BIOCHEMICAL STUDIES ON THE EXPRESSION OF OVERDOMINANCE AT THE PHOSPHOGLUCOMUTASE-2 LOCUS IN THE PACIFIC OYSTER, *CRASSOSTREA GIGAS* (THUNBERG)

by

GRANT H. POGSON

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Department of Zoology

The University of British Columbia
Vancouver, Canada

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ABSTRACT

Numerous studies have documented significant associations between multiple-locus heterozygosity and fitness-related traits in natural populations, but the explanations for these patterns remain unknown. The objective of the present study was to examine the merits of the overdominance hypothesis as the mechanism responsible for a positive correlation between adult body weight and heterozygosity involving the phosphoglucomutase-2 (Pgm-2) locus in the Pacific oyster, Crassostrea gigas.

The kinetic and structural properties of seven Pgm-2 genotypes were examined over physiological ranges of temperature and pH. Significant differences were detected between Pgm-2 genotypes in a variety of enzymic parameters, but these were largely confined to genotypes possessing the Pgm-2-92 allele, and heterozygotes displayed strict intermediacy for all functional and structural properties examined. The expression of marginal overdominance at the Pgm-2 locus was considered unlikely because of the limited scope of the observed variation between allozymes, and its incompatibility with allelic frequencies in natural populations.

The three most common heterozygotes at the Pgm-2 locus displayed the extremely unusual property of overdominant enzyme activities. The magnitude of this overdominance was similar in the mantle and adductor muscle tissues, and was consistently

observed in population samples from two intertidal positions in three different seasons. A physiological impact of the Pgm-2 polymorphism was demonstrated on the metabolism of glycogen, the biochemical pathway in which PGM functions. Pgm-2 genotypes exhibited different concentrations of glycogen in their mantle, but not their adductor muscle tissues, which were directly associated with variation in their PGM activity levels. It was suggested that Pgm-2 genotype-dependent enzyme activity variation may affect rates of glycogen synthesis by a partitioning effect at the glucose-6-phosphate branch point.

Non-random associations were detected between the PGM activities of Pgm-2 genotypic groups and the activities of adjacent glycogen synthesis pathway enzymes, but none that could clearly account for the differing glycogen concentrations observed between genotypes. The expression of overdominance for PGM activity, and its impact on mantle glycogen levels, provided direct evidence favoring the overdominance explanation as the cause of the larger body weights of heterozygotes at the Pgm-2 locus in Crassostrea gigas.

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CHAPTER 1

GENERAL INTRODUCTION

The evolutionary process may be defined as the conversion of variation among individuals within a population into variation between populations and species, both spatially and temporally (Lewontin 1974, p. 12). Irrespective of mechanism, all evolutionary change has, and will continue, to depend on the existence of genetic variation affecting the physiological, morphological and behavioral attributes that constitute the distinctiveness of biological species. As argued eloquently by Mayr (1982), the conceptual revolution pioneered by Darwin (1859) involved the replacement of a "typological" with a "populational" world-view that recognized the importance of this naturally-occurring variation between individuals as one of the fundamental characteristics of biological life. The crucial role played by genetic variation in the adoption of this new perspective is a prime reason that its study has been, and will always be, a principal focus of evolutionary studies.

All evolutionary theories rely directly on the presence of intra- or inter-populational variation, but differ in how these changes occur. A major source of past and present controversies concerns the relative importance of Darwinian natural selection as an agent of the evolutionary process. Early in the neo-synthetic period a significant role was attributed to random

genetic drift in producing what were then interpreted as non-adaptive differences between subspecies and geographic races (e.g. Robson and Richards 1936). These sentiments were reflected in the writings of the founders of the neo-Darwinist movement at this time (e.g. Dobzhansky 1937; Simpson 1944). Gould (1983) has pointed out how later publications of these same authors shifted from a pluralistic to a strict adaptationist interpretation of the same patterns that he termed the "hardening of the modern synthesis". A similar trend occurred in the work of Sewall Wright over this same period (Provine 1986). Only recently has the adaptationist paradigm (cf. Gould and Lewontin 1979) given way to allow the consideration of alternative modes of evolutionary change.

In addition to disagreements over its relative importance in directing the evolutionary process, another long-standing dispute has centered on how selection operates in natural populations. Dobzhansky (1955) summarized these opposing views in what he termed the "classical" and the "balanced" hypotheses of population structure. Proponents of the classical school contend that natural selection mainly serves a "purifying" function, by removing deleterious mutants from populations. In contrast, the balanced view holds that the major role of natural selection is to actively maintain genetic variation within populations through various types of balancing selection (e.g. overdominance, frequency-dependent selection, variable selection over time and space). Genic heterozygosity is thus viewed by the

"balanced" school as adaptive and stable (given specific environmental circumstances), whereas the "classical" school predicts that heterozygosity is transient and contributes little to population adaptedness.

Lewontin (1974) has argued that the current controversy over the adaptive significance of the high levels of enzyme polymorphism observed in natural populations, uncovered by electrophoretic procedures, is simply a continuation of this dispute, but now waged at the molecular level. Arguments based on the importance of purifying selection in determining various aspects of protein structure and function form a cornerstone of the neutral theory of molecular evolution (Kimura 1983). Neutral theory has stimulated heated discussion in the fields of population and evolutionary genetics. Comparisons of the quantities and distributions of allozymic (i.e. allelic isozymes) variation within and between species to predictions of various neutral and selective models has dominated these areas of study for many years (see discussions in Lewontin 1974; Ayala et al. 1974; Nei 1975; Ewens 1977; Gillespie 1978; Nevo 1978; Wills 1981; Kimura 1983; Nei and Koehn 1983; Ohta and Aoki 1985). A review of the evidence that has been garnered for and against various neutral predictions will not be presented here. Suffice it to say that indirect (cf. McDonald 1983) or statistical approaches have not been successful in resolving this controversy, since both neutral and selection theory are sufficiently robust to account for virtually any observed

distribution of allelic polymorphism.

An alternative research strategy that evolved over the same period involved the direct measurement of natural selection on allozymic variation through the comparison of the biochemical and physiological attributes of enzyme genotypes which, through their interactions with environmental variables, may give rise to fitness differences relevant to the adaptive significance of the polymorphism studied (cf. Clarke 1975; Koehn 1978). Although concerned initially with testing the validity of neutral theory, this approach has developed into a separate discipline of evolutionary genetics (see Watt 1985a) and has been successfully applied to polymorphisms at the aminopeptidase-1 locus in the blue mussel, Mytilus edulis (Koehn, Newell and Immerman 1980; Koehn and Immerman 1981; Koehn and Siebenaller 1981; Hilbish, Deaton and Koehn 1982; Hilbish and Koehn 1985; reviewed by Koehn and Hilbish 1987), the lactate dehydrogenase-B locus in the killifish, Fundulus heteroclitus (Place and Powers 1979, 1984a, 1984b; DiMichele and Powers 1982a, 1982b; reviewed by Powers, DiMichele and Place 1983), and the phosphoglucose isomerase locus in Colias butterflies (Watt 1977, 1983; Watt, Cassin and Swan 1983; Watt, Carter and Blower 1985; reviewed in Watt 1985a, 1985b). The great strength of these studies lie in their mechanistic linking of allelic variation onto phenotypic "character states" (cf. Lewontin 1972) that may in turn be exposed to the selective process.

Examination of the impact of enzyme polymorphisms on phenotypic characters has recently expanded to consider multiple-locus effects. In these studies, individuals are scored for their genotypes at a small number of electrophoretic loci (usually 5 to 7) and pooled into a series of discrete heterozygosity classes. Relationships between the degree of enzyme heterozygosity and various phenotypic and physiological parameters are then examined by standard linear regression procedures. Following this basic protocol, a large number of studies have demonstrated significant correlations between multiple-locus heterozygosity and different morphological, physiological and fitness-related traits in a wide diversity of organisms (reviewed by Mitton and Grant 1984; Zouros and Foltz 1987). These associations have been most extensively documented in marine bivalves. In these organisms, multiple-locus heterozygosity has been positively correlated with growth rate (Singh and Zouros 1978; Zouros, Singh and Miles 1980; Fujio 1982; Green et al. 1983; Koehn and Gaffney 1984; Koehn, Diehl and Scott 1988), viability (Zouros et al. 1983; Diehl and Koehn 1985), and fecundity (Rodhouse et al. 1986), and negatively correlated with rates of oxygen consumption (Koehn and Shumway 1982; Garton, Koehn and Scott 1984; Diehl et al. 1985), protein turnover (Hawkins, Bayne and Day 1986), and weight loss under nutritive stress (Rodhouse and Gaffney 1984).

The underlying cause(s) of these relationships is unknown. One possibility is that heterozygotes at these enzyme loci are

functionally superior to homozygotes (overdominance). The overdominant effects at each locus are additive, and hence when pooled together result in a more efficient and buffered phenotype. An alternative explanation is that these correlations are not caused by the electrophoretic loci themselves, but instead by tightly-linked loci that are segregating for deleterious recessive alleles (associative overdominance). If linkage disequilibrium exists between these loci, a proportion of homozygotes for these deleterious alleles are expected to be present within the homozygous, but not the heterozygous, genotypic groups at the electrophoretic loci examined. Therefore, detrimental phenotypic effects would be manifested in the homozygote classes that are reflected in the multiple-locus relationship, but no direct advantage is expected by heterozygosity per se.

A related hypothesis is that the patterns are caused by inbreeding, which in several species of marine bivalves has been shown to result in decreased larval viability and growth (e.g. Longwell and Stiles 1973; Beattie et al. 1987). According to this explanation, the reduced performance of enzyme homozygotes is simply a manifestation of inbreeding depression. Obviously, this hypothesis requires the expression of significant inbreeding coefficients in the study population. This corollary is certainly met by marine bivalves, in which marked heterozygote deficiencies at electrophoretic loci are a common feature of their population stuctures, particularly at the early

post-settlement stage (see Zouros and Foltz 1984; Singh and Green 1984). A final explanation for these patterns is that they are caused by the undetected presence of null alleles (producing non-functional enzyme products) at the electrophoretic loci studied. The misclassification of null heterozygotes as homozygotes could thus account for 1) the heterozygote deficiencies, and 2) the superior properties of heterozygotes for two functional alleles, because homozygote classes would contain a percentage of these low activity null heterozygotes.

Establishing the cause of relationships between multiple-locus heterozygosity and fitness-related traits is a matter of theoretical and practical importance. On the theoretical side, demonstrating that the electrophoretic loci are responsible could simultaneously prove the functional significance of a large number of enzyme polymorphisms that would strain the credibility of the neutral theory. Understanding the potential selective basis for the maintenance of this genetic variation is particularly relevant, considering the large diversity of species involved in these studies. Distinguishing between these alternative hypotheses is also of vital importance for selective breeding programs. Breeding methodologies for the improvement of commercially valuable traits would differ greatly if overdominance, rather than associative overdominance, was expressed at the loci examined in these studies.

The objective of my study was to determine if evidence

favoring the overdominance hypothesis could be obtained for an enzyme locus involved in a multiple-locus heterozygosity relationship through an examination of the biochemical and physiological properties of homozygous and heterozygous genotypes. I chose to study the phosphoglucomutase-2 locus in the Pacific oyster, Crassostrea gigas.

THE STUDY ANIMAL

The Japanese, or Pacific oyster, Crassostrea gigas (Thunberg) is a temperate, intertidal species native to the western Pacific where its geographic range extends from China to the southern U.S.S.R., and includes the Japanese archipelago (Stenzel 1971). This species was introduced to the west coast of North America at the turn of the century for commercial purposes and has become well established in isolated pockets of British Columbia and Washington state (Quayle 1969). The principal factor limiting the colonization of this new habitat by C. gigas has been its inability to spawn successfully. Reproduction in the Pacific oyster requires warmer summer water temperatures (21°C or higher) than usually experienced in this geographic region (Quayle 1969). The study population was located on a private oyster lease in Nanoose Bay, situated on the eastern side of Vancouver Island 20 km north of Nanaimo, British Columbia. Originally established from Pendrell Sound breeding stock, continuous production from this lease is dependent on supplementing the natural reproduction of the bay with seed from

local suppliers (P. MacClelland, personal communication).

Sex determination in Crassostrea gigas, as in other oysters, is complex, responding to both genetic and environmental influences (Galtsoff 1964; Haley 1977; Buroker 1983). The sexes are usually separate, but hermaphroditic individuals are generally detected at low frequencies. The prevailing pattern of sex change in the genus Crassostrea is protandrous; young animals beginning life as males but switching to females as they grow older. Fertilization occurs externally, and after a planktonic larval stage ranging from 15 to 30 days, the larvae select a suitable substrate to which they adhere, metamorphose, and begin their sessile adult life. Excellent descriptions of the biology of this species, and the closely related C. virginica, may be found in Yonge (1960), Galtsoff (1964), and Quayle (1969).

The Pacific oyster is a facultative anaerobe, capable of surviving for prolonged periods in the complete absence of oxygen. The ability of marine bivalves to tolerate anoxic conditions is accomplished by 20-fold reductions in their basal metabolic rates and the utilization of novel metabolic pathways generating ATP by substrate-level phosphorylations (reviewed by de Zwaan 1983). Virtually all aspects of metabolism in C. gigas undergo pronounced seasonal fluctuations which are integrated with prevailing abiotic conditions, the availability of food, and the annual cycle of reproduction (reviewed by Gabbott 1983).

A dominant seasonal cycle in marine bivalves, closely tied to reproduction, involves the synthesis and degradation of glycogen. The timing of this cycle varies between different species depending on their spawning season. In C. gigas, glycogen accumulates in the mantle and digestive gland in the fall and early spring and is degraded in the early summer for gametogenesis prior to spawning in late July and August (Quayle 1969). Since phosphoglucomutase functions in glycogen metabolism, a physiological effect of the Pgm-2 polymorphism must be expressed through the differential abilities of genotypes to synthesize or degrade glycogen. Fitness-related differences between Pgm-2 genotypes may in turn be expressed through these effects on tissue glycogen levels because of the central role played by this carbohydrate in the energy metabolism of oysters (Gabbott 1975).

As generally found for other marine invertebrates, Crassostrea gigas possesses substantial levels of allozymic variation. In five electrophoretic studies on this species summarized by Ozaki and Fujio (1985), an average of 53% of the enzyme loci examined were polymorphic and the mean heterozygosity per individual was 19.4%. Circumstantial evidence favoring a net heterozygote advantage for growth and/or viability has been suggested for a number of polymorphic loci in C. gigas. These include leucine aminopeptidase (Nagaya, Sasaki and Fujino 1978), catalase (Fujio, Nakamura and Sugita 1979), an unidentified muscle protein (Buroker 1979), and aspartate

aminotransferase (Sugita and Fujio 1982). Multiple-locus relationships have not been extensively studied in C. gigas. However, after pooling data from 20 wild Japanese populations, Fujio (1982) observed a significant positive correlation between heterozygosity at five enzyme loci and adult body weight. The Pgm-2 locus was involved in this relationship; heterozygotes exhibited greater body weights than homozygotes in 18 of the 20 populations. Based on the results of Fujio's (1982) study, I set out to determine if overdominance was indeed responsible for the higher growth rates of Pgm-2 heterozygotes.

