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THE INITIATION AND CONTROL OF FREE AMINO ACID REGULATION OF CELL VOLUME IN SALINITY-STRESSED MARINE BIVALVES*

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INTRODUCTION

Intracellular free amino acid regulation as a measure of cellular volume control in salinity-stressed marine invertebrates is, by now, commonly known. Still, the details of the mechanisms by which free amino acids are regulated have been examined in only a few animals. For instance, in bivalves stressed by low salinities intracellular free amino acids cross the distended cell membranes in company with osmotically obligated water, and cell volume is thus restored (Pierce & Greenberg, 1972). Gastropod tissues, on the other hand, may synthesize protein as a method of removing the amino acids from the intracellular osmotic solute pool (Bedford, 1971).

The stimulus initiating amino acid regulation in molluscs remains unknown. A salinity decrease causes two coincident changes in the extracellular medium: the osmotic pressure decreases and the concentrations of the constituent ions decrease. Either change might trigger the regulatory response. To differentiate between these two factors we compared the effects of salinity decrease, and of isosmotic deletion of particular ions, on amino acid release from isolated *Modiolus* hearts. This comparison has indicated that, while the initiation of the amino acid release is probably due to osmotic pressure decrease, the magnitude and duration of the response is controlled by divalent cation concentration. In addition, the divalent ions exert their effect at the external surface of the cell membrane.

MATERIALS AND METHODS

Mussels

Specimens of *Modiolus demissus demissus* (Dillwyn) were collected from two locations: Little Sippewisset Marsh (Cape Cod, Massachusetts), and a salt marsh on the Chincoteague Bay side of Assateague Island (Maryland). The Cape Cod mussels were kept in running sea water (salinity = 31 $^0/_{00}$) on sea tables at the Marine Biological Laboratory, Woods Hole, Massachusetts. The Assateague mussels were maintained

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in aerated artificial sea water (Instant Ocean; salinity = 28 ‰) in a constant-temperature room (15 °C) at College Park, Maryland.

Test solutions

The full-strength sea water used in the experiments at the Marine Biological Laboratory is pumped out of Vineyard Sound and circulated through the Laboratory's sea-water system (osmolality = 930 m-osmoles/kg H_2O). The various ion-free solutions, as well as the artificial sea water used in the Assateague mussel experiments, were compounded from reagent-grade salts and glass-distilled water. The composition of the artificial sea water was unexceptional (Pierce, 1970); but the various isosmotic solutions lacking specific ions were especially formulated for isolated *M. demissus* hearts (Wilkens, 1972). In preparing the ion-free solutions, Na^+ was usually substituted for Ca^{2+}, Mg^{2+}, or K^+; Tris–Cl or Li^+ was substituted for Na^+; and propionate ($C_3H_5O_2^-$) was used to replace Cl^-.

All of the test solutions, as well as the artificial sea water, were made isosmotic or slightly hyperosmotic to the sea water of acclimation (930 m-osmoles/kg H_2O for Cape Cod mussels; 890 m-osmoles/kg H_2O for Assateague mussels). Osmotic concentrations were determined with a freezing-point depression osmometer (Precision Systems Osmette).

Ion-deletion experiments: typical procedure

Four *M. demissus* ventricles were isolated and suspended in sea water in 1 ml aerated organ baths (described by Pierce & Greenberg, 1972). When all four hearts were beating regularly they were washed rapidly with 10 bath volumes of sea water. After 30 min in this solution the fluid in each of the baths was sampled with haematocrit tubes as previously described (Pierce & Greenberg, 1972). In three of the baths the sea water was then replaced with a test solution; the fourth heart served as the control and was perfused with sea water throughout the experiment. The bath fluid was re-sampled, and the ventricles re-washed with the test solution at 30 min intervals, for 90 min. After the third test-sample was taken the ventricles were again perfused with normal sea water and were further sampled and washed at 30 min intervals for 1 h. In some experiments the test solution was a 50% dilution of full-strength sea water (hereinafter, 50% SW). Isolated *M. demissus* hearts immersed in 50% SW produce a vigorous amino acid efflux terminating in only 90 min (Pierce & Greenberg, 1972).

The samples in the haematocrit tubes were spotted on filter paper and sprayed with ninhydrin solution (0·5 g ninhydrin; 100 ml acetone; 2 ml acetic acid; 8 ml distilled water). The colour was developed by heating the filter paper in an oven (70 °C). The resulting chromatograms were photocopied (Luxacopy CMB–3) and the ninhydrin-positive spots (NPS) were assigned quantal response values based on their densities (Fig. 1).

