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INFLUX, NET FLUX AND TRANSEPITHELIAL FLUX OF AMINO ACIDS IN THE HARDSHELL CLAM *MERCENARIA MERCENARIA* (LINNE): INFLUENCE OF SALINITY*

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Abstract—1. The effects of salinity on the uptake and internal distribution of alanine and other free amino acids (FAA) in hardsell clams, *Mercenaria*, was studied by radiochemical and high performance liquid chromatography (HPLC) techniques.

2. Exposure of animals to reduced salinity does not alter the rate of unidirectional alanine influx.

3. In 34‰ salinity, entry of labeled alanine reflects the net flux of the amino acid. However, in 17‰ salinity, there is a net loss of alanine and other acids, mainly taurine, to the medium.

4. Reduced salinity induces greater incorporation of radiolabeled FAA into macromolecular fractions throughout the animal.

5. The major factors in reducing intracellular pools of FAA are loss to the external medium and incorporation into macromolecules.

INTRODUCTION

Uptake of free amino acids (FAA) by marine bivalves has been studied extensively since the appearance of Pütter's monograph on uptake and utilization of dissolved organic material by aquatic animals (1909). Jørgensen (1976) and Wright (1982) review this work and present evidence for interpreting entry of labeled FAA as net influx of substrate from the environment in these animals. Work subsequent to these reviews using high-performance liquid chromatography (HPLC) has demonstrated that entry of labeled substrates reflects the net entry of FAA in *Mytilus edulis* (Manahan *et al.*, 1982, 1983) and *Crassostrea gigas* (Rice and Stephens, 1987, 1988). Net entry of FAA occurring naturally in the normal environment of bivalve molluscs, annelids and larval and adult echinoderms has also been demonstrated (see reviews by Stephens, 1985, in press).

The present study reports uptake of ^{14}C-alanine by *Mercenaria mercenaria* at salinities of 34 and 17‰. Although *Mercenaria* is known to be less tolerant of low salinity than other bivalve species, including *Mytilus* and *Crassostrea*, it has been reported to survive and grow at salinities as low as 15‰ (Davis, 1969). Entry of labeled alanine is compared with net changes in ambient alanine as assessed by HPLC at the two different salinities. The distribution of labeled carbon in the trichloroacetic acid soluble and insoluble fractions of various tissues is reported and related to the FAA content of the tissues at the two salinities studied.

MATERIALS AND METHODS

Mercenaria were purchased locally after commercial collection in Massachusetts and air shipment to California. Shell-free wet weight ranged from 8.5 to 17.2 g. Animals were maintained unfed in natural seawater aquaria at 34 or 17‰ at 17°C for 5–7 days before use. Animals were acclimated for at least 2 hr to room temperature (21°C) prior to observation of uptake. Animals that failed to extend their siphons during the acclimation period were discarded.

Observations were carried out in artificial seawater (ASW) prepared according to Cavanaugh (1956) using reagent grade salts and HPLC grade water. Steady rates of water transport by *Mercenaria* were induced by artificial irrigation of the mantle cavity as follows. A 0.5 cm hole was drilled at the junction of the valves just anterior to the location of the incurrent siphon. A Pasteur pipet was introduced between the mantle edges via the hole. The mantle chamber was irrigated at 2.5 l/hr using a peristaltic pump to recirculate ASW from the 250 ml experimental vessel. This sufficed to induce steady pumping and reproducible rates of amino acid uptake throughout the 2-hr period during which samples were collected for analysis (see Fig. 1). Although this cannulation–perfusion procedure does not necessarily assure perfusion of the gills and other epithelial surfaces as in normally pumping animals, it does standardize water flow through the mantle cavity, facilitating interpretation of amino acid transport data. After irrigation had begun, sufficient ^{14}C-alanine was added to provide an initial activity of 320 Bq/ml (10 nCi/ml). ^{12}C-alanine (Sigma) was added to produce an initial concentration of 2 μM. Labeled alanine was obtained from New England Nuclear and had an initial specific activity in excess of 100 mCi/mmol (37 MBq/mmol).

