J. exp. Biol. (1976), 65, 553–563
With 8 figures
Printed in Great Britain

THE EFFECTS OF OSMOTIC STRESS ON THE ELECTRICAL PROPERTIES OF THE AXONS OF A MARINE OSMOCONFORMER (*MAIA SQUINADO.* BRACHYURA: CRUSTACEA)

BY Y. PICHON* AND J. E. TREHERNE†
Institut de Biologie Marine, Arcachon, Gironde, France

(*Received* 9 *May* 1976)

SUMMARY

In contrast to the depolarization observed in hyperosmotic media, exposure of peripheral nerve to hyposmotic conditions induced pronounced axonal hyperpolarization. It is suggested that this hyperpolarization resulted from increased potassium and chloride permeabilities which could assist axonal volume regulation in hyposmotic conditions. The hyperpolarization was readily reversible, but the spike-generating mechanism suffered irreversible damage at hyposmotic concentrations below 665 m-osmoles. It is suggested that this axonal damage contributes to the lethal effects of hyposmotic stress in this crustacean osmoconformer and, possibly, in some euryhaline osmoregulators.

INTRODUCTION

The cells of some marine invertebrates may be subjected to considerable osmotic and ionic stress. This is most dramatically seen in estuarine osmoconformers, such as the annelids *Arenicola marina* (Robertson, 1949) and *Mercierella enigmatica* (Skaer, 1974*a*, *b*, *c*, *d*) or the lamellibranch *Mytilus edulis* (Potts, 1964), in which the tissues can be subjected to massive dilution during exposure to hyposmotic media. More limited changes in osmotic concentration also occur during osmotic stress in euryhaline animals (cf. Potts & Parry, 1964) and a regulation of the intracellular osmotic concentration and cell volume has been demonstrated in the nerve and muscle cells of crustaceans (cf. Gérard & Gilles, 1972; Shaw, 1955; see also review by Schoffeniels, 1976), of fishes (Lasserre & Gilles, 1971) and in amphibian brain (Baxter & Ortiz, 1966; Shank & Baxter, 1973). Little appears to be known, however, of the effects of osmotic stress on nervous function in marine invertebrates. It seems not to be known, for example, whether the lethal effects of hyposmotic stress (observed in both stenohaline osmoconformers and some euryhaline regulators) are associated with irreversible disruption of nervous function. Such an effect cannot be assumed *a priori*, for axonal function has been shown to persist in nerves of a serpulid worm (A. D. Carlson & J. E. Treherne, unpublished observations), of a

* Laboratoire de Neurobiologie Cellulaire, Centre National de la Recherche Scientifique, 91190-Gif-sur-Yvette, France.
† Department of Zoology, University of Cambridge, U.K.

marine (P. G. Willmer, personal communication) and a freshwater lamellibranch (Treherne, Mellon & Carlson, 1969) after extreme hyposmotic stress. This study attempts to elucidate the effects of osmotic stress on the electrical responses of the peripheral axons of an osmoconformer, the spider crab (*Maia squinado*), a marine species which can withstand only limited dilution of the body fluids (Duval, 1925).

MATERIALS AND METHODS

Spider crabs (*Maia squinado* L.) were obtained from local fishermen at Arcachon and kept alive in circulating sea water of 21–22 °C. The legs were most often severed from the body by reflex autotomy, induced by a moderate twisting of the leg. This procedure allowed a good survival of the animal which could be used for successive experiments. Several cm of physiologically viable leg nerve could be isolated from each appendage by pulling from the limb after cracking the first joint. Each nerve consisted of several bundles, each of which contained a large number of small fibres. According to Abbott, Hill & Howarth (1958) the fibre diameters of the claw and leg nerves of *Maia* range from 0·25 to 20 μm with a majority of very small fibres (less than 1·2 μm). Small bundles were isolated over a length of about 5 cm and were tied at both ends with cotton threads before transfer to the recording chamber.

The nerve chamber used in these experiments consisted of five parallel compartments, which were isolated from one another by vaseline-seals as used previously (Treherne *et al.* 1970; Pichon & Treherne, 1970). Since the length of the preparation was significantly larger than in insect nerve cords, slightly larger compartments and larger seals were used, resulting in a reduced short circuit and better stability. The central compartment, which contained the test solution, was connected via an agar bridge to the indifferent Ag/AgCl electrode. The level of the fluid in this compartment was adjusted by raising or lowering the suction micropipette. Complete replacement of the fluid was achieved within 15–20 s. The right-hand compartment which contained isosmotic KCl solution was connected via an agar bridge to a high impedance negative capacitance amplifier.

