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A STUDY OF THE METABOLIC ADAPTATIONS
OF A BIVALVE MOLLUSC
TO ENVIRONMENTAL STRESSES

by

SANDRA ALISON KORYCAN, B.Sc. (Hons. Biochemistry)

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

Department of Biology
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Ottawa, Ontario

November 15, 1984

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in partial fulfilment of the requirements for
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ABSTRACT

The effects of environmental stresses on the metabolism of isolated gill and mantle tissues of Mercenaria mercenaria revealed certain of the biochemical adaptations of this bivalve.

a) The major response to hypoosmotic stress is excretion of free amino acids with no alteration of intermediary metabolism.

b) Accumulation of free amino acids during hyperosmotic stress appears to be from an alteration of intracellular aerobic metabolic processes.

c) Utilization of glycogen and aspartate as anaerobic substrates and accumulation of the anaerobic end products, succinate and alanine changes over time during anaerobiosis. Phosphofructokinase and pyruvate kinase appear to control carbon flow during anaerobic metabolism.

d) The free amino acid pool is not enlarged during hyperosmotic stress under anoxic conditions. The changes in glycolysis are typical of the isolated tissue metabolic response to anoxia stress.

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LIST OF ABBREVIATIONS AND SYMBOLS

ADP	- adenosine diphosphate
AMP	- adenosine monophosphate
ATP	- adenosine triphosphate
°C	- degrees centigrade
CoA	- coenzyme-A
DHAP	- dihydroxyacetone phosphate
EC	- energy charge
EDTA	- ethylene diamine tetraacetic acid
EGTA	- (ethylenedis(oxyethylenenitrilo)) tetraacetic acid
euryhaline-	able to live in water of a wide range of salinity
euryoxic	- able to live in a wide range of oxygen partial pressures
FAA	- free amino acids
FDP	- fructose-1,6-diphosphate
G6P	- glucose-6-phosphate
g wet wt	- gram wet weight
h	- hours
I_{50}	- inhibitor concentration producing 50% decrease in enzyme velocity
$K_{0.5}$	- substrate concentration producing 50% of enzyme maximal velocity
mg	- milligram
MgCl ₂	- magnesium chloride
MgSO ₄	- magnesium sulphate
min	- minutes
μl	- microlitre

μ M	- micromolar
ml	- millilitre
mM	- millimolar
mOsmol	- milliosmolar
N	- normal
n_H	- Hill coefficient or slope of Hill plot
NAD ⁺	- nicotinamide adenine dinucleotide
NADP ⁺	- nicotinamide adenine dinucleotide phosphate
NADH	- nicotinamide adenine dinucleotide (reduced)
NaF	- sodium fluoride
O.D. ₃₄₀	- optical density @ 340 nm
PEP	- phosphoenolpyruvate
PEPCK	- phosphoenolpyruvate carboxykinase
PFK	- phosphofructokinase
PK	- pyruvate kinase
P _i	- orthophosphate
PMSF	- phenylmethylsulfonyl fluoride
s.e.m.	- standard error of the mean
TCA	- tricarboxylic acid
Tris	- tris (hydroxymethyl) amino methane
U	- units
V	- volume

INTRODUCTION

Molluscs are one of the major phyla present in the littoral zone. An important characteristic of this habitat is the tidal cycle which subjects the animals to oscillating changes in environmental conditions, such as large fluctuations in oxygen availability, salinity and temperature. Intertidal species may undergo two daily periods of exposure to air. Under these circumstances, bivalved molluscs may close their valves to avoid dessication. This rapidly leads to anoxia as blood and tissue reserves of oxygen are minimal and are quickly used up. Hypoxic or anoxic stress would also be commonly encountered by bivalve molluscs: 1) in the sand or mud bottom in which they are buried, particularly at low tide when oxygen supplies would be rapidly depleted as they can no longer circulate with surface water, and 2) in tide pools when the amount of oxygen dissolved can vary dramatically depending on temperature, salinity and the presence of organic decaying matter. Tidal waters, particularly those in estuaries or trapped in tidal pools, are also characterized by sudden and extensive changes in salinity. Intertidal animals may be exposed to 1) rainfall during low tide, 2) low salinity groundwater in the sand or mud bottom in which they are buried, and 3) hypersaline waters during evaporation of tidepool and coastal lagoons. Adaptation to

these environmental stresses occurs at behavioral, physiological and biochemical levels and it is this last aspect which is of primary interest in this thesis.

To cope with life in the intertidal environment, it is now well established that the remarkable tolerance to oxygen deprivation seen in bivalve molluscs arises from a well developed capacity for anaerobic energy production. When oxygen-based metabolism can no longer function, the bivalve modifies its intermediary metabolism in order to continue to meet tissue energy requirements. Anaerobic metabolism has been extensively investigated in the sea mussel, Mytilus edulis, (de Zwaan et al., 1976; de Zwaan, 1977; Kluytmans et al., 1977; Zurburg and Kluytmans, 1980; Ebberink and de Zwaan, 1980; Zurburg and Ebberink, 1981; de Zwaan et al., 1982) while others have examined aspects of anaerobiosis in various other bivalves (Stokes and Awapara, 1968; Collicutt and Hochachka, 1977; Eberlee et al., 1983; Korycan and Storey, 1983). It is well established that glycogen is the main substrate source for anaerobiosis, and is present in large quantities (de Zwaan and Wijsman, 1976). Amino acids are also involved as fuel substrates (de Zwaan, 1977). Lactate, the usual end product of carbohydrate fermentation in working vertebrate muscle, is not often a major end product of anaerobic metabolism in bivalves (Zandee et al., 1980). Instead, anaerobic breakdown of glycogen results in the formation of a variety of end products such as succinate, volatile fatty acids (mainly propionate and

acetate), alanine and the imino acids octopine, alanopine and strombine. Recently, strombine formation has been demonstrated in M. edulis adductor muscle during environmental anoxia and in the subsequent recovery period (de Zwaan et al., 1982; de Zwaan et al., 1983), however, strombine levels were not significantly elevated in tissues of Mercenaria mercenaria in either situation (Korycan and Storey, 1983). The end products produced differ considerably between species and between tissues of individual species and also fluctuate quantitatively with time during anoxia.

Figure 1 is an overall scheme incorporating most of the known elements of anaerobic metabolism in bivalves. Glycogen reserves appear to be the primary energy reserve for anaerobiosis. Of amino acids, aspartic acid has been shown to be an important substrate in some species (Collicutt and Hochachka, 1977; de Zwaan et al., 1982; Korycan and Storey, 1983). During the early stages of anoxia, glycogen is converted to pyruvate by glycolysis and pyruvate is transaminated to alanine with aspartate serving as the source of amino groups; the deamination of aspartate produces oxaloacetate which is reduced to malate in the cytosol using the reducing equivalents generated by the glyceraldehyde-3-phosphate dehydrogenase reaction. During later stages of anoxia, carbon-flow from glycogen is diverted at the level of phosphoenolpyruvate (PEP) away from alanine production and through the phosphoenolpyruvate

FIGURE 1: Scheme for anaerobic energy metabolism
in bivalve molluscs (Zurburg, 1981).

carboxykinase (PEPCK) reaction to produce oxaloacetate which is similarly reduced to malate. The carbon skeleton for malate (and therefore succinate) therefore can originate from either aspartate or glycogen depending on the stage of anaerobiosis and redox-balance is maintained in the cytosol irrespective of the distribution of flux. Malate passes into the mitochondrion and is converted to succinate via a reversal of 2 Krebs cycle reactions. The reduction of fumarate is coupled to ATP formation (Livingstone, 1982) making succinate production energetically advantageous over the synthesis of various cytoplasmic end products derived from pyruvate including octopine, alanopine, strombine and alanine. The catabolism of succinate to produce propionate in some species further increases ATP yield per glucose utilized. This enhancement of energetic efficiency of anaerobic metabolism in these animals, compared to the lactate formation typical of vertebrate systems, combined with a strong depression of metabolic rate during anoxia enables these animals to withstand long periods of anoxia. Several studies on various bivalves have shown that the anoxic metabolic rate is only a fraction (5-10%) of the rate in oxygenated seawater (de Zwaan and Wijsman, 1976; Pamatmat, 1979; Famme et al., 1981; Shick et al; 1983). Energy demand during anaerobiosis is greatly reduced (de Zwaan and Wijsman, 1976; Holwerda and de Zwaan, 1980; Zurburg, 1981) through a combination of such factors as a strong reduction of cardiac activity and decreases in

filtration rate and muscular activity.

In euryoxic bivalves that have significant quantities of both pyruvate kinase and phosphoenolpyruvate carboxykinase (PEPCK), a major branchpoint in glycolysis occurs at the level of PEP. According to the scheme in Figure 1, from PEP two conversions are possible leading to the formation of either pyruvate or oxaloacetate. The anaerobic production of succinate from glycogen reserves requires a differential regulation of the fate of PEP under aerobic versus anaerobic conditions and depends largely on the regulatory properties of pyruvate kinase and PEPCK. Most studies on molluscs have shown that PEPCK is not particularly active during the initial stage of anaerobiosis (de Zwaan, 1977; Ebberink and de Zwaan, 1980; Nicchitta and Ellington, 1983; Gade, 1983) and pyruvate kinase is predominant at the PEP branchpoint. Initially, glycolytic carbon is directed mainly towards pyruvate which in turn is primarily converted to alanine; meanwhile aspartate reserves are mobilized for succinate production. As the time of shell valve closure increases, succinate production from aspartate becomes gradually less important. Instead, succinate is increasingly derived from glycogen. This metabolic transition, aerobic to anaerobic, involves a transition at the PEP branchpoint to increase activity of PEPCK (Ebberink et al., 1979) and depress pyruvate kinase activity (Holwerda et al., 1981; Ebberink and de Zwaan, 1980). Studies of pyruvate kinase (PK) from tissues of anoxia-tolerant marine molluscs indicated that it

is an allosteric enzyme (Mustafa and Hochachka, 1971; Holwerda and de Zwaan, 1973; Livingstone and Bayne, 1974). Pyruvate kinase activity was shown to be inhibited by L-alanine, ATP and H^+ and activated by fructose-1,6- P_2 . In particular, with the onset of anoxia, the decrease in intracellular pH as a consequence of the accumulation of organic acids (Wijsman, 1975) combined with increasing L-alanine concentration was believed to inhibit PK. Recently, it has been shown that PK, in addition to modulation by pH and other effectors, is modified during anaerobiosis by phosphorylation/dephosphorylation mechanisms with phosphorylation during anoxia producing a less active enzyme form (Holwerda et al., 1981; Siebenaller, 1979; Plaxton and Storey, 1984). PEPCK, however, is activated during anoxia; the enzyme shows a pH optimum of about 6.0 and is activated by alanine (Zammitt and Newsholme, 1978). Formerly, it was assumed that the catalytic requirements and control properties of these two enzymes at the PEP branchpoint were arranged to function on a reciprocal, either-or basis (Hochachka et al., 1973). Recently, however, the opinion that both reactions can proceed together, the predominance of one reaction over the other being determined by the degree of hypoxia, has gained support (Hochachka and Somero, 1976; de Zwaan, 1977; Holwerda and de Zwaan, 1980). Thus the simultaneous production, from glycogen reserves, of succinate as well as alanine (or octopine, alanopine, strombine) may occur.

Life in the intertidal zone requires biochemical adaptation for coping with changing salinity as well as for surviving periods of anoxia. The degree of euryhalinity exhibited by any species of marine invertebrates is directly dependent on the ability of that organism to regulate cell volume in response to external salinity change. Only a few species control cell volume by extracellular osmotic regulation over the entire nonlethal salinity range. Rather, the osmotic concentration of the blood of most species varies with that of the environment (osmoconformity) over at least a part of the nonlethal salinity range (Pierce, 1971; Lockwood, 1976). When placed in a hypoosmotic or hyperosmotic medium, the tissue water content of various bivalves changed considerably less than would be expected based on the change in the external osmotic pressure which has been interpreted as evidence of cell volume regulation (Pierce, 1971; Otto and Pierce, 1981; Matsushima, 1982). Since the cells of these osmoconformers do not simply shrink or swell passively with the osmotic changes of the extracellular fluids, mechanisms must exist for cell volume regulation (Gilles, 1979; Pierce and Amende, 1981). The intracellular mechanism utilized involves modulation of the intracellular pool of free amino acids (FAA) and other low molecular weight nitrogenous solutes such as betaine and taurine. Accordingly, when confronted by the stress of changing environmental salinity, these animals minimize cell volume alterations by adjusting free

amino acid concentrations to increase or decrease intracellular osmotic pressure (Figure 2). In hypoosmotic environments the intracellular concentrations of particular amino acids are decreased while hyperosmotic stress causes their accumulation. The mechanisms that control the size of the FAA pool are not fully understood. Three different possibilities for controlling the amino acid pool can be considered: 1) modification of the amino acid-protein equilibrium (Gilles, 1979), 2) changes in the rate of transport of the amino acids through the cell membranes (Pierce and Amende, 1981; Crowe, 1981) or 3) variation in the steady state between synthesis and degradation of amino acids (Henry et al., 1980; Bishop et al., 1981).

The mechanisms involved in the response to hypoosmotic stress are reasonably well-understood. Two factors cause a drop in the intracellular free amino acid pool: 1) FAA leave the cell and 2) the catabolism of free amino acids is enhanced. Studies concerning the transport of amino acids have shown that in vitro many tissues respond to hypoosmotic stress by increased amino acid efflux, decreased influx or both (Otto and Pierce, 1981; King et al., 1980; Henry and Mangum, 1980; Matsushima, 1982). Divalent cations, Ca^{2+} and Mg^{2+} , have been implicated in the control of membrane osmotic properties and permeability (Otto and Pierce, 1981). Under hypoosmotic stress there is a decrease of these cations in the extracellular fluids hence the cellular membrane will become permeable to amino acids and an efflux

FIGURE 2: Diagrammatic representation of cell volume regulation mediated by intracellular solute regulation. The circles represent a single cell and the dots represent intracellular solute particles (in particular, free amino acids) (Pierce and Amende, 1981).

LOW SALINITY

HIGH SALINITY

of amino acids into the body fluids will result. High concentrations of FAA in blood have been observed in both Rangia cuneata (Henry and Mangum, 1980) and Corbicula japonica (Matsushima, 1982) upon their transference to dilute media. While these findings indicate regulation of transmembrane movement of amino acids as an important mechanism in regulating the FAA pool, their fate subsequent to being released from the tissue is not clear.

The excretion of these amino acids intact would represent a substantial loss of potential metabolic energy (King et al., 1980). Studies on marine invertebrates have indicated that although isolated tissues may show a net efflux of amino acids during hypoosmotic volume regulation, the whole animal excretes nitrogen as ammonia (King et al., 1980; Henry and Mangum, 1980; Zurburg and de Zwaan, 1981; Matsushima, 1982), indicating some degree of amino acid catabolism prior to excretion. Blood FAA have been reported to actually decrease at the time when ammonia levels begin to rise and this relationship supports the suggestion that deamination in a specialized organ follows FAA transport to that organ from multiple sites of origin. Reduction of the FAA pool during low salinity adaptation is closely correlated in time with the active resumption of ventilation and oxidative metabolism (Henry and Mangum, 1980). The synthesis of blood protein from formerly intracellular FAA, if it occurs, must be a transient mechanism of reducing pool size, because protein values after 14 days in dilute media

do not differ significantly from those in concentrated media (Henry et al., 1980).

Regulation of cell volume during high salinity stress in marine invertebrates is accomplished by increasing the intracellular concentration of free amino acids. In order to distinguish the control mechanisms involved studies have examined the time course of changes in amino acid content in tissues of various bivalves during exposure to high salinity. Generally, the results showed that the response of the free amino acid pool to increased salinity is complex and involves more than a sudden addition of all amino acids to the total pool. Rather, although the total amino acid pool rapidly increased to a stable high level, the time course of accumulation is unique for each amino acid. Alanine is among the chief osmotic effectors in several bivalves: Modiolus demissus demissus (Baginski and Pierce, 1975), Mya arenaria (Du Paul and Webb, 1970) and R. cuneata (Henry et al., 1980), while in a second group of bivalves taurine dominates the pool: M. edulis (Kluytmans et al., 1980), M. mercenaria and Crassostrea gigas (Shumway et al., 1977). The pattern of free amino acid accumulation in M. d. demissus (Baginski and Pierce, 1977), M. arenaria (Du Paul and Webb, 1970), and M. edulis (Lange, 1963) involves an initial increase in alanine and other amino acids followed by a slower accumulation of taurine. Thus taurine appears to be the major osmotic solute utilized in these bivalves for long term salinity acclimation and thereby exerts a

sparing effect on the use of essential amino acids in osmoregulation.

The role of protein hydrolysis in enlarging the FAA pool has not been fully evaluated. Siebers et al. (1972) and Gilles (1977) have suggested that extracellular (serum) protein hydrolysis and cellular transport of FAA may be an important mechanism of high salinity acclimation. However, according to Henry et al. (1980) blood protein does not change. Information on intracellular protein changes appears to be contradictory (Gilles, 1979) and the changes required to explain increases in FAA are small. A uniform protein hydrolysis without extensive transaminations would not produce the striking changes in the composition of the pool, which is always biased in favor of a few amino acid species. Moreover, organic compounds such as taurine and betaine, which also play an important part in isoosmotic regulation in molluscs cannot be considered constituents of proteins.

The uptake of amino acids from outside the cell can be an additional source of these amino acids. However, animals and isolated tissues held in artificial seawater with no exogenous source of amino acids or ammonia accumulate the same specific amino acids as those taken from nature at high salinity (Baginski and Pierce, 1975). The efflux and influx experiments discussed by Crowe (1981) suggest that there may be considerable exchange (uptake and release) of amino acids among the tissues within the animal as the whole animal

adapts to prolonged hyperosmotic conditions. Otto and Pierce (1981) have established the important role of Ca^{2+} and Mg^{2+} and membrane permeability in the accumulation and stabilization of amino acids at high salinity. The divalent cation studies indicate that the intracellular FAA control requires a critical external concentration of $\text{Ca}^{2+}/\text{Mg}^{2+}$ to prevent FAA efflux.