Samples for analysis were withdrawn from the medium periodically for 2 hr. Depletion of radioactivity was followed using a Beckman 3801 scintillation counter. Samples (0.5 ml) were counted using Aquasol (New England Nuclear) added after acidification to volatilize any labeled

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carbon dioxide. Counting rates were corrected for background and quenching. Alanine concentration in the medium was determined by HPLC. The method used is essentially that of Jones *et al.* (1981) with minor modifications as described by Davis and Stephens (1984).

Clams were sacrificed after the 2-hr exposure to alanine as described. Hemolymph was sampled with a syringe as it collected in the pallial space after the adductor muscles were cut. Extrapallial fluid (EPF) was collected by slipping a syringe needle between the mantle and the shell. Radioactivity in the hemolymph and EPF was determined directly by scintillation counting. Samples of other tissues were taken and weighed. Tissues were minced and extracted for 24 hr in 5% trichloroacetic acid (TCA) made up in 50% ethanol. The TCA insoluble precipitates were washed in fresh ethanol-TCA and digested in a quaternary ammonium based tissue solubilizer (TS-2; Research Products International). Radioactivity in the TCA-soluble and insoluble fractions was determined by scintillation counting.

Metabolic conversion of labeled alanine to other amino acids was assessed by spotting 15 μl of the TCA-soluble extract on 12.7 × 12.7 cm cellulose TLC plates (Polygram CEL-300; Machery-Nagel). Amino acids were separated using the solvents and procedure described by Clark (1968). Spots were visualized with ninhydrin and the chromatograms were exposed to X-ray film (Kodak Omat) for 3–5 weeks.

The free amino acids present in samples of the gills, mantle tissue, hemolymph and EPF of *Mercenaria* were extracted in ethanol-TCA and analysed by HPLC after maintaining clams in seawater at 34 and 17‰ for 5 days. Peaks were identified by elution time and quantified by area, comparing them with chromatograms of authentic standards.

RESULTS

The data show that there is a steady exponential depletion of labeled alanine in both 100 and 50% ASW (Fig. 1). First-order depletion constants were determined by plotting the log of radioactivity vs time. In the case of 100% ASW, the depletion constant was 0.74/hr ($r = 0.996$). This yields an estimate of initial influx of alanine from the 2 μM medium of 0.37 μmol/hr or 26.1 nmol/g-hr. In the case of 50% ASW, the depletion constant was 0.51/hr ($r = 0.975$) and the initial depletion rate was 0.26 μmol/hr or 24.2 nmol/g-hr. Analysis of covariance (ANCOVA) of the log transforms indicates no significant difference ($P > 0.05$) in the weight-specific initial rates. The mean total radiolabel removed from 100% ASW at the end of 2 hr was 60.4 kBq or 377.5 nmol of alanine. In 50% ASW, the corresponding totals are 51.2 kBq and 320 nmol of alanine.

The net change in alanine in the medium as determined by HPLC analysis is different at the two salinities (Fig. 1). In 100% ASW, net removal of alanine corresponds closely to the depletion of labeled alanine in the medium. A semilog transform of the data gives an estimate of the first-order depletion constant of 0.64/hr ($r = 0.993$). The initial depletion rate is 0.32 μmol/hr or 22.9 nmol/g-hr. This is not significantly different from the influx of labeled alanine, 26.1 nmol/g-hr ($P > 0.05$; ANCOVA). In 50% ASW, there is a net loss of alanine to the medium. At the end of 1 hr of incubation, alanine in the medium has increased to approximately 4 μM after which there is no significant further change in alanine concentration in the medium.

Other amino acids are also lost to the medium when *Mercenaria* is placed in fresh 50% ASW (Table 1). The β-amino acid, taurine, is listed separately in the table. It is also the major amino acid of *Mercenaria* FAA pools. It is also the major amino acid lost to the medium, and its concentration increases through the 2 hr period of analysis. The medium concentration of the α-amino acids does not change significantly after 30 min.

Data concerning the distribution of labeled carbon in various tissues of *Mercenaria* after incubation in ^{14}C-alanine for 2 hr are presented in Table 2. As indicated, labeled carbon appears in all of the tissues as well as the hemolymph and EPF. Recovery of radioactivity lost from the medium is 84.5 (100% ASW) and 82.8% (50% ASW). Of the labeled carbon recovered, approximately 22% is in the TCA-insoluble fraction in 100% ASW while more than 53% is TCA-insoluble in clams incubated in 50% ASW.