THE STUDY ENZYME

Phosphoglucomutase (PGM, E.C. 2.7.5.1, alpha-D-glucose-1-phosphate: alpha-D-glucose-1,6-diphosphate phosphotransferase) catalyzes the interconversion of glucose-1-phosphate (G1P) and glucose-6-phosphate (G6P) in presence of glucose-1,6-diphosphate and magnesium ion. A comprehensive review of its stuctural and functional properties may be found in Ray and Peck (1972). PGM is a monomeric enzyme, exhibiting molecular weights from different organisms ranging from 62,000-67,000 daltons. The rabbit muscle enzyme's complete sequence of 561 amino acids has recently been determined by Ray et al. (1983). Najjar and Pullman (1954) first demonstrated that PGM may exist in phosphorylated and dephosphorylated states, representing active and inactive forms of the enzyme, respectively. The discovery that glucose-1,6-diphosphate's sole function was to convert the

dephospho into the phosphoenzyme led to uncertainty surrounding PGM's reaction mechanism. Ray and Roscelli (1964a) showed that the dephosphoenzyme of rabbit muscle PGM was formed once every 20 catalytic cycles, hence, the reaction in this species approximates a "uni-uni" mechanism. However, Hanabusa et al. (1966) found that PGM extracted from bacteria displayed a "ping-pong" mechanism: the diphosphate dissociated frequently from the enzyme's central reaction complex, thus acting as the "first product" and the "second substrate" in the scheme proposed by Cleland (1963). Similar patterns have been described during the less efficient interconversion of other 5- and 6-carbon sugar phosphates by PGM (Passonneau et al. 1969). Therefore, phosphoglucomutase functions along a continuum between these extremes depending on its phylogenetic origin, the substrates involved, and the particular assay conditions (Ray and Peck 1972).

The reaction catalyzed by PGM is freely reversible, but its equilibrium constant strongly favors the conversion of glucose-1-phosphate to glucose-6-phosphate (Keq = [G6P]/[G1P] = 17.2). Because of its reversibility, PGM participates in both the synthesis and degradation of glycogen. Lacking any regulatory properties, PGM's metabolic role is to respond efficiently to flux rates determined by three closely-positioned enzymes whose activities are under stringent regulatory control: glycogen synthetase, glycogen phosphorylase, and phosphofructokinase. The enzymic parameter most relevant for PGM's catalytic function is

its Vmax/Km ratio, since the enzyme is unlikely to be saturated *in vivo* (see Atkinson 1977), and via its composite nature Vmax simultaneously incorporates steady-state activity levels (Hoffman 1981; Watt 1983). Therefore, functional differences between *Pgm*-2 genotypes of physiological significance are most likely to be expressed through these Vmax/Km ratios.

Phosphoglucomutase is a widely polymorphic enzyme, genetic variants having been reported in a diversity of organisms (see Gauldie 1984). Allelic isozymes of PGM have been characterized from *Drosophila melanogaster* (Fucci et al. 1979) and the anemone, *Metridium senile* (Hoffman 1985). The biochemical differences detected between *Pgm* genotypes in both species were limited and thus provided little support for the adaptive significance of either polymorphism. PGM is a highly polymorphic enzyme in marine bivalves, and has been included in virtually all studies involving multiple-locus heterozygosity in these organisms. In two of these studies, locus-specific comparisons of homozygotes and heterozygotes detected significant differences only between *Pgm* genotypes (e.g. Rodhouse and Gaffney 1984; Diehl et al. 1985). The significant effects of heterozygosity for this enzyme in other bivalve species suggests that biochemical differences could exist between allelic variants at the *Pgm*-2 locus in *C. gigas* that may account for the larger body weights of heterozygotes reported by Fujio (1982).

FORMAT OF THE THESIS

The thesis has been organized into a series of discrete chapters. Chapter 2 presents the purification and biochemical properties of seven Pgm-2 genotypes. Chapter 3 compares the mantle and adductor muscle specific activities of Pgm-2 genotypes sampled from two intertidal positions in three seasons from the Nanoose Bay study population. An assessment of the physiological effects of this polymorphism on glycogen metabolism is the subject of Chapter 4. Chapter 5 analyzes the activity structure of the glycogen synthesis pathway to allow a more detailed examination of the effects of the Pgm-2 locus on the synthesis of glycogen. A general discussion of the major findings of each chapter and their implications for multiple-locus heterozygosity relationships is presented in Chapter 6.

CHAPTER 2

BIOCHEMICAL CHARACTERIZATION OF PGM-2 GENOTYPES

INTRODUCTION

Overdominance, or heterozygote superiority, has remained a controversial explanation for the maintenance of genetic variation in natural populations since its theoretical formulation by Fisher (1922). The popularity of overdominance peaked in the 1940s and 1950s largely through the empirical work of Dobzhansky and his colleagues (e.g. Dobzhansky and Levene 1948, 1951), the provocative ideas of Lerner (1954), and the British ecological genetics school led by E.B. Ford (Ford 1965). After the discovery and examination of the patterns of allozymic variation in natural populations, a variety of experimental and theoretical studies have discredited the role played by overdominance in maintaining balanced polymorphisms. Evidence against overdominance has included: discrepancies from predicted additive and dominance components of viability estimates (e.g. Mukai et al. 1974); the unobserved large depressions in fitness expected upon inbreeding (e.g. Lewontin 1974, p. 207); the asymmetrical frequency distributions of electrophoretic alleles at polymorphic loci (e.g. Yamazaki and Maruyama 1972; Coyne 1976); the high levels of genetic variation displayed by haploids such as *E. coli* (e.g. Selander and Levin 1980); the inherent difficulties in establishing stable multi-allelic

equilibria by overdominance (e.g. Lewontin, Ginzburg and Tuljapurkar 1978); the potential roles of environmental heterogeneity and frequency-dependent selection in maintaining polymorphisms (Hedrick, Ginevan and Ewing 1976; Clarke 1979; Hedrick 1986); and, perhaps most importantly, a paucity of examples demonstrating its occurrence. The case against overdominance appears so overwhelming that Kimura (1983, p. 282) stated that "only blind faith can maintain it".

Renewed interest in overdominance has been sparked by recent studies, on a wide variety of organisms, documenting significant correlations between multiple-locus heterozygosity and various morphological, physiological and fitness-related traits (reviewed by Mitton and Grant 1984; Zouros and Foltz 1987). In marine bivalves, associations involving multiple-locus heterozygosity, and the potentially related deficiencies of heterozygotes, have been most extensively studied and discussed in the American oyster Crassostrea virginica (Singh and Zouros 1978; Zouros, Singh and Miles 1980; Singh 1982; Koehn and Shumway 1982; Zouros et al. 1983; Foltz, Newkirk and Zouros 1983; Zouros and Foltz 1984; Singh and Green 1984; Foltz 1986a, 1986b). Despite extensive work, the underlying cause of these relationships remains unknown. Although consistent with single-locus overdominance, these results could also be produced by inbreeding, the undetected presence of null alleles, or associative overdominance.

Inbreeding was originally discounted as an explanation by Zouros, Singh and Miles (1980) because the heterogeneity of the inbreeding coefficients, calculated for each of the seven loci studied, was significantly greater than expected from a process that should affect all loci equally. Further evidence against the inbreeding hypothesis was provided by Mitton and Pierce (1980) and Chakraborty (1981) who showed that individual heterozygosity at a small subset of loci does not accurately reflect overall genomic heterozygosity, a conclusion also emphasized by Smouse (1986) (see however discussion in Zouros and Foltz 1987). Recently, Foltz (1986a, 1986b) detected the segregation of null alleles at two loci in C. virginica, thus providing support for a hypothesis originally considered unlikely by Zouros, Singh and Miles (1980). However, the explanatory power of null alleles still appears limited because they were observed at one locus (Lap-2) but not at four others involved in these earlier studies (Pgi, Got, Pgm, Xdh). The null allele explanation also suffers from an important theoretical limitation: the absence of null homozygotes in the large sample of Zouros, Singh and Miles (1980) implies an unrealistically high selective advantage of null heterozygotes over genotypes possessing two functional alleles that is difficult to reconcile. At present, neither the inbreeding or null allele hypotheses appear capable of providing a general explanation for the relationships between heterozygosity and fitness-related traits, at least for oysters.

Distinguishing between the overdominance and associative overdominance hypotheses is much more difficult. Are correlations involving multiple-locus heterozygosity produced by overdominance at the enzyme loci scored, or are they a consequence of associative overdominance caused by the presence of deleterious recessive alleles segregating at a number of tightly-linked loci (cf. Ohta 1971)? The distinction between these alternatives is directly analogous to the dispute between the "dominance" and "overdominance" explanations for heterosis, or hybrid vigor (Zouros and Foltz 1987), which remains unresolved despite decades of study (see Gowen 1952; Wright 1977, p. 9-46; Sedcole 1981). Indirect approaches to determining the cause of heterozygosity-growth rate relationships in several Pinus species have not helped to solve this question. Comparison of inbred and crossbred progeny of P. attenuata by Strauss (1986, 1987) or the application of the "adaptive distance" model of Smouse (1986) to heterozygosity relationships in a number of P. rigida populations by Bush, Smouse and Ledig (1987) only tested the predictions of the inbreeding versus the overdominance hypotheses. Any evidence for overdominance provided by these approaches cannot discriminate between genuine or associative overdominance.

Perhaps the only way to provide concrete evidence for the overdominance explanation and simultaneously distinguish between these competing hypotheses, is to study these enzyme polymorphisms directly and produce the biochemical and

physiological support for these results in mechanistic terms (cf. Clarke 1975; Koehn 1978; Watt 1985b). To succeed, this research strategy must meet three requirements. First, it must demonstrate that functional differences are expressed at the biochemical level between allelic variants at the locus chosen for study. These differences must be sufficient in magnitude to confer a net advantage to heterozygotes through either 1) marginal overdominance arising from heterozygote intermediacy (cf. Wallace 1959), or 2) genuine overdominance produced by superior kinetic properties of heterozygotes, thus eliminating the potential contribution of unknown tightly-linked loci. Second, the functional superiority of heterozygotes must be manifested at the phenotypic level through some physiological effect attributable to the locus in question. The physiological impact of this allelic variation must be capable of explaining the involvement of the locus in the heterozygosity relationship. Third, the fitness array of genotypes at the locus under study must be compatible with conditions producing a stable equilibrium state. Given the difficulties of maintaining stable multi-allelic equilibria by overdominance (cf. Lewontin, Ginzburg and Tuljapurkar 1978), this requirement is especially pertinent because most enzyme loci in these studies involving oysters possess 3 to 5 alleles at moderate frequencies.

I have taken this direct approach with the phosphoglucomutase-2 locus (PGM; E.C. 2.7.5.1) in the Pacific oyster, Crassostrea gigas. PGM catalyzes the interconversion of

glucose-1-phosphate and glucose-6-phosphate in the presence of the cofactors glucose-1,6-diphosphate and magnesium ion, and functions metabolically in the synthesis and degradation of glycogen. In the Pacific oyster, Fujio (1982) observed a significant positive relationship between heterozygosity and adult body weight in a study on 20 wild populations around Japan. The Pgm-2 locus was was one of the five enzymes involved in this correlation. Pgm-2 heterozygotes exhibited greater mean weights than homozygotes in 18 of the 20 populations sampled. The objective of this chapter was to determine if biochemical differences exist between the four most common allozymes at the Pgm-2 locus in C. gigas that provide evidence in support of the overdominance hypothesis. To assess the presence of these functional differences, the kinetic properties of seven Pgm-2 genotypes were measured over physiologically important ranges of temperature and pH. Oysters situated in the intertidal zone experience large fluctuations in both temperature, through seasonal variation and daily changes associated with the tidal cycle, and intracellular pH, as a consequence of the transitions between aerobic and anaerobic metabolism (e.g. Wijsman 1975; Walsh, McDonald and Booth 1984). Kinetic differences may exist between Pgm-2 allozymes over these broad ranges of temperature or pH that could be responsible for imparting a net advantage to heterozygotes that could provide a foundation for explaining their superior rates of growth reported by Fujio (1982).

MATERIALS AND METHODS

Chemicals. All buffers, substrates, cofactors, proteins, and coupling enzymes used for enzymatic assays were obtained from Sigma. Sephadex G-100 and G-25 were supplied by Pharmacia, DEAE-Cellulose (DE32, microgranular) from Whatman, and ultrafiltration membrane cones (CF 25) from Amicon. Coomassie blue G-250 and standards used for protein determinations were provided by Bio-Rad, and the electrostarch for electrophoresis from Connaught Laboratories.

Animals. Oysters were collected over a three year period from the intertidal zone of a private oyster farm located in Nanoose Bay on Vancouver Island, British Columbia. Sampling stations were established at two tidal heights on the lease; a low water site situated at 0.5-0.8 m above mean low water (MLW), and a high water site at 1.7-2.0 m above MLW. Animals were returned to the laboratory on ice, and after a small piece of mantle was dissected for electrophoresis, immediately frozen at -40°C.

Electrophoresis. Horizontal starch gel electrophoresis was performed on 12% gels (w/v) according to the methods of Kristjansson (1963). Approximately 0.5 g of frozen mantle tissue was homogenized in an equal volume of 10 mM Tris, 10 mM maleic acid, 1 mM EDTA, 1 mM MgCl2, pH 7.4 (buffer A of Fucci et al. 1979) and centrifuged for 5 min at top speed in a clinical centrifuge. Supernatant was applied to the gels with Whatman No.

2 filter paper wicks. Gels were run at 200 V (35 mA) for approximately 5 h and PGM was stained visually as outlined by Shaw and Prasad (1970). For improved resolution of the allozymic banding patterns, two different running conditions were routinely employed. Under "standard" conditions the electrode buffer was 100 mM Tris, 100 mM borate, 5 mM EDTA, pH 8.3. The gel buffer was a 10-fold dilution of the electrode buffer. Under "catalytic" conditions, the following saturating concentrations of substrate and cofactors were added to the standard gel and electrode buffers to make the PGM allozymes catalytically active while migrating in the electric field: 1 mM glucose-1-phosphate, 5 μM glucose-1,6-diphosphate, and 1.5 mM MgCl2.

Two techniques were used to examine the presence of hidden variation within the PGM electromorphic classes as described for both Drosophila melanogaster (Trippa, Loverre and Catamo 1976; Trippa et al. 1978) and Mytilus edulis (Beaumont and Beveridge 1983). Following Trippa et al. (1978), two slices of the same gel, run under standard conditions, were treated in the following manner. One slice, serving as a control, was incubated for 15 min in a water bath at room temperature and, the other, for the same length of time in a water bath at 50°C. The slices were immediately transferred to staining solutions and subsequently scored for differential staining intensities. 300 homozygotes for the most common allele were treated in this fashion. The pH-dependent resolution of cryptic alleles was examined with the Tris-maleic acid buffer system described in

Beaumont and Beveridge (1983). A sample of 120 oysters, composed of the 8 most frequent Pgm-2 genotypes, was examined electrophoretically employing a 10 mM Tris, 10 mM maleic acid, pH 7.4 gel buffer, and a 100 mM Tris, 100 mM maleic acid electrode buffer adjusted to either pH 7.4 or 6.0. Gels run at each pH were compared under the above and "catalytic" conditions described previously.