Data obtained in various studies on R. cuneata and M. demissus (Baginski and Pierce, 1975, 1977, 1978; Henry et al., 1980; Bishop et al., 1981) indicate that the mechanism of intracellular accumulation of FAA during initial stages of acclimation to high salinity rests with an alteration of intracellular metabolism. First, the amino acid source must be located intracellularly since accumulation occurs in isolated tissues incubated in solution free of amino acids (Baginski and Pierce, 1975). Second, upon exposure to high salinity, glycogen metabolism is altered resulting in increased concentrations of intracellular soluble compounds (Henry et al., 1980). For these bivalves, the accumulation of alanine, reduction in metabolic rate (O_2 consumption) and labeling of alanine by ^{14}C -glucose during a hyperosmotic challenge lead to the proposal that carbohydrate is the major source of carbon for alanine production and that the hyperosmotic challenge generates the same sort of response as that seen with anaerobiosis (Baginski and Pierce, 1975). However, the accumulation of alanine during hyperosmotic stress is different from that occurring during anaerobiosis

as it is not accompanied by an accumulation of succinate (Baginski and Pierce, 1978). Henry et al. (1980), however, measured the changes in FAA pool during high salinity adaptation under anaerobic conditions in R. cuneata and showed that the FAA pool can be enlarged without the need for oxygen and that non-oxidative reactions occurring in a modified form of glycolysis are capable of effecting the accumulation of FAA during high salinity adaptation. Depending on the inducing exogenous stimulus, either limited oxygen availability or a high salinity stress, qualitatively different anaerobic mechanisms may be involved.

It is probable that the carbon skeletons of increased FAA during hyperosmotic stress are derived from degradation of glucose or glycogen but the source of the amino groups must also be determined. Zurburg and de Zwaan (1981) have suggested the participation of ammonia in alanine formation either by a direct action of an alanine dehydrogenase or a glutamate-pyruvate transferase/glutamate dehydrogenase complex. The hypothesis of a reductive amination of pyruvate to form alanine requires an alanine dehydrogenase which is unknown in animal tissues. The exact biochemical mechanism underlying amino acid synthesis during high salinity acclimation remains unknown (Bishop et al., 1981; Zurburg and de Zwaan, 1981; Baginski and Pierce, 1978) although the activation of the GDH system must be examined as a possibility especially as the initial stimulus for amino acid accumulation.

The gain in cellular taurine during hyperosmotic regulation can be due to an increased influx of the compound from the extracellular fluid and/or to a synthesis within the cells (Vislie, 1983). No data, however, are available concerning the pathway and the control mechanisms implicated in the increased taurine production in bivalves during a hyperosmotic stress.

Previous research on the biochemical response of bivalves to environmental stresses has extensively dealt with tissue specific substrate utilization and end product accumulation and the regulation of select enzymes. However, in comparison, very little research has examined the reported pathways of synthesis. The present study was designed to examine metabolism and metabolic regulation with respect to the sites of control and the controlling metabolites in tissues of an intertidal bivalve in response to commonly encountered environmental stresses. In order to study these metabolic activities, tissues were excised and incubated in vitro to allow for experimental manipulation of the chemical and physical environment and to exclude uptake from and/or release to the blood, of metabolites. Isolated tissue preparations serve very well for the study of in vivo metabolism provided that it can be demonstrated that the isolated tissues retain the sensitivity to physiological changes observed in vivo. Four natural stresses were under study: 1) hypoosmotic stress, 2) hyperosmotic stress, 3) anoxia and 4) hyperosmotic stress under anoxic conditions.

This thesis reports the effects of environmental stresses on the metabolism of isolated tissues of the intertidal bivalve, Mercenaria mercenaria, to answer the following questions:

1) Hypoosmotic stress

What is the fate of the FAA?

What are the metabolic alterations coincident with the decrease in the FAA pool?

Is there evidence for gluconeogenesis utilizing the carbon skeletons of FAA?

2) Hyperosmotic stress

What are the metabolic alterations leading to accumulation of FAA?

What are the sites of control in glycolysis during FAA synthesis and are they under regulation by allosteric effectors or covalent modification?

3) Anoxia

What are the control points in glycolysis and how are they regulated?

Is glycolytic flux increased or decreased?

4) Hyperosmotic stress under anoxic conditions

Is the FAA pool enlarged by anaerobic pathways of metabolism during adaptation to a hyperosmotic stress?

Of which stress are the metabolic changes typical?

The present study uses as an experimental animal the cherrystone clam, Mercenaria mercenaria. This bivalve

mollusc is a major commercial species on the Atlantic Coast of North America and inhabits the intertidal zone where it experiences many environmental changes. The clam leads a relatively sedentary existence and therefore has evolved a method of filter feeding on microscopic organisms in the surrounding seawater. The powerful foot of this burrowing clam enables it to dig into the mud or sand bottom. Many bivalves of the intertidal zone generally show high anoxia tolerance and M. mercenaria has been shown to routinely survive 96 h of anoxia. Bivalve molluscs are not structurally well adapted for aerial gas exchange (Widdows et al., 1979). The gills show extensive modifications for filter feeding and secondarily for gas exchange. The inner surface of the shell is covered by the mantle. The mantle tissue is unique in being a major site for stored energy reserves and of gametogenic development (Zaba and Davies, 1980). Both the gill and mantle are superficial tissues which are primarily epithelial and highly vascularized. Isolated gill and mantle tissues were monitored in this study since these tissues are intimately involved with the environment and, therefore, potentially very important in adjusting to the environmental stresses. Also, the metabolic activities of these tissues might be expected to reflect their distinctive physiological function. Other advantageous features of gill and mantle tissues are: 1) these tissues are easily accessible and may be excised with minimal damage to the tissue, and 2) they occur naturally as

relatively thin tissues with a large surface area in contact with the media and tissue slicing would not be necessary.

The results of this investigation, herein, demonstrated that each environmental stress elicited a unique metabolic response in the isolated gill and mantle tissues of M. mercenaria. In answer to the previously posed questions:

1) Hypoosmotic stress

The major response is excretion of the free amino acids with no alteration of intermediary metabolism.

2) Hyperosmotic stress

Activation of glycolysis at the pyruvate kinase locus in isolated mantles indicates that carbohydrate metabolism is responsible, at least partly, for the increase in FAA. This activation of PK appears to be of an allosteric nature rather than due to covalent modification.

3) Anoxia

Phosphofructokinase and pyruvate kinase control the carbon flow during anaerobic metabolism.

A relief of inhibition due to decreasing ATP and arginine-P levels may be the key factor increasing the activity of PFK and speeding up glycolysis. Pyruvate kinase is inhibited during anaerobiosis. Kinetic studies indicate that the inhibition of PK is not due to covalent modification but instead due to changes in the

levels of allosteric effectors such as increasing alanine, decreasing pH and reduced energy charge.

4) Hyperosmotic stress under anoxic conditions

The FAA pool is not enlarged during hyperosmotic stress by anaerobic metabolism.

The changes in glycolysis are typical of the isolated tissue metabolic response to anoxia stress.

MATERIALS AND METHODS

Animals and Chemicals: Cherrystone clams, Mercenaria mercenaria, were purchased from a local seafood retailer and were held, without feeding, in an aerated, recirculating seawater tank at 100% seawater (SW; ~1000 mOsmol) and at 18°C for at least 2 weeks before use.

All biochemicals were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp.. Succinyl-thiokinase was a gift from Dr. W. Bridger, University of Alberta.

Preparation and Incubation of Isolated Mantle and Gill Tissue: The valves of clams were opened by severing the adductor muscles. Pairs of mantles and gills (right and left) were rapidly excised from each clam. The lobulated mantle borders were removed with scissors and the remaining, central portions of the mantles were used. Gills were trimmed of any contaminating tissue. Both gill and mantle, handled separately, were rinsed in 100% seawater and divided into two groups. Each group, consisting of one half of each pair, was put into incubation flasks. The flask was a 25 ml erlenmeyer into which 10 ml of the appropriate seawater solution had been added. Solutions were flushed with either N₂ gas or air before addition of tissues. Flasks were stoppered and were bubbled with either N₂ gas or air throughout the incubation. The tissues were incubated, shaking, for 1, 2 or 4 h at room temperature. They were

then removed from the saline, blotted and frozen in liquid nitrogen. Tissues at 0 h were sampled directly from animals taken from the seawater tank and were excised, rinsed and blotted as above and then frozen in liquid N₂. Tissues were stored at -80°C until use. The incubation medium was collected, stored frozen and analyzed without further processing.

The media in which the control and experimental groups were incubated is as follows:

EXPERIMENT 1: As a control one group was placed in aerated 100% SW (~1000 mOsmol). The other group was placed in aerated 50% SW (~500 mOsmol).

EXPERIMENT 2: As a control one group was placed in aerated 100% SW (~1000 mOsmol). The other group was placed in aerated 150% SW (~1500 mOsmol).

EXPERIMENT 3: As a control one group was placed in aerated 100% SW (~1000 mOsmol). The other group was placed in oxygen-free 100% SW (~1000 mOsmol) under a nitrogen atmosphere.

EXPERIMENT 4: As a control one group was placed in aerated 150% SW (~1500 mOsmol). The other group was placed in oxygen-free 150% SW (~1500 mOsmol) under a nitrogen atmosphere.

Metabolite Studies

Sample Preparation: Frozen tissues were ground to a powder under liquid nitrogen using a mortar and pestle and were then transferred to preweighed, acid-washed, polypropylene centrifuge tubes stored on dry ice. After a second weighing to determine tissue weight, tubes were transferred one at a time to a dry ice-methanol bath at -8°C . Five volumes (w/v) of 6% perchloric acid containing 1 mM EDTA (chilled at -8°C) were quickly added and the powdered tissue was then rapidly homogenized using a Polytron PT 10 homogenizer. A 50 μl sample of the homogenate was withdrawn for glycogen determination. Homogenates were centrifuged at 5900g for 15 min at 4°C to pellet the precipitated protein. The protein pellet was retained for protein determination. The acid supernatant was removed, transferred to a second tube and neutralized by the addition of 3 N KOH- 0.3 M imidazole- 0.4 M KCl. After a second centrifugation to remove precipitated KClO_4 , neutralized samples were stored at -80°C until analysis (Williamson, and Corkey, 1969).

Glycogen Determination: The reserved 50 μl of homogenate was added to a solution of 0.75 ml acetate buffer (0.2 M; pH 4.8). Potassium bicarbonate (0.05 ml of 1 M solution) was then added to readjust the pH to 4.8. Amyloglucosidase (20 U) was added and digestion of sample glycogen was allowed to proceed overnight at room

temperature. After enzymatic hydrolysis of glycogen to glucose, the samples were then stored at -80°C until analysis (Keppler and Decker, 1974).

Protein Assay: The perchloric acid-precipitated protein pellets were resuspended in 0.2 N NaOH. Protein concentration was measured by the method of Bradford (1976) using the Bio-Rad prepared reagent and bovine gamma globulin as the standard.

Measurement of Metabolite Levels: Metabolite levels were measured spectrophotometrically or fluorometrically in enzyme assays coupled to the reduction or oxidation of pyridine nucleotides. In each metabolite assay, the amount of pyridine nucleotide reacted is directly proportional to the amount of substrate reacted. To conduct each assay, sample was added to a cuvette containing 1 ml of the appropriate reagent mixture. After mixing, an initial reading was recorded. Reactions were then started by the addition of enzyme. Upon completion of the reaction, a final reading was taken. Blanks (containing no added sample) and standards (of known metabolite concentration) were run together with samples for each metabolite measured.

For the spectrophotometric assays the amount of pyridine nucleotide reacted is calculated via the formula:

$$\mu\text{moles} = \Delta\text{O.D.} \times \text{vol.} / \epsilon$$

where: Δ O.D. is the change in absorbance of the pyridine nucleotide at 340 nm, vol. is the total volume in the cuvette, and ϵ is the molar extinction coefficient of the pyridine nucleotides at 340 nm = $6.22 \text{ ml } \mu\text{mol}^{-1} \text{ cm}^{-1}$ (Williamson and Corkey, 1969) using cuvettes of 1 cm light path length. The change in absorbance of the pyridine nucleotides was measured at 340 nm in a Pye-Unicam SP8-100 recording spectrophotometer.

For the fluorometric assays changes in sample fluorescence were compared to a standard curve relating known concentrations of substrate (or NADH) to fluorescence. Standard curves were run with each group of samples. With this curve, the change in fluorescence could be converted to the concentration of NADH which had been used or produced in the cuvette. The amount of pyridine nucleotide used could then be calculated via the formula:

$$\mu\text{moles} = \text{conc.} \times \text{vol.}$$

where conc. is the concentration of pyridine nucleotide used as determined from the standard curve and vol. is the total volume in the cuvette. The change in fluorescence of the pyridine nucleotide was measured in a Turner Model 111 Fluorometer.

The concentration of the metabolite of interest in the tissue, expressed as $\mu\text{mol/g}$ wet weight, was then determined as follows:

$$\mu\text{mol/g} = \mu\text{moles} \times \frac{V_1}{V_2} \times \frac{V_2 + V_3}{V_4} \times \frac{V_1 + W}{W}$$

where: μ moles: number of μ moles of metabolite in cuvette

V_5 : 1000 μ l

V_4 : volume of sample added to cuvette in μ l

V_3 : volume of KOH solution required to
neutralize acid supernatant

V_2 : volume of acid supernatant recovered in ml

V_1 : volume of perchloric acid solution required
to homogenize powdered tissue in ml

W: tissue wet weight

Metabolite Assays:

ADP and AMP (fluorometer)

AMP + ADP $\xrightarrow{\text{MK}}$ 2 ADP

ADP + PEP $\xrightarrow{\text{PK}}$ ATP + pyruvate

Pyruvate + NADH + H^+ $\xrightarrow{\text{LDH}}$ lactate + NAD^+ + H_2O

Pyruvate kinase (PK, 0.6 U) is added to a cuvette containing buffer (50 mM Phosphate, pH 7.0), NADH (5 μ M), MgSO_4 (2 mM), KCl (75 mM), ATP (5 μ M), PEP (20 μ M), lactate dehydrogenase (LDH, 0.4 U), and neutralized extract and the change in fluorescence due to ADP utilization recorded. Then myokinase (MK, 0.36 U) is added and the amount of AMP is measured (Lowry and Passonneau, 1972).

Alanine (spectrophotometer)

Glutamate-pyruvate transaminase (GPT, 4 U) is added to a cuvette containing buffer (100 mM Tris-HCl, pH 8.1), NADH (150 μM), α -ketoglutarate (200 μM), lactate dehydrogenase (LDH, 0.4 U) and neutralized extract (Lowry and Passonneau, 1972).

Aspartate (spectrophotometer)

Glutamate-oxaloacetate transaminase (GOT, 1.8 U) is added to a cuvette containing buffer (100 mM Imidazole-HCl, pH, 7.0), NADH (150 μM), α -ketoglutarate (200 μM), malate dehydrogenase (MDH, 1.4 U) and neutralized extract (Lowry and Passonneau, 1972).

ATP and Arginine-P (spectrophotometer)

Hexokinase (HK, 0.28 U) is added to a cuvette containing buffer (100 mM Tris-HCl, pH 8.1), NAD^+ (500 μM), MgSO_4 (1 mM), glucose (1 mM), glucose-6-phosphate dehydrogenase (G6PDH, 0.07 U) and neutralized extract and the change in OD_{340} due to ATP utilization recorded (Lowry and Passonneau, 1972). Then ADP (500 μM) and arginine kinase (AK, 0.9 U) are added and the change in OD_{340} for arginine is recorded (Storey and Storey, 1978).

Dihydroxyacetone phosphate, Glyceraldehyde-3-phosphate and Fructose-1,6-diphosphate (fluorometer).

Glycero-phosphate dehydrogenase (GDH, 0.04 U) is added to a cuvette containing buffer (50 mM Phosphate, pH 7.0), NADH (5 μM) and neutralized extract and the change in fluorescence recorded. Then triose phosphate isomerase (TIM, 1.2 U) is added and the change in fluorescence due to glyceraldehyde-3-phosphate utilization recorded. Finally aldolase (0.004 U) is added and the amount of FDP measured. Each of the three reactions is allowed to go to completion before enzyme for the next assay is added (Lowry and Passonneau, 1972).

Glucose (spectrophotometer)

Hexokinase (HK, 0.28 U) is added to a cuvette containing buffer (100 mM Tris-HCl, pH 8.1), NAD^+ (500 μM), MgSO_4 (1 mM), ATP (500 μM), glucose-6-phosphate dehydrogenase (G6PDH, 0.08 U) and neutralized extract (Lowry and Passonneau, 1972).

Glucose-6-phosphate, Fructose-6-phosphate and Glucose (fluorometer)

Glucose-6-phosphate dehydrogenase (G6PDH, 0.02 U) is added to a cuvette containing buffer (50 mM Tris-HCl, pH 8.1), NAD^+ (100 μM), MgSO_4 (1 mM), ATP (300 μM) and neutralized extract and the change in fluorescence recorded. Then phosphoglucose isomerase (PGI, 0.35 U) is added and the change in fluorescence for fructose-6-phosphate recorded. Finally hexokinase (HK, 0.14 U) is added and the amount of glucose measured. Each of the three reactions is allowed to

go to completion before the enzyme for the next assay is added (Lowry and Passonneau, 1972).

Pyruvate and Phosphoenolpyruvate (fluorometer)

Lactate dehydrogenase (LDH, 0.4 U) is added to a cuvette containing buffer (50 mM Phosphate, pH 7.0), NADH (5 μM), MgSO_4 (2 mM), ADP (200 μM) and neutralized extract and the change in fluorescence recorded. Then pyruvate kinase (PK, 0.15 U) is added and the change in fluorescence due to phosphoenolpyruvate utilization is recorded (Lowry and Passonneau, 1972).

Succinate and Arginine (spectrophotometer)

Succinyl-thiokinase (STK, 0.09 U) is added to a cuvette containing buffer (100 mM Imidazole-HCl, pH 7.0), NADH (150 μM), MgSO_4 (2 mM), KCl (75 mM), ATP (300 μM), CoA (300 μM),

PEP (300 μ M), lactate dehydrogenase (LDH, 0.4 U), pyruvate kinase (PK, 0.3 U) and neutralized extract, and the change in OD₃₄₀ recorded (Williamson and Corkey, 1969). Then arginine kinase (AK, 4 U) is added and the change in OD₃₄₀ for arginine recorded (Storey and Storey, 1978).

