J. comp. Physiol. 108, 233–242 (1976)

Journal of Comparative Physiology · B

The Effects of Salt Depletion on Blood and Tissue Ion Concentrations in the Freshwater Mussel, *Ligumia subrostrata* (Say)

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Received December 22, 1975

Summary. The extracellular and intracellular fluid volumes of pondwater acclimated *Ligumia subrostrata* are equal (3.9 ml/g dry tissue). Total blood solute is 47 mOsm and is composed primarily of Na (19.1 mM), Cl (10.6 mM), HCO_3 (12.7 mM), Ca (4.3 mM), and K (0.5 mM). Major intracellular solutes are K (14.0 mM), Na (7.0 mM) and Cl (2.4 mM). *L. subrostrata* continuously exposed to deionized water at 20 °C exhibit a maximum decrease of 23% in extracellular fluid total solute within 30 days. The maximum [Na] and [Cl] losses are 40% and 76% respectively, while [Ca] and [HCO_3] increase by 44% and 37% respectively. No apparent change in extracellular [K] occurs. Intracellular [Na] decreases 53% and [Cl] decreases 79%, but [K] declines only 15%. Intracellular fluid volume, extracellular fluid volume, and total body water decrease 17%, 31%, and 22% respectively. Inulin clearance is 0.41 ml/g dry tissue·h for pondwater acclimated mussels and declines to 0.24 ml/g dry tissue·h during salt depletion. When salt depleted mussels are returned to solutions containing Na or Cl, they experience a net uptake of salt. The accumulated ions are about equally distributed in the extra- and intracellular compartments.

Introduction

The total blood solute concentration of many freshwater bivalves is unusually low compared to other freshwater metazoans (Krogh, 1939; Potts, 1954a; Little, 1965a; Bedford, 1973). Additionally, several of these molluscs are able to maintain their blood NaCl concentrations within narrow limits over a range of external NaCl concentrations of 0.1 to 14 mM (Hiscock, 1953; Chaisemartin et al., 1968). When external NaCl concentrations exceed 15 mM, blood solute concentration increases to a maximum tolerated level of 150 mOsm (Dietz and Branton, 1975). In deionized water the animals lose blood solutes.

Under normal conditions mussels are able to obtain ions from food and through epithelial ion transport systems, thereby compensating for ion losses.

With prolonged exposure to deionized water and in the absence of food, mussels lose ions via diffusion across epithelia and renal efflux, and the blood solute concentration is reduced (Krogh, 1939). When salt depleted animals are returned to pondwater the inward transport of Na and Cl is accelerated and the animals accumulate salt (Dietz and Branton, 1975; Krogh, 1939). However, there is no information pertaining to the changes occurring in mussels during the process of salt depletion. This study was undertaken to determine: the degree to which the unionid *Ligumia subrostrata* (Say) is able to maintain blood and cellular solute concentrations during exposure to deionized water; the role of the shell in supplying blood solutes; the uptake and distribution of Na or Cl when provided to salt depleted animals.

Materials and Methods

Ligumia subrostrata ranging from 22 to 57 g total weight (1.45–3.73 g dry tissue) were collected from a pond near Baton Rouge, Louisiana. The animal shells were washed with tap water and the mussels placed directly in deionized water or in artificial pond water (Prosser, 1973). Deionized water baths were changed daily at the outset of depletion and usually twice a week thereafter. Bath temperatures were maintained at 20 ± 1 °C by placing animal containers in a temperature controlled water table. No food was provided. Except in long term experiments, animals were used within 1–6 weeks of collection. Animals maintained in deionized water for 2 to 3 months suffered no mortality. Although some animals would die after several months in deionized water others have survived 10 months.

Inulin Clearance

Inulin-^{14}COOH (0.5 μCi in 25 μl) was injected into the foot of each mussel and the mussel was returned to an appropriate bath to equilibrate overnight (< 18 h). After equilibrium the valves were opened, the mantle cavity was drained of water, and the mussel transferred to an individual bath. After approximately 1 h (time allowed for siphons to extend), a bath sample was taken for liquid scintillation counting; two successive samples were taken at 0.5 h intervals. The mussel was immediately removed from the bath, valves opened, and mantle cavity drained of water. The posterior mantle tissue was separated from the shell at the pallial line and reflected anteriorly. A 1.5 ml sample was collected from the blood that accumulated in the shell cavity due to rupture of mantle sinuses (Dietz, 1974). The sample was centrifuged (5 min; $8{,}000 \times g$) and an aliquot taken for liquid scintillation counting. The remaining blood was used for ion analyses.

