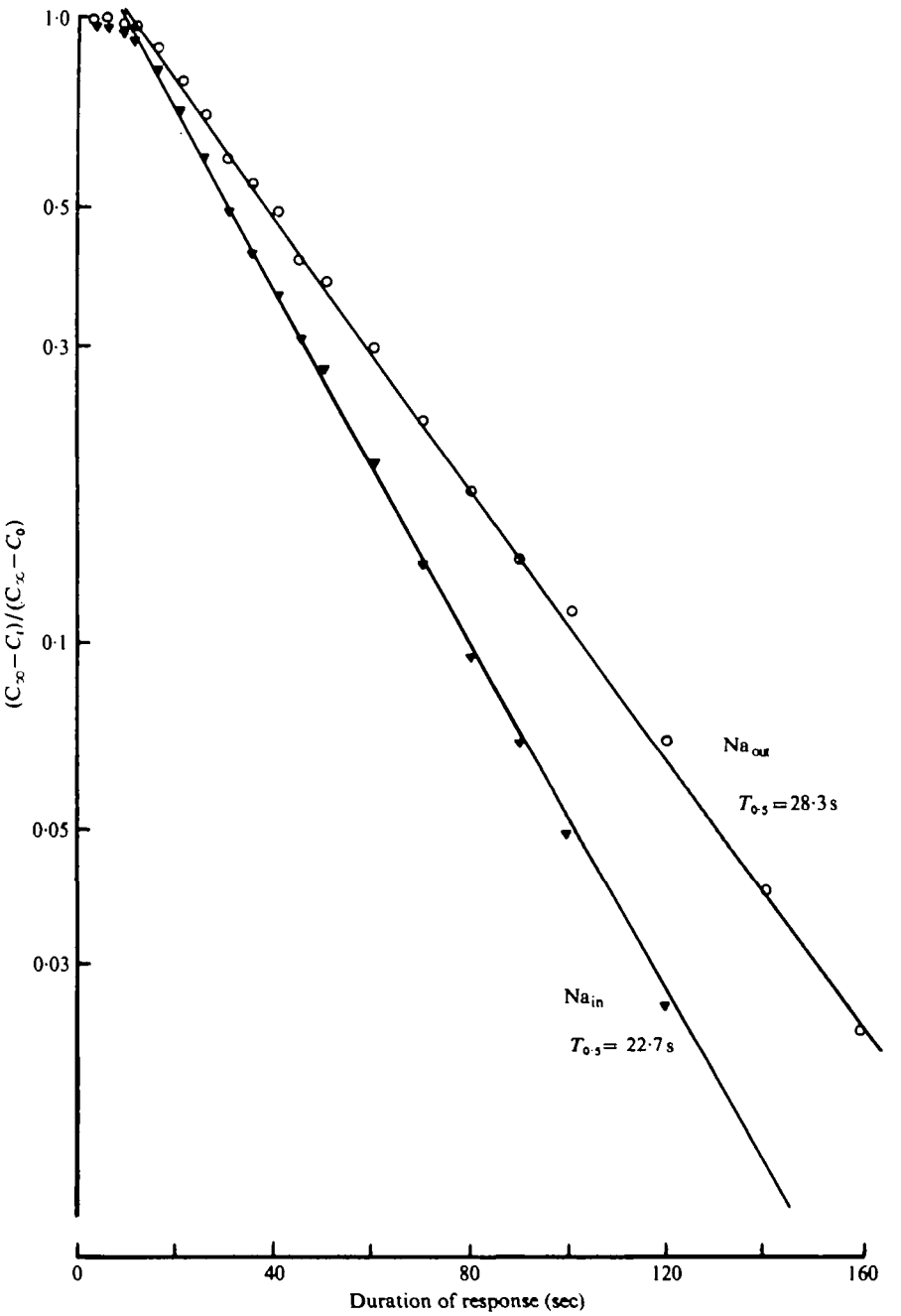

Fig. 3. An example of sodium movements (determined from the electrophysiological effects on the nerve) in a 100%-adapted *Mytilus* connective, during exposure to 221 mM-Na and subsequent recovery in normal solutions (442 mM-Na). Ordinate is relative concentration; subscripts refer to concentrations (C) at time o, t and infinity. ▼, Sodium entry; ○, sodium exit. Values for $T_{0.5}$ in these experiments are summarized in Table 1.

Table 1. *Half-times of influx (I) and efflux (O) for sodium in connectives from* Mytilus *acclimated to different salinities*

Results are given as mean ± 2 S.E.M. for the linear phase of the ion movements (cf. Fig. 3), during exposures to solutions of varying sodium concentration.

% Initial Na		70 %		50 %		30 %		10 %	
$T_{0.5}$ (min)		I	O	I	O	I	O	I	O
100 % nerves	Intact (n = 7)	33·1 ±2·0	36·2 ±1·8	29·9 ±3·0	33·7 ±1·9	32·2 ±2·7	37·1 ±4·0	33·5 ±3·3	34·2 ±2·2
	Desheathed (n = 5)	30·0 ±1·8	42·5 ±3·3	31·4 ±2·7	43·5 ±2·4	34·6 ±2·6	46·2 ±1·2	31·4 ±3·9	50·1 ±4·4
25 % nerves	Intact (n = 6)	34·2 ±2·3	39·2 ±1·8	30·1 ±3·4	38·7 ±1·9	34·0 ±4·0	41·1 ±4·1	35·3 ±3·2	43·2 ±2·9

(2) *Effects of potassium ions*

Resting potentials recorded by depolarizations with isosmotic KCl were of a similar range in both normal and 25 %-adapted connectives, the mean values being 31·1 mV and 30·3 mV respectively. Applications of Ringers containing increments of potassium resulted in graded depolarizations, indicating that the resting potential is conventionally dependent on this ion. Examples of the effects obtained appear in Fig. 4 and show that the A.P. is also abolished at high $[K^+]_0$; thus the effect is probably of cellular origin, rather than being an extraneural potential change such as occurs in insects (Pichon & Treherne, 1970). By arbitrarily using the depolarization in the highest K concentration (321 mM for normal nerves, 80·5 mM for 25 %-adapted nerves) as a reference point, the mean effects of potassium are presented in Fig. 5 as relative Nernst slopes; the relation again appears to be simple logarithmic over most of the concentration range, and gradients were in the range of 22–29 mV per decade change in $[K^+]_0$ above 20 mM K. However, application of zero-potassium Ringers produced no polarization changes, so the Nernst slope must flatten off markedly over its lower range: a similar effect has been reported in *Mytilus* myocardium (Irisawa, Shigeto & Otani, 1967).

The time-courses of potassium responses were calculated as before, and an example for 233 mM $[K^+]_0$ appears in Fig. 6. Again this shows initial complexity succeeded by linear diffusional efflux; the half-times in different tissues are listed in Table 2.

As with sodium movements, potassium entry and loss for all nerves, whether or not dilute adapted, and whether intact or desheathed, have similar time-courses; the neural lamella does not constitute a barrier to either of these ions, and there is no greater restriction to the access of ions after adaptation to 25 % salinity.

(3) *Other ionic effects*

Since calcium may carry part of the inward current in some nerves and could in theory become a convenient inward charge carrier in dilute media, the effects of excluding this cation, and also of excluding Mg, were tested. As chelating agents were not used, small amounts of these divalent cations probably remained; but the lack of effects of 'Ca-free' and 'Mg-free' Ringers (each ion being substituted by the other), seems to exclude a major role for them as direct current-bearing agents at either salinity (Fig. 7).