The mechanical activity of each heart was monitored throughout each experiment with a 4-channel oscillograph (Grass Model 7) recording the output of the force-displacement transducer (Grass Model FT.03-C) attached to each heart.

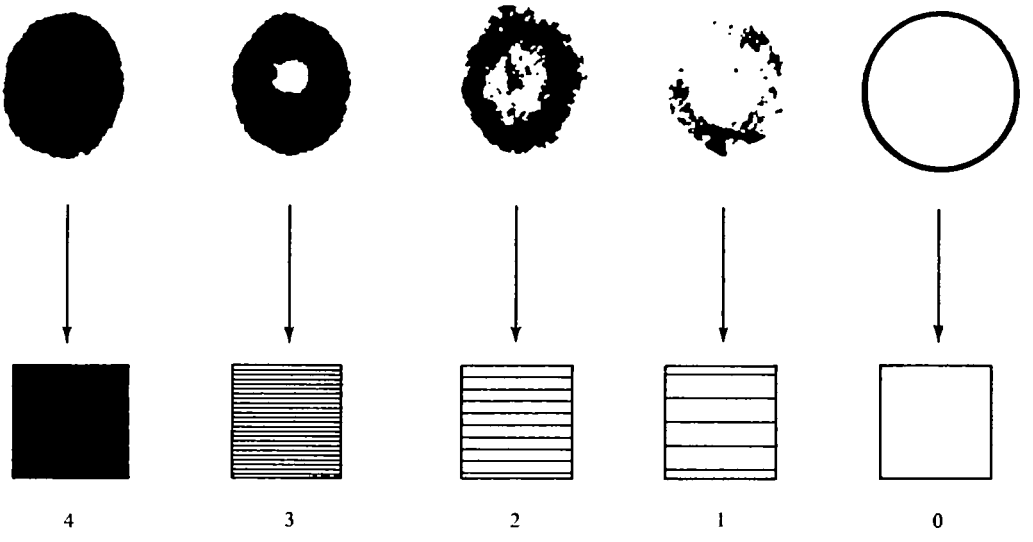

Fig. 1. Quantal conversion of NPS chromatographic spots to diagrammatic representations.

Quantitative analysis of the amino acid efflux

The amino acid efflux in response to the test solution was quantified as follows. Ten *M. demissus* ventricles were excised, washed in sea water, blotted, and placed in 1 ml of the test solution. After 120 min the hearts were removed from the bath, frozen on dry ice, lyophilized, and weighed. The bath fluid was extracted in hot ethanol (Pierce & Greenberg, 1972) and lyophilized. The residue was dissolved in an appropriate volume of sodium citrate buffer (pH 2·2) and tested with an amino acid analyser (JEOL-Model JLC-6AH).

RESULTS

Deletion of monovalent ions

The deletion of Na^+, K^+ or Cl^- from sea water did not produce an NPS efflux from the isolated *M. demissus* ventricles, albeit the mechanical properties of the beat were altered (Fig. 2). The immediate response of the ventricles to either Na^+-free or K^+-free SW was an increase in tone and frequency, accompanied by a decrease in amplitude. After 90 min in the test solution, however, the beat recovered the characteristics of the control period, except that the tone increase persisted in Na^+-free SW.

Cl^--free SW produced a gradual depression of ventricular beat, usually culminating in diastolic arrest during the first 30 min. During the ensuing 60 min most hearts recovered spontaneously. All the hearts beat normally following their return to sea water (Fig. 2).

Deletion of divalent ions

Ca^{2+}-free SW and Mg^{2+}-free SW each effected a moderate, but persistent, NPS efflux from the isolated ventricles. When both Ca^{2+} and Mg^{2+} were omitted from the