Amino Acid Analyses: Amino acids were measured in the neutralized extracts and incubation media using a Beckman 119BL amino acid analyzer.

Enzyme Studies

Tissue Preparation and Enzyme Assay: Frozen tissues were ground to a powder under liquid nitrogen using a mortar and pestle and were then weighed and homogenized (1/5, w/v) in ice-cold 50 mM imidazole-HCl buffer (pH 7.5) containing 0.1 mM phenylmethylsulphonyl fluoride, 15 mM 2-mercaptoethanol, 100 mM NaF, 5 mM EGTA and 5 mM EDTA, using a PT 10 homogenizer. Homogenates were centrifuged at 27,000g for 30 min at 4°C. Supernatants were removed and a demi-portion of this was dialysed against 4 l of 50 mM imidazole-HCl buffer (pH 7.5) containing 15 mM 2-mercaptoethanol, 100 mM NaF and 5 mM EDTA for 2 h at 4°C. The pellets were resuspended in 5 volumes of ice-cold homogenization buffer. Glycogen phosphorylase activities were determined in the undialysed supernatants and the resuspended pellets. Dialysed and undialysed portions of the supernatants were each used for the determination of the

activities and the kinetic properties of pyruvate kinase.

All enzyme activities were determined by following the reduction or oxidation of pyridine nucleotides at 340 nm using a Gilford recording spectrophotometer attached to a circulating water bath for temperature control of cuvettes. Assay temperature was maintained at 21°C. Enzyme activities were corrected for non-specific NADP or NADH utilization (by subtraction of blanks run minus substrate) when present. All assays were initiated by addition of enzyme preparation. One unit of enzyme activity is defined as the amount of enzyme resulting in the utilization of 1 μ mol of NADP or NADH per min at 21°C.

Glycogen Phosphorylase Assay: Optimal assay conditions for glycogen phosphorylase in the glycolytic direction were 50 mM phosphate buffer (pH 7.0), 2 mg/ml dialysed glycogen, 10 μ M glucose-1,6-phosphate, 0.5 mM NADP, 0.25 mM EDTA, 15 mM MgSO_4 , and excess dialysed phosphoglucomutase and glucose-6-phosphate dehydrogenase in a final volume of 1 ml. Phosphorylase activity was determined in the absence (phosphorylase a) and presence (phosphorylase a+b) of 1 mM AMP.

Pyruvate Kinase Assay and Kinetic Studies: Standard assay conditions for PK were 50 mM imidazole-HCl buffer (pH 7.0), 1 mM PEP, 5 mM ADP, 100 mM KCl, 10 mM MgCl_2 , 0.10 mM NADH and excess dialysed lactate dehydrogenase. $K_{0.5}$ values and Hill coefficients were calculated from Hill plots. I₅₀

values (inhibitor concentration producing 50% inhibition of enzyme activity) were determined from plots of $v(\text{uninhibited})/v(\text{inhibited})$ versus inhibitor concentrations by the method of Job et al. (1978).

Data Calculations and Statistics: Data is reported as mean $\pm$ standard error of the mean. All data points in Figures 3-18 that have no indication of the size of the standard error of the mean (s.e.m.) had a s.e.m. that was within the limits of the symbols of the plot on the scale, used. Statistical significance was assessed by the student's t-test (Mendenhall, 1971).

Wet Weight versus Dry Weight: For all the experimental tissues types (hyposaline, hypersaline, anoxic), wet weight (fresh weight) versus dry weight data was obtained. In all cases the value of % H_2O was about 90% and did not change with experimental procedure.

RESULTS

HYPOOSMOTIC STRESS

Experiment 1:

Gill: When transferred from 100% SW to 50% SW and incubated for 4 h, the total free amino acid (FAA) pool size of the isolated gills of M. mercenaria decreased to 50% of the pre-incubation level, approximately 70 $\mu\text{mol/g}$ wet wt (Table 1). A dramatic reduction in the gill intracellular taurine pool accounted for approximately 95% of the total drop. The amino acids, aspartic acid, glutamic acid, glycine and alanine together largely accounted for the remaining 5% decrease in the FAA pool of the hypoosmotically stressed gills. Increases in the levels of FAA in the hyposaline incubation fluid of the isolated gills over 4 h corresponded closely to the decreases recorded for these compounds in the tissues. Taurine content in the dilute medium was elevated by approximately 81 $\mu\text{mol/g}$ wet wt in the 10 ml of incubation media, accounting for the measured decrease (70 $\mu\text{mol/g}$ wet wt) in gill intracellular taurine pools. The decrease in the concentration of tissue alanine (Table 1; Figure 3A) was accompanied by the corresponding increase in alanine concentration of the incubation fluid. Of the remaining major amino acids only the decrease in tissue glycine content could be wholly accounted for by amino acids in the incubation medium. Under low salinity

TABLE 1: Concentrations of amino compounds of tissue and incubation medium of isolated gills of M. mercenaria incubated in 100% SW and 50% SW.

Amino Compound	Tissue ($\mu\text{mol/g wet wt}$)			Incubation Medium ($\mu\text{mol/g wet wt} \cdot 10 \text{ ml}$)		
	0 h	4 h	4 h	0 h	4 h	4 h
		100% SW	50% SW		100% SW	50% SW
Taurine	157.61	189.94	87.30	0.83	2.08	81.55
Aspartate	2.16	3.87	2.77	0.04	0.18	0.56
Glutamate	4.60	5.23	2.09	0.07	0.18	0.81
Glycine	1.83	2.31	1.23	0.09	0.16	1.32
Alanine	3.81	3.92	2.00	0.10	0.40	1.83
Threonine	0.05	0.06	0.03	0.01	0.08	0.74
Serine	0.05	0.06	0.03	0.04	0.09	0.70
Glutamine	0.18	0.14	0.19	0.01	0.03	0.00
Valine	0.06	0.05	0.10	0.00	0.14	0.85
Cystine	0.16	0.17	0.13	0.07	0.08	0.01
Methionine	0.06	0.06	0.05	0.00	0.01	0.01
Isoleucine	0.07	0.08	0.09	0.01	0.16	0.61
Leucine	0.04	0.06	0.11	0.02	0.05	0.05
Tyrosine	0.05	0.07	0.05	0.00	0.01	0.01
Ornithine	0.06	0.08	0.07	0.04	0.01	0.02
Lysine	0.14	0.15	0.31	0.03	0.01	0.01
Histidine	0.05	0.11	0.10	0.00	0.01	0.01
Arginine	0.51	0.73	0.64	0.00	0.01	0.01
Ammonia	2.02	3.28	3.70	0.29	1.81	4.94
Total FAA +Taurine	167.49	207.09	97.29	1.36	3.50	88.99
Total FAA -Taurine	13.88	17.12	10.00	0.53	1.41	7.46

Results are the means of n=2.

stress, the ammonia concentration was elevated in the isolated tissue and incubation fluid by $0.4 \mu\text{mol/g wet wt}$ and $3.0 \mu\text{mol/g wet wt}$ in 10 ml respectively within 4 h. The levels of amino compounds in the media of gills incubated for 4 h at 100% SW were very low after the experiment, indicating that the tissues were essentially intact and not 'leaky' after dissection from the animals. The relationship of the changes in FAA content of the isolated gills and surrounding incubation fluid indicates that exposure of the gills to hypoosmotic conditions stimulated the efflux of these amino compounds.

Figures 3A and 4 show the changes in selected metabolite levels during exposure of isolated gills to hyposaline conditions over the course of 4 h. Alanine levels were significantly reduced over 4 h to approximately 45% of the 0 h level. Alanine gradually accumulated in the incubation medium such that the level at 4 h was ~10 fold that at time zero. Aspartate pools increased significantly ($P < 0.01$) in gills incubated at 100% and at 50% SW. As shown by the amino acid analysis data, the increase was greater for tissues at 100% SW ($1.6 \mu\text{mol/g wet wt}$) than for osmotically stressed tissues ($1.0 \mu\text{mol/g wet wt}$). At 4 h, control and experimental intracellular arginine contents were significantly reduced ($P < 0.01$) to about 40% of pre-incubation levels. This, however, correlates with the observed increase in arginine phosphate concentration throughout the incubation. Glycogen reserves remained

FIGURE 3: The changes in concentration of selected metabolites in isolated gills of M. mercenaria incubated in 100% SW (●) and 50% SW (○) over 4 h.

- A. Alanine, aspartate, arginine, glycogen, succinate and glucose.
- B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.
- C. ATP, ADP, AMP, total adenylates, adenylate energy charge and arginine phosphate.

Metabolites were measured as described in MATERIALS AND METHODS. Glycogen is expressed as μ mol glucose after enzymatic hydrolysis.

Adenylate energy charge is calculated as

$$([ATP] + \frac{1}{2}[ADP])/([ATP] + [ADP] + [AMP]).$$

Results are expressed as means $\pm$ s.e.m. for n=3 samples (3 animals per sample) at each point.

FIGURE 4: The changes in concentration of alanine and glucose in the incubation media surrounding isolated gills of M. mercenaria incubated in 100% SW (●) and at 50% SW (○) over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (3 animals per sample) at each point.

constant in the osmotically stressed gills. Pre-incubation levels of succinate ($\sim 0.8 \mu\text{mol/g wet wt}$) were low in the gills and continued as such throughout the incubation period in tissues at 100% and at 50% SW. Succinate, an end product of anaerobic metabolism, was not detected in the hypoosmotic or isoosmotic incubation media. The absence of succinate accumulation suggests that the isolated gills maintained an aerobic metabolic state. Glucose content in gills incubated in hypoosmotic media was significantly reduced ($P < 0.01$) from 0.16 ± 0.02 to $0.07 \pm 0.01 \mu\text{mol/g wet wt}$ representing a decrease of 60%. The levels of glucose were low in control and dilute incubation media and continued as such over the course of 4 h except for a significant ($P < 0.05$) inflection in the hyposaline solution at 2 h representing an increase of $1.2 \mu\text{mol/g wet wt}$ in 10 ml.

Figure 3B shows the effects of a hypoosmotic stress on the levels of selected glycolytic intermediates in isolated gills over the course of 4 h. Glucose-6-P ($0.07 \pm 0.01 \mu\text{mol/g wet wt}$) and fructose-6-P ($0.022 \pm 0.005 \mu\text{mol/g wet wt}$) levels remained low and constant throughout at either 100% SW or 50% SW. Within 1 h of incubating the isolated gills at 100% or 50% SW, fructose-1,6-P₂ content declined sharply by 80% from about 0.056 to about $0.011 \mu\text{mol/g wet wt}$. Levels of dihydroxyacetone-P, a product of the reversible aldolase reaction, also decreased significantly by 40% within 2 h under both control and low salinity conditions. This decrease in the concentration of the

products of the phosphofructokinase (PFK) reaction and the subsequent equilibrium enzyme, suggests an inhibition of PFK during incubation of isolated gills in isoosmotic and hypoosmotic solutions. . . Dihydroxyacetone-P and glyceraldehyde-3-P are interconvertible in a reaction catalyzed by triose phosphate isomerase and at equilibrium the majority of the triose phosphate is dihydroxyacetone-P. Consequently the perturbation at the PFK locus did not affect the glyceraldehyde-3-P ($0.026 \pm 0.008 \mu\text{mol/g wet wt}$) levels significantly. No significant alterations in phosphoenolpyruvate ($0.060 \pm 0.009 \mu\text{mol/g wet wt}$) and pyruvate ($0.036 \pm 0.005 \mu\text{mol/g wet wt}$) levels were seen during the experimental salinity change.

Figure 3C shows the responses of the adenylate and phosphagen pools of isolated M. mercenaria gills to incubation in isoosmotic and hypoosmotic seawater over 4 h. At 100% SW and 50% SW, ATP content remained relatively constant between 0.8 and 1.1 $\mu\text{mol/g wet wt}$ throughout the experiment. No significant alterations in the ADP ($\sim 0.24 \mu\text{mol/g wet wt}$) and AMP ($\sim 0.04 \mu\text{mol/g wet wt}$) levels were seen during the 4 h incubation period in isoosmotic or hypoosmotic solutions. Total adenylate pool sizes did not vary significantly during the experiment, but the total adenylate content was slightly lower for gills in 50% SW than in 100% SW. The adenylate energy charge (EC) values of gills incubated at both 100% and 50% SW were high and consistently remained between 0.8 and 0.9 over the 4 h

incubation. Control and experimental arginine phosphate levels increased significantly ($P < 0.01$) by 90% from about 0.25 $\mu\text{mol/g}$ wet wt to about 0.47 $\mu\text{mol/g}$ wet wt over the incubation period. Arginine content of the incubated gills decreased in an opposite pattern to the decline in the phosphagen. This improved energy state over the course of incubation of isolated gills is a strong indication that the gills remained viable throughout the in vitro incubation.

The water content of isolated gills during hypoosmotic stress may increase thus increasing the wet weight of the tissue which could account for, in part, the observed decrease in FAA content. An increase or decrease in water content of the tissue would be indicated by an increase or decrease, respectively, of the mg protein per g wet weight tissue. Water uptake occurred in the isolated gills incubated in 50% SW for 4 h as the protein content of the tissue was reduced from 57.9 ± 1.8 to 51.1 ± 1.8 mg protein/g wet wt. The gain of tissue water under experimental conditions accounted for only 11% of the decrease in FAA concentration in hypoosmotic gills.

Mantle: Table 2 shows FAA concentrations in the isolated mantles of M. mercenaria and the surrounding incubation media after 4 h of low salinity stress. Total free amino acid content in mantles is approximately 80 $\mu\text{mol/g}$ wet wt at 0 h. Taurine is the predominant amino acid in the mantle and composes 85% of the total FAA pool. Other

TABLE 2: Concentrations of amino compounds of tissue and incubation medium of isolated mantles of M. mercenaria incubated in 100% SW and 50% SW.

Amino Compound	Tissue ($\mu\text{mol/g}$ wet wt)			Incubation Medium ($\mu\text{mol/g}$ wet wt $\cdot$ 10 ml)		
	0 h	4 h	4 h	0 h	4 h	4 h
		100% SW	50% SW		100% SW	50% SW
Taurine	67.87	74.00	44.44	0.83	1.47	19.29
Aspartate	1.36	1.32	1.07	0.05	0.02	0.00
Glutamate	3.42	5.83	3.89	0.08	0.06	0.49
Glycine	1.59	1.84	1.01	0.09	0.12	1.28
Alanine	4.19	3.90	1.34	0.10	0.15	1.75
Threonine	0.08	0.07	0.04	0.01	0.03	0.19
Serine	0.14	0.15	0.13	0.05	0.06	0.07
Glutamine	0.27	0.18	0.14	0.01	0.03	0.49
Valine	0.02	0.04	0.03	0.00	0.00	0.00
Cystine	0.16	0.15	0.10	0.07	0.08	0.16
Methionine	0.13	0.09	0.05	0.00	0.00	0.09
Isoleucine	0.10	0.05	0.04	0.01	0.03	0.25
Leucine	0.08	0.04	0.03	0.02	0.04	0.36
Tyrosine	0.04	0.05	0.02	0.00	0.00	0.15
Ornithine	0.03	0.02	0.02	0.04	0.04	0.06
Lysine	0.12	0.12	0.12	0.03	0.05	0.31
Histidine	0.05	0.06	0.03	0.00	0.02	0.11
Arginine	0.46	0.42	0.34	0.00	0.00	0.18
Ammonia	2.11	2.38	2.06	0.30	0.50	1.75
Total FAA +Taurine	80.11	88.33	52.84	1.39	2.20	25.23
Total FAA -Taurine	12.24	14.33	8.44	0.56	0.73	5.94

Results are the means of n=2.

major amino acid contributions (~13% of total FAA) come from aspartic acid, glutamic acid, glycine and alanine. After 4 h of hypoosmotic stress, the cellular FAA pool size decreased substantially by 34% (27 $\mu\text{mol/g}$ wet wt) of the mantle content at zero time. Approximately 85% of the total drop was due to a decrease in taurine content. Prominent amino acids, aspartic acid, glutamic acid, glycine and alanine, together accounted for an additional 13% of the total pool size reduction. Free amino acids accumulated in the hyposaline incubation media such that the levels at 4 h were 20 fold the pre-incubation level. The total increase from the 0 h level was accounted for largely by taurine (~75%), alanine (~10%) and glycine (~7%). The rise in taurine content of the dilute media represents about 70% of the intracellular taurine decrease. Under hypoosmotic conditions, tissue ammonia concentration decreased by 0.05 $\mu\text{mol/g}$ wet wt whereas the ammonia content of the incubation media increased by 1.4 $\mu\text{mol/g}$ wet wt in 10 ml. Free amino acid excretion, as a mechanism of cell volume regulation, appears to be the major response of isolated mantles to dilution of the seawater medium.

Figures 5A and 6 show the effects of hypoosmotic stress on metabolite levels in isolated mantles over a time course of 4 h. Tissue alanine pool sizes gradually declined from 1.8 ± 0.5 to 0.84 ± 0.09 $\mu\text{mol/g}$ wet wt over 4 h at low salinity. A corresponding rise in alanine content of the dilute medium, 0.8 $\mu\text{mol/g}$ wet wt in 10 ml, accounted for 80%

FIGURE 5: The changes in concentration of selected metabolites in isolated mantles of M. mercenaria incubated in 100% SW (●) and 50% SW (○) over 4 h.

- A. Alanine, aspartate, arginine, glycogen, succinate and glucose.
- B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.
- C. ATP, ADP, AMP, total adenylates, adenylate energy charge and arginine phosphate.

Metabolites were measured as described in MATERIALS AND METHODS. Glycogen is expressed as μmol glucose after enzymatic hydrolysis.

Adenylate energy charge is calculated as $([\text{ATP}] + \frac{1}{2}[\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$.

Results are expressed as means $\pm$ s.e.m. for $n=3$ samples (3 animals per sample) at each point.