Potential difference between the central and right-hand compartments were recorded across an 'oil-gap'. This technique was used in preference to the usual 'sucrose-gap' which caused axonal damage. This was seen from the effects on the action potentials which, after a transient increase in size (due to the reduction of the short circuit between the fibres), developed a pronounced after-depolarization and after about 1 min slowly declined and eventually disappeared. Isotonic sucrose was, however, used routinely to wash the bundle during the first 30–60 s and then replaced with a vaseline-oil mixture. Under these conditions, the action potentials retained their size and remained stable for several hours. Stable DC recordings were also obtained with this system.

The two adjacent compartments on the left side of the nerve chamber were filled with sea water and connected to the output of a pulse generator (Farnell) with silver wires and an optically coupled stimulus isolation unit. Continuous DC changes and action potentials elicited by electrical stimulation were monitored on a Tektronix 502A oscilloscope and recorded respectively on a Sefram servoscribe recorder and a Nihon Kohden continuous recording camera.

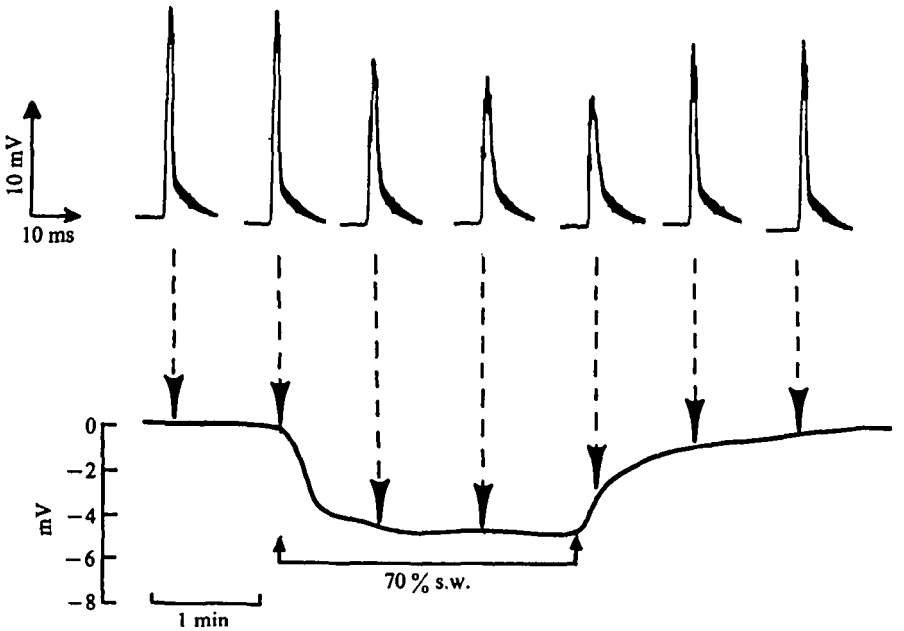

Fig. 1. The effects of 70 % sea water (665 m-osmols) on the resting and action potentials recorded from an axon bundle from a leg nerve of *M. squinado*.

Normal Arcachon sea water was used as a standard saline and had the following ionic composition: Na^+, 430; K^+ 9·8; Ca^{2+}, 9·6; Mg^{2+}, 42·0; Cl^-, 520 mM (P. Lasserre, personal communication), an osmotic concentration of 950 m-osmoles and a pH of 8·0.

Isosomotic sucrose solutions were made by diluting crystalline sucrose (Merck) with distilled water. Hyperosmotic solutions were obtained by adding NaCl to the sea water. Hyposmotic solutions were obtained by mixing sea water with distilled water. Isosmotic solutions with changed ionic compositions were made in a similar way by mixing sea water with isosmotic sucrose or choline chloride.

Osmotic concentrations were routinely checked, from the freezing point depression, using a Halbmikro osmometer.

Experiments were carried out at room temperature (25–30 °C).

RESULTS

Exposure of axon bundles to dilute hyposmotic sea water caused a clear and reversible hyperpolarization and reduction in the size of the action potentials (Fig. 1). Both effects were correlated with the change in osmotic concentration but were not clearly correlated with each other: the change in the DC potential level being small compared with the change in the amplitude of the spike. The time courses of the two changes also differed during exposure to hyposmotic and subsequent return to normal sea water (see Fig. 6). For these reasons, the effects on the DC and action potentials will be described and discussed separately.