Purification. All purification steps were carried out on ice or at 4°C. The following procedure was identical for the 4 Pgm-2 allozymes purified, and starting material for each represented pooled tissue from 5-8 individuals homozygous for the appropriate allele. Approximately 70-80 g of mantle and adductor muscle tissue was minced and homogenized with an Ultra-Turrax homogenizer in 3 volumes of buffer A and stirred for 30 min. The homogenate was centrifuged at 42,000 x g for 1 h and the supernatant passed through a filter of glass wool. The crude homogenate was slowly brought to 50% saturation with an ice-cold saturated solution of ammonium sulfate. After stirring for 1 h the suspension was centrifuged at 12,000 x g for 20 min. The supernatant was brought to 75% saturation with ammonium sulfate as before and equilibrated with stirring for 1 h. After a final centrifugation at 12,000 x g for 20 min, the pellets were resuspended in buffer A and dialyzed overnight against three 100 vol changes of the same buffer.

The dialyzed sample was adjusted to pH 7.6 and loaded onto

a DEAE-Cellulose column (1.5 x 30 cm) equilibrated with 5 mM Tris, 5 mM maleic acid, 0.5 mM MgCl2, 0.1 mM EDTA, pH 7.6 (buffer B) at a flow rate of 5.6 ml/h. After washing with a minimum of 3 column volumes, the enzyme was eluted with a linear gradient (0-200 mM) of NaCl (200 ml in total). Fractions containing the greatest specific activity were combined (total vol 15-25 ml) and concentrated to approximately 1.5 ml on an Amicon CF 25 ultrafiltration cone by centrifugation at 2,000 x g. The concentrated sample was applied to a Sephadex G-100 column (1.5 x 60 cm) equlibrated with buffer A. Elution was carried out at a flow rate of 8.4 ml/h and the peak fractions were pooled (total vol 7-9 ml). The sample was adjusted to pH 7.6 and glucose-1-phosphate (G1P) was added to achieve a concentration of 2 mM. The sample was loaded onto a second DEAE-Cellulose column (0.9 x 15 cm), washed, and eluted with a linear gradient (0- 200 mM) of NaCl (total vol 150 ml) at a flow rate of 7.2 ml/h as before, except the equilibration and elution buffer (B) also contained 2 mM G1P. Peak fractions were again combined (total vol 7-14 ml) and the sample was concentrated to approximately 1 ml by centrifugation at 2,000 x g on an Amicon CF 25 ultrafiltration cone. The preparation was passed through a Sephadex G-25 column (0.9 x 15 cm) equilibrated with buffer A at a flow rate of 8.4 ml/h and peak fractions were pooled. The purified enzyme was brought to 20% glycerol (v/v) and frozen at -70°C. The PGM allozymes stored in this manner were extremely stable, exhibiting on average a 10% loss in activity over a 2 month period. Electrophoretic examination of these partially

purified preparations verified the correct allozymic composition and showed that they were free of enzyme encoded by the Pgm-1 locus. General protein concentration was determined in triplicate at room temperature after each purification step on a Pye Unicam SP8-400 UV/visible spectrophotometer by the method of Bradford (1976) using gamma globulin as a standard.

Enzyme Assays and Kinetics. All enzyme assays were conducted at 340 nm on a Pye Unicam SP 1800 UV/visible spectrophotometer. Assay temperature was controlled by a Lauda K-2/RD circulating water bath attached to a constant temperature cuvette holder and was monitored by a YSI model 46 thermometer probe inserted into a blank cuvette. PGM activity was measured using a coupled assay modified from Joshi et al. (1967). The standard assay medium contained 50 mM imidazole-HCl, 3 mM MgCl2, 2 mM glucose-1-phosphate (G1P), 16 μM glucose-1,6-diphosphate (G16diP), 0.4 mM NADP, 1 unit glucose-6-phosphate dehydrogenase (G6PDH), pH 7.0 (20°C) in a final volume of 1 ml. The assay mixture, containing an aliquot of PGM, was preincubated for a minimum of 10 min and the reaction started by the addition of G1P. One unit of PGM activity is defined as the quantity of enzyme required to convert 1 μmol of substrate to product per minute at 15°C.

Preliminary kinetic analyses were carried out on each of the 4 Pgm-2 allozymes following earlier purification trials. Kinetic parameters for the forward reaction (G1P to G6P) were determined by measuring initial rates in triplicate at 11

concentrations of G1P ranging from 10 μM to 2 mM at a saturating G16diP concentration of 16 μM. These experiments were performed at 5° intervals over the temperature range of 5 to 30°C allowing the pH of the imidazole buffer to vary with temperature (ΔpKa/°C = -0.017). The effect of pH on the kinetic parameters was studied by measuring initial rates as before at pH 6.5, 7.0 and 7.5 at a constant temperature of 20°C. Apparent Km and Vmax values were estimated by the direct linear plot of Eisenthal and Cornish-Bowden (1974). Kinetic parameters for the cofactor G16diP were evaluated by measuring initial reaction rates in triplicate at 8 concentrations of the diphosphate in a geometric series from 0.125 to 16 μM at saturating levels of G1P (1 mM). Initial rates were measured over the same ranges of temperature and pH as before and the kinetic parameters again estimated by the direct linear plot.

These initial kinetic experiments enabled construction of an optimal kinetic design that minimized the variances of the parameter estimates (cf. Duggleby 1979; Endrenyi and Chan 1981; Currie 1982). For the forward reaction direction, initial rate measurements were replicated 8 times at each of two G1P concentrations (2 mM and 20 μM) at a diphosphate concentration of 16 μM. Kinetic parameters for the cofactor G16diP were determined similarly by repeating initial rate measurements 8 times at each of two diphosphate concentrations (16 and 0.70 μM) at a G1P concentration of 1 mM. Several additional precautions were taken to minimize experimental error. For the kinetic study

the 4 Pgm-2 allozymes were purified sequentially, stored frozen, and subsequently studied together under identical conditions at each temperature and pH. For all initial rate measurements the allozymes were diluted to equal activities in buffer A. Three heterozygote preparations were made by mixing equal activities of the most common Pgm-2 allozyme with each of the three less frequent allozymes. Although synthetic in nature, these heterozygote preparations ensured that similar quantities of the two monomers were present, a condition justified by the staining patterns of these genotypes, and not guaranteed by the purification of enzyme from naturally occurring heterozygotes. The same substrate solutions, maintained at -40°C in small aliquots, were used throughout the study. Substrate conversion was kept to within 20% to ensure strictly linear reaction time courses (Ray and Peck 1972; Hoffman 1985). Since the kinetic parameters determined by this experimental design represent single estimates, the extent of day-to-day variability was assessed prior to the start of the study and was found to be negligible, providing the activities of the daily preparations were kept constant. Replicate Km determinations across 5 days exhibited a coefficient of variation of only 2.9%.

Kinetic parameters for G1P and G16diP from this optimal design were estimated by the method of biweight regression with the Fortran program NATO written by Cornish-Bowden (1985). The kinetic parameters were examined by two-way analysis of variance (ANOVA) treating Pgm-2 genotype and either temperature or pH as

independent variables. Means expressed over these ranges of temperature and pH were compared by *a posteriori* Bonferroni multiple range tests. Rationale for the statistical comparison of the apparent Km values of these seven *Pgm*-2 genotypes at each individual pH or temperature interval by the analysis of covariance (ANCOVA) is as follows. First, for all genotypes the apparent Km and Vmax values were determined a second time by ordinary least-squares regression. These estimates were found to be identical with those obtained by the more robust biweight procedure. This analysis was repeated after changing the linear transformation of the Michaelis-Menten equation in the program from the Lineweaver-Burk plot (1/v *vs.* 1/[s]) to the Eadie-Hofstee plot of v *vs.* v/[s], which produces a regression line with a y-intercept of Vmax and a slope of -Km. These different transformations yielded identical kinetic parameters for both regression procedures, although the standard errors obtained from the Eadie-Hofstee plot were slightly larger. The absence of outliers in the kinetic data and the independence of the parameter estimates on the linear transformation allowed the statistical comparison of the apparent Km values of different genotypes by ANCOVA, treating v/[s] as the covariate and v as the dependent variable. Differences between the slope estimates (-Km) for these *Pgm*-2 genotypes were tested by Bonferroni *a posteriori* multiple range tests. This procedure is thus analogous to the unplanned comparison of a series of regression coefficients (Sokal and Rohlf 1981, p. 507), irrespective of the validity of treating v/[s] as a covariate in the ANCOVA.

Kinetic parameters for the reverse reaction direction (G6P to G1P) were estimated by the procedure of Ray and Roscelli (1964b) through the constraints imposed by the Haldane equation (Haldane 1930):

$$Keq = \frac{Vmax(f)\ \ app\ Km\ (G6P)}{Vmax(r)\ \ app\ Km\ (G1P)} \qquad ...(1)$$

where Keq is the reaction's equilibrium constant ([G6P]/[G1P]); Vmax(f) and Vmax(r) are the maximum velocities of the forward and reverse reaction directions, respectively; and app Km (G6P) and app Km (G1P) are the apparent Michaelis constants for glucose-6-phosphate and glucose-1-phosphate, respectively. Equation 1 may be rearranged to allow estimation of app Km (G6P):

$$app\ Km\ (G6P) = \frac{Keq\ \ app\ Km\ (G1P)}{Vmax(f)/Vmax(r)} \qquad ...(2)$$

Therefore, determination of the Vmax(f)/Vmax(r) ratios of different Pgm-2 genotypes, employing identical enzyme concentrations for both reaction directions, enables indirect measurement of their app Km's for G6P, providing values of Keq and app Km (G1P) were attained under similar conditions.

Maximum velocities for both reaction directions were

determined for the four Pgm-2 allozymes at 5° intervals over the temperature range of 5 to 30°C. The assay medium contained 50 mM imidazole-HCl pH 7.0 (20°C), 3 mM MgCl2, 16 μM G16diP, and either 4 mM G1P (forward) or G6P (reverse) in a final volume of 1 ml. The four allozyme preparations were diluted to equal activities in buffer A. Eight replicate reaction tubes for both reaction directions were equilibrated to the appropriate temperature in a shaking water bath, and were initiated by the addition of substrate. At 3, 6, and 9 min intervals, 250 μl aliquots were removed from each reaction mixture. For assays proceeding in the forward direction, these were transferred into an equal volume of 1N HCl; for the reverse direction into a similar volume of 1N NaOH. The quantity of G6P produced at each time interval from the forward reaction was measured in duplicate as described by Lang and Michal (1974). The G1P formed from the reverse reaction was assayed in a similar fashion by the procedure of Bergmeyer and Michal (1974).

For both reaction directions, Vmax was estimated as the y-intercept of an unweighted linear regression of the product formed/time versus time. Equilibrium constants were determined at each temperature by incubating 10 reaction tubes prepared as above for 2 h with a sample of the Pgm-2-100 allozyme. The equilibrium was approached from both directions with starting concentrations of 2 mM G1P or G6P. The reactions were stopped by immersion in a boiling water bath for 2 min, and the concentrations of G1P and G6P measured in duplicate as described

above. Apparent Michaelis constants for G6P for the four allozymes were estimated from equation 2 by substituting the appropriate Vmax(f)/Vmax(r) ratio, Keq, and app Km (G1P) estimate for the forward reaction direction determined previously.

Thermostability Studies. The four purified allozymes were diluted to equal activities in 50 mM sodium phosphate, 0.5 mM MgCl2, 0.1 mM EDTA, pH 7.0 buffer containing 0.1% (w/v) bovine serum albumin. As in the kinetic study, the same three heterozygote preparations were made and studied alongside the four homozygote samples. For each genotype 50 µl aliquots were pipetted into a set of 21 glass culture tubes (10 x 75 mm). After being sealed with parafilm, 18 of the tubes were incubated with shaking in a water bath at 50°C. At 5 min intervals over a 30 min period triplicate tubes were removed and immediately transferred to an ice bath. Three control tubes were maintained in the ice bath throughout the experiment. PGM activities were measured in duplicate on all samples by the standard assay at 25° and converted to the proportion of activity remaining at each time interval relative to the control. After Place and Powers (1984a) and Hall (1985), the thermal denaturation of these enzyme genotypes was treated as a first-order exponential decay process. Least-squares regression of the logarithm of fractional activity remaining at time t against time yielded a straight line with a negative slope estimating the denaturation constant, kd. Replication of the experiment produced estimates

of kd for all genotypes that did not differ significantly from before, so the data from both trials was pooled. Differences between genotypes in these denaturation constants were tested statistically by examining the heterogeneity of these slopes by ANCOVA, and performing pair-wise comparisons with *a posteriori* Bonferroni multiple range tests.

pH optima. Initial reaction rates were measured under saturating conditions at 0.2 pH unit intervals from pH 6.0 to 8.0 at 20°C by the standard assay on preparations of the four allozymes diluted to equal activities in buffer A. All assay components were dissolved in 50 mM imidazole-HCl solutions adjusted to the appropriate pH. At each pH interval activity measurements were replicated four times after ensuring that the coupling enzyme G6PDH was not rate-limiting. The proportion of activity expressed at each interval relative to that observed at the pH optimum was determined for each genotype. The experiment was repeated a second time and because the results did not differ significantly (by paired t-tests on the angular transformed data), the data from both trials was pooled. Statistical comparison of the four genotypes was carried out for the entire pH range by two-way ANOVA and at each interval by one-way ANOVA on the angular transformed proportions and tested by *a posteriori* Bonferroni multiple range tests.

Effect of Magnesium Ion. The four *Pgm*-2 allozymes were diluted to equal activities in 50 mM imidazole-HCl pH 7.0 (20°C) buffer

and passed through a Sephadex G-25 column (0.9 x 15 cm, equilibrated with the same buffer) to remove free magnesium ions. Initial reaction rates were measured by the standard assay at 15°C on four replicates at magnesium concentrations of 0.5, 1, 3, 5, and 10 mM. Reaction velocities at each cofactor concentration were expressed as proportional activities relative to the maximum rate measured. The experiment was replicated and the data from both trials was pooled for statistical analysis. The four allozymes were compared by two-way ANOVA on the angular transformed proportions for the entire range of Mg concentrations, and by one-way ANOVA for each level separately. Means were compared by *a posteriori* Bonferroni multiple range tests.