At the end of 2 hr exposure, the bulk of the labeled carbon remained in the form of alanine according to examination of TLC autoradiograms of clams maintained in 100% ASW. Minor conversion to glutamate and aspartate was noted. In 50% ASW, the major portion of the radiolabel remained in the form of alanine, but there was greater conversion of radiolabel to glutamate and glycine.

The FAA content of mantle and gill tissues and of hemolymph and EPF of *Mercenaria* maintained in 100 and 50% seawater was determined by HPLC of

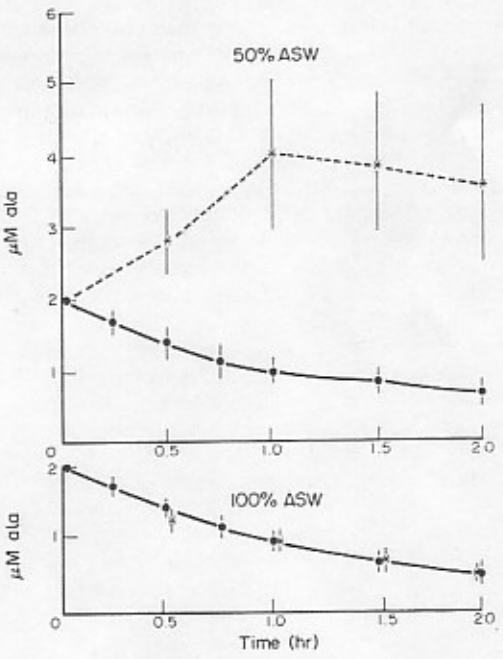

Fig. 1. Medium depletion of radioactivity and alanine concentration by *Mercenaria* was monitored by scintillation counting and HPLC, respectively. The first order depletion constants were estimated by linear regression of the natural log of remaining alanine in the medium versus time. Details of the regression results are in the text. Error bars are SEM ($n = 6$) for radiochemical determinations (closed circles and solid lines) and SEM ($n = 4$) for HPLC determinations (stars and hatched lines).

Table 1. Amino acids appearing in the 50% ASW medium. Initial non-zero values refer to the supplied (2 μM) alanine in the medium

Amino acid	Time (min) μmol ± SEM (n = 4) 0	30	60	90	120
Taurine	0	1.39 ± 0.29	2.11 ± 0.31	2.60 ± 0.42	3.41 ± 0.56
Other FAA	0.5	2.88 ± 0.65	3.56 ± 1.24	2.81 ± 1.18	2.57 ± 0.80
Total	0.5	4.26 ± 0.80	5.41 ± 1.43	5.41 ± 1.44	5.98 ± 1.21

Table 2. Radiolabel originally supplied as alanine in the external medium was determined in various tissues. Radioactivity was determined in the ethanol-TCA soluble and insoluble fractions of the tissues in response to normal (34‰) salinity and reduced (17‰) salinity. Radioactivity was determined directly in the fluids without ethanol-TCA

Tissue	kBq mean ± SEM (n = 6) 100% ASW Soluble	Insoluble	50% ASW Soluble	Insoluble
Gill	14.57 ± 3.54	2.31 ± 0.53	4.68 ± 0.74	5.03 ± 0.59
Mantle	8.25 ± 1.80	2.47 ± 0.25	3.67 ± 0.59	4.41 ± 0.39
Viscera	7.25 ± 1.97	1.36 ± 0.22	2.10 ± 0.69	2.94 ± 0.27
Adductor	2.12 ± 0.20	1.96 ± 0.08	2.48 ± 0.41	3.11 ± 0.13
Foot	2.62 ± 0.18	2.14 ± 0.13	3.12 ± 0.56	3.33 ± 0.29
Hemolymph	1.50 ± 0.18	—	0.60 ± 0.06	—
EPF	0.34 ± 0.02	—	0.13 ± 0.02	—
Total	36.66 ± 5.15	10.25 ± 0.66	17.12 ± 2.45	19.88 ± 2.09
Recovery %	65.2 ± 5.9	19.3 ± 2.7	40.7 ± 7.4	42.1 ± 6.6

ethanol-TCA extracts. Data are presented in Table 3. Total amino acids decrease in intracellular pools of the two tissues and increase in the extracellular fluids of clams maintained in 50% seawater.

