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THE NATURE OF CELLULAR VOLUME REGULATION IN MARINE BIVALVES*

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INTRODUCTION

The salinity-induced regulation of intracellular free amino acids in the tissues of marine invertebrates has been the subject of a great deal of research during the last decade and a half. Although the process has been demonstrated in every marine animal tested, from ciliates to arthropods and fishes (for references, see Pierce, 1971*b*), the theory explaining the molecular basis of free amino acid regulation rests on crustacean data alone (Florkin & Schoffeniels, 1965, 1969; Schoffeniels, 1968). Florkin and Schoffeniels and their co-workers have demonstrated that several enzymes, associated both with amino acid synthesis and the electron transport system in the decapod crustaceans, are sensitive to external ion concentrations. An increase in salinity caused a simultaneous increase in amino acid synthesis (or a decrease in amino acid degradation) and a concomitant decrease in ammonia excretion. A decrease in salinity had the opposite effects (most recent review in Florkin & Schoffeniels, 1969); the ammonia produced diffused out of the cell in company with its osmotically obligated water, and the cell recovered its normal volume.

This hypothetical mechanism notwithstanding, amino acids might leave the osmotically active portion of the intracellular solute pool in at least two other obvious ways: incorporation into large colloidal protein, or excretion of the unchanged acids. In fact, proline, in addition to ammonia, is released from isolated crab nerve, in response to lowered salinities (Gilles & Schoffeniels, 1969).

The occurrence, in the Mollusca, of volume regulation mediated by free amino acids is established (Pierce, 1971*b*). The present study examines the time course of events occurring during the volume regulation of an isolated molluscan tissue. Isolated ventricles from two species of the bivalve genus *Modiolus* were chosen for several reasons. First, the physiological responses of intertidal and subtidal species of *Modiolus* to reduced salinities have been thoroughly examined (Pierce, 1970, 1971*a*, *b*). Secondly, the myocardium has a relatively uniform cell type compared to the intact

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animal; thus a uniform response is assured. Thirdly, isolation precludes effects of other organ systems on the responses being studied. Finally, the isolated bivalve heart exhibits an easily recordable normal response – spontaneous beat – which is modified by salinity changes. We have found that the ventricular cells of *Modiolus* extrude intact free amino acids, from the intracellular osmotic pool to the extracellular space, during volume regulation. Thus, the responses of molluscan and decapod crustacean cells, to osmotic pressure changes, differ.

A preliminary report of this investigation was communicated to the American Society of Zoologists (Pierce & Greenberg, 1970).

MATERIALS AND METHODS

Animals and sea water

Three forms of mussels were used in the experiments. Subtidal horse mussels, *Modiolus modiolus* (Linné) were collected at Manomet, Massachusetts. Specimens of the northern subspecies of the ribbed mussel, *Modiolus demissus demissus* (Dillwyn), were collected at Great Sippewisset Marsh on Cape Cod, Massachusetts. The southern subspecies of this mussel, *Modiolus demissus granosissimus* (Sowerby), were collected in a salt marsh on Alligator Point, Franklin County, Florida. The northern mussels were maintained on sea tables, in running sea water from Vinyard Sound (salinity 31‰; temperature 21 °C), at the Marine Biological Laboratory, Woods Hole, Massachusetts. The southern mussels were kept in aerated, constant-temperature aquaria (18 °C) at the Florida State University, Tallahassee, Florida.

Sources and composition of both natural and artificial sea waters were unexceptional and were described previously (Pierce, 1970).

Total water in ventricles from acclimated mussels

Modiolus demissus granosissimus were acclimated to various salinities between 3 and 48‰ for at least 3 weeks. In an earlier study (Pierce, 1970) this salinity range was shown to be non-lethal and the time period adequate for acclimation. Following acclimation, the mussels were opened, the ventricle was removed, and the rectum, in turn, was removed from the ventricle. The ventricle was quickly blotted on filter paper and weighed to the nearest mg. Next, the tissue was placed in a 10 ml Erlenmeyer flask, and frozen in a dry ice–acetone bath (temperature = −40 °C). The frozen heart was lyophilized overnight, and the dry tissue was re-weighed immediately following removal from the freeze-dryer. The amount of water in the tissues was then calculated using the following formula:

$$\text{total water as \% wet wt} = \frac{(\text{wet wt}) - (\text{dry wt})}{(\text{dry wt})} \times 100.$$

The water contents of all ventricles from each salinity were averaged, and standard deviations were computed.