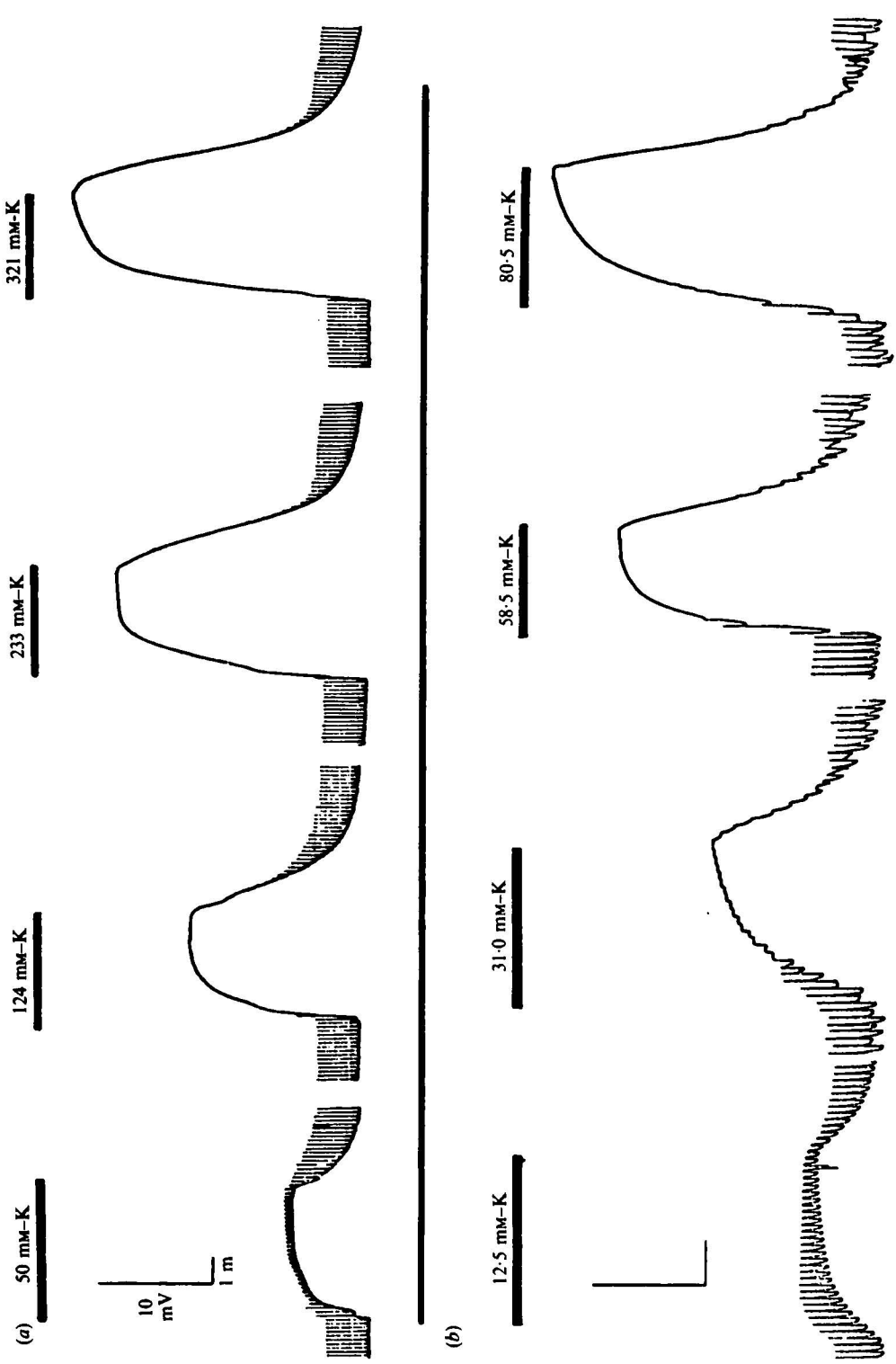

Fig. 4. The depolarizing effects of raised potassium concentrations on (*a*) 100 %-adapted and (*b*) 25 %-adapted *Mytilus* connectives. Normal $[K^+]_o$ values are 12·4 mM for (*a*) and 3·1 mM for (*b*).

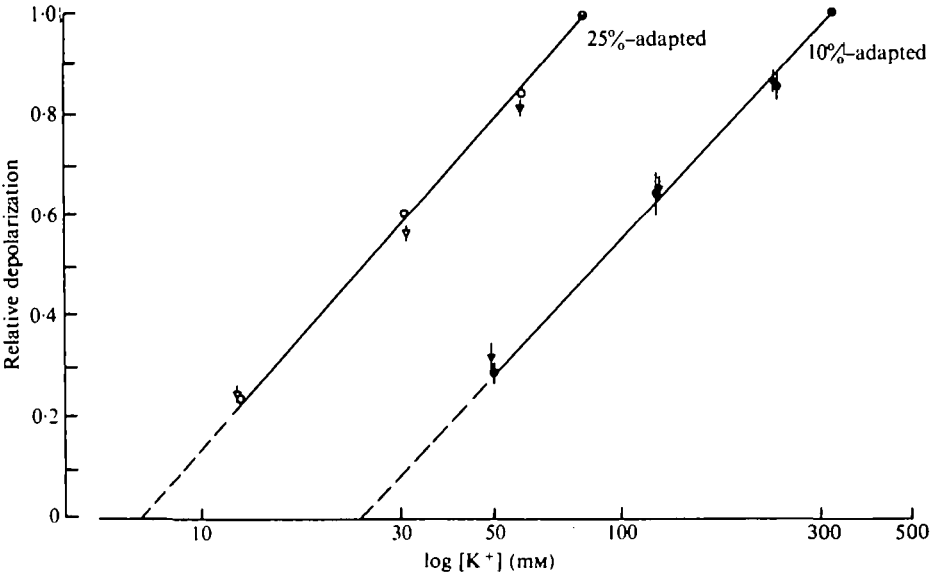

Fig. 5. Nernst plots for the effects of $[K^+]_o$ on recorded d.c. potentials, against a relative amplitude scale, comparing 100 %-adapted animals (closed symbols: ▼, intact; ●, desheathed), and 25 %-adapted mussels (open symbols: ▽, intact; ○, single desheathed preparation).

Table 2. *Half-times of influx (I) and efflux (O) for potassium in* Mytilus *connectives*

Figures represent means ± 2 S.E.M., at the levels of $[K^+]_o$ indicated for 100 %- and 25 %-adapted mussels.

	K^+ (mM)	50		124		233		321	
	$T_{0.5}$ (min)	I	O	I	O	I	O	I	O
100 % nerves	Intact (n = 7)	26·1 ±1·7	33·1 ±3·2	27·2 ±1·8	32·8 ±2·0	29·9 ±1·5	31·3 ±2·3	28·9 ±3·9	32·5 ±1·9
	Desheathed (n = 6)	28·2 ±0·9	30·2 ±1·2	27·5 ±3·3	31·1 ±2·5	27·8 ±1·4	33·6 ±2·8	29·0 ±3·7	37·2 ±3·1
	K^+ (mM)	12·5		31·0		58·5		80·5	
25 % nerves	Intact (n = 6)	30·2 ±1·2	35·1 ±3·2	31·0 ±3·7	33·7 ±2·8	30·6 ±3·3	36·4 ±2·6	34·2 ±4·1	35·8 ±2·6

Also shown in Fig. 7 are the effects of replacing over 90 % of the chloride in normal Ringers with methyl sulphate ions. There were no detectable alterations in A.P. size or shape, but a small hyperpolarization generally occurred. To check the possible effects of chloride ions on the d.c. potentials, isosmotic solution of KCl and $KMeSO_4$ were applied, and the latter did produce marginally greater depolarizations in each case (mean = 32·5 mV). Thus while the divalent cations and major anion present in sea water have little or no function in the production of action potentials, chloride may have a minor role in determining the resting potential in *Mytilus*.

