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**THE EFFECT OF SEASONAL CHANGE AND PROLONGED ANOXIA
ON METABOLIC ENZYMES OF MARINE MOLLUSCS**

by

Steven Clive Greenway, B Sc.

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
Carleton University
Ottawa, Ontario

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**"THE EFFECT OF SEASONAL CHANGE AND PROLONGED ANOXIA
ON METABOLIC ENZYMES OF MARINE MOLLUSCS"**

submitted by
Steven Clive Greenway, B.Sc.

in partial fulfillment of the requirements for
the degree of Master of Science

Chair, Department of Biology

Thesis Supervisor

Carleton University
1995

ABSTRACT

This thesis investigated the effects of seasonal change (summer versus winter) and prolonged anoxia on the activities of 20 different metabolic enzymes and the kinetic properties of phosphofructokinase (PFK) and pyruvate kinase (PK) from the tissues of two marine molluscs, the periwinkle *Littorina littorea* and the oyster *Crassostrea virginica*. Seasonal change and prolonged anoxia induced alterations in enzyme activities which were animal- and tissue-specific. PFK displayed only minor seasonal variation but PK was present in seasonal forms. Adaptation to anoxia was seasonally dependent with changes in enzyme properties indicating a more effective depression of metabolic rate in response to anoxia during the winter. In both molluscs PK was the key regulatory enzyme of glycolysis and in *C. virginica* anoxia induced a drastic decrease in the activity of this enzyme via a dephosphorylation reaction.

ACKNOWLEDGMENTS

I must first thank Ken Storey for his guidance and advice during the past two years. Thanks also to Jan Storey for her constructive criticisms and insights. Thanks to everyone in the Storey lab (past and present), I know that I learned something from each of you. Computer programs supplied by Dr. Stephen Brooks were invaluable in completing this work. Finally, thanks to my family and friends for their support at a distance.

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LIST OF ABBREVIATIONS

AK	Adenylate kinase
AlaAT	Alanine aminotransferase
ArgK	Arginine kinase
AspAT	Aspartate aminotransferase
ATP, ADP, AMP	Adenosine tri-, di- and mono-phosphate
DHAP	Dihydroxyacetone phosphate
EDTA	Ethylenediamine tetraacetate
EGTA	Ethyleneglycol bis(β -aminoethyl ether) tetraacetate
F1,6P ₂	Fructose-1,6-bisphosphate
F2,6P ₂	Fructose-2,6-bisphosphate
F6P	Fructose-6-phosphate
FBPase	Fructose-1,6-bisphosphatase
G1P	Glucose-1-phosphate
G3PDH	Glycerol-3-phosphate dehydrogenase
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GAP	Glyceraldehyde-3-phosphate
GP	Glycogen phosphorylase
GP _a	Glycogen phosphorylase <u>a</u>
GP _b	Glycogen phosphorylase <u>b</u>
GS	Glycogen synthetase

HK	Hexokinase
I ₅₀	Concentration of inhibitor that inhibits the maximal activity of an enzyme by 50%
IU/gww	International units per gram wet weight
K _a	Concentration of activator that produces half-maximal enzyme activity
K _m	Concentration of substrate at which the enzyme reaction proceeds at 50% of its maximal velocity
KP _i	Potassium phosphate
LDH	Lactate dehydrogenase
MDH	Malate dehydrogenase (using NADH as a coenzyme)
MDH-NADP	Malate dehydrogenase (using NADPH as a coenzyme)
ME	Malic enzyme
Mg.ATP	Mg ²⁺ and ATP complex
NAD ⁺ , NADH	oxidized and reduced forms of nicotinamide adenine dinucleotide
NADP ⁺ , NADPH	oxidized and reduced forms of nicotinamide dinucleotide phosphate
NaF	Sodium fluoride
n _H	Hill coefficient
OXA	Oxaloacetate
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
6PGDH	6-Phosphogluconate dehydrogenase
PFK	Phosphofructo-1-kinase

PFK-2	Phosphofructo-2-kinase
PGM	Phosphoglucomutase
P _i	Inorganic phosphate
PK	Pyruvate kinase
PMSF	Phenylmethylsulfonyl fluoride
S _{0.5}	Concentration of substrate at which the enzyme reaction proceeds at 50% of its maximal velocity
SDH	Serine dehydratase
TCA Cycle	Tricarboxylic acid cycle
TPI	Triose phosphate isomerase
UDPG	Uridine diphosphate glucose
V _{max}	maximal enzyme velocity

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INTRODUCTION

Part I) SEASONAL CHANGES IN METABOLISM OF MARINE MOLLUSCS

An organism obtains energy for its metabolic processes from the hydrolysis of ATP to ADP. Since ATP is not stored within the cell it must be continually resynthesized via processes of fuel oxidation to satisfy immediate energy demands. These demands and the capacity for ATP production are altered in many animals and plants for extended periods of time due to seasonal variations in environmental factors (i.e. temperature, nutrient availability, salinity), reproductive requirements, and behavioural changes (Newsholme and Crabtree, 1986). Both of the experimental animals used in this study, the marine oyster *Crassostrea virginica* and the marine periwinkle *Littorina littorea*, are subjected to wide variations in several environmental parameters and also undergo metabolic reorganization associated with reproduction. By synchronizing modifications in the metabolic pathways with the progression of the seasons organisms are able to maximize their benefits during favorable conditions and survive when conditions become unfavorable. Seasonal changes occur in the metabolism of carbohydrate, lipid, nucleic acids, amino acids, and protein in marine molluscs, as well as in anaerobic metabolism and respiration rates (Gabbott, 1983).

Seasonal changes in metabolism are often closely linked to the reproductive cycle; for example, as demonstrated by changes in the levels of stored glycogen over the course of a year in tissues of marine molluscs. Although glycogen is an important energy reserve for all molluscs, the cycle of glycogen utilization may be coincident with the gametogenic cycle (e.g. *C. virginica*; Shumway, 1994), clearly separated (e.g. *Mytilus edulis*; Gabbott, 1983), or the energy for gametogenesis may come from both stored reserves and ingested nutrients (e.g. *Placopecten magellanicus*; Gabbott, 1983). In *C. virginica* glycogen is used as an energy source for gonad development and spawning. Glycogen reserves accumulate following spawning in the spring and reach their maximum in late autumn but

accumulation may continue during the winter; the process of glycogen synthesis can be rapid or can occur gradually over several months (Galtsoff, 1964). Glycogen levels remain high until May and then glycogen is rapidly consumed during the proliferation of the sex cells and is at a minimum by the end of the reproductive cycle (Galtsoff, 1964; Shumway, 1994). In general the lowest content of glycogen occurs during the summer and the length of low glycogen levels depends on the length of the reproductive period with oysters in warmer waters (which show a longer reproductive cycle) having lower levels of glycogen for a longer period of time (Galtsoff, 1964). This reproductive pattern of oysters contrasts with that of *M. edulis* which shows high levels of stored glycogen during the summer following spring spawning whereas glycogen content is at a minimum during the winter (Gabbott, 1983).

The pattern of carbohydrate and lipid accumulation and utilization in the gastropod *L. littorea* is slightly different from that seen for the bivalve *C. virginica*. The periwinkle displays an inverse relationship between growth and gonad development. Following spawning in May growth occurs during the summer and early autumn but ceases from October/November until April. However, once growth and feeding have stopped maturation of the gonads occurs from December until April (Williams, 1970). From May to September *L. littorea* actively feed and excess nutrients accumulate as lipid and carbohydrate reserves which show large increases from July-September. Carbohydrate levels may begin to decline before lipid levels as they are preferentially used to support activity during feeding. Once feeding stopped lipid and carbohydrate reserves were gradually used up over the winter and levels were low from February till June (Williams, 1970).

A correlation has been demonstrated between the rate of respiration, which is indicative of overall metabolic rate, and the cycle of gametogenesis in molluscs. The gametogenic resting phase of *C. virginica* occurs during the winter (Bayne et al., 1976) and, accordingly, the respiration rate is lowest in the active tissues during the winter.

When respiration rates of excised tissues from *C. virginica* collected in July and November were measured *in vitro* at a constant 20°C gill and mantle exhibited significantly decreased respiration rates during the winter months (Percy *et al.*, 1970). This seasonal depression of respiration in isolated tissues was qualitatively the same as that seen for intact oysters (Galtsoff, 1964) indicating, therefore, that metabolic depression was a property of individual cells and tissues and not the result of decreased ventilation or nervous control (Percy *et al.*, 1970). These observations seem to indicate a "hardwired" adaptation to environmental change that involves a hibernation effect in the more active tissues, indicated by the depressed metabolic rate during the colder months (Percy *et al.*, 1970). However, in *M. edulis*, respiration rates are higher in the winter and lower in the summer (Bayne *et al.*, 1976). In the sea mussel, however, gametogenesis is in resting phase during the summer but active in the winter with consumption of glycogen stores (Bayne *et al.* 1976). The correlation between respiration rate and gametogenesis indicates that the gametogenic cycle, directly or indirectly, may determine the seasonal metabolic pattern (Bayne *et al.*, 1976). Since these metabolic changes can be quite large (e.g. in *M. edulis* mantle glycogen reserves change by 10-fold over the year) there must be correspondingly large seasonal changes in flux through various metabolic pathways (Livingstone, 1981).

Reactions in a metabolic pathway can be categorized as being either near-equilibrium or nonequilibrium reactions. The forward and reverse rates of near-equilibrium reactions are much greater than the overall flux through the pathway whereas the forward rate of a nonequilibrium reaction is much greater than the reverse reaction rate (Newsholme and Crabtree, 1986). Regulatory enzymes catalyzing nonequilibrium reactions will naturally be important loci of control in an animal's adaptation to seasonal change due to their role in limiting and directing metabolic flux. Seasonal alterations in the activities of regulatory enzymes could be accomplished by; (1) quantitative changes in the amount of enzyme present via changes in the processes of synthesis and degradation, (2)

production of molecular variants of the enzymes (isozymes) possessing more appropriate kinetic properties, or (3) post-translational modification (i.e. protein phosphorylation or dephosphorylation) (Livingstone, 1981; Storey, 1992a). Which regulatory mechanism used will depend on other limiting factors of metabolism such as the concentration of substrates and the substrate affinities of enzymes. For example, the concentration of glucose-6-P (G6P) greatly exceeds the K_m of glucose-6-phosphate dehydrogenase (G6PDH) for this substrate; therefore, the enzyme is substrate-saturated and increases in enzyme activity are likely effected by changes in the quantity of enzyme (Livingstone, 1981). Seasonal changes in enzyme function are more likely to occur by these three mechanisms than via control by the production of small molecular effectors since seasonal changes in flux occur over months and require long-term changes in control as opposed to limited, short-term regulation (Livingstone, 1981). In general, "coarse" control over metabolism for acclimatization to seasonal change is brought about by changes in the synthesis of regulatory enzymes and "fine" control over daily fluctuations is provided through the action of regulatory molecules (Livingstone, 1981; Gabbott, 1983).

Part II) ANAEROBIC METABOLISM IN MARINE MOLLUSCS

Molluscs that live in the intertidal zone of the world's oceans, such as *L. littorea*, are regularly exposed to large fluctuations in oxygen availability, salinity, and temperature with the ebb and flow of the tides. Although *C. virginica* live in fairly shallow but subtidal waters they, like other bottom dwellers (e.g. whelks), possess excellent anoxia tolerance. For oysters, environmental oxygen availability can vary due to silting or summer eutrophication and, in addition, these animals can voluntarily close their valves in response to environmental insults (e.g. salinity change, effluent run-off) or when winter water temperatures fall below about 10°C. Thus, both of these gill-breathing animals must be able to tolerate prolonged periods of oxygen deprivation. The oyster and periwinkle, like many other marine molluscs, have developed several key biochemical adaptations that make them excellent facultative anaerobes including. (1) high levels of tissue glycogen, (2) use of alternative fermentative pathways to increase ATP yield, (3) enhanced buffering capacity of body fluids to minimize metabolic acidosis, and (4) metabolic rate depression (Hochachka and Somero, 1984; Storey and Storey, 1990). Of these adaptations, metabolic rate depression has the most significant impact on long-term survival.

For a facultative anaerobe, oxygen consumption decreases as oxygen availability decreases but an inverse response by fermentative glycolysis can maintain normal ATP production over a wide range of oxygen tensions. However, if pO_2 falls below a critical minimum, a strong reduction in metabolic rate is initiated which serves to ration the supply of metabolic fuel and reduce energy demand to maximize anaerobic survival time (Storey and Storey, 1990). This situation is dramatically different from the response of an oxygen conformer to decreasing pO_2 . These animals lack the metabolic depression response and when O_2 levels drop below the critical minimum, their only response is an attempt to compensate for the loss of ATP production by oxidative phosphorylation by greatly increasing fermentative ATP production. The large activation of glycolysis that results is known as the Pasteur effect but in most animals this fermentative ATP output cannot

nearly match the unrestricted rate of cellular ATP use and hence, cellular energetics are rapidly compromised. Facultative anaerobes do not show a Pasteur effect; indeed, the reverse effect is seen, low oxygen stimulating a reduction in the rate of glycolysis and of all other metabolic functions (Storey, 1985a). Thus, during anoxia, intertidal marine invertebrates depress overall metabolic rate to only 5-10% of the normal aerobic resting rate at the same temperature (as measured by calorimetry) (Famme *et al.*, 1981; Shick *et al.*, 1983) so that energy requirements can be satisfied by the ATP production of fermentative pathways alone. This 10-20 fold decrease in metabolic rate results in a stable, viable, hypometabolic state during anoxia that also extends the anoxic survival time by the same 10 to 20-fold factor (Storey and Storey, 1990). However, during this time, energy can only come from endogenous supplies and the organism must be able to tolerate the accumulation of metabolic end-products in body fluids (Hochachka and Somero, 1984; Storey and Storey, 1990).

While enduring anoxia physical activities such as movement, feeding and digestion, heart rate, and respiration are minimized or cease altogether and the animals remove themselves as much as possible from the environment (e.g. closing the valves or withdrawing into the shell) so as to avoid or limit the impact of changes in environmental conditions (Storey and Storey, 1990). These physical mechanisms contribute to the depressed state but the most important controls occur at the cellular level. In order to accomplish a sustained metabolic suppression, a new balance must be struck between the few reactions that generate ATP and the multitude of reactions that use ATP. Limiting the availability of ATP and pathway precursors helps to regulate ATP-utilizing reactions but there must also be regulation at the point of entry into complicated, energetically-expensive, biosynthetic pathways. Furthermore, ATP-independent functions that impact upon net ATP use must also be suppressed; e.g. the rate of facilitated diffusion of ions through membrane ion channels must be reduced and matched with the opposing rates of ATP-dependent ion pumps (Storey and Storey, 1990). A new, lower, steady-state

metabolic rate must be established and this involves a general reduction of all cellular processes as well as the initiation of selected adjustments specific to the depressed state. In order to establish this new state, intracellular regulatory mechanisms must be able to respond to extracellular signals and be able to induce widespread, readily reversible, coordinated changes to metabolic processes throughout the cell. The activities of key regulatory enzymes or functional proteins in the cell are suppressed in a coordinated fashion to effect a reduction in the activity of the processes they control. Several mechanisms for regulating glycolytic rate depression have been identified and are found not only in marine molluscs responding to environmental anoxia but also in other animals that depress their metabolic rate under environmental stress (e.g. hibernating mammals, anoxia-tolerant turtles, estivating snails and toads). These mechanisms include: (1) covalent modification of enzymes by reversible protein phosphorylation, (2) allosteric regulation of key enzymes (e.g. suppression of fructose-2,6-bisphosphate [F2,6P₂] levels to reduce phosphofructokinase [PFK] activity), and (3) reversible association of enzymes with subcellular structures (Storey and Storey, 1990; Storey, 1992b).

Post-translational modification, via the addition or removal of covalently bound phosphate from proteins, is one of the fastest and most powerful mechanisms available for changing the activity of an enzyme, and thereby the flux through a pathway. Frequently, reversible phosphorylation effectively switches an enzyme between active and inactive states. The easy reversibility of this mechanism is also important to allow a rapid return to normal metabolism once the stress has been removed. The identification of reversible phosphorylation as a key mechanism of stress-induced metabolic suppression began, in fact, with studies of anoxia tolerance in marine molluscs. Siebenaller (1979) first demonstrated that two separable forms of pyruvate kinase (PK) in the adductor muscle of *M. edulis* were interconvertible *in vitro* by a cAMP-dependent protein kinase and that the proportions of the two forms varied *in vivo* in response to anoxia exposure or temperature change. Initial studies focused on reversible phosphorylation control of PK as the means of

regulating phosphoenolpyruvate (PEP) use by aerobic (via PK) versus anaerobic (via PEP-carboxykinase) pathways of carbohydrate catabolism but subsequent research soon showed that the activities of several enzymes of carbohydrate catabolism were regulated in this fashion during anoxia. For example, anoxia induces changes in the phosphorylation state of GP (Storey, 1988; Brooks *et al.*, 1991), PFK (Storey, 1984; Brooks *et al.*, 1991; Michaelidis and Storey, 1991), PFK-2 (Bosca and Storey 1991), and PK (Plaxton and Storey, 1984a, 1984b; Holwerda *et al.*, 1989; Gaitanaki *et al.*, 1990) in marine molluscs.

As opposed to the limited role of allosteric effectors in adaptations to seasonal variations there are numerous metabolites in the cell that are able to activate or inhibit key enzymes during the adaptation to the anaerobic state. For example, alanine control of PK and F2,6P₂ control of PFK are well known for anoxia in molluscs. Indeed, the coupling of covalent modification and metabolite changes can effectively shut off enzymes as seen for PK from *Busycotypus canaliculatum* where the effects of rising alanine levels (resulting from fermentative glycolysis) are enhanced by the phosphorylation of PK which greatly increases the sensitivity of the enzyme towards alanine inhibition (Storey and Storey, 1990).

Metabolic regulation may also occur through the reversible binding of enzymes to the F-actin skeleton of the cell or other macromolecular structures (Storey, 1992a). These associations could function to increase flux through a pathway by optimizing the availability of substrate, positioning sequential enzymes, and directing product to where it is needed and will be rapidly used (Storey, 1992a).

Even with an aggressive depression of metabolic rate a basal level of activity must continue to maintain vital processes but, since the animal is removed from its favored environment, the energy necessary to support the animal must come from an internal reservoir. In the case of marine molluscs the primary endogenous fuel that sustains prolonged anaerobic metabolism is glycogen with aspartate being consumed during the first hours of anoxia. Under aerobic conditions in living systems, energy is produced by the

oxidation (loss of electrons) of organic substrates. The potential energy of the substrate is released stepwise by a series of oxidation-reduction reactions in the electron transport chain with oxygen acting as the terminal electron acceptor. In the absence of oxygen, electron transport chain function is halted but ATP can still be generated from substrate-level phosphorylations linked to the catabolism of carbohydrate; lipids, by contrast, cannot be oxidized except by O_2 -dependent mechanisms (de Zwaan, 1977). Use of most amino acids and proteins is limited by the presence of carboxyl groups and the lack of hydroxyl groups whereas its multiple hydroxyl groups make carbohydrate an excellent storage substrate for coupled oxidoreduction reactions (Livingstone, 1991).

Fermentation reactions are oxidation reactions that produce NADH but they are linked to "substrate-level" phosphorylations of ADP, not to ATP generation by oxidative phosphorylation and it is these reactions which provide the mollusc with energy during anaerobiosis. In order to improve energy yield from the fermentation of endogenous glycogen stores marine invertebrates use alternative pathways other than the classical glucose to lactate fermentation (which yields 2 moles ATP/mole glucose). These energetically improved fermentations include:

- 1) Glucose $\rightarrow$ Imino acid, energy yield 2 moles ATP/mole glucose.
- 2) Glucose $\rightarrow$ Succinate, energy yield 4 moles ATP/mole glucose.
- 3) Glucose $\rightarrow$ Propionate, energy yield 6 moles ATP/mole glucose.
- 4) Aspartate $\rightarrow$ Succinate, energy yield 1 mole ATP/mole glucose.
- 5) Aspartate $\rightarrow$ Propionate, energy yield 2 moles ATP/mole glucose.

(Hochachka and Somero, 1984)

The relative contributions of these pathways during environmental anaerobiosis are animal-, tissue-, and season-specific and, as well, are dependent upon the degree of hypoxia stress. The loss of efficient energy production (a decrease from an aerobic rate of 37 ATP/glucosyl unit to 2-6 ATP/glucosyl unit) is more than compensated for by a strong

reduction in energy demand (metabolic depression) during anoxia and this is why a Pasteur effect or increased rate of glycogen catabolism are not seen (de Zwaan, 1983)

Unlike freshwater species, marine molluscs maintain high concentrations of intracellular free amino acids which contribute to intracellular osmolality which is maintained isosmotic with seawater. Some of these amino acids also participate in energy metabolism, either as fuels (aspartate, sometimes also glutamate) or as end-products (alanine) of anaerobic metabolism. Both the glucose-succinate pathway and aspartate-succinate pathways of fermentation are found in most tissues of marine molluscs as well as other invertebrates and hence, both aspartate or glycogen may be the precursor for succinate synthesis (Collicutt and Hochachka, 1977; de Zwaan, 1983; de Zwaan and Dando, 1984). Generally, when both pathways are present there is a temporal separation of function with the aspartate-succinate pathway providing energy in the early stages of environmental anaerobiosis and the glucose-succinate pathway becoming the primary energy-producing pathway later in anoxia. The use of aspartate is advantageous in that it provides an immediate fuel source for substrate-level phosphorylation by fumarate reductase upon reversal of the succinate-oxaloacetate arm of the Krebs cycle at the onset of anoxia (de Zwaan and Dando, 1984).

In the initial stages of anoxia when succinate is being produced from aspartate, utilization of glycogen and aspartate are indirectly coupled together by two aminotransferase reactions (see Figure 1). Carbon from glycogen is catabolized by the glycolytic pathway (Figure 2) to produce pyruvate which is then converted to alanine by alanine aminotransferase (AlaAT), at the expense of glutamate conversion to α -ketoglutarate (α -KG). Glutamate is replenished by the action of aspartate aminotransferase (AspAT) which, by transferring an amino group from aspartate to α -KG, releases oxaloacetate (OXA) which then enters the reactions of succinate synthesis. Malate dehydrogenase (MDH) reduces OXA to form malate and this is transported into

the mitochondria where it is converted to fumarate and then to succinate, with ADP phosphorylation occurring at the fumarate reductase reaction.

During prolonged anoxia exposure, the aspartate pool is depleted and the continuing production of ATP at the fumarate reductase reaction is fueled instead by OXA derived from glycolysis. To accomplish this, the flow of glycolytic carbon is shifted at the PEP branchpoint; instead of PEP conversion to pyruvate via PK, PEP is channeled via PEP-carboxykinase (PEPCK) into OXA production. This shift is regulated by a sharp decrease in PK activity which is primarily caused by enzyme inactivation via protein phosphorylation (Plaxton and Storey, 1984b) or dephosphorylation (Russell and Storey, 1994a) augmented by changes in the levels of amino acid allosteric effectors (i.e. accumulation of the PK inhibitor alanine and depletion of the activator, aspartate) (Livingstone and Bayne, 1974; Storey, 1986). After this point glycogen becomes the sole fuel for continued anaerobic metabolism.

However, this shift in the fuel of anaerobiosis from aspartate to glycogen may not occur at certain times of the year. PEPCK in the posterior adductor muscle of *M. edulis* only contributed to succinate production in the summer with the aspartate-succinate pathway responsible for all succinate production (at least up to 18 hours of anoxia) during the winter (de Zwaan *et al.*, 1983). Eberlee *et al.* (1983), studying *C. virginica*, suggested that aspartate was the sole source of succinate in the adductor muscle (phasic and catch combined) for the first 24 hours of anoxia whereas in gill and mantle continuing aspartate depletion could account for all of the succinate produced for at least 96 hours. These observations indicate that in many tissues a switch at the PEP-branchpoint may occur much later in anoxia exposure than was previously thought. Furthermore, since in many systems glycolytic carbon may not be routinely converted into succinate, the phosphorylation-mediated block on PK may have less to do with regulating the PEP branchpoint and more to do with overall glycolytic rate depression. Metabolism may be slowed to such a great extent that the "small" aspartate pool is indeed sufficient to supply

succinate for at least 96 hours of anoxia and the glycogen-succinate pathway may not need to be activated till after 4 days of anoxia in some tissues.

Seasonal differences in end-product accumulation have also been determined for marine molluscs. Anaerobic propionate production was higher during the summer in the mussels *M. edulis* (Kluytmans *et al.*, 1980; Gabbott, 1983) and *Geukensia demissus* (Ho and Zubkoff, 1982) and the accumulation of alanine, aspartate, glutamate, fumarate, malate, and succinate was higher in July than November in the muscle and mantle of *M. edulis* air-exposed for 24 hours (Ahmad and Chaplin, 1979).