Intracellular amino acid concentrations in hearts of acclimated mussels

A different group of *M. d. granosissimus* was acclimated to various salinities between 3 and 48‰ for at least 3 weeks. Following this period, the ventricles were

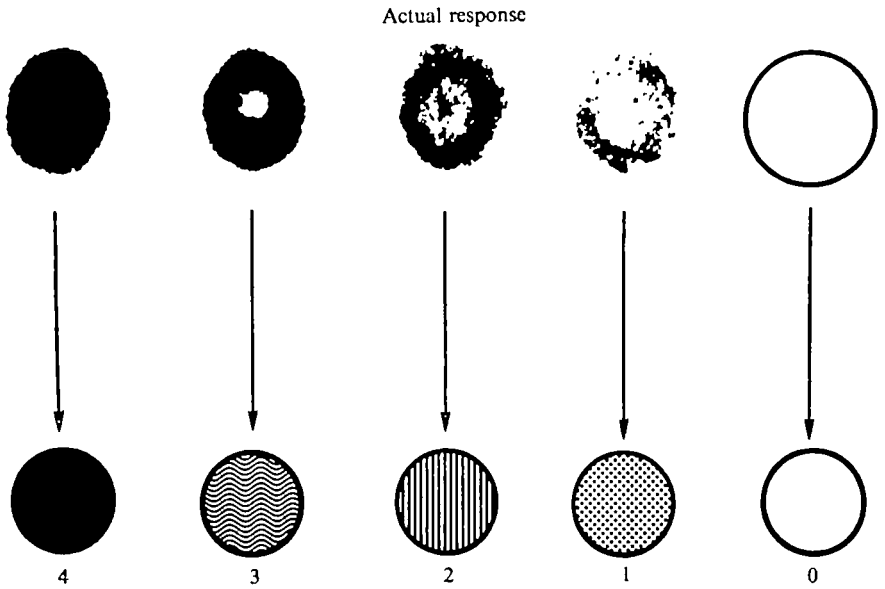

Fig. 1. Quantal conversion of NPS chromatographic spots to diagrammatic representations.

removed, frozen, freeze-dried and weighed. Intracellular free amino acids were then extracted from the dried tissue. The tissue was placed into 80% ethanol (EtOH) (1 vol EtOH: 1 vol tissue) and brought to a boil. After 30 min of extraction the tissue was homogenized (Sorvall Omnimixer). The homogenate was centrifuged at 20000 *g* for 30 min and the supernatant retained. The EtOH was evaporated off with a vacuum freeze-dryer, and the resultant residue was dissolved in an appropriate volume of sodium citrate buffer (pH 2·2). The amino acid composition of this solution was determined on an amino acid analyser (Beckman-Spinco Model 120C).

Qualitative measurement of the amino acid efflux

Following the classical procedure of Welsh & Taub (1948), ventricles from each of the three species were isolated from animals which had been kept in normal sea water. After isolation, the hearts were suspended in 1 ml aerated, temperature-controlled organ baths designed to facilitate rapid sampling and changing of the bath fluid. In each experiment mechanical activities of three experimental hearts and one control heart were recorded simultaneously with a 4-channel oscillograph (Grass Model 7) and force-displacement transducers (Grass Model FT 03).

After suspension in the organ baths the hearts were kept in normal sea water until all were beating rhythmically (usually 30–60 min). All hearts were then washed with 10 bath volumes of normal sea water (36‰).

After 30 min the bath water was sampled according to the procedure below. After sampling the hearts were washed and the salinity in three of the organ baths were changed. At 30 min intervals thereafter, until 60 min after the spontaneous beat had returned to its normal control appearance, each of the baths was sampled and washed. While the three experimental hearts were being washed at each interval with a solution

of the test salinity, the control heart was treated similarly but with normal sea water.

All bath fluids were sampled with glass capillary tubes (75 mm, un-heparinized), as follows. At the end of each 30 min interval, two capillary tubes were lowered vertically to the bottom of each bath. The fluid drawn up in the tubes by capillarity was spotted on filter paper (Whatman No. 4) mounted on a clean glass plate. The spots were concentrated by repeated application of sample, followed by drying with hot air. At the end of the experiment, ninhydrin was sprayed on the filter paper, and the spots were developed in an 80 °C oven for 3–4 h.