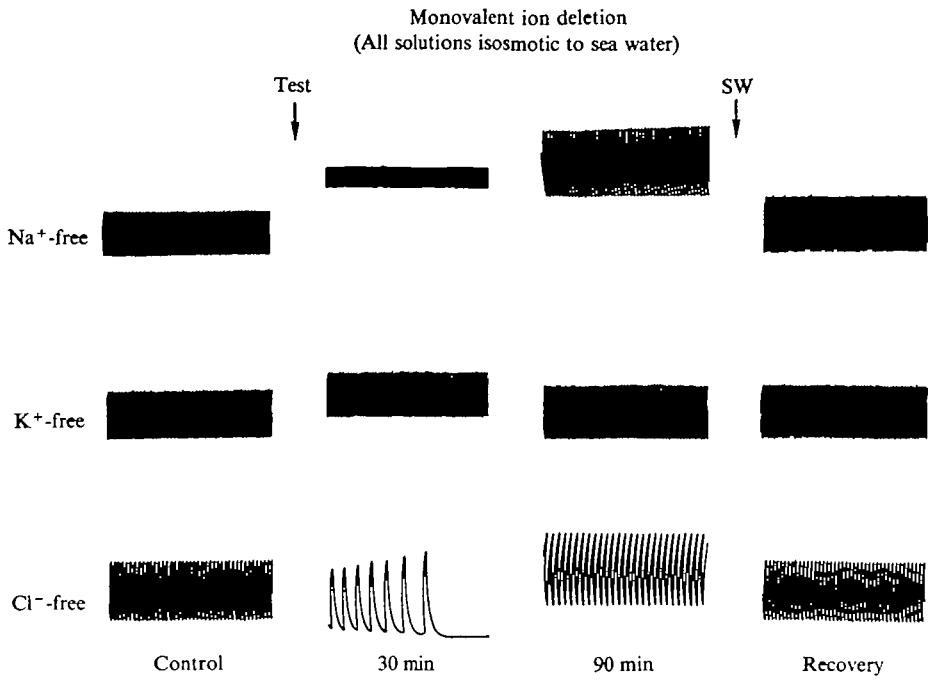

Fig. 2. The effects of monovalent ion deletion on the mechanical activity of the *M. demissus* ventricle. No NPS were recovered at the 30 min sampling periods. Position of recordings reflects base-line changes relative to control base-line. Test indicates introduction of the test solution into the organ baths and SW indicates the replacement of the test solution with sea water.

bathing solution (Ca^{2+}- and Mg^{2+}-free SW), a vigorous NPS efflux began and continued unabated until the hearts were returned to normal sea water (Figs. 3, 6).

The mechanical effects of Ca^{2+}-free SW were an initial depression of beat followed by the appearance of slow rhythmical contractions of low frequency and amplitude. Mg^{2+}-free SW had no marked effect on rhythmicity, although an irregularity – prolongation of systole during each beat – was observed in some preparations (Fig. 3). Ca^{2+}- and Mg^{2+}-free SW caused complete cardiac arrest, and the quiescent ventricles exhibited a marked loss of tone. All hearts in any of the divalent ion-free solutions recovered normal rhythmical activity upon return to sea water (Fig. 3).

Blocking of the NPS efflux

Since Ca^{2+}- and Mg^{2+}-free SW effects an NPS efflux, we attempted to block the hypo-osmotic NPS efflux with a high concentration of divalent ions (Fig. 4). Hearts exposed to 50% SW produced a large initial NPS efflux which tapered off and finally ceased after 90 min (Fig. 4; top line). A solution osmotically equivalent to 50% SW, but containing 150 mM-Ca^{2+} and 50 mM-Mg^{2+}, partially blocked this osmotically induced efflux (Fig. 4; middle line).

Low concentrations of lanthanum ion (La^{3+}) in a number of excitable tissues have been shown to act like much higher concentrations of Ca^{2+} (barnacle muscle, Hagiwara & Takahashi, 1967; guinea-pig ileum, Weiss & Goodman, 1969; squid giant axon, van Breemen & de Weer, 1970; frog sartorius, Weiss, 1970). Therefore, the