HOURS

FIGURE 6: The changes in concentration of alanine and glucose in the incubation media surrounding isolated mantles of M. mercenaria incubated in 100% SW (●) and at 50% SW (○) over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (3 animals per sample) at each point.

umol/g wet weight · 10 ml

ALANINE

GLUCOSE

of the observed decrease in cellular alanine pools. No significant alterations in aspartate ($1.5 \pm 0.1 \mu\text{mol/g wet wt}$) levels were detected during 4 h at 50% SW. Decreases in the arginine pool of mantles in 100% SW or 50% SW of $0.41 \mu\text{mol/g wet wt}$ (75%) and $0.27 \mu\text{mol/g wet wt}$ (60%), respectively, corresponded inversely to the changes in the arginine-P content over 4 h. Mantle glycogen reserves were large ($\sim 72 \mu\text{mol/g wet wt}$ measured as glucose equivalents) and stable over the experimental period. Intracellular succinate levels dropped by 75% from 0.55 ± 0.09 to $0.129 \pm 0.004 \mu\text{mol/g wet wt}$ during acclimation of the mantles to reduced salinity. Succinate was not detected in the hyposaline solution. The glucose content increased only slightly in control mantles and their respective incubation media. The glucose content of isolated mantles in 50% SW doubled from 0.18 ± 0.05 to $0.37 \pm 0.06 \mu\text{mol/g wet wt}$ over the 4 h incubation. Similarly, glucose content of the incubation media was significantly enlarged ($P < 0.05$) by $7.6 \mu\text{mol/g wet wt}$ in 10 ml.

Figure 5B shows the effects of a hypoosmotic stress on the levels of selected intermediary metabolites in isolated mantles over the course of 4 h. Glucose-6-P ($0.080 \pm 0.005 \mu\text{mol/g wet wt}$) and fructose-6-P ($0.019 \pm 0.001 \mu\text{mol/g wet wt}$) levels did not vary significantly during the incubation. Under isoosmotic and hypoosmotic conditions, fructose-1,6-P₂ levels rose significantly by $\sim 100\%$ from about $0.035 \mu\text{mol/g wet wt}$ to about $0.064 \mu\text{mol/g wet wt}$ over the experimental

period. Similarly, isolated mantles showed a significant (~2 fold) elevation of dihydroxyacetone-P levels from about 0.037 $\mu\text{mol/g}$ wet wt to about 0.064 $\mu\text{mol/g}$ wet wt over 4 h in 100% SW and 50% SW. These responses indicate an activation (positive cross-over) of phosphofructokinase during in vitro incubation of mantles in both control and test solutions. Levels of glyceraldehyde-3-P (0.014 ± 0.009 $\mu\text{mol/g}$ wet wt), phosphoenolpyruvate (0.052 ± 0.004 $\mu\text{mol/g}$ wet wt) and pyruvate (0.029 ± 0.009 $\mu\text{mol/g}$ wet wt) remained low and constant throughout the incubation period.

Figure 5C shows the changes in the energy status of isolated mantles incubated in 100% SW and 50% SW over 4 h. Under experimental conditions, ATP levels increased by 40% from 0.59 ± 0.06 to 0.82 ± 0.07 $\mu\text{mol/g}$ wet wt over 4 h. The increase in the ATP pool size of isolated mantles in 100% SW paralleled the experimental response except for a significant inflection at 2 h representing an increase of 0.391 $\mu\text{mol/g}$ wet wt over the time zero level. ADP content changed oppositely, decreasing by ~30% from about 0.25 $\mu\text{mol/g}$ wet wt to about 0.17 $\mu\text{mol/g}$ wet wt over the incubation period. No significant alterations in AMP (0.020 ± 0.015 $\mu\text{mol/g}$ wet wt) levels were detected throughout the 4 h in 100% SW and 50% SW. Within 2 h in 100% SW, isolated mantles showed a significant rise in total adenylates, a 40% increase from 0.84 ± 0.13 to 1.19 ± 0.08 $\mu\text{mol/g}$ wet wt. Total adenylates remained essentially constant for tissues in dilute solution over 4 h. Energy charge values of

mantles were high (~ 0.83) throughout the incubation period. At 2 h the energy charge value peaked to ~ 0.90 in isolated mantles in isoosmotic solutions. In both isoosmotic and hypoosmotic solutions, mantles showed a significant (3 fold) elevation of arginine phosphate levels over time. Arginine concentrations followed an inverse pattern to that of the phosphagen. Isolation and incubation of the mantles did not have a detrimental effect on either EC or any of the other adenine nucleotide variables which indicates that the mantles remained viable throughout the experiment.

After 4 h of low salinity acclimation, the isolated mantles showed no significant changes in tissue water content from control mantles as the protein contents of experimental and control mantles were 32.1 ± 4.6 and 29.1 ± 2.9 mg protein/g wet wt, respectively. When placed in a dilute medium the total body water content of bivalves of the genus Modiolus changes considerably less than that of the medium which has been interpreted as evidence of cell volume regulation (Pierce, 1971).

HYPEROSMOTIC STRESS

Experiment 2:

Gill: When transferred from 100% SW to 150% SW and incubated for 4 h, the total FAA pool size of the isolated gills of M. mercenaria increased by 50% ($\sim 87 \mu\text{mol/g wet wt}$) from 167.5 to $254.5 \mu\text{mol/g wet wt}$ (Table 3). Taurine, the most abundant amino acid representing 95% of the FAA pool, increased by 45% from 157.6 to $228.9 \mu\text{mol/g wet wt}$. This increase accounted for $\sim 80\%$ of the rise in the total FAA content in the hyperosmotic gills over 4 h. Other amino acids that are normally present in larger amounts, alanine, aspartic acid and glycine, also increased in content by 200%, 100% and 60%, respectively, compared to pre-incubation levels. In hypersaline solutions, glutamic acid levels decreased by 30% from 4.6 to $3.3 \mu\text{mol/g wet wt}$ over 4 h. Isolated gills enlarged the FAA content (excluding taurine and glutamic acid) 2.5 fold from 9.3 to $22.3 \mu\text{mol/g wet wt}$ during incubation in high salinity. Increases in alanine, aspartic acid and glycine accounted for 65%, 15% and 10%, respectively, of this increase in FAA pool size from time zero. The levels of the remaining amino acids, present in low concentrations in the gills, doubled during hyperosmotic stress. After 4 h, only small amounts of free amino acids had accumulated in the 150% SW incubation medium. The FAA content of the hypersaline incubation fluid doubled from 1.39 to $3.01 \mu\text{mol/g wet wt}$ in 10 ml and 55% of this increase

TABLE 3: Concentrations of amino compounds of tissue and incubation medium of isolated gills of M. mercenaria incubated in 100% SW and 150% SW.

Amino Compound	Tissue ($\mu\text{mol/g}$ wet wt)			Incubation Medium ($\mu\text{mol/g}$ wet wt $\cdot$ 10 ml)		
	0 h	4 h	4 h	0 h	4 h	4 h
		100% SW	150% SW		100% SW	150% SW
Taurine	157.61	130.44	228.89	0.83	0.35	1.74
Aspartate	2.16	2.44	4.19	0.05	0.04	0.10
Glutamate	4.60	3.32	3.32	0.08	0.04	0.07
Glycine	1.83	1.47	3.07	0.09	0.12	0.25
Alanine	3.81	3.38	12.29	0.10	0.05	0.56
Threonine	0.05	0.04	0.14	0.01	0.02	0.00
Serine	0.05	0.04	0.09	0.05	0.04	0.00
Glutamine	0.18	0.34	0.20	0.01	0.01	0.01
Valine	0.06	0.06	0.05	0.00	0.00	0.00
Cystine	0.16	0.13	0.46	0.07	0.07	0.03
Methionine	0.06	0.05	0.18	0.00	0.00	0.00
Isoleucine	0.07	0.06	0.14	0.01	0.02	0.04
Leucine	0.04	0.03	0.08	0.02	0.03	0.04
Tyrosine	0.05	0.05	0.11	0.00	0.00	0.00
Ornithine	0.06	0.05	0.11	0.04	0.03	0.06
Lysine	0.14	0.10	0.18	0.03	0.02	0.06
Histidine	0.05	0.07	0.15	0.00	0.00	0.05
Arginine	0.51	0.54	0.84	0.00	0.00	0.00
Ammonia	2.02	2.69	3.53	0.30	2.12	1.65
Total FAA +Taurine	167.49	142.61	254.49	1.39	1.19	3.01
Total FAA -Taurine	13.88	12.17	25.60	0.56	0.84	1.27

Results are the means of n=2.

during 4 h was due to changes in taurine levels. Alanine, aspartic acid and glycine, together, accounted for an additional 40% of the total pool size increase. The ammonia concentration in isolated gills exposed to high salinity for 4 h, increased by 75% from 2.0 to 3.5 $\mu\text{mol/g}$ wet wt. The amount of ammonia in the incubation media was elevated 5 fold from 0.3 to 1.65 $\mu\text{mol/g}$ wet wt in 10 ml. The FAA content of isolated gills in 100% SW was not significantly altered in the tissue or in the surrounding incubation medium. During high salinity stress, accumulation of intracellular FAA as osmotic solutes appears to be the mechanism responsible for cell volume regulation in isolated gills of M. mercenaria.

Figures 7A and 8 show the time course of changes in selected metabolites in isolated gills and the surrounding media during exposure to 150% SW. Following transfer to high salinity, cellular alanine levels rapidly increased in the first 2 h from 4.2 ± 0.6 $\mu\text{mol/g}$ wet wt to a new steady state at 8.2 ± 1.6 $\mu\text{mol/g}$ wet wt. Alanine content was not elevated significantly in the 150% SW during the experiment nor did alanine content change in the tissue or incubation medium of control gills in isoosmotic saline. The concentration of aspartate in isolated gills incubated in 100% SW for 4 h increased by 40% from 2.51 ± 0.08 to 3.49 ± 0.16 $\mu\text{mol/g}$ wet wt. Aspartate content in isolated gills decreased by 35% from 2.78 ± 0.05 to 1.76 ± 0.05 $\mu\text{mol/g}$ wet wt over 4 h under hyperosmotic conditions. Arginine levels

FIGURE 7: The changes in concentration of selected metabolites in isolated gills of M. mercenaria incubated in 100% SW (●) and 150% SW (○) over 4 h.

A. Alanine, aspartate, arginine, glycogen, succinate and glucose.

B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.

C. ATP, ADP, AMP, total adenylates, adenylate energy charge and arginine phosphate.

Metabolites were measured as described in MATERIALS AND METHODS. Glycogen is expressed as μmol glucose after enzymatic hydrolysis.

Adenylate energy charge is calculated as $([\text{ATP}] + \frac{1}{2} [\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$.

Results are expressed as means $\pm$ s.e.m. for $n=3$ samples (3 animals per sample) at each point.

A.

B.

FIGURE 8: The changes in concentration of alanine and glucose in the incubation media surrounding isolated gills of M. mercenaria incubated in 100% SW (●) and at 150% SW (○) over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (3 animals per sample) at each point.

μmol/g wet weight · 10 ml

decreased in control tissues over 4 h by 40% from 0.52 ± 0.02 to $0.32 \pm 0.05 \mu\text{mol/g wet wt}$ in an inverse pattern to the changes in arginine phosphate levels. Arginine content in experimental tissues, however, did not vary significantly over 4 h. No significant changes in the glycogen ($\sim 15 \mu\text{mol/g wet wt}$) reserves were seen during exposure of isolated gills to hypersalinity over 4 h. The isolated gills significantly reduced the cellular succinate pools by 70% from 0.71 ± 0.06 to $0.21 \pm 0.04 \mu\text{mol/g wet wt}$ during 4 h in 150% SW. Succinate was not detected in the hyperosmotic incubation media during the experiment. Glucose levels of isolated gills in concentrated media decreased over 4 h to 60% of the level at zero time. No significant amounts of glucose appeared over 4 h in the media of increased salinity containing isolated gills.

Figure 7B shows the changes in the concentrations of glycolytic intermediates in isolated gills during hyperosmotic stress. Glucose-6-P ($0.11 \pm 0.02 \mu\text{mol/g wet wt}$) and fructose-6-P ($0.030 \pm 0.005 \mu\text{mol/g wet wt}$) levels remained low and constant throughout the 4 h incubation in 100% SW and 150% SW. Isolated gills incubated in 100% SW and in 150% SW showed a significant decrease in fructose-1,6-P₂ (by 60% and 50%, respectively) with an accompanying decrease in dihydroxyacetone-P (by 30% and 55%, respectively) which suggests an inhibition (negative cross-over) of phosphofructokinase in gills during incubation under isosmotic and hyperosmotic conditions.

Glyceraldehyde-3-P levels were not significantly altered, over the 4 h incubation in either 100% SW or 150% SW. No significant changes in phosphoenolpyruvate (0.083 ± 0.011 $\mu\text{mol/g}$ wet wt) and pyruvate (0.041 ± 0.003 $\mu\text{mol/g}$ wet wt) contents were detected.

The effects of acclimation to high salinity on adenylate and phosphagen pools of isolated gills are shown in Figure 7C. Levels of ATP (1.12 ± 0.04 $\mu\text{mol/g}$ wet wt), ADP (0.32 ± 0.04 $\mu\text{mol/g}$ wet wt) and AMP (0.02 ± 0.01 $\mu\text{mol/g}$ wet wt) were unaffected by the experimental salinity change as was arginine phosphate (0.42 ± 0.17 $\mu\text{mol/g}$ wet wt) content. The total adenylate pool size of the isolated gills remained essentially constant at 1.45 ± 0.09 $\mu\text{mol/g}$ wet wt over the 4 h exposure to 150% SW. The energy charge of hyperosmotic gills remained consistently high ($\sim 0.86-0.88$) throughout the experiment. Gills under control conditions (100% SW) also maintained a sound energy status, showing high ATP and arginine-P levels, over the course of the 4 h incubation.

The protein content of experimental gills, 53.8 ± 6.9 mg protein/ g wet wt., was not significantly different from that of the control tissues, 55.5 ± 4.6 mg protein/ g wet wt. Hence isolated gills in 150% SW showed no significant changes in tissue water content after 4 h of incubation and loss of tissue water content could not account for the observed increase in FAA content.

* Mantle: Table 4 shows the concentrations of free amino

TABLE 4: Concentrations of amino compounds of tissue and incubation medium of isolated mantles of M. mercenaria incubated in 100% SW and 150% SW.

Amino Compound	Tissue ($\mu\text{mol/g}$ wet wt)			Incubation Medium ($\mu\text{mol/g}$ wet wt $\cdot$ 10 ml)		
	0 h	4 h	4 h	0 h	4 h	4 h
		100% SW	150% SW		100% SW	150% SW
Taurine	83.22	93.14	30.45	0.83	2.31	3.70
Aspartate	1.20	1.85	1.38	0.04	0.04	0.07
Glutamate	2.78	3.36	2.74	0.07	0.05	0.14
Glycine	1.36	1.32	1.51	0.09	0.25	0.40
Alanine	3.36	4.01	6.31	0.10	0.24	0.80
Threonine	0.07	0.08	0.09	0.01	0.08	0.11
Serine	0.11	0.12	0.10	0.04	0.12	0.23
Glutamine	0.05	0.24	0.09	0.01	0.08	0.08
Valine	0.01	0.04	0.02	0.00	0.03	0.08
Cystine	0.16	0.28	0.53	0.07	0.07	0.03
Methionine	0.11	0.11	0.14	0.00	0.00	0.09
Isoleucine	0.07	0.06	0.06	0.01	0.05	0.13
Leucine	0.05	0.08	0.09	0.02	0.07	0.20
Tyrosine	0.04	0.02	0.03	0.00	0.00	0.00
Ornithine	0.03	0.01	0.04	0.04	0.05	0.14
Lysine	0.12	0.19	0.11	0.03	0.09	0.16
Histidine	0.07	0.08	0.08	0.00	0.04	0.06
Arginine	0.47	0.42	0.47	0.00	0.00	0.00
Ammonia	2.10	2.11	1.88	0.29	1.11	1.24
Total FAA +Taurine	93.28	105.39	44.24	1.36	3.57	6.42
Total FAA -Taurine	10.06	12.25	13.79	0.53	1.26	2.72

Results are the means of $n=3$.

acids in the tissue and incubation fluid of isolated mantles of M. mercenaria following 4 h of high salinity acclimation compared with pre-incubation and control amino acid concentrations. The total FAA pool size in mantles in 150% SW decreased over 4 h by 50% from 93.28 to 44.24 $\mu\text{mol/g}$ wet wt. This decrease can be wholly accounted for by the reduction in taurine content by 65% from 83.22 to 30.45 $\mu\text{mol/g}$ wet wt. FAA levels when taurine is not considered (total less taurine), increased by 40% from 10.06 to 13.79 $\mu\text{mol/g}$ wet wt in mantles in 150% SW over 4 h. Alanine, a major amino acid composing 30% of the time zero FAA pool without taurine, increased by 90% from 3.36 to 6.31 $\mu\text{mol/g}$ wet wt. The elevation of alanine levels represented about 80% of this increase in FAA (-taurine) content. Aspartate and glycine, other prominent amino acids, accounted for an additional 10% of the FAA minus taurine pool size increase. Free amino acids accumulated in the hypersaline solutions such that the level at 4 h was 5 fold the pre-incubation level. 55% of the total increase in FAA during 4 h was due to the changes in taurine content, however, the rise in taurine content of the 150% SW represents only about 5% of the intracellular taurine decrease. Alanine and glycine increases, by 7 fold and 3.5 fold respectively, were responsible for 45% of the residual (total FAA - taurine) increase or 20% of the total FAA increase. Under high salinity, the cellular ammonia pool was reduced by 0.22 $\mu\text{mol/g}$ wet wt (10%) from 2.10 to 1.88 $\mu\text{mol/g}$ wet wt, whereas

the ammonia concentration of the incubation media (150% SW) was enlarged by $0.95 \mu\text{mol/g}$ wet wt in 10 ml (~3.5 fold). Taurine levels in the mantles did not show a similar response to hyperosmotic stress as was demonstrated in isolated gills. However, similar results have been reported for clam adductor muscle (Shumway et al., 1977) and for various tissues of other bivalves (Baginski and Pierce, 1977; Strange and Crowe, 1979; Powell et al., 1982). These decreases were generally compensated for by over-riding increases in alanine and glycine and/or attributed to the high variability of taurine content. As in various tissues for other marine bivalves, the isolated mantles responded to hyperosmotic stress with a rise in the concentrations of non-essential amino acids such as alanine and glycine.