Hypotheses

The following hypotheses will be tested in this thesis:

1. Metabolic reorganization occurs in tissues of both mollusc species on a seasonal basis.
2. Metabolic reorganization occurs in tissues of both mollusc species in response to anoxia exposure.
3. Metabolic responses to anoxia exposure vary on a seasonal basis.

Rationale

Seasonal changes in aerobic metabolism (i.e. for gametogenesis) and anaerobic metabolism are known in marine molluscs (Gabbott, 1983). Seasonal differences in metabolism must be due, to a large extent, to seasonal modifications ("coarse" control) of the enzymes that constitute metabolic pathways. Metabolic depression, arguably the most important factor for anoxia survival, is known to occur as a result of enzyme modification ("fine" control) (Storey and Storey, 1990). Measuring the maximal activities of both equilibrium and nonequilibrium enzymes and examining the kinetic properties of key regulatory enzymes will allow an assessment of the extent of seasonal modifications on the basic metabolism of marine molluscs and compare the degree of this control, and the mechanisms of regulation, to the modifications occurring as the result of anoxia. This

study will also compare the efficacy and regulation of anaerobic metabolism during different seasons.

Summary of Proposed Studies

This thesis will investigate the effects of seasonal change (summer versus winter) and prolonged anoxia on the metabolism of tissues from *C. virginica* and *L. littorea*. Maximal activities of equilibrium and nonequilibrium enzymes from several different pathways (glycogenolysis, glycogenesis, pentose phosphate shunt, TCA cycle) and different aspects of metabolism (carbohydrate and amino acid metabolism) will be measured to determine the effects of season and anoxia on the metabolic reorganization of individual tissues. The effect of seasonal change on the kinetic properties of PFK and PK from both *C. virginica* and *L. littorea* will be examined and column chromatography will be used to search for the presence of seasonal forms of these enzymes. The effect of prolonged anoxia on the kinetic properties of PFK and PK from both *C. virginica* and *L. littorea* will also be examined. Those tissues that demonstrate anoxia-induced changes in enzyme kinetic properties will be subjected to column chromatography to separate aerobic, control and anoxic forms on the basis of charge differences such as occur due to the addition or removal of phosphate. This study will reveal the relative importance of these enzymes in imposing the hypometabolic state necessary for anoxia survival and determine the importance of regulation by reversible covalent modification. Seasonal differences in the metabolic reorganization occurring as the result of prolonged anoxia will also be studied by comparing the anoxia-induced changes in the maximal activities of various enzymes during the winter and summer seasons and comparing seasonal differences in anoxia-induced kinetic changes. This comparative study will discuss seasonal alterations in metabolism, the existence and purpose of seasonal enzyme forms, anoxia-induced regulation of metabolism, and seasonal differences in this regulation.

Animal Models Used

Two marine animals belonging to the phylum Mollusca, the bivalve oyster *Crassostrea virginica* and the gastropod periwinkle *Littorina littorea* were used in this study. Figure 3 shows the general anatomy of an oyster and a gastropod. The tolerance for prolonged anoxia of both of these species is well known (Eberlee et al., 1983; Russell and Storey, 1994a) and seasonal fluctuations in metabolite concentrations have been documented for both the oyster and periwinkle (Gabbott, 1983). The use of two species allows for a comparative study between a sessile, subtidal bivalve and a mobile, intertidal gastropod. This comparison may reveal interesting divergences that have arisen over biological time due to their different physiologies and environments.

Figure 1. Pathways involved in anaerobic metabolism in marine molluscs (de Zwaan and Dando, 1984).

Aspartate (ASP) or glycogen are oxidized to oxaloacetate (OAA) which is converted to malate (MAL) by MDH for translocation into the mitochondrion where it enters the TCA cycle operating "in reverse" and is converted to fumarate (FUM) and subsequently, succinate (SUC) which accumulates as a metabolic end-product. Additionally, propionate (PROP) may also be produced as an end-product. The classical pathway of glycolysis found in mammalian skeletal muscle is indicated by the heavy arrows.

Figure 2. The reactions involved in glycogenolysis (Russell, 1993).

Enzymes are: 1a) glycogen phosphorylase*, 1b) glycogen synthetase*, 2) phosphoglucomutase, 3) hexokinase and glucokinase, 4) phosphoglucoisomerase, 5) phosphofructokinase*, 6) aldolase, 7) triose phosphate isomerase, 8) phosphoglyceraldehyde dehydrogenase, 9) phosphoglycerate kinase, 10) phosphoglyceratemutase, 11) enolase, 12) pyruvate kinase*, 13) lactate dehydrogenase, 14) pyruvate dehydrogenase.

* Enzymes investigated in this thesis.

Figure 3. (A) Anatomy of the oyster *Crassostrea virginica* (Galtsoff, 1964) and (B) a generalized gastropod representing the periwinkle *Littorina littorea* (Sherman and Sherman, 1970).

Extracts of the gill, hepatopancreas, mantle, phasic adductor, and catch adductor tissues of the bivalve *C. virginica* and of the foot muscle and hepatopancreas (intestine) of the gastropod *L. littorea* were used in this study.

A

B

MATERIALS AND METHODS

Chemicals and Animals

All biochemicals and coupling enzymes were obtained from Sigma Chemical Co., St. Louis, MO or Boehringer-Mannheim Corp., Montreal, PQ. Distilled, deionized water was used throughout for the preparation of biochemical solutions. ATP stock solutions contained added MgCl_2 in 1:1 molar amounts. Marine periwinkles, *Littorina littorea*, and Malpeque oysters, *Crassostrea virginica*, both imported from Prince Edward Island, were obtained from a local seafood supplier. Winter animals were purchased in the first weeks of November and summer animals were obtained in July or August. Winter *L. littorea* were acclimated at 5°C in aerated artificial seawater and all other animals were acclimated at 10°C in aerated artificial seawater (1000 mOsmol/l) for at least 1 week prior to experimentation. No attempt was made to separate the animals on the basis of sex.

Preparation of Experimental Animals

Control animals were taken directly from the aerated seawater. To dissect the snails, the shell was broken open and the foot muscle and hepatopancreas were rapidly removed and immediately frozen in liquid nitrogen. To dissect the oysters, the valves were pried apart and the adductor muscle was severed and then the gills, mantle, hepatopancreas, phasic adductor (the larger translucent portion), and catch adductor (white, fibrous muscle) were quickly excised and immediately frozen in liquid nitrogen. All tissues were held at -70°C until use. To induce experimental anoxia a small amount of seawater was placed in a container to prevent dehydration. Nitrogen gas (100%) was then bubbled through the water for at least 20 minutes to displace any oxygen present. Animals were removed from the aerated seawater and placed in the container. The container was then flushed with nitrogen gas for a further 30 minutes, after which the lid was sealed, and the container was returned to the 10°C incubator. *C. virginica* were held in this state of

anoxia for 96 hours and *L. littorea* for 6 days after which time the animals were rapidly dissected as described above.

Tissue Extraction

Enzyme extracts were prepared by homogenizing frozen tissue samples with an Ultra-Turrax homogenizer or a Pro200 homogenizer (Diamed Lab Supplies Inc. Mississauga, Ont.) using a weight to volume ratio ranging from 1:4 to 1:6 depending on the tissue. The same homogenization buffer was used for all tissues and enzymes and contained 50 mM imidazole-HCl buffer (pH 7.2), 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 15 mM 2-mercaptoethanol. A few crystals of phenylmethylsulfonyl fluoride (PMSF) were added prior to homogenization.

For the analysis of glycogen phosphorylase (GP) and glycogen synthetase (GS) homogenates were allowed to settle on ice for a minimum of 30 minutes and then a sample of the supernatant was used for the assay. However, homogenates of the oyster adductor muscles were centrifuged at low speed in a benchtop centrifuge for 30 seconds at room temperature prior to assaying.

The homogenates used for the analysis of pyruvate kinase (PK) and phosphofructokinase (PFK) kinetic properties were centrifuged for 20 minutes at 25,000g in a Sorvall RC-5B refrigerated centrifuge at 5°C or in a Heraeus Biofuge 15 (Baxter Canlab, Toronto, ON) at 18,000g for 20 minutes at 5°C. The supernatant was removed and desalted by centrifugation (benchtop centrifuge at top speed for 1 min) through a 1 x 5 ml column of fine Sephadex G-25 equilibrated in homogenization buffer. Filtrates were kept on ice at all times.

Analysis of Enzyme Activities

The percentage of the enzyme in the active form was determined for GP and GS and the kinetic properties of PK and PFK were investigated. Coupled enzyme reactions

were used in all cases to monitor enzyme activity. These reactions couple the formation of an enzyme's product to other reactions whose products are more easily detectable

The following assay conditions were used:

Glycogen Phosphorylase (GP; EC 2.4.1.1): Assay conditions for GP_a (the active form) were 50 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM EDTA, 10 mM MgCl₂, 0.4 mM NADP, 10 μ M glucose-1,6-bisphosphate, 2 mg/ml oyster glycogen (previously dialyzed), and 0.7 U/ml phosphoglucomutase and 0.2 U/ml glucose-6-phosphate dehydrogenase. For measurements of total phosphorylase (a + b), assays were conducted in the presence of 1.6 mM AMP.

Glycogen Synthetase (GS; EC 2.4.1.11): Assay conditions for the active I form were 20 mM imidazole-HCl buffer (pH 7.0), 20 mM KCl, 5 mM MgCl₂, 0.15 mM NADH, 5 mM uridine 5' diphosphoglucose (UDPG), 2 mg/ml oyster glycogen (previously dialyzed), 1 mM phosphoenolpyruvate (PEP), 1 U/ml pyruvate kinase and 1 U/ml lactate dehydrogenase. For total synthetase activity (I + D), 5 mM glucose-6-phosphate (G6P) was included in the assay mixture.

Phosphofructokinase (PFK; EC 2.7.1.11): Assay conditions for determining the kinetic properties of *L. littorea* and *C. virginica* PFK were 100 mM imidazole-HCl buffer (pH 7.2) containing 50 mM KCl, 5 mM MgCl₂, 0.15 mM NADH, 0.1% v/v rotenone-saturated ethanol and 0.2 U/ml aldolase, 1.2 U/ml α -glycerophosphate dehydrogenase (α GPDH) and 0.16 U/ml triosephosphate isomerase (TPI) with varying concentrations of Mg.ATP and fructose-6-phosphate (F6P) as described in Results.

Pyruvate Kinase (PK; EC 2.7.1.40): Assay conditions for determining the kinetic properties of PK from *L. littorea* and *C. virginica* were 100 mM imidazole-HCl buffer (pH 7.2), 50 mM KCl, 5 mM MgCl₂, 2 mM ADP, 0.15 mM NADH, 0.2% v/v rotenone-saturated ethanol, and 1 U/ml lactate dehydrogenase with varying concentrations of PEP as described in Results.

All enzyme assays were conducted by monitoring changes in the concentration of NADH or NADPH at 340 nm using a Gilford 240 recording spectrophotometer and a Kipp and Zonen BD 111 chart recorder or a MR 5000 Microplate Reader (Dynatech Laboratories, Chantilly, VA) connected to a 486 computer running the Biolinx version 2.0 software (Dynatech Laboratories, Chantilly, VA). Assays were initiated by addition of the enzyme preparation and performed at $21 \pm 1^\circ\text{C}$ with a final cuvette volume of 1 ml plus added homogenate or a microplate well volume of 0.250 ml plus added homogenate.

Enzyme Survey

Maximal activities of 18 different enzymes were determined in *L. littorea* foot and hepatopancreas and *C. virginica* gill, mantle, hepatopancreas, phasic adductor and catch adductor. Frozen tissue samples were homogenized (1:6 w/v) as previously described and centrifuged for 20 min at 18,000 g at 5°C in a Heraeus Biofuge 15 centrifuge (Baxter Canlab, Toronto, ON). Supernatants were not desalted and were kept on ice until use. Controls for non-specific activity were run for all assays and any blank value subtracted to yield final activity values. Optimal conditions for maximal activities were determined to be:

Hexokinase (HK; EC 2.7.1.1): 100 mM Tris buffer (pH 8.0), 1 mM EDTA, 2 mM MgCl_2 , 5 mM glucose, 1 mM Mg.ATP, 0.2 mM NADP^+ , and excess NADP^+ -dependent G6PDH.

Phosphofructokinase (PFK; EC 2.7.1.11): 100 mM imidazole-HCl buffer (pH 7.2), 50 mM KCl, 5 mM MgCl_2 , 10 mM fructose-6-phosphate, 0.1 mM ATP.Mg, 0.15 mM NADH, 0.1% (v/v) rotenone-saturated ethanol and excess aldolase, TPI, and α GPDH.

Aldolase (EC 4.1.2.13): 50 mM imidazole-HCl buffer (pH 7.0), 2 mM MgCl_2 , 0.2 mM fructose-1,6- P_2 , 0.15 mM NADH, and excess TPI and α GPDH.

Glycerol-3-phosphate dehydrogenase (G3PDH; EC 1.1.1.8): 500 mM triethanolamine buffer (pH 7.9), 5% (w/v) bovine serum albumin, 26 mM glyceraldehyde-3-phosphate, 0.15 mM NADH, and excess TPI.

Pyruvate kinase (PK; EC 2.7.1.40): 100 mM imidazole-HCl buffer (pH 7.2), 50 mM KCl, 5 mM MgCl_2 , 10 mM PEP, 2 mM ADP, 0.15 mM NADH, 0.2% (v/v) rotenone-saturated ethanol, and excess LDH.

Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32): 100 mM imidazole-HCl buffer (pH 6.6), 30 mM 2-mercaptoethanol, 5 mM PEP, 50 mM NaHCO_3 , 1.25 mM IDP, 1 mM MnCl_2 , 0.15 mM NADH, and excess MDH. Solutions were degassed prior to assaying.

Lactate dehydrogenase (LDH; EC 1.1.1.27): 50 mM imidazole-HCl buffer (pH 7.0), 2 mM MgCl_2 , 4 mM pyruvate, and 0.15 mM NADH.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11): 25 mM imidazole-HCl buffer (pH 7.0), 5 mM MgSO_4 , 1.4 mM fructose-1,6- P_2 , 0.2 mM NADP^+ , and excess PGI and G6PDH.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49): 100 mM Tris-HCl buffer (pH 7.5), 3.3 mM glucose-6-phosphate, 6 mM MgCl_2 , and 0.4 mM NADP^+ .

6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44): 50 mM Tris-HCl (pH 7.0), 0.5 mM 6-phospho-D-gluconate, and 0.4 mM NADP^+ .

Alanine aminotransferase (AlaAT; EC 2.6.1.2): 500 mM imidazole-HCl buffer (pH 7.3), 30 mM 2-mercaptoethanol, 500 mM L-alanine, 0.11 mM pyridoxal phosphate, 15 mM 2-oxoglutarate, 0.15 mM NADH, and excess LDH.

Aspartate aminotransferase (AspAT; EC 2.6.1.1): 500 mM imidazole-HCl buffer (pH 7.8), 30 mM 2-mercaptoethanol, 250 mM L-aspartate, 0.11 mM pyridoxal phosphate, 15 mM 2-oxoglutarate, 0.15 mM NADH, and excess MDH.

Malic enzyme (ME; EC 1.1.1.40): 150 mM Triethanolamine buffer (pH 7.4), 5 mM L-malate, 4 mM MnCl_2 , and 0.2 mM NADP^+ .

Serine dehydratase (SDH; EC 4.2.1.13): 200 mM K_2HPO_4 buffer (pH 8.0), 2 mM EDTA, 100 mM L-serine, 0.11 mM pyridoxal phosphate, 0.15 mM NADH, 0.2% (v/v) rotenone-saturated ethanol, and excess LDH.

NAD⁺-dependent malate dehydrogenase (MDH-NAD; EC 1.1.1.37): 50 mM imidazole-HCl buffer (pH 7.2), 10 mM MgCl₂, 20 mM oxaloacetate, and 0.15 mM NADH. This assay determines the maximal activity of malate dehydrogenase using its preferred coenzyme NADH.

NADP⁺-dependent malate dehydrogenase (MDH-NADP; EC 1.1.1.37): 50 mM imidazole-HCl buffer (pH 7.2), 10 mM MgCl₂, 15 mM oxaloacetate, and 0.15 mM NADPH. This assay determines the maximal activity of malate dehydrogenase using NADPH which is a less efficient coenzyme.

Arginine kinase (ArgK; EC 2.7.3.3): 50 mM imidazole-HCl (pH 7.2), 10 mM MgCl₂, 15 mM arginine phosphate, 15 mM glucose, 2 mM ADP, 0.45 mM NADP⁺, and excess hexokinase and G6PDH.

Adenylate Kinase (AK; EC 2.7.4.3): 50 mM potassium phosphate buffer (pH 7.4), 20 mM KCl, 5 mM MgSO₄, 2 mM AMP, 2 mM ATP, 2 mM PEP, 0.15 mM NADH, and excess PK and LDH.

All assays were conducted using a MR 5000 Microplate Reader (Dynatech Laboratories, Chantilly, VA) connected to a 486 computer running the Biolinx version 2.0 software (Dynatech Laboratories, Chantilly, VA). The assays were initiated by addition of the enzyme preparation and performed at $21 \pm 1^\circ\text{C}$ with a final microplate well volume of 0.250 ml plus added homogenate.

Coupling Enzymes

Commercial coupling enzymes are stored in the presence of ammonium sulfate. This substance is an activator of PFK and therefore had to be removed prior to assaying. In order to remove the ammonium sulfate the coupling enzymes, aldolase, TPI and α -GPDH, were combined and centrifuged for 2 minutes in a Brinkman 5412 microcentrifuge. The supernatant was removed and the coupling enzyme pellet resuspended in assay buffer (100 mM imidazole-HCl buffer, pH 7.2, 50 mM KCl and 5

mM MgCl_2). This suspension was then desalted by centrifugation (benchtop centrifuge at top speed for 1 min) through a 5 ml column of fine Sephadex G-25 equilibrated in assay buffer.

Coupling enzymes used for the enzyme survey were diluted in coupling enzyme buffer (50 mM imidazole-HCl buffer, pH 7.0, 5 mM EDTA, 20% glycerol v/v and 30 mM 2-mercaptoethanol), desalted by centrifugation and kept at 4°C until use (for a maximum of 1 week).

Hydroxylapatite Chromatography of Pyruvate Kinase

To determine whether multiple forms of PK occurred in oysters (seasonally expressed isozymes or phosphorylated/dephosphorylated forms) tissue extracts were subjected to hydroxylapatite (Bio-Rad) chromatography. Gill, mantle, hepatopancreas, phasic adductor and catch adductor samples were homogenized 1:5 w/v in 50 mM imidazole-HCl buffer (pH 7.2), 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 15 mM 2-mercaptoethanol. A few crystals of PMSF were added prior to homogenization. The homogenates were centrifuged for 20 min at 25,000g in a Sorvall RC-5B refrigerated centrifuge at 5°C. The supernatant was then desalted by centrifugation (benchtop centrifuge at top speed for 1 minute) through a 1 x 5 ml column of fine Sephadex G-25 equilibrated in column buffer consisting of 10 mM potassium phosphate buffer (pH 6.5), 50 mM NaF, 2 mM EDTA, 2 mM EGTA, and 15 mM 2-mercaptoethanol. The filtrate was placed on a hydroxylapatite column (1.5 cm x 3 cm) equilibrated in column buffer, and washed with 20 ml of column buffer. The column was then developed with a linear phosphate gradient of 0-500 mM KPi in column buffer and 1 ml fractions were collected and assayed for PK activity.

***In Vitro* Dephosphorylation**

To prepare enzyme extracts for *in vitro* dephosphorylation, tissue samples were homogenized 1:5 w/v in dephosphorylation buffer consisting of 20 mM potassium

phosphate (pH 6.5), 50 mM KCl, 5 mM MgCl_2 , and 15 mM 2-mercaptoethanol (a few crystals of PMSF were added immediately prior to homogenization). Homogenates were then centrifuged as described above and supernatants were incubated overnight at 4°C. Incubates were desalted as previously described but with the G-25 pre-equilibrated in dephosphorylation buffer prior to hydroxylapatite chromatography (detailed above).

Protein Determination

Protein concentration was determined by the Bio-Rad protein assay kit based on the Coomassie Blue G-250 dye-binding method with bovine serum albumin as the standard (Bradford, 1976).

Kinetics and Statistics

The substrate affinity constant, $S_{0.5}$, was defined as the concentration of substrate at which the enzyme reaction proceeds at 50% of its maximal velocity. I_{50} was defined as the concentration of inhibitor required to reduce enzyme activity to one half its value in the absence of inhibitor. K_a was defined as the concentration of activator at which the effector produces 50% of its maximal activity.

$S_{0.5}$ values were determined by fitting data to the Hill equation using a non-linear least square regression program (Brooks, 1992). A modified Hill equation that introduced a V_0 term (rate at zero activator concentration) was used for determining K_a values. The inhibitor constant values (I_{50}) were obtained from plots of rate vs. [inhibitor]. All data are presented as means $\pm$ S.E.M. with statistical testing using Student's t-test (2-tailed).

For determination of enzyme activity as units per gram-wet-weight (IU/gww), kinetic data from Biolinx 2.0 were imported into the Microplate Analysis Program (Brooks, 1994) which performed a linear regression on the OD/time readings. One unit of enzyme activity was defined as the amount that uses 1 μmol substrate per minute at 21°C.

RESULTS

Part I) SEASONAL CHANGES IN ENZYMES OF MARINE MOLLUSCS

Seasonal Changes in the Kinetic Properties of Phosphofructokinase and Pyruvate Kinase from *Littorina littorea*

Phosphofructokinase

Table 1 summarizes the kinetic properties of PFK from the foot muscle of *L. littorea* collected in winter (early November) versus summer (July/August). The enzyme was assayed in high speed supernatant extracts of muscle after removal of low molecular weight metabolites and ions by passage through Sephadex G-25. Enzyme affinity for one substrate, fructose-6-phosphate (F6P), did not change between the seasons nor did the sensitivity to inhibition by the second substrate, ATP. However, compared with the enzyme from winter snails, PFK in foot of summer snails showed increased sensitivity to two allosteric activators, AMP and fructose-2,6-bisphosphate (F2,6P₂), with K_a values 49% less than the corresponding winter values. Summer PFK also showed an increase in the Hill coefficient for activation by AMP, indicating increased cooperativity between the enzyme subunits, and an increase in the fold-activation by F2,6P₂.

PFK from hepatopancreas showed extensive seasonal changes in enzyme properties (Table 2). PFK from summer animals showed a significantly greater Hill coefficient for F6P, compared with the winter enzyme. A 40% decrease in the I_{50} for ATP in the summer meant that the effective concentration of ATP required to produce the same degree of enzyme inhibition was substantially reduced. Unlike foot PFK, the enzyme in hepatopancreas of summer snails was less sensitive to metabolite activators. Compared with the winter enzyme, summer PFK showed a 2.4-fold increase in the K_a for AMP and a 3-fold increase in the K_a for F2,6P₂. However, the fold activation of PFK by AMP was higher in summer hepatopancreas than in winter.

Pyruvate Kinase

The kinetic properties of PK from foot muscle (Table 3) and hepatopancreas (Table 4) of winter and summer periwinkles were also assessed in crude extracts. Foot PK from summer animals showed properties that would make it a less active enzyme compared with the winter form. The affinity for PEP was reduced ($S_{0.5}$ increased by 45%) and sensitivity to alanine inhibition was increased (I_{50} was reduced by 48%). However, fructose-1,6-bisphosphate (F1,6P₂) produced a greater fold activation of the summer enzyme.

Oppositely, PK in hepatopancreas of summer periwinkles showed properties that would make it more active than the winter form (Table 4). Summer PK showed an 87% decrease in the $S_{0.5}$ for PEP and a reduced Hill coefficient. I_{50} for alanine was decreased by 30% but summer PK was more sensitive to activation by F1,6P₂ (K_a was only 40% of the winter value) although the fold activation by F1,6P₂ was less for the summer enzyme than the winter enzyme.

Effect of Seasonal Change on the Maximal Activities of Enzymes in *L. littorea*

The maximal activities of 18 different enzymes were measured in crude extracts of *L. littorea* foot and hepatopancreas from winter and summer animals (kept under aerobic control conditions at 5 or 10°C). The enzymes represented pathways including glycolysis, gluconeogenesis, amino acid metabolism, phosphagen/adenylate metabolism and the anaplerotic reactions of the TCA cycle.