Rate of inulin clearance was determined from the change in bath radioactivity divided by the blood specific activity and expressed as ml/g dry tissue·h. The maximum experimental error considering possible timing, pipetting, liquid scintillation counting, and bath volume measurement errors is between 15 and 20% for this calculation, assuming sources of error are additive for each variable. Since blood specific activity (cpm/ml) decreased with time, clearance rates calculated from blood specific activities obtained at the end of the experiment may overestimate the clearance during the first interval. This error is minimized by sampling over short intervals.

Samples of inulin in the bath and blood were chromatographed to ensure no significant degradation occurred. The recovered isotope (89–93%) corresponded to the inulin standard.

Inulin Space

After blood collection, all tissue and body fluids were removed from the valves, placed in a tared pan, and dried at 90 ± 3 °C for a minimum of 24 h. The dry tissue was ground and a 1.00 g sample of tissue was transferred to a flask containing 0.1 N HNO_3. The tissue solutes

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were extracted at 4 °C for at least 24 h. The extraction solution was centrifuged (5 min; 8,000 × *g*) and a sample taken for liquid scintillation counting. The remainder of the extract was used for ion analyses.

Inulin space or extracellular fluid was determined by dividing the total tissue radioactivity by the blood specific activity and expressed as ml/g dry tissue. Wet tissue was determined by subtracting dry shell weight from total wet weight; total water was found by subtracting dry tissue from wet tissue. The intracellular fluid was then estimated from the difference between total tissue water and extracellular fluid.

Blood and Tissue Ion Analyses

Chloride was estimated, in duplicate, using an Aminco chloride titrator. Sodium and potassium were determined from diluted samples using a Coleman flame photometer. Calcium was determined on a Perkin-Elmer atomic absorption spectrophotometer using blood diluted with 1% LaO_3–5% HCl solution and the concentration expressed assuming all Ca was ionized. Total solute was determined on diluted centrifuged blood using a Precision Systems osmometer.

Bicarbonate was determined using a previously described method (Dietz and Branton, 1975; Potts, 1954a). This method utilized pH, total blood CO_2, the apparent dissociation constants of carbonic acid (4.46×10^{-7}) and bicarbonate (1.70×10^{-10}) to determine the HCO_3^- ion concentration.

Total CO_2 is the sum of dissolved CO_2, HCO_3^-, CO_3^{--}, and H_2CO_3, and was determined using a Gilson differential respirometer. Each respirometer flask contained 1.5 ml CO_2-free distilled water and 0.5 ml of blood in the chamber with 0.5 ml of 3.6 N sulfuric acid in the sidearm. The flasks were allowed to temperature equilibrate (29 °C) before sealing the system. The system was sealed and the sulfuric acid was tipped into the blood from the flask sidearm and CO_2 evolution measured manometrically. The pH was determined anaerobically on blood samples, obtained by cardiac puncture, (Fyhn and Costlow, 1975) using a Corning expanded pH meter in conjunction with a Beckman microsensor assembly with an asbestos fiber electrode (24 ± 1 °C).

Intracellular fluid ion concentrations were calculated from the difference between total tissue ions and extracellular fluid ions and expressed as mM/l intracellular fluid. There may be some error in the calculated intracellular fluid concentrations due to solubilization of complexed ions in the extraction process.

Unidirectional Ion Flux

Unidirectional ion influxes (J_i) were estimated by the disappearance of isotope from the bathing medium (Dietz and Branton, 1975). Bath samples were collected at specific intervals and the radioactivity detected by a liquid scintillation counter. Net fluxes (J_{net}) were calculated from changes in bath ion concentration and the efflux (J_o) estimated by the difference ($J_i - J_{net}$).

All data are expressed as means ± one standard error with the number of animals given in parentheses. The Student "*t*" test was used to compare means.

Results

Pondwater acclimated *L. subrostrata* have a total blood solute of 47.3 ± 1.2 mOsm. The major ion constituents of the blood are Na, HCO_3, Cl and Ca, with K making a minor contribution (Fig. 1, Table 1). Although total solute of the intracellular fluid must equal the blood, the specific composition was not determined. Of the intracellular ions measured, K is highest followed by Na and Cl respectively.