Figures 9A and 10 show the responses of selected metabolites in isolated mantles and the surrounding media to hyperosmotic stress over the course of 4 h. Cellular alanine levels rapidly climbed from 3.0 ± 0.2 to $6.3 \pm 0.2 \mu\text{mol/g}$ wet wt. The hypersaline solutions showed an increase in alanine content of about $1.06 \mu\text{mol/g}$ wet wt in 10 ml. The aspartate pool size of the osmotically stressed mantles was reduced by 60% from about 1.9 to $0.8 \mu\text{mol/g}$ wet wt over 4 h. Arginine levels decreased significantly in mantles in 100% SW and in 150% SW by 0.588 and $0.369 \mu\text{mol/g}$ wet wt respectively. The decline in arginine pools over 4 h followed an inverse pattern to the rise in arginine-P content. Glycogen reserves were reduced overall at 4 h

FIGURE 9: The changes in concentration of selected metabolites in isolated mantles of

M. mercenaria incubated in 100% SW (●)

and 150% SW (○) over 4 h.

A. Alanine, aspartate, arginine, glycogen, succinate and glucose.

B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.

C. ATP, ADP, AMP, total adenylates, adenylate energy charge and arginine phosphate.

Metabolites were measured as described in

4 MATERIALS AND METHODS. Glycogen is expressed as μ mol glucose after enzymatic hydrolysis.

Adenylate energy charge is calculated as

$([ATP] + \frac{1}{2} [ADP]) / ([ATP] + [ADP] + [AMP])$.

Results are expressed as means $\pm$ s.e.m. for n=3 samples (3 animals per sample) at each point.

A.

B.

HOURS

FIGURE 10: The changes in concentration of alanine and glucose in the incubation media surrounding isolated mantles of M. mercenaria incubated in 100% SW (●) and at 150% SW (○) over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (3 animals per sample) at each point.

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umol/g wet weight · 10 ml

although the content was variable over the time course. Succinate levels decreased in both control and test mantles by $0.26 \mu\text{mol/g wet wt}$ (~40%) and $0.47 \mu\text{mol/g wet wt}$ (~60%), respectively. No significant amounts of succinate were detected in the seawater medium. Mantles in 100% SW for 4 h showed a significant elevation in glucose pool size from 0.17 ± 0.01 to $0.25 \pm 0.03 \mu\text{mol/g wet wt}$ whereas mantles in 150% SW showed a significant reduction in glucose pools from 0.172 ± 0.025 to $0.100 \pm 0.004 \mu\text{mol/g wet wt}$. Glucose content was low in isoosmotic and hyperosmotic media and continued as such throughout the incubation of the isolated mantles except for a significant inflection ($P < 0.05$) at 2 h in the 150% SW representing a increase of $5.0 \mu\text{mol/g wet wt}$ in 10 ml.

Figure 9B shows the effects of a hyperosmotic stress on the levels of selected glycolytic intermediates in isolated mantles of M. mercenaria during 4 h. No significant alterations in the glucose-6-P ($0.082 \pm 0.008 \mu\text{mol/g wet wt}$) or fructose-6-P ($0.026 \pm 0.005 \mu\text{mol/g wet wt}$) pools were seen during the 4 h at 150% SW. Isolated mantles under control and under test conditions showed a significant rise ($P < 0.1$) in fructose-1,6-P₂ levels, a 60% increase from about $0.05 \mu\text{mol/g wet wt}$ to about $0.08 \mu\text{mol/g wet wt}$. This increase in the product of the phosphofructokinase reaction suggests an activation of this enzyme during incubation of isolated mantles in 100% SW and in 150% SW. Hyperosmotic tissues showed an accompanying increase in

dihydroxyacetone-P levels from 0.039 ± 0.006 to 0.07 ± 0.01 $\mu\text{mol/g}$ wet wt whereas dihydroxyacetone-P levels in isoosmotic tissues did not change significantly. Glyceraldehyde-3-P (0.022 ± 0.006 $\mu\text{mol/g}$ wet wt) and phosphoenolpyruvate (0.070 ± 0.001 $\mu\text{mol/g}$ wet wt) levels remained low and constant throughout the experiment. Levels of pyruvate in mantles increased rapidly (by ~3 fold) from about 0.04 to about 0.15 $\mu\text{mol/g}$ wet wt within 2 h of the switch to the higher salinity. These results show a positive cross-over at the pyruvate kinase reaction suggesting an acceleration of glycolytic flux through the pyruvate kinase locus.

Figure 9C shows the effects of an environmental salinity change from 100‰ SW to 150‰ SW on the adenylate and phosphagen pools in isolated mantles over 4 h. ATP content of the mantles remained constant at about 0.8 $\mu\text{mol/g}$ wet wt over the 4 h in concentrated media. ADP content remained relatively constant in hyperosmotic tissues over 4 h whereas ADP content of control tissues was significantly reduced at 2 h by 30% of time zero content. No significant alterations in AMP (0.046 ± 0.025 $\mu\text{mol/g}$ wet wt) pools were detected in stressed mantles over 4 h. The sum of the adenylate phosphates remained fairly constant at about 1.09 $\mu\text{mol/g}$ wet wt over the incubation period. The adenylate energy charge of the isolated gills incubated in both 100‰ SW and in 150‰ SW were high and held consistently between 0.82 and 0.90 over 4 h. The arginine-P pool of mantles under control and

test conditions increased by 3 fold and 2 fold respectively. The improved energy state over the course of the incubation of isolated mantles in 150% SW indicates that the mantles remain viable throughout the in vitro incubation.

After 4 h under hypersaline conditions the tissue water content of isolated mantles was not altered significantly from the tissue water content of control mantles as the protein contents of experimental and control mantles were 34.0 ± 2.9 and 34.9 ± 2.6 mg protein/ g wet wt, respectively. Water loss from the hyperosmotic mantles could not account for the observed rise in the FAA level.

ANOXIAExperiment 3:

Gill: Figures 11A and 12 show the changes in selected metabolite levels in isolated gills of M. mercenaria incubated in 100% SW under anoxic conditions over the course of 0 to 4 h. The immediate response of this tissue to an oxygen-free environment was the accumulation of succinate and alanine. Tissue succinate content increased linearly over 4 h whereas tissue alanine accumulated rapidly over the first hour, then plateaued. Succinate levels rapidly increased intracellularly by $3.9 \mu\text{mol/g wet wt}$ and in the surrounding incubation media by $3.5 \mu\text{mol/g wet wt}$ in 10 ml while alanine pools of the tissue and incubation media increased by $2.7 \mu\text{mol/g wet wt}$ and $0.27 \mu\text{mol/g wet wt}$ in 10 ml, respectively. Succinate was the predominant product of anaerobic metabolism with the ratio succinate:alanine produced at 4 h of anoxia being 2.5:1. The increase observed in these products was accompanied by the corresponding dramatic decrease in the aspartate concentration. After a 4 h anoxic period, the reduction of aspartate levels ($\sim 3 \mu\text{mol/g wet wt}$) accounted for approximately 40% of carbon for succinate synthesis and virtually all of the nitrogen groups for alanine synthesis. Glycogen reserves decreased by $\sim 8 \mu\text{mol/g wet wt}$ (in glucose equivalents) at 4 h of anoxia to 60% of the pre-incubation

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FIGURE 11: The changes in concentration of selected metabolites in isolated gills of M. mercenaria incubated in 100% SW under normoxic (●) and anoxic (○) conditions over 4 h.

A. Alanine, aspartate, glycogen, succinate and glucose.

B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.

C. ATP, ADP, AMP, total adenylates, adenylate energy charge, arginine phosphate and arginine.

Metabolites were measured as described in MATERIALS AND METHODS. Glycogen is expressed as μmol glucose after enzymatic hydrolysis.

Adenylate energy charge is calculated as
$$([\text{ATP}] + \frac{1}{2} [\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}]).$$

Results are expressed as means $\pm$ s.e.m. for $n=3$ samples (6 animals per sample) at each point.

B.

TOTAL
ADENYLATES ENERGY
CHARGE

ARGININE-P

ARGININE

ATP

ADP

AMP

FIGURE 12: The changes in concentration of alanine, and succinate in the incubation media surrounding isolated gills of M. mercenaria incubated in 100% SW under normoxic (●) and anoxic (○) conditions over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (6 animals per sample) at each point.

level. The carbon derived from glycogen degradation more than accounted for the increases observed in alanine and succinate over 4 h of anoxia. The rate of aspartate utilization was high during the initial hours (0-2 h) whereas glycogen utilization only became evident between 2 to 4 h of anoxia. Glucose levels in the isolated gills declined sharply during the first hour of incubation under normoxic and anoxic conditions to a level (~30% of 0 h) which was essentially maintained for the duration of the incubation.

Figure 11B shows the effects of an anoxic stress on the levels of intermediary metabolites in isolated gills over 4 h. Glucose-6-P (0.08 ± 0.02 $\mu\text{mol/g wet wt}$) levels remained low and constant throughout the experiment. Anoxic gills showed a rapid decrease in fructose-6-P with accompanying increases in fructose-1,6-P₂ and dihydroxyacetone-P levels. These results produced a positive cross-over at the phosphofructokinase reaction suggesting an anoxic activation of PFK. No significant alterations in glyceraldehyde-3-P (0.041 ± 0.014 $\mu\text{mol/g wet wt}$) pools were detected in gills under a nitrogen atmosphere over 4 h. When isolated gills were transferred to an oxygen-free environment, phosphoenolpyruvate was dramatically elevated (~2 fold) while pyruvate levels did not vary significantly, which produced a negative cross-over at the pyruvate kinase reaction. This suggests a block at the pyruvate kinase reaction in gills under anoxic conditions.

Figure 11C shows the changes in the energy status of isolated gills incubating in O_2 -free 100% SW under a nitrogen atmosphere over the course of 4 h. Under experimental conditions, ATP linearly declined by 50% from 1.35 ± 0.24 to 0.64 ± 0.03 $\mu\text{mol/g wet wt}$ over 4 h. Both ADP and AMP contents were elevated within the first hour of anoxia by 123% and 177%, respectively, to levels which were held over the remainder of the incubation period. The decrease in ATP could be accounted for by the increase in concentrations of AMP plus ADP and therefore the total adenylate pool (1.96 ± 0.30 $\mu\text{mol/g wet wt}$) was hardly affected. The gill energy charge, initially high (0.83), plummeted in the first hour to 0.66 and reached 0.61 after 4 h under a nitrogen atmosphere. Anoxia resulted in the reduction of arginine-P pools such that after 4 h only 20% of phosphagen reserves remained. Arginine pools did not show significant differences from 0 h levels (1.9 ± 0.2 $\mu\text{mol/g wet wt}$) during anoxia. Gills under control conditions maintained a sound energy status, showing increases in ATP and arginine-P pools, over the course of the 4 h incubation.

Mantle: Figures 13A and 14 show the effects of an anoxic environment on metabolite levels in isolated mantles of M. mercenaria over the course of 4 h. Alanine and succinate were products of anaerobic metabolism in the mantles. Like gill, succinate was the major product of this

FIGURE 13: The changes in concentration of selected metabolites in isolated mantles of M. mercenaria incubated in 100% SW under normoxic (●) and anoxic (○) conditions over 4 h.

- A. Alanine, aspartate, glycogen, succinate and glucose.
- B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.
- C. ATP, ADP, AMP, total adenylates, adenylate energy charge, arginine phosphate and arginine.

Metabolites were measured as described in MATERIALS AND METHODS. Glycogen is expressed as μmol glucose after enzymatic hydrolysis. Adenylate energy charge is calculated as
$$([ATP] + \frac{1}{2}[ADP]) / ([ATP] + [ADP] + [AMP])$$
. Results are expressed as means $\pm$ s.e.m. for $n=3$ samples (6 animals per sample) at each point.

A.

B.

HOURS

FIGURE 14: The changes in concentration of alanine, and succinate in the incubation media surrounding isolated mantles of M. mercenaria incubated, in 100% SW under normoxic (●) and anoxic (○) conditions over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (6 animals per sample) at each point.

tissue, the ratio succinate:alanine produced at 4 h of anoxia being 7.3:1 (including both tissue and incubation medium contents). Succinate content sharply increased in the mantle and in the incubation media by $3.0 \mu\text{mol/g wet wt}$ and $8.1 \mu\text{mol/g wet wt}$ in 10 ml, respectively. Intracellular alanine pools remained essentially constant whereas alanine accumulated ($1.6 \mu\text{mol/g wet wt}$ in 10 ml) in the seawater. Anoxia stimulated the catabolism of aspartate ($2 \mu\text{mol/g wet wt}$) which contributes about 20% of the carbon required for succinate synthesis. In the isolated mantles the decline in aspartate levels was near stoichiometric with the increase in alanine levels throughout anoxia suggesting aspartate as the source of NH_4^+ groups for alanine synthesis. In contrast to the change previously described in isolated gills, the glycogen reserves in the mantles were not significantly altered over the 4 h anoxic period. However, owing to the large glycogen content ($\sim 165 \mu\text{mol/g wet wt}$ in glucose equivalents) and the high variability in glycogen reserves, the standard deviations were large ($\sim 10 \mu\text{mol/g wet wt}$) and may have obscured any degradation of glycogen which would account for the rises in succinate and alanine (i.e. 3% of 0 h levels). Glucose levels exhibited a dramatic decrease within the first hour of incubation under normoxic and anoxic conditions to about 50% of the levels at time zero where they held throughout the experiment.

Figure 13B shows the changes in levels of glycolytic intermediates in isolated mantles subjected to anoxic

stress. No significant alterations in glucose-6-P ($0.081 \pm 0.018 \mu\text{mol/g wet wt}$) and fructose-6-P ($0.006 \pm 0.002 \mu\text{mol/g wet wt}$) levels were detected throughout the 4 h anoxic incubation. As in Experiments 1 and 2, isolated mantles incubated in 100% SW under normoxic conditions showed a significant rise in fructose-1,6-P₂ levels by 50% over 4 h indicating an activation of phosphofructokinase under these circumstances. Fructose-1,6-P₂ and dihydroxyacetone-P levels were significantly elevated by 226% and 243% respectively, in mantles incubated under a nitrogen atmosphere. These results suggest an activation of glycolytic flux through the phosphofructokinase locus in anoxic mantles. Glyceraldehyde-3-P ($0.039 \pm 0.007 \mu\text{mol/g wet wt}$) pools in mantles were unaffected by the anoxic conditions. Phosphoenolpyruvate and pyruvate content dramatically increased by 250% and 190% respectively. These results, as the increase in phosphoenolpyruvate was substantially greater than that in pyruvate, produce a displaced negative cross-over at the pyruvate kinase reaction as opposed to a classic negative cross-over which normally intersects the 100% axis (pre-incubation level). This displaced negative cross-over indicates an inhibition of the pyruvate kinase reaction and perhaps also an inhibition of a subsequent reaction that utilizes pyruvate as a substrate in anoxic mantles.

Figure 13C shows the responses of the adenylate and phosphagen pools of isolated M. mercenaria mantles to

incubation in an oxygen-free environment over 4 h. The concentrations of ATP decreased by 55% from 1.05 ± 0.13 to 0.47 ± 0.02 $\mu\text{mol/g}$ wet wt over 4 h of anoxia. ADP content was significantly elevated ($P < 0.05$) by ~60% of the pre-incubation content by 4 h of exposure to anoxic conditions. Levels of AMP (0.13 ± 0.02 $\mu\text{mol/g}$ wet wt) remained relatively constant in the anoxic mantles over 4 h. Total adenylates (1.58 ± 0.18 $\mu\text{mol/g}$ wet wt) did not vary significantly over the O_2 -free incubation period. Energy charge was high (0.80) prior to incubation and decreased dramatically to 0.67 by 1 h of anoxia and to 0.57 over the remainder of the experiment. Arginine-P levels gradually decreased over 4 h of anoxia to 13% of time zero levels. The arginine content of anoxic mantles did not vary significantly over the incubation period. Arginine pools of mantles under control conditions were gradually diminished over 4 h by 30% of the 0 h value in a pattern inverse to the changes in the arginine-P concentration. The control mantles showed increases in the energy charge and phosphagen pools indicating that the mantles remained viable throughout the in vitro incubation.

HYPEROSMOTIC STRESS UNDER ANOXIC CONDITIONS

Experiment 4:

Gill: Table 5 shows FAA concentrations in isolated gills of M. mercenaria following 4 h of high salinity acclimation under aerobic versus anaerobic conditions. Under an O₂ atmosphere the total FAA pool increased greatly by 120% (as seen previously in Experiment 2) whereas under a N₂ atmosphere the total FAA pool decreased slightly by 11%. Taurine, in each case, was the most abundant amino acid and composed about 80% of the total FAA content. The rise in taurine levels over 4 h of normoxia represented 87% of the total FAA pool enlargement as was seen in Experiment 2. The reduction in taurine levels over 4 h of anoxia accounted for virtually all of the observed decrease in the total FAA pool, thus the decrease may be attributed to the variability of taurine content. When taurine is not considered, the FAA pool of normoxic gills increased by 70% over 4 h while the FAA pool of anoxic gills essentially remained constant. Aspartate levels decreased under aerobic and anaerobic conditions by 17% and 81%, respectively. The elevations in alanine and glycine concentrations under normoxic conditions were substantially greater than the rises observed for these amino acids under anoxic conditions by 5 fold and 2.5 fold, respectively. Glutamate content declined by 25% in gills under an O₂ atmosphere, as in Experiment 2, whereas glutamate content remained relatively constant in anoxic

TABLE 5: Concentrations of amino compounds in isolated gills of *M. mercenaria* incubated in 150‰ SW under normoxic and anoxic conditions.