As soon as the chromatograms were developed, they were reproduced on a photocopier (Luxacopy), and quantal response values were assigned to the individual spots (Fig. 1).

The experiments on *M. demissus* hearts were carried out at room temperature (24 °C). However, *M. modiolus* hearts did not beat well at that temperature, and experiments with this species were carried out at 17 °C. The experiments with *M. demissus* were also repeated at 17 °C to ensure that species differences in efflux pattern were not due to differences in experimental temperature.

Quantitative measurement of the amino acid efflux and the intracellular amino acid concentrations of acclimated isolated hearts

Ventricles of *M. d. granosissimus* were isolated and suspended in organ baths. After all hearts were beating normally, they were washed in normal sea water, and the salinity of the bath fluid was changed to 48, 36, 18 or 3‰. After 60 min the hearts were removed from the baths, freeze-dryed and weighed. The free amino acid composition of the dry tissue was then determined by the procedure described above.

The water from the organ baths was also recovered, frozen and lyophilized. The resulting dry residue was taken up in an appropriate amount of sodium citrate buffer (pH 2·2), and the amino acid composition was determined with the amino acid analyser. In order to obtain a sufficient quantity of material for analysis in this experiment all of the ventricles acclimated to each salinity, and all samples of bath water, were pooled.

RESULTS

Ventricles from acclimated mussels: water of hydration and size of amino acid pool

The variation in the water content of ventricles, isolated from *M. demissus* acclimated to different salinities, is shown in Table 1. As might be expected, tissue hydration increases as external salinity decreases; but, while the external salinity varied by about 95 %, tissue water increased by only about 4 %. Therefore, volume regulation occurs in the ventricular tissue, as well as in the entire organism, in response to external salinity variation.

The concentration of intracellular free amino acids in the ventricles of acclimated mussels is represented by the histograms of Fig. 2. The concentrations of taurine, alanine, glycine and proline decrease markedly with decreasing salinity, while the rest of the major constituent of the free amino acid pool (glutamic acid, aspartic acid and serine) remained relatively constant. Proline was not detected in any test

Table 1. *Total water in isolated ventricles from* Modiolus demissus *acclimated to various salinities*

Salinity (‰)	Total water as % wet weight* (±S.E.)
48	82·88 (±1·08)
36	83·40 (±0·77)
18	85·37 (±0·79)
3	87·23 (±0·39)

* $\frac{\text{(Wet weight)} - \text{(dry weight)}}{\text{(Wet weight)}} \times 100.$