DISCUSSION

M. mercenaria was one of the invertebrates examined in the early study of Stephens and Schinske (1961). In this work, net entry of amino acid was demonstrated colorimetrically by ninhydrin analysis. However, amino acid was supplied at an initial concentration of 2 mM which is far more than FAA concentrations expected in the natural habitat. Clams are buried in the sediment where the total FAA concentration in the interstitial water may be considerably higher than that in the water column (Henrichs and Raffington, 1979; Davis *et al.*, 1985; O'Dell and Stephens, 1986) but their siphons presumably draw water through the mantle cavity from the overlying water where FAA concentrations are in the range of 1–2 μM or lower (Jørgensen, 1982; Macko and Green, 1982; Braven *et al.*, 1984; Poulet *et al.*, 1985). In the present study, the net entry of alanine from the medium corresponds closely to the influx of labeled alanine in 100% ASW (Fig. 1). This is consistent with previous reports of net entry of FAA from seawater into marine bivalves based on HPLC analysis of net influx as noted in the introduction.

Clams placed in substrate-free ASW at 17‰ lose both taurine and α-amino acids during the 2 hr observation period used in this study (Fig. 1; Table 1). The animals had been maintained in seawater at the reduced salinity for 5–7 days. There is no reason to accept the rate of loss reported here as indicative of initial rates of loss on transfer to reduced salinity. Heavers and Hammen (1985) report that 29% of the FAA lost from tissues of intact oysters, *C. virginica*, appears in the medium in 1 hr, 89% of which is taurine. If the rate of loss of taurine in Table 1 is assumed to continue in a linear fashion for seven days, the total loss would amount to about 220 μmol. This is approximately 15% of the difference for gill and mantle tissue noted in Table 3. If the taurine content of these tissues is accepted as representative of the change in intracellular FAA of tissues of *Mercenaria*, the rate of loss observed in the present work does not suffice to account for the observed difference in intracellular pools of FAA at the two salinities (Table 3).

The loss of amino acids to the medium is puzzling in light of the report by Davis (1969). Either the 5–7 day period of acclimation employed in this study is inadequate for complete acclimation of *Mercenaria* to reduced salinity or the population studied by Davis is physiologically distinct from the population from which our animals were obtained. Loss of amino acids to the medium is not observed in euryhaline bivalves such as *Mytilus* which are fully adapted to 50% ASW (approximately 17‰) (Jørgensen, 1983). Thus *Mercenaria* would appear to be at a competitive disadvantage and therefore maladapted to hyposaline environments based on the present work.

Reduction of ambient salinity reduces intracellular pools of FAA in euryhaline invertebrates (see Bishop *et al.*, 1983 for a review). The response of uptake of FAA to changes in salinity has been investigated in several invertebrate groups. Stephens (1964) showed that uptake of glycine by euryhaline annelids, *Nereis limnicola* and *Nereis succinea*, was completely inhibited when salinity was reduced to levels where osmotic regulation of body fluids supervened. In contrast, studies of other osmoconforming invertebrates such as the bivalves *Mytilus* and *Rangia* and the brittlestar *Ophiactis*, show no sharp cessation of uptake of labeled FAA as salinity is lowered (Stephens and Virkar, 1966; Anderson and Bedford, 1973; Anderson, 1975). In these forms, there is no evidence for a well-defined onset of osmotic regulation of body fluids at reduced salinities.

The authors cited also determined the distribution of radioactivity in ethanol-soluble and ethanol-

Table 3. Free amino acids in selected tissues and fluids after extraction in ethanolic TCA and analysis by HPLC