Foot Muscle

The first seven enzymes shown in Figure 4 (HK, PFK, aldolase, G3PDH, PK, PEPCK, and LDH) are enzymes associated with glycolysis. Although typically associated with gluconeogenesis in animal tissues, PEPCK is considered here as a glycolytic enzyme because of its role in marine invertebrates in the anaerobic conversion of phosphoenolpyruvate (PEP) to oxaloacetate (OXA) as the means of channeling glycolytic

carbon into the reactions of succinate synthesis. The only significant seasonal change observed was an increase in the activity of LDH in muscle of summer animals. PEPCK levels were extremely low (winter control level = 0.44 ± 0.04 U/gww), especially when compared to PK levels which were about 61-fold higher during the winter and 53-fold higher during the summer. This ratio provided an indication of the degree to which PK function must be arrested in order for PEP to be processed anaerobically by PEPCK. Levels of FBPase in the winter and summer were not significantly different (0.0378 ± 0.00602 and 0.0528 ± 0.00486 IU/gww, respectively).

G6PDH activity was 52% less in the summer than in the winter but 6PGDH showed no seasonal change. The activities of AlaAT and AspAT did not change with the progression of the season. Activities of SDH (winter control activity = 0.18 ± 0.01 U/gww) and ME (winter control level = 0.37 ± 0.02 U/gww) were also unaffected seasonally. MDH was assayed in the presence of its preferred coenzyme NAD^+ and the less efficient NADP^+ and, as shown in Figure 4, *L. littorina* MDH still showed substantial activity even with NADP^+ as its cofactor (11% of the NAD^+ -dependent activity). Activities of MDH (using NAD^+ or NADP^+) increased slightly in foot muscle during the summer. Arginine kinase (ArgK) which interconverts phosphagen (arginine phosphate) and ATP pools was present in extremely high activities in *L. littorea* foot muscle, which is generally true of phosphagen kinases in skeletal muscles, and its activity decreased significantly by 34% during the summer. Adenylate kinase (AK), responsible for interconverting ATP, ADP and AMP, did not change in activity with the seasons

Hepatopancreas

Seasonal changes in the activities of enzymes in hepatopancreas of periwinkles are shown in Figure 5. During the summer, activities of PFK, aldolase, G3PDH, PK, LDH, and PEPCK (0.148 ± 0.015 U/gww in winter vs. 0.285 ± 0.030 U/gww in summer) were significantly higher than during the winter. FBPase activities did not change seasonally with an activity of 0.124 ± 0.0166 U/gww during the winter and a summer activity of

0.155 ± 0.0140 U/gww. Overall, glycolytic enzyme activities in hepatopancreas were about one-half of the corresponding values in foot muscle (except for HK and G3PDH).

Activities of pentose phosphate shunt enzymes (G6PDH and 6PGDH) did not change with season in hepatopancreas and activities were about 2-fold greater than the corresponding values for these enzymes in foot muscle. AspAT activity did not change with season but other enzymes of amino acid metabolism, AlaAT and SDH increased during the summer (SDH rose from 0.353 ± 0.058 to 0.663 ± 0.058 U/gww).

Hepatopancreas ME activity also increased in the summer from 0.096 ± 0.012 to 0.218 ± 0.027 U/gww. Activities of these four enzymes were similar between the two tissues. Unlike in foot, MDH (with both coenzymes) and ArgK activities were not affected by seasonal changes and AK activities remained constant. Although MDH activities were similar in both tissues, ArgK and AK activities were 10- and 3-fold higher, respectively, in foot than in hepatopancreas.

Effect of Seasonal Change on Enzymes from *Crassostrea virginica*

Glycogen Phosphorylase

Table 5 shows the total phosphorylase activity (active a + inactive b forms) and the percentage of the enzyme in the active a form for five different tissues from winter and summer *C. virginica*. In the summer, four of the five tissues (gill, hepatopancreas, phasic adductor, and catch adductor) showed significantly lower total GP activity (by 18-49%), compared with the winter, the mantle being the only tissue that did not exhibit a seasonal change. The percentage of GP in the active a form was also lower in gill, mantle, hepatopancreas, and phasic adductor in summer animals compared with winter oysters. The largest decreases occurred in the hepatopancreas (%a dropped from 70% to 18%) and in the phasic adductor (%a fell to 46% during the summer compared to 81% during the winter) with more modest decreases occurring in the gill (from 66% to 53%) and

mantle (from 89% to 72%) tissues. By contrast %a rose in catch adductor from 74% in winter to 95% in summer.

Glycogen Synthetase

Table 6 shows the total activity of GS (the sum of the active G6P-independent I form and the inactive G6P-dependent D form) as well as the percentage of activity in the I form in tissues of winter and summer *C. virginica*. In contrast to the generally lower GP activities in oyster tissues in the summer, GS activity was higher in three tissues during the summer. Activities in gill, mantle, and catch adductor were 60, 106, and 74% higher in summer, compared with winter, oysters. However, in phasic adductor, GS activity was lower in summer, only 35% of the winter value. Despite the seasonal changes in total GS activity, no winter versus summer change was detected in the percentage of GS in the active I form in any of the tissues.

Phosphofructokinase

The kinetic properties of PFK in crude tissue extracts from winter and summer *C. virginica* are summarized in Tables 7-11. In all tissues, PFK from control, aerobic tissues exhibited distinct seasonal changes in kinetic properties. Table 7 shows that gill PFK from summer oysters displayed a slightly higher Hill coefficient for F6P, a 3-fold higher K_a for AMP, and a reduced (by 56%) K_a F2,6P₂, compared with the enzyme from winter animals. In mantle (Table 8), summer PFK had a lower affinity for F6P (73% higher $S_{0.5}$) along with a slightly higher Hill coefficient, compared with the winter enzyme. The K_a AMP was also 42% higher in summer, as was the fold activation by AMP. PFK in the hepatopancreas showed significantly higher values for both $S_{0.5}$ and n_H for F6P of summer, compared with winter animals (Table 9). Furthermore, the K_a for AMP was 2.6-fold higher and both AMP and F2,6P₂ showed higher fold activation values during the summer. Table 10 shows the kinetic properties of PFK from phasic adductor. In the

summer, phasic adductor PFK showed an elevated $S_{0.5}$ for F6P (double the winter value) and a higher Hill coefficient for AMP activation; however, other parameters were not affected. PFK from catch adductor PFK showed a higher Hill coefficient for F6P, a 2-fold higher K_a AMP and a higher fold activation by AMP in summer, compared with winter, oysters (Table 11). In addition, the enzyme was more sensitive to activation by F2,6P₂ during the summer, K_a F2,6P₂ being only half of the winter value.

Pyruvate Kinase

The kinetic properties of PK in crude tissue extracts from winter and summer *C. virginica* are summarized in Tables 12-16. In gill (Table 12), PK showed large seasonal changes. The $S_{0.5}$ for PEP was 5.5-fold higher in the summer, accompanied by a significant increase in the Hill coefficient. Sensitivity to inhibition by L-alanine was increased, the I_{50} alanine for summer PK being only 27% of the winter value. However, sensitivity to the activator, F1,6P₂, was reduced in the summer, the K_a being 26-fold higher for summer, versus winter, PK. Summer gill PK also showed significantly higher Hill coefficient and fold activation values for F1,6P₂ than did winter PK. Mantle PK showed similar changes in PK properties in the summer (Table 13). Compared with winter PK, $S_{0.5}$ PEP of summer PK was 5.7-fold higher, I_{50} for alanine was 25% lower (with a lower n_H), and K_a for F1,6P₂ was 8-fold higher along with a 9-fold increase in the fold activation by this activator. PK from the hepatopancreas of summer *C. virginica* also showed a higher $S_{0.5}$ for PEP (by 2.5-fold) and reduced sensitivity to F1,6P₂ (K_a was 7-fold higher), compared with the winter enzyme (Table 14). Phasic and catch adductor PK both followed the same pattern, the summer enzyme from both tissues showing a 4-fold higher $S_{0.5}$ for PEP as well as much higher K_a values for F1,6P₂ (63- and 22-fold higher, respectively), compared with the winter enzyme (Tables 15 and 16). The fold activation by F1,6P₂ was also significantly higher for summer PK from both muscles, compared with the winter enzyme.

Hydroxylapatite Chromatography of Pyruvate Kinase from *C. virginica*

As an aid to determining whether the kinetic differences between winter and summer PK were due to seasonal changes in the enzyme form expressed in oyster tissues, hydroxylapatite chromatography of tissue extracts was employed. Elution from this column is dependent upon the charge characteristics of an enzyme and changes in the elution pattern of an enzyme off hydroxylapatite indicate that some modification of the enzyme, that affects net enzyme charge, has occurred. This form of chromatography is particularly effective for separating enzyme forms that differ in their content of covalently bound phosphate. Extracts of four tissues from winter and summer oysters were subjected to hydroxylapatite chromatography and eluted with a 0-500 mM potassium phosphate (KPi) gradient. As Figures 6-9 show, in all four tissues the peak of PK activity was eluted at a considerably lower salt concentration in extracts from winter tissues than in summer extracts. In gill the activity peak of winter PK was eluted at 110 mM KPi whereas summer PK eluted at 210 mM (Figure 6). Similarly, mantle PK from winter oysters eluted at 150 mM KPi whereas summer PK eluted at 210 mM (Figure 7). Figure 8 shows that winter PK from phasic adductor muscle eluted at 70 mM KPi and summer PK at 200 mM KPi whereas the values for catch adductor PK were 100 and 200 mM KPi, respectively (Figure 9).

Seasonal Changes in the Maximal Activities of Enzymes in *C. virginica* Tissues

The maximal activities of various enzymes involved in glycogenolysis, glycogenesis, the pentose phosphate shunt, amino acid metabolism, phosphagen and adenylate metabolism, and anaplerotic reactions of the TCA cycle were quantified in tissues of winter and summer oysters under aerobic, control conditions at 10°C (Figures 10-14). The extremely low FBPase activities are presented separately (Figure 15)

Gill

Figure 10 shows the results of the enzyme survey for gill tissue from winter and summer oysters. The first nine enzymes are important in the metabolism of glycogen (as well as FBPase, shown in Figure 15). GP activity decreased significantly in summer, accompanied by an opposite rise GS activity. There were no seasonal changes in the activities of HK, PFK, aldolase, G3PDH, or LDH. However, PK activity decreased substantially during the summer, falling to 41% of the winter value, and the activity of PEPCK also decreased significantly. FBPase activity increased during the summer complementing the increase in GS. Activities of 10 other enzymes involved in the pentose phosphate cycle, amino acid metabolism, and adenylate and phosphagen metabolism in gill did not change seasonally.

Mantle

Figure 11 summarizes enzyme activities in mantle from both winter and summer oysters. In general, mantle enzyme activities were very stable between seasons, the activities of only three enzymes changing significantly from winter to summer. Thus, GS increased by 2.1-fold in mantle of summer oysters, although GP activity did not change seasonally. G3PDH activity also showed a small increase during the summer as did serine dehydratase. FBPase activity was also unaffected by season in mantle (Figure 15).

Hepatopancreas

Seasonal changes in enzyme activities were more prominent in oyster hepatopancreas (Figure 12). GP activity decreased by 43% in the summer although GS activity did not change. HK increased by 33% and PFK increased by 44% although activity of the gluconeogenic enzyme FBPase also increased by 63% (Figure 15). By contrast, PEPCK activity decreased significantly (by 29%) and, coupled to the large increase in PK activity (2.5-fold), this might serve to preferentially direct the flow of carbon into the aerobic TCA cycle in the summer. The activity of G6PDH also increased in the summer although 6PGDH activity remained constant. SDH activity also increased in

summer (from 0.068 ± 0.008 U/gww in winter to 0.113 ± 0.019 U/gww in summer) and arginine kinase activity increased by 31% in the summer

Phasic Adductor Muscle

Figure 13 shows the effect of seasonal change on enzymes from the phasic adductor muscle. In the summer, activities of enzymes of glycogen metabolism decreased significantly; GP activity was reduced by 50% and GS by 65%. However, enzymes of glycolysis were largely unaffected except for a significant reduction in LDH activity in the summer by 25%. PEPCK activity increased by 31% during the summer but FBPase activity did not change seasonally (Figure 15). G6PDH and 6PGDH activities behaved oppositely, the former decreasing by 23% in the summer whereas the latter increased by 21% during the summer. ArgK activity decreased slightly (by 11%) in the summer. SDH activity could not be detected in the phasic adductor (< 0.04 U/gww).

Catch Adductor Muscle

Enzymes of glycogen metabolism showed oppositely directed changes with season in the catch adductor (Figure 14); in the summer, GP activity decreased by 57% whereas GS activity rose by 42%. HK activity also increased in the summer, as did G3PDH but PFK activity was strongly reduced (by 57%) in summer muscle. Summer and winter FBPase activities were not significantly different (Figure 15). Activities of G6PDH did not change between seasons in catch adductor but 6PGDH increased by 29% in the summer. Two enzymes of amino acid metabolism, AlaAT and AspAT, also increased significantly in activity during the summer, by 24% and 19% respectively. As in the phasic adductor, SDH activity was again below the limit of detection in catch muscle (activity < 0.04 U/gww).

Effect of Seasonal Change on Tissue Soluble Protein

Figure 16 shows the amount of soluble protein (mg protein/gww) in foot and hepatopancreas of *L. littorea* in the winter and summer. No significant changes in soluble

protein content occurred on a seasonal basis. Protein concentration in the hepatopancreas was nearly 2-fold greater than in the foot but neither tissue demonstrated a seasonal change in the level of protein

Soluble protein concentrations (mg protein/gww) in the tissues of *C. virginica* are presented in Figure 17. A comparison of protein contents in aerobic, control tissues of winter versus summer oysters revealed only a small decrease by 10% in protein content in the hepatopancreas in the summer. Other tissues lacked seasonal changes in protein content.

Table 1. Kinetic properties of PFK from foot muscle of winter and summer *L. littorea*.

	Winter	Summer
$S_{0.5}$ F6P, mM	1.94 ± 0.07	2.06 ± 0.05
n_H F6P	2.53 ± 0.23	2.32 ± 0.074
I_{50} Mg.ATP, mM	2.42 ± 0.09	2.50 ± 0.16
n_H Mg.ATP	1.78 ± 0.09	1.95 ± 0.09
K_a AMP, mM	0.047 ± 0.011	0.022 ± 0.002^a
n_H AMP	1.12 ± 0.06	1.79 ± 0.26^a
fold activation by AMP	2.33 ± 0.22	3.48 ± 0.24
K_a F2,6P ₂ , μ M	0.57 ± 0.05	0.28 ± 0.01^a
n_H F2,6P ₂	1.12 ± 0.07	1.22 ± 0.06
fold activation by F2,6P ₂	3.78 ± 0.41	5.73 ± 0.36^a

Results are means $\pm$ SEM, $n=5-8$ different animals. All parameters (except I_{50} Mg.ATP) were determined at 0.1 mM Mg.ATP. I_{50} Mg.ATP was determined at 4 mM F6P. K_a AMP and K_a F2,6P₂ were determined at 1 mM F6P.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 2. Kinetic properties of PFK from hepatopancreas of winter and summer *L. littorea*.

	Winter	Summer
$S_{0.5}$ F6P, mM	2.07 ± 0.09	2.29 ± 0.07
n_H F6P	2.56 ± 0.05	3.04 ± 0.07^a
I_{50} Mg.ATP, mM	3.24 ± 0.25	1.28 ± 0.08^a
n_H Mg.ATP	2.48 ± 0.29	3.18 ± 0.09
K_a AMP, mM	0.034 ± 0.006	0.082 ± 0.01^a
n_H AMP	1.35 ± 0.10	1.35 ± 0.15
fold activation by AMP	2.01 ± 0.09	3.99 ± 0.31^a
K_a F2,6P ₂ , μ M	0.45 ± 0.06	1.38 ± 0.11^a
n_H F2,6P ₂	1.02 ± 0.01	1.17 ± 0.08
fold activation by F2,6P ₂	7.80 ± 0.49	7.58 ± 1.02

Results are means $\pm$ SEM, $n=3-8$ different animals. All parameters (except I_{50} Mg.ATP) were determined at 0.1 mM Mg.ATP. I_{50} Mg.ATP was determined at 4 mM F6P. K_a AMP and K_a F2,6P₂ were determined at 1 mM F6P.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 3. Kinetic properties of PK from foot muscle of winter and summer *L. littorea*.

	Winter	Summer
$S_{0.5}$ PEP, mM	0.22 ± 0.01	0.32 ± 0.03^a
n_H PEP	1.06 ± 0.03	1.06 ± 0.04
I_{50} L-alanine, mM	2.42 ± 0.26	1.16 ± 0.14^a
n_H L-alanine	1.06 ± 0.04	1.23 ± 0.07
K_a F1,6P ₂ , μ M	0.043 ± 0.008	0.053 ± 0.007
n_H F1,6P ₂	1.00 ± 0.001	1.00 ± 0.001
fold activation by F1,6P ₂	2.68 ± 0.17	4.52 ± 0.25^a

Results are means $\pm$ SEM, n=4-11 different animals. All parameters were determined at 2 mM ADP. Winter I_{50} L-alanine was determined at 1 mM PEP. Summer I_{50} L-alanine was determined at 2 mM PEP. K_a F1,6P₂ values were determined at 0.1 mM PEP.

^a - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Table 4. Kinetic properties of PK from hepatopancreas of winter and summer *L. littorea*.

	Winter	Summer
S _{0.5} PEP, mM	1.40 ± 0.11	0.18 ± 0.006 ^a
n _H PEP	1.37 ± 0.09	1.09 ± 0.02 ^a
I ₅₀ L-alanine, mM	1.18 ± 0.13	0.83 ± 0.08 ^a
n _H L-alanine	1.00 ± 0.001	1.26 ± 0.12
K _a F1,6P ₂ , μM	3.77 ± 0.47	1.53 ± 0.16 ^a
n _H F1,6P ₂	1.05 ± 0.04	1.00 ± 0.001
fold activation by F1,6P ₂	5.23 ± 0.26	2.99 ± 0.21 ^a

Results are means ± SEM, n=5-11 different animals. All parameters were determined at 2 mM ADP. Winter I₅₀ L-alanine was determined at 4 mM PEP. Summer I₅₀ L-alanine was determined at 0.5 mM PEP. Winter K_a F1,6P₂ was determined at 1 mM PEP. Summer K_a F1,6P₂ was determined at 0.1 mM PEP.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), P < 0.05.

Figure 4. Activities of enzymes in *L. littorea* foot muscle in winter and summer. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Figure 5. Activities of enzymes in *L. littorea* hepatopancreas in winter and summer. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Table 5. Total glycogen phosphorylase (**a** + **b**) activity and the percentage of the active **a** form in tissues of winter and summer *C. virginica*.

	Winter U/gww	Winter % a	Summer U/gww	Summer % a
Gill	1.14 ± 0.09	66.3 ± 12.1	0.93 ± 0.04 ^{a}	53.1 ± 2.40 ^{a}
Mantle	1.14 ± 0.05	88.5 ± 3.30	1.22 ± 0.11	71.7 ± 2.68 ^{a}
Hepatopancreas	0.99 ± 0.11	70.3 ± 5.78	0.56 ± 0.05 ^{a}	18.3 ± 1.53 ^{a}
Phasic Adductor	3.40 ± 0.15	81.0 ± 3.40	1.73 ± 0.25 ^{a}	45.7 ± 5.84 ^{a}
Catch Adductor	2.05 ± 0.10	74.2 ± 0.99	1.17 ± 0.10 ^{a}	95.2 ± 2.27 ^{a}

Results are means ± SEM. n=6-8 different animals. Total activity was determined at 1.6 mM AMP for all tissues and both conditions.

^{**a**} - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 6. Total glycogen synthetase (I + D) activity and the percentage of the active I form in tissues of winter and summer *C. virginica*.

	Winter U/gww	Winter % I	Summer U/gww	Summer % I
Gill	0.60 ± 0.06	68.7 ± 6.81	0.96 ± 0.07 ^a	61.1 ± 3.38
Mantle	0.67 ± 0.06	40.1 ± 8.66	1.38 ± 0.08 ^a	48.4 ± 3.70
Hepatopancreas	1.12 ± 0.08	43.5 ± 3.43	1.36 ± 0.08	34.9 ± 2.34
Phasic Adductor	1.66 ± 0.05	24.5 ± 4.49	0.58 ± 0.07 ^a	36.6 ± 4.79
Catch Adductor	0.99 ± 0.10	25.5 ± 5.34	1.72 ± 0.15 ^a	41.8 ± 8.13

Results are means ± SEM, n=5-8 different animals. Total activity was determined at 5 mM G6P for all tissues and both conditions.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), P < 0.05.

Table 7. Kinetic properties of PFK from gill of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ F6P, mM	1.72 ± 0.19	1.94 ± 0.20
n_H F6P	1.40 ± 0.10	1.95 ± 0.12^a
K_a AMP, mM	0.02 ± 0.005	0.06 ± 0.005^a
n_H AMP	1.37 ± 0.10	1.23 ± 0.08
fold activation by AMP	2.30 ± 0.19	3.17 ± 0.30
K_a F2,6P ₂ , μ M	2.58 ± 0.16	1.14 ± 0.15^a
n_H F2,6P ₂	1.09 ± 0.05	1.16 ± 0.09
fold activation by F2,6P ₂	2.30 ± 0.23	3.01 ± 0.08^a

Results are means $\pm$ SEM, n=3-9 different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 8. Kinetic properties of PFK from mantle of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ F6P, mM	1.28 ± 0.20	2.22 ± 0.09^a
n_H F6P	1.47 ± 0.06	1.87 ± 0.17^a
K_a AMP, mM	0.024 ± 0.002	0.034 ± 0.002^a
n_H AMP	1.13 ± 0.09	1.38 ± 0.12
fold activation by AMP	1.95 ± 0.06	2.80 ± 0.15^a
K_a F2,6P ₂ , μ M	1.44 ± 0.17	2.41 ± 0.37
n_H F2,6P ₂	1.07 ± 0.05	1.21 ± 0.08
fold activation by F2,6P ₂	2.14 ± 0.10	2.42 ± 0.08

Results are means $\pm$ SEM, $n=3-6$ different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P.

^a - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Table 9. Kinetic properties of F_{1,6}P₂ from hepatopancreas of winter and summer *C. virginica*.

	Winter	Summer
S _{0.5} F6P, mM	1.50 ± 0.13	2.08 ± 0.13 ^a
n _H F6P	1.37 ± 0.11	2.43 ± 0.20 ^a
K _a AMP, mM	0.033 ± 0.002	0.085 ± 0.010 ^a
n _H AMP	1.28 ± 0.07	1.32 ± 0.05
fold activation by AMP	2.27 ± 0.09	3.80 ± 0.41 ^a
K _a F2,6P ₂ , μM	1.54 ± 0.25	1.47 ± 0.06
n _H F2,6P ₂	1.18 ± 0.02	1.08 ± 0.03
fold activation by F2,6P ₂	2.01 ± 0.18	3.83 ± 0.24 ^a

Results are means ± SEM, n=3-9 different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P

^a - Significantly different from the corresponding winter values by Student's t-test (2-tailed), P < 0.05.