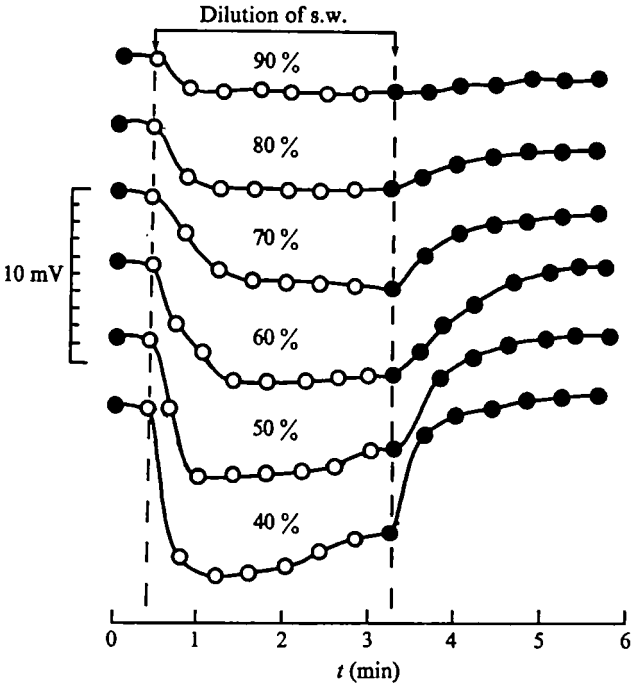

Fig. 2. Effects of exposure to various dilutions of sea water on axonal resting potential.

1. *Effects of hyposmotic sea water on DC potential*

The hyperpolarization induced by hyposmotic media consisted of a transient fast component which was usually followed by a plateau or a slow hyperpolarization for dilution of 90–60 % (Fig. 2, also Fig. 6). For larger dilutions the membranes showed a tendency to repolarize after 1–2 min (Fig. 2).

The initial, transient, hyperpolarizations were of short duration ($15 < t_{0.5} < 25$s) and their amplitude was linearly related to the percentage dilution ($r = 0.997, n = 6$). Such a correlation could not be found after 3 min exposure, indicating that a secondary process was probably involved after this time.

The observed hyperpolarizations could result from changes in osmotic concentration or from the decrease in the external ionic concentrations. The following three series of experiments were therefore performed in an attempt to elucidate the origin of the hyperpolarization. In the first, the osmotic concentration was kept constant by diluting sea water with an isosomotic sucrose solution (i.e. isosmotic and low ionic strength). In the second, the effects of external K^+ concentration in hyposmotic sea water was measured. In the third series of experiments, the osmotic concentration was maintained using choline chloride to maintain the external chloride concentration at a constant level.

2. *Effects of isosmotic (sucrose-diluted) sea water on DC potential*

In contrast to the responses in hyposmotic solutions, change in the external ionic concentration in isosmotic conditions induced a transient depolarization followed, during long exposures, by gradual hyperpolarizations (Fig. 3). On return to normal

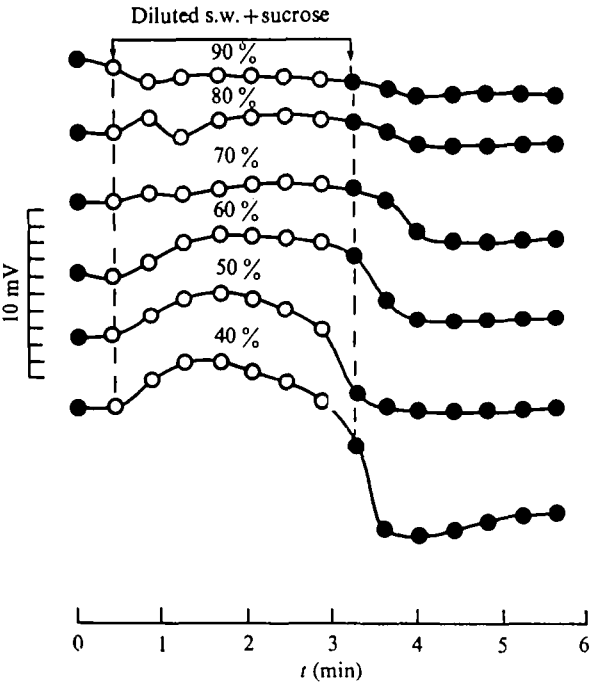

Fig. 3. Changes in axonal resting potential during exposure to diluted sea water in which isosmicity (950 m-osmoles) was maintained by the addition of sucrose.

sea water, these complex responses were followed by a transient hyperpolarization, the amplitude of which (2 min after return to normal sea water, the value at 90% omitted) were proportional to the degree of dilution (r = 0·974, n = 6). This hyperpolarization was eventually followed by a return to the original level.