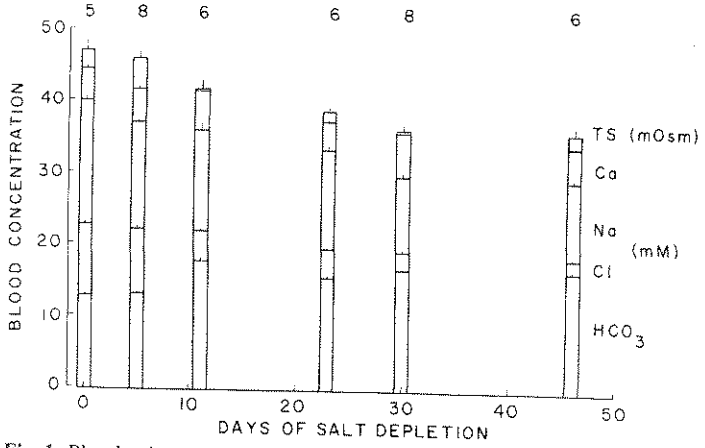

Fig. 1. Blood solute composition in *L. subrostrata* subjected to deionized water. The column height represents total solute (*TS*) and specific ion concentrations are represented by the subdivisions within a column. Vertical lines indicate one SEM. Number of sampled animals is given above each bar

Table 1. Effect of salt depletion on extracellular (ECF) and intracellular (ICF) fluid volumes and ionic concentrations in *L. subrostrata*

Days of salt depletion	ECF (ml/g dry wt)	ICF (ml/g dry wt)	g dry wt / g wet wt	ECF Na (mM)	ECF Cl (mM)	ICF Na (mM)	ICF Cl (mM)	ICF K (mM)
0	3.89* ±0.19 (15)	3.90 ±0.18 (15)	0.116 ±0.003 (16)	19.1 ±0.4 (16)	10.6 ±0.4 (16)	7.0 ±0.2 (14)	2.4 ±0.2 (15)	14.0 ±0.6 (15)
4	3.66 ±0.24 (16)	3.58 ±0.12 (16)	0.124 ±0.004 (16)	15.8[c] ±0.6 (10)	8.2[c] ±0.2 (16)	5.2[b] ±0.6 (10)	2.3 ±0.1 (16)	15.2 ±0.5 (16)
7	3.45 ±023 (6)	3.25 ±0.23 (6)	0.132[b] ±0.004 (16)	14.0[c] ±0.4 (16)	4.9[c] ±0.3 (16)	6.5 ±0.6 (6)	0.8[c] ±0.1 (6)	13.6 ±1.2 (6)
14	3.50 ±0.18 (15)	3.41 ±0.21 (15)	0.131[a] ±0.005 (16)	13.2[c] ±0.4 (16)	4.2[c] ±0.2 (16)	5.1[b] ±0.5 (15)	0.7[c] ±0.1 (15)	13.1 ±0.8 (15)
23	2.63[c] ±0.09 (15)	3.25[b] ±0.08 (15)	0.146[c] ±0.002 (16)	12.5[c] ±0.4 (16)	2.7[c] ±0.1 (16)	4.8[c] ±0.2 (15)	0.8[c] ±0.2 (15)	12.2[a] ±0.4 (15)
30	2.69[c] ±0.15 (8)	3.24[a] ±0.09 (8)	0.145[c] ±0.002 (8)	11.5[c] ±0.5 (8)	2.6[c] ±0.2 (8)	3.3[c] ±0.3 (8)	0.5[c] ±0.0 (8)	11.9[a] ±0.3 (8)

* Mean ± one standard error of the mean, and (*N*)

[a, b, c] Significantly different from pondwater acclimated animals: ($P<0.05$)[a], ($P<0.01$)[b], ($P<0.001$)[c]

(mOsm)

(mM)

CO_3

0

onized water. The column height
represented by the subdivisions
sampled animals is given above

cellular (ICF) fluid volumes and

	ICF Na (mM)	ICF Cl (mM)	ICF K (mM)
	7.0 ±0.2 (14)	2.4 ±0.2 (15)	14.0 ±0.6 (15)
	5.2[b] ±0.6 (10)	2.3 ±0.1 (16)	15.2 ±0.5 (16)
	6.5 ±0.6 (6)	0.8[c] ±0.1 (6)	13.6 ±1.2 (6)
	5.1[b] ±0.5 (15)	0.7[c] ±0.1 (15)	13.1 ±0.8 (15)
	4.8[c] ±0.2 (15)	0.8[c] ±0.2 (15)	12.2[a] ±0.4 (15)
	3.3[c] ±0.3 (8)	0.5[c] ±0.0 (8)	11.9[a] ±0.3 (8)

$P<0.05$)[a],($P<0.01$)[b], ($P<0.001$)[c]

Total tissue Na in *L. subrostrata* exposed to deionized water for 30 days declines 59% from 103 to 42 μeq/g dry tissue. Total tissue Cl decreases 83% from 51 to 8.5 μeq/g dry tissue, while total tissue K declines 29% from 56.5 to 39.9 μeq/g dry tissue.