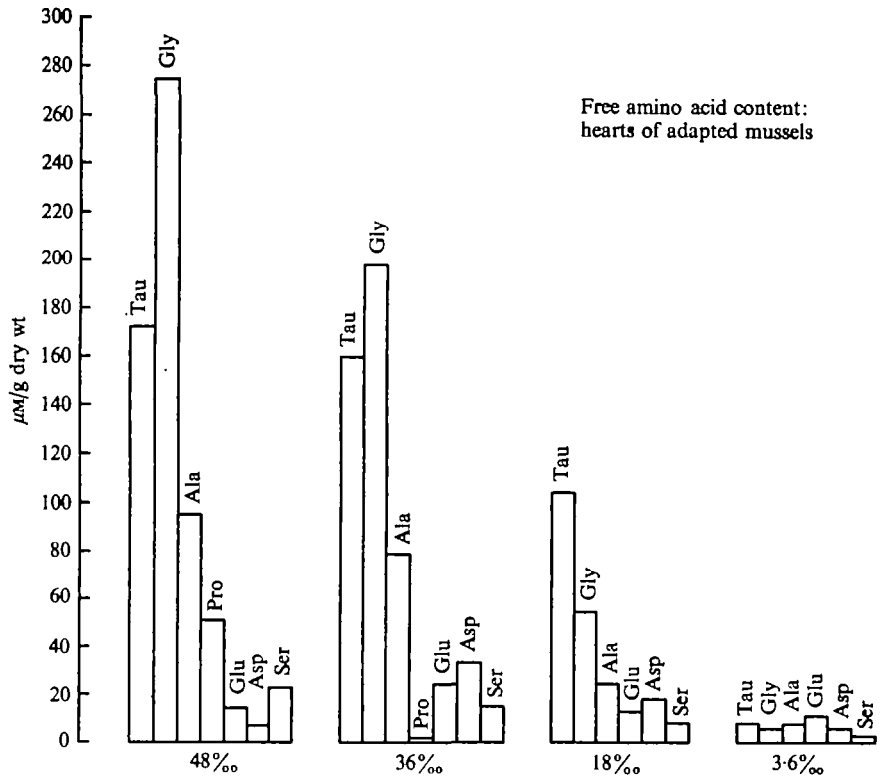

Fig. 2. Intracellular free amino acid concentrations in ventricles taken from *Modiolus demissus granosissimus* acclimated to various salinities.

salinity below 36‰. In the concentrated salinity (48‰), on the other hand, the tissue concentrations of taurine, alanine, glycine and proline increased while the rest of the pool remained unchanged.

Responses of isolated hearts to lowered salinities

Isolated, spontaneously active *Modiolus* hearts, challenged with a decrease in external salinity, stop beating temporarily. During this quiescence an efflux of ninhydrin positive substances (NPS) occurs, from the tissue into the bath fluid. A

Table 2. *The time course of both the mechanical response and the NPS efflux of isolated* Modiolus *ventricles during acclimation to various salinities*

		Mechanical response		NPS efflux	
Species	Salinity (‰)	Time to recovery of mechanical activity (min)	Time to recovery of normal beat (min)	Sequential quantal NPS efflux*	Time to end of NPS efflux (min)
Modiolus demissus demissus (23 °C)	23	No beat loss	30–60	1, 0	60
	18	10	60–90	3, 2, 1, 0	120
	18 (17°)	10	60–90	3, 2, 1, 0	120
	3	120–150	150–180	4, 4, 3, 2, 2, 1, 0	210
Modiolus demissus granosissimus (23 °C)	16	15	60	3, 2, 1, 0	120
	3	90–120	150–180	4, 3, 2, 2, 1, 0	180
Modiolus modiolus (17 °C)	27	No beat loss	30–60	1, 0	30
	23	10	30–60	2, 2, 0	90
	18	60–90	120–150	3, 4, 2, 1, 0	180

* Quantal efflux measured at 30 min intervals starting 30 min from the salinity decrease. See also Figs. 1, 3 and 4.

mechanical record and NPS efflux pattern typical of those obtained in this study is shown for isolated *M. d. demissus* ventricles (Fig. 3) and for *M. modiolus* ventricles (Fig. 4), both from a comparable salinity (18‰). The characteristic time courses of both the NPS efflux and the mechanical response of isolated ventricles exposed to lowered salinities are summarized for each species tested in Table 2.

The duration of both mechanical disruption and NPS efflux from isolated *M. d. demissus* ventricles depends upon the magnitude of the salinity decrease; the longest period of quiescence and efflux occurs in the lowest salinity tested. Regardless of salinity, the NPS released is always smaller in each successive 30 min sampling period.

Experiments with isolated ventricles of *M. d. granosissimus*, the southern subspecies, yielded similar results (Table 2). For instance, *M. d. granosissimus* hearts were exposed to 16‰ sea water; the time courses of both the mechanical events and the NPS efflux were similar to those occurring after treatment of *M. d. demissus* hearts with 18‰. However, in 3‰ sea water *M. d. granosissimus* ventricles characteristically recovered as much as 30 min earlier than those of *M. d. demissus*. This undoubtedly reflects the slightly narrower salinity tolerance of *M. d. demissus*; the lower limit of survival of this subspecies is 8‰, while that of *M. d. granosissimus* is 3‰ (Pierce, 1970).

The mechanical response and NPS efflux from *M. modiolus* ventricles in 27‰ are virtually identical to the responses shown by *M. demissus* in 23‰. In 23‰ sea water the ventricular beat of *M. modiolus* stopped, usually for about 5 min, and by the end of the first 30 min had recovered completely. NPS was detected in bath fluid samples after 30 and 60 min.