Amino acid	Mantle 100% SW mean ± SEM ($n = 3$) (μmol/g)	Mantle 50% SW mean ± SEM ($n = 3$) (μmol/g)	Gill 100% SW mean ± SEM ($n = 3$) (μmol/g)	Gill 50% SW mean ± SEM ($n = 3$) (μmol/g)
Aspartate	14.4 ± 0.7	5.2 ± 0.8	5.5 ± 1.4	6.1 ± 1.6
Glutamate	30.4 ± 1.6	10.3 ± 1.0	12.1 ± 3.5	10.7 ± 0.8
Asparagine	2.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
Serine	11.0 ± 5.6	1.4 ± 0.4	3.3 ± 0.4	0.5 ± 0.3
Glutamine	6.4 ± 1.9	0.6 ± 0.1	1.2 ± 0.2	0.6 ± 0.3
Glycine	44.1 ± 7.8	13.3 ± 2.6	12.8 ± 2.2	4.9 ± 1.5
Arginine	8.4 ± 0.4	4.5 ± 0.2	2.2 ± 0.5	1.8 ± 0.2
Taurine	186.0 ± 26.8	65.5 ± 3.6	223.3 ± 39.6	82.1 ± 5.4
Alanine	66.8 ± 16.1	17.6 ± 1.4	23.8 ± 4.3	11.7 ± 0.7
Tyrosine	0.9 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
Methionine	15.1 ± 1.2	4.9 ± 0.6	4.5 ± 1.3	4.0 ± 0.6
Valine	3.6 ± 1.4	0.7 ± 0.1	1.5 ± 0.2	0.5 ± 0.1
Phenylalanine	3.0 ± 1.1	0.5 ± 0.1	1.2 ± 0.2	0.4 ± 0.1
Leucine	2.1 ± 1.4	0.5 ± 0.1	1.0 ± 0.1	0.3 ± 0.1
Isoleucine	1.7 ± 0.4	0.7 ± 0.1	1.5 ± 0.2	0.5 ± 0.1
Lysine	2.0 ± 0.2	0.9 ± 0.2	1.2 ± 0.1	0.9 ± 0.1
Total	415.9 ± 60.7	128.1 ± 1.1	296.3 ± 32.0	126.3 ± 5.9
Amino acid	Hemolymph 100% SW mean ± SEM ($n = 3$) (nmol/ml)	Hemolymph 50% SW mean ± SEM ($n = 3$) (nmol/ml)	EPF 100% SW mean ± SEM ($n = 3$) (nmol/ml)	EPF 50% SW mean ± SEM ($n = 3$) (nmol/ml)
Aspartate	140.2 ± 21.4	191.9 ± 60.2	53.4 ± 23.5	93.0 ± 21.1
Glutamate	134.5 ± 19.7	482.5 ± 206.4	50.2 ± 25.0	126.8 ± 39.7
Asparagine	14.5 ± 3.6	21.4 ± 7.7	6.6 ± 2.0	12.7 ± 1.3
Serine	90.2 ± 18.7	153.3 ± 17.1	38.5 ± 14.4	124.1 ± 3.5
Glutamine	83.2 ± 19.7	103.9 ± 31.2	42.6 ± 19.7	40.8 ± 13.4
Glycine	532.9 ± 103.9	1109.1 ± 367.6	117.3 ± 41.8	314.6 ± 153.4
Arginine	61.8 ± 28.8	139.5 ± 46.4	25.0 ± 14.3	47.3 ± 18.3
Taurine	955.4 ± 89.4	3404.9 ± 1264.9	467.8 ± 221.7	1990.5 ± 353.7
Alanine	618.6 ± 153.3	1416.2 ± 477.2	291.4 ± 156.6	657.1 ± 85.5
Tyrosine	19.6 ± 7.3	35.4 ± 5.0	7.7 ± 2.3	15.8 ± 4.7
Methionine	68.3 ± 12.2	164.6 ± 62.4	27.4 ± 10.5	64.3 ± 12.5
Valine	25.4 ± 2.2	59.6 ± 7.2	11.8 ± 1.9	42.1 ± 5.6
Phenylalanine	17.4 ± 1.4	38.4 ± 6.3	7.1 ± 1.3	29.5 ± 4.4
Leucine	21.6 ± 4.7	40.8 ± 3.7	9.4 ± 1.7	32.6 ± 3.9
Isoleucine	26.9 ± 3.3	54.8 ± 5.5	12.7 ± 2.4	44.8 ± 5.7
Lysine	50.0 ± 22.0	112.6 ± 52.7	24.2 ± 12.1	50.0 ± 27.3
Total	2861.4 ± 404.1	7528.7 ± 2529.0	1212.1 ± 491.9	3670.7 ± 527.5

insoluble fractions of whole body or gill homogenates. At reduced salinities, increased radioactivity was found in the ethanol-insoluble fractions. This presumably reflected increased incorporation of FAA into macromolecules such as protein thus rendering them osmotically inactive. This was interpreted as a compensatory mechanism sparing the loss of amino nitrogen and reduced carbon in *Ophiactis* (Stephens and Virkar, 1966) and *Rangia* (Anderson, 1975). Bedford (1971) reports a similar increase in radioactivity in the TCA-insoluble nitrogen of the euryhaline gastropod, *Melanopsis*. In this case, the increase in radioactivity in the TCA-insoluble nitrogen could be blocked by puromycin, an inhibitor of protein synthesis. However, it was not possible at the time of these studies to examine the net entry of FAA in these animals since techniques for quantitative analysis of amino acids in seawater by HPLC (Lindroth and Mopper, 1979) were not available.