Changes in tissue ion content can be partitioned into concentration changes occurring in the extracellular and intracellular fluid compartments when compartment volumes and blood concentrations are known (Table 1). After 30 days of salt depletion blood [Na] decreases 39.8% and blood [Cl] drops 75.5%. Blood [K] for pondwater acclimated mussels is approximately 0.4 to 0.7 mM (Potts, 1954a; Dietz and Branton, 1975). Contamination resulting from tissue damage during blood collection usually resulted in apparent blood [K] values of 1.0 mM or more, therefore blood [K] determinations were omitted from routine analyses. However, an extracellular [K] value of 0.48 ± 0.05 mM ($N=6$) was determined from blood samples carefully obtained by cardiac puncture on 60 day salt depleted animals. This suggests that extracellular [K] remains constant. Intracellular [Na] and [Cl] drop significantly over the 30 day interval, 52.9% and 79.2% respectively. Intracellular [K] remains relatively high exhibiting a decrease of only 15%.

Extracellular and intracellular fluid volumes decline progressively during salt depletion exhibiting 30.9% and 16.9% reductions respectively, after 30 days of depletion. Correspondingly, the dry to wet tissue weight ratio change indicates a progressive water loss of 25% during the same period of time.

The time dependent changes in blood solute composition are displayed in Figure 1. Total solute concentration declines gradually during 30 days of salt depletion and becomes stabilized between 30 and 46 days with total solute 23% less than pondwater acclimated animals. Rapid loss of blood Na and Cl during the first two weeks contribute to the decline in blood solute. An increase in blood HCO_3, from 13 to 17 mM, is responsible in minimizing the change in blood total solute concentration. Calcium concentration also increases 44% after 30 days of salt depletion, but the [Ca] changes are masked by variability. The pH of pondwater acclimated *L. subrostrata* blood is 7.927 ± 0.062 ($N=5$); blood from salt depleted specimens does not differ significantly from this value.

Inulin clearance (Table 2) declines from 0.41 ml/g dry tissue·h for pondwater acclimated mussels to 0.24 ml/g dry tissue·h for 30 day salt depleted mussels. The differences between the first and second interval are probably due to the decrease in blood specific activity as noted above.

When 45 day salt depleted mussels are returned to either 0.5 mM N_2SO_4 or 1.0 mM choline chloride there is a large net influx of Na or Cl during the first 2 h (Table 3). The animals were left in the respective solutions for 24 h and then blood was collected (Table 4). There was a significant increase in blood Na or Cl above the salt depleted controls.

The average amount of Na taken up was 13 ± 3 μeq/g dry wt in 24 h. The animals accumulating the greatest Na reduced the external bath Na concentration to 0.07 mM. The average amount of Cl accumulated during the 24 h was 11 ± 2 μeq/g dry wt. The lowest bath Cl concentration at 24 h was 0.1 mM.

Table 2. Inulin clearance during two consecutive 30 min periods by *L. subrostrata*. ECF = extracellular fluid

Days of salt depletion	ml ECF cleared/g dry wt·h		
	I	II	Avg.
0	0.50[a] ±0.06 (20)	0.33 ±0.03 (21)	0.41 ±0.03 (41)
4	0.63 ±0.13 (15)	0.47 ±0.09 (14)	0.55 ±0.08 (29)
7	0.43 ±0.04 (11)	0.34 ±0.05 (11)	0.39 ±0.03 (22)
14	0.55 ±0.03 (13)	0.47 ±0.05 (14)	0.51 ±0.03 (27)
23	0.38 ±0.05 (12)	0.30 ±0.03 (11)	0.34 ±0.03 (23)
30	0.25 ±0.03 (12)	0.23 ±0.04 (13)	0.24[b] ±0.02 (25)

[a] Mean ± one standard error of the mean; (*N*)
[b] Significantly different from pondwater acclimated mussels ($P < 0.001$)

Table 3. Unidirectional fluxes of Na from Na_2SO_4 and Cl from choline chloride by salt depleted *L. subrostrata*