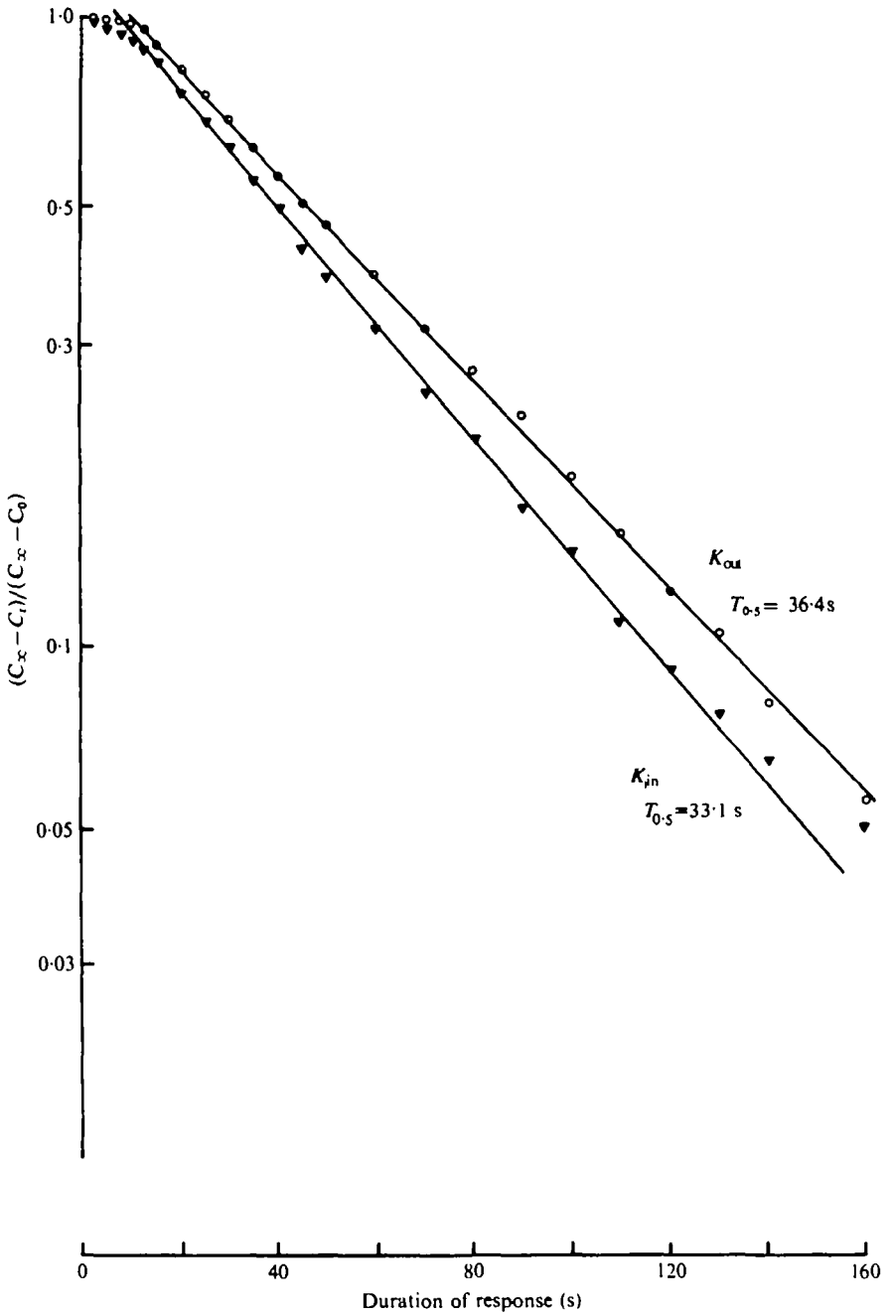

Fig. 6. Rate of potassium movements in a 100%-adapted *Mytilus* connective during exposure to 233 mM-K^+ and subsequent recovery in 12·4 mM-K^+. ▼, Entry of K^+; ○, exit of K^+. Half-times are summarized in Table 2.

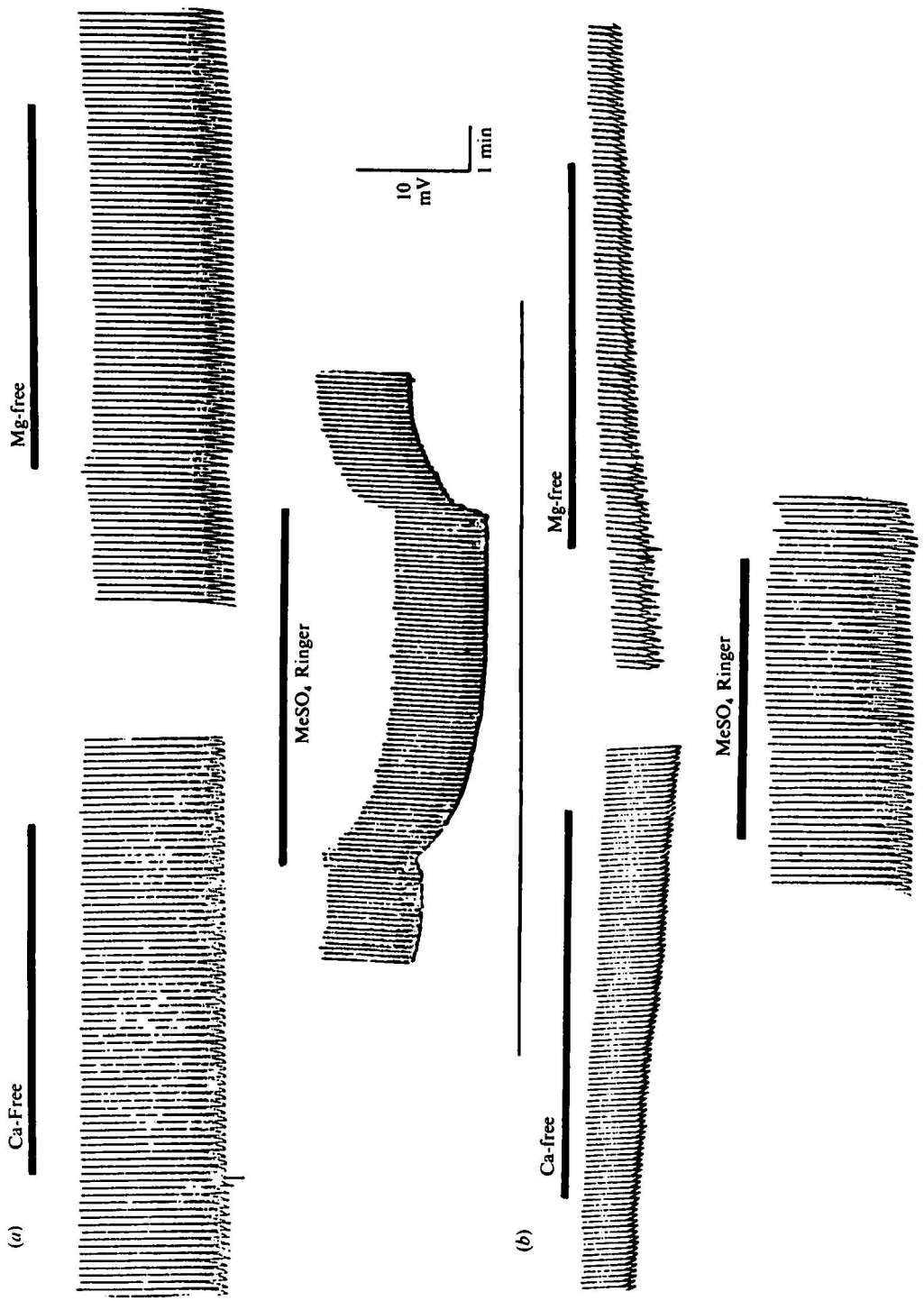

Fig. 7. Effects of divalent cation and of anion substitutions on (*a*) 100 %-adapted and (*b*) 25 %-adapted *Mytilus* connectives.

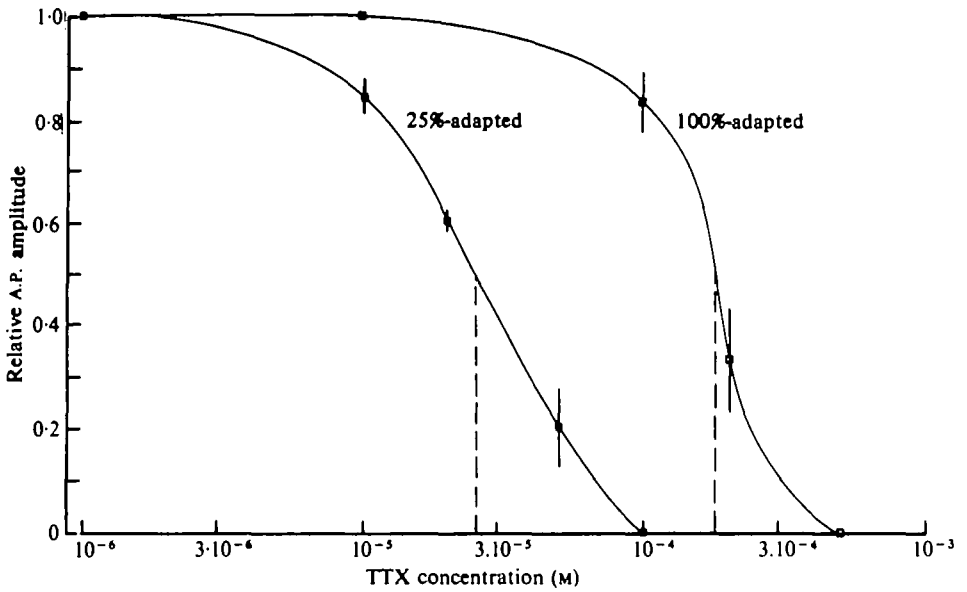

Fig. 8. Dose–response curves for the effects of tetrodotoxin at high concentrations on *Mytilus* nerves, using a relative scale for A.P. amplitude. □, 100%-adapted nerve; ■, 25%-adapted nerve. Concentrations for half maximal inhibition ($C_{0.5}$) are $1{\cdot}8 \times 10^{-4}$ M and $2{\cdot}5 \times 10^{-5}$ M respectively.