The present investigation of incorporation of radioactivity into TCA-soluble and insoluble fractions of tissues of *Mercenaria* supports the earlier work cited. Table 2 reports a significant increase in incorporation of radioactivity into the TCA-insoluble fraction of gill, mantle, viscera and adductor muscle. Overall, the TCA-insoluble radioactivity at the end of a 2-hr exposure increases from 22% of the total in 34‰ ASW to 53% in 17‰ ASW.

Table 3 presents the data for changes in FAA content of mantle tissue, gill, hemolymph and EPF after incubation in the two salinities employed. Total FAA in mantle and gill tissues show a dramatic decrease after incubation in 17‰ salinity and a decrease in each individual amino acid with the exception of aspartate, glutamate, methionine and tyrosine in gill tissue. The reduction of intracellular FAA with reduced salinity is consistent with changes reported in other bivalves such as *Mya arenaria* (Virkar and Webb, 1970) and *M. edulis* (Gilles, 1972). In the present study, FAA in the hemolymph and EPF more than double. However, this shift in FAA intracellular pools to extracellular fluids accounts for only a minor fraction of the decrease in intracellular FAA (approximately 0.2% of the total change). Thus, the bulk of the decrease in intracellular FAA is due to incorporation into macromolecules and losses to the ambient medium.

The decrease of radiolabel recovered in the extracellular fluids under reduced salinity conditions (Table 2) apparently contradicts the findings of increased extracellular FAA in reduced salinity (Table 3). However, the data in Tables 2 and 3 are not

directly comparable. There is no reason to expect that radiolabeled alanine appearing in the hemolymph after the 2-hr incubation will have attained the same distribution in the animal that it would over the 5-day acclimation period.

Finally, labeled alanine entering from the ambient medium is rapidly and effectively translocated to deeper tissues (adductor muscles, viscera, hemolymph and EPF) at both salinities employed in this study (Table 2). Gomme (1982, 1985) and Wright (1985) have suggested that the primary role of amino acid uptake is to sustain the level of organic osmolytes (primarily FAA) in epidermal epithelia and that translocation to deeper tissues, if it occurs at all, is quantitatively trivial. It is clear that in the case of *Mercenaria*, there is rapid translocation of alanine to internal tissues of the animal after uptake from the medium. At high salinity (in this case 34‰), entry of labeled alanine reflects the net entry of alanine as estimated by HPLC. Therefore, FAA taken up from the medium are both translocated to deeper tissues and incorporated into TCA-insoluble macromolecules in these tissues. Presumably, they can also be deaminated and oxidized. This may possibly account for a portion of the discrepancy between disappearance of labeled substrate and recovery of labeled compounds (Table 2). Translocation and incorporation also occur at the lower salinity employed in this study, but the animals suffer a net loss of substrate as discussed above.

In summary, exposure to reduced salinity does not modify the rate of influx of labeled alanine into *M. mercenaria*. In 100% ASW (34‰), entry of labeled alanine accurately reflects the net flux of the amino acid. At both salinities employed in this study, there is rapid and effective translocation of alanine to, and incorporation into, deeper tissues of the animal. However, at 50% ASW (17‰), there is a net loss of alanine and other amino acids, mainly taurine, to the medium. At the lower salinity, increased incorporation of labeled alanine into macromolecules in tissues of the animal is observed. FAA in the body fluids (hemolymph and EPF) increase, but this accounts for only a minor fraction of the large reduction in intracellular FAA of the tissues. The major factors in reducing the intracellular pools of FAA are increased incorporation into macromolecules at reduced salinity and net losses of FAA to the medium after 5–7 days of acclimation to reduced salinity suggests that the population of *Mercenaria* studied in the present work may be poorly adapted to hyposaline environments.

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