Table 10. Kinetic properties of PFK from phasic adductor of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ F6P, mM	1.13 ± 0.18	2.17 ± 0.26^a
n_H F6P	1.50 ± 0.09	1.36 ± 0.10
K_a AMP, mM	0.052 ± 0.011	0.033 ± 0.002
n_H AMP	1.05 ± 0.04	1.26 ± 0.04^a
fold activation by AMP	2.96 ± 0.36	2.43 ± 0.09
K_a F2,6P ₂ , μ M	1.77 ± 0.61	1.90 ± 0.24
n_H F2,6P ₂	1.14 ± 0.06	1.11 ± 0.05
fold activation by F2,6P ₂	2.87 ± 0.08	2.66 ± 0.24

Results are means $\pm$ SEM, $n=3-7$ different animals. All parameters were determined at 0.1 mM Mg.ATP. Winter and summer K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 11. Kinetic properties of PFK from catch adductor of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ F6P, mM	1.75 ± 0.22	1.94 ± 0.05
n_H F6P	1.23 ± 0.04	1.75 ± 0.04^a
K_a AMP, mM	0.028 ± 0.004	0.055 ± 0.006^a
n_H AMP	1.73 ± 0.21	1.53 ± 0.1
fold activation by AMP	1.85 ± 0.15	2.67 ± 0.18^a
K_a F2,6P ₂ , μ M	1.93 ± 0.34	0.89 ± 0.19^a
n_H F2,6P ₂	1.05 ± 0.02	1.16 ± 0.09
fold activation by F2,6P ₂	2.08 ± 0.21	2.69 ± 0.22

Results are means $\pm$ SEM, n=3-6 different animals. All parameters were determined at 0.1 mM Mg.ATP. Winter and summer K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P.

a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 12. Kinetic properties of PK from gill of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ PEP, mM	0.45 ± 0.04	2.46 ± 0.23^a
n_H PEP	1.45 ± 0.17	2.01 ± 0.12^a
I_{50} L-alanine, mM	0.28 ± 0.01	0.076 ± 0.008^a
n_H L-alanine	1.43 ± 0.19	1.08 ± 0.05
K_a F1,6P ₂ , (μ M)	0.036 ± 0.01	0.94 ± 0.11^a
n_H F1,6P ₂	1.00 ± 0.002	1.49 ± 0.12^a
fold activation by F1,6P ₂	1.68 ± 0.07	16.6 ± 2.5^a

Results are means $\pm$ SEM, n=3-5 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values were determined at 1 mM PEP.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 13. Kinetic properties of PK from mantle of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ PEP, mM	0.50 ± 0.06	2.86 ± 0.15^a
n_H PEP	1.31 ± 0.06	1.56 ± 0.15
I_{50} L-alanine, mM	0.24 ± 0.01	0.18 ± 0.02^a
n_H L-alanine	1.31 ± 0.06	1.04 ± 0.04^a
K_a F1,6P ₂ , μ M	0.098 ± 0.023	0.81 ± 0.15^a
n_H F1,6P ₂	1.03 ± 0.03	1.22 ± 0.10
fold activation by F1,6P ₂	2.23 ± 0.07	20.7 ± 3.14^a

Results are means $\pm$ SEM, n=3-5 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values were determined at 1 mM PEP.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 14. Kinetic properties of PK from hepatopancreas of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ PEP, mM	1.27 ± 0.31	3.23 ± 0.35^a
n_H PEP	1.26 ± 0.15	1.30 ± 0.03
I_{50} L-alanine, mM	0.19 ± 0.02	0.15 ± 0.03
n_H L-alanine	1.29 ± 0.07	1.45 ± 0.06
K_a F1,6P ₂ , μ M	0.092 ± 0.016	0.61 ± 0.07^a
n_H F1,6P ₂	1.33 ± 0.13	1.27 ± 0.07
fold activation by F1,6P ₂	4.73 ± 1.53	17.98 ± 3.19^a

Results are means $\pm$ SEM, n=3-6 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values were determined at 1 mM PEP.

^a - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Table 15. Kinetic properties of PK from phasic adductor of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ PEP, mM	1.16 ± 0.26	4.17 ± 1.36^a
n_H PEP	1.25 ± 0.11	1.29 ± 0.14
I_{50} L-alanine, mM	0.14 ± 0.01	0.19 ± 0.03
n_H L-alanine	1.17 ± 0.08	1.06 ± 0.02
K_a F1,6P ₂ , μ M	0.011 ± 0.002	0.69 ± 0.08^a
n_H F1,6P ₂	1.27 ± 0.19	1.08 ± 0.05
fold activation by F1,6P ₂	2.15 ± 0.31	8.56 ± 1.10^a

Results are means $\pm$ S.E.M, n=3-9 different animals. All parameters were determined at 1 mM ADP. Winter I_{50} L-alanine values were determined at 1 mM PEP. Summer I_{50} L-alanine values were determined at 5 mM PEP. K_a F1,6P₂ values were determined at 1 mM PEP.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Table 16. Kinetic properties of PK from catch adductor of winter and summer *C. virginica*.

	Winter	Summer
$S_{0.5}$ PEP, mM	0.81 ± 0.22	3.24 ± 0.29^a
n_H PEP	1.57 ± 0.15	1.39 ± 0.14
I_{50} L-alanine, mM	0.16 ± 0.01	0.21 ± 0.06
n_H L-alanine	1.15 ± 0.07	1.04 ± 0.01
K_a F1,6P ₂ , μ M	0.044 ± 0.015	0.98 ± 0.11^a
n_H F1,6P ₂	1.00 ± 0.001	1.22 ± 0.12
fold activation by F1,6P ₂	2.01 ± 0.27	7.04 ± 0.86^a

Results are means $\pm$ SEM, n=3-8 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine values were determined at 5 mM PEP. K_a F1,6P₂ values were determined at 1 mM PEP.

^a - Significantly different from the corresponding winter value by Student's t-test (2-tailed), $P < 0.05$.

Figure 6. Representative plot of hydroxyapatite chromatography of PK from gill of winter and summer *C. virginica* eluted using a linear gradient of 0-300 mM KPi (dashed line). Peak activity of PK from winter animals (closed circles) eluted at approximately 110 mM KPi and from summer animals (open circles) at 210 mM KPi.

Figure 7. Representative plot of hydroxyapatite chromatography of PK from mantle of winter and summer *C. virginica* eluted using a linear gradient of 0-300 mM KPi (dashed line). Peak activity of PK from winter animals (closed circles) eluted at approximately 150 mM KPi and from summer animals (open circles) at 210 mM KPi.

Figure 8. Representative plot of hydroxyapatite chromatography of PK from phasic adductor of winter and summer *C. virginica* eluted using a linear gradient of 0-300 mM KPi (dashed line). Peak activity of PK from winter animals (closed circles) eluted at approximately 70 mM KPi and from summer animals (open circles) at 200 mM KPi

Figure 9. Representative plot of hydroxyapatite chromatography of PK from catch adductor of winter and summer *C. virginica* eluted using a linear gradient of 0-300 mM KPi (dashed line). Peak activity of PK from winter animals (closed circles) eluted at approximately 100 mM KPi and from summer animals (open circles) at 200 mM KPi

Figure 10. Activities of enzymes in *C. virginica* gill in winter and summer. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. * - Significantly different from the corresponding winter values by Student's t-test (2-tailed). $P < 0.05$

Figure 11. Activities of enzymes in *C. virginica* mantle in winter and summer. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. * - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Figure 12. Activities of enzymes in *C. virginica* hepatopancreas in winter and summer
Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. * - Significantly different from
the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$

Figure 13. Activities of enzymes in *C. virginica* phasic adductor muscle in winter and summer. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. * - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Figure 14. Activities of enzymes in *C. virginica* catch adductor muscle in winter and summer. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. * - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Figure 15. Activities of fructose-1,6-bisphosphatase in gill, mantle, hepatopancreas, phasic adductor, and catch adductor of winter and summer *C. virginica*. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. * - Significantly different from the corresponding winter values by Student's t-test (2-tailed), $P < 0.05$.

Figure 16. Soluble protein content (mg protein/gram wet weight) of the hepatopancreas and foot muscle of winter and summer *L. littorea* under aerobic, control conditions and after 6 days of anoxia exposure. Data are mean $\pm$ SEM, n=6 different animals. There were no significant seasonal differences between protein levels in either tissue.

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Figure 17. Soluble protein content (mg protein/gram wet weight) of gill, mantle, hepatopancreas, phasic adductor, and catch adductor of winter and summer *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Data are mean $\pm$ SEM, n=5 different animals. ^a - Significantly different from the corresponding winter control value by Student's t-test (2-tailed), $P < 0.05$.

RESULTS

Part II) THE EFFECTS OF PROLONGED ANOXIA

Effects of Anoxia on Phosphofructokinase and Pyruvate Kinase from *Littorina littorea*

Phosphofructokinase

Crude extracts of the foot and hepatopancreas were used to analyze the kinetic properties of PFK from summer and winter *L. littorea* held under aerobic, control conditions or given anoxia exposure for 6 days. Table 17 summarizes the kinetic properties of foot muscle PFK. Anoxia exposure elicited significant changes in enzyme properties in both seasons. With PFK from winter animals, anoxia exposure resulted in a decrease in the K_a value for AMP and an enhanced fold activation by this adenylate. The enzyme from summer animals also showed the significantly higher fold activation by AMP but F2,6P₂ activation parameters were oppositely affected: the K_a value increased by 2-fold and the fold activation was reduced, as compared with PFK from aerobic controls.

The effects of anoxia on the kinetic properties of PFK from hepatopancreas of winter and summer snails are shown in Table 18. Anoxia exposure significantly altered numerous PFK kinetic parameters in both seasons. In winter, anoxia exposure resulted in a significant increase (by 34%) in the $S_{0.5}$ for F6P as well as a decrease in the Hill coefficient for F6P. K_a for AMP was also increased by 3-fold, accompanied by a decrease in the Hill coefficient for AMP activation and a greater fold activation by AMP of the anoxic enzyme. Activator effects by F2,6P₂ were also altered by anoxia exposure, the Hill coefficient increasing significantly whereas the fold activation was reduced. PFK from hepatopancreas of summer animals did not show a change in substrate affinity as the result of anoxia exposure but the Hill coefficient for F6P was reduced. Unlike the enzyme from winter animals, the K_a for AMP and the fold activation produced by AMP were both

decreased during anoxia for the summer enzyme. These same two parameters were also significantly reduced when F2,6P₂ activation was assessed.

Pyruvate Kinase

Crude extracts of foot muscle (Table 19) and hepatopancreas (Table 20) were also used to analyze the effects of anoxia on the kinetic properties of PK from winter and summer *L. littorea*. Unlike the situation with PFK, the qualitative responses by foot PK to anoxia were the same in both seasons (Table 19). Anoxia exposure greatly reduced enzyme affinity for PEP; the $S_{0.5}$ for PEP rose by 6.4-7.4 fold and Hill coefficient increased significantly in both cases. The anoxic enzyme also showed much greater inhibition by L-alanine, the I_{50} dropping to only 5% in winter and 7% in summer of the corresponding aerobic values. PK from anoxic foot was also much less sensitive to F1,6P₂ activation, the K_a values rising by 61- and 74-fold for the winter and summer enzymes, respectively. The fold activation by F1,6P₂ was also greater for PK from anoxic tissues although the difference in this parameter between the aerobic and anoxic enzymes was more pronounced for the winter enzyme. Overall, the changes in PK kinetic properties during anoxia were consistent with a strong suppression of enzyme activity during anoxia in both seasons.

The effects of anoxia exposure on the properties of PK from hepatopancreas of winter *L. littorea* were similar to those seen for foot PK (Table 20). For winter PK, anoxia exposure resulted in reduced affinity for PEP substrate ($S_{0.5}$ increased by 64%), increased inhibition by L-alanine (I_{50} decreased to 30% of the aerobic value and n_H increased), and reduced activation by F1,6P₂ (K_a rose by 60%). By contrast, anoxia exposure affected only one kinetic parameter of PK from hepatopancreas of summer snails, causing a 70% increase in the K_a for F1,6P₂.

The Effect of Anoxia on the Maximal Activity of Enzymes from *L. littorea*

Crude extracts of *L. littorea* foot and hepatopancreas were used to assay the activities of 17 different enzymes involved in glycolysis, gluconeogenesis, amino acid metabolism, adenylate/phosphagen metabolism, or the anaplerotic reactions of the TCA cycle. The effect of prolonged anoxia exposure was determined by comparison to aerobic controls and documented for both winter and summer animals.

Foot muscle

Figure 18 summarizes the activities of enzymes from the foot muscle of *L. littorea* under control and anoxic conditions in the winter. Of the seven enzymes associated with glycolysis in marine molluscs (HK, PFK, aldolase, G3PDH, PK, PEPCK, and LDH), the only effect of anoxia exposure was a significant decrease in the activity of LDH. PEPCK activities were very low (winter control activity = 0.439 ± 0.043 U/gww) and the activity of FBPase did not change significantly from the control value of 0.0378 ± 0.00602 U/gww as a result of anoxia exposure in the winter. Maximal activities of the enzymes of the pentose phosphate shunt were also unaffected by anoxia. However, anoxia exposure resulted in a significant reduction in the activities of AlaAT and AspAT whereas MDH activity (NADP⁺-dependent) increased slightly. Winter control levels of SDH and ME were also very low, 0.182 ± 0.013 and 0.371 ± 0.015 U/gww, respectively. ArgK activities in foot muscle were extremely high but neither this enzyme nor AK were affected by anoxia.

The effect of anoxia exposure on the maximal activities of enzymes in foot muscle of summer *L. littorea* is shown in Figure 19. In the summer, anoxia exposure had minimal effects on enzyme activities. The activity of HK increased slightly from 0.751 ± 0.04 to 0.944 ± 0.07 U/gww but activities of other glycolytic enzymes were not altered as a result of anoxia. Anoxia exposure also resulted in a small increase in the maximal activity of 6PGDH but FBPase activity did not change (summer control activity of 0.0528 ± 0.00486 U/gww). As in the foot muscle of winter snails, anoxia induced a decrease (by 32%) in the

activity of AlaAT during the summer but AspAT activity did not change. SDH and ME activities in foot of summer snails were again low with control values of 0.192 ± 0.029 and 0.429 ± 0.039 U/gww, respectively.

Hepatopancreas

Enzyme activities from the hepatopancreas of winter *L. littorea* under control and anoxic conditions are shown in Figure 20. Of the glycolytic enzymes, anoxia exposure had dramatic effects on the activities of PK and LDH, causing a 55% decrease in the activity of PK and a 62% decrease in LDH activity. Winter control PEPCK activity was 0.148 ± 0.015 U/gww and was not affected by anoxia; FBPase activity was also unchanged as a result of anoxic exposure (0.124 ± 0.0166 U/gww in control animals). There were no significant changes in the activities of any other enzymes in hepatopancreas during anoxia. Activities of SDH and ME were also very low in hepatopancreas of winter snails, aerobic control values being 0.353 ± 0.058 and 0.0964 ± 0.0124 U/gww, respectively.

The situation in hepatopancreas of summer *L. littorea* was different (Figure 21). Anoxia exposure had no significant effect on the activities of any of the enzymes measured. Activities of PEPCK and ME were very low, with control values of 0.285 ± 0.0305 and 0.218 ± 0.027 U/gww, respectively. FBPase activity was also unchanged from a summer control activity of 0.155 ± 0.0140 U/gww as a result of anoxia.

Effects of Prolonged Anoxia on Enzyme Properties in *Crassostrea virginica*

Glycogen Phosphorylase

Table 21 shows the effect of prolonged anoxia (96 hours under N₂ atmosphere) on the total activity (active a + inactive b forms) of GP and the percent a in five tissues of winter *C. virginica*. Total GP activity was not affected by anoxia in any of the tissues. However, anoxia exposure resulted in significant decrease in the percentage of the enzyme in the active a form in hepatopancreas (by nearly 3-fold) and phasic adductor. Comparable measurements on summer animals revealed no significant effect of anoxia exposure on

either the total ($a + b$) GP activity or the % a in any of the tissues (see Table 5 for summer control data, anoxic data not shown).

Glycogen Synthetase

Table 22 shows the total activity (active G6P-independent I form + inactive G6P-dependent D form) of GS and the percentage of GS in the I form in the tissues of control and anoxic winter *C. virginica*. The percentage of the enzyme in the active form was not significantly affected by anoxia in any tissue. However, anoxia exposure resulted in nearly a 2-fold decrease in total GS in the hepatopancreas and, oppositely, led to a 30% increase in total GS activity in the phasic adductor. However, as for GP, anoxia exposure of summer oysters had no effect on either the total ($I + D$) activity or the % I of GS in any of the oyster tissues (see Table 6 for summer control data; anoxic data not shown)

Phosphofructokinase

The effects of anoxia exposure on the properties of PFK from winter and summer *C. virginica* were analyzed in crude extracts of five tissues. In general, anoxia exposure had very little effect on the kinetic properties of PFK in either season. Table 23 shows the results for gill PFK; the only effect of anoxia exposure was a 50% decrease in the K_a for $F_{2,6}P_2$ of the enzyme from winter animals. Anoxic stress had no effect on the kinetic properties of mantle PFK in either season (Table 24). PFK from hepatopancreas showed modification during anoxia in both seasons (Table 25); in both cases, anoxia exposure resulted in a decrease in affinity for F6P ($S_{0.5}$ increased by 42% in winter and 67% in summer animals). The enzyme from summer oysters also showed an accompanying decrease in the Hill coefficient for F6P as well as an increase in the Hill coefficient for AMP activation. Data for phasic adductor PFK are shown in Table 26. Anoxia exposure did not change the kinetic properties of the enzyme from winter oysters but with summer animals, anoxia led to a 2.3-fold increase in the K_a for AMP and a 40% decrease in the K_a

for F1,6P₂. Properties of catch adductor PFK, however, were unaffected by anoxia exposure in either season (Table 27).

Pyruvate Kinase

The S_{0.5} for PEP, the I₅₀ for L-alanine, the K_a and fold activation for F1,6P₂ were analyzed in five tissues from both winter and summer oysters to examine the effects of prolonged anoxia on the kinetic properties of PK from *Crassostrea virginica*. Table 28 shows that anoxia exposure had no effect on the kinetic properties of PK in the gill of summer oysters but the properties of winter PK changed substantially in anoxic animals. Overall, these anoxia-induced kinetic changes were consistent with the conversion of PK to a less active enzyme form during anoxia. Thus, under anoxia, the S_{0.5} for PEP increased by 4-fold whereas the I₅₀ for L-alanine decreased by one-half. In addition, the K_a for F1,6P₂ increased by 7-fold and was also accompanied by a 4-fold increase in the fold-activation caused by F1,6P₂. By contrast with the gill, however, anoxia exposure had no effect on the kinetic properties of mantle PK from either winter or summer oysters (Table 29). In the oyster hepatopancreas, anoxia exposure changed PK properties differently in two seasons. In the winter, anoxia exposure resulted in a slight decrease in the Hill coefficient for alanine inhibition, a 4-fold increase in the K_a for F1,6P₂ and nearly a 3-fold increase in the fold-activation by F1,6P₂ (Table 30). In summer animals, however, anoxic exposure altered the Hill coefficient for PEP of hepatopancreas PK and reduced the I₅₀ for L-alanine by 50%. In the phasic adductor, anoxia exposure resulted in a major change in the activation of the enzyme by F1,6P₂ from winter animals but did not affect the enzyme from summer animals (Table 31). Winter PK from anoxic phasic adductor showed a 26-fold increase in the K_a for F1,6P₂ as well as a 5-fold increase in the fold-activation caused by F1,6P₂, compared with the aerobic control enzyme. Table 32 shows the effect of anoxia on winter and summer catch adductor PK. In both seasons, anoxia stimulated an increase in the S_{0.5} for PEP (by nearly 5-fold in winter but a more modest

1.7-fold in summer). However, the additional effects of anoxia were only seen with winter PK: the K_a for F1,6P₂ increased by 21-fold along with an increase in the Hill coefficient and a 10-fold increase in the fold-activation by F1,6P₂.

Hydroxylapatite Chromatography of Pyruvate Kinase from *C. virginica* Tissues

Hydroxylapatite chromatography was carried out on extracts of tissues from aerobic and anoxic winter oysters to determine whether PK from *C. virginica* existed in multiple molecular forms. Extracts of gill, hepatopancreas, phasic adductor, and catch adductor were subjected to chromatography and, in all cases, PK activity in control and anoxic extracts eluted at distinctly different salt concentrations. The control and anoxic forms of gill PK eluted at approximately 110 and 140 mM KPi, respectively (Figure 22A). By contrast, the anoxic form of hepatopancreas PK eluted at 150 mM KPi, a lower value than the control peak which eluted at 220 mM KPi (Figure 23A). The major control PK peak from phasic adductor muscle eluted at 70 mM KPi with the anoxic peak detected at 210 mM KPi (Figure 24A). The catch adductor control and anoxic peaks eluted at 100 and 220 mM KPi, respectively (Figure 25A). PK in all four of these tissues had shown anoxia-induced kinetic changes. By contrast, PK from oyster mantle did not show any kinetic changes as a result of prolonged anoxic exposure and, correspondingly, PK in both control and anoxic extracts eluted at the same point, 150 mM KPi (Figure 26).

PK from the hepatopancreas and catch adductor of summer oysters demonstrated kinetic changes and these tissues were therefore analyzed by hydroxyapatite chromatography. The patterns of elution were similar to those observed for the winter tissues but the separation between the control and anoxic peaks was much narrower for both tissues. In hepatopancreas extracts, the anoxic form of PK eluted first at 200 mM KPi before the control peak which was eluted at 220 mM KPi (Figure 27). For catch adductor PK the control form was eluted at 200 mM KPi and the anoxic form at 250 mM KPi (Figure 28). The lack of clear separation for the control and anoxic peaks from the

summer tissues may be correlated with the minor kinetic changes observed and it is possible that anoxia-induced covalent modification of oyster PK did not occur during the summer.

***In Vitro* Dephosphorylation and Hydroxylapatite Chromatography**

To determine whether *C. virginica* PK could be modified by reversible phosphorylation, extracts of gill, hepatopancreas, phasic adductor, and catch adductor tissues from winter animals (all of which demonstrated a separation of aerobic and anoxic forms on hydroxylapatite chromatography) were subjected to *in vitro* conditions (incubation overnight at 4°C with 5 mM MgCl₂ in the absence of phosphatase inhibitors) that would promote dephosphorylation of the enzyme. After incubation, extracts were analyzed by hydroxylapatite chromatography to determine whether a shift in the elution peak had occurred. As shown in Figures 22B, 23B, 24B, and 25B, *in vitro* dephosphorylation of the enzyme resulted in a major shift in the elution patterns of the enzyme, in all cases changing from two separate peaks eluting at different salt concentrations to both peaks eluting at the same salt concentration. In all four cases the control peak, when subjected to dephosphorylating conditions, shifted its position, eluting at a salt concentration corresponding to the location of the anoxic peak. For three tissues this was a higher salt concentration whereas for hepatopancreas extracts, the control peak shifted to a lower salt concentration (although still corresponding to the position of the anoxic form).

The Effect of Anoxia on the Maximal Activity of Enzymes from *C. virginica*

The maximal activities of enzymes involved in either glycogenolysis, glycogenesis, the pentose phosphate shunt, amino acid metabolism, or anaplerotic reactions of the TCA cycle were measured in tissues of winter and summer animals held under control conditions or subjected to 96 hours of anoxia. Enzyme activities in winter and summer

oysters are presented separately for each tissue (Figures 29-38) and FBPase levels are presented in Figures 39 (winter) and 40 (summer) due to their extremely low activity relative to the other enzymes measured.

Gill

Figure 29 shows the maximal activities of enzymes in gill tissue from winter oysters under control or anoxic conditions. Of the eight enzymes associated with glycogen catabolism, HK, PFK, PK, and PEPCCK all showed significant decreases in activity as the result of prolonged anoxia. HK declined from 0.681 ± 0.042 to 0.500 ± 0.016 U/gww whereas PFK activity dropped by 31% from a control level of 1.57 ± 0.077 U/gww. PK showed the largest reduction in activity (from 2.65 ± 0.18 to 0.82 ± 0.081 U/gww) and PEPCCK decreased 32% from 0.664 ± 0.058 to 0.454 ± 0.029 U/gww. GS activity did not change but FBPase (Figure 39) increased significantly by 50% during anoxic stress. Of the two pentose phosphate cycle enzymes, only G6PDH decreased significantly (by 19%). Among the amino acid metabolizing enzymes, AlaAT activity decreased by 25% in anoxia whereas the activities of AspAT and SDH remained unchanged. ME activity declined slightly during anoxia, from 0.488 ± 0.024 to 0.399 ± 0.018 U/gww. Anoxia did not affect MDH activity using either coenzyme (NAD^+ or NADP^+) but both ArgK activity (38% decrease) and AK (32% decrease) changed during anoxia.

Figure 30 shows the comparable results for gill tissue from control and anoxic summer oysters. Anoxia had far fewer effects on the enzyme activities of summer animals. Of the glycolytic enzymes, only LDH activity decreased significantly from 0.321 ± 0.024 to 0.181 ± 0.039 U/gww. The only other enzyme to show a significant change in activity was AspAT which decreased by 32% during anoxia.

Mantle

During the winter, oyster mantle showed little metabolic reorganization during anoxia (Figure 31). However, two key changes were observed. The activity of PK was

reduced by 57% whereas PEPCK activity increased slightly from 0.408 ± 0.0503 to 0.620 ± 0.036 U/gww.

Anoxia exposure during the summer (Figure 32) similarly resulted in few changes in mantle. Again a strong reduction in PK activity was seen to only about one-third of the aerobic, control activity. The only other change during anoxia was a 50% decrease in the activity of SDH which was reduced from 0.112 ± 0.009 to 0.057 ± 0.01 U/gww.

Hepatopancreas

The maximal activities of several enzymes in hepatopancreas of winter animals changed as the result of anoxia exposure (Figure 33). As was also seen in the other two soft tissues, there was a strong depression of PK activity during anoxia (a 72% decrease). In addition, LDH activity decreased by 33% from 0.669 ± 0.067 to 0.442 ± 0.070 U/gww. Of the gluconeogenic enzymes, GS activity decreased significantly during anoxia but FBPase activity showed a sharp 3-fold increase. For the enzymes of the pentose phosphate, only 6PGDH activity changed, decreasing by 50% from 0.787 ± 0.099 to 0.375 ± 0.0259 U/gww. There were no other significant changes in enzyme activities in the hepatopancreas of winter oysters.

Figure 34 shows that anoxia exposure during the summer caused a 62% reduction in PK activity in the hepatopancreas. However, the only other significant change during anoxia was a decrease in AlaAT activity (by 15%).