3. *Effects of potassium concentration on DC potential in hyposmotic sea water*

Since crustacean axon membranes approximate to potassium electrodes (cf. Dalton, 1958, 1959) it is conceivable that the observed hyperpolarizations could have resulted from the reduced potassium concentration in diluted sea water. For example, from the data of Abbott, Moreton & Pichon (1975) it can be calculated that a change from *ca.* 10 mM-K^+ (normal sea water) to *ca.* 6 mM-K^+ (60% sea water) would produce a hyperpolarization of 5 mV, which approximates to the observed values (Fig. 2). However, the hyperpolarizing responses obtained in hyposmotic (60%) sea water in which the potassium concentration was elevated to that of normal sea water (10 mM) (Fig. 4) eliminate this possibility.

4. *Effect of isosmotic (choline diluted) sea water on DC potential*

When isosmicity was maintained with sucrose a secondary effect could have arisen from the reduced concentration of chloride ions in the external medium. To test this possibility choline chloride was used to maintain isosmicity in diluted sea water. This replacement caused hyperpolarizing responses which were of

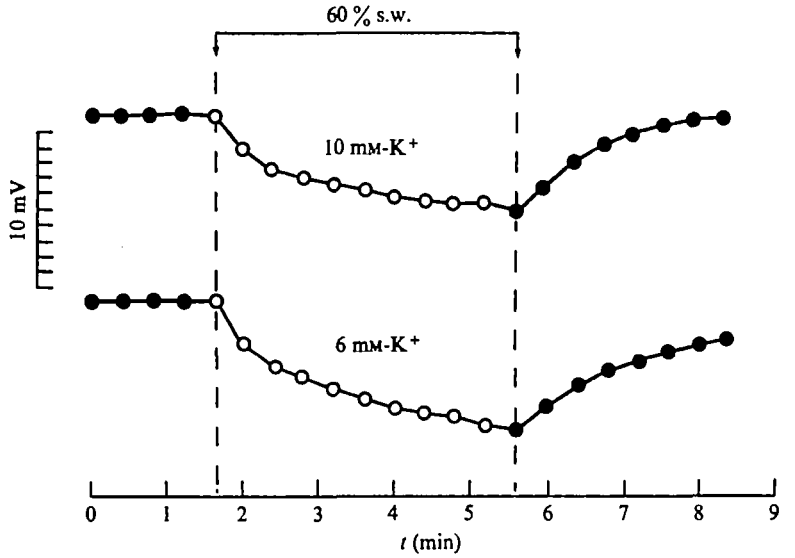

Fig. 4. Effects of potassium concentration on the changes in axonal resting potential in hypotonic media.

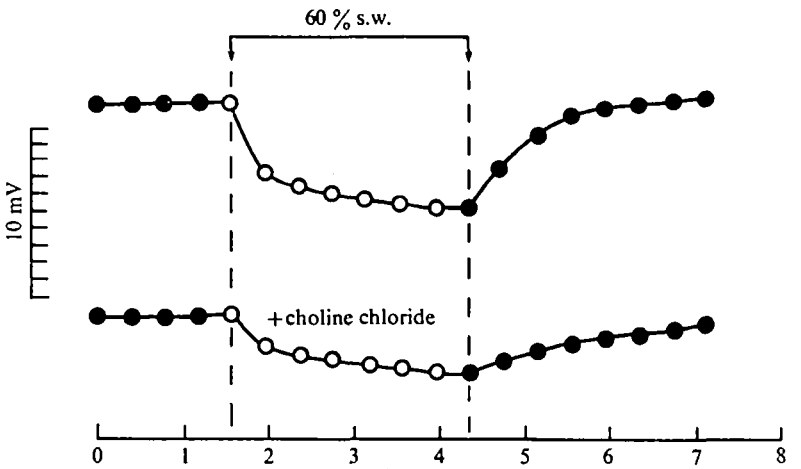

Fig. 5. Hyperpolarizing response in 60 % sea water (570 m-osmoles) and in 60 % sea water in which isosmicity (950 m-osmoles) was maintained with choline chloride.

apparently similar time course, but of smaller magnitude (about 50 %) of that observed in hyposmotic solution (Fig. 5).