RESULTS

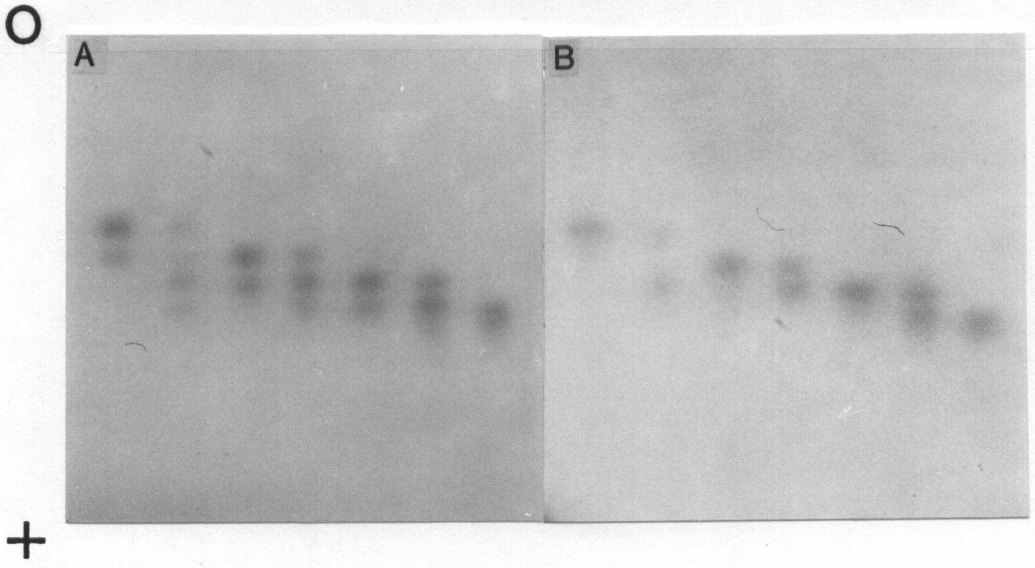
ELECTROPHORESIS AND *PGM*-2 ALLELE FREQUENCIES

Two phosphoglucomutase loci were detected electrophoretically in *Crassostrea gigas*. The more cathodal *Pgm*-1 locus appeared to be polymorphic, but since its activity was only a fraction of that expressed at the *Pgm*-2 locus it could not be reliably scored. Genotypes at the more anodal *Pgm*-2 locus exhibited double-banded staining patterns similar to those described for this enzyme in other species (e.g. Ward and Beardmore 1977; Hoffman 1985). Under standard electrophoretic conditions homozygotes displayed a two-banded phenotype and

heterozygotes were either three- or four-banded depending on the mobilities of the alleles comprising them (Figure 1A). This double-banding was found to result from the simultaneous presence of faster migrating phosphorylated (active) and slower migrating dephosphorylated (inactive) forms of the enzyme, which differed in mobility because of the presence or absence of this negatively charged phosphate group. When electrophoresis was carried out under catalytic running conditions, known to convert PGM entirely to the active phosphoenzyme (Ray and Roscelli 1964b), this double-banding was eliminated and the patterns became consistent with those expected for a monomeric enzyme (Figure 1B).

Evidence consistent with the conversion of the presumed slower migrating dephosphoenzyme to the faster migrating phosphoenzyme was provided when the cofactor G16diP alone was added to the gel buffer at a concentration of 5 μM (not shown). This resulted in the near elimination of the slower migrating band in all genotypes, as predicted from the ability of G16diP to react with the dephosphoenzyme and convert it to the phosphorylated state (Najjar and Pullman 1954). These staining patterns did not result as an artifact of storage because freshly ground tissue and that from oysters stored for months at -40°C behaved identically. It is not known whether the resolution of these different phosphorylated forms of PGM was a consequence of the homogenization and/or electrophoretic procedures, or if they reflect actual <u>in vivo</u> proportions.

Figure 1. Electrophoretic staining patterns of oyster PGM under (A) standard and (B) catalytic running conditions. Pgm-2 genotypes presented from left to right are: 92/92, 92/100, 96/96, 96/100, 100/100, 100/104, and 104/104.


O
A
B
+

Catalytic running conditions aided in the scoring of Pgm-2 genotypes and allowed unequivocal identification of homozygotes selected for enzyme purification.

No hidden variation was uncovered within the most common electromorphic class at the Pgm-2 locus for a temperature-sensitive (Ts) allele as demonstrated in D. melanogaster by Trippa, Loverre and Catamo (1976) and Trippa et al. (1978). Incubation of gels containing individuals homozygous for the Pgm -2-100 allele for 15 min at 50°C resulted in the consistent loss of approximately 50% of the PGM activity observed on the control gels. No evidence of Ts heterozygotes was observed, nor were any Ts homozygotes detected in the sample of 300 oysters. Although heterozygotes for a Ts allele would be hard to identify by this crude method, the complete absence of Ts homozygotes indicates that the frequency of such an allele, if present, is so low ($p < .08$) that it would not seriously influence kinetic properties of this genotypic class presented in the next section. The variable pH system of Beaumont and Beveridge (1983) also failed to detect any heterogeneity within the four most common electromorphic classes when electrophoresis was carried out under either standard or catalytic conditions. However, in agreement with these authors the Tris-maleic acid system at pH 7.4 was unable to accurately resolve two alleles (Pgm-2-96 and 104) that could be more easily identified by the electrode buffer at pH 6.0, or by the standard Tris-borate system run at a higher pH.

A total of 8 alleles were detected at the Pgm-2 locus, in agreement with that reported by Buroker, Hershberger and Chew (1979a). Although pair crosses were not performed in this study, several of these alleles have been shown to segregate in a Mendelian fashion in C. gigas by Wilkins (1976), and at the presumably homologous Pgm locus in C. virginica by Foltz (1986b). Numerical values have been assigned to these alleles expressing their mobilities relative to the most common 100 allele. Their observed frequencies at the low and high water sampling sites over a two year period are presented in Table I. A contingency table analysis indicated that these frequencies were homogeneous over time (χ^2 = 34.2 with 35 df, P > . 50). To examine the potential effect of microenvironmental conditions on these frequencies, a heterogeneity Chi-square analysis was carried out comparing allelic distributions between the two tidal heights. No significant differences were found between tidal heights at any of the four sampling dates, on the pooled totals, or for the computed interaction term.

The Chi-square values listed in Table I test the agreement between the observed genotypic proportions and Hardy-Weinberg expectations. Statistically significant deviations were detected for 3 of the 8 samples, and an additional two (10/83 High and 11/85 high) were nearly significant (P < .10). A heterogeneity Chi-square on these genotypic proportions yielded a significant interaction term (χ^2 = 57.3 with 35 df, P < .05), caused by the 3/85 sample which exhibited the largest deficiency of

Table I. *Pgm*-2 allele frequencies, conformity to Hardy-Weinberg expectations, and heterozygote deficiencies at the four sampling dates.

	Frequency at Date and Tidal Height									
	10/83		6/84		3/85		11/85		Totals	
Allele	Low	High	Low	High	Low	High	Low	High	Low	High
84	.004	.002	.003	.003	---	.002	.002	.002	.002	.003
88	.004	.005	.007	.006	---	.004	.007	.006	.006	.005
92	.133	.125	.091	.146	.108	.131	.118	.115	.112	.129
96	.096	.075	.111	.097	.075	.097	.100	.094	.094	.093
100	.596	.610	.601	.538	.652	.552	.605	.609	.617	.578
104	.142	.133	.157	.166	.141	.180	.134	.155	.141	.163
108	.025	.050	.027	.038	.019	.032	.034	.013	.027	.027
112	---	---	.003	.006	.005	.002	---	.006	.002	.002
N	121	100	149	160	205	222	220	265	695	747
x^2	9.05	10.5	4.73	2.40	27.4***	20.4**	13.0*	9.72	na	na
He	.597	.586	.593	.651	.537	.635	.591	.583	.577	.613
Ho	.579	.550	.557	.631	.454	.550	.550	.562	.528	.572
D	-.030	-.061	-.061	-.031	-.155	-.134	-.069	-.036	-.085	-.067

na = not applicable
* P < .05
** P < .01
*** P < .001

heterozygotes as measured by D = (Ho-He)/He (where Ho and He are the observed and expected heterozygosities, respectively). D values calculated for the other samples were also negative, but generally much lower than previously reported for this locus [D averaging -0.122 for 6 Washington state populations (Buroker 1975) and -0.172 for 20 Japanese populations (Fujio 1982)]. Given the allelic frequency distributions in Table I, individuals at the Pgm-2 locus are dominated by genotypes homozygous for the Pgm-2-100 allele and heterozygotes between this allele and either the Pgm-2-92, 96, and 104 alleles. Therefore, the biochemical properties of these three heterozygotes and the homozygotes for the four common alleles were singled out for study to examine the potential expression of overdominance at the Pgm-2 locus.

BIOCHEMICAL PROPERTIES OF PGM-2 GENOTYPES

A purification summary for the Pgm-2-104 allozyme is shown in Table II. The four step procedure resulted in a 300-fold purification and recovered 39% of the starting activity. The presence of saturating levels of substrate in the final DEAE-substrate step caused an earlier elution of the saturated enzyme in the salt gradient, presumably because of altered binding and/or charge properties. Hall (1985) has reported a similar observation in the purification of two phosphoglucose isomerase allozymes from the mussel, Mytilus edulis. The final specific activities of the three other Pgm-2 allozymes were similar to

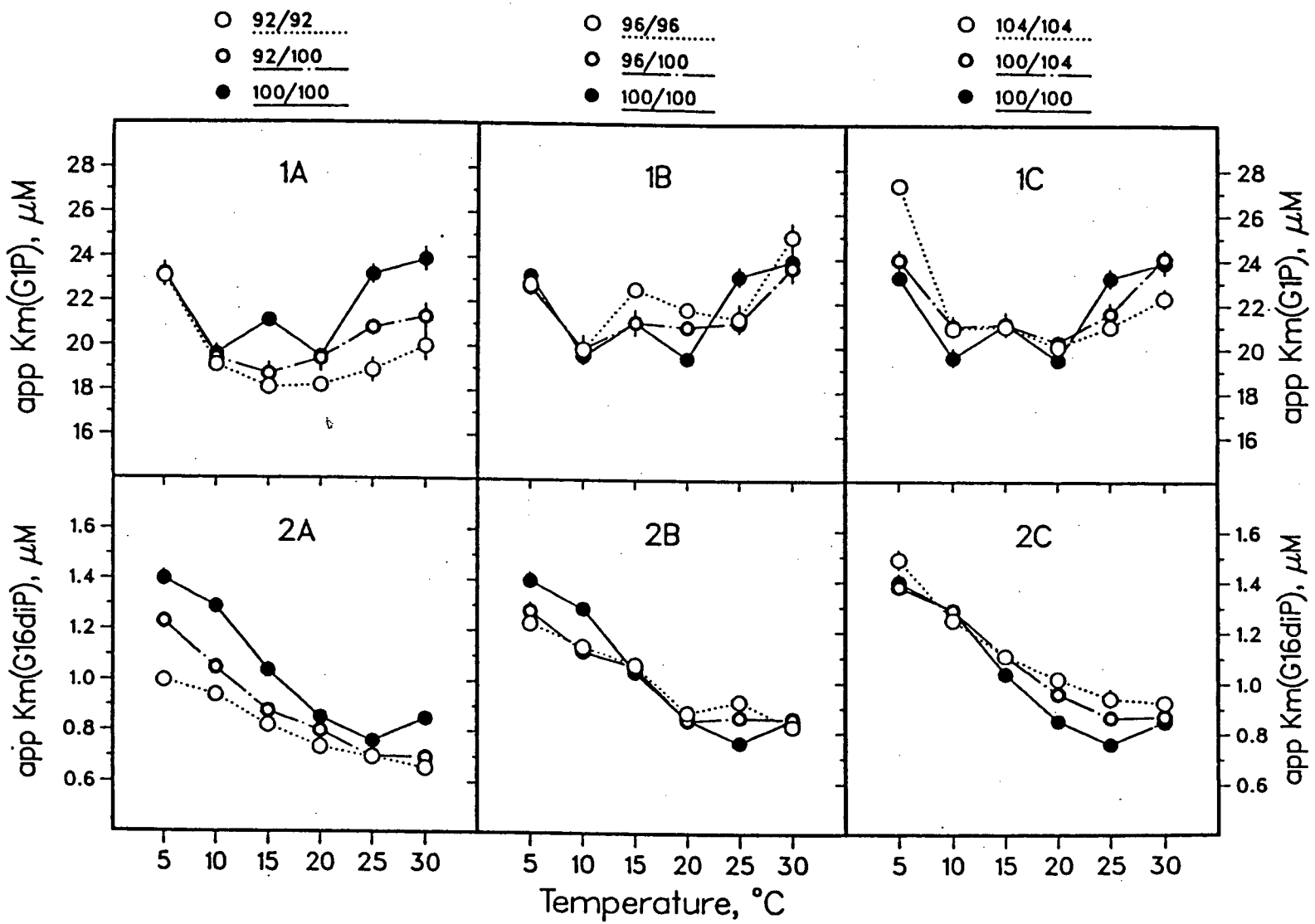
Table II. Purification summary for the Pgm-2-104 allozyme.

Fraction	Volume (ml)	Spec. Activity (units/mg)	Purification (fold)	Recovery (%)
Crude Homogenate	168.0	0.0533	1	100
Ammonium Sulphate Precipitation	10.0	0.175	3.28	94.3
DEAE-Cellulose	20.2	2.25	42.2	90.3
Sephadex G-100	6.7	6.94	130	53.2
DEAE-Substrate	13.2	15.9	298	39.3

that shown in Table II for the Pgm-2-104 allozyme, and although these preparations were not homogeneous (as determined by SDS gels), all were found to be free of enzymes capable of interfering with the PGM assay (UDP-glucose pyrophosphorylase and phosphoglucose isomerase).

The apparent Michaelis constants of seven Pgm-2 genotypes for glucose-1-phosphate (G1P) and glucose-1,6-diphospate (G16diP) over the temperature range of 5 to 30°C are presented graphically in Figure 2. A two-way analysis of variance (ANOVA) on the app Km (G1P) values (Figure 2, upper half) revealed that temperature exerted a highly significant effect ($F(5,30) = 17.6$, $P < .001$). A posteriori Bonferroni multiple range tests indicated that the app Km's measured at 5 and 30°C were significantly larger than those at 10, 15, or 20°C, but the values obtained at 25 and 30°C were indistinguishable. This analysis also uncovered a significant effect of Pgm-2 genotype ($F(6,30) = 5.21$, $P < .001$). Multiple range tests comparing the app Km values of different genotypes showed that this result was due entirely to the Pgm-2-92/92 homozygote (1A) which exhibited significantly lower app Km's for G1P than the Pgm-2-96/96 and Pgm-2-104/104 homozygotes, and the Pgm-2-100/104 heterozygote. Genotypic comparisons at individual temperatures by ANCOVA showed that the significantly lower app Km's for G1P of the Pgm-2-92/92 genotype arose from its performance over the 15 to 30°C range. The three heterozygotes were strictly intermediate in their app Km (G1P) values, as expected for a monomer like PGM,

Figure 2. Effect of temperature on the apparent Michaelis constants (in μM) for glucose-1-phosphate (1; upper half) and glucose-1,6-diphosphate (2; lower half) of seven Pgm-2 genotypes. In each grid the Pgm-2-100/100 homozygote (closed circle) is compared to a different homozygote (open circle) and the corresponding heterozygote (partially closed circle). Bars represent ±1 standard error where visible or fall within the plotted symbol.


92/92
92/100
100/100
96/96
96/100
100/100
104/104
100/104
100/100
1A
1B
1C
2A
2B
2C
app Km(G1P), μM
app Km(G16diP), μM
Temperature, °C
5
10
15
20
25
30
16
18
20
22
24
26
28
0.6
0.8
1.0
1.2
1.4
1.6

and were kinetically indistinguishable from each other except at 15°C where the Pgm-2-92/100 heterozygote expressed a significantly lower app Km (G1P) than the other two.