(4) *Effects of inhibitors*

To verify the proposed involvement of certain ions in the resting state and active membrane response of *Mytilus* nerves, various inhibitors and poisons could be used. The best understood of these are STX and TTX, but many bivalves show a considerable degree of immunity to both toxins (Twarog, Hidaka & Yamaguchi, 1972). Since the mechanism and extent of this resistance are not fully understood, tetrodotoxin was tested on *Mytilus* preparations at high concentrations; dose–response curves for its blocking effect are shown in Fig. 8, for both normal and 25%-adapted connectives. The blocking action of TTX was clear and reversible, and controls indicated that it was not due simply to the presence of citrate (cf. Kao, 1966), so it seems likely that the drug is having a specific inhibitory effect on the Na channel as expected. Yet the concentrations required were so high that the present results cannot be accepted until more information about TTX specificity at such levels is available. Thus, the apparent sevenfold decrease in the concentration needed for half-maximal inhibition after adaptation may be an artefact arising from reduced non-specific drug-binding in the adapted tissue.

The action of 20 mM-TEA-Cl on a 100%-adapted stimulated nerve is shown in Fig. 9; no effects were produced with 10 mM-TEA-Cl. The A.P. was at first visibly prolonged, with an accompanying depolarization of 5–8 mV; within 5 min the spike was reversibly abolished. These responses clearly accord with the view that the R.P. is potassium-based and cannot be fully restored in a stimulated nerve when gK is blocked, and that the restoring current of the A.P. is probably largely carried by potassium ions.

The slower-acting drugs 2,4-dinitrophenol and ouabain were also tested on normal

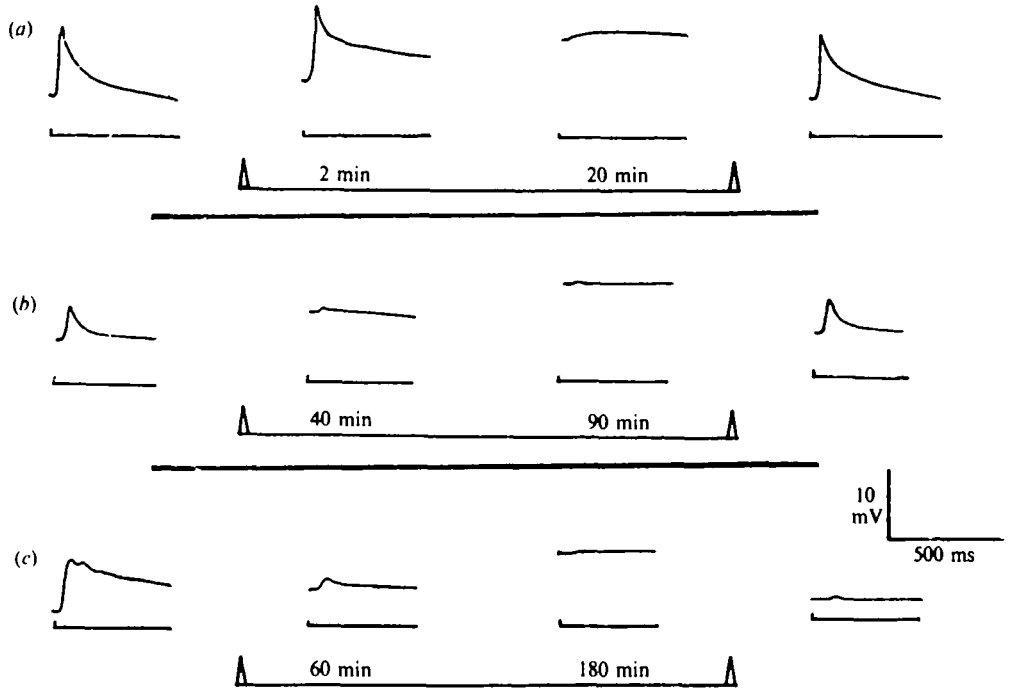

Fig. 9. The effects of (*a*) 20 mM-TEA, (*b*) 10^{-4} M DNP and (*c*) 10^{-3} M ouabain on 100 %-adapted *Mytilus* connectives. In each case the first A.P. is that obtained in normal Ringer, the second and third traces show the A.P. at stated times during exposure, and the final trace indicates the extent of maximal recovery on return to normal Ringer.

Mytilus nerves (though not on 25 %-adapted tissues) but both poisons required rather high concentrations and long exposures to achieve conduction block. DNP gave slow but complete inhibition at 10^{-4} M (with irreversible rapid deleterious effects at 10^{-3} M), and ouabain produced a blocking effect at 10^{-3} M after 2–3 h, though in the latter case the action was not readily reversible. These inhibitor effects are also summarized in Fig. 9. They suggest the involvement of some active metabolic process in the long-term maintenance of neural activity in *Mytilus*, which, from the action of the specific glycosidic blocker ouabain, is likely to be a conventional Na/K exchange pump mediated by an ATPase enzyme (Skou, 1957).

(*b*) *Osmotic effects on* Mytilus *nerves*

(1) *Hyposmotic solutions*

The effects of a series of hyposmotic dilutions of normal Ringer on the relative amplitude of both normal and 25 %-adapted connectives are shown in Fig. 10. A single example of the effects on a normal desheathed nerve is also included; in view of the similar behaviour of intact and desheathed preparations in all experiments, use of the latter was not routinely continued. In each of the tissues tested here, reduced osmotic concentrations caused a marked decline in the action potential; but these responses were not directly predictable from the known effects of reducing sodium alone (cf. Fig. 2), the A.P. persisting above that for a given value of $[Na^+]_O$.

Recovery from low salinity exposure is analysed in Figs. 11 and 12, in normal and

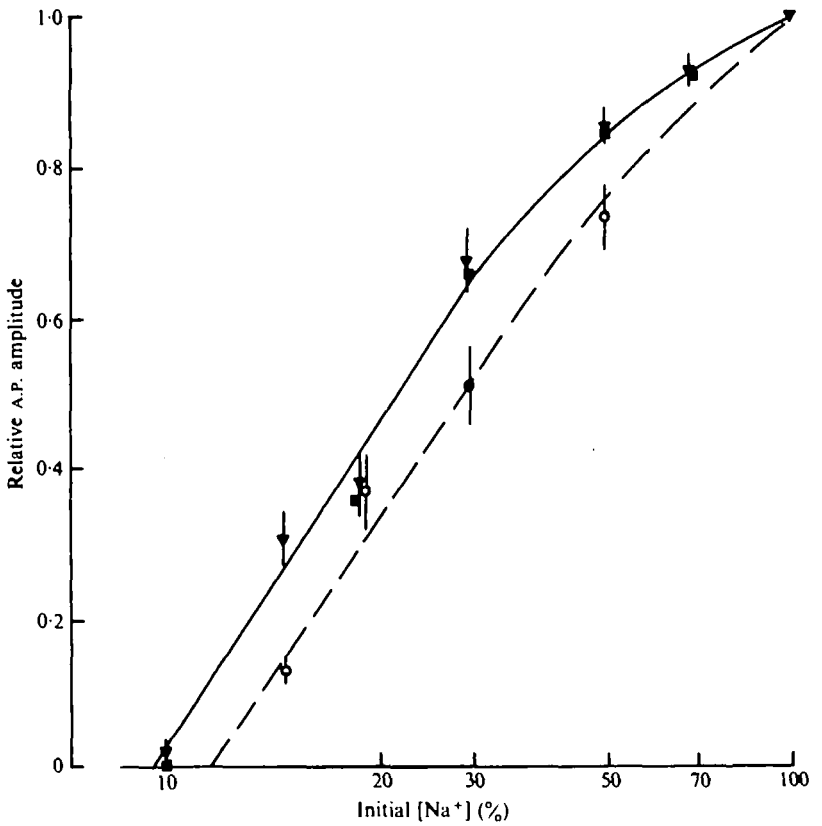

Fig. 10. The effects of hyposmotic solutions (Ringer+H_2O) on recorded A.P. height in *Mytilus* connectives, shown against a percentage concentration scale for Na. Solid symbols indicate 100%-adapted nerves; ▼, intact; ■, single desheathed preparation; open symbols and broken line indicate 25%-adapted nerves; ○, intact.

adapted tissues. Connectives from unadapted mussels showed almost complete recovery down to 20% salinity, irrespective of exposure time, but exposure to 15% or less resulted in impairment of the A.P. after return to normal Ringers, suggesting that the critical salinity for *Mytilus* is roughly equal to its lowest survival limit (at about 20% salinity). For the 25%-adapted tissues, this critical point for recovery was at about 3·25% salinity (that is, 15% of initial concentration). A single desheathed connective from each group of mussels was also tested, and the critical salinities for these were in each case 5–10% higher, with permanent loss of function at 25–30% initial concentrations.