Phasic Adductor

Figure 35 shows the enzyme activities in phasic adductor muscle from winter *C. virginica* under control and anoxic conditions. The only significant effect of anoxia on the glycolytic enzymes was a 77% decrease in the measurable activity of PK. G6PDH activity decreased by 22% during anoxia but the activities of two gluconeogenic enzymes, GS and FBPase (Figure 39) increased significantly during anoxia. FBPase activity rose from 0.047 ± 0.010 to 0.084 ± 0.011 U/gww.

The phasic adductor from summer oysters also showed a large decrease (72%) in PK activity during anoxia (Figure 36). However, activities of four other enzymes increased in the anoxic muscle. The 25% increase in aldolase activity was the first time that this enzyme had been observed to change during anoxia exposure in any tissue. G6PDH increased by 44% during anoxia along with a small increase in the activity of NAD⁺-dependent MDH. The activity of AK nearly doubled (96% increase) during anoxia exposure in the summer.

Catch Adductor

In the catch adductor muscle of winter *C. virginica* (Figure 37) anoxia resulted in a decrease in the activities of PFK and PK, by 56% and 77%, respectively. The only other change seen was a small decrease in NAD⁺-dependent MDH activity.

In the summer, anoxia also led to a large decrease in PK activity (by 63%) in catch adductor (Figure 38). In addition, AspAT also decreased significantly (by 23%) in anoxia. No other significant changes in enzyme activity were detected.

Effect of Prolonged Anoxia on Tissue Soluble Protein

Figure 16 shows the effects of season and anoxia exposure on the concentration of soluble protein in foot muscle or hepatopancreas of *L. littorea*. Neither experimental parameter had an effect on the protein content of either tissue.

Comparable results for the soluble protein content of *C. virginica* tissues are shown in Figure 17. Anoxia exposure led to a significant decrease in the protein content of the gill in winter animals although this effect was not observed in summer oysters. In the remaining tissues anoxia did not cause any change in the amount of protein in either season.

Table 17. Kinetic properties of PFK from foot muscle of aerobic, control and 6 days anoxic winter and summer *L. littorea*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ F6P, mM	1.94 ± 0.07	1.70 ± 0.11	2.06 ± 0.06	2.05 ± 0.06
n_H F6P	2.53 ± 0.23	2.37 ± 0.20	2.32 ± 0.07	2.44 ± 0.15
I_{50} Mg.ATP, mM	2.42 ± 0.09	2.74 ± 0.20	2.50 ± 0.16	2.08 ± 0.19
n_H Mg ATP	1.78 ± 0.09	1.87 ± 0.07	1.95 ± 0.09	1.89 ± 0.07
K_a AMP, mM	0.05 ± 0.01	0.02 ± 0.001^a	0.02 ± 0.002	0.03 ± 0.004
n_H AMP	1.12 ± 0.06	1.34 ± 0.12	1.79 ± 0.26	1.36 ± 0.07
fold activation by AMP	2.33 ± 0.22	3.20 ± 0.18^a	3.48 ± 0.24	4.76 ± 0.33^a
K_a F2,6P ₂ , μ M	0.57 ± 0.05	0.80 ± 0.20	0.28 ± 0.01	0.45 ± 0.08^a
n_H F2,6P ₂	1.12 ± 0.07	1.08 ± 0.08	1.22 ± 0.06	1.20 ± 0.10
fold activation by F2,6P ₂	3.78 ± 0.41	4.69 ± 0.40	5.73 ± 0.36	3.81 ± 0.45^a

Results are means $\pm$ SEM, n=5-8 different animals. All parameters (except I_{50} Mg.ATP) were determined at 0.1 mM Mg.ATP. I_{50} Mg.ATP was determined at 4 mM F6P. K_a AMP and K_a F2,6P₂ were determined at 1 mM F6P.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$.

Table 18 Kinetic properties of PFK from hepatopancreas of aerobic, control and 6 days anoxic winter and summer *L. littorea*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ F6P, mM	2.07 ± 0.09	2.77 ± 0.09^a	2.29 ± 0.07	2.37 ± 0.10
n_H F6P	2.56 ± 0.05	2.12 ± 0.05^a	3.04 ± 0.07	2.45 ± 0.18^a
I_{50} Mg.ATP, mM	3.24 ± 0.25	2.98 ± 0.16	1.28 ± 0.08	1.31 ± 0.08
n_H Mg.ATP	2.48 ± 0.29	2.44 ± 0.29	3.18 ± 0.09	3.65 ± 0.70
K_a AMP, mM	0.03 ± 0.006	0.10 ± 0.015^a	0.08 ± 0.01	0.04 ± 0.01^a
n_H AMP	1.35 ± 0.10	1.07 ± 0.05^a	1.35 ± 0.15	1.24 ± 0.13
fold activation by AMP	2.01 ± 0.09	3.63 ± 0.40^a	3.99 ± 0.31	2.67 ± 0.23^a
K_a F2,6P ₂ , μ M	0.45 ± 0.06	0.41 ± 0.03	1.38 ± 0.11	0.95 ± 0.07^a
n_H F2,6P ₂	1.02 ± 0.01	1.12 ± 0.03^a	1.17 ± 0.08	1.09 ± 0.06
fold activation by F2,6P ₂	7.80 ± 0.49	6.17 ± 0.34^a	7.58 ± 1.02	4.41 ± 0.51^a

Results are means $\pm$ SEM, $n=3-8$ different animals. All parameters (except I_{50} Mg.ATP) were determined at 0.1 mM Mg.ATP. I_{50} Mg.ATP was determined at 4 mM F6P. K_a AMP and K_a F2,6P₂ were determined at 1 mM F6P.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$

Table 19. Kinetic properties of foot muscle PK from aerobic control and 6 days anoxic winter and summer *L. littorea*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ PEP, mM	0.22 ± 0.01	1.41 ± 0.10^a	0.32 ± 0.03	2.37 ± 0.16^a
n_H PEP	1.06 ± 0.03	1.40 ± 0.09^a	1.06 ± 0.04	1.67 ± 0.06^a
I_{50} L-alanine, mM	2.42 ± 0.26	0.12 ± 0.01^a	1.16 ± 0.14	0.08 ± 0.01^a
n_H L-alanine	1.06 ± 0.04	1.26 ± 0.08	1.23 ± 0.07	1.18 ± 0.06
K_a F1,6P ₂ , μ M	0.04 ± 0.008	2.45 ± 0.45^a	0.05 ± 0.007	3.69 ± 0.36^a
n_H F1,6P ₂	1.00 ± 0.001	1.02 ± 0.007	1.00 ± 0.001	1.04 ± 0.02
fold activation by F1,6P ₂	2.68 ± 0.17	19.01 ± 0.54^a	4.52 ± 0.25	11.08 ± 0.45^a

Results are means $\pm$ SEM, n=4-11 different animals. All parameters were determined at 2 mM ADP. Winter I_{50} L-alanine was determined at 1 mM PEP. Summer I_{50} L-alanine was determined at 2 mM PEP. K_a F1,6P₂ values were determined at 0.1 mM PEP.

a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$.

Table 20. Kinetic properties of PK from hepatopancreas of aerobic, control and 6 days anoxic winter and summer *L. littorea*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
S _{0.5} PEP, mM	1.40 ± 0.11	2.29 ± 0.08 ^a	0.18 ± 0.006	0.19 ± 0.02
n _H PEP	1.37 ± 0.09	1.42 ± 0.11	1.09 ± 0.02	1.02 ± 0.008
I ₅₀ L-alanine, mM	1.18 ± 0.13	0.36 ± 0.03 ^a	0.83 ± 0.08	0.68 ± 0.08
n _H L-alanine	1.00 ± 0.001	1.20 ± 0.09 ^a	1.26 ± 0.12	1.30 ± 0.13
K _a F1,6P ₂ , μM	3.77 ± 0.47	6.07 ± 0.72 ^a	1.53 ± 0.16	2.59 ± 0.29 ^a
n _H F1,6P ₂	1.05 ± 0.04	1.04 ± 0.03	1.00 ± 0.001	1.07 ± 0.03
fold activation by F1,6P ₂	5.23 ± 0.26	7.14 ± 0.70 ^a	2.99 ± 0.21	2.54 ± 0.21

Results are means ± SEM, n=5-11 different animals. All parameters were determined at 2 mM ADP. Winter I₅₀ L-alanine was determined at 4 mM PEP. Summer I₅₀ L-alanine was determined at 0.5 mM PEP. Winter K_a F1,6P₂ was determined at 1 mM PEP. Summer K_a F1,6P₂ was determined at 0.1 mM PEP.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), P < 0.05.

Figure 18. Activities of enzymes in foot muscle of winter *L. littorea* under aerobic, control conditions and after 6 days of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 19. Activities of enzymes in foot muscle of summer *L. littorea* under aerobic, control conditions and after 6 days of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 20. Activities of enzymes in hepatopankreas of winter *L. littorea* under aerobic, control conditions and after 6 days of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 21. Activities of enzymes in hepatopancreas of summer *L. littorea* under aerobic, control conditions and after 6 days of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. No significant differences were detected between experimental groups.

Table 21. Total glycogen phosphorylase (**a** + **b**) activity and the percentage of the active **a** form in tissues of winter *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure.

	Winter Control IU/gww	Winter Anoxic IU/gww	Winter Control % a	Winter Anoxic % a
Gill	1.03 ± 0.13	1.25 ± 0.10	66.3 ± 12.1	74.2 ± 1.1
Mantle	1.08 ± 0.04	1.20 ± 0.09	88.5 ± 3.3	76.7 ± 3.6
Hepatopancreas	1.12 ± 0.20	0.87 ± 0.08	70.3 ± 5.8	25.6 ± 3.6 ^{a}
Phasic Adductor	3.21 ± 0.24	3.59 ± 0.15	81.0 ± 3.4	58.7 ± 5.7 ^{a}
Catch Adductor	2.00 ± 0.19	2.09 ± 0.12	74.2 ± 1.0	72.0 ± 2.3

Results are means ± SEM, n=3 different animals. Total activity was determined at 1.6 mM AMP for all tissues.

a - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.025$.

Table 22. Total glycogen synthetase (I + D) activity and the percentage of the active I form in tissues of winter *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure.

	Winter Control IU/gww	Winter Anoxic IU/gww	Winter Control % I	Winter Anoxic % I
Gill	0.60 ± 0.06	0.86 ± 0.16	76.3 ± 9.1	76.8 ± 8.1
Mantle	0.67 ± 0.06	0.53 ± 0.04	56.8 ± 9.0	69.0 ± 4.2
Hepatopancreas	1.12 ± 0.08	0.58 ± 0.08 ^a	63.9 ± 3.2	73.2 ± 5.7
Phasic Adductor	1.66 ± 0.05	2.14 ± 0.07 ^a	44.5 ± 9.7	36.8 ± 5.9
Catch Adductor	0.99 ± 0.10	0.76 ± 0.12	42.1 ± 6.3	53.1 ± 5.1

Results are means ± SEM, n=3 different animals. Total activity was determined at 5 mM G6P for all tissues. ^a - Significantly different from the corresponding control values by Student's t-test (2-tailed), P < 0.01.

Table 23. Kinetic properties of PFK from gill of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ F6P, mM	1.72 ± 0.19	*	1.94 ± 0.20	2.21 ± 0.24
n_H F6P	1.40 ± 0.10	1.44 ± 0.10	1.95 ± 0.12	2.01 ± 0.11
K_a AMP, mM	0.017 ± 0.005	0.018 ± 0.001	0.06 ± 0.005	0.049 ± 0.004
n_H AMP	1.37 ± 0.10	1.54 ± 0.21	1.23 ± 0.08	1.32 ± 0.07
fold activation by AMP	2.30 ± 0.19	1.92 ± 0.09	3.17 ± 0.30	3.15 ± 0.19
K_a F2,6P ₂ , μ M	2.58 ± 0.16	1.35 ± 0.26^a	1.14 ± 0.15	1.22 ± 0.12
n_H F2,6P ₂	1.09 ± 0.05	1.22 ± 0.11	1.16 ± 0.09	1.05 ± 0.05
fold activation by F2,6P ₂	2.30 ± 0.23	2.02 ± 0.10	3.01 ± 0.08	2.73 ± 0.23

Results are means $\pm$ SEM, n=3-9 different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P. * - No significant difference was detected between experimental groups so pooled control value combines n=3 control and n=2 anoxic samples.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$.

Table 24. Kinetic properties of PFK from mantle of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter	Summer Control	Summer Anoxic
$S_{0.5}$ F6P, mM	1.28 ± 0.20	2.22 ± 0.09	2.13 ± 0.14
n_H F6P	1.47 ± 0.06	1.87 ± 0.17	2.21 ± 0.11
K_a AMP, mM	0.024 ± 0.002	0.034 ± 0.002	0.038 ± 0.004
n_H AMP	1.13 ± 0.09	1.38 ± 0.12	1.43 ± 0.06
fold activation by AMP	1.95 ± 0.06	2.80 ± 0.15	2.68 ± 0.09
K_a F2,6P ₂ , μ M	1.44 ± 0.17	2.41 ± 0.37	2.32 ± 0.29
n_H F2,6P ₂	1.07 ± 0.05	1.21 ± 0.08	1.09 ± 0.06
fold activation by F2,6P ₂	2.14 ± 0.10	2.42 ± 0.08	2.48 ± 0.14

Results are means $\pm$ SEM, n=3-6 different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P. No significant difference was detected between control (n=3) and anoxic (n=2) experimental groups for winter animals so data for these were pooled.

Table 25. Kinetic properties of PFK from hepatopancreas of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
S _{0.5} F6P, mM	1.50 ± 0.13	2.13 ± 0.08 ^a	2.08 ± 0.13	3.48 ± 0.39 ^a
n _H F6P	1.37 ± 0.11	1.37 ± 0.07	2.43 ± 0.20	1.67 ± 0.13 ^a
K _a AMP, mM	0.033 ± 0.002	*	0.085 ± 0.01	0.076 ± 0.007
n _H AMP	1.28 ± 0.07	*	1.32 ± 0.05	1.75 ± 0.10 ^a
fold activation by AMP	2.27 ± 0.09	*	3.80 ± 0.41	3.24 ± 0.48
K _a F2,6P ₂ , μM	1.54 ± 0.25	*	1.47 ± 0.06	1.38 ± 0.14
n _H F2,6P ₂	1.18 ± 0.02	*	1.08 ± 0.03	1.24 ± 0.08
fold activation by F2,6P ₂	2.01 ± 0.18	*	3.83 ± 0.24	3.67 ± 0.28

Results are means ± SEM, n=3-9 different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P. * - No significant difference was detected between experimental groups so pooled control value combines n=3 control and n=2 anoxic samples.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), P < 0.05.

Table 26. Kinetic properties of PFK from phasic adductor of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ F6P, mM	1.13 ± 0.18	1.19 ± 0.05	2.17 ± 0.26	1.66 ± 0.12
n_H F6P	1.50 ± 0.09	1.40 ± 0.09	1.36 ± 0.10	1.67 ± 0.07
K_a AMP, mM	0.052 ± 0.01	0.031 ± 0.004	0.033 ± 0.002	0.075 ± 0.004^a
n_H AMP	1.05 ± 0.04	1.39 ± 0.18	1.26 ± 0.04	1.08 ± 0.08
fold activation by AMP	2.96 ± 0.36	2.06 ± 0.08	2.43 ± 0.09	2.51 ± 0.17
K_a F2,6P ₂ , μ M	1.77 ± 0.61	0.99 ± 0.36	1.90 ± 0.24	1.18 ± 0.14^a
n_H F2,6P ₂	1.14 ± 0.06	1.14 ± 0.05	1.11 ± 0.05	1.30 ± 0.14
fold activation by F2,6P ₂	2.87 ± 0.08	2.37 ± 0.023	2.66 ± 0.24	2.90 ± 0.23

Results are means $\pm$ SEM, n=3-7 different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$.

Table 27. Kinetic properties of PFK from catch adductor of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter	Summer Control	Summer Anoxic
$S_{0.5}$ F6P, mM	1.75 ± 0.22	1.94 ± 0.05	1.89 ± 0.11
n_H F6P	1.23 ± 0.04	1.75 ± 0.04	1.69 ± 0.12
K_a AMP, mM	0.028 ± 0.004	0.055 ± 0.006	0.056 ± 0.009
n_H AMP	1.73 ± 0.21	1.53 ± 0.10	1.27 ± 0.10
fold activation by AMP	1.85 ± 0.15	2.67 ± 0.18	2.58 ± 0.24
K_a F2,6P ₂ , μ M	1.93 ± 0.34	0.89 ± 0.19	0.87 ± 0.11
n_H F2,6P ₂	1.05 ± 0.02	1.16 ± 0.09	1.18 ± 0.08
fold activation by F2,6P ₂	2.08 ± 0.21	2.69 ± 0.22	2.18 ± 0.09

Results are means $\pm$ SEM, n=3-6 different animals. All parameters were determined at 0.1 mM Mg.ATP. K_a AMP and K_a F2,6P₂ values were determined at 1 mM F6P. No significant difference was detected between control (n=3) and anoxic (n=2) experimental groups for winter animals so data for these were pooled.

Table 28. Kinetic properties of PK from gill of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ PEP, mM	0.45 ± 0.04	1.84 ± 0.18^a	2.46 ± 0.23	2.49 ± 0.22
n_H PEP	1.45 ± 0.17	1.17 ± 0.08	2.01 ± 0.12	2.13 ± 0.31
I_{50} L-alanine, mM	0.28 ± 0.01	0.13 ± 0.01^a	0.08 ± 0.008	0.09 ± 0.005
n_H L-alanine	1.43 ± 0.19	1.53 ± 0.38	1.08 ± 0.05	1.15 ± 0.04
K_a F1,6P ₂ , μ M	0.04 ± 0.01	0.28 ± 0.03^a	0.94 ± 0.11	0.85 ± 0.12
n_H F1,6P ₂	1.00 ± 0.002	1.06 ± 0.05	1.49 ± 0.12	1.37 ± 0.11
fold activation by F1,6P ₂	1.68 ± 0.07	6.57 ± 0.96^a	16.6 ± 2.50	15.4 ± 2.30

Results are means $\pm$ SEM, n=3-5 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values were determined at 1 mM PEP.

^a - Significantly different from the corresponding winter control values by Student's t-test (2-tailed), $P < 0.05$.

Table 29. Kinetic properties of PK from mantle of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter	Summer Control	Summer Anoxic
$S_{0.5}$ PEP, mM	0.50 ± 0.06	2.86 ± 0.15	2.76 ± 0.18
n_H PEP	1.31 ± 0.06	1.56 ± 0.15	1.49 ± 0.08
I_{50} L-alanine, mM	0.24 ± 0.01	0.18 ± 0.02	0.17 ± 0.001
n_H L-alanine	1.31 ± 0.06	0.99 ± 0.054	1.06 ± 0.037
K_a F1,6P ₂ , μ M	0.098 ± 0.023	0.81 ± 0.15	0.91 ± 0.12
n_H F1,6P ₂	1.03 ± 0.03	1.20 ± 0.11	0.96 ± 0.10
fold activation by F1,6P ₂	2.23 ± 0.07	20.7 ± 3.1	14.6 ± 0.9

Results are means $\pm$ SEM, n=3-5 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values were determined at 1 mM PEP. No significant difference was detected between control (n=3) and anoxic (n=2) experimental groups for winter animals so data for these were pooled.

Table 30. Kinetic properties of PK kinase from hepatopancreas of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ PEP, mM	1.27 ± 0.31	3.41 ± 0.85	3.23 ± 0.35	4.61 ± 0.49
n_H PEP	1.26 ± 0.15	1.21 ± 0.06	1.30 ± 0.03	1.40 ± 0.02^a
I_{50} L-alanine, mM	0.19 ± 0.02	*	0.15 ± 0.03	0.07 ± 0.01^a
n_H L-alanine	1.29 ± 0.07	1.03 ± 0.01^a	1.45 ± 0.06	1.59 ± 0.34
K_a F1,6P ₂ , μ M	0.092 ± 0.016	0.39 ± 0.05^a	0.61 ± 0.07	0.73 ± 0.09
n_H F1,6P ₂	1.33 ± 0.13	1.56 ± 0.18	1.27 ± 0.067	1.33 ± 0.094
fold activation by F1,6P ₂	4.73 ± 1.53	12.26 ± 1.19^a	17.98 ± 3.19	29.55 ± 6.86

Results are means $\pm$ SEM, n=3-6 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values were determined at 1 mM PEP. * - No significant difference was detected between experimental groups so pooled control value combines n=3 control and n=2 anoxic samples.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$.

Table 31. Kinetic properties of PK from phasic adductor of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ PEP, mM	1.16 ± 0.26	*	4.17 ± 1.36	3.67 ± 0.75
n_H PEP	1.25 ± 0.11	*	1.29 ± 0.14	1.35 ± 0.16
I_{50} L-alanine, mM	0.14 ± 0.01	*	0.19 ± 0.03	0.21 ± 0.02
n_H L-alanine	1.17 ± 0.08	*	1.06 ± 0.02	1.12 ± 0.06
K_a F1,6P ₂ , μ M	0.011 ± 0.002	0.29 ± 0.07^a	0.69 ± 0.08	0.71 ± 0.09
n_H F1,6P ₂	1.27 ± 0.19	1.17 ± 0.17	1.08 ± 0.05	1.02 ± 0.02
fold activation by F1,6P ₂	2.15 ± 0.31	10.7 ± 2.12^a	8.56 ± 1.10	6.71 ± 0.58

Results are means $\pm$ SEM, n=3-6 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values determined at 1 mM PEP. * - No significant difference was detected between experimental groups so pooled control value combines n=3 control and n=2 anoxic samples.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$.

Table 32. Kinetic properties of PK from catch adductor of aerobic, control and 96 hours anoxic winter and summer *C. virginica*.

	Winter Control	Winter Anoxic	Summer Control	Summer Anoxic
$S_{0.5}$ PEP, mM	0.81 ± 0.22	3.74 ± 0.82^a	3.24 ± 0.29	5.62 ± 0.68^a
n_H PEP	1.57 ± 0.15	1.31 ± 0.08	1.39 ± 0.14	1.43 ± 0.14
I_{50} L-alanine, mM	0.16 ± 0.01	*	0.21 ± 0.06	0.15 ± 0.04
n_H L-alanine	1.15 ± 0.07	*	1.04 ± 0.01	1.15 ± 0.13
K_a F1,6P ₂ , μ M	0.044 ± 0.015	0.85 ± 0.04^a	0.98 ± 0.11	0.75 ± 0.15
n_H F1,6P ₂	1.00 ± 0.001	1.15 ± 0.08^a	1.22 ± 0.12	1.11 ± 0.05
fold activation by F1,6P ₂	2.01 ± 0.27	20.78 ± 3.67^a	7.04 ± 0.86	7.98 ± 1.29

Results are means $\pm$ SEM, n=3-8 different animals. All parameters were determined at 2 mM ADP. I_{50} L-alanine and K_a F1,6P₂ values were determined at 1 mM PEP.

* - No significant difference was detected between experimental groups so pooled control value combines n=3 control and n=2 anoxic samples.

^a - Significantly different from the corresponding control value by Student's t-test (2-tailed), $P < 0.05$.

Figure 22. Representative plots of hydroxyapatite chromatography of PK from aerobic (closed circles) and 96 hours anoxic (open circles) winter *C. virginica* gill eluted using a linear gradient of 0-300 mM KPi (dashed line). (A) Chromatography was carried out under conditions that stabilized the phosphorylation state of the enzyme (50 mM imidazole-HCl (pH 7.2), 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 15 mM 2-mercaptoethanol). The peak of PK activity from control animals eluted at approximately 110 mM KPi and anoxic PK eluted at 140 mM KPi. (B) Effect of *in vitro* incubation under conditions promoting dephosphorylation on the elution profile of control and anoxic gill PK from winter oysters (conditions were 20 mM KPi (pH 7.2), 5 mM MgCl₂, 15 mM 2-mercaptoethanol). After incubation the control and anoxic peaks eluted at approximately 150 mM KPi.

Figure 23. Representative plots of hydroxyapatite chromatography of PK from aerobic (closed circles) and 96 hours anoxic (open circles) winter *C. virginica* hepatopancreas eluted using a linear gradient of 0-300 mM KPi (dashed line). (A) Chromatography was carried out under conditions that stabilized the phosphorylation state of the enzyme (conditions were as in Figure 22). The peak of PK activity from anoxic animals eluted at approximately 150 mM KPi and control PK eluted at 220 mM KPi. (B) Effect of *in vitro* incubation under conditions promoting dephosphorylation on the elution profile of control and anoxic gill PK from winter oysters (conditions were as in Figure 22). After incubation the control and anoxic peaks eluted at approximately 150 mM KPi.