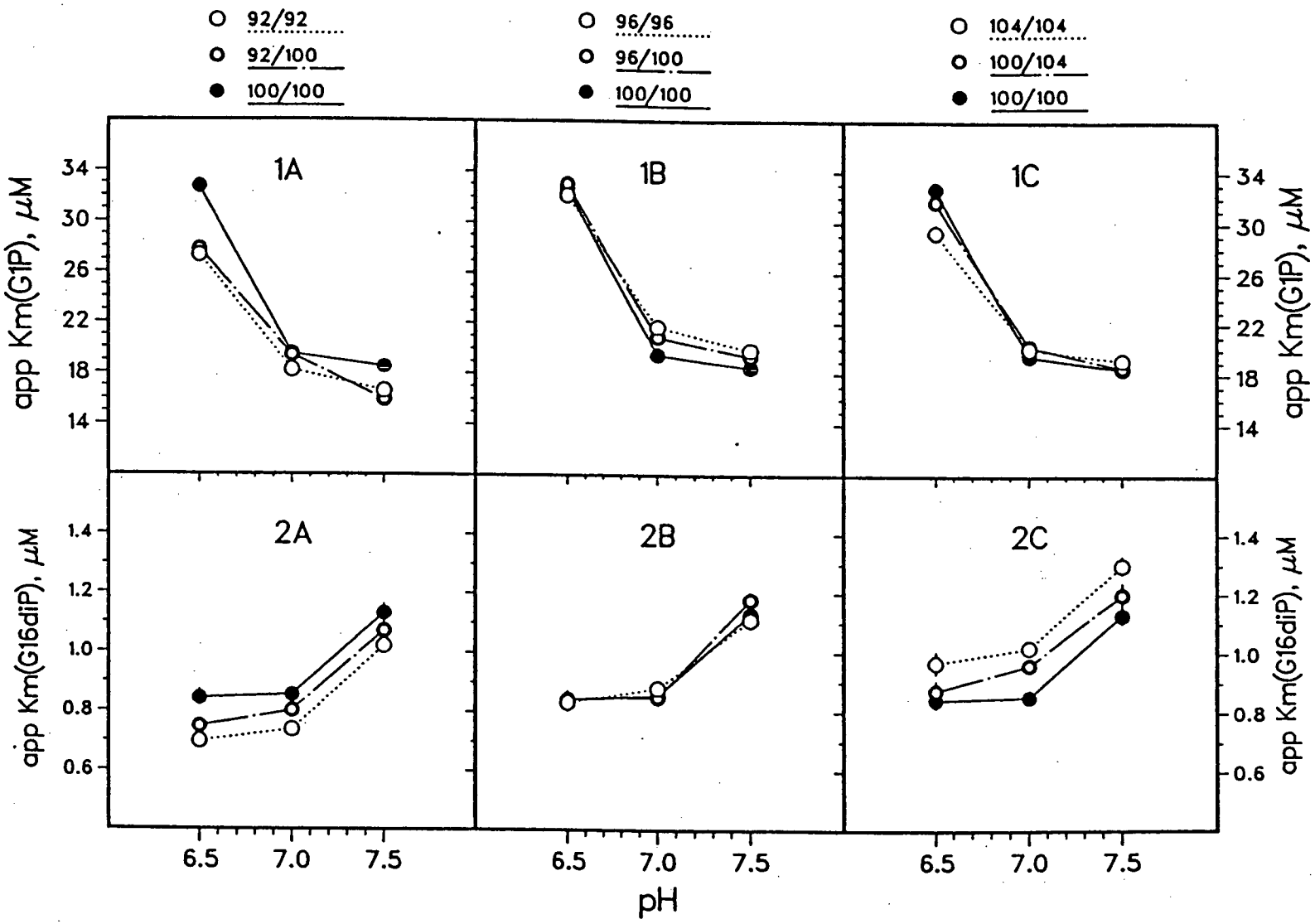
The apparent Michaelis constants for the cofactor glucose-1,6-diphosphate decreased with increasing temperature (Figure 2, lower half), and again a two-way ANOVA found this effect of temperature to be significant ($F(5,30) = 76.8$, $P < .001$). Multiple range tests indicated that these app Km values were homogeneous over the upper (20 to 30°C) and lower (5 to 10°C) temperature ranges, and that both were distinct from those measured at 15°C. A significant effect was again attributable to Pgm-2 genotype ($F(6,30) = 18.0$, $P < .001$). As found for the substrate G1P, multiple range tests showed this effect to be caused primarily by the Pgm-2-92/92 homozygote (2A) which displayed significantly lower app Km (G16diP) values than the three other homozygotes. This was found by ANCOVA to hold true at each temperature except 25°C where the Pgm-2-92/92 homozygote did not differ significantly from the Pgm-2-100/100 homozygote. Overall, the Pgm-2-92/100 heterozygote displayed significantly lower apparent Michaelis constants for G16diP than Pgm-2-100/104, but not Pgm-2-96/100. This occurred because the Pgm-2-92/100 and Pgm-2-96/100 heterozygotes differed at 15, 25, and 30°C, but not at 5, 10 or 20°C.

Since the pH of the imidazole buffer used in this study varies inversely with temperature (Yancey and Somero 1978), the

influence of temperature on the kinetic functioning of these PGM allozymes contained a confounding effect of the simultaneous variation in buffer pH. To examine the effect of pH alone, apparent Michaelis constants for G1P and G16diP were measured at pH 6.5, 7.0, and 7.5 at a constant temperature of 20°C (Figure 3). pH exerted a highly significant effect on the app Km's for both G1P ($F(2,12) = 308.4$, $P < .001$) and G16diP ($F(2,12) = 517.0$, $P < .001$). For both kinetic parameters, multiple range tests showed that the means expressed at each pH differed significantly from each other. Pgm-2 genotype also contributed significantly to the variation with pH of both app Km (G1P) ($F(6,12) = 7.14$, $P < .05$) and app Km (G16diP) ($F(6,12) = 61.4$, $P < .001$). Once more, the Pgm-2-92/92 homozygote was largely responsible for this significant effect of genotype. Multiple range tests comparing the app Km (G1P) values of the genotypes uncovered a significant difference only between the Pgm-2-92/92 and Pgm-2-96/96 homozygotes. For the app Km (G16diP) estimates, the Pgm-2-92/92 genotype displayed significantly lower values than the three other homozygotes. The Pgm-2-100/100 and Pgm-2-96/96 genotypes were identical, although each was significantly different from the Pgm-2-104/104 homozygote. For the heterozygotes, these tests showed Pgm-2-92/100 to be significantly different from the two, which again were indistinguishable over this pH range.

A comparison of the patterns produced by variation in pH on the apparent Michaelis constants for G1P and G16diP in Figure 3

Figure 3. Effect of pH on the apparent Michaelis constants (in μM) for glucose-1-phosphate (1; upper half) and glucose-1,6-diphosphate (2; lower half) of seven Pgm-2 genotypes. Genotypic comparisons are shown at the top of the figure. Bars represent ±1 standard error where visible or fall within the plotted symbol.


92/92
92/100
100/100
96/96
96/100
100/100
104/104
100/104
100/100
1A
1B
1C
2A
2B
2C
app Km(G1P), μM
app Km(G16diP), μM
34
30
26
22
18
14
1.4
1.2
1.0
0.8
0.6
6.5
7.0
7.5
pH

shows that one is virtually a mirror image of the other. An increase in pH from 6.5 to 7.0 caused a marked decline in app Km (G1P) but only a slight increase in app Km (G16diP). A further increase in pH from 7.0 to 7.5 produced only a small decrease in app Km (G1P) but a substantial increase in app Km (G16diP). This antagonistic effect of pH on the affinity of the enzyme for substrate and cofactor is suggestive of a role played by the dissociation state of the imidazole side chain of a histidine residue on the binding of these reactants [the alpha-imidazole hypothesis of Reeves (1972)]. Under standard physiological conditions the imidazole moiety is present in roughly equal proportions of protonated and deprotonated forms (Somero 1981). Active site sequences of PGM isolated from rabbit (Milstein and Sanger 1961), human (Joshi and Handler 1969), flounder (Hashimoto, del Rio and Handler 1966), and E. coli (Joshi and Handler 1964) all possess a histidine adjacent to the catalytic phosphoserine residue. If oyster PGM also has this histidine present, Figure 3 suggests that the deprotonated form of this residue favors binding of G1P to the phosphoenzyme (above pH 7.0) and the protonated state favors binding of G16diP to the dephosphoenzyme (below pH 7.0), an effect of pH not previously described for this enzyme.

These pH-induced effects are capable of explaining some of the variation in the app Km (G1P) and (G16diP) estimates in Figure 2 that were apparently caused by temperature. Since the pH of imidazole buffer decreases 0.017 pH units/°C, and was

initially adjusted to pH 7.0 at 20°C, as temperature was increased from 5 to 30°C the pH of the assay buffer decreased linearly from approximately 7.3 to 6.8. For the observed variation in app Km (G1P) with temperature, the negligible changes from 10 to 20°C (pH 7.2 to 7.0) and the increases from 20 to 30°C (as pH fell below 7.0) are largely predicted from these changes in pH alone; only the decreases from 5 to 10°C appear to be an effect attributable to temperature. The variation in app Km (G16diP) with temperature is also consistent with these changes in pH. The declines in app Km (G16diP) over the temperature range of 5 to 20°C were expected from the drop in buffer pH from 7.3 to 7.0, as were the minor changes observed from 20 to 30°C as pH decreased below 7.0. These observations suggest that the in vivo functioning of these allozymes may be more sensitive to variation in intracellular pH than to changes in ambient temperature.

Maximum velocity (Vmax) estimates obtained by biweight regression were standardized to a common protein content at 5°C (to correct for the slight differences in activities between allozyme preparations) and compared between genotypes over the temperature range of 10 to 30°C. Plots of log (Vmax) against temperature were curved slightly downward at higher temperatures (not shown) as found previously for PGM isolated from rabbit muscle (Ray and Peck 1972) and the sea anemone, Metridium senile (Hoffman 1985). Two-way analysis of variance found no significant differences between genotypes in the sensitivity of

log (Vmax) to either temperature ($F(5,24) = 1.15$, $P > .40$) or pH ($F(6,12) = 1.56$, $P > .20$). Vmax/Km ratios of the seven Pgm-2 genotypes for the forward reaction direction as functions of temperature and pH are presented in Figures 4 and 5, respectively. Statistical comparisons of these ratios by two-way ANOVA revealed significant differences between genotypes associated with both temperature ($F(6,30) = 6.90$, $P < .001$) and pH ($F(6,12) = 9.07$, $P < .001$) that were produced by the differences in the apparent Michaelis constants for glucose-1-phosphate shown in Figures 2 and 3, respectively. In Figure 4 this significant result is caused entirely by the Pgm-2-92/92 homozygote (A) which displayed a larger overall Vmax/Km ratio than all other genotypes except the Pgm-2-92/100 heterozygote. Over the pH range of 6.5 to 7.5 the Pgm-2-92/92 homozygote also had a Vmax/Km ratio that was significantly greater than the Pgm-2-96/96 homozygote but not the Pgm-2-100/100 or Pgm-2-104/104 genotypes. The intermediate behavior of heterozygotes again resulted in the Pgm-2-92/100 genotype being significantly different from the Pgm-2-96/100 but not the Pgm-2-100/104 heterozygote. Since Vmax/Km ratios most accurately reflect *in vivo* catalytic function (Hoffman 1981; Watt 1983), these results show that only one allozyme, Pgm-2-92, consistently displays divergent kinetic properties.

Ratios of Vmax(f)/Vmax(r) for the four Pgm-2 homozygotes from 5 to 30°C are presented in Table III. The rate of the forward reaction exceeded the reverse by a factor of three,

Figure 4. Effect of temperature on the Vmax/Km ratios of seven *Pgm*-2 genotypes for the forward reaction direction. Bars represent ±1 standard error where visible or fall within the plotted symbol.

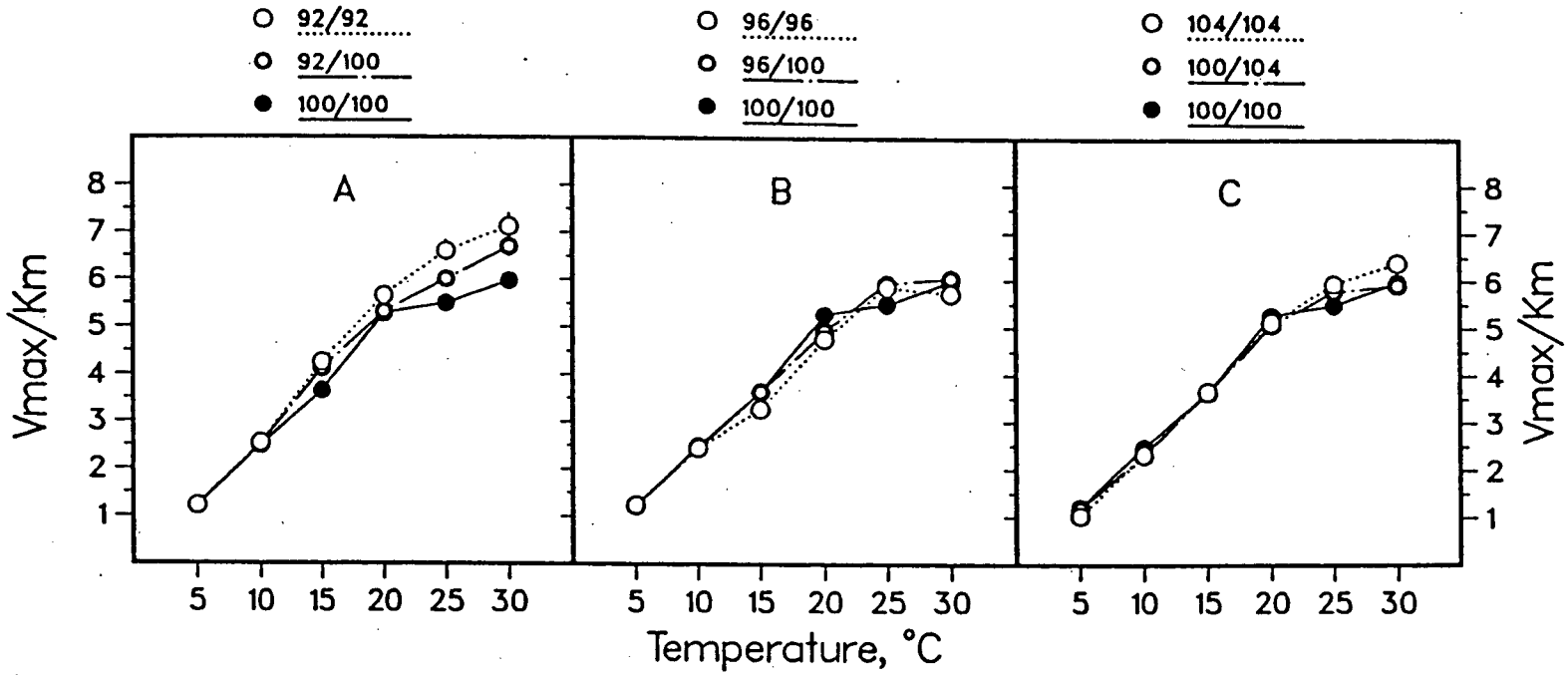

92/92
92/100
100/100
96/96
96/100
100/100
104/104
100/104
100/100
A
B
C
Vmax/Km
Vmax/Km
1
2
3
4
5
6
7
8
5
10
15
20
25
30
Temperature, °C

Figure 5. Effect of pH on the Vmax/Km ratios of seven Pgm-2 genotypes for the forward reaction direction. Bars represent ±1 standard error where visible or fall within the plotted symbol.