In all tests at low salinity, a further effect observed was the presence of consistent shifts in the d.c. potential recordings. Usually there was an initial small hyperpolarization, but at lower salinities this reversed into a net depolarization. The eventual steady changes are plotted in Fig. 13; comparison of normal and adapted connectives indicates just significantly reduced values of maximum depolarization in the latter for equivalent dilutions. Such depolarizations could result from excess cation entry into axons, or from anion leakage, but the latter proposition is more likely when ionic levels are falling externally. The third plot in Fig. 13 shows the result of testing this hypothesis

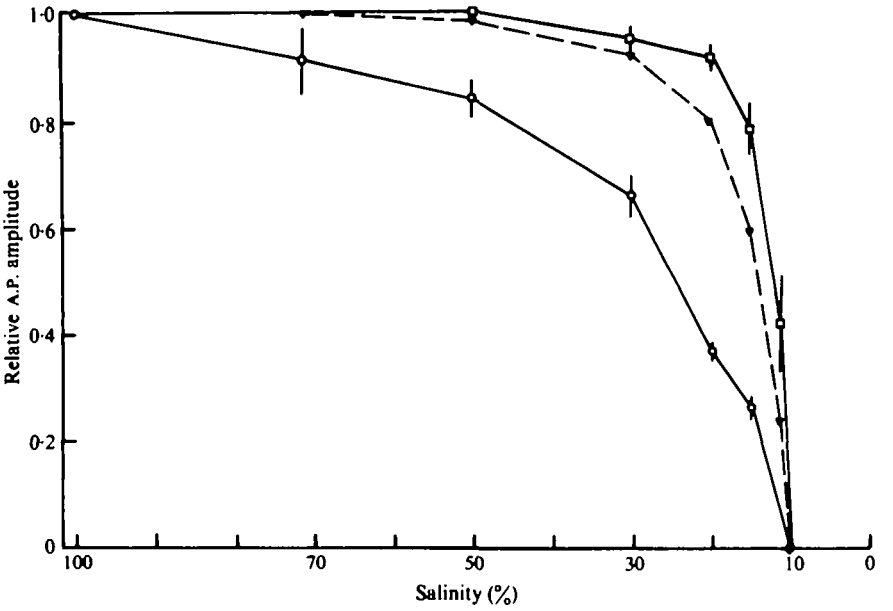

Fig. 11. Analysis of the ability to recover from hypotonic solutions in intact 100 %-adapted nerves. ○, A.P. height during dilute exposures; □, A.P. height after recovery in normal Ringer. ▽, single desheathed connective, after recovery.

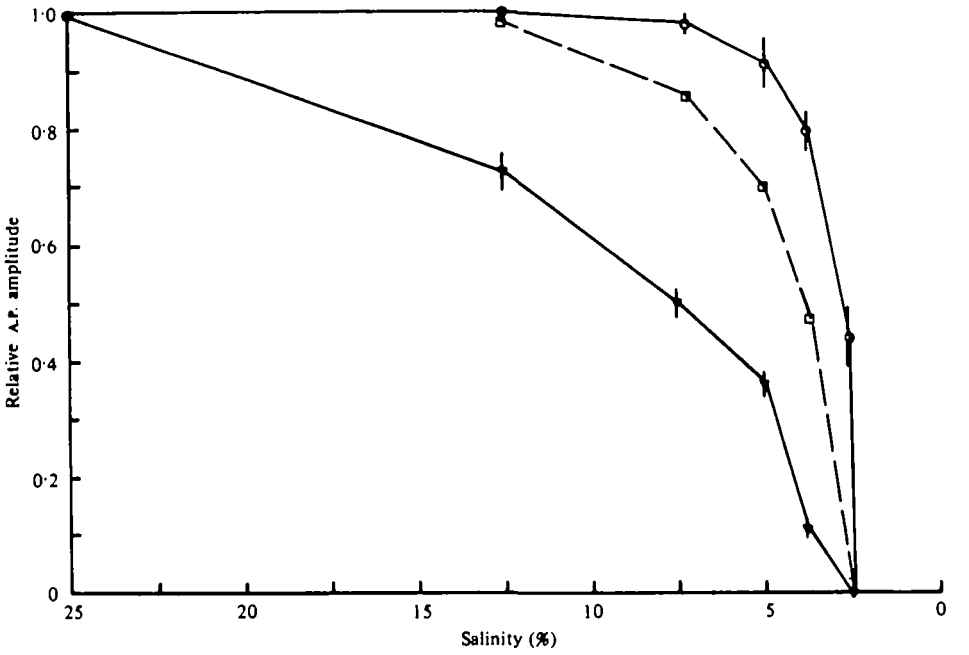

Fig. 12. Recovery from hypotonic solutions in 25 %-adapted nerves. A.P. heights are shown during low salinity exposure, ▼, and after recovery in normal (25 %) Ringer, ○. Recovery in a single desheathed nerve is also shown, □.

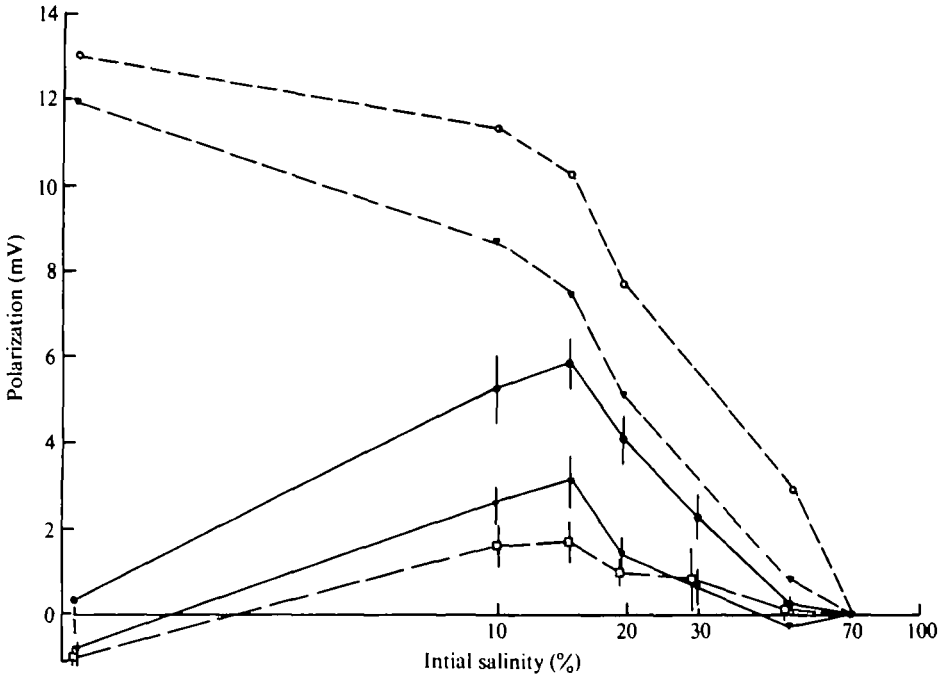

Fig. 13. Depolarizations recorded in hyposmotic media from *Mytilus* connectives, ▼, 100%-adapted nerves; □, 25%-adapted nerves, at equivalent dilutions. The effects of hyposmotic reduced-chloride Ringers on 100%-adapted nerves are also shown, ●. The significance of the broken lines is discussed in the text, p. 201).

on 100%-adapted nerves by substituting most of the chloride in normal Ringers with methyl sulphate ions. As predicted, there were significant increases in the recorded polarizations, consistent with an 'excess' chloride loss into low-chloride media. The complete effects of hyposmotic solutions, with and without normal chloride, are summarized by the examples in Fig. 14.

(2) *Isosmotic solutions*

The effects of salinity dilutions with maintained osmotic concentration (that is, sea water diluted with isosmotic mannitol) on connectives from normal or adapted mussels are shown in Fig. 15. The effects of isosmotic and hyposmotic dilutions are very similar (cf. Fig. 10), the A.P. apparently responding to some aspect of ionic concentration (principally to Na) rather than to total osmotic strength.

Recovery from gross isosmotic dilution was not impaired even after prolonged exposures, in contrast to the apparent damage incurred in hyposmotic conditions, suggesting that cellular swelling was responsible for impaired functioning in the earlier experiments.