Figure 24. Representative plots of hydroxyapatite chromatography of PK from aerobic (closed circles) and 96 hours anoxic (open circles) winter *C. virginica* phasic adductor eluted using a linear gradient of 0-350 mM KPi (dashed line). (A) Chromatography was carried out under conditions that stabilized the phosphorylation state of the enzyme (conditions were as in Figure 22). The peak of PK activity from control animals eluted at approximately 70 mM KPi and anoxic PK eluted at 210 mM KPi (B) Effect of *in vitro* incubation under conditions promoting dephosphorylation on the elution profile of control and anoxic gill PK from winter oysters (conditions were as in Figure 22). After incubation the control and anoxic peaks eluted at approximately 160 mM KPi.

Figure 25. Representative plots of hydroxyapatite chromatography of PK from aerobic (closed circles) and 96 hours anoxic (open circles) winter *C. virginica* catch adductor eluted using a linear gradient of 0-300 mM KPi (dashed line). (A) Chromatography was carried out under conditions that stabilized the phosphorylation state of the enzyme (conditions were as in Figure 22). The peak of PK activity from control animals eluted at approximately 100 mM KPi and anoxic PK eluted at 220 mM KPi (B) Effect of *in vitro* incubation under conditions promoting dephosphorylation on the elution profile of control and anoxic gill PK from winter oysters (conditions were as in Figure 22). After incubation the control and anoxic peaks eluted at approximately 150 mM KPi.

Figure 26. Representative plot of hydroxyapatite chromatography of PK from aerobic (closed circles) and 96 hours anoxic (open circles) winter *C. virginica* mantle eluted using a linear gradient of 0-300 mM KPi (dashed line). Chromatography was carried out under conditions that stabilized the phosphorylation state of the enzyme (conditions were as in Figure 22). Peak PK activity from both control and anoxic animals eluted at approximately 150 mM KPi.

Figure 27. Representative plot of hydroxyapatite chromatography of PK from aerobic (closed circles) and 96 hours anoxic (open circles) summer *C. virginica* hepatopancreas eluted using a linear gradient of 0-350 mM KPi (dashed line). Chromatography was carried out under conditions that stabilized the phosphorylation state of the enzyme (conditions were 50 mM imidazole-HCl (pH 7.2), 100 mM NaF, 5 mM EDTA, 5 mM EGTA, and 15 mM 2-mercaptoethanol). The peak of PK activity from anoxic animals eluted at approximately 200 mM KPi and control PK eluted at 220 mM KPi.

Figure 28. Representative plot of hydroxyapatite chromatography of PK from aerobic (closed circles) and 96 hours anoxic (open circles) summer *C. virginica* catch adductor eluted using a linear gradient of 0-350 mM KPi (dashed line). Chromatography was carried out under conditions that stabilized the phosphorylation state of the enzyme (conditions were as in Figure 27). The peak of PK activity from control animals eluted at approximately 200 mM KPi and anoxic PK eluted at 250 mM KPi.

Figure 29. Activities of enzymes in gill of winter *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 30. Activities of enzymes in gill of summer *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$

Figure 31. Activities of enzymes in mantle of winter *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 32. Activities of enzymes in mantle of summer *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 33. Activities of enzymes in hepatopancreas of winter *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 34. Activities of enzymes in hepatopancreas of summer *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 35. Activities of enzymes in phasic adductor of winter *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 36. Activities of enzymes in phasic adductor of summer *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 37. Activities of enzymes in catch adductor of winter *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n= 5-6 * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$

Figure 38. Activities of enzymes in catch adductor of summer *C. virginica* under aerobic, control conditions and after 96 hours of anoxia exposure. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-6. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$

Figure 39. Activities of fructose-1,6-bisphosphatase in tissues of winter *C. virginica* under aerobic versus 96 hours anoxic conditions. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. * - Significantly different from the corresponding control values by Student's t-test (2-tailed), $P < 0.05$.

Figure 40. Activities of fructose-1,6-bisphosphatase in tissues of summer *C. virginica* under aerobic versus 96 hours anoxic conditions. Activities are IU/gram wet weight, means $\pm$ SEM, n=3-5. There were no significant differences in activity between control and anoxic conditions.

DISCUSSION

Part I) SEASONALITY

Seasonal changes extend to most aspects of metabolism in molluscs including carbohydrate, lipid, nucleic acid, protein, amino acid, and anaerobic metabolism (Gabbott, 1983). Regulatory enzymes catalyzing nonequilibrium reactions will naturally be important loci of control in an animal's adaptation to seasonal change due to their role in limiting and directing metabolic flux. Seasonal alterations in the regulatory enzymes could be accomplished by: (1) quantitative changes in the amount of enzyme present via changes in the processes of synthesis and degradation, (2) production of molecular variants of the enzymes (isozymes) possessing more appropriate kinetic properties, or (3) post-translational modification (i.e. protein phosphorylation or dephosphorylation) (Livingstone, 1981; Storey, 1992b). Different enzymes may be subject to different modes of seasonal regulation and which regulatory mechanism is used with each enzyme could also depend on other factors such as seasonal changes in the concentrations of enzyme substrates, allosteric effectors, and tissue-type. Seasonal changes are more likely to occur by these three mechanisms rather than the production of small molecular effectors since seasonal changes in flux occur over months and require long-term changes in control as opposed to limited, short-term regulation (Livingstone, 1981). In general, "coarse" control over metabolism for acclimatization to seasonal change is brought about by changes in the synthesis of regulatory enzymes and "fine" control over daily fluctuations is provided by the production of regulatory molecules (Livingstone, 1981; Gabbott, 1983). This section will discuss the seasonal changes in enzymes activities involved in glycogenolysis, glycogenesis, the pentose phosphate shunt, phosphagen/adenylate metabolism, amino acid metabolism, and the anaplerotic reactions of the TCA cycle in *C. virginica* and *L. littorea*. In both animals there were tissue-specific alterations in enzyme activities in response to the change of season from summer to winter.

Seasonal Change in Enzymes of Glycogenolysis

Glycogen Phosphorylase

GP has an important role controlling the mobilization of glycogen reserves into glucosyl units that can be catabolized by glycolysis. The enzyme exists in two interconvertible forms, an active a form and an inactive b form. In *C. virginica* total GP activity (a + b) was generally higher in all tissues during the winter (Table 5) and given the reliance of oyster tissues on endogenous glycogen reserves as the primary fuel for winter survival this result was not surprising. The lower GP activity in the summer was also coordinated with a decrease in the percentage of GP in the active a form. The most dramatic decrease in GPa activity occurred in the hepatopancreas, the primary glycogenic tissue in the oyster. Only the catch adductor showed an increase in GPa in summer although the total GP activity decreased by half. The decrease in GP activity during the summer complemented the increased GS activity (Table 6) and overall allowed the process of glycogenesis to proceed. GP activity is controlled by changes in the percentage of the active a form in both mammals (Cohen, 1980) and molluscs (Michaelidis and Athanasiadou, 1994; Brooks et al., 1991), but the seasonal differences in total GP suggest that processes of enzyme synthesis and degradation (which may also show seasonal patterns) also contribute to change the overall potential for glycogenolysis versus glycogenesis.

During the summer, *M. edulis* derives almost all of its G6P from exogenous glucose despite the extremely high levels of tissue glycogen but during the winter, glycogen breakdown increases and use of exogenous glucose decreases (Gabbott and Whittle, 1986). During the summer when food availability is high, nutrients are transferred from the digestive gland to the mantle where they can be used for "maintenance metabolism" and glycogen is conserved for winter catabolism and gametogenesis (Gabbott and Whittle, 1986). Summer *C. virginica* also probably follow a similar pattern although their seasonal glycogen levels are opposite to those of *M. edulis*. A reliance on exogenous

glucose for energy during the summer could also explain why summer GP activity was generally lower. With a large available food supply in the summer, glycogen catabolism is reduced but becomes important again in the winter. Seasonal changes in *L. littorea* GP activity were not analyzed.

Hexokinase

HK catalyzes the ATP-dependent phosphorylation of glucose and other sugars to produce G6P, the substrate for glycolysis. In *M. edulis*, HK is an equilibrium enzyme in the adductor muscle (which relies primarily on endogenous glycogen for energy) (Ebberink and de Zwaan, 1980) but a nonequilibrium enzyme in the mantle (Livingstone and Clarke, 1983). The activity of HK was significantly higher in the summer in the hepatopancreas and catch adductor of oysters (Figures 12 and 14). In the hepatopancreas (also known as the digestive diverticula) from summer oysters, the activity of HK was also higher than the activity of GP which adds further support to the idea that the metabolic fuel during the summer is primarily glucose coming from digested nutrients, rather than carbon from stored glycogen. This strategy would help conserve the glycogen stores which are being rebuilt during the summer (activities of GS were nearly 2-fold higher than HK in the hepatopancreas). A similar metabolic pattern may occur in the catch adductor but there was still a high activity of GP in this tissue during the summer. The other tissues (gill, mantle, phasic adductor) may have a high reliance on their own endogenous glycogen stores throughout the year as indicated by the lack of seasonal change in activities of HK and the relatively higher activities of GP in all cases (Figures 10, 11, and 13).

The activity of HK in *L. littorea* foot muscle or hepatopancreas did not change seasonally. The total activity of GP ($\bar{a} + \bar{b}$) in the foot of winter control *L. littorea* was 2.09 ± 0.15 U/gww (Russell and Storey, 1994a) which is approximately 2-fold higher than the activity of HK in the same tissue. The GP/HK ratio suggests a greater use of GP and, therefore, a reliance on endogenous glycogen stores for energy. However, in the

hepatopancreas of winter control *L. littorea* total GP only had an activity of 0.26 ± 0.015 U/gww (Russell and Storey, 1994a) which is half the amount of HK in this tissue. Unlike the foot, then, the hepatopancreas may be relying on exogenous glucose that is being produced by glycogenolysis in another tissue during the winter and transported via the hemolymph to the hepatopancreas.

Phosphofructokinase

PFK is an important regulatory enzyme whose activity typically reflects the rate of flux through glycolysis. Although there were no seasonal differences in the total activity of PFK from the gill, mantle, and phasic adductor of *C. virginica*, significant seasonal differences in the kinetic properties of PFK from these tissues were detected. In these three tissues summer and winter PFK possessed distinctly different properties with regards to their interactions with F6P substrate and the small molecular weight effectors, AMP and F2,6P₂ (Tables 7, 8, and 10). These different kinetic properties, which were tissue-specific, indicated that PFK was not regulated by changes in the amount of enzyme present but by seasonal adjustments in the properties of the enzyme, perhaps due to the production of season-specific enzyme forms. In the hepatopancreas and catch adductor of *C. virginica* (Tables 9 and 11) significantly different seasonal changes in the quantity of PFK were found and the kinetic studies also suggested the presence of seasonal forms of the enzyme. In the hepatopancreas the total activity of PFK increased in the summer, possibly to support increased glycolytic flux for biosynthesis and conversely in the winter the lower activity of PFK may contribute to rationing the stored glycogen. The higher $S_{0.5}$ (1.4-fold increase) and K_a AMP (2.6-fold higher) of summer PFK from this tissue may reflect an adaptation to higher glycolytic flux (the enzyme remains unsaturated) that could support a higher energy charge present during the summer such as has been found in the hepatopancreas of *Mytilus galloprovincialis* where the summer energy charge was 1.6 times greater than the winter value (Ibarguren *et al.*, 1990). These kinetic changes during

the summer would allow PFK in the hepatopancreas to retain a similar relative sensitivity to an activator under conditions where there was a stable increase in activator concentration. In the catch adductor, PFK activity was lower in the summer and the enzyme showed opposing regulation by AMP (less sensitive to activation) and F2,6P₂ (more sensitive). The decrease in the activity of PFK may accomplish a general reduction in glycolytic flux during the summer with kinetic changes to account for short-term fluctuations.

A seasonal change in the activity of *L. littorea* PFK was observed in the hepatopancreas but not in the foot (Figures 4 and 5). However, in both tissues there were kinetic differences between winter and summer PFK suggesting the presence of seasonal forms of the enzyme (Tables 1 and 2). The summer form of foot PFK is more active than the winter form and this may create a greater glycolytic capacity to support the greater physical activity of the muscle associated with summer foraging. In the hepatopancreas PFK activity increased during the summer but the enzyme might be less active *in vivo* since it was more sensitive to inhibition by ATP and less sensitive to activation by both AMP and F2,6P₂. An increased level of a less active enzyme may act as an important regulatory site during the summer to modulate glycolytic flux which, judging by the increased activity of all the glycolytic enzymes measured (except HK), would probably be higher during the summer than during the winter.

Aldolase

The activity of oyster aldolase was not significantly affected by seasonal change. However, although aldolase, as an equilibrium enzyme, is generally present in amounts much higher than PFK, the enzyme activity in all five oyster tissues was less than that of PFK (Figures 10-14). As has been shown in locust flight muscle (Storey, 1980), aldolase can assume a regulatory role in some invertebrate tissues due to its sensitivity to citrate inhibition, a function which is associated with PFK in vertebrate tissues. The insensitivity

of *C. virginica* PFK towards citrate (Storey, 1976) suggests that aldolase may also have this regulatory function in oyster tissues.

The activity of aldolase in *L. littorea* only changed seasonally in the hepatopancreas, the activity of the enzyme increasing during the summer as a part of a general increase in the activity of the glycolytic enzymes. Unlike the oyster, levels of aldolase in the tissues of the periwinkle were higher than PFK which is consistent with a role as an equilibrium enzyme.

Glycerol-3-Phosphate Dehydrogenase

Summer activities of G3PDH were significantly higher in the mantle and catch adductor of the oyster and G3PDH activity increased in the hepatopancreas of *L. littorea* but did not change in the foot muscle. Increased G3PDH activity could reflect elevated triglyceride synthesis in the summer or, if the alpha-glycerophosphate shunt is the mechanism of transferring cytosolic reducing equivalents into the mitochondria in this species (as opposed to the malate-aspartate shuttle), then it could also indicate higher summer shuttle activity consistent with higher rates of oxidative metabolism in mitochondria due to elevated temperature and elevated physical activity.

Pyruvate Kinase

Tissue-specific seasonal changes in the activity and/or kinetic properties of oyster PK were identified. In gill there was a decrease in total PK activity present in the summer (Figure 10) as well as changes in the kinetic properties of the enzyme that would create a less active enzyme (Table 12). PK from mantle also showed seasonal differences in enzyme kinetics (Table 13) but did not show a change in $V_{\max}$ activity (Figure 11). In the hepatopancreas there was a larger amount of PK present in summer (Figure 12) with season-specific kinetics (Table 14). There were no seasonal changes in maximal PK activities in the adductor muscles (Figures 13 and 14) but both enzymes showed

significantly different seasonal kinetic parameters (Tables 15 and 16). In all tissues summer PK showed a higher $S_{0.5}$ for PEP and there was a general decrease in sensitivity to activation by F1,6P₂. Modification of F1,6P₂ sensitivity may act to disengage PK from feed-forward activation by the product of PFK at the time when a high rate of gluconeogenic flux is desired. In addition, summer PK in two tissues was more strongly inhibited by L-alanine. These changes in PK during the summer may represent a mechanism to decrease glycolytic flux which would support the general gluconeogenic activity occurring in the oyster during the summer following spawning.

The maximal activity of PK did not change in foot muscle of *L. littorea* but seasonal differences in enzyme form were found (Table 3). Compared with winter PK, the properties of the summer form (higher $S_{0.5}$ PEP, reduced I_{50} alanine) should create a less active form *in vivo*. The maximal activity of PK from the hepatopancreas increased by 2-fold during the summer (Figure 5) and the summer form also showed properties that should make it more active *in vivo* including an 8-fold greater affinity for PEP as well as a 50% decrease in the K_a for F1,6P₂ although the enzyme was slightly more sensitive to alanine inhibition (Table 4). Activities of enzymes that can utilize the product of PK, pyruvate, also increased in the summer, LDH by nearly 3-fold and alanine aminotransferase by 10%. The increased activity of glycolysis in the periwinkle hepatopancreas during the summer could support a higher level of biosynthesis in preparation for winter.

Hydroxylapatite chromatography was able to separate the winter and summer forms of *C. virginica* PK from four tissues (Figures 6-9). When equilibrated in phosphate buffer hydroxylapatite has a net negative charge (Gorbunoff, 1985) and the clear separation of winter and summer PK activity peaks demonstrated that the winter and summer forms possessed significant charge differences in addition to their measurable kinetic differences. The seasonal difference in the overall charge of the enzyme could

result from the presence of distinct gene products (seasonal isozymes) or from differences in the phosphorylation state of the enzyme.

PEP-Carboxykinase

Under aerobic conditions PEPCK aids in the process of gluconeogenesis by converting oxaloacetate (OXA) to PEP but during anaerobiosis in molluscs PEPCK utilizes PEP to produce OXA (with the concomitant generation of GTP) resulting in the "PEP-branchpoint". However, for the aerobic mollusc (including both *C. virginica* and *L. littorea*) the high PK/PEPCK ratio ensures that there is no real competition for PEP and virtually all PEP will be converted to pyruvate rather than OXA. Oyster PEPCK activity showed seasonal changes in the gill, hepatopancreas, and phasic adductor. In the gill and hepatopancreas the decline in PEPCK activity during the summer may indicate decreased gluconeogenesis via this route and an increased reliance on glycogen production from ingested glucose (consistent with the increased level of GS in the gill). In the phasic adductor, PEPCK activity increased in the summer and thus PEPCK may be acting in an increased gluconeogenic capacity to increase carbohydrate reserves in this tissue. The activity of PEPCK changed seasonally only in the hepatopancreas of *L. littorea* where it increased during the summer. Similarly to oyster phasic adductor the increased PEPCK in this tissue may be contributing to glycogen production during the summer.

Lactate Dehydrogenase

The conversion of pyruvate to lactate is catalyzed by LDH. This enzyme is found in very low activities in *C. virginica* compared with the activities of the opine dehydrogenases, alanopine dehydrogenase or strombine dehydrogenase (Eberlee *et al.*, 1983). The only significant seasonal change in LDH activity occurred in the phasic adductor of summer oysters although, given the limited role of LDH in the oyster, it is doubtful that this decrease represents an important metabolic rearrangement.

However, lactate is a significant anaerobic end-product in marine gastropods (Kluytmans and Zandee, 1983). The activity of *L. littorea* LDH increased significantly in both tissues during the summer which would appear to be an adaptation to a high rate of anaerobic glycolysis. A higher level of physical activity in the summer during active foraging could require some amount of ATP generation by anaerobic glycolysis.

Seasonal Change in Enzymes of Glycogenesis

Glycogen Synthetase

GS contributes to glycogen biosynthesis by catalyzing the addition of uridine diphosphate glucose (UDP-glucose) to the nonreducing end of a glycogen branch. GS exists as both an inactive G6P-dependent D form and an active G6P-independent I form with conversion to the active I form being brought about by dephosphorylation of the D form (opposite to the activation of GP). Swift *et al.* (1988) noticed an activation of *C. virginica* GS by glucose and an inverse relationship between the level of endogenous glycogen and glycogenesis was demonstrated in the gills of the European oyster, *Ostrea edulis* (L-Fando *et al.*, 1972). The high levels of glycogen stored in the winter inhibited the incorporation of glucose into glycogen but as glycogen was consumed in the spring and summer the rate of glycogenesis increased (L-Fando *et al.*, 1972). This feedback inhibition of GS activity by high levels of tissue glycogen has also been noticed by Gabbott and Whittle (1986) in the mantle of *M. edulis* and inhibition of glycogen synthase phosphatase by glycogen has been proposed for the regulation of rat liver GS (Watts and Malthus, 1980).

The total activities (I + D) of GS in *C. virginica* tissues were generally higher in all tissues (except for phasic adductor) during the summer and this indicates higher glycogenic activity in the summer (Table 6). This is consistent with observations that marine bivalves actively accumulate glycogen during the summer to act as an endogenous fuel for winter survival and to support gametogenesis (Gabbott and Whittle, 1986, Swift

et al., 1988) Gabbott and Whittle (1986) found that the I activity of GS reached a maximum at the end of May in the mantle of *M. edulis* and in July the percentage of GS in the active I form declined rapidly and reached a minimum in December. They concluded that this decrease may be the result of inhibition by high glycogen levels. If the I form of GS had already begun to decline in July then that could explain why no significant differences were observed in any of the tissues between the %I of July (summer) and November (winter) oysters (Table 6). Gabbott and Whittle (1986) suggested that the I form was more sensitive to inhibition by glycogen and this would explain why the percentage of GS in the active I form is not significantly different from the winter levels but that there are still differences in the total GS activity. Seasonal changes in *L. littorea* GS activity were not measured.

Fructose-1,6-Bisphosphatase

The presence of FBPase in all tissues (Figure 15) suggests that glycogen synthesis goes on in all tissues which is generally consistent with a greater general reliance on endogenous reserves in tissues of many invertebrates. Whereas the oyster tissues showed an increased use of glycogen during the winter there was increased gluconeogenesis in the summer in both the gill and hepatopancreas. Seasonal differences in both the activities and kinetics of FBPase have been found in the mantle of *Mytilus galloprovincialis* and, as also seen here for *C. virginica*, there were no seasonal changes in FBPase from the adductor muscle (Barcia et al., 1992). Whereas the mantle is the primary tissue for the storage of glycogen in *Mytilus* sp., the hepatopancreas has a significant role in glycogen storage in the oyster (Gabbott, 1983). Seasonal changes in *L. littorea* FBPase activity were not detected. However, like the oyster, the presence of FBPase in the foot and hepatopancreas indicates that there is the potential for gluconeogenesis in both tissues and, like the oyster, the activity of FBPase in the hepatopancreas was much higher than that detected in the muscle tissue.

Seasonal Change in Enzymes of the Pentose Phosphate Shunt

Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase

The pentose phosphate pathway produces pentose phosphates for nucleotide synthesis and NADPH which is important in fatty acid and sterol synthesis. G6PDH channels carbon into this pathway but 6PGDH is the first committed step due to the large change in free energy of this reaction which is functionally irreversible *in vivo* due to the loss of CO₂. Consistent with the roles of these two enzymes, maximal activities of G6PDH in *M. edulis*, *O. edulis*, and *C. gigas* exceeded those of 6PGDH by a factor of about three (Gabbott and Head, 1980) which has also been seen in rat liver (Sapag-Hagar *et al.*, 1973). A similar discrepancy was also seen in all five tissues of *C. virginica* but interestingly did not occur in the tissues of *L. littorea* where G6PDH and 6PGDH activities were similar.

The activity of G6PDH increased in the hepatopancreas of the oyster during the summer. Gabbott and Head (1980) also found that activities of G6PDH in *M. edulis*, *O. edulis*, and *Crassostrea gigas* hepatopancreas were highest in summer when food availability was high. This increased activity may be related to increased biosynthesis of nucleotides or the production of new lipid reserves at this time since in oysters (including *C. virginica*) there is a positive correlation between glycogen and lipid accumulation under favorable conditions, just as under unfavorable conditions both decrease together (Walne, 1970). Livingstone (1981) observed that whereas there were large seasonal changes in G6PDH activities, there were no significant changes in the enzyme K_m values for G6P and NADP⁺. This may indicate the significance of a quantitative change (varying amounts of enzyme) as opposed to a qualitative change (production of isoenzymes) for regulation of seasonal G6PDH activity in the hepatopancreas of *M. edulis* and possibly *C. virginica* and other bivalves. G6PDH activity in the phasic adductor of *C. virginica* decreased during the summer and this may function to reduce flux through the pentose phosphate pathway and to channel G6P into other uses. The activities of G6PDH reported in this study are

similar to, but slightly higher than, those measured by Ballantyne and Berges (1991) at 10° C in several tissues of summer (June) *C. virginica* and these differences are probably due to variability in assay conditions or heterogeneity of the animals rather than seasonal differences between June and July oysters. The only change in the activity of *L. littorea* G6PDH was a decrease in foot muscle during the summer.

The activity of oyster 6PGDH was not seasonally altered in the gill, mantle, or hepatopancreas although the activity of 6PGDH increased in both adductor muscles during the summer suggesting increased flux through the pentose phosphate shunt in muscle tissue. There were no seasonal differences in the activity of 6PGDH in either tissue of *L. littorea*.

Seasonal Change in Enzymes of Amino Acid Metabolism

Alanine Aminotransferase and Aspartate Aminotransferase

In the gill, mantle, hepatopancreas, and phasic adductor of *C. virginica* there were no significant seasonal changes in the activity of either AlaAT or AspAT. However, in the oyster catch adductor muscle the activities of both transaminase activities were higher in the summer. In *L. littorea* the activity of AlaAT increased in the hepatopancreas during the summer. There were no seasonal differences in the activity of AspAT in either tissue of *L. littorea*.