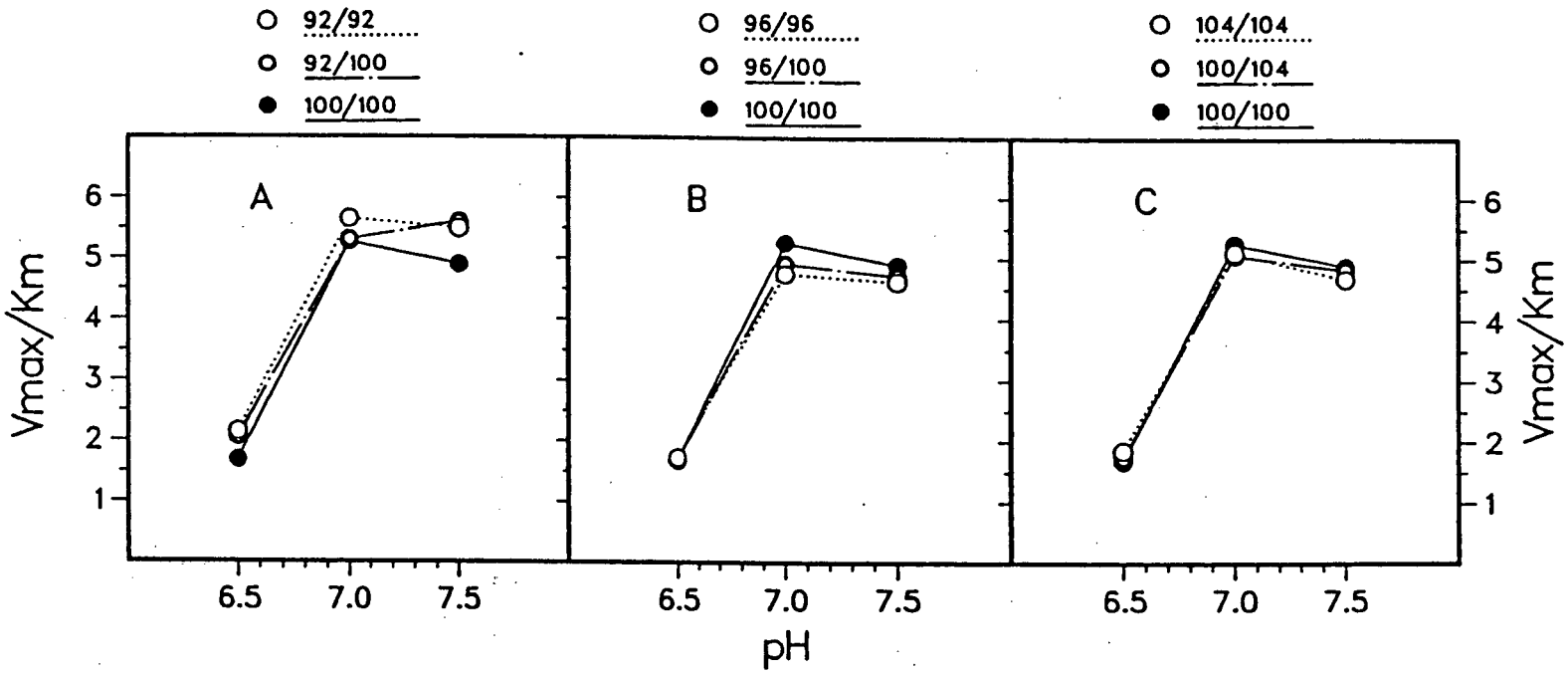

92/92
92/100
100/100
96/96
96/100
100/100
104/104
100/104
100/100
A
B
C
Vmax/Km
Vmax/Km
6
5
4
3
2
1
6.5
7.0
7.5
pH

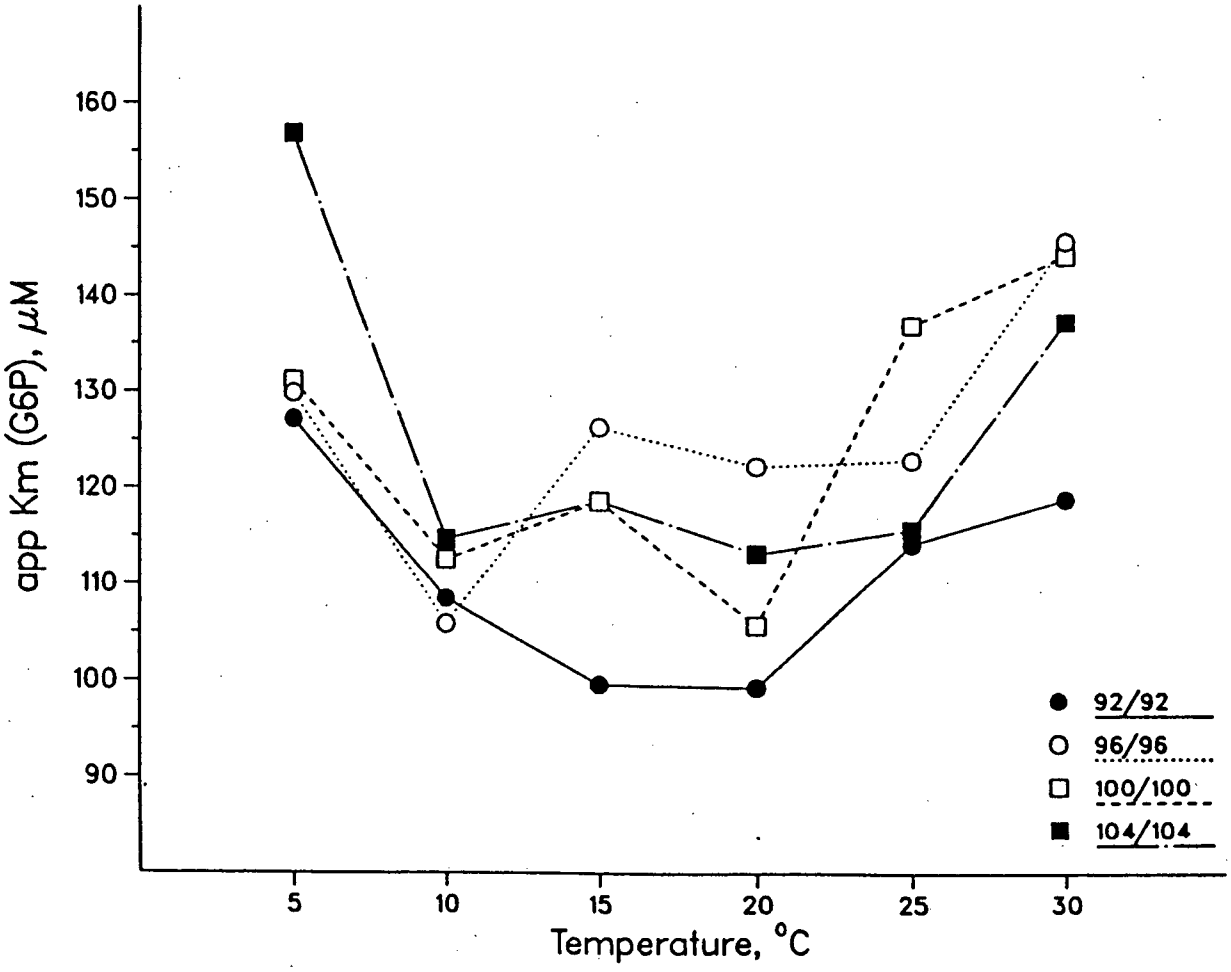
Table III. Effect of temperature on the Vmax(f)/Vmax(r) ratios of four Pgm-2 homozygotes.

Temperature	Pgm-2 Genotype			
	92/92	96/96	100/100	104/104
5	3.07±.16	2.99±.10	3.08±.14	2.97±.11
10	3.01±.08	2.92±.09	2.93±.09	3.13±.10
15	3.12±.06	3.06±.06	3.04±.05	3.07±.07
20	3.09±.05	3.01±.05	3.10±.06	3.03±.05
25	2.84±.07	2.96±.06	3.02±.06	3.10±.07
30	2.87±.05	2.93±.05	2.83±.05	2.77±.06

similar to that reported for rabbit muscle PGM by Ray and Roscelli (1964b). Two-way analysis of variance on the Vmax ratios detected a significant effect of temperature ($F(5,15) = 4.09$, $P < .05$), that multiple range tests found to be caused by the higher ratios measured at 15°C compared to 30°C. No significant differences were observed between the four Pgm-2 homozygotes ($F(3,15) = 0.17$, $P > .90$). The regressions of product formed/time vs. time used to calculate these ratios were always highly significant. The coefficients of determination for the Vmax(f) and Vmax(r) regressions averaged 82.0 and 83.4%, respectively. The slopes of the regression lines from the forward reaction direction approximated unity, thus confirming a linear conversion of substrate to product over the assay period. However, as found by Ray and Roscelli (1964b), the slopes from the reverse direction were always negative, indicating departures from linearity even though substrate conversion was 1% or lower.

Equilibrium constants for the PGM reaction showed a tendency to decline with increasing temperature. At 5°C, Keq was estimated as 18.0±0.45, while at 30°C it was calculated to be 17.3±0.32. Because these values were slightly larger than previously reported for this reaction, Colowick and Sutherland's (1942) estimate of 17.2 was accepted for the entire temperature range. Estimates of the apparent Michaelis constants for G6P of the four Pgm-2 homozygotes were calculated from equation 2 and are presented in Figure 6. Since the Vmax(f)/Vmax(r) ratios of

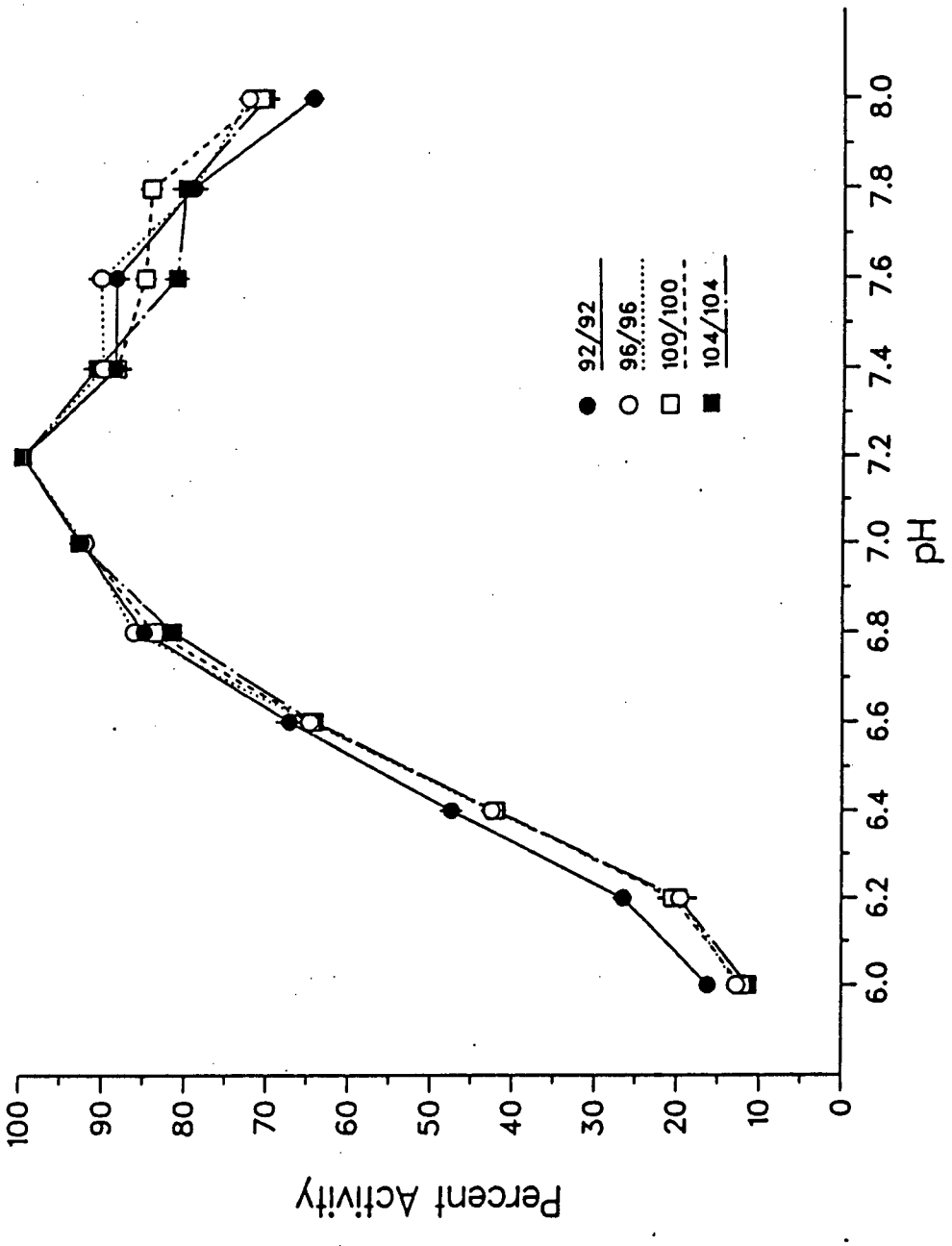
Figure 6. Apparent Michaelis constants (in μM) for glucose-6-phosphate of four Pgm-2 homozygotes estimated from the Haldane equation.


app Km (G6P), μM
160
150
140
130
120
110
100
90
5
10
15
20
25
30
Temperature, °C
92/92
96/96
100/100
104/104

these genotypes were indistinguishable, their app Km (G6P) values over this temperature range were very similar to the patterns observed for their app Km's for G1P seen in Figure 2. The ability to detect kinetic differences between Pgm-2 genotypes for the reverse reaction direction by this indirect approach is severely restricted. Therefore, these values must be viewed as provisional, and all that may be concluded is that there is no evidence for further differentiation through the constraints imposed by the Haldane relationship.

Over the pH range of 6.0 to 8.0 all four Pgm-2 allozymes exhibited optimal activity at pH 7.2 (Figure 7), which is slightly lower than the pH optima for PGM isolated from Metridium senile (Hoffman 1985), D. melanogaster (Fucci et al. 1979), humans (Joshi and Handler (1969), flounder and shark (Hashimoto and Handler 1966), and two bacterial species (Hanabusa et al. 1966). Although these pH profiles were very similar in form, a two-way ANOVA on the angular transformed proportional activities showed that significant differences existed between these four homozygotes ($F(3,308) = 15.3$, $P < .001$). Multiple range tests revealed that the overall activity of the Pgm-2-92/92 homozygote significantly exceeded Pgm-2-100/100 and Pgm-2-104/104 but not Pgm-2-96/96. Decomposition of this overall analysis into 11 individual one-way ANOVA's at each pH interval showed that this was caused by the greater activity of the Pgm-2-92/92 homozygote over the pH range of 6.0 to 6.6. In the standard physiological pH range of 7.0 to 7.4 the four

Figure 7. PH-dependent activities of four Pgm-2 homozygotes at 20°C. Bars represent ±1 standard error where visible or fall within the plotted symbol.