The effects of Ringer/mannitol solutions on the d.c. potentials are plotted in Fig. 16. At all salinities below 70% there were steady hyperpolarizations, roughly proportional to the magnitude of the dilution involved and of identical size in 100%- and 25%-adapted connectives. Hyperpolarizations of similar magnitude also occurred in the experiments reported above (section 1*A*), where sodium levels were reduced and

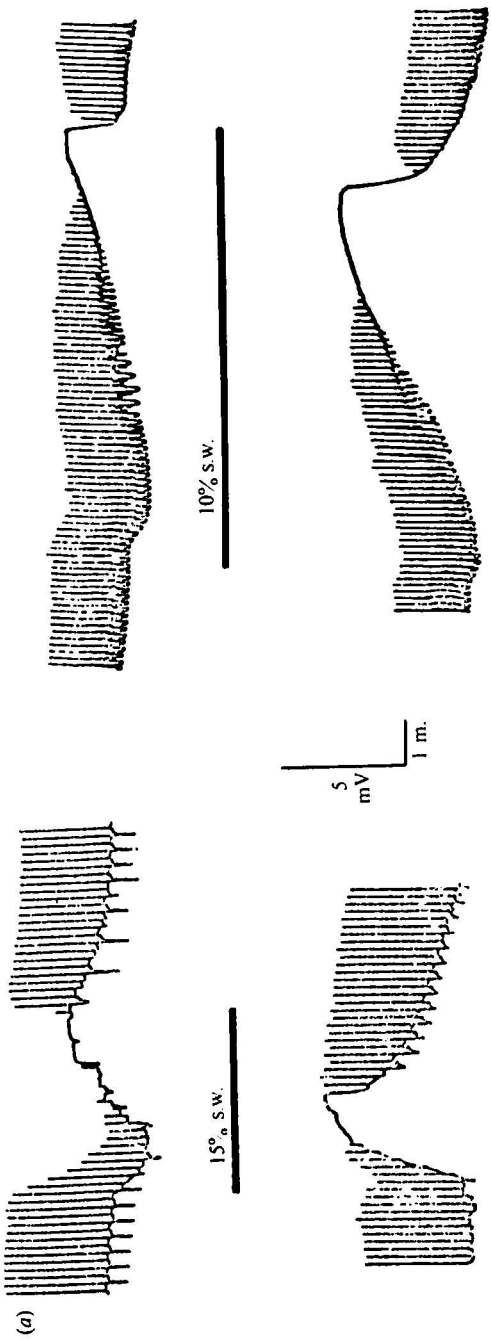

Fig. 14. Examples of the effects of hyposmotic solutions on (*a*) 100 %-adapted and (*b*) 25 %-adapted *Mytilus* connectives. The enhanced depolarizations in reduced-chloride ($MeSO_4$) Ringers are also shown for (*a*).

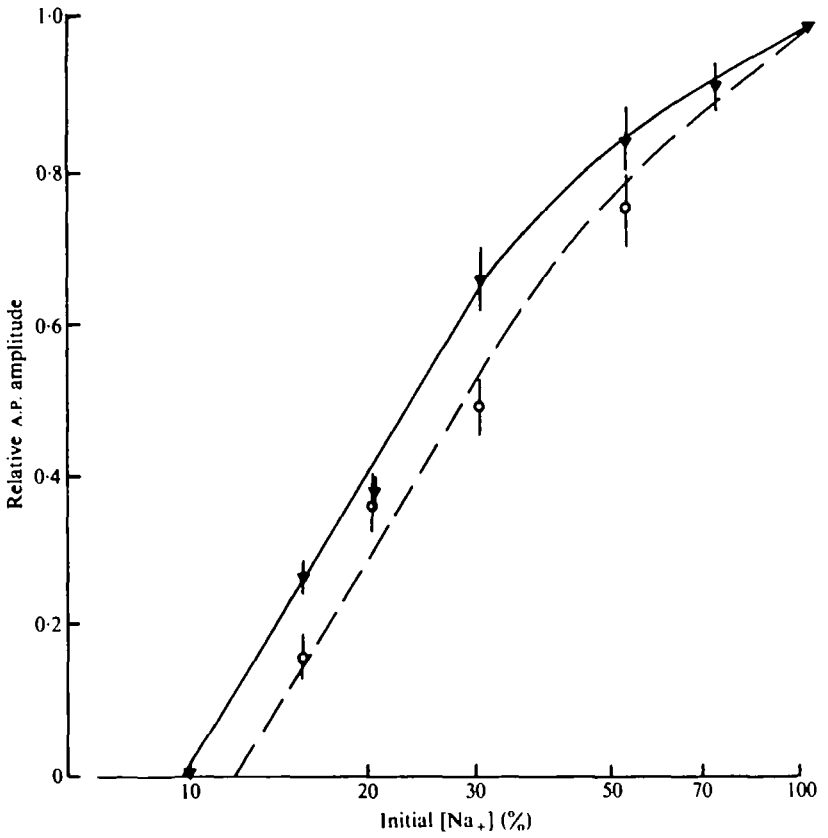

Fig. 15. Effects of isosmotic dilutions (Ringer + mannitol) on the A.P. amplitude of *Mytilus*. ▼, 100%-adapted connectives; ○, 25%-adapted connectives.

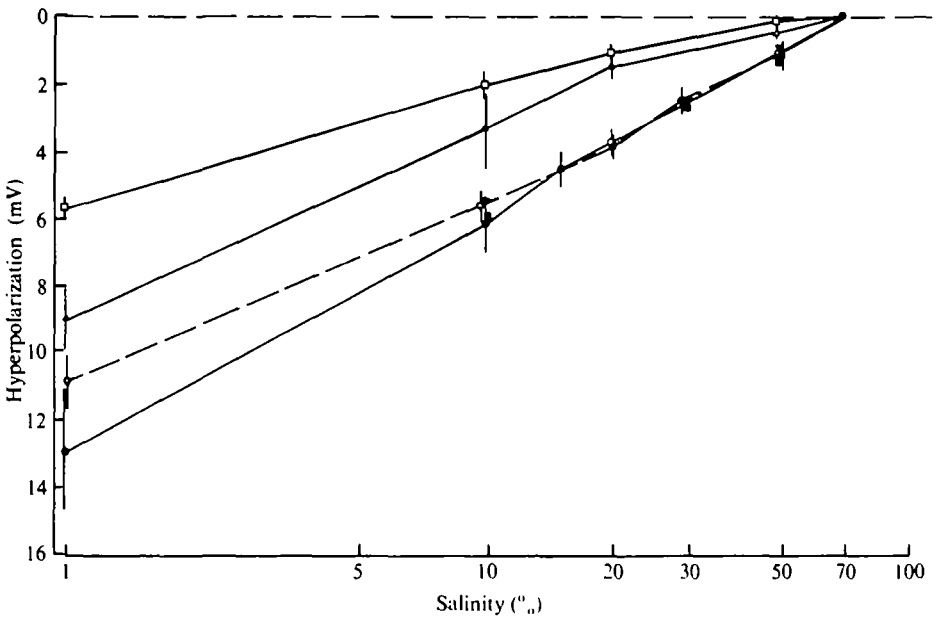

Fig. 16. A summary of the hyperpolarizing effects of all isosmotic reductions in Na concentration on *Mytilus* connectives. Solid lines represent 100%-adapted nerves: ●, Ringer diluted with mannitol; ▼, Na substituted with Tris; ■, Na substituted with mannitol; △, Ringer/mannitol + K (see text) and □, Ringer/mannitol + TEA. Dotted lines, ○, indicate effects on 25%-adapted nerves of Ringer/mannitol dilutions.