The responses of *M. modiolus* ventricles in 18‰ were markedly different from those of *M. demissus*. The mechanical activity stopped immediately upon exposure to 18‰ sea water, and did not reappear until between 60 and 90 min following the

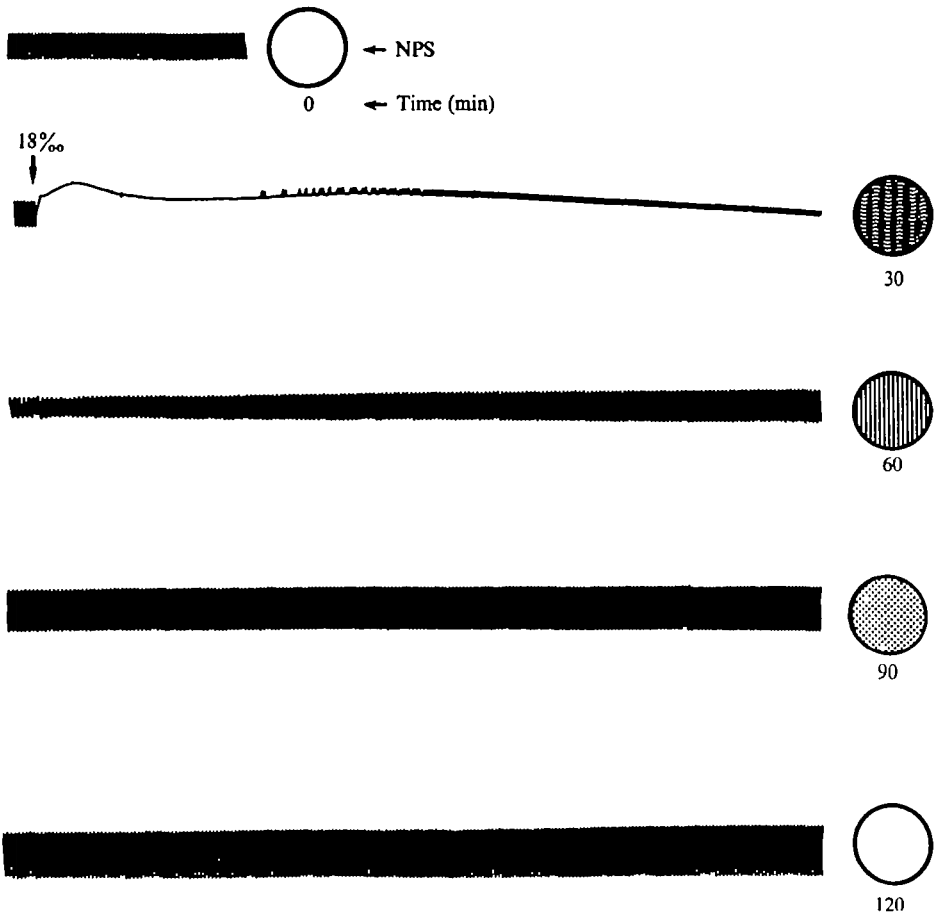

Fig. 3. The NPS efflux and mechanical response of isolated *Modiolus demissus demissus* ventricles to 18 ‰ sea water. The arrow indicates introduction of the test salinity.

salinity reduction. In addition, the peak of the NPS efflux from *M. modiolus* hearts occurred during the second sampling period (30–60 min) following the salinity change. Moreover, it continued for at least 30 min longer (120 min) than the efflux from *M. demissus* ventricles in the same salinity (compare Figs. 3 and 4 and Table 2).

Since *M. modiolus* hearts did not beat well at room temperature (23–24 °C), all experiments with this species were carried out at 17 °C. The possibility that the observed differences between the responses of *M. demissus* and *M. modiolus* were simply a function of experimental temperature was tested. As is shown in Table 2, the NPS efflux may be slightly retarded by the lowered temperature, but both the pattern and the time course of the NPS efflux and beat recovery are similar to those seen at the higher temperature.

In summary, the ventricles of the subtidal animal, *M. modiolus*, are less adaptive to low salinities than those of *M. demissus*.