Percent Activity
100
90
80
70
60
50
40
30
20
10
0
6.0
6.2
6.4
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
pH
92/92
96/96
100/100
104/104

allozymes were homogeneous. Above pH 7.4 each curve displayed a "shoulder" which for the Pgm-2-92 and Pgm-2-96 allozymes occurred between pH 7.4 and 7.6 and for the Pgm-2-100 and Pgm-2-104 allozymes between pH 7.6 and 7.8.

Marked differences existed between Pgm-2 genotypes in their temperature stabilities at 50°C (Figure 8). Thermal denaturation constants, determined for each genotype by regressing log (fractional activity x 10) against incubation time, were compared statistically by ANCOVA and found to be significantly heterogeneous ($F(6,448) = 383.0$, $P < .001$). The Pgm-2-100/100 genotype was the most stable (kd = -0.0143), and multiple range tests indicated that it was significantly different from the Pgm-2-104/104 homozygote (kd = -0.0176). These two homozygotes were significantly more stable than the Pgm-2-92/92 and Pgm-2-96/96 genotypes (exhibiting kd's of -0.0283 and -0.0295, respectively). Because of the intermediate properties of heterozygotes, the Pgm-2-100/104 genotype exhibited a significantly lower denaturation constant (kd = -0.0159) than either Pgm-2-92/100 (kd = -0.0206) or Pgm-2-96/100 (kd = -0.0212) which, as expected, were indistinguishable.

The effect of magnesium ion on the initial reaction velocities of the four Pgm-2 allozymes is shown in Figure 9. Each variant displayed optimal activity at 3 mM and a slight tendency to decline at higher cofactor concentrations. Statistical comparison of the angular transformed proportional

Figure 8. Thermal inactivation plots of seven Pgm-2 genotypes at 50°C. Bars represent ±1 standard error where visible or fall within the plotted symbol.

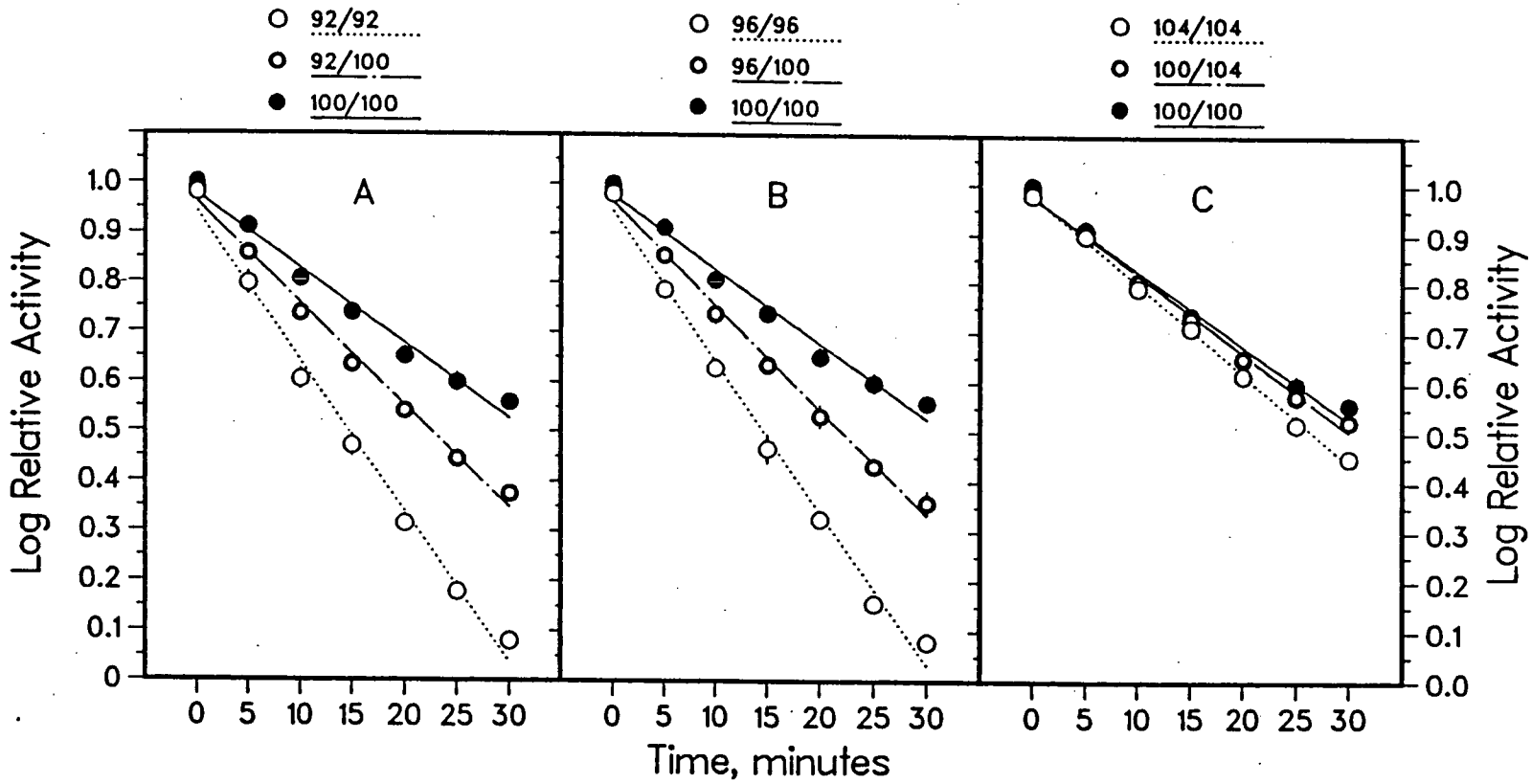
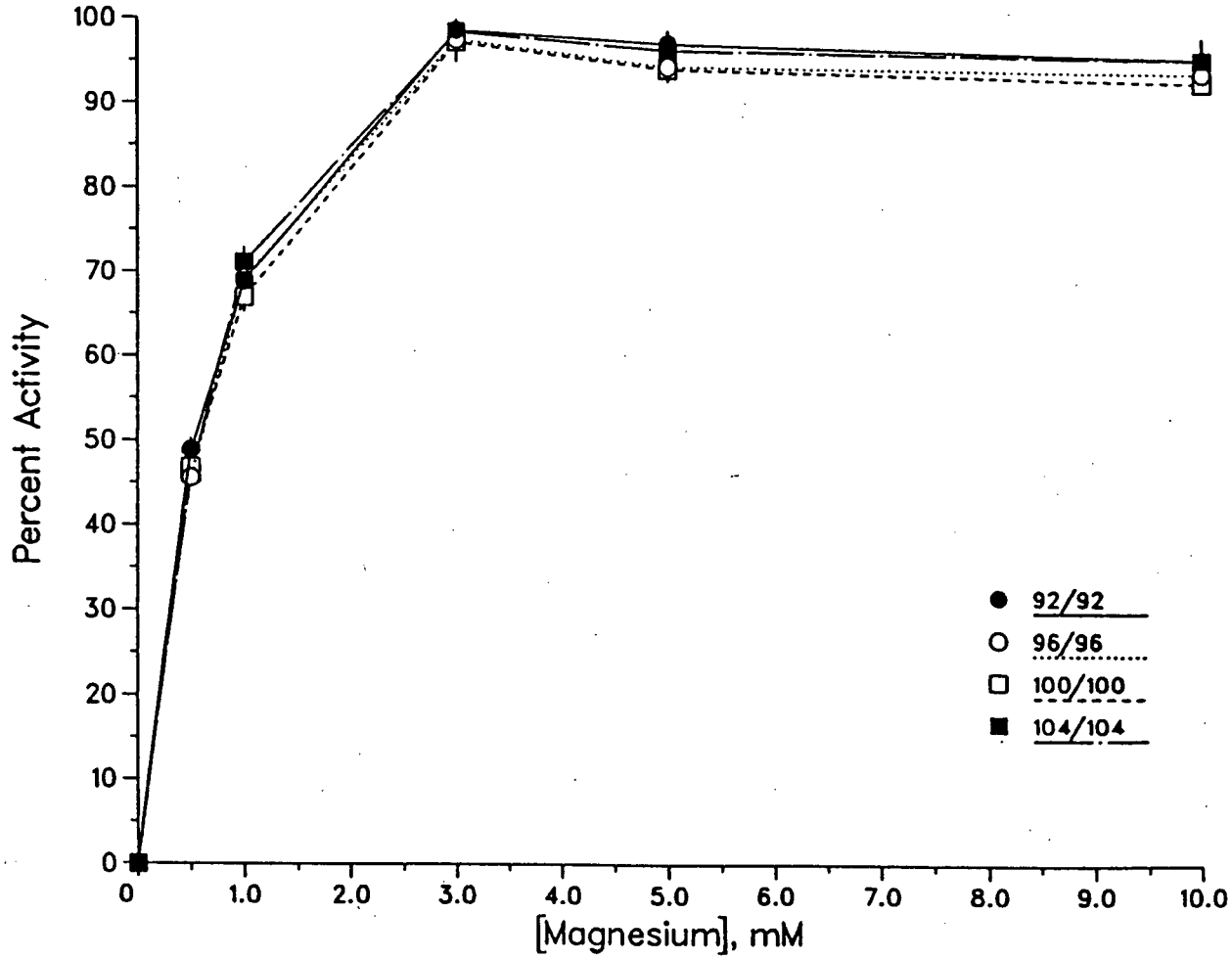

92/92
92/100
100/100
96/96
96/100
100/100
104/104
100/104
100/100
A
B
C
Log Relative Activity
Time, minutes
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0
5
10
15
20
25
30

Figure 9. Effect of magnesium ion on the activities of four *Pgm*-2 homozygotes. Bars represent ±1 standard error where visible or fall within the plotted symbol.


Percent Activity
100
90
80
70
60
50
40
30
20
10
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
[Magnesium], mM
92/92
96/96
100/100
104/104

activities by two-way ANOVA detected no significant differences between Pgm-2 allozymes ($F(3,140) = 1.22$, $P > .20$).

DISCUSSION

A selection-based explanation for the associations between multiple-locus heterozygosity and various phenotypic traits must be founded upon the existence of biochemical differences between the allelic products of the specific loci involved in these relationships (cf. Clarke 1975; Koehn 1978). Once these functional differences have been established, however, verification of the overdominance hypothesis places stringent requirements on the properties of heterozygotes relative to their constituent homozygotes concerning both the manifestation of overdominance and the conditions allowing a stable polymorphic equilibrium (e.g. Mandel 1959; Lewontin, Ginzburg and Tuljapurkar 1978). For the Pgm-2 locus in Crassostrea gigas, kinetic and structural differences exist between the four most common allozymes. Significant differences were observed between Pgm-2 genotypes in their apparent Michaelis constants for glucose-1-phosphate and glucose-1,6-diphosphate over ranges of temperature (Figure 2) and pH (Figure 3), Vmax/Km ratios (Figures 4 and 5), pH-dependent activities (Figure 7), and thermostabilities (Figure 8). It must now be determined if these biochemical differences are sufficient to account for the overdominance for adult body weight described at this locus by Fujio (1982). Several lines of argument suggest that they are

not.

First, consideration of the catalytic reaction mechanism of phosphoglucomutase suggests that the observed kinetic differences may not be as pronounced, or even expressed at all, under in vivo conditions. Previous studies on PGM have established that it functions along a continuum between "uni-uni" and "ping-pong" kinetics depending on the specific sugar phosphates interconverted and the organism from which it was extracted (reviewed by Ray and Peck 1972). This variation is caused by differences in the dissociation rate of the diphosphate from the enzyme's central reaction complex: if this occurs frequently, glucose-1,6-diphosphate acts as "first product" as well as "second substrate" in the classic "ping-pong" mechanism of Cleland (1963). Despite this variation in reaction mechanism, double reciprocal plots of initial velocity against G1P concentration at various levels of G16diP in the vicinity of Km (G16diP) produce a series of parallel lines in accordance with the ping-pong mechanism (Ray and Roscelli 1964a; Joshi and Handler 1966, 1969; Hashimoto and Handler 1966). For an enzyme obeying ping-pong kinetics, Ray and Roscelli (1964a) have verified experimentally that the true or "realized" Michaelis constant for G1P is related to the apparent Km's for both G1P and G16diP by the following equation:

$$K'm\ (G1P) = \frac{app\ Km\ (G1P)[G16diP]}{app\ Km\ (G16diP) + [G16diP]} \quad ...(3)$$

where K'm (G1P) is the realized Michaelis constant for G1P, app Km (G1P) and app Km (G16diP) are the apparent Michaelis constants for G1P and G16diP, respectively, and [G16diP] is the concentration of G16diP.

In mammalian tissues, G16diP concentrations have been observed to range from 5 μM (Beitner, Haberman and Nordenberg 1978) to as high as 80 μM (Passonneau et al. 1969). These levels are several orders of magnitude above PGM's Km for G16diP in these organisms (Ray and Roscelli 1964b; Passonneau et al. 1969), suggesting that the enzyme exists *in vivo* entirely in the active phosphorylated state. However, a number of recent studies have demonstrated activations in the activity of PGM by increases in G16diP concentrations (reviewed by Beitner 1984) which are unexpected if the cofactor exists at saturating levels. These results have been interpreted as a deinhibitory effect of G16diP, but from what is known about PGM, this can only occur if some proportion of the enzyme exists in the dephosphorylated state (see Ray and Peck 1972). The electrophoretic detection of both phosphorylated and dephosphorylated forms of oyster PGM is consistent with these suggestions. Therefore, assuming an unsaturating G16diP concentration of 1 μM the realized Michaelis constants for G1P and corresponding Vmax/K'm ratios of the 7 *Pgm*-2 genotypes as

functions of temperature and pH have been calculated from equation 3 and are presented in Figures 10 and 11, respectively.

Comparison of these modified parameters with those presented in Figures 2, 3, 4 and 5 shows that the K'm (G1P) estimates are reduced by approximately 50% and hence the Vmax/K'm ratios have increased by roughly the same amount. More importantly, two-way analysis of variance on these new estimates found that the significant effects previously attributable to Pgm-2 genotype had either been eliminated or substantially reduced. In contrast to the results for the app Km (G1P) and app Km (G16diP) estimates individually, these analyses were unable to detect any significant differences between genotypes in their realized Michaelis constants for G1P ($F(6,30) = 0.913$, $P > .40$) or Vmax/K'm ratios ($F(6,30) = 1.75$, $P > .40$) over the temperature range of 5 to 30°C (Figure 10). Over the pH range studied (Figure 11), significant differences were still present between genotypes in these modified K'm values ($F(6,12) = 4.70$, $P < .05$) and Vmax/K'm ratios ($F(6,12) = 4.04$, $P < .05$), but their magnitude was diminished and a posteriori multiple range tests were unable to detect any differences between the genotypes.