Amino Compound	Tissue Concentration ($\mu\text{mol/g}$ wet weight)		
	0 h	4 h O_2	4 h N_2
Taurine	128.49	300.12	109.61
Aspartate	5.87	4.88	1.09
Glutamate	5.66	4.27	5.78
Glycine	5.59	12.00	7.46
Alanine	16.64	34.74	19.53
Threonine	0.06	0.23	0.08
Serine	0.07	0.25	0.14
Glutamine	0.15	0.15	0.00
Valine	0.05	0.63	0.38
Cystine	0.08	0.08	0.18
Methionine	0.07	0.09	0.10
Isoleucine	0.06	0.18	0.08
Leucine	0.06	0.47	0.20
Tyrosine	0.06	0.21	0.13
Ornithine	0.04	0.21	0.05
Lysine	0.06	0.24	0.17
Histidine	0.05	0.18	0.05
Arginine	0.58	0.86	0.42
Ammonia	5.56	4.39	4.24
Total FAA +Taurine	163.58	359.80	145.45
Total FAA -Taurine	35.09	59.68	35.84

Results are the means of $n=2$.

gills. In both cases, the concentration of ammonia was reduced by approximately 23% over 4 h. The results demonstrate that the accumulation of FAA in isolated gills in response to a hyperosmotic stress is severely limited under anaerobic conditions.

Figures 15A and 16 show the effects of anoxia on metabolite levels in isolated gills incubated in 150% SW over a time course from 0 to 4 h. Under anaerobic conditions, alanine gradually increased intracellularly and in the incubation media by 5.4 $\mu\text{mol/g}$ wet wt and 0.63 $\mu\text{mol/g}$ wet wt in 10 ml, respectively. Anoxia also stimulated succinate synthesis. During the 4 h anaerobic incubation, succinate accumulation in the tissue and the seawater was 2.5 $\mu\text{mol/g}$ wet wt and 4.1 $\mu\text{mol/g}$ wet wt in 10 ml, respectively. The ratio succinate:alanine produced at 4 h of anoxia in 150% SW was 1.1:1. Aspartate pools were rapidly depleted by 5 $\mu\text{mol/g}$ wet wt at 4 h to 10% of the pre-incubation level. The reduction in this amino acid's content accounted for approximately 80% of the rise in each product of anaerobic metabolism. The glycogen reserves of the isolated gills were not significantly altered over the 4 h anoxic period. Consequently, endogenous glycogen could not account for the observed accumulation of products of anaerobic metabolism. Under an O_2 atmosphere, alanine increased by 12.7 $\mu\text{mol/g}$ wet wt in the tissue and by 1.86 $\mu\text{mol/g}$ wet wt in 10 ml of incubation media, while no significant changes in succinate and aspartate pools were

FIGURE 15: The changes in concentration of selected metabolites in isolated gills of M. mercenaria incubated in 150‰ SW under normoxic (●) and anoxic (○) conditions over 4 h.

A. Alanine, aspartate, arginine, glycogen, succinate and glucose.

B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.

C. ATP, ADP, AMP, total adenylates, adenylate energy charge and arginine phosphate.

Metabolites were measured as described in MATERIALS AND METHODS. Glycogen is expressed as μmol glucose after enzymatic hydrolysis.

Adenylate energy charge is calculated as

$$([\text{ATP}] + \frac{1}{2}[\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}]).$$

Results are expressed as means $\pm$ s.e.m. for $n=3$ samples (6 animals per sample) at each point.

A.

C.

FIGURE 16: The changes in concentration of alanine, glucose and succinate in the incubation media surrounding isolated gills of M. mercenaria incubated in 150% SW under normoxic (●) and anoxic (○) conditions over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (6 animals per sample) at each point.

seen over 4 h. Arginine levels doubled in gills under a N_2 atmosphere over 4 h, changing oppositely to arginine-P levels. Glucose ($0.22 \pm 0.06 \mu\text{mol/g wet wt}$) content did not vary significantly in gills and no significant amounts of glucose were detected in the seawater over 4 h of aerobic or anaerobic incubation.

Figure 15B shows the effects of an anaerobic incubation on levels of intermediary metabolites in isolated gills in 150% SW. As in Experiment 2, the levels of glucose-6-P, fructose-6-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate remained low and constant in hyperosmotically-stressed gills under aerobic conditions (controls) over 4 h. However, in this case, fructose-1,6- P_2 and dihydroxyacetone-P content also remained constant whereas in Experiment 2 the levels of these glycolytic intermediates decreased. The switch to an anoxic environment produced an immediate drop in glucose-6-P levels which remained low (approximately $0.010 \pm 0.004 \mu\text{mol/g wet wt}$) over the 4 h. No significant alterations in fructose-6-P ($0.003 \pm 0.001 \mu\text{mol/g wet wt}$) pools were seen during the 4 h under a nitrogen atmosphere. A large increase in fructose-1,6- P_2 and dihydroxyacetone-P levels, 311% and 168% respectively, in anoxic gills suggested an activation of phosphofructokinase with a transition from aerobic to anaerobic conditions. Glyceraldehyde-3-P ($0.043 \pm 0.004 \mu\text{mol/g wet wt}$) content remained low and constant throughout the anaerobic incubation period. An elevation

(~3 fold) of phosphoenolpyruvate levels, but no detectable change in pyruvate levels indicates an inhibition of flux (negative cross-over) at the pyruvate kinase locus in gills upon exposure to an O_2 -free environment. The cross-overs shown here, in anaerobic gills in 150% SW, are the same as those demonstrated in anaerobic gills in 100% SW (Experiment 3).

Figure 15C shows the changes in the energy status of isolated gills incubating in O_2 -free 150% SW over the course of 4 h. During the anaerobic incubation period, ATP pools steadily declined by 40% from 1.41 ± 0.05 to 0.84 ± 0.05 $\mu\text{mol/g}$ wet wt. Anaerobic gills showed a slight elevation in ADP levels at 4 h by about 30% over pre-incubation levels. AMP content doubled within 1 h of anoxia, and remained elevated (0.405 ± 0.007 $\mu\text{mol/g}$ wet wt) over the remainder of the 4 h. As the reduction in ATP corresponds closely to the rise in the AMP plus ADP pools, the total adenylates held constant at 2.2 ± 0.2 $\mu\text{mol/g}$ wet wt in gills under a nitrogen atmosphere over 4 h. Within the first hour of the anaerobic incubation, the energy charge of gills dramatically dropped from 0.78 to 0.65 and then declined further to 0.60 over the remainder of the 4 h. The arginine-P pools in anoxic gills were rapidly depleted, decreasing to 22% of the levels at time zero. The changes in energy status of anaerobic gills under hyperosmotic stress paralleled those determined in anaerobic gills in 100% SW (Experiment 3). ATP and phosphagen pools increased

in aerobic gills (controls) by 36% and 125%, respectively, while ADP and AMP and total adenylate levels as well as the energy charge were essentially unaltered over 4 h, as in Experiment 2.

Mantle: When exposed to 150% SW under anaerobic conditions and incubated for 4 h, the total FAA pool size of the isolated mantles of M. mercenaria decreased by approximately 10 $\mu\text{mol/g}$ wet wt (Table 6), equivalent to 8% of the initial pool size. Taurine, the major amino acid representing about 70% of the FAA pool in each case, decreased by 13% from 87.4 to 76.3 $\mu\text{mol/g}$ wet wt over 4 h of anoxia. This decrease accounts for the reduction in total FAA content produced by the anaerobic mantles over 4 h. However, under aerobic conditions taurine content increased by 4% to 91.1 $\mu\text{mol/g}$ wet wt over 4 h, which disagrees with the results of Experiment 2 where taurine decreased in mantles after 4 h of hyperosmotic stress. These contradictory results suggest that the unexpected drop in mantle taurine content in Experiment 2 may indeed be attributed to the high variability of taurine content in individual mantles. FAA levels, when taurine is not considered, increased by ~4% in mantles in 150% SW under anaerobic conditions over 4 h compared to ~30% under aerobic conditions. Under a nitrogen atmosphere, aspartate and glutamate content decreased by 87% and 32%, respectively. Isolated mantles enlarged the residual FAA pool (excluding

TABLE 6: Concentrations of amino compounds in isolated mantles of *M. mercenaria* incubated in 150‰ SW under normoxic and anoxic conditions.

Amino Compound	Tissue Concentration ($\mu\text{mol/g}$ wet weight)		
	0 h	4 h O_2	4 h N_2
Taurine	87.39	91.10	76.28
Aspartate	2.84	1.88	0.37
Glutamate	7.04	5.34	4.78
Glycine	4.55	7.00	5.46
Alanine	17.06	25.91	22.12
Threonine	0.13	0.24	0.21
Serine	0.29	0.56	0.60
Glutamine	0.33	0.31	0.18
Valine + Cystine + Methionine	0.11	0.22	0.20
Isoleucine	0.03	0.14	0.11
Leucine	0.06	0.26	0.22
Tyrosine	0.07	0.10	0.11
Ornithine	0.05	0.00	0.00
Lysine	0.18	0.21	0.20
Histidine	0.09	0.14	0.05
Arginine	0.54	0.72	0.58
Ammonia	2.97	3.91	3.39
Total FAA +Taurine	120.76	134.13	111.47
Total FAA -Taurine	33.37	43.03	35.19

Results are the means of $n=2$.

taurine, aspartate and glutamate) by 28% during incubation in 150% SW under a nitrogen atmosphere which was approximately half the increase observed for aerobic mantles in 150% SW. In anoxic mantle tissues, the amino acids that are normally present in larger amounts, alanine and glycine, were elevated to 130% and 120%, respectively, compared to the level at time zero. In normoxic mantle tissues, alanine and glycine increases were greater (152% and 155%, respectively). However, under both anoxia and normoxia the increases in alanine and glycine concentrations accounted for 77% and 14%, respectively, of the rise in the residual FAA pool size. Ammonia content of anaerobic mantles increased by 14% from 3.0 to 3.4 $\mu\text{mol/g}$ wet wt over 4 h. Aerobic mantles showed a 32% increase in ammonia content compared to the 10% decrease noted for mantles under the same conditions in Experiment 2. While the pattern of elevations and reductions of the free amino acids (except taurine) was similar under both aerobic and anaerobic incubations in 150% SW, the intensity of changes was different. Anaerobic mantles showed larger decreases in aspartate and glutamate and smaller increases in the remaining amino acids, and hence the overall accumulation of amino acids was considerably less than for aerobic mantles.

Figures 17A and 18 show the time course of changes in selected metabolites in isolated mantles and the surrounding media during acclimation to 150% SW under aerobic or anaerobic conditions. Following transfer to O_2 -free

FIGURE 17: The changes in concentration of selected metabolites in isolated mantles of M. mercenaria incubated in 150% SW under normoxic (●) and anoxic (○) conditions over 4 h.

- A. Alanine, aspartate, arginine, glycogen, succinate and glucose.
- B. Glucose-6-P, fructose-6-P, fructose-1,6-P₂, dihydroxyacetone-P, glyceraldehyde-3-P, phosphoenolpyruvate and pyruvate.
- C. ATP, ADP, AMP, total adenylates, adenylate energy charge and arginine phosphate.

Metabolites were measured as described in MATERIALS AND METHODS. Glycogen is expressed as μmol glucose after enzymatic hydrolysis.

Adenylate energy charge is calculated as $([\text{ATP}] + \frac{1}{2}[\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$.

Results are expressed as means $\pm$ s.e.m. for $n=3$ samples (6 animals per sample) at each point.

A.

B.

C.

FIGURE 18: The changes in concentration of alanine, glucose and succinate in the incubation media surrounding isolated mantles of M. mercenaria incubated in 150% SW under normoxic (●) and anoxic (○) conditions over 4 h. Metabolites were measured as described in MATERIALS AND METHODS. Results are expressed as means $\pm$ s.e.m. for n=3 samples (6 animals per sample) at each point.

conditions, cellular alanine levels gradually increased by 50% from about 7.2 to 10.8 $\mu\text{mol/g}$ wet wt. Alanine accumulated in the incubation media from essentially zero to 1.1 $\mu\text{mol/g}$ wet wt in 10 ml over 4 h of anoxia. Anoxic mantles produced succinate, increasing cellular pools by 164% from approximately 1.1 to 2.9 $\mu\text{mol/g}$ wet wt over 4 h. Succinate was also excreted into the incubation media such that the level at 4 h of anoxia was 3.2 $\mu\text{mol/g}$ wet wt in 10 ml. The ratio succinate:alanine produced at 4 h of anoxia in 150% SW was 1.06:1. The increase in alanine content was significantly greater in aerobic mantles in 150% SW as cellular and media levels rose by 4.5 $\mu\text{mol/g}$ wet wt and 2.9 $\mu\text{mol/g}$ wet wt in 10 ml, respectively, over 4 h. No significant changes in the concentration of succinate of mantle tissue and media were seen for aerobic mantles in 150% SW over 4 h as in Experiment 2. Anoxia stimulated the degradation of aspartate such that the decrease in aspartate concentration (2.2 $\mu\text{mol/g}$ wet wt) represented approximately 45% of the increase in each of the end products of anaerobic metabolism. Under aerobic conditions, aspartate pools decreased by 40% in hyperosmotic mantles within 2 h and subsequently increased to pre-incubation levels by 4 h. Glycogen reserves in mantles remained high (199 ± 21 $\mu\text{mol/g}$ wet wt) and essentially constant over 4 h under an oxygen or a nitrogen atmosphere. Consequently, glycogen utilization did not appear to be a carbon source for alanine and/or succinate synthesis. During incubation of isolated mantles,

arginine pools were reduced by 65% under aerobic conditions and elevated by 73% under anaerobic conditions. In their respective environments, arginine pools changed in an opposite pattern to the changes in arginine-P pools. The concentration of glucose in mantles in both cases declined by 53% of the pre-incubation level. Glucose was not detected in the aerobic media, whereas glucose content was slightly elevated to $0.78 \pm 0.33 \mu\text{mol/g wet wt}$ in 10 ml of anaerobic media.

Figure 17B shows the changes in the concentration of glycolytic intermediates in isolated mantles in 150% SW under aerobic or anaerobic conditions. Within 1 h of aerobic incubation, glucose-6-P pools doubled to $0.13 \pm 0.02 \mu\text{mol/g wet wt}$ but then decreased to and remained at an average of $0.090 \pm 0.008 \mu\text{mol/g wet wt}$ over the remainder of the incubation. Under anoxia glucose-6-P levels decreased to 20% of the pre-incubation level. No significant alterations in fructose-6-P ($0.005 \pm 0.004 \mu\text{mol/g wet wt}$) content were detected over 4 h under control or experimental conditions. Anoxic mantles incubated in 150% SW showed a significant rise in fructose-1,6-P₂ and dihydroxyacetone-P levels, an 8 fold and 1.5 fold increase respectively, over the 4 h. These results show a positive cross-over at the phosphofructokinase reaction suggesting an activation of glycolytic flux through the phosphofructokinase locus in anoxia. As in Experiment 2, aerobic mantles in 150% SW showed a positive cross-over at the PFK locus as

fructose-1,6-P₂ levels increased. It appears, then, that in all cases for isolated mantles in this study PFK is activated. Glyceraldehyde-3-P (0.039 ± 0.004 $\mu\text{mol/g}$ wet wt) levels remained low and constant in mantles under an oxygen or a nitrogen atmosphere over 4 h. Phosphoenolpyruvate and pyruvate dramatically increased by 164% and 92% respectively. As in Experiment 3, this displaced negative cross-over indicates a block at the pyruvate kinase reaction and perhaps at a subsequent reaction in the pathway in anoxic mantles. However, pyruvate kinase was activated in hyperosmotic mantles under aerobic conditions both in the present case and in Experiment 2. Therefore, the controls exerted under anoxia appear to override those exerted due to a hyperosmotic stress.

Figure 17C shows the responses of the adenylate and phosphagen pools of isolated M. mercenaria mantles to incubation in 150% SW under anaerobic conditions. ATP content was reduced by approximately 40% from 1.14 ± 0.03 to 0.70 ± 0.09 $\mu\text{mol/g}$ wet wt over 4 h of O₂-free incubation. No significant alterations in ADP (0.55 ± 0.02 $\mu\text{mol/g}$ wet wt) and AMP (0.18 ± 0.03 $\mu\text{mol/g}$ wet wt) levels were seen during the 4 h anoxic incubation period. The total adenylate pool size declined slightly from 1.89 ± 0.08 to 1.50 ± 0.14 $\mu\text{mol/g}$ wet wt over 4 h of anoxia due to the apparent absence of increases in AMP or ADP levels to match the observed decrease in ATP concentration. The energy charge of anaerobic mantles fell from the time zero value,

0.75, to 0.66 at 4 h. The concentration of arginine-P was reduced after 4 h of anoxia to about 20% of the pre-incubation level. The changes in energy status of the anoxic mantles in 150% SW paralleled those of anoxic mantles in 100% SW (Experiment 3). Aerobic mantles showed increases in the phosphagen pool (by 387%) and the energy charge (from 0.76 to 0.85) while the concentration of the individual adenine nucleotides and hence the total adenylates remained essentially constant over 4 h as in Experiment 2.

ENZYME STUDIES

Glycogen Phosphorylase: The levels of phosphorylase a and b in isolated gills and mantles prior to incubation and after a 4 h incubation under aerobic isoosmotic, aerobic hyperosmotic or anaerobic isoosmotic conditions are summarized in Table 7. Phosphorylase in the a form was detected at very low levels in both tissues in all cases. At time zero and after 4 h under control or experimental conditions, phosphorylase was mainly in the b form in isolated gills and mantles as evidenced by the low ratios of activity in the absence and presence of AMP. There was no detectable conversion of glycogen phosphorylase b to a in isolated gill and mantle tissues after 4 h of exposure to hyperosmotic or anaerobic stress.

Pyruvate kinase: Early studies of pyruvate kinase from muscle tissues of anoxia-tolerant molluscs indicated that it is an allosteric enzyme and that its activity was modulated by fructose-1,6-P₂, alanine, ATP and pH. Recently it has been shown that, during anoxia and salinity changes, pyruvate kinase is also regulated by covalent modification, possibly through a phosphorylation/dephosphorylation mechanism (Siebenaller, 1979; Holwerda et al., 1981; Plaxton and Storey, 1984). The crossover studies described in Experiments 2-4 suggested that pyruvate kinase of M. mercenaria gill and mantle tissues plays a predominant role in the regulation of glycolytic flux under various stresses.