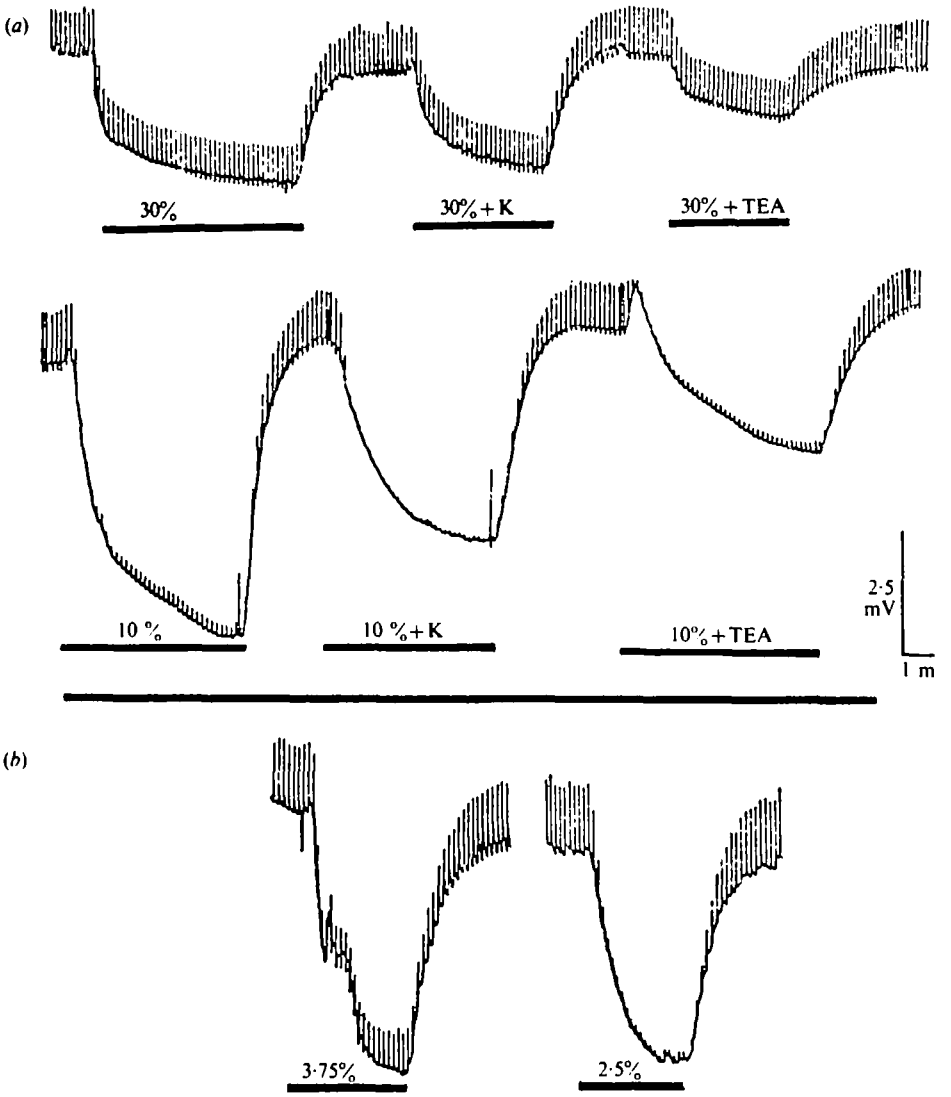

Fig. 17. Examples of the complete effects of isosmotic dilutions on (*a*) 100 %-adapted and (*b*) 25 %-adapted connectives. For (*a*), the reduced hyperpolarizations when K^+ or TEA were added are also shown.

osmotic concentrations maintained (cf. Fig. 2), and these changes are included in Fig. 16. Again the most feasible cause of such an effect is an ionic leakage into dilute media, so tests were performed (on unadapted nerves only) to estimate the role of potassium, the most important cellular cation, in such changes. Firstly, aliquots of KCl were added to s.w./mannitol Ringers to bring $[K^+]_o$ to the normal level of 12·4 mM, thus reducing the gradient for K loss; since the Nernst slope is almost flat between 0 mM and 12·4 mM K, additions of potassium over this range would normally have no depolarizing effect. As a further check, Ringers containing 20 mM-TEA were used (by substituting for Na), since this ion may affect the passive leakage of potassium ions.

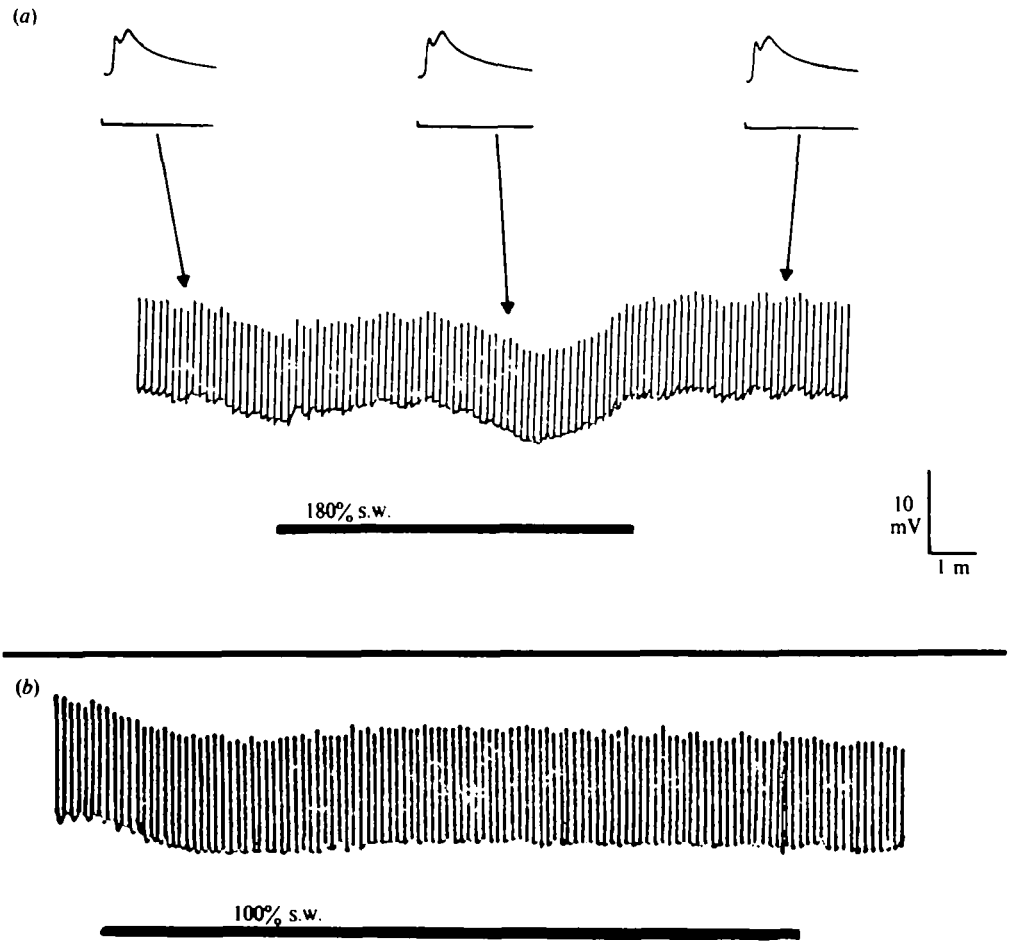

Fig. 18. The effects of hyperosmotic solutions on (*a*) 100% and (*b*) 25%-adapted *Mytilus* connectives.

The results of both these treatments are included in Fig. 16, and examples are given in Fig. 17 together with the normal responses. Each of the tests produced a significant reduction in the hyperpolarizations, the TEA effect being particularly marked. It is therefore likely that in *all* solutions of reduced $[Na^+]_o$ a degree of potassium leakage is induced in proportion to the sodium concentration change.

(3) *Hyperosmotic solutions*

The effects of Ringers containing excess NaCl are shown in Fig. 18. Concentrations of up to 180% salinity were tested on normal animals, and of up to 100% salinity on dilute-adapted mussels; these solutions produced initial hyperpolarizations, followed by recovery within 10 min. There were no further changes, and the A.P. was unaffected throughout. The changes in ionic gradient which must be incurred are not reflected in the gross electrical behaviour of the tissue, indicating a levelling of the Nernst slope above normal values of $[Na^+]_o$, and thus a relative insensitivity to high osmotic concentrations.

DISCUSSION

The results presented in the first section of this paper clearly suggest that *Mytilus* axons have a conventional basis for their excitability irrespective of the salinity to which they are adapted. The action potential probably depends upon an inward Na current and a restoring K current, blocked by TTX and TEA respectively, and the resting membrane potential is primarily determined by the potassium gradient, perhaps with a small contribution from chloride ions. Metabolic energy is required to restore full excitability after prolonged stimulation, an effect likely to involve a classic (i.e. ouabain-sensitive) Na–K exchange system. Ionic movements within the connective are relatively fast and apparently unrestricted, a situation which is probably common to most non-insect invertebrates (see Abbott & Treherne, 1977). The connective is fairly insensitive to all the usual pharmacological tools, though; since this is unlikely to be due to any physiological barrier or restriction of access (the presence or otherwise of an intact neural lamella being immaterial to conduction), it may reflect a lowered frequency or affinity of the various binding sites. This view is supported by the reports of Treherne *et al.* (1969) and Rutherford & Dunham (1970), which have indicated a similar insensitivity in the bivalves *Anodonta* and *Unio* respectively.