Serine Dehydratase

Pyruvate and ammonia are formed from the breakdown of serine in a reaction catalyzed by SDH. Activities of SDH increased in the mantle and hepatopancreas of *C. virginica* during the summer but SDH was below the level of detection in the phasic and catch adductor muscles (activity < 0.04 U/gww). SDH activity increased significantly in the hepatopancreas of summer *L. littorea* but did not change in the foot. Based on their studies of *M. demissus*, Bishop *et al.* (1983) have suggested that the low activity of SDH

and its high K_m for serine preclude it from having a significant part in a transdeamination system although they do not eliminate the possibility of SDH having a role in serine catabolism. Increased levels of serine breakdown in the summer could provide pyruvate as a substrate for gluconeogenesis with the tissue distribution indicating that serine catabolism occurs mainly in the soft tissues of molluscs.

Malic Enzyme

ME catalyzes the oxidation of malate to pyruvate and this reaction direction is favored *in vivo* (Hochachka and Mustafa, 1973; Brodey and Bishop, 1992). Activities of ME were low and there were no significant seasonal changes in ME activity in any of the tissues of *C. virginica*. ME activity increased significantly in the hepatopancreas of summer *L. littorea* but did not change in the foot.

Malate Dehydrogenase

As shown in this study MDH is capable of using NADPH as a coenzyme although enzyme activity is reduced substantially in all tissues (to approximately 5% or 11% of the activity of NADH-dependent MDH in oysters and periwinkles, respectively). Activities of MDH in all five oyster tissues examined were similar (approximately 20 U/gww in winter and summer control conditions) and no seasonal differences were observed. In *L. littorea* the activity of MDH (using NADPH or NADH) remained unchanged in the hepatopancreas but showed a slight increase in the foot muscle during the summer.

Arginine Kinase

ArgK catalyzes a near-equilibrium reaction (Ebberink and de Zwaan, 1980) transferring a phosphate group from phosphoarginine to ADP to produce ATP. The high levels of phosphoarginine found in invertebrate muscle (i.e. adductor muscle of *Pecten maximus*) function as a energy reserve and the high activities of arginine kinase ensure that

ATP is rapidly regenerated from ADP whenever there is demand (Newsholme *et al.*, 1978). The increased activities of ArgK in the hepatopancreas of *C. virginica* during the summer may indicate that the capacity to mobilize phosphoarginine is enhanced in this tissue during the active summer months. The activity of ArgK in the hepatopancreas of *L. littorea* did not exhibit any seasonal changes. In the phasic adductor of *C. virginica* and the foot of *L. littorea* there was a decrease in ArgK activity during the summer. The foot muscle of the mobile snail has an ArgK activity nearly 2.5-times greater than the phasic adductor of the sessile oyster and this may indicate the relative importance of this additional energy source for these two animals.

Adenylate Kinase

AK is responsible for the conversion of two ADP molecules to ATP + AMP and this reaction is considered to be in near-equilibrium (Ebberink and de Zwaan, 1980). This mechanism supplies additional ATP when the ADP/ATP ratio becomes high. Activities of the enzyme were highest in the adductor muscles of the oyster and the foot muscle of the periwinkle which is consistent with findings in most animal species and with the role of the enzyme in buffering ATP levels during muscle exercise. There were no seasonal differences observed in AK activity in any of the *C. virginica* or *L. littorea* tissues.

Effect of Seasonal Change on Tissue Protein Content

The demonstration that soluble protein levels in the tissues of both *L. littorea* and *C. virginica* (Figures 16 and 17) remained constant in the summer and winter (except for a slight decrease in the hepatopancreas) indicates that the observed changes in the activities of specific enzymes are the result of specific targeting of individual enzymes and not a result of seasonal changes in the overall protein concentration.

Conclusions

Seasonal change in *L. littorea* had tissue-specific effects. In the foot muscle the activities of individual enzymes increased or decreased but there was no real overall change in metabolic activity. This contrasts with the hepatopancreas where there was a definite increase in the activity of metabolic enzymes during the summer. Increased flux through glycolysis and increased amino acid catabolism could support summer biosynthetic activity. Seasonal differences in kinetic properties were observed for PFK and PK in both tissues and this may indicate the presence of seasonal enzyme forms.

In the oyster tissue-specific seasonal differences were also detected with generally increased enzyme activity during the summer when active feeding is underway to replace glycogen reserves consumed during the winter and during spawning. During the summer glycogenesis was favored over glycogenolysis in all tissues with increased use of exogenous glucose in the hepatopancreas and catch adductor. The summer forms of PFK and PK were generally less active in all oyster tissues and the seasonally-induced changes in PK activity were substantially larger than those seen for PFK.

Livingstone (1981) raised the question as to whether the differences in metabolic make-up of marine bivalves seen over the course of the year were only transitory changes in response to changing environmental factors (for example, changes occurring due to fluctuating food availability) or whether the changes were more fundamental and associated with true seasonal differences in metabolism. Making this distinction may be impossible without additional information about the metabolic state of the animal. For example, based on his results for *M. edulis*, Livingstone (1981) concluded that changes in G6PDH in the mantle were related to the seasonal storage of lipid and the reproductive cycle because the specific activity of G6PDH was low when food availability was high and increased when lipid was being deposited in the gametes and when increased nucleic acid synthesis was needed for gametogenesis. However, in the hepatopancreas, changes in G6PDH activity could be associated with either possibility (i.e. related to nutritional

changes or metabolic reorganization) since activity decreased with the decrease in food supply (Livingstone, 1981).

From the present study it can be concluded that seasonal differences in enzyme activities are both animal- and tissue-specific and that the presence of these differences, along with the existence of seasonal enzyme forms, demonstrates that metabolism is a process undergoing continuous modification in response to yearly cycles of external events (e.g. temperature changes, food availability) or in anticipation of genetically "hardwired" cycles (e.g. gametogenesis).

Part II) THE EFFECTS OF PROLONGED ANOXIA

Intertidal marine invertebrates depress their metabolic rate during periods of anoxia so that energy requirements can be satisfied by the ATP production of fermentative pathways alone. In the hypometabolic state metabolic rate is reduced to only 5-10% of the normal resting aerobic rate at the same temperature and this prolongs the survival time of an animal enduring unfavorable environmental conditions (Storey and Storey, 1990). However, during this time energy can only come from an endogenous supply and the organism must be able to tolerate the accumulation of metabolic end-products (Hochachka and Somero, 1984; Storey and Storey, 1990). While there is some evidence of oxygen uptake from the air by respiratory surfaces (e.g. gill) during aerial exposure in some species, the fact that *C. virginica* and *L. littorea* were given anoxia exposure under N₂ gas precludes the possibility of aerobic pathways contributing to metabolism in these experiments. In marine invertebrates large tissue reserves of glycogen act as the primary endogenous fuel to sustain anaerobic metabolism. To improve energy yield from the fermentation of glycogen, alternative pathways to the classical glucose-to-lactate fermentation are used. Although the energy produced from these pathways (2-6 moles ATP/mole glucose) is only a fraction of the 37 ATP/mole available from the complete oxidation of glucose, the overall metabolic depression of the animal more than compensates for the reduction in energy production. Strong control over fuel catabolism limits the availability of ATP and pathway precursors which helps to regulate ATP-utilizing reactions but there must also be regulation at the point of entry of intermediates into complicated, energetically-expensive, biosynthetic pathways. Post-translational modification, via the addition or removal of covalently bound phosphate from proteins, is frequently employed as the mechanism that regulates the switch between the active and inactive states of enzymes and is one of the quickest and most powerful mechanisms available for controlling an enzyme's activity. As opposed to the limited role of allosteric effectors in seasonal acclimatization, there are numerous metabolites in the cell that are

able to activate or inhibit key enzymes during the adaptation to the anaerobic state. This section will discuss the effects of prolonged anoxia on the regulatory enzymes of glycogenolysis, glycogenesis, and the enzymes involved in anaerobic metabolism in two marine molluscs, the oyster *Crassostrea virginica* and the periwinkle *Littorina littorea*.

Glycogen Phosphorylase

The two interconvertible forms of GP (the active GP_a and the inactive GP_b) have both been purified from the adductor muscle of the Pacific oyster, *Crassostrea gigas* and have been shown to have a structure, mode of activation, and allosteric regulation similar to that found for mammalian GP (Hata *et al.*, 1993). When entering the anoxic state, the activity of GP in each tissue must be modified to coincide with the general anoxia-induced metabolic depression and to meet the ATP demands specific to that tissue. Accordingly, tissue-specific and seasonal differences in the control of GP during 96 hours of anoxia were noted in *C. virginica*. In the summer, anoxia did not cause any significant change in the activity of GP (data not shown, summer control activities are presented in Table 5). In the winter there were no changes in total phosphorylase activity ($\bar{a} + \bar{b}$) and only the hepatopancreas and phasic adductor muscle demonstrated a decrease in active phosphorylase during anoxia (Table 21). This regulation is similar to that seen in mammals where changes in GP are exerted by changing the percentage of the active form with the total phosphorylase activity typically remaining constant (Cohen, 1980). Similarly, in the ventricle of the freshwater mussel, *Anodonta cygnea*, the percentage of GP_a began to decline when the pO₂ fell below 90 mm Hg and continued as oxygen tension fell further whereas total GP ($\bar{a} + \bar{b}$) remained constant (Michaelidis and Athanasiadou, 1994). During anoxia the activity of GP_a also decreased in the foot muscle of the clam *Scapharca inaequivalvis* but not in *Venus gallina* and this difference may be correlated with the greater anoxia tolerance shown by *S. inaequivalvis* (Brooks *et al.*, 1991). For *C. virginica*, activities of GP and PFK were similar in all tissues and glycolytic flux was not altered at

the GP locus by anoxia in the gill, mantle, or catch adductor of winter animals. The hepatopancreas has a strong gluconeogenic and biosynthetic function and decreasing the availability of substrate for biosynthetic pathways may be a priority during anoxia-induced metabolic depression in this organ. Hence, the suppression of GP_a content in *C. virginica* might be adaptive in this light. The phasic adductor is only responsible for emergency, rapid closure of the shell and therefore, once the catch adductor has taken over keeping the valves shut, a high rate of glycogenolysis is not necessary and a decrease in GP_a would reduce glycolytic flux in this tissue. In the foot of winter *L. littorea* total GP decreased after 24 h anoxia but the %_a did not change and in the hepatopancreas both total GP and the %_a were unchanged from control activities (Russell and Storey, 1994a).

Phosphofructokinase

The next important regulatory enzyme in the pathway of glycogenolysis is PFK which catalyzes a nonequilibrium, irreversible reaction in the conversion of F6P to F1,6P₂ which is also the first ATP investment step of glycolysis (when G6P is derived from glycogen). PFK is an allosteric enzyme and is also a target for regulation by phosphorylation in many animals, including molluscs (Biethinger *et al.*, 1991; Michaelidis and Storey, 1991). PFK from *C. virginica* has been purified from the adductor muscle (phasic and catch muscles combined) and is a tetramer with a net molecular weight of 340,000 (Storey, 1976). AMP has been shown to greatly decrease the S_{0.5} for F6P and appears to be a necessary metabolite for activity in the adductor muscle whereas ATP was shown to be a weak inhibitor and citrate, a traditional inhibitor in mammalian systems, had no effect on oyster PFK (Storey, 1976). However, arginine-phosphate was found to be a novel and important inhibitor of PFK activity in the oyster adductor (Storey, 1976). During muscle work or in the early stages of shell valve closure, a reduction in arginine-phosphate levels, coupled with a rise in AMP, would permit PFK activation to support ATP generation by glycolysis (de Zwaan, 1983). Interestingly, arginine-phosphate did not

affect the activity of PFK from the mussel *Mytilus edulis* or the scallop *Placopecten magellanicus* (de Zwaan, 1983).

Fructose-2,6-bisphosphate (F2,6P₂) is a potent activator of PFK and inhibitor of FBPase and this metabolite regulates the balance between glycolysis and gluconeogenesis (Storey and Storey, 1990). Seasonal fluctuations in the level of F2,6P₂ have also been documented in the mantle, hepatopancreas, and posterior adductor muscle of *Mytilus galloprovincialis* where winter concentrations of F2,6P₂ are roughly 2-fold greater than summer levels (Ibarguren *et al.*, 1990). High levels of F2,6P₂ signal the presence of high amounts of glucose that can be used for biosynthesis. However, levels of this effector drop rapidly during times of starvation or anoxia when carbohydrate reserves must be rationed (Hue and Rider, 1987). During anoxia the dephosphorylation of PFK-2 produces a less active enzyme (Vazquez-Illanes and Ramos-Martinez, 1991) and the resulting decrease in F2,6P₂ production contributes to the overall metabolic depression (decreased PFK activity) since the activities of numerous anabolic pathways are extremely limited without carbohydrate substrate. As expected, the greatest reduction in the concentration of F2,6P₂ occurred in the biosynthetic tissues as shown by the rapid and strong decrease in the level of F2,6P₂ in the soft tissues of *O. edule*, *M. edulis*, and *L. littorea* in anoxia (Storey, 1985b).

During the winter, prolonged anoxia did not alter the kinetic properties of PFK in three tissues of *C. virginica* and the anoxia-induced changes seen in gill and hepatopancreas were quite minor and different in the two tissue types (Tables 23-27). Anoxia exposure resulted in an increased sensitivity of gill PFK to F2,6P₂ activation (although gill F2,6P₂ levels are typically sharply suppressed during anoxia) whereas hepatopancreas PFK showed a reduced affinity for F6P which is consistent with anoxia-induced metabolic depression. Anoxic exposure during the summer also affected hepatopancreas PFK, again reducing F6P affinity and also affecting the Hill coefficient for AMP activation. PFK in phasic adductor also showed reduced sensitivity to AMP

activation (although if AMP levels are elevated in anoxia, the net effect of this change on enzyme function *in vivo* is questionable) but increased sensitivity to F2,6P₂. The lack of major kinetic changes in oyster PFK during anoxia correlates with an analysis of PFK from another bivalve (*Geukensia demissus*) by DE-52 chromatography which found that PFK from gill, mantle, hepatopancreas, and adductor eluted in a single peak and anoxia exposure did not alter the elution profile (Russell and Storey, 1994b). This indicated that multiple forms of PFK were not present and suggested that the enzyme was not subject to anoxia-induced covalent modification. These observations support the idea that PFK may not be an important point of regulation during prolonged anoxia in bivalves. At the beginning of valve closure PFK regulates glycolytic flux but, as the duration of anoxia increases, PFK moves towards equilibrium while PK moves away from equilibrium (de Zwaan, 1983). After 1 hour and 4 hours of anoxia glycolytic intermediates before PFK (G1P, G6P, and F6P) accumulated (consistent with PFK regulation of glycolytic flux early in anoxia) whereas intermediates after PFK but before PK (F1,6P₂, DHAP, GAP, 3-PG, 2-PG, and PEP) showed an initial decrease but then accumulated to high levels after 24 hours of anoxia (Zandee *et al.*, 1979; Ebberink and de Zwaan, 1980) as flux through the PK locus became limiting.

During the winter, prolonged anoxia exposure caused PFK from the foot muscle of *L. littorea* (Table 17) to be slightly more sensitive to AMP activation which may make the enzyme more sensitive to the energy demands of the anoxic cell. Primary effects of anoxia on summer PFK were on the enzyme's sensitivity to F2,6P₂ activation (K_a increased). PFK from periwinkle hepatopancreas (Table 18) also showed seasonal differences in the enzyme's response to anoxia. Anoxia in the winter resulted in kinetic changes that produced a less active enzyme as indicated by the increased $S_{0.5}$ for F6P and K_a for AMP. Summer PFK, by contrast, was more sensitive to activation by both AMP and F2,6P₂ although the fold activation by both effectors was reduced. Whereas kinetic differences were detected for *L. littorea* PFK from foot muscle and hepatopancreas,

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analysis of PFK from both tissues of winter animals by DE-52 chromatography revealed only a single enzyme form in each tissue (tissue-specific isozymes) whose elution profile was unaltered by *in vivo* anoxia exposure or *in vitro* incubations under conditions promoting either phosphorylation or dephosphorylation (Russell and Storey, 1994a). Hence, the basis of either the seasonal or the anoxia-induced changes observed in *L. littorea* PFK does not appear to be changes in the phosphorylation state of PFK. Regulation of PFK by reversible phosphorylation has been observed in the tissues of the land snail *Otala lactea* (Whitwam and Storey, 1991) and the marine whelk *Busycon canaliculatum* (Whitwam and Storey, 1989) and by association with subcellular macromolecules in *B. canaliculatum* (Plaxton and Storey, 1986) but PFK from *L. littorea* and *C. virginica* may not undergo stress-induced covalent modification.

Pyruvate Kinase

The fermentation of aspartate followed by glycogen catabolism to provide energy during anoxia has been documented in several tissues of numerous marine invertebrates (de Zwaan and Dando, 1984). It was generally assumed that when both pathways were present there was a temporal separation of function with the aspartate-succinate pathway providing energy in the early stages of environmental anaerobiosis and the glycogen-succinate pathway becoming the primary energy-producing pathway later in anoxia as the small aspartate pool was consumed (Livingstone, 1991). However, observations by de Zwaan *et al.* (1983) of seasonal differences in the activity of PEPCK from *M. edulis* and by Eberlee *et al.* (1983) that aspartate has the potential to support anaerobic metabolism for at least 96 hours in some tissues of *C. virginica* seem to oppose the view of aspartate as a rapidly consumed, short-term energy source, at least in some species, and indicate that a switch at the PEP-branchpoint may occur much later in anoxia than previously thought. Thus, the decreased PK activity during anoxia may function not just to redirect carbon flow but, along with the suppression of other enzymes, to reduce overall metabolic

rate. The relative use of aspartate and glycogen during anaerobiosis has not been determined for marine gastropods.

Aspartate has been found to be a potent activator of PK with especially pronounced effects in anoxia-tolerant bivalves and gastropod molluscs, including *C. virginica* and *L. littorea* (Storey, 1986). In the aerobic state aspartate and other effectors (i.e. alanine, F1,6P₂) will regulate PK activity in response to demands for glycolysis or gluconeogenesis but in the anoxic state the progressive depletion of aspartate will gradually lessen the activation of PK and assist in the rerouting of carbon flow through PEPCK (Storey, 1986). Thus, there will not be an "either/or" situation at the PEP-branchpoint but rather PK and PEPCK can operate simultaneously and which reaction dominates will depend on the tissue and the degree of hypoxia/anoxia.

In adductor muscle of *M. edulis* seasonal differences in the S_{0.5} PEP of PK have been documented as well as seasonal differences in the response of PK to increasing anoxic duration (Holwerda *et al.*, 1984). Tissue-specific responses to anoxia were also noted; for example, gill and mantle PK displayed a much greater reduction in activity during the first 4 hours of anoxia than adductor muscle PK. This correlated with the observation that levels of aspartate and alanine did not change significantly in the mantle unlike the situation for adductor muscle (Ebberink *et al.*, 1979, Holwerda *et al.*, 1984).

Tissue-specific isozymes of oyster (*C. gigas*) PK, occurring in at least two forms (mantle and adductor muscle) and with distinct kinetic properties and electrophoretic mobilities, have been previously demonstrated (Mustafa and Hochachka, 1971). As with mammalian PK isozymes, the kinetic properties of the isozymes are designed for optimal function under the metabolic conditions of a particular tissue. In the oyster *C. virginica* both tissue-specific and seasonal differences in the modification of PK during anoxia were noted. During the winter, anoxia resulted in a general decrease in measureable PK activity in gill, hepatopancreas, phasic adductor, and catch adductor (Figures 29, 33, 35, 37) although the kinetic differences between the aerobic and anoxic forms of PK were tissue-

specific (i.e. all three kinetic parameters showed significant changes in gill but only one or two parameters changed in the other tissues) (Tables 28, 30-32). For gill PK, anoxia exposure reduced enzyme affinity for PEP, increased inhibition by alanine, and decreased sensitivity to F1,6P₂ activation, all effects that would create a less active enzyme form in the anoxic state. During the summer only PK from the hepatopancreas and catch adductor showed any significant differences in the kinetic properties between the control and anoxic forms of the enzyme. Gill, mantle, and phasic adductor showed no indication of the presence of aerobic and anoxic forms of PK in the summer.

Distinct winter versus summer differences were seen in the kinetic properties of PK from all tissues of *C. virginica*. These could be due to either seasonal isozymes or seasonal changes in the phosphorylation state of the enzyme (Tables 12-16). For oyster gill and phasic adductor the seasonal differences in the effects of anoxia on PK kinetics (Tables 28 and 31) strongly support the contention that the winter and summer forms of PK are seasonal isozymes. Thus, in the winter, PK in these tissues showed major changes in kinetic properties during anoxia exposure whereas the summer enzyme was unaffected by the stress. Furthermore, the changes in PK kinetic properties induced by anoxia exposure in winter did not create an enzyme that kinetically resembled the summer PK variant. For mantle also, the distinct winter/summer differences, with no effects of anoxia exposure on either variant (Table 29), again suggested that these are seasonal isozymes which differ distinctly in properties, but neither of which are subjected to anoxia-induced covalent modification. PK from oyster hepatopancreas (Table 30), however, showed elements of both behaviours. Anoxia exposure in the winter changed enzyme properties in a way that made the enzyme look more like the summer form but anoxia also affected summer and winter enzymes somewhat differently so they could be isozymes. However, in the catch adductor (Table 32) anoxia exposure in winter altered PK properties to those that were virtually identical with the properties of summer PK and hence, the winter and summer control forms may be phosphorylated variants of the same enzyme. Thus, it

appears that seasonal modifications to enzyme activity can occur by completely different mechanisms in different tissues of the same animal (i.e. by the production of isozymes versus post-translational modification) as shown by the tissue-specific seasonal modifications of PK in the oyster.

The initial demonstration that two separable forms of PK in the adductor muscle of *M. edulis* were interconvertible *in vitro* by a cAMP-dependent protein kinase (Siebenaller, 1979) has been followed by much work that has documented the importance of protein phosphorylation in regulating the activity of PK during anaerobiosis in numerous marine mollusc species (Plaxton and Storey, 1984a, 1984b; Holwerda *et al.*, 1989; Gaitanaki *et al.*, 1990). In general, it has been found that anoxia induced the phosphorylation of the enzyme and converted it to a less active enzyme form. Common features of the less active, anoxic variant of PK (compared with the aerobic form) include a reduced affinity for the substrate PEP, a greater sensitivity to inhibition by L-alanine, and reduced sensitivity to the activator F1,6P₂.

Anoxia-induced changes in PK properties in the tissues of winter *C. virginica* are consistent with those induced by protein phosphorylation. However, the lack of anoxia effects on the summer isozymes of PK in the gill, mantle, and phasic adductor may indicate that these are not modified by phosphorylation *in vivo* (at least in response to this stress). This seasonal difference in the ability of anoxia to induce changes in enzyme properties provides additional evidence of the existence of seasonal isozymes; an isoform that is subject to covalent modification occurs during the winter but is replaced by an enzyme that is not subject to reversible phosphorylation in the summer. The kinetic properties of summer PK in all of the tissues were generally consistent with reduced enzyme activity *in vivo* compared to winter PK (lower substrate affinity, lower I₅₀ for alanine, higher K_a for F1,6P₂) and this reduced activity would decrease glycolytic flux while supporting summer glycogenesis. Therefore, the summer isozymes of PK may

already be less active and require less regulation than their winter counterparts with the higher summer alanine levels sufficient to depress PK activity during anoxia.

Those tissues of *C. virginica* that demonstrated changes in PK kinetic properties as a result of anoxia exposure (gill, hepatopancreas, phasic adductor, and catch adductor) were used to test the hypothesis that anoxia had induced a protein kinase-mediated phosphorylation of the enzyme. Chromatography of winter PK from aerobic and anoxic animals revealed that PK existed in two forms which were eluted at different KP_i concentrations (Figures 22A, 23A, 24A, and 25A). Chromatographic separation of aerobic and anoxic forms of PK has also been reported for numerous marine animals such as the crab *Carcinus maenas* (Giles *et al.*, 1976), the mussels *M. edulis* (Holwerda *et al.*, 1983) and *M. galloprovincialis* (DeVoos and Holwerda, 1986), the limpet *Patella caerulea* (Michaelidis *et al.*, 1988; Gaitanaki *et al.*, 1990), and the periwinkle *L. littorea* (Russell and Storey, 1994a). For *C. virginica*, the aerobic (control) form of PK from gill, phasic adductor, and catch adductor eluted from the hydroxylapatite column at a lower KP_i concentration than did the anoxic form whereas the anoxic form of hepatopancreas PK eluted before the aerobic form. When hydroxylapatite is equilibrated in KP_i buffer it assumes a net negative charge (Gorbunoff, 1985). Therefore, these patterns of elution would suggest that aerobic PK from the gill and both adductor muscles was the phosphorylated form of the enzyme since the negative phosphate groups would decrease the enzyme's affinity for the negatively charged hydroxylapatite substrate. Chromatography of mantle PK from aerobic and anoxic winter oysters revealed no separation of enzyme forms (Figure 26) consistent with the absence of altered kinetics (Table 29). Mantle acted as a negative control and supported the results found in the other oyster tissues which linked differences in kinetic properties (induced by anoxia exposure) with charge separation on hydroxylapatite.