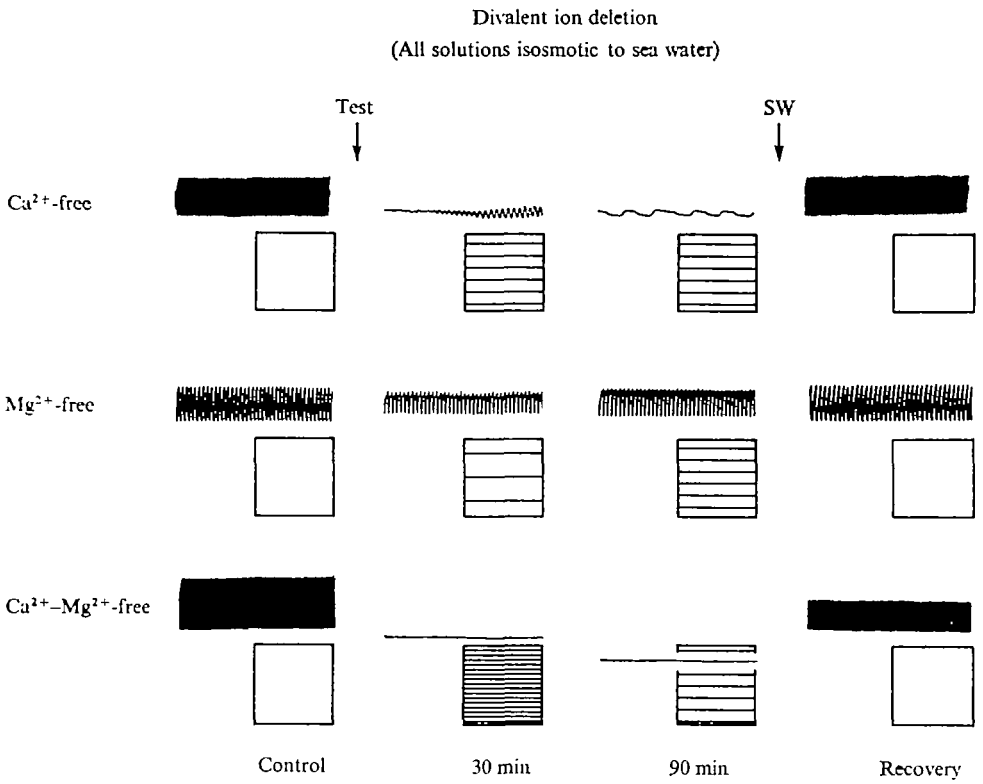

Fig. 3. The effects of divalent ion deletion on the NPS efflux and mechanical activity of the *M. demissus* ventricle. Position of recording reflects base-line changes relative to control base-line. Squares below recordings indicate quantal NPS efflux (Fig. 1) at each 30 min sampling period. Test indicates introduction of test solution into organ baths and SW indicates replacement of test solution with sea water.

effects of 50% SW containing 5 mM-La^{3+} were tested. This solution markedly reduced the NPS efflux (Fig. 4; bottom line). In addition, the mechanical activity of the heart was completely suppressed; but La^{3+} alone, even at lower concentrations, obliterated rhythmicity (Fig. 5).

Finally, the NPS efflux produced by Ca^{2+}- and Mg^{2+}-free SW was also effectively blocked by 5 mM-La^{3+} (Fig. 6).

Quantitative analysis of amino acid efflux

Amino acid analyses of the NPS effluxes obtained under various conditions are presented in Fig. 7. Taurine, glycine, and alanine are the major components of the NPS efflux, both in 50% SW (see also Pierce & Greenberg, 1972) and in Ca^{2+}- and Mg^{2+}-free SW. In addition, glutamic acid, threonine, serine, and aspartic acid were detected, but in amounts smaller than 1 μM/g dry weight of ventricular tissue. Clearly, the efflux in 50% SW is larger than that in Ca^{2+}- and Mg^{2+}-free SW over a 2 h period; but the proportional concentrations of individual amino acids are similar in both cases.

Fig. 7 shows, quantitatively, that La^{3+} almost completely blocks the Ca^{2+}- and