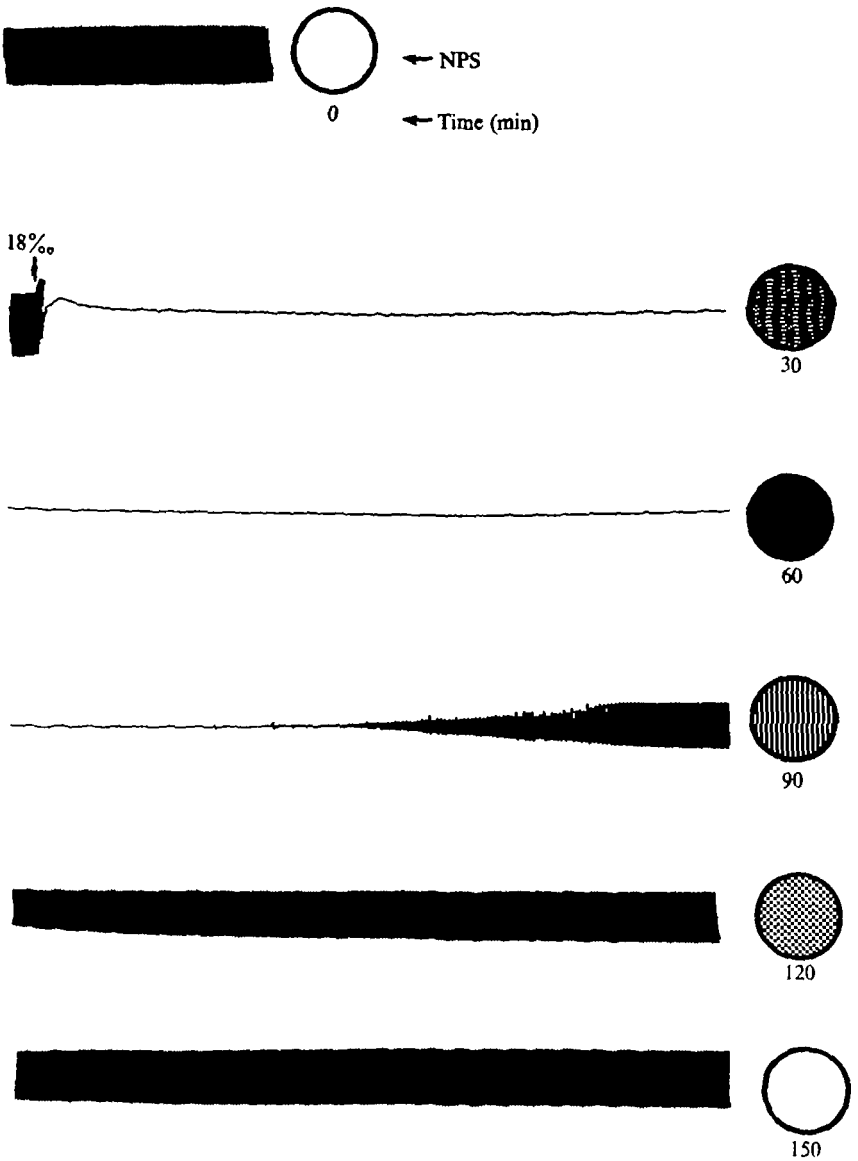

Fig. 4. The NPS efflux and mechanical response of isolated *Modiolus modiolus* ventricles to 18‰ sea water. The arrow indicates introduction of the test salinity.

Free amino acid pool of isolated acclimated hearts

The change in free amino acid concentration of isolated *M. demissus* ventricles acclimated to four different salinities is illustrated in Fig. 5. Taurine, alanine, glycine, and proline decreased sharply with decreasing osmotic pressure. Changes in other free amino acids were small. Again, proline was not observed in any salinity below 36‰.

Finally, we determined the composition of NPS released into the bath fluid (3‰)