5. *Effects of hyposmotic sea water on the action potential*

Exposure of axon bundles to hyposmotic sea water resulted in a progressive, non-exponential, decrease in the amplitude of the action potentials (Fig. 6). The decrease in amplitude of the action potentials was reversible down to 70 % (665 m-osmoles) sea water, irreversible damage becoming apparent at 60 % (570 m-osmoles) and below (Fig. 7*a*).

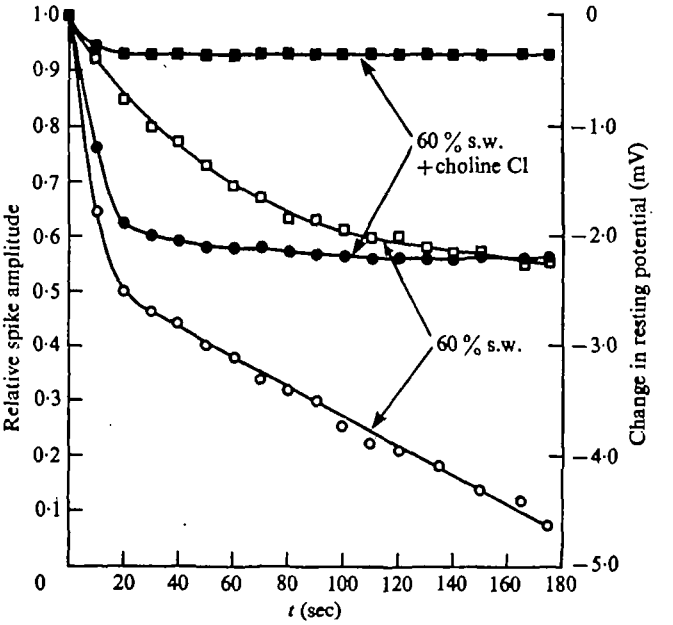

Fig. 6. Changes in the resting potential (circles) and the relative amplitude of the action potentials (squares) following exposure of axons to diluted, 60% (570 m-osmoles) sea water (open symbols) and to 60% sea water in which isosmicity (950 m-osmoles) was maintained by the addition of choline chloride (filled symbols).

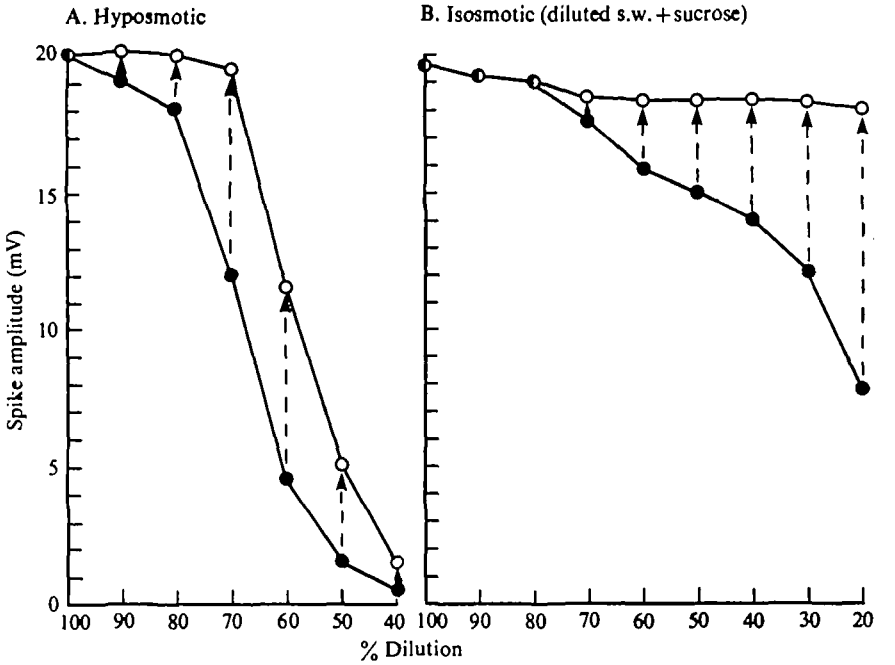

Fig. 7. (*a*) Effects of successive exposures to progressively diluted sea water on the amplitude of axonal action potentials (closed circles) and their recovery on exposure to normal sea water (open circles). (*b*) Effects of successive exposures to diluted sea water, in which isosmicity was maintained with sucrose, on the amplitude of axonal action potentials (closed circles) and their recovery on exposure to normal sea water (open circles).