To determine if the aerobic and anoxic enzyme forms were really phosphorylated versus dephosphorylated variants of the enzyme, as has been found in other species

(Siebenaller, 1979, Plaxton and Storey, 1984a, 1984b, Whitwam and Storey, 1990a, 1990b), extracts of control and anoxic tissues were incubated under conditions which promoted dephosphorylation of the enzyme (5 mM MgCl_2 and the absence of protein phosphatase inhibitors). When subsequently chromatographed on hydroxylapatite, PK in extracts from aerobic, control gill, phasic adductor and catch adductors shifted to elute at a higher KPi concentration and eluted in a peak that overlapped with that of the anoxic enzyme form. The corresponding elution patterns of the anoxic enzyme forms, however, did not change. These results indicated that the control form of the enzyme was dephosphorylated by this *in vitro* treatment and, hence, that it was the control enzyme which was the high phosphate form (Figures 22B, 23B, 24B, and 25B). The lack of change in the elution pattern of the anoxic enzyme after incubation under dephosphorylating conditions indicated that this enzyme was already in the low phosphate form. The aerobic form of PK from the hepatopancreas also moved toward the anoxic peak (which eluted at the same KPi concentration in both cases) demonstrating that the charge of the enzyme was altered by dephosphorylation and that the aerobic form of the enzyme was also phosphorylated. However, other charges on the enzyme must cause its elution profile to be the opposite of that seen for PK in the other tissues. From these experiments, then, it was concluded that the aerobic form of PK in oyster tissues was the high phosphate form and that anoxia stress resulted in a dephosphorylation of the enzyme. This is exactly the opposite to the situation that has been reported in some other marine molluscs; in species such as *M. edulis*, *B. canaliculatum*, and *Anodonta cygnea* (Siebenaller, 1979; Plaxton and Storey, 1984b; Michaelidis and Athanasiadou, 1994) anoxia clearly induced the phosphorylation of PK although enzyme kinetic properties changed in the same manner as described here (i.e. K_m rises, I_{50} alanine decreases, K_a F1,6P_2 rises). However, dephosphorylation leading to decreased activity of PK has also been demonstrated in *L. littorea* (Russell and Storey, 1994a). Thus, the development of anoxia-induced PK inactivation as a means of both metabolic rate depression and PEP-

branchpoint control clearly always involves reversible phosphorylation control of the enzyme but in different species this has been achieved by either phosphorylation or dephosphorylation of the enzyme protein. Cohen (1980) concluded that phosphorylation inactivates enzymes involved in biosynthetic pathways but activates enzymes involved in degradation. *C. virginica* PK fits into this scheme since it is primarily involved in regulating the degradation of glycogen.

Those tissues from summer animals that demonstrated kinetic differences between control and anoxic PK were also subjected to hydroxylapatite chromatography (Figures 27 and 28). Aerobic and anoxic forms of PK from the hepatopancreas and catch adductor eluted at slightly different salt concentrations although the separation between the forms was much less than the separation observed for the winter forms and this may correlate with the relatively minor anoxia-induced kinetic changes observed in these tissues during the summer. The elution profile of the two tissues was the same as that seen for the winter tissues with the anoxic form of PK eluting before the control form in the hepatopancreas and the opposite pattern of elution for the catch adductor

In *L. littorea* PK activity during prolonged anoxia was much more rigorously controlled in the foot than in the hepatopancreas and seasonal differences in the degree of this control were only seen in the hepatopancreas. PK in the foot muscle of *L. littorea* showed a dramatic reduction in activity as a result of prolonged anoxia during both the winter and summer. Contributing to the decreased PK activity were an increased $S_{0.5}$ for PEP, increased sensitivity to inhibition by alanine, and decreased sensitivity to activation by $F_{1,6}P_2$, the changes being almost identical in both seasons (Table 19). However, the response of PK from *L. littorea* hepatopancreas was seasonally dependent (Table 20). In the winter 6 days of anoxia exposure resulted in large changes in enzyme properties (2-fold increases in $S_{0.5}$ F6P and K_a $F_{1,6}P_2$ and a 3-fold decrease in I_{50} alanine), all of which are consistent with a change to a less active enzyme form during anoxia. In the

summer, however, only the K_a for F1,6P₂ was modified during anoxia. This tissue-specific seasonal difference may indicate that PK is not a regulatory site for metabolic depression in this tissue during the summer or, like the anoxia-tolerant land snail *Helix pomatia* (Livingstone and de Zwaan, 1983), *L. littorea* may be more tolerant of anoxia during the winter. Observations of a stress-response by periwinkles (mucus secretion) and an increased mortality rate during summer anoxic exposure (Livingstone and de Zwaan, 1983) support this hypothesis.

The seasonally-dependent response to anoxia by PK from *L. littorea* hepatopancreas may be based on the presence of seasonal isozymes in this tissue. Summer PK showed hyperbolic PEP kinetics and a high PEP affinity, comparable to the non-regulatory form of PK in mammalian skeletal muscle. In addition, the general lack of anoxia effects on the summer enzyme suggest that this form may not be subject to reversible phosphorylation *in vivo*. The winter form, however, showed a high $S_{0.5}$ for PEP and sigmoidal PEP kinetics (characteristic of regulatory PK's such as the mammalian liver form which is subject to reversible phosphorylation) and also showed large changes in its kinetics during anoxia that suggested anoxia-induced covalent modification of the enzyme (either protein phosphorylation or dephosphorylation). Furthermore, the effects of anoxia made the kinetic properties of winter PK even more different than the kinetics of the summer form. By contrast, PK from the foot muscle of *L. littorea* appeared to be the same enzyme in both winter and summer. Although there were some minor winter-summer differences (Table 3), the relative effect of anoxia on winter and summer PK was virtually the same with anoxia making the kinetic properties of the winter and summer enzymes more similar. Furthermore, for foot PK, anoxia in both seasons had a major effect on enzyme properties and all properties changed in the same way in both seasons whereas for the hepatopancreas, the winter enzyme showed large anoxia-induced changes but the summer enzyme was largely unaffected by anoxia. Thus, the effect of prolonged anoxia on

PK activity is tissue-specific and seasonally-dependent in the hepatopancreas and the properties of the winter PK isozyme may enhance anoxia survival

The Effect of Prolonged Anoxia on Glycogenesis:

Glycogen Synthetase and Fructose 1,6-Bisphosphatase

The response of GS to anoxia, like that of GP, was tissue-specific and only the winter form of the enzyme was affected. However, unlike GP, which was controlled through decreases in the percentage of the enzyme in the active form, GS activity in the oyster during prolonged anoxia was controlled by changes in the total ($I + D$) amount of the enzyme. In order to depress metabolism during anoxic stress there should be a general decrease in the activity of GS since the use of ATP by anabolic pathways such as glycogen synthesis should be restricted when ATP availability is limiting. Furthermore, glycogen synthesis is typically restricted under conditions where glycogen is being utilized as a major fuel and such is the case during anoxia in molluscs. However, in the gill of *O. edulis*, L-Fando *et al.* (1972) found that although glycogen synthesis was inhibited by total anoxia (100% N_2 atmosphere) the incorporation of glucose into glycogen was the same under O_2 contents ranging from 5-100% of an atmospheric $O_2:N_2$ mixture. In *C. virginica*, the total activity of GS decreased by half during anoxia in the hepatopancreas, which is an important biosynthetic organ and also a storage depot for glycogen. The total activity of GS in the phasic adductor increased slightly during anoxia. In *Geukensia demissus* a similar decrease in total GS ($I + D$) was seen in anoxic hepatopancreas although adductor muscle GS activity was not altered by anoxia and mantle % I decreased to counteract an increase in the total mantle GS (Russell and Storey, 1994b). Seasonal differences in the response of *L. littorea* GS to anoxia were not assessed.

FBPase has been purified from the mantle of *M. galloprovincialis* (Barcia *et al.*, 1991) and phosphorylation by a cAMP-dependent protein kinase results in a more active enzyme (Barcia *et al.*, 1993). Contrary to the expectation of decreased anabolic functions

during anaerobiosis, FBPase activity increased in the gill, hepatopancreas, and phasic adductor from anoxic winter oysters (Figure 39). F2,6P₂ is a known inhibitor of FBPase and the decrease in the concentration of this effector during anoxia (Storey, 1985b) would relieve the inhibition of FBPase. Hence, this might account for the increased activity of this enzyme during anoxia. In the summer the activities of FBPase did not change significantly as a result of anoxia exposure. FBPase activity was found in the foot and hepatopancreas of *L. littorea* indicating gluconeogenic potential in both of these tissues although the enzyme activity was not affected by anoxia in either tissue or either season.

Effect of Prolonged Anoxia on the Maximal Activity of Enzymes

Given the limited time frame of response to anoxic stress and the importance of metabolic depression during anoxia it is unlikely that changes in enzyme maximal activity would result from changes in enzyme amount (via protein synthesis and/or degradation) or from the *de novo* synthesis of a new isozyme, unlike seasonal acclimatization. Anoxia-induced metabolic depression is more likely to be accomplished by changes in the levels of allosteric effectors (e.g. decreased F2,6P₂) or through the covalent modification of proteins (i.e. phosphorylation or dephosphorylation). However, the survey of the maximal activities of enzymes from aerobic versus anoxic tissues did reveal selected changes in a few enzymes suggesting that the activities of some enzymes may be modified by differential rates of synthesis versus degradation during anoxia.

The Glycolytic Pathway

Hexokinase

In both seasons HK from *C. virginica* was less active than both GP and PFK in all tissues and the only effect of anoxia exposure was a significant decrease in HK activity in the gill of winter oysters. Since the activity of HK under conditions of saturating substrate is indicative of the maximum potential for glucose utilization (Livingstone and Clarke,

1983), the lower activities of HK, compared with GP, would seem to indicate a general reliance on endogenous glycogen catabolism during anoxia to provide energy for tissue metabolism. Each tissue appears to function autonomously with its own internal reserves of glycogen and aspartate as substrate and with little or no intertissue transport of fuels (e.g. as glucose) such as occurs in vertebrates. Activities of HK were similar in all tissues and were in agreement with measurements by Zammit and Newsholme (1976) for HK from the phasic adductor of *O. edulis* and within the same order of magnitude as those obtained by Ballantyne and Berges (1991) for several *C. virginica* tissues assayed at 10°C.

For *L. littorea* the only change in the activity of HK was an increase in activity in foot muscle during anoxia of summer animals. This could reflect an increased reliance on blood glucose (increased due to summer feeding) to sustain foraging metabolism when food availability is high during the summer and these animals are known to be actively accumulating glycogen (Livingstone and de Zwaan, 1983).

Phosphofructokinase, Aldolase, and Glycerol-3-Phosphate Dehydrogenase

PFK is an important regulatory enzyme in the early stages of anoxia but loses this regulatory role as the duration of anoxia increases (de Zwaan, 1983). During the winter maximal PFK activity decreased (gill and catch adductor) or remained constant as a result of anoxic stress and there was no correlation between the observed kinetic changes and changes in enzyme activity except in gill where decreased sensitivity to F_{2,6}P₂ occurred concomitant with a decrease in maximal activity. During the summer there was no change in the maximal activity of PFK as a result of anoxia in any of the tissues. PFK activities did not change significantly in the foot or hepatopancreas of *L. littorea* as a result of anoxic stress in either season.

Prolonged anoxia did not induce a change in aldolase maximal activity in any of the tissues from winter animals and the only change observed in summer animals was an increase in activity in the phasic adductor to match the activity of PFK. Aldolase activity

did not change significantly in the foot or hepatopancreas of *L. littorea* as a result of anoxic stress in either season

Although not directly involved in the pathway of glycolysis dihydroxyacetone phosphate, a glycolytic intermediate, is the substrate for G3PDH. The activity of G3PDH did not change as a result of anoxic stress in either season or in any of the tissues of both *C. virginica* and *L. littorea*.

Pyruvate Kinase

During the winter anoxia resulted in a dramatic decrease in the maximal activity of PK in all oyster tissues (Figures 29, 31, 33, 35, and 37). The importance of regulating PK during the winter was emphasized by the coordination of kinetic changes induced by the dephosphorylation of the enzyme with the general decrease in the activity of the enzyme (either through decreased synthesis or increased degradation). There was also a general decrease in the activity of PK in the tissues of the oyster during the summer although the kinetic changes affecting PK activity during the summer were limited

A 2.25-fold decrease in the activity of PK in hepatopancreas of winter *L. littorea* was the only anoxia-induced change in PK activity seen in this species (Figure 20). This decrease in the activity of PK occurred concomitantly with large phosphatase-induced kinetic changes in the enzyme (Table 20) and this dual regulation emphasizes the importance of regulating this enzyme during anoxia stress.

PEP-Carboxykinase

PK and PEPCK both compete for the same substrate at the "PEP-branchpoint" and the reduction of PK activity as anoxia progresses is generally seen as allowing an increased flux of carbon to be directed through the PEPCK reaction and subsequently onwards to succinate. In general, there is a correlation between the presence of an allosteric PK and the presence of PEPCK because, when PEPCK is absent, PK shows hyperbolic kinetics

such as those seen in scallop muscle or skeletal muscle (de Zwaan, 1977). The presence of PEPCK activity indicated that a glucose-succinate pathway was present in all tissues of oysters and snails. Activities of PEPCK decreased or remained the same during anoxia in oysters although it is interesting to note that PEPCK activities were much closer to PK activities during the winter than during the summer. Activities of PEPCK were low in both tissues of *L. littorea* and the activity of this enzyme was not altered by anoxia in either season.

Lactate Dehydrogenase

LDH is found in very low activities in *C. virginica* compared to the activities of alanopine dehydrogenase or strombine dehydrogenase (Eberlee *et al*., 1983). Given the limited role of LDH in the metabolism of *C. virginica* it is not surprising that the already low activities either remained the same or decreased during anoxia in both seasons. Given the strict suppression of PK activity that occurs as anoxia progresses and the initial production of alanine by transamination with pyruvate by AlaAT there is not much substrate available for LDH. When anaerobic end-product accumulation in sessile marine bivalves (*M. edulis*, *O. edulis*, and *Crassostrea angulata*) was compared to marine gastropods (*L. littorea*, *Buccinum undatum*, and *Crepidula fornicata*) it was found that although some lactate accumulated in all animals a much larger amount was found in the marine gastropods (Kluytmans and Zandee, 1983).

In contrast to the low activities of LDH in *C. virginica* and the limited role of this enzyme in marine bivalves, LDH was present in extremely high activities in the tissues of *L. littorea* and has an important role in the anaerobic metabolism of marine gastropods with activities comparable to alanopine dehydrogenase (the only opine dehydrogenase present in these snails) in the foot muscle (Plaxton and Storey, 1982). LDH activity decreased in the foot and hepatopancreas during anoxia exposure during the winter and this may contribute to an overall metabolic depression and act to minimize the

accumulation of lactate. Summer activities of LDH were unchanged after 6 days of anoxia in both tissues. D-lactate was also found to accumulate in *L. littorea* after 72 hours of anoxia (Churchill and Storey, 1994).

Pentose Phosphate Pathway

Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase

Decreasing flux through the complex and energetically expensive pentose phosphate pathway would contribute to the overall metabolic depression required for prolonged anaerobic survival. Activities of G6PDH in *C. virginica* either decreased significantly or remained the same in both seasons and all tissues after 96 hours of anoxia except for an increase in G6PDH activity in the phasic adductor of summer animals. G6PDH did not change significantly in the foot or hepatopancreas of *L. littorea* as a result of anoxic stress in either season.

The already low activities of 6PGDH in *C. virginica* were unaltered by anoxic stress although there was a significant decrease in 6PGDH in the hepatopancreas of winter oysters which would help contribute to the overall metabolic rate depression in this tissue. 6PGDH did not change significantly in the hepatopancreas of *L. littorea* as a result of anoxic stress in either season but the activity of 6PGDH in the foot increased significantly in the summer only.

Amino Acid Metabolism

Alanine Aminotransferase and Aspartate Aminotransferase

Amino acids in marine molluscs serve a dual function. In addition to contributing to the osmotic balance of the cells, certain amino acids can be catabolized or accumulated to aid in the survival of environmental anaerobiosis. AlaAT, also known as glutamate-pyruvate transaminase, catalyzes the formation of alanine and α -ketoglutarate (α -KG) from pyruvate and glutamate and has an important role in supplying aspartate

aminotransferase (AspAT) with α -KG to allow the conversion of aspartate to oxaloacetate (also regenerating glutamate) which can then be catabolized anaerobically to succinate. Hence, these coupled transaminase reactions are important for mobilizing aspartate reserves as a fuel supply for anaerobic fermentation in molluscan tissues. AlaAT activity remained high during anoxia and only decreased significantly in the gill of winter oysters and the hepatopancreas of summer animals. AlaAT may be required in the muscles for hydrogen translocation during anoxia and levels must therefore remain high but the decreased alanine production during prolonged anoxia in the non-muscular tissues would allow AlaAT activities to decrease. However, alanine is not excreted during recovery from anoxia (Ellington, 1983). The accumulated alanine could either be used as a cosubstrate (pyruvate + alanine) for alanopine dehydrogenase or be reconverted to pyruvate for oxidation or gluconeogenesis since the AlaAT reaction is reversible. If alanine is used during recovery, then changes in AlaAT activity during anoxia would not be desired since this enzyme would be needed again immediately when aerobic conditions were reestablished. In *L. littorea* anoxia induced a decrease in the activity of foot AlaAT in both seasons but the activity of AlaAT was unaltered in the hepatopancreas.

Like AlaAT, the aspartate transamination reaction would also have an important role during the recovery period. A reversal of the AspAT reaction is necessary to replenish the depleted aspartate pool and since the activity of this enzyme will naturally become limited during anoxia by the progressive depletion of aspartate pools, it may be neither necessary or desirable to change the content of the enzyme during anoxia. Accordingly, there were no significant decreases in the activity of AspAT in the tissues of winter oysters during anoxia and AspAT activities only decreased in the gill and the catch adductor of summer animals. Anoxia induced a decrease in the AspAT activity in the foot muscle of winter *L. littorea* but no other significant changes in periwinkle AspAT activity were observed.

Malate Dehydrogenase

MDH has an important role in connecting the breakdown of aspartate or glycogen to the production of energy by the alternate pathways of fermentation. During anaerobiosis the catabolism of aspartate or glycogen produces OAA which is converted to malate by MDH for transport into the mitochondria where it is further reduced to produce succinate, propionate and acetate, the end-products of anaerobic fermentation in marine molluscs. Under anaerobic conditions, then, MDH functionally replaces LDH (and opine dehydrogenases) in reoxidizing cytoplasmic NADH (Livingstone, 1976). The ratio of MDH to LDH is always high in succinate-forming organisms (Livingstone, 1976) and this high activity argues against MDH having a role as a regulatory enzyme. MDH was capable of low activity (approximately 5% of NAD-MDH in *C. virginica* and 11% in *L. littorea*) using NADPH as the coenzyme but anoxia had no effect on the NADP-dependent MDH activity in any tissue and only caused a slight decrease in the NADH-dependent activity in the catch adductor of winter animals and a slight increase in enzyme activity in phasic adductor of summer oysters. *L. littorea* MDH activities were generally unaffected by anoxia stress. The activity of foot MDH using NADPH as the coenzyme did increase in the winter but since there was no corresponding increase in the activity of NADH-dependent MDH activity this change was probably not significant.

Malic Enzyme

ME activity in *C. virginica* was generally unaffected by anoxia in all tissues and both seasons with the only significant change being a decrease in winter gill tissue. ME activities did not change significantly in the foot or hepatopancreas of *L. littorea* as a result of anoxic stress in either season.

Serine Dehydratase

SDH activity was extremely low in the soft tissues of winter and summer *C. virginica* and was below the level of detection (less than 0.04 U/gww) in the adductor muscles. SDH activities were generally unaffected by anoxia although there was a significant decrease in activity in the mantle of summer oysters. The activity of SDH did not change significantly in the foot or hepatopancreas of *L. littorea* as a result of anoxic stress in either season.

Arginine Kinase

Activities of ArgK in the oyster were highest in the phasic adductor, maximal activities in this tissue were higher than the 147 U/gww seen in *Ostrea edulis* and approached the 930 U/gww found in the scallop *Pecten maximus* phasic adductor (Zammit and Newsholme, 1976). It is highly likely, therefore, that arginine phosphate is the primary, if not sole, supplier of the ATP required for valve closure. Activities of ArgK were more modest in other oyster tissues where the metabolic demands for rapid ATP production to support intense work are not as great. In *M. edulis* the level of arginine phosphate decreased during the early hours of anoxia in the posterior adductor muscle, foot, and mantle as it contributed to the ATP supply in these tissues (Zurburg and Ebberink, 1981). The activity of ArgK was generally unaffected by anoxia in all tissues and both seasons except for a significant decrease in activity seen in the gill tissue of winter oysters. ArgK activities did not change significantly in the foot or hepatopancreas of *L. littorea* as a result of anoxic stress in either season. ArgK activities were highest in the periwinkle foot and arginine phosphate may be an important phosphate reservoir for emergency locomotion to escape unfavorable conditions.

Adenylate Kinase

As ATP is consumed during muscular work, the AK reaction will cause an increase in the concentration of AMP which will activate glycolysis and increase energy production (Beis and Newsholme, 1975). However, the low glycolytic activity of the tissues, the low ATP/AMP ratio, and the decreased ATP utilization during anoxia preclude this reaction from having a significant effect on oyster metabolism. AK was generally unaffected by anoxia in all tissues and in both seasons except for a decrease in activity in winter gill tissue and a large increase in activity in summer phasic adductor from summer oysters. AK activities did not change significantly in the foot or hepatopancreas of *L. littorea* as a result of anoxic stress in either season.

Effect of Prolonged Anoxia on Soluble Protein Content

As expected soluble protein levels were generally unaffected by anoxia in either season or for either animal (Figures 16 and 17) since regulation of metabolic depression occurs via changes in small molecular effectors, covalent modification of enzymes (Storey, 1992b), or changes in the activity of only a few key regulatory enzymes as shown in this thesis.

Conclusion

The presence in gastropods of key enzymes known to contribute to anoxia tolerance in bivalves (e.g. AlaAT, AspAT) and a similar regulation of key glycolytic enzymes (e.g. PFK, PK) during anoxia in both species indicates that the basic metabolic mechanisms responsible for conferring anoxia tolerance are very similar between *C. virginica* and *L. littorea*. However, differences between them (e.g. importance of LDH and other tissue-specific differences in enzyme activities) hint at interesting divergences from a common process over evolutionary time. These differences may reflect specific adaptations to unique pressures exerted by their different physiologies (e.g. the constraints

of a sessile existence for the bivalve compared with the mobile lifestyle of the gastropod) or by differences in environmental parameters (e.g. the shallow, subtidal existence of the oyster and the intertidal environment of the periwinkle)

Prolonged anoxia exposure induced changes in enzyme properties and activities that were animal- and tissue-specific although most changes resulted in a decrease in enzyme activity thus contributing to metabolic depression. In addition adaptation to anoxia at the molecular level was seasonally dependent with both species exhibiting enzymatic changes that were indicative of a more effective depression of metabolic rate in response to anoxia during the winter. For example, in periwinkle hepatopancreas strong control was implemented at two key points (PK and LDH) during winter anoxia but during summer anoxia there was no regulation via the synthesis or degradation of enzymes in this very active tissue. Similarly, in the oyster gill and hepatopancreas anoxia caused the activity of numerous enzymes to decrease during the winter but summer decreases in enzyme activity were very limited. In *L. littorea* the kinetic properties of PFK from both tissues only underwent slight modifications as the result of anoxia and these changes were largely restricted to altering sensitivity to effectors although there were changes in both seasons. PK from foot muscle underwent nearly identical changes in both seasons as a result of anoxia which would contribute to a greatly reduced enzyme activity *in vivo* and these changes were much larger than those seen for PFK. Seasonal differences in anoxia effects on PK were seen in the hepatopancreas. PK activity was greatly reduced during the winter but was basically unaltered during similar anoxia exposure during the summer. Oyster PFK and PK showed tissue-specific, seasonally varied responses to anoxia. Anoxia induced much larger changes in the kinetic properties of PK than for PFK and PK kinetics indicate a more effective depression of activity during the winter. In both molluscs PK was the key regulatory enzyme of glycolysis during anoxia and in *C. virginica* anoxia induced a drastic decrease in the activity of this enzyme brought about by a dephosphorylation reaction

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