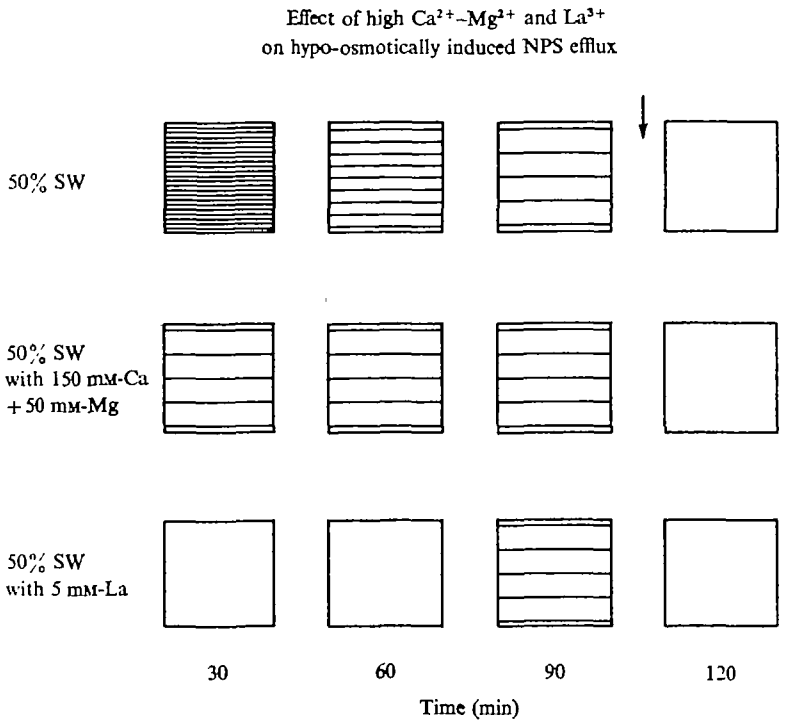

Fig. 4. Blocking of the hypo-osmotically induced NPS efflux by high divalent ion concentration and by La^{3+}. Squares indicate quantal NPS efflux at each sampling interval. Arrow indicates replacement of test solution with 50% SW.

Mg^{2+}-free induced amino acid efflux, and also retards, markedly, the efflux in 50% SW.

Amino acid analyses of the bath fluid from hearts left in Na^+-free SW, K^+-free SW, Cl^--free SW and full-strength sea water indicated small amino acid effluxes, less than 10 μM total amino acid/g dry weight, in all solutions.

DISCUSSION

Cellular volume regulation in *Modiolus* is accomplished by isosmotic release of intracellular free amino acids. The amino acid efflux reflects a change in cell membrane permeability, and is responsive both to the external osmotic pressure and to the external divalent ion concentration. These two factors are apparently independent. The hypo-osmotic efflux is transient and occurs at low external salinity, even if Ca^{2+} and Mg^{2+} are at normal, or even higher, concentrations; but a continuous efflux takes place in sea water lacking Ca^{2+} and Mg^{2+}, even if the osmotic pressure is unchanged.

A possible mechanism relating osmotic pressure, divalent ion concentration, amino acid efflux, and membrane permeability to amino acids has emerged. The cell membrane is seen as containing specific sites of amino acid permeability. The sites exist in two conformations in steady state equilibrium. In the open conformation (S_p) amino acids can pass from the inside to the outside of the cell; in the closed conformation (S_n)

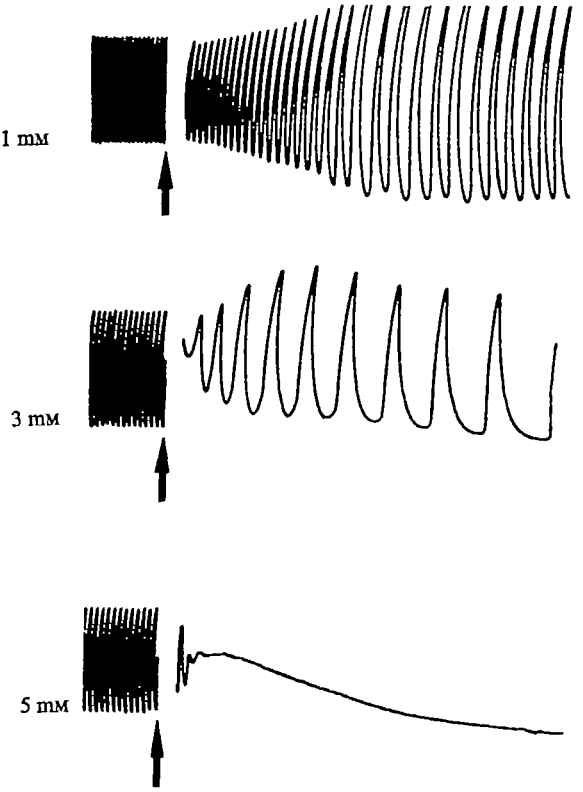

Fig. 5. The effects of La^{3+} on the mechanical activity of the isolated *M. demissus* ventricle. Arrows indicate point of application of the test solution indicated.