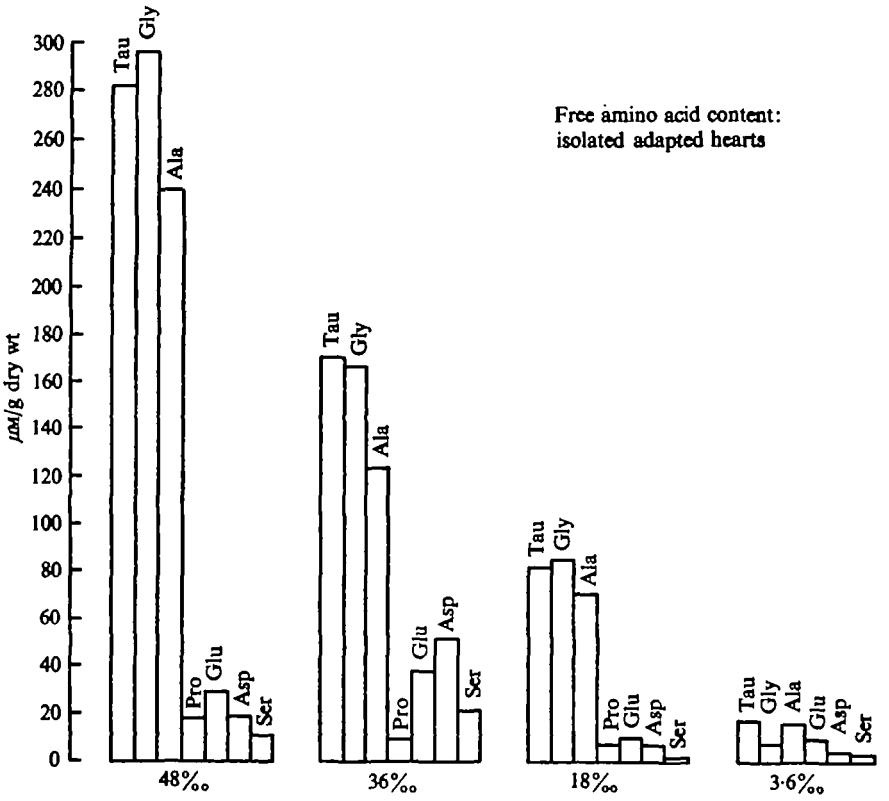

Fig. 5. The intracellular free amino acid concentrations of isolated ventricles of *Modiolus demissus granosissimus* following spontaneous beat recovery in various salinities. All ventricles were taken from mussels acclimated to 36 ‰ sea water.

by isolated *M. d. granosissimus* ventricles during their period of acclimation. These data, together with the free amino acid composition of the acclimated hearts, are presented in Fig. 6. Free amino acids were the only NPS detected in the bath fluid. Moreover, the decrease in intracellular free amino acids was equivalent to the amounts of these substances released into the bath water. As expected (Figs. 2, 5), taurine, alanine, glycine and proline were the major components of the efflux, although serine, glutamic acid and aspartic acid were also detected in small amounts (Fig. 6).

DISCUSSION

The volumes of the ventricular cells of *Modiolus* are regulated by a mechanism similar to that demonstrated in the intact mussels (Pierce, 1971*b*). In salinities more concentrated than the control level, intracellular free amino acid concentrations increase. This phenomenon has been described before in several bivalve species (Allen, 1961; DuPaul & Webb, 1970; Pierce, 1971*b*). In response to dilute salinities, taurine, alanine, glycine and proline are released from the intracellular free amino acid pool, and leave the cells, accompanied by osmotically obligated water. This regulatory process, controlling the amount of water in the cells, is independent of any