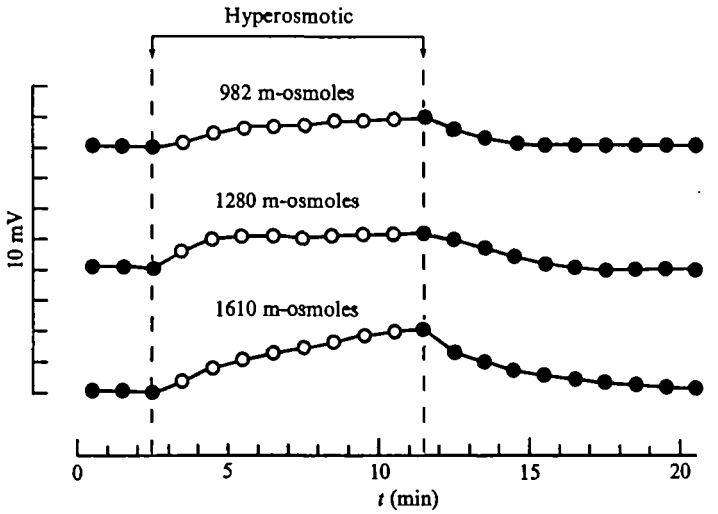

Fig. 8. The effects of hyperosmotic saline (100% sea water+NaCl) on axonal resting potentials.

6. *Effects of isosmotic (choline or sucrose diluted) sea water on action potentials*

When isosmicity was maintained with choline chloride, the time course in the change in the amplitude of the spike followed that of the DC change (Fig. 6).

The effects of diluted sea water (in which isosmicity was maintained by the addition of sucrose) differed from those of hyposmotic sea water in two respects: (1) the reduction in the size of the action potentials was significantly smaller and (2) the reversibility was almost complete even following a large dilution (Fig. 7*b*).

7. *Effects of hyperosmotic solutions on DC and action potentials*

Moderate increase of the osmotic concentration of the bathing medium, by addition of sodium chloride to sea water, resulted in a progressive depolarization followed by a complete recovery if the osmotic concentration did not exceed 2000 m-osmoles (Fig. 8). For larger osmotic concentrations, irreversible damage was caused. No appreciable change in the amplitude of the action potential was observed at osmotic concentrations lower than 2000 m-osmoles.

DISCUSSION

The relatively rapid electrical responses of the axons to reduced salinity confirm earlier observations which indicate that the axonal surfaces in crustacean peripheral nerves are accessible, there being little restriction to the diffusion of small water-soluble ions and molecules between the blood, or bathing medium, and the interstitial fluid (Baker, 1965*a*, *b*; Abbott *et al.* 1975; Lane & Abbott, 1975).

In contrast to the depolarization induced by hyperosmotic media exposure of peripheral axons to hyposmotic conditions produced a marked hyperpolarization of the axon membrane, the extent of the hyperpolarization increasing with decreasing

osmotic concentration. This hyperpolarization is unlikely to have arisen from dilution of the external potassium ions. It was, for example, not observed when isosmicity was maintained with sucrose and was unaffected by the addition of potassium ions to maintain the normal concentration of this cation in hyposmotic conditions. The hyperpolarization can only be partly attributed to the reduced inward gradient of sodium ions across the axon membrane, for only a relatively small potential change occurred in diluted sea water in which isosmicity was maintained with choline chloride. It is, also, difficult to attribute the hyperpolarization to the reduction in the external concentration of divalent cations in the absence of a significant effect in diluted sea water in which the normal osmotic concentration was maintained by sucrose.

The electrical responses of these crustacean axons to hyposmotic conditions differ from those obtained with perfused squid axons, in which appreciable depolarization was obtained when the osmotic concentration was simultaneously reduced on each side of the axon membrane (Kukita & Yamagishi, 1975). The present results suggest that a significant proportion of the hyperpolarization that occurs on exposure to hyposmotic media results from the rapid axonal swelling which has been shown to occur in crustacean peripheral axons during hyposmotic stress (Gérard & Gilles, 1972). In this case, the hyperpolarization could result from an increase in net potassium efflux caused by a change in the permeability of the axon membrane to this cation as has been shown, for example, in erythrocytes during exposure to hypotonic media (Kregenow, 1971). It is conceivable that such an increase in the relative permeability of the membrane could, by increasing the potassium leakage from the axon, contribute to volume regulation as has already been suggested on the basis of the increased efflux of amino acids observed during hyposmotic stress in crustacean axons (Gérard & Gilles, 1972).