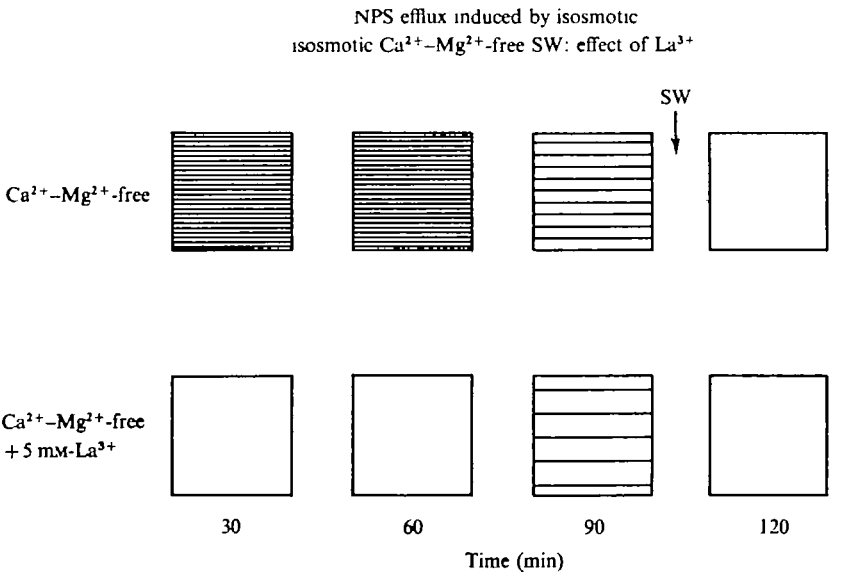

Fig. 6. Blocking by La^{3+} of the NPS efflux induced by Ca^{2+}- and Mg^{2+}-free SW. Squares indicate quantal NPS (Fig. 1) at each 30 min sampling period. SW indicates replacement of test solution with sea water.

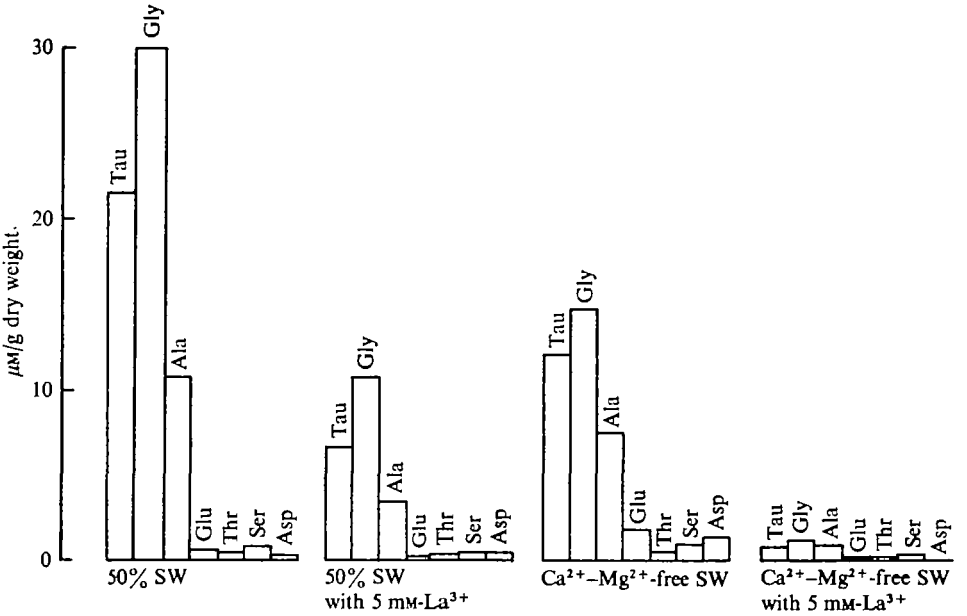

Fig. 7. Amino acid analysis of the NPS efflux from *M. demissus* hearts after 120 min in various test solutions.