The second section of the results described above concerns short-term effects of osmotic stress on the nerve, over a time span probably insufficient to permit any adaptation. These tests revealed interesting effects both on A.P. amplitude and on the recorded d.c. potentials. In the first case, there were significant differences between the action potential sizes recorded with reduction in Na alone (tris substitution) or Na and Cl concomitantly (mannitol substitution) (Fig. 2), and reductions in all ions simultaneously, with or without maintenance of osmotic concentration (Figs. 10, 14); these differences cannot be attributed solely to changes in external conductance. The A.P. was abolished more readily in the former tests, and only then was the relationship conventionally logarithmic. *Mytilus* axons therefore appear to respond both to Na and to total ionic strength, such that a true Nernst relation only applies if a single ion species is varied. Furthermore, in these experiments the 25%-adapted connectives always showed slightly steeper gradients of response, perhaps indicative of greater selectivity for sodium after acclimation.

With respect to d.c. resting potentials, large hyperpolarizations have been recorded with isosmotic media, of similar size for both normal and adapted nerves at equivalent concentrations. Since these effects occur even in solutions in which only Na has been varied (i.e. after tris substitution), it seems likely that the change in potassium permeability which apparently causes them is itself triggered by declining $[Na^+]_o$. If this view is correct, it could be the underlying reason for the complex concentration-dependence of the responses to hyposmotic solutions, the depolarizations due to the reduced osmotic strength being superimposed on hyperpolarizations resulting from decreased $[Na^+]_o$. It would also explain why recorded depolarizations were generally preceded by small hyperpolarizing shifts. Subtraction of the known voltage change due to cation leakage should therefore reveal the true depolarizing effect of hyposmotic media; this operation results in the dotted curves shown in Fig. 13 (for normal and reduced-chloride Ringers), indicating a relatively smooth depolarizing trend with declining salinity.

The changes in chloride permeability responsible for these depolarizations (perhaps accompanied by loss of other negative radicals such as the anionic amino-acids) only occur in dilute media and may therefore be a specific effect of swelling incurred in the connective; their time course accords with the volume changes described previously (Willmer, 1978*a*). Since the responses in dilute-adapted tissues were just significantly smaller at equivalent dilutions, this would also agree with the findings of an earlier paper (Willmer, 1978*a*) that adapted connectives are less prone to short-term swelling due to modifications in the neural lamella.

It is pertinent to compare the present results with those obtained with other osmoconformers having conventional action potentials. Considering first the short-term polarization changes, the effects recorded in *Mytilus* are reversed in the crustacean *Maia squinado* (Pichon & Treherne, 1976), which shows hyperpolarizations in dilute media due to increased gK, and marked depolarizations in seawater/sucrose media. Similarly, hyperpolarizing responses to both hyposmotic and isosmotic dilute media have been described in *Mercierella enigmatica*, due in this case to decreased $[K^+]_O$ (Benson & Treherne, 1978*a*, *b*); these were of greater amplitude and persistence in isosmotic solutions. A third osmoconformer, *Sabella penicillus*, shows only small polarization changes in hyposmotic media (Treherne & Pichon, 1978). The phenomenon of hyperpolarization due to increased potassium permeability in hyposmotically treated cells is well known, having been recorded in isolated erythrocytes (Kregenow, 1971) and in many other vertebrate tissues (see reviews by Macknight & Leaf, 1977; Hoffmann, 1977); *Mytilus* is apparently unique amongst those preparations so far studied in showing the opposite effect, with anionic loss normally exceeding potassium leakage. But in isosmotic media, *Mytilus* shares with other osmoconformers the tendency to hyperpolarize, due to the alterations either in potassium gradient across the excitable membranes or in their permeability to this ion.

In long-term acclimation, all three of the osmoconformers mentioned above lose intracellular potassium and thereby achieve a more negative resting potential; in *Mercierella* in particular, this is of importance in permitting a maintained total spike amplitude in the face of reduced overshoot. However, *Mytilus* appears to exhibit a similar resting potential before and after dilute adaptation; although the cells do lose potassium (Willmer, 1978*a*) this loss must be accompanied by equivalent losses of chloride or amino-acids.

The short-term alterations in the spike of those osmoconformers so far examined are usually rather similar to those of *Mytilus*, although the salinity at which irreversible damage to the A.P. occurs is very variable. The exception to the general pattern is *Sabella*, where hyposmotic media cause swelling and thus restricted access, so that initial effects of such treatments are considerably slower than the effects of isosmotic dilutions.

With regard to long-term responses that would serve to maintain a reasonable spike height in an adapted nervous tissue, *Sabella* shows an increase in gNa (with constant $[Na^+]_I$) after 50% acclimation (Treherne & Pichon, 1978), while *Mercierella* incurs a 50% decrease in $[Na^+]_I$ with constant sodium permeability at 25% dilution (Benson & Treherne, 1978*b*). By comparison, *Mytilus* certainly dilutes its cellular sodium, from 100 mM to 40 mM in 25% media (Willmer, 1978*a*), and probably also shows an increased sodium permeability, thereby utilizing both of the possible adaptations which have been shown in annelid nerves.

Thus, while there are certain standard responses to reduced salinity which can assist in both short- and long-term survival of the nerves in osmoconformers, the degree to which each of these is adopted varies considerably, with each of the four osmoconformers so far analysed (which include representatives from three different phyla) using a slightly different pattern of responses. Such differences could be related in part to the varying time-courses of adaptation in the animals concerned. To consider only the extremes, *Mercierella* adapts fully to dilute media within hours and could be thought of as 'pre-adapted' by virtue of its potassium sensitivity and capacity for rapid reduction in $[Na^+]_I$; whereas *Mytilus* requires at least 12 days for acclimation, which probably involves both ion permeability changes and, as a further paper will show (Willmer, 1978*b*), more drastic biochemical and structural alterations in the excitable membranes.

Mytilus axons, in common with those of other osmoconformers, can clearly adapt successfully to dilute solutions. At 25% salinity they continue to produce A.P.s having a conventional ionic basis at levels of $[Na^+]_O$ which would cause total conduction block prior to adaptation. All ionic and osmotic responses are thus 'reset' to new positions on the concentration axes. But there are apparently no mechanisms in these invertebrates to permit maintenance of the action potential during brief and acute hyposmotic exposures, so that the animals might be at least temporarily prostrated if meteorological or tidal factors caused such a drastic change of conditions. However, in *Mytilus* such an experience can be efficiently avoided by immediate apposition of the valves, with subsequent very gradual dilution of the medium directly surrounding the tissues. It is curious that a bivalve, with this inherent escape response available to it, is in fact far less drastically affected by acute hyposmotic stress than the even more euryhaline serpulid *Mercierella*; for whereas *Mytilus* can readily recover from exposures as low as 15% salinity, *Mercierella* axons suffer irreversible damage if exposed directly to salinities of less than 50%. Perhaps the ability of *Mytilus* cells to lose both potassium and anions, with a net depolarization and moderate swelling, is actually a more efficient adaptive mechanism in face of acute stress than the tendency in *Mercierella* to hyperpolarize (implying relatively limited loss of anions) and to resist swelling. The greater short-term sensitivity of the annelid may also reflect the larger size of its axons, since as pointed out elsewhere (Willmer, 1978*a*) the tension on a cell membrane during osmotic stress is proportional to its radius.

In conclusion, *Mytilus* axons appear to adopt several strategies to limit the deleterious effects of hyposmotic media while maintaining adequate electrical signalling capacities. Firstly, internal sodium and potassium concentrations are reduced, though not in proportion to the external medium. In each case this reduction is likely to reflect a balance between at least three factors: the need to maintain ionic gradients for determining resting and action potentials; the need to reduce intracellular concentrations and restore osmotic equilibrium; and the opposing problem of retaining sufficient Na and K in the cells to ensure efficient enzyme functioning. Secondly, *Mytilus* retains a roughly constant resting potential, probably by balancing losses of potassium with losses of anions; that is, of chloride (which has been shown to efflux from cells during short-term osmotic stress) and of amino-acids such as glutamate and aspartate. Finally the action potential is maintained at its full pre-acclimation height, due in part to the increased sodium permeability reflected in the Nernst slopes.

The long-term alterations in the excitable membranes which could underlie such

effects are not at present clear. Conceivable models might involve changes in ionic channels, mediating the postulated permeability changes, or in the sodium/potassium exchange pumps on whose activity fine adjustments of the trans-membrane ion gradients may depend. While the first of these possibilities is difficult to test in an animal showing considerable insensitivity to the classic pharmacological tools such as tetrodotoxin, the second hypothesis is more amenable to study, and experiments designed to elucidate the role of sodium pumps in long-term neural osmotic adjustment are described in the paper which follows.

The work described in this paper represents part of a Ph.D thesis submitted to the University of Cambridge. The author would particularly like to thank Dr J. E. Treherne for his help and advice throughout, and for valuable comments on the present manuscript. This work was supported by an SRC grant, and by New Hall, Cambridge.

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