An increased influx of chloride ions could also contribute to the observed hyperpolarization if it is not counterbalanced by an equivalent increase in the influx of Na^+ ions. Such an increase in net chloride influx could result from an increased leak permeability and, also, if chloride ions are dragged into the cell with water through the (presumed) water channels in the axon membrane.

The depolarization observed when isosmicity was maintained by sucrose can be explained by the generally accepted hypothesis that chloride ions are distributed across the membrane according to a passive Donnan equilibrium. According to the Donnan theory, a decrease in the external ionic strength will result in a redistribution of internal anions and cations. With a relatively large conductance of the resting membrane to chloride, these ions will leave the axoplasm, leading to a depolarization of the membrane. A transient hyperpolarization following return to normal sea water (Fig. 2) can be predicted on the same basis.

The electrical response to diluted, isosmotic sea water, differed when a non-electrolyte or an electrolyte was used to maintain isosmicity, sucrose causing a depolarization and and choline chloride a slight hyperpolarization. The depolarization in the presence of added sucrose could have resulted from the effects of changed ionic strength on the passive permeability of the unstretched axonal membrane, conceivably from a reduction in the net influx of chloride ions.

The reversible effects of hyposmotic stress on the resting potential suggest that

osmotic swelling does not induce permanent changes in the relative ion permeabilities of the resting axon membrane. The irreversible effects on the action potential observed on exposure to osmotic concentrations lower than 665 m-osmoles suggest, on the other hand, that osmotic stretching of the axon membrane causes permanent damage to the mechanisms involved in carrying the action current in the active membrane.

The origin of the observed block remains to be investigated. It is likely that one of the effects of the osmotic stretching is to increase the leak of the membrane as observed following mechanical stretching in isolated insect nerve fibres (Pichon, unpublished observations). This increase in the leak, if sufficient, can be responsible for the observed conduction block, the outwardly flowing leak current counteracting the depolarizing effects of inwardly-moving sodium ions. Kukita & Yamagishi (1975) have reported that in perfused squid axons, a 75 % decrease in the osmotic pressure of either the external or the internal solution results in an irreversible loss of excitability. In the same series of experiments, Kukita & Yamagishi observed a rapid decrease in the action potential associated with a drop in membrane resistance when the inside solution was made hypertonic with respect to the outside. A progressive increase in the axoplasmic Na^+ concentration resulting from both the unspecific leakage of Na^+ ions into the axon and the decreased efficacy of the Na^+/K^+ pump would also raise the threshold and induce conduction block.

A direct effect of osmotic stretching on the sodium system itself cannot be excluded *a priori*. Such an effect could be due either to a mechanical disruption of the channels due to the massive inward movement of water across the membrane or to conformation change induced by a swelling of the membrane as suggested by Singer & Tasaki (1968). A voltage-clamp analysis of these effects of reduced osmotic pressure on the nerve membrane presently in progress is expected to provide more information on this phenomenon.

Irreversible damage was caused to the spike-generating system, when the osmotic concentration of the fluid bathing the axon surfaces was reduced from 665 to 570 m-osmoles, which approximates to the critical lethal concentrations of the blood for this osmoconformer and for the euryhaline osmoregulator *Carcinus maenas*, during gradual exposure to hypotonic conditions (Duval, 1925). The osmoregulating abilities of the latter crustacean, for example, enabled it to survive indefinitely at an external concentration of 294 m-osmoles when, the blood concentration averaged 605 m-osmoles. Lower blood concentrations proved lethal in *C. maenas* as in *M. squinado* (Duval, 1925). The present results suggest that the lethal effects of hyposmotic stress in *M. squinado*, and possibly *C. maenas*, are likely to involve permanent disruption of nervous function.

We are grateful to the Royal Society for a travel grant and to Dr P. Lassere and Mme B. Lassere for their generous help during this investigation.

REFERENCES

ABBOTT, B. C., HILL, A. V. & HOWARTH, J. V. (1958). The positive and negative heat production associated with a nerve impulse. *Proc. Roy. Soc.* B **148**, 149–87.

ABBOTT, N. J., MORETON, R. B. & PICHON, Y. (1975). Electrophysiological analysis of potassium and sodium movements in crustacean nervous systems. *J. exp. Biol.* **63**, 85–115.

BAKER, P. F. (1965*a*). Phosphorus metabolism of intact crab nerve and its relation to active transport of ions. *J. Physiol. Lond.* **180**, 383–423.

BAKER, P. F. (1965*b*). A method for location of extracellular space in crab nerve. *J. Physiol. Lond.* **180**, 529–42.