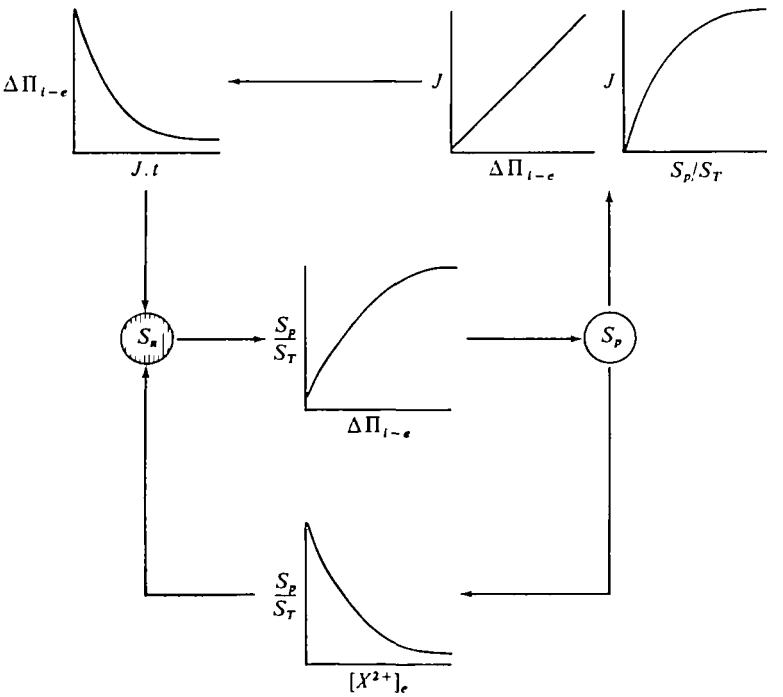

Fig. 8. Control of amino acid permeability in the bivalve myocardium. As the extracellular osmolarity decreases with respect to the cytoplasm ($\Delta\pi_{i-e}$), the fraction of permeable amino acid efflux sites (S_p/S_T, where S_T = total amino acid efflux sites) increases. Both the increased number of 'open' sites and the osmotic pressure differential augment the amino acid efflux (J; moles/cm²/h). As the flux continues ($J.t$), $\Delta\pi_{i-e}$ drops toward the value of the colloid osmotic pressure, and the number of amino acid sites in the non-permeable conformation (S_n) increases. S_p/S_T is also reduced by a process depending on the external Ca^{2+} and Mg^{2+} concentrations ($[X^{2+}]_e$).

they cannot. The S_p conformation is favoured by any decrement in extracellular osmotic pressure relative to that of the cytoplasm. S_n is maintained by an unspecified cellular process requiring Ca^{2+} and Mg^{2+}. In summary:

$$S_n \underset{-\Delta\pi}{\overset{[Ca^{2+}, Mg^{2+}]}{\rightleftharpoons}} S_p.$$

In cells acclimated to a particular osmotic concentration most, but not all, of the sites are in the S_n conformation; and the outward amino acid permeability is small, but not zero. For example, a small efflux from acclimated hearts in normal sea water was consistently observed.

This distribution of open and closed sites in osmotically equilibrated cells is in dynamic balance. The persistence of the divalent-ion-dependent process in acclimated hearts is most clearly demonstrated by the sustained amino acid efflux occurring when Ca^{2+} and Mg^{2+} are omitted from the bathing medium. The continuation of this process in the resting cell could be in compensation for the colloid osmotic pressure of the cytoplasm or for the thermodynamic preferability of the S_p conformation.

The consequences of a decrease in the ambient osmolarity follow from the foregoing model, and are shown diagrammatically in Fig. 8. A low external salinity shifts the acclimated equilibrium to the right; a larger fraction of the sites assume the S_p conformation; the outward permeability to amino acids increases; and the observed amino acid efflux occurs. The size of the efflux, reflecting the fraction of open sites, is dependent on the magnitude of the fall in salinity. As the flux continues the difference in osmotic pressure across the cell membrane decreases and – in the presence of divalent ions – the amino acid permeability sites may return to the closed conformation. Thus the efflux will be transient, as we have reported, and the length of the pulse, as well as its size, will be dependent on the osmotic pressure change.

In addition, according to the model (Fig. 8), the time course of the amino acid efflux will depend on the external divalent ion concentration. When the external medium is diluted, the concentrations of Ca^{2+} and Mg^{2+} are, perforce, also reduced. If the conversion of S_p to S_n depends on these ionic concentrations, as proposed, then longer effluxes would be expected in greater sea-water dilutions. If all Ca^{2+} and Mg^{2+} are removed then the efflux should go on indefinitely; and it does. At the other extreme the model suggests that, no matter how high the divalent ion concentration, if the salinity is lowered some efflux will occur; and that its time course will be limited by the maximum rate of the S_p- to S_n-conversion. In fact, not only high Ca^{2+} but also 5 mM-La^{3+}, failed to block completely the amino acid efflux in 50% SW.

The La^{3+} inhibition of both the hypo-osmotic and Ca^{2+}- and Mg^{2+}-free induced amino acid effluxes indicates, in part, that the site of action of the divalent ions in controlling the efflux is at the external surface of the membrane. Lanthanum does not pass through the cell membrane; it has been long used as an extracellular space marker in electron microscopy (e.g. Laszlo, Ekstein, Lewin & Stern, 1952). In addition, La^{3+}, due to its high charge density and specific activity, competes with Ca^{2+}- for Ca^{2+}-binding sites at the external membrane surface in numerous tissues (see references in Results section).