Ion	*N*	μEq/g dry tissue·h	
		J_i	J_o
Na	5	2.24 ± 0.22	0.56 ± 0.15
Cl	7	1.69 ± 0.20	0.53 ± 0.09

Table 4. Blood NaCl concentrations of salt depleted *L. subrostrata* after 24 h in Na_2SO_4 or choline chloride

Treatment	*N*	mM/l	
		Na	Cl
Salt depleted	6	10.9 ± 0.5	1.8 ± 0.3
Na_2SO_4	6	12.7 ± 0.3[a]	1.1 ± 0.2
Choline chloride	7	10.4 ± 0.3	3.4 ± 0.4[a]

[a] Significantly different from salt depleted controls, $P < 0.05$

Discussion

L. subrostrata subjected to salt depletion experience significant changes in solute concentration. Blood [Na] and [Cl] decrease when the animals are exposed to deionized water, however, there is simultaneous increase in [Ca] and [HCO_3]. The decline in blood [Na] and [Cl] exceeds the increase in blood [Ca] and [HCO_3] resulting in a net decline in total blood solute concentration. Similar results have been noted in the freshwater gastropod *Viviparus viviparus* in response to "Washing out" in deionized water (Little, 1965a). Blood Ca and HCO_3 increased in approximately equivalent amounts; a major source is probably from the calcareous shell of the mussel (Dugal, 1939) although the contraction of the extracellular fluid volume could also contribute. The alkaline pH of *L. subrostrata* blood is similar to values reported for other molluscs (Wilbur, 1964). Increases in blood HCO_3 at constant pH will be associated with small increases in calculate blood P_{CO_2}. Using the Henderson-Hasselbach equation, estimated blood P_{CO_2} in pondwater animals is 6 mm Hg and increases to 8 mm Hg with salt depletion.

Greater than 90% of the total blood solute was due to the sum of Na, Cl, Ca, and HCO_3. The remaining solute is comprised of K and probably minor contributions of PO_4, SO_4, Mg and free amino acids (Potts, 1954a). The blood solute concentrations obtained for pondwater acclimated *L. subrostrata* are similar to other bivalves such as *Anodonta cygnea* (Potts, 1954a) and *Margaritifera* (=*Margaritana*) *margaritifera* (Chaisemartin, 1968a).

Intracellular ion concentration changes parallel those of the blood. It is interesting that both intracellular and blood [K] remain relatively constant. Burton (1968) suggests that dilution of body fluids during the vertebrate invasion of freshwater was limited by the necessity of maintaining optimal levels of cellular K. Freshwater bivalves have adapted to the lowest solute levels known, but are also limited to maintaining blood and cellular ion gradients for proper cell function.

The use of salt depletion to stimulate ion transport has been widespread since the studies of Krogh (1939). However, there have been few studies of the physiology of salt depletion. The decrease in blood and tissue solutes in salt depleted gastropods is similar in magnitude and time course to *L. subrostrata* (Little, 1965a; Greenaway, 1970). Larval insects (Wright, 1975) and annelids (unpublished) display a substantial decrease in blood [Na] and [Cl]. Some crustaceans are more resistant to blood ion loss (Shaw, 1959), as are salamanders (Alvarado and Dietz, 1970).

The elevated J_i of Na and Cl when salt depleted animals were returned to Na_2SO_4 and choline chloride solutions is similar in magnitude to an earlier study (Dietz and Branton, 1975). Of interest is the redistribution of accumulated ions in the body fluids. If all Na and Cl returned to the blood compartment the expected blood concentrations would be 16 mM Na/l and 6 mM Cl/l. However, less than 40% appeared in the blood (Table 4). Since blood represents about 45% of the body fluids this suggests that accumulated Na and Cl were about equally distributed in the blood and cellular compartments.

The use of inulin for determining extracellular fluid volumes is common

(Thorson, 1964). Presently there is no evidence that inulin is absorbed or metabolized by cells and is probably restricted to the extracellular space. Additionally, low concentrations of inulin have no known effects.

As salt depletion progresses, the extracellular fluid volume decreases by 31% and the intracellular fluid volume decreases by 17%. Simultaneously, there is a net loss of total water by the mussels. A 23% drop in total blood solute concentration occurred after 30 days of depletion. However, a reduction of water volume in each fluid compartment tends to minimize the decline in solute concentration due to ion loss from the animal. It is noteworthy that cellular volume is regulated at the expense of extracellular volume.