TABLE 7: Activities of glycogen phosphorylase in isolated gill and mantle tissues of M. mercenaria at time zero and after 4 h of incubation under various conditions. Enzyme activities were measured under standard conditions as described in MATERIALS AND METHODS. Phosphorylase a: -AMP. Phosphorylase a + b: +AMP. Results are means $\pm$ s.e.m. for n=3 preparations.

Tissue and incubation conditions	Phosphorylase $\times 10^{-3}$ units/ g wet wt		
	phosphorylase a	phosphorylase (a+b)	% a
Gill 0 h	40 $\pm$ 9	227 $\pm$ 16	18
Gill 4 h-O ₂ 100% SW	73 $\pm$ 1	403 $\pm$ 29	18
Gill 4 h-O ₂ 150% SW	40 $\pm$ 7	334 $\pm$ 44	12
Gill 4 h-N ₂ 100% SW	34 $\pm$ 7	275 $\pm$ 87	12
Mantle 0 h	26 $\pm$ 2	268 $\pm$ 73	10
Mantle 4 h-O ₂ 100% SW	21 $\pm$ 7	302 $\pm$ 55	7
Mantle 4 h-O ₂ 150% SW	30 $\pm$ 9	250 $\pm$ 23	12
Mantle 4 h-N ₂ 100% SW	24 $\pm$ 1	192 $\pm$ 35	13

Consequently, kinetic studies of pyruvate kinase of isolated gills and after mantles prior to incubation and 4 h of incubation with or without various environmental stresses were undertaken in order to determine whether regulation of the clam gill and mantle pyruvate kinases was due to covalent modification of the enzyme.

Generally, the effect of phosphorylation on PK kinetics is to alter the allosteric behaviour, namely an increase of the apparent $K_{0.5}$ for phosphoenolpyruvate, an increase of the Hill coefficient and an increased sensitivity to modulators. Plaxton and Storey (1984) have shown this effect for aerobic and anoxic variants of PK from the red muscle of the channelled whelk. Affinity for PEP was more than 10 fold greater for PK-aerobic ($K_{0.5} = 0.067 \pm 0.002$ mM, $n_H = 0.99 \pm 0.10$) whereas the cooperative effect for PEP binding was greatly enhanced for PK-anoxic ($K_{0.5} = 0.85 \pm 0.02$ mM, $n_H = 2.57 \pm 0.01$). PK-anoxic showed much stronger regulation by the allosteric inhibitor alanine. No such significant differences or trends were observed in the kinetic parameters for gill-PK or mantle-PK after 4 h of incubation of the isolated tissues under hyperosmotic or anaerobic conditions (Table 8). Both gill and mantle-PK showed hyperbolic kinetics as the Hill coefficient was about 1 in all cases. The $K_{0.5}$ for phosphoenolpyruvate did not show any dramatic differences indicative of a change in the phosphorylation state of PK. Pyruvate kinase of both tissues after 4 h of environmental stress did not appear to

TABLE 8: Kinetic Parameters of Clam PK and Whelk PK

Tissue Preparation	Parameter	0 h	Whole Animal 96 h - N ₂	4 h - O ₂ 100% SW ²	Isolated Tissues 4 h - O ₂ 150% SW ²	4 h - N ₂ 100% SW ²
CLAM gill	K _{0.5} (mM PEP)	0.61 ± 0.06	0.75 ± 0.06	0.32 ± 0.02	0.34 ± 0.02	0.66 ± 0.08
	η _H	0.75 ± 0.10	0.84 ± 0.10	0.98 ± 0.07	0.91 ± 0.03	1.16 ± 0.27
	I ₅₀ (mM Ala)	2.08 ± 0.37	1.04 ± 0.36	2.78 ± 0.33	2.68 ± 0.23	0.93 ± 0.27
	K _{0.5} (mM PEP)	0.67 ± 0.08	0.73 ± 0.10	0.92 ± 0.07	0.53 ± 0.05	0.77 ± 0.10
	η _H	1.01 ± 0.16	0.89 ± 0.06	0.86 ± 0.08	0.88 ± 0.06	1.23 ± 0.18
	I ₅₀ (mM Ala)	1.11 ± 0.16	1.85 ± 0.11	2.94 ± 0.86	1.95 ± 0.12	1.04 ± 0.54
mantle						
WHELK heart		0 h	24 h - N ₂			
	K _{0.5} (mM PEP)	0.193	3.09			
	η _H	1.23	2.97			
	I ₅₀ (mM Ala)	29.20	0.026			

Enzyme activities of dialysed preparations measured under standard assay conditions as described in Materials and Methods. Results are means ± s.e.m. for n=3 preparations of each clam tissue and n=1 for whelk hearts.

be more sensitive to alanine inhibition. However, as experimental procedure for the isolation and assay of pyruvate kinase was that of Plaxton and Storey (1984) characterization of anoxic and aerobic variants of whelk heart PK was also performed as a control and predicted results were obtained (Table 8). In order to ascertain whether the apparent lack of covalent modification of clam soft tissue-PK was due to the isolation of the tissues from the whole animal or the short exposure to environmental stress, PK from gills and mantles directly sampled from clams that had been made anoxic for 96 h was also studied. Again, the kinetic parameters were similar to those determined at time zero. It appears, then, that the regulation of the PK reaction in gills and mantles of M. mercenaria is of an allosteric nature rather than due to covalent modification.

DISCUSSION

The cherrystone clam, Mercenaria mercenaria, inhabits the intertidal zone where the animal frequently experiences large fluctuations in salinity and oxygen availability. The biochemical adaptations and metabolic 'strategies' underlying the remarkable tolerance displayed by many bivalves to changing salinity and environmental anoxia are to date not well understood. This study examined metabolism and metabolic regulation with respect to the sites of control and the controlling metabolites in tissues of the clam in response to these commonly encountered environmental stresses. The results of these investigations demonstrated that each environmental stress elicited a unique metabolic response in the isolated gill and mantle tissues of M. mercenaria.

Before considering the effects of environmental stresses on the isolated gill and mantle tissues some estimation of the effects of dissection, handling and incubation can be made by examining the tissues incubated in aerated 100% SW in Experiments 1, 2 and 3. The low levels of amino acids in the incubation media of these isolated tissues indicate that leakage from broken cells caused by dissection is minimal. The improvement in energy status over the early incubation times, reflected by the energy charge and arginine-P levels, of isolated tissues is a strong indication that the tissues remained viable throughout the in vitro incubation.

Isolated tissues incubated in aerated 100% SW also maintained an aerobic metabolism as succinate did not accumulate intracellularly or in the incubation media. In addition, the levels of the glycolytic intermediates generally held constant in these tissues. However, there is some effect of incubation at the PFK locus as indicated by changes in the levels of fructose-1,6-P₂ and/or dihydroxyacetone-P in both isolated gills and mantles. Isolated gills incubated in aerated 100% SW (except in Experiment 3) showed an inhibition of phosphofructokinase whereas the isolated mantles showed an activation of the enzyme under these conditions. A faster metabolic rate in an in vitro experiment compared with an in vivo experiment was observed for oyster heart (Collicutt and Hochachka, 1977) and a similar response may have been invoked in the isolated clam mantles.

The experimental design used in this thesis relies on "internal controls", i.e. pairs of tissues from each individual animal were separated such that one half of the pair was used as the control and one half as the experimental. This allows us to circumvent any animal variation encountered due to the different populations supplied by the commercial retailers or due to seasonal variation in the biochemical composition of the animals. Particularly, because of the well known seasonal variation in fuel levels and in ratios of end products produced during anaerobiosis (Zandee et al., 1980) we cannot expect exactly

comparable results between experiments (experiments 2 vs. 4, 3 vs. 4 for example) although to a large extent when experimental protocol was repeated, results were very similar.

Hypoosmotic stress

It is well established that intracellular free amino acids are utilized to regulate cell volume during salinity changes in osmoconforming bivalves. Regulation of intracellular FAA pools during hypoosmotic stress has been generally attributed to control of amino acid efflux and/or amino acid degradation. The present study examined the role of amino acid metabolism in the regulation of intracellular FAA in isolated gill and mantle tissues of M. mercenaria in 50% SW.

When isolated gill and mantle tissues of M. mercenaria were exposed to hypoosmotic stress the FAA content of the tissues decreased while that of the surrounding incubation media increased. As the FAA loss from the isolated tissues approximately equals the FAA output into the surrounding incubation media, intratissue oxidation of amino acids could not have occurred to any great extent. The apparent lack of amino acid deamination and degradation is supported by the fact that the isolated tissues did not accumulate an appreciable amount of ammonia nor was ammonia output by isolated tissues greatly increased. Hypoosmotic stress did not appear to alter glycolytic rate (neither increase or

decrease) as the levels of glycolytic intermediates did not differ significantly from control levels. There was also no evidence of gluconeogenesis in gill tissues but mantle tissues may have utilized some amino acids as gluconeogenic substrates as glucose levels rose significantly in the tissue and the incubation media without an apparent decrease in glycogen levels. The process of adaptation to low salinity did not stress the energy state of the tissues; the concentration of adenine nucleotides and arginine-P and the energy charge remained constant in the hypoosmotic gill and mantle tissues. Therefore free amino acid excretion, with no other effects on intermediary metabolism, appears to be the major response of isolated gills and mantles to dilution of the seawater medium.

Studies of isolated adductor muscle, gill and mantle tissues of R. cuneata (Henry and Mangum, 1980) and isolated foot tissues of C. japonica (Matsushima, 1982) also show that the primary response of isolated tissues to a hypoosmotic stress is excretion of FAA while ammonia output is minimal. These two reports also included studies on hypoosmotic stress on the whole animal. The latter report in conjunction with that of Bartberger and Pierce (1976) has shown that during acclimation to low salinity, FAA are released intact from muscle tissue cells into the hemolymph and are subsequently degraded elsewhere, by specific body tissues. Thus the primary response of isolated tissues is the release of FAA while studies of whole animals show

ammonia as the ultimate product released into the environment during hypoosmotic stress. Amino acid degradation must take place in one or more tissues, therefore. Other studies have implicated both mantle and gill in this role. Bartberger and Pierce (1976) have suggested the possibility that the degradation of amino acids and the excretion of ammonia occurs in mantle tissues of M. demissus. Their suggestion depends upon the observations that mantle tissue is highly vascularized and that the FAA pool in mantle did not decrease during hypoosmotic stress. In the present study, however, the FAA concentration in the mantle of M. mercenaria decreased as well as in the gill. Henry and Mangum (1980) and Matsushima (1982) also showed a decrease in the FAA content of mantle, gill and muscle tissues in low salinity. On the other hand, Henry and Mangum (1980) have suggested for R. cuneata that the gill should be considered to be a site of deamination and ammonia excretion due to the greater oxidative capacity of this branchial tissue. However, in the present study neither gill nor mantle showed a capacity for amino acid catabolism in an isolated preparation over 4 h. Henry and Mangum (1980) suggest that the possibility of an appreciable level of in situ deamination in various tissues, concomitant with FAA excretion does not appear to be likely during the early phase of low salinity adaptation. Generally, isolated tissues such as gill, mantle, foot and adductor muscle exposed to hyposaline media show an almost immediate loss of

intracellular FAA. On the other hand, the blood ammonia level and ammonia excretion rate increases significantly in intact animals after ventilation is resumed and the blood FAA have peaked (16-20 h), (Henry and Mangum, 1980; Matsushima, 1982) suggesting this mechanism may become somewhat more important in late stages of adaptation (a secondary process).

The present data show the effects of hypoosmotic stress on isolated gill and mantle tissues of the clam and it may be concluded that the primary mechanism of cell volume regulation under these conditions is an efflux of FAA into the surrounding medium. Since the change in the tissue water content in both cases was much smaller than would be expected based on the changes in the external osmotic pressure, it may be suggested that cell volume regulation indeed occurred and is associated with amino acid efflux. There is no evidence of concomitant changes in glycolytic rate or energy status needed for this process. Both gill and mantle tissues excreted FAA intact, although it was thought that mantle, as a gluconeogenic tissue, and gill, as a site of ammonia excretion, might be prime candidates for sites of deamination and amino acid degradation. The results suggest that another organ serves as the site of deamination or possibly, as suggested above, the deamination process may only be initiated during later stages of adaptation to low salinity.

Hyperosmotic stress

Various aspects of amino acid-mediated volume regulation during hyperosmotic stress have been studied in a variety of bivalve species over the years, yet the details of the biochemical control mechanisms underlying the volume regulatory process have not been completely demonstrated in any system. The objective of the continuing study in this thesis was an elucidation of the metabolic mechanisms regulating the concentrations of the amino acids in isolated gill and mantle tissues of M. mercenaria in response to hyperosmotic stress.

Under hyperosmotic conditions, the total FAA (minus taurine) pool rapidly increased in both gill and mantle tissues of M. mercenaria. Alanine and, to a lesser degree, glycine were significant osmotic effectors. These amino acids, particularly alanine, have been shown to be the major osmotic effectors of R. cuneata adductor muscle (Henry et al., 1980) and isolated ventricles and gills of M. demissus (Baginski and Pierce, 1977). However, taurine appears to be the major osmotic solute utilized in isolated gill tissues of M. mercenaria. Taurine content of gill rapidly increased during aerobic hyperosmotic stress (in both Experiments 2 and 4). Mantle taurine content behaved differently, however, increasing slightly in Experiment 4 but decreasing in Experiment 2. The reasons for this are not known; however one factor may be a large individual variation in tissue taurine levels as has been shown in previous studies

(Shumway et al., 1977; Powell et al., 1982). Taurine is certainly not excreted into the incubation medium as it is during hypoosmotic stress. Overall, gill tissues showed a stronger response to a 4 h hyperosmotic stress as the total FAA (less taurine) content increased by 85% and taurine by 45% while FAA (less taurine) content increased by only 40% in mantle tissues. In both gill and mantle tissues the initial response (0-2 h) to high salinity was accumulation of intracellular alanine. Perhaps, in the isolated gills, subsequent to the rapid alanine increase, the slower accumulation of taurine continues which is the pattern of FAA that has been found in a number of bivalves (Lange, 1963; Du Paul and Webb, 1970; Baginski and Pierce, 1977). Therefore, it may be suggested, for isolated gill, that alanine provides the main osmotic solute during short term salinity stress and taurine serves as the major osmotic solute for long term salinity acclimation. Perhaps gill, as a primary tissue in contact with changing salinity in the natural environment, is set up to respond very rapidly to salinity change, inducing mechanisms for endogenous synthesis or production of taurine. Mantle may have the capacity for the synthesis of amino acids derived from glycolysis, alanine and glycine, but may not be able to synthesize its own taurine or may only induce taurine production at a much later stage in its adaptation to high salinity. Similarly, Baginski and Pierce (1977) found that mantle tissue of M. demissus showed a different pattern of

amino acid accumulation from gill tissue and that the increase in total amino acid pool of gills occurred much faster than the increase in the mantles. Initially alanine concentration increased rapidly followed by a slower accumulation of glycine and taurine in gill tissues whereas although alanine accumulated first, followed by glycine, taurine did not increase in mantle tissues.

The results obtained here, as well as other data (Baginski and Pierce, 1977; Matsushima, 1982) indicate that the source of alanine, glycine and taurine must be from an alteration in intracellular metabolism since accumulation occurs in isolated tissues incubated in solutions free of amino acids. Negligible amounts of FAA accumulated in the hypersaline media of isolated gill and mantle tissues. Thus, the volume regulatory increase in FAA in these M. mercenaria tissues during high salinity stress required not only intracellular biosynthetic mechanisms to produce the FAA, but also a strongly inhibited FAA efflux. Intracellular free amino acids appear to be synthesized by aerobic metabolic processes as; 1) succinate, an anaerobic end product, does not accumulate; 2) aspartate content is not reduced; and 3) the energy charge remains high.

Both the sources of the amino group and the carbon skeleton must be known before the mechanism of FAA accumulation can be elucidated. It has been postulated that the carbon skeletons for FAA synthesis during hyperosmotic stress are derived from degradation of glycogen or glucose

(de Zwaan and Marrewijk, 1973; Baginski and Pierce, 1978; Henry et al., 1980). In the present study, glycogen reserves of isolated gills were not degraded and even if the total glycogen reserve had been mobilized there would not have been sufficient carbon produced to account for the increase in taurine ($\sim 43 \mu\text{mol/g}$ wet wt in glucose equivalents). An alternate carbon source for de novo synthesis of taurine is therefore required or alternately taurine must be derived from some other cellular reserve. Despite the widespread occurrence of taurine in marine invertebrates, little is known about its biosynthesis in species of most invertebrate phyla. The source of sulphur groups must also be determined and the invertebrate mucus has been suggested to be the source as it is known to have a high sulphate anion content (Powell et al., 1982). Glycogen and glucose pools were reduced in hyperosmotically stressed mantles and could account for the observed increase in FAA (minus taurine). Activation of glycolysis at the PK locus in mantle also indicates that carbohydrate metabolism is responsible for the increase in FAA. This activation of PK appears to be, from the kinetic studies, of an allosteric nature rather than due to covalent modification.

The amino groups required for FAA synthesis could be derived from ammonia fixation or from protein degradation and subsequent transamination. Two problems arise with respect to the first hypothesis; 1) the source of ammonia for the initial reductive amination is unknown and 2) the

pathway by which ammonia fixation takes place is unknown. Henry et al. (1980) suggest that much of the ammonia required for the initial reductive amination could be produced in normal nitrogen metabolism and that fixation of toxic ammonia into amino acids may be indicated by a much diminished ammonia excretion (Matsushima, 1982). The ammonia content of the isolated gill and mantle tissues does not provide sufficient nitrogen to account for the increase in FAA, hence the results of the present study do not clearly indicate that ammonia fixation is indeed occurring. The existence of a glutamate dehydrogenase/glutamate-pyruvate transaminase complex controlling alanine (amino acid) accumulation during high salinity exposure in the present study does not appear to be in evidence as the precursor, glutamate, does not accumulate. The role of protein hydrolysis in enlarging the FAA pool has not been fully evaluated and the available information on intracellular protein provides no evidence in support of this hypothesis (Henry et al., 1980). In addition, the changes in the protein content required to explain the observed increases in FAA are only a fraction of the pool size.