BAXTER, C. F. & ORTIZ, C. L. (1966). Amino acids and the maintenance of osmotic equilibrium in brain tissue. *Life Sci.* **5**, 2321–9.

DALTON, J. C. (1958). Effects of external ions on membrane potentials of a lobster giant axon. *J. gen. Physiol.* **41**, 529–42.

DALTON, J. C. (1959). Effects of external ions on membrane potentials of a crayfish giant axon. *J. gen. Physiol.* **42**, 971–82.

DUVAL, M. (1925). Recherches physico-chemique et physiologiques sur le milieu interieur des animaux aquatiques. Modifications sous l'influence du milieu exterieur. *Ann. Inst. oceanogr.* **2**, 232–407.

GÉRARD, J. F. & GILLES, R. (1972). Modification of the amino acid efflux during osmotic adjustment of isolated axons of *Callinectes sapidus*. *Experientia* **28**, 863–4.

KREGENOW, F. M. (1971). The response of duck erythrocytes to nonhemolytic hypotonic media. Evidence for volume controlling mechanism. *J. gen. Physiol.* **58**, 372–95.

KUKITA, F. & YAMAGISHI, S. (1975). Excitability of squid giant axons in hypertonic and hypotonic solutions. *Proc. Japan Acad.* **51**, 68–73.

LANE, N. J. & ABBOTT, N. J. (1975). The organization of the nervous system in the crayfish *Procambarus clarkii*, with emphasis on the blood-brain interface. *Cell & Tissue Res.* **156**, 173–87.

LASSERRE, P. & GILLES, R. (1971). Modification of the amino pool in the parietal muscle of two euryhaline teleosts during osmotic adjustment. *Experientia* **27**, 1434–5.

PICHON, Y. & TREHERNE, J. E. (1970). Extraneuronal potentials and potassium depolarization in cockroach giant axons. *J. exp. Biol.* **53**, 485–93.

POTTS, W. T. (1964). Quoted in Potts & Parry (1964).

POTTS, W. T. & PARRY, G. (1964). *Osmotic and Ionic Regulation in Animals*. Oxford: Pergamon.

ROBERTSON, J. D. (1949). Ionic regulation in some marine invertebrates. *J. exp. Biol.* **26**, 182–200.

SCHOFFENIELS, E. (1976). Biochemical approaches to osmoregulatory processes in Crustacea. In *Perspectives in Experimental Biology I* (ed. P. Spencer Davies), pp. 107–24. Oxford: Pergamon.

SHANK, R. P. & BAXTER, C. F. (1973). Metabolism of glucose, amino acids, and some related metabolites in the brain of toads (*Bufo boreas*) adapted to fresh water or hyperosmotic environments. *J. Neurochem.* **21**, 301–13.

SHAW, J. (1955). Ionic regulation in the muscle fibres of *Carcinus maenas*. II. The effect of reduced blood concentration. *J. exp. Biol.* **32**, 664–80.

SINGER, I. & TASAKI, I. (1968). Nerve excitability and membrane macromolecules. In *Biological Membranes: Physical Fact and Function* (ed. D. Chapman), pp. 347–410. London and New York: Academic Press.

SKAER, H. LE B. (1974*a*). The water balance of a serpulid polychaete, *Mercierella enigmatica* (Fauvel). I. Osmotic concentration and volume regulation. *J. exp. Biol.* **60**, 321–30.

SKAER, H. LE B. (1974*b*). The water balance of a serpulid polychaete, *Mercierella enigmatica* (Fauvel). II. Ion concentration. *J. exp. Biol.* **60**, 331–8.

SKAER, H. LE B. (1974*c*). The water balance of a serpulid polychaete, *Mercierella enigmatica* (Fauvel). III. Accessibility of the extracellular compartment and related studies. *J. exp. Biol.* **60**, 339–49.

SKAER, H. LE B. (1974*d*). The water balance of a serpulid polychaete, *Mercierella enigmatica* (Fauvel). IV. The excitability of the longitudinal muscle cells. *J. exp. Biol.* **60**, 351–70.

TREHERNE, J. E., LANE, N. J., MORETON, R. B. & PICHON, Y. (1970). A quantitative study of potassium movements in the central nervous system of *Periplaneta americana*. *J. exp. Biol.* **53**, 109–36.

TREHERNE, J. E., MELLON, D. & CARLSON, A. D. (1969). The ionic basis of axonal conduction in the central nervous system of *Anodonta cygnea* (Mollusca: Eulamellibranchia). *J. exp. Biol.* **50**, 711–22.