Martin et al. (1958) determined an inulin space of $49.0 \pm 6.0\%$ (space as per cent wet tissue without shell) for *M. margaritifera*; for *Mytilus californianus* a blood volume of $50.8 \pm 7.6\%$ and a dry tissue to wet tissue ratio of 11% were found. Values of $49 \pm 1.8\%$ for hemolymph, $39 \pm 2.9\%$ cellular water, and 89% total water have also been determined for *M. margaritifera* (Chaisemartin et al., 1970). Potts (1954b) found an inulin space of 55% of wet weight (without shell) for *A. cygnea*. These data agree with the values obtained for pondwater acclimated *L. subrostrata* (3.9 ml/g dry tissue $\simeq$ 0.45 ml/g wet tissue without shell). Chloride space is significantly higher (0.55 ml/g wet tissue) than inulin reflecting the presence of intracellular chloride.

Pericardial fluid results from filtration of the blood through the walls of the heart. This ultrafiltrate passes through the kidney nephrostome and into the tubule where significant salt reabsorption occurs. The final urine which is excreted is hypoionic to the blood and the pericardial fluid (Picken, 1937). Inulin is assumed to be neither secreted nor reabsorbed and therefore provides an excellent means for estimation of filtration rate and an approximation of urine flow. Inulin clearance is not a true indication of urine flow because some water is reabsorbed from the urine in conjunction with salt reabsorption during the formation of a hypoionic urine. Urine flow has not been measured in *L. subrostrata* because the nephropore opens into the anterior portion of the suprabranchial chamber and cannot be cannulated without tissue damage.

The inulin clearance of 0.41 ml/g dry tissue·h ($\sim$0.048 ml/g wet tissue·h) for pondwater acclimated mussels is of the same order of magnitude as reported values for *A. cygnea* (Potts, 1954b) and *M. margaritifera* (Chaisemartin et al., 1970; Martin et al., 1958). As salt depletion progresses, a 40% decrease in filtration rate (inulin clearance) occurs. This probably reflects the decrease in osmotic water uptake due to the lower osmotic gradient.

The measured inulin clearance indicates a water filtration rate of 115% of the mussels soft tissue per day. Little (1965b) reported similar rates for *V. viviparus*, however, he noted that 73% of the filtered water was reabsorbed. It is noteworthy that molluscs have the highest filtration rates of freshwater animals (Kirschner, 1967). This observation is even more interesting considering that the bivalves maintain a low blood solute concentration. The combination of high filtration rate and low blood solute suggests freshwater bivalves have an epithelial water permeability 5–10 times greater than other freshwater animals.

Although the urine in mussels is hypoionic to the blood, the rapid water turnover contributes to the loss of blood solutes when mussels are in deionized

water. However, the changes in ion concentrations are less than would be predicted from first order kinetics. The efflux of Na and Cl is about 1.5 μeq/g dry tissue·h (Dietz and Branton, 1975). Based on total tissue Na and Cl, the predicted turnover time would be 16 days for Na and 6 days for Cl. The measured solute concentrations decline but approach a steady state within 30 days. Therefore, some mechanisms must exist for reducing ion loss. There are several potential routes of ion loss and one or more may be controlled.

Behaviorally, the mussels are able to close their valves and markedly reduce their heart rate (Koch, 1917). When the valves are closed, water is impeded from entering the mussel osmotically and, if water balance is to be maintained, urine flow will be interrupted (Hiscock, 1953). Mussels in deionized water for prolonged periods of time have been observed to be open and siphons extended only periodically.

Finally, the ion transport mechanisms increase their activity. Thus, urinary loss of salt could be significantly reduced. Diffusive loss of ions across epithelial tissue may also be reduced (see Table 3). The exact method through which transport functions are controlled is not known. Hormonal regulation of salt transport in snails has been suggested (Chaisemartin, 1968b) but has not been explored in mussels.

In conclusion, it appears that *L. subrostrata* has a well developed osmo-ionoregulatory mechanism as indicated by its marked resistance to salt depletion, which includes behavioral adaptations, as well as possible renal and epithelial ion transport adaptations.

Part of this paper was submitted by William A. Murphy to the Graduate Faculty at Louisiana State University in partial fulfillment of the requirements for the M.S. degree. We wish to thank Ms. Mary Bozeman and Avia Dimattia for typing the manuscript. This investigation was supported in part by National Science Foundation Grant BMS 75-05483.

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