During high salinity stress, accumulation of intracellular FAA as osmotic solutes appears to be the mechanism responsible for cell volume regulation in both isolated gill and mantle tissues of M. mercenaria. Since the amount of water in the tissues did not change

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significantly compared to the magnitude of change of the external salinity, it appears that cell volume regulation indeed occurred in both gill and mantle tissues. However, exposure to hyperosmotic stress invokes a stronger response from gill tissues than mantle tissues, particularly with respect to endogenous taurine production. Mantle may not have the capacity to synthesize its own taurine or may require a longer exposure to high salinity to induce taurine synthesis. Alanine and glycine are likely derived from carbohydrate degradation in both tissues. This is particularly apparent in mantle tissue where glycolysis is activated at the PK locus. Research to date, including this study, has not determined the source of amino groups required to account for the FAA increase although the source is obviously endogenous. The process of FAA accumulation appears to be aerobic as alanine formation is not linked to succinate production.

From the salinity experiments (1 and 2) it is apparent that free intracellular amino acids play an important part in the regulation of the osmolarity of the intracellular medium. Amino acids and taurine account for 17% of the total osmolarity in the gill and for 10% in the mantle of M. mercenaria. Under hyposaline and hypersaline conditions the contribution of the FAA pool to the intracellular osmolarity of isolated gills remains constant. That is, when the osmolarity of the incubation medium was decreased or increased by 50% the levels of FAA decreased or increased by

50%, respectively, therefore the FAA pool maintained the same proportion of the total osmolarity in the gill. Similarly, there were no significant alterations in the contribution of FAA to the total osmolarity in the mantle under hypoosmotic conditions.

Anoxia

Succinate and alanine were the major products of anaerobic metabolism in the isolated gill and mantle tissues of M. mercenaria as they are in these and other tissues of the whole animal under anoxia (Korycan and Storey, 1983). These end products have also been demonstrated to accumulate in several other bivalve species such as the oyster, Crassostrea gigas (Collicutt and Hochachka, 1977), the sea mussel, M. edulis (Zurburg and Kluytmans, 1980) and the clam R. cuneata (Stokes and Awapara, 1968). Succinate accumulated linearly to high levels in both gill and mantle tissues and their respective incubation media over the course of anaerobic exposure. The accumulation of alanine, however, occurred within the first 2 h of anoxia to a new steady state level. Succinate was the major anaerobic end product in these tissues which differs from the oyster and sea mussel which accumulate more alanine than succinate under most circumstances (Collicutt and Hochachka, 1977; Eberlee et al., 1983; de Zwaan et al., 1982). Hochachka et al. (1973) suggested that alanine accumulation is an ultimate condition for the generation of NADH which serves

to reduce fumarate into succinate and this is characterized by a 1:1 ratio of succinate to alanine accumulation. The present data does not support this proposal as the ratio of succinate:alanine accumulation varies between tissues (for example, gill- 2.5:1; mantle- 7.3:1). The NADH oxidized in the formation of succinate may be generated by a still operating TCA cycle (Zurburg, 1981).

Anoxic isolated gill and mantle tissues exported succinate to the external incubation media. Similarly, succinate was released by tissues in vivo into the mantle cavity fluid (Korycan and Storey, 1983). The formation of propionate, from the further catabolism of succinate, was not tested in the present study but is unlikely to occur, Kluytmans et al. (1983) having shown that Mediterranean Venus species (M. mercenaria formerly known as V. mercenaria) do not produce propionate from accumulated succinate. Succinate appears to be the terminal organic acid produced by M. mercenaria tissues and is excreted from cells. When considering the whole animal, succinate in the extracellular fluid can be buffered during anoxia by calcium derived from the shell (Crenshaw and Neff, 1969).

It is generally accepted that the main fuel substrate in bivalves under anaerobic conditions is glycogen. Direct measurement of changes in concentration of the substrate glycogen is difficult after short periods of anaerobiosis because the amount consumed is only a very small fraction of the total pool. Glycogen consumption, however, was noted

for the anoxic gills providing sufficient carbon to account for the synthesis of the anaerobic end products. Aspartic acid was also found to be utilized by these isolated tissues during anaerobiosis. The role of aspartic acid as an additional fuel during anaerobiosis has been previously reported for M. mercenaria (whole animal study) (Korycan and Storey, 1983) and for other bivalve species (Collicutt and Hochachka, 1977; Zurburg and Kluytmans, 1980; Zurburg and Ebberink, 1981; Eberlee et al., 1983). The aspartate content of both tissues declined rapidly within the first 2 h of anoxia. The catabolism of aspartate probably follows the route given in Figure 1 and as described by Collicutt and Hochachka (1977). Aspartate provides the carbon skeleton for succinate synthesis and the amino group is used in alanine formation via a transaminase. Once aspartate levels become depleted, further alanine formation is minimal and succinate carbon is then derived from glycogen.

The isolated gill and mantle tissues in the present study provide a closed system in which to study the interconversion between metabolites. Whole animal studies (Collicutt and Hochachka, 1977; Korycan and Storey, 1983) have suggested that in the in vivo experiments aspartate and glucose might be taken up from the blood and, therefore, that intertissue transport of substrates via the blood may contribute to anoxic energy production. The present in vitro study indicates that gill and mantle tissues have the capacity to respond individually to anoxic stress not only

in terms of possessing sufficient endogenous substrates to maintain anaerobic metabolism but also in terms of the control of metabolic processes as hormonal and nervous control are not involved. However, intertissue transport of substrates and hormonal control mechanisms may play a key role in the coordination and regulation of anaerobiosis between tissues in the whole animal.

From the changes in concentrations of glycolytic intermediates and the crossover plots (for example, gill; Figure 19) it is concluded that phosphofructokinase and pyruvate kinase control the carbon flow during anaerobic metabolism in isolated gill and mantle tissues of M. mercenaria. Glycogenolysis does not appear to be activated in short term anoxia as 1) glucose-6-P did not significantly accumulate and 2) activation of glycogen phosphorylase (conversion of phosphorylase b to a by covalent modification) did not occur. The increasing levels of AMP, however, could activate glycogen phosphorylase b to a small extent.

Elevated levels of fructose-1,6-P₂, the product of phosphofructokinase, indicated a facilitation of this reaction during the period of anaerobic metabolism. PFK may be activated during anoxia by 1) the relief of the inhibition by high arginine-P concentrations (Storey, 1976; Storey, 1981), 2) reduction of arginine-P inhibition by lowered pH, 3) the relief of inhibition by high ATP concentrations and 4) increasing ADP and AMP (activators)

FIGURE 19: Crossover plot of the levels of glycolytic intermediates of isolated gills of M. mercenaria under anoxic conditions. The levels of glycolytic intermediates of gills at time zero (pre-incubation) serve as the 100% level.

CROSSOVER PLOT

levels. Hence, the decreasing ATP and arginine-P levels that accompany the onset of anaerobiosis as has been found in the present study would therefore increase the activity of PFK and speed up glycolysis. During burst activity, skeletal muscle becomes anoxic and the anaerobic energy metabolism is entirely glycogen based with lactate as the sole end product (classical glycolysis). Metabolism during short time anaerobiosis such as vigorous muscle activity can be characterized as having a low efficiency (reduced ATP yield) but a high rate of energy production in order to meet the increased energy demand. The higher glycolytic flux in working, vertebrate muscles exhibiting a much higher carbohydrate consumption rate (a Pasteur effect) is caused by activation of PFK. However, various authors point to the fact that most molluscs fail to show a clear Pasteur effect during anaerobiosis (Gade, 1975; de Zwaan, 1977; Ebberink and de Zwaan, 1980) and that the absence of a Pasteur effect is due to the alteration of the glycolytic pathway in combination with the strong reduction in energy demand. Anaerobic metabolism in bivalves may be characterized as having a relatively high efficiency but low rate of energy production. In other words, because of the reduction in overall metabolism in bivalves, utilization and production of substrates and end products, respectively, are low and a Pasteur effect need not occur. On the other hand, Gade (1983) suggests that the apparent absence of a Pasteur effect may be due to lack of good measurements because the

consumption of carbohydrates is only a fraction of the pool size. Furthermore, Livingstone and Bayne (1977) and Ebberink (1980) suggest that during the first hours of anoxia a Pasteur effect can be observed. Famme et al. (1981) recently determined that anaerobic heat production of M. edulis increased at low oxygen partial pressures indicating that there may indeed be some activation of glycolysis. The present data, however, indicate that an activation of glycolysis at the PFK locus indeed occurred in isolated gill and mantle tissues during anaerobiosis.

Data reported by Ebberink (1980) on the posterior adductor muscle of M. edulis has produced a number of anomalies, 1) the presence of a Pasteur effect during the first hours of anaerobiosis (Ebberink et al., 1979), 2) the concentrations of a number of modulators of PFK (adenine nucleotides, inorganic phosphate, arginine-P and citrate) change in such a way that an increase in glycolysis would be expected (Ebberink and de Zwaan, 1980), 3) based on the changes in concentrations of the glycolytic intermediates it was concluded that PFK is inhibited during the first hours after valve closure (Ebberink and de Zwaan, 1980) and 4) to explain the absence of a Pasteur effect properties of PFK were investigated and it was found that PFK activity was kept low by the combined effect of an increased inhibition by ATP due to a decrease in pH during valve closure and the inhibition by the increased PEP concentration (Ebberink, 1982). Kinetic studies of PFK from the gill and mantle

tissues of M. mercenaria would reveal useful information perhaps explaining the differences observed with respect to regulation of the PFK locus during anoxia in isolated tissues.

Pyruvate kinase from isolated gill and mantle tissues of M. mercenaria appears to be inactivated in anoxia. PK has been established as an allosteric enzyme and is modulated by the energy charge, fructose-1,6-P₂, alanine and pH (Holwerda and de Zwaan, 1973; Livingstone and Bayne, 1974). This enzyme is also regulated by phosphorylation/dephosphorylation mechanisms in other marine molluscs (Plaxton and Storey, 1984) but this did not appear to occur in the present study. The inhibition of pyruvate kinase in isolated gill and mantle tissues during anaerobiosis appears to play a key role in the redirection of carbon flow at the PEP branchpoint.

From the data reported in Experiment 3, anaerobiosis may follow the route suggested by Zurburg and Ebberink (1981) where two phases of anaerobic metabolism were postulated (Figure 20) involving the PEP branchpoint. In the first hours of anaerobiosis the formation of succinate was achieved by the catabolism of aspartate reserves; alanine production arises from glycogen catabolism (Figure 20A). ATP and arginine-P reserves of the tissues are also depleted as an additional energy supply. It was proposed that the importance of the aspartate utilization was the bypass of the PEPCK reaction and therefore it allows the extra

FIGURE 20 A: Scheme of carbon flow during the
first hours of anaerobiosis.

B: Scheme of carbon flow after longer
periods of anaerobiosis.

(Zurburg and Ebberink, 1981).

A.

B.

energy-yielding reaction (via fumarate reductase) to operate without delay (Gade, 1983). Later when aspartate reserves can no longer be depleted PEPCK activity gradually increases and PK activity is reduced (as indicated by rising PEP levels) and succinate will be formed from glycogen via the fixation of CO_2 to PEP (Figure 20B). Regulation of PK at the PEP branchpoint in the isolated gill and mantle tissues appears to be of an allosteric nature and the inhibition of this enzyme is likely due to increasing alanine concentration, decreasing pH and reduced energy charge. The enhanced levels of PEP and CO_2 (Gade, 1983) and the lower pH will positively influence PEPCK activity. The continued rise in alanine in isolated mantles over the anoxic period and the continued aspartate decrease may indicate that some flux through the PK locus continues unlike in gill tissues. It is obvious from the experiments on isolated tissues reported above that a hormonal control mechanism is not required for the regulation of anaerobic metabolism. Such a process, however, might be involved in the whole animal adaptation to anoxic conditions.

Hyperosmotic stress under anoxic conditions

Baginski and Pierce (1975) found that the rate of oxygen consumption of isolated ventricles from M. demissus decreased during high salinity and suggested that the FAA pool is enlarged by anaerobic mechanisms. Later, they modified this original assumption from the evidence of a

difference in the pattern of end product accumulation and in the metabolic alterations during anaerobiosis and hyperosmotic stress regulation (Baginski and Pierce, 1978). However, Henry et al. (1980) who measured the changes in the FAA pool during high salinity adaptation under anaerobic conditions in R. cuneata showed that enlargement of the FAA pool can occur anaerobically in the long term (24-48 h) but not in the short term (0-6 h) and that oxygen is critical to survival during high salinity adjustment. Thus the question of whether the FAA pool is enlarged by anaerobic pathways of metabolism has not been answered. In addition, a number of ~~recent~~ studies suggest that two stresses presented concurrently might superimpose two distinct metabolic phenomena, though possibly modifying each to some extent, resulting in a FAA pattern different from that produced by either one alone (Powell et al., 1982). The relationship between anaerobiosis and high salinity acclimation is explored in this study on isolated tissues of the clam.

Exposure of isolated gill and mantle tissues of M. mercenaria to hyperosmotic stress under anoxic conditions yielded changes in metabolites and glycolytic intermediates that were indicative of adaptations to anoxic stress rather than to hyperosmotic stress or to a combination of the stresses (i.e. additive or synergistic effects). The major metabolic events in glycolysis, activation of PFK and inhibition of PK, that occurred in isolated tissues incubated in 150‰ SW under a nitrogen atmosphere paralleled

those noted for anoxic tissues in 100% SW. It was particularly apparent at the PK locus of isolated mantles that regulation of this enzyme in response to anoxic stress was dominant to regulation in response to hyperosmotic stress; during hyperosmotic stress alone (Experiments 2 and 4) PK is activated while during anoxia stress (Experiment 3) PK is inhibited. The cellular changes in concentrations of amino acids and succinate observed in isolated gill and mantle tissues during exposure to high salinity stress under anaerobic conditions were similar to the changes determined under solely anaerobic conditions. The changes in the energy status of anaerobic gill and mantle tissues in 150% SW paralleled those of the anaerobic tissues in 100% SW. Adaptation to high salinity (Experiments 2 and 4) did not stress the energy state of the tissues. These results suggest that although both conditions induce alanine formation it is unlikely that the pathways and control systems induced are the same. The metabolic response of the tissues indicates that the effects from anoxia stress are predominant. Consequently, there would be a drastic reduction in the energy (ATP) yield per equivalent of fuel and likely a depression of the metabolic rate of the tissues. The results of this study demonstrate that the accumulation of FAA in the isolated tissues in response to hyperosmotic stress was severely limited under anaerobic conditions. Therefore, as adaptation to high salinity by enlarging the intracellular FAA pool of these tissues cannot

be carried out anaerobically and as the catabolic output of ATP is dramatically reduced, it may be suggested that the production of FAA and taurine has a high energy requirement.

When exposed to a sinusoidal salinity fluctuation, M. mercenaria reacts by closing its valves and isolating itself temporarily from the environment (Shumway et al., 1977). The interaction of shell valve closure and volume regulation allows bivalves a period of grace, following a possible transient environmental salinity change, before a physiologically committing acclimation must be made. During valve closure, the animal maintains an anaerobic metabolism which could lead to increases in alanine levels. Shumway et al. (1977) points out that in this context, however, the role of anaerobiosis should not be confused with the suggestion that anaerobic metabolism is the source of osmotic solutes (mainly alanine) during high salinity acclimation of marine bivalves. After a period of valve closure, the animal interacts with the environment for food, oxygen and waste removal (Pierce, 1971). At this time, aerobic metabolism is resumed and the animal then regulates cell volume by extensive alterations in the intracellular concentrations of FAA (Hoyaux et al., 1976).

The results of this study indicate that the biochemical adjustments of the clam tissues to the two stresses, hyperosmotic and anoxia, are different with anoxia stress eliciting the primary metabolic response. Although different from the response to anaerobiosis, the mechanism

of intracellular FAA accumulation during the initial stages of high salinity acclimation also depends on an alteration of the intracellular metabolism.

Summary

Marine invertebrates of the intertidal zone are adapted behaviourally, physiologically and biochemically to wide-ranging external conditions and their survival in such circumstances is dependent on the breadth and flexibility of response. Biochemical adaptations are a key requirement for adaptation to any environment. This thesis has outlined certain of the major adaptive patterns or metabolic 'strategies' which appear most important in tailoring the biochemistry of the clam, M. mercenaria, to function well under the particular environmental conditions it experiences. The set of experiments, herein, gives a survey of the changes in the biochemical composition and energy metabolism of isolated gill and mantle tissues of M. mercenaria during exposure to environmental stresses and the results of these investigations allow us to draw certain conclusions about the biochemical adaptations.

1) Hypoosmotic stress:

Free amino acid excretion, as a mechanism of cell volume regulation, with no alteration in intermediary metabolism appears to be the major response.

2) Hyperosmotic stress:

Accumulation of FAA as osmotic solutes appears to be due to an alteration of intracellular aerobic metabolic processes. Activation of PK in isolated mantles indicates that carbohydrate metabolism is responsible, at least in part, for the increase in FAA. This activation of PK appears to be of an allosteric nature rather than due to covalent modification.

3) Anoxia:

Utilization of glycogen and aspartate as anaerobic substrates and accumulation of the anaerobic end products, succinate and alanine changes over time during anaerobiosis. PFK and PK control the carbon flow during anaerobic metabolism. A relief of inhibition due to decreasing ATP and arginine-P levels may be the key factor increasing the activity of PFK and speeding up glycolysis. Carbon flow from glycogen is directed to succinate formation at the PEP branchpoint due to inhibition of PK. Kinetic studies indicate that this inhibition of PK is not due to covalent modification but rather due to changes in allosteric effectors such as increasing alanine levels, decreasing pH and reduced energy charge.

4) Hyperosmotic stress under anoxic conditions

The FAA pool is not enlarged during hyperosmotic stress by anaerobic metabolism. The changes in glycolysis are

typical of the isolated tissue metabolic response to anoxia stress. .

The isolated tissue preparations described in this thesis have proved to be robust and convenient to use under a variety of experimental conditions and have served very well in these metabolic investigations. Perhaps further metabolic and enzymic investigations with selected isotopes, inhibitors and hormones using such tissue preparations may provide answers to some of the additional questions that are raised by this thesis and are to date unresolved.

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