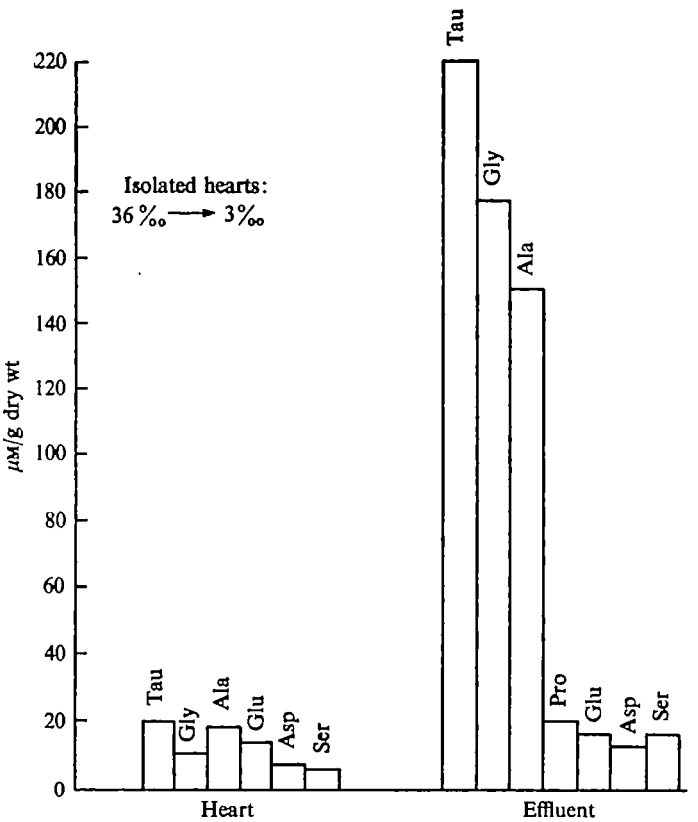

Fig. 6. Composition of the NPS efflux from isolated *Modiolus demissus granosissimus* ventricles in 3‰ sea water. The heart histogram indicates the concentration of free amino acids left in the tissues. The effluent histogram shows the free amino acids which diffused into the bath water.

extracellular osmoregulatory system. Since the free amino acids leave the intracellular pool and cross the membrane intact, the existing hypothetical description of salinity-dependent free amino acid regulation must be re-examined.

The current hypothesis, based on observations of euryhaline crustaceans, holds that certain intracellular dehydrogenases, such as glutamate dehydrogenase, catalysing the formation of amino acids from ammonia and α-keto acids, are inhibited by low salt concentrations. The results of this inhibition are an increase in the ammonia excretion rate, an increase in oxygen consumption, and a decrease in the size of the intracellular free amino acid pool (Florkin & Schoffeniels, 1969). This analysis implies that the ammonia diffuses out of the cell, and is the source of osmotic solute for cellular volume regulation.

This mechanism can not explain free amino acid regulation in molluscs. First, when challenged with a reduced salinity, molluscan cells release free amino acids rather than ammonia. Although our experimental system did not allow for the measurement of ammonia, the sum of the amino acids released from the tissue and the amino acids left in the tissue closely approximates the amino acids in the control hearts. This fact precludes amino acid degradation to ammonia as a major mechanism of amino acid regulation. Furthermore, although the crustaceans are ammonotelic (Parry, 1960)

molluscan nitrogenous waste is a mixture of amino acids, ammonia, and occasionally purines and uric acid (Potts, 1967; Hammen, 1968; Campbell & Bishop, 1970). Secondly, glutamate dehydrogenase, the enzyme so important to crustacean free amino acid regulatory processes, is either undetectable, or present in very small quantities, in molluscan tissue (reviewed by Campbell & Bishop, 1970). Thirdly, taurine is highly concentrated in molluscs, and appears to be more important as an intracellular osmoregulating component in molluscan (Bricteux-Grégoire *et al.* 1964*a*, *b*; Lynch & Wood, 1966; Virkar & Webb, 1970; Pierce, 1971*b*), than in crustacean systems (Schoffeniels, 1964; Vincent-Marique & Gilles, 1970); still, its fate in invertebrate tissues has scarcely been examined (Jacobsen & Smith, 1968). Furthermore, Florkin and Schoffeniels do not consider taurine in their theory.

Finally, removal of free amino acids from the intracellular osmotic solute pool via protein synthesis is apparently an unlikely mechanism in molluscs, inasmuch as the amino acid concentrations of the control tissue, and of the NPS efflux into low salinity, are similar. None the less, protein synthesis has been proposed as the main mechanism of salinity-induced free amino acid regulation in the isolated foot of the gastropod *Melanopsis trifasciata* (Bedford, 1971).

In conclusion, the regulation of the intracellular free amino acid pools of molluscs and crustaceans occurs by different mechanisms.

SUMMARY

1. Regulation of cell volume utilizing intracellular free amino acids has been studied in isolated ventricles from marine bivalves of the genus *Modiolus*.

2. As in the intact animal, ventricles taken from *Modiolus* acclimated to various salinities show only a slight change in tissue hydration. This control over cell volume is accomplished by isosmotic intracellular regulation of taurine, alanine, glycine and proline concentrations.

3. When stressed with decreased external salinities the isolated spontaneously beating ventricle becomes quiescent for a period, and then resumes activity. During the period of quiescence ninhydrin-positive substances (NPS) are released. The duration of quiscence and the amount of NPS released increase with increasing dilution of the external medium.

4. The salinity-induced NPS efflux is composed of taurine, alanine, glycine and proline.

5. In molluscs, the amino acids utilized for volume regulation are released from the cells unchanged and are not degraded into keto-acids and ammonia as they are in the crustaceans.

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