The mechanism whereby lowered external osmotic pressure induces the efflux would appear to be related to the increase in turgor pressure as water enters the cell.

For example, the resultant increase in tension in the cell membrane could change the conformation of the macromolecules comprising the amino acid permeability sites (Flory, 1956). However, the nature of the divalent-ion-dependent process, which restores the S_n conformation, remains obscure. Several mechanisms are possible: external divalent ion binding to the membrane might affect, directly, the conformation of the permeability sites; Ca^{2+} permeability could be increased inducing membrane contraction; an amino acid transport system could be involved.

Ample experimental and theoretical evidence from other studies supports the above possibilities. Permeability changes resulting from osmotic stretching and membrane contraction are well known in erythrocytes (Palek, Curby & Lionetti, 1971*a*). A Ca^{2+}-activated adenosine triphosphatase (ATPase) has been implicated in the restoration of both cell volume and osmotic behaviour in osmotically swollen erythrocyte ghosts (Wins & Schoffeniels, 1966*a*; Rosenthal, Kregenow & Moses, 1970; Palek, Curby & Lionetti, 1971*b*). In addition, a Ca^{2+}- and Mg^{2+}-activated ATPase, causing membrane contraction and decreased permeability, has also been found in isolated kidney-tubule membranes (Rorive & Kleinzeller, 1972), as well as in the red cell membrane (Wins & Schoffeniels, 1966*b*). Finally, a membrane-bound Mg^{2+}-activated ATPase has been implicated in the control of the passive ionic permeability of crayfish abdominal muscle (Bowler & Duncan, 1967). These systems have in common a divalent-ion-requiring membrane-bound enzyme functioning in permeability control and membrane contraction. We are currently examining the bivalve heart cells for a similar mechanism regulating amino acid permeabilities.

Amino acid transport seems to be unrelated to divalent ion concentrations. In most tissues amino acid transport seems to be directly dependent upon simultaneous Na^+ movements (for a recent review see Christensen, 1970). At present, however, the possibility of a divalent-ion-dependent amino acid transport system in the *M. demissus* heart cells cannot be ruled out.

In euryhaline Crustacea the enzymic reactions associated with amino acid synthesis and degradation are controlled by monovalent cation concentration (reviewed by Florkin & Schoffeniels, 1969). Frequently, this mechanism has, on theoretical grounds, been extended to include other invertebrate phyla (most recently Mollusca – Schoffeniels & Gilles, 1972); but experimental data supporting this extrapolation are lacking. While it cannot be denied that the maintenance and turnover of the free amino acid pool must be associated with these enzyme systems, it is obvious that the utilization of amino acids for volume regulation in the bivalve heart is independent of their metabolic origins. In fact, evidence for alternative mechanisms of amino acid regulation have been found in molluscs (Bedford, 1971; Pierce, 1971; Pierce & Greenberg, 1972). This report now strongly suggests that volume regulation in molluscan cells mediated by free amino acids is dependent upon the permeability characteristics of the membrane which, in turn, are controlled by the external osmolarity and divalent ion concentration.

SUMMARY

1. Molluscan cells maintain constant volume in decreasing salinities by releasing intracellular free amino acids as osmotic solute.

2. During a salinity decrease both osmotic pressure and ion concentrations decline simultaneously. Either condition might initiate the amino acid release.

3. Under constant osmotic conditions a decrease in Na^+, K^+ or Cl^- concentration in the medium surrounding the isolated bivalve heart, did not result in an amino acid efflux.

4. On the other hand, removal of Ca^{2+} and Mg^{2+} resulted in a vigorous amino acid efflux which continued until the divalent ion concentration was restored.

5. The hypo-osmotically induced amino acid efflux could be partially blocked by raising the external divalent ion concentration. Similarly, lanthanum proved to be an effective blocking agent, both in low osmotic pressures and in Ca^{2+}- and Mg^{2+}-free SW.

6. These results show that salinity-induced free amino acid regulation is initiated by a decrease in external osmotic pressure. The time course of the efflux is dependent on external divalent ion concentration.

7. Finally, the site of divalent ion action is at the external membrane surface.

8. A hypothetical membrane model is proposed.

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