The homogenization of the K'm (G1P) estimates of Pgm-2 genotypes appears to be a consequence of the covariation of their app (G1P) and app Km (G16diP) values. Apparently, the amino acid substitution(s) giving rise to the differences in

Figure 10. Effect of temperature on the estimated *in vivo* apparent Michaelis constants (in μM) for glucose-1-phosphate (1; upper half) and corresponding Vmax/K'm ratios (2; lower half) of seven *Pgm*-2 genotypes.

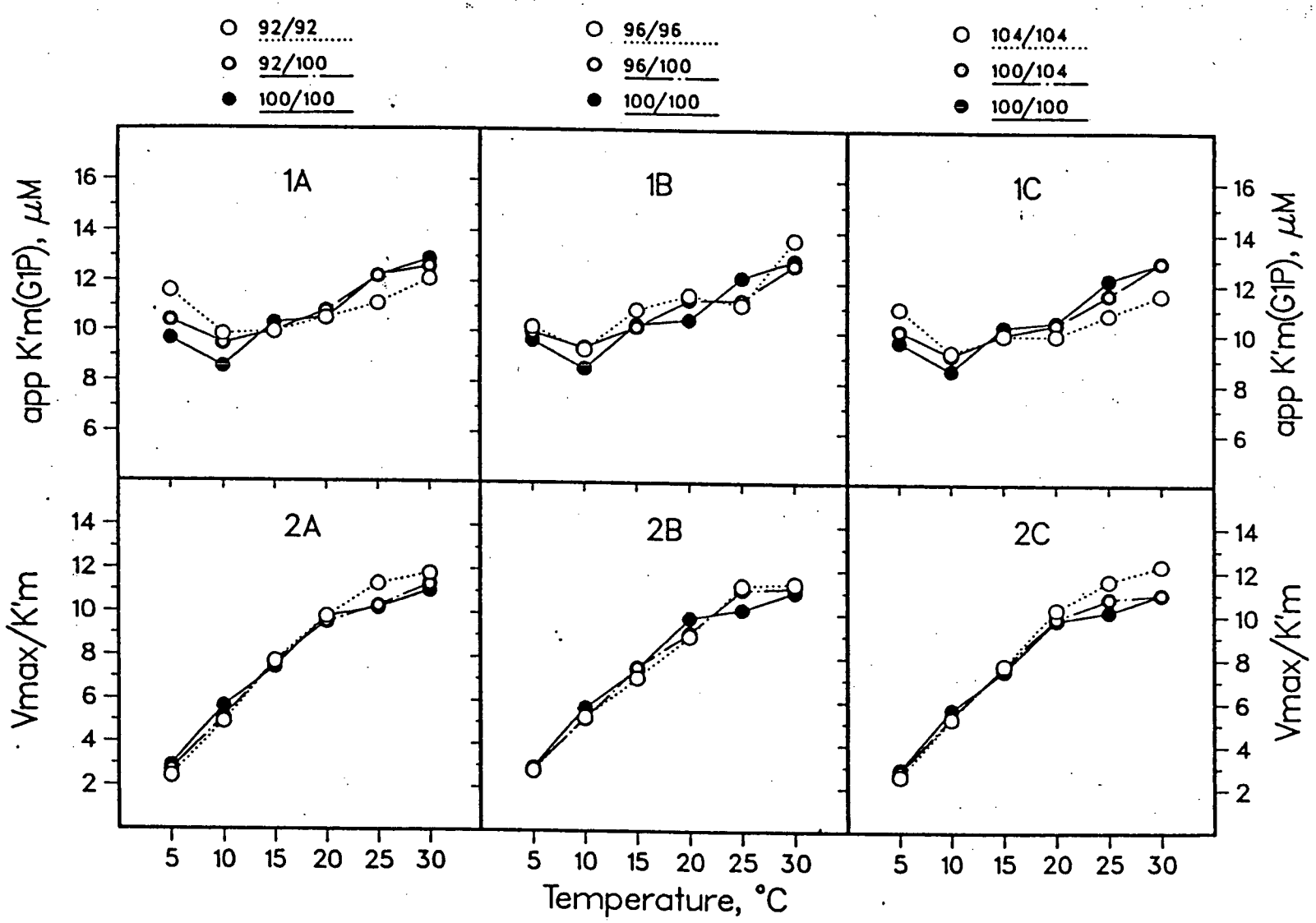
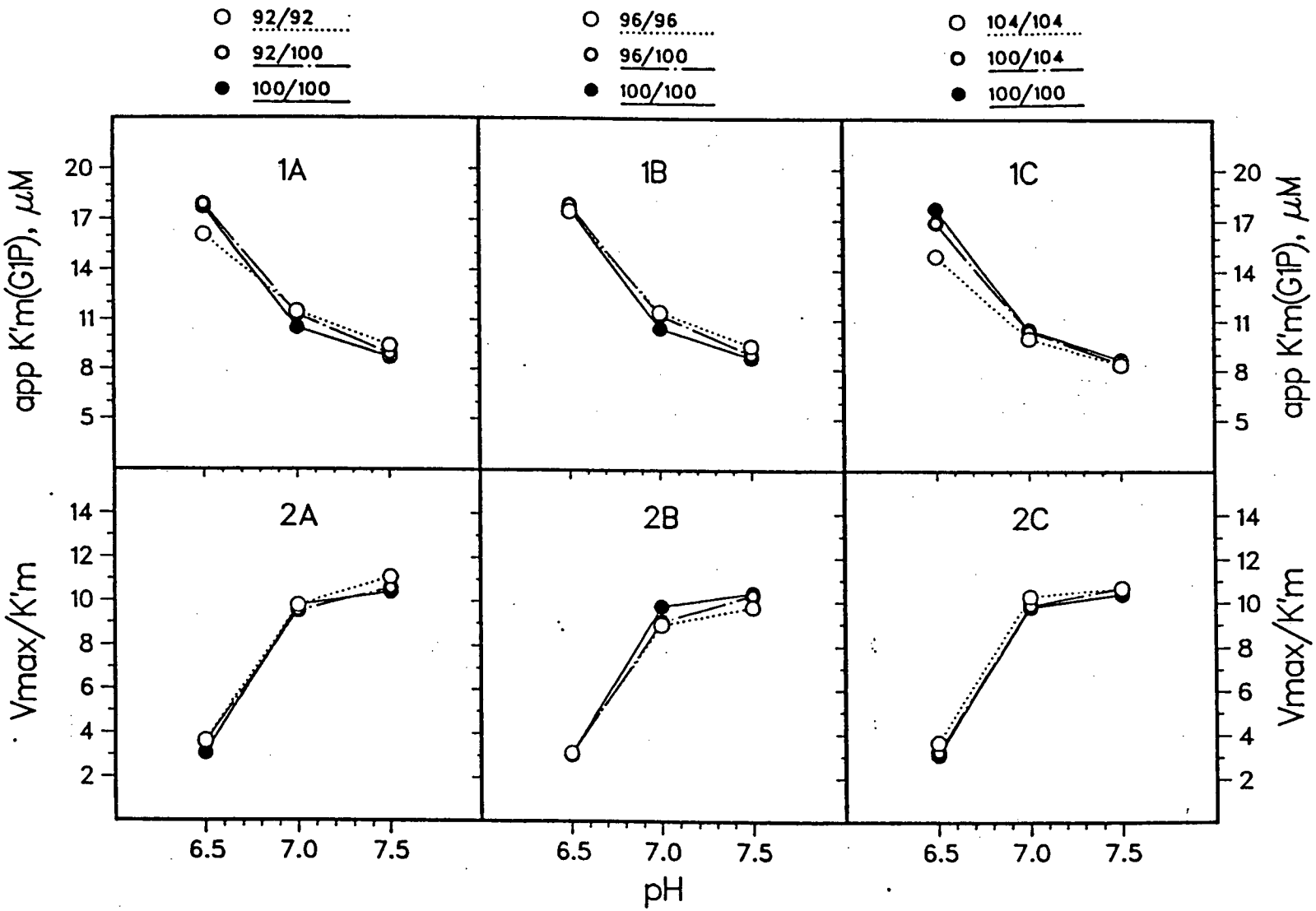

92/92
92/100
100/100
96/96
96/100
100/100
104/104
100/104
100/100
1A
1B
1C
2A
2B
2C
app K'm(G1P), μM
Vmax/K'm
Temperature, °C
5
10
15
20
25
30
2
4
6
8
10
12
14
16

Figure 11. Effect of pH on the estimated *in vivo* apparent Michaelis constants (in μM) for glucose-1-phosphate (1; upper half) and corresponding Vmax/K'm ratios (2; lower half) of seven *Pgm*-2 genotypes.


92/92
92/100
100/100
96/96
96/100
100/100
104/104
100/104
100/100
1A
1B
1C
2A
2B
2C
app K'm(G1P), μM
Vmax/K'm
pH
6.5
7.0
7.5
5
8
11
14
17
20
2
4
6
8
10
12
14

electrophoretic mobility between these allozymes affect the binding and/or internal reaction pathways for G1P and G16diP in a similar fashion. This trend is most apparent for the Pgm-2-92/92 homozygote which exhibited apparent Michaelis constants for both G1P and G16diP that were smaller than most other genotypes over the range of temperatures studied (see Figure 2). Upon substitution into equation 3, these lower parameter values act to cancel each other out such that the K'm (G1P) estimates for the Pgm-2-92/92 homozygote become indistinguishable from the other genotypes. The covariation of these kinetic parameters is also exhibited over the pH range of 6.5 to 7.5 (Figure 3) with the exception however of the app Km (G1P) values of genotypes possessing the Pgm-2-104 allele. The anomalous behavior of these genotypes may in part explain the significant effect of Pgm-2 genotype expressed for these K'm (G1P) estimates and Vmax/K'm ratios over this pH range. However, the important point being stressed here is that by taking into consideration the ping-pong reaction mechanism of PGM, and thus the interdependence of app Km (G1P) and app Km (G16diP) on catalytic function, the kinetic differences expressed in vivo between these Pgm-2 genotypes may be much less than expected from the differences shown by the parameters considered separately.

It should be emphasized that these kinetic differences may still hold under in vivo conditions if oyster PGM does not conform to the ping-pong mechanism or the intracellular concentration of G16diP is in fact saturating. However, even if

these functional differences are expressed the biochemical properties of Pgm-2 genotypes do not appear compatible with either the manifestation of overdominance or the maintenance of a stable polymorphic equilibrium. Molecular characterizations of phosphoglucomutase from a variety of organisms have established it as a monomer with a molecular weight ranging from 62,000 to 67,000 daltons (reviewed by Ray and Peck 1972). The electrophoretic staining patterns of oyster PGM, in addition to its mobility on SDS gels and elution patterns on gel filtration columns, are all in agreement with these previous studies. For a monomeric enzyme, the expression of overdominance via the superior properties of heteromultimers (e.g. Schwartz and Laughner 1969; Singh, Hubby and Lewontin 1974; Watt 1977, 1983), is not possible. Heterozygotes at the Pgm-2 locus were expected to display the strictly intermediate properties that were in fact observed over the range of conditions examined. If biochemical overdominance is expressed at this locus by these differences in catalytic function, it must be of the type termed by Wallace (1959) as "marginal", i.e., the mean performance of heterozygotes exceeds that of homozygotes only when averaged over different conditions.

Heterozygotes at the Pgm-2 locus in C. gigas are dominated by the Pgm-2-92/100, 96/100 and 100/104 genotypes (representing 74% of all heterozygotes). Therefore, it is these heterozygotes that must be responsible for overdominant effects attributed to this locus. The expression of marginal overdominance by the

three most common Pgm-2 heterozygotes places restrictive conditions on the biochemical properties of the Pgm-2-92, 96 and 104 allozymes relative to the most common Pgm-2-100 allozyme. Because of the similar frequencies of these less frequent alleles, the functional properties of their respective homozygotes must diverge by roughly equal amounts from the Pgm-2-100/100 genotype. Furthermore, the functional parameters of the less common homozygotes must fluctuate in value above and below that of the Pgm-2-100/100 genotype in such a manner, and with sufficient magnitude, that superior properties are conferred upon the average behavior of the Pgm-2-92/100, 96/100 and 100/104 heterozygotes. Variation in the selective values of these homozygotes could arise through either 1) variable selection across different seasons, microenvironmental conditions, or physiological states (e.g. Koehn and Immerman 1981), 2) reversals of the kinetic and/or structural advantages expressed over different ranges of temperature or pH (e.g. Place and Powers 1979), or 3) trade-offs between different catalytic or structural properties (e.g. Walsh 1981).

The overall performance of Pgm-2 homozygotes do not seem capable of meeting the above requirements for the production of marginal overdominance. The Pgm-2-92 allozyme possesses a suite of characteristics that differ from the Pgm-2-100 allozyme, but the same does not hold for either the Pgm-2-96 or Pgm-2-104 allozymes. Consequently, a case could be made for the expression of marginal overdominance by the Pgm-2-92/100 heterozygote by

invoking a variable selection regime, or from trade-offs between catalytic function and thermostability. However, the greater similarity of the Pgm-2-96 and Pgm-2-104 allozymes relative to the Pgm-2-100 allozyme precludes a similar argument for the Pgm-2-96/100 and Pgm-2-100/104 heterozygotes. Although there is some evidence for reversals of performance involving the apparent Michaelis constants for G1P and G16diP of Pgm-2-96 and Pgm-2-104 relative to Pgm-2-100 over different ranges of temperature and pH (see Figures 2 and 3), the magnitude of these fluctuations seem far too small to produce a net heterozygote advantage. The differential behavior of these heterozygotes is also incompatible with the maintenance of a stable multi-allelic polymorphism by overdominance. In their numerical analyses, Lewontin, Ginzburg and Tuljapurkar (1978) found that the conditions for such an equilibrium require that the variance in fitness of heterozygotes be much lower than that expressed between homozygotes and heterozygotes. Although kinetic differentiation may be weakly associated with fitness, the distinctiveness of the Pgm-2-92 allozyme and hence the Pgm-2-92/100 heterozygote compared to both the Pgm-2-96/100 and 100/104 genotypes causes both of these conditions to be violated.

The biochemical differences uncovered between these allozymes are also contradictory to their observed frequency distributions. The Pgm-2-92 allozyme displayed lower app Km (G1P) values and therefore larger catalytically important

Vmax/Km ratios from 15 to 30°C and at all pH's compared to the three others. This allozyme also exhibited significantly greater activities under low pH conditions, which have been demonstrated to occur in marine bivalves as a consequence of prolonged anaerobic metabolism (Wijsman 1975; Walsh, McDonald and Booth 1984). Because of these superior properties the Pgm-2-92 allele might be expected to enjoy a selective advantage that could eventually lead to its fixation in populations of C. gigas. The low frequency of this allele is in conflict with these advantageous catalytic properties unless these are somehow balanced by its significantly greater thermolability. This argument however would predict the selective removal of the Pgm-2-96 allele because this allozyme was equally sensitive to thermal denaturation, but indistinguishable from the Pgm-2-100 allozyme in all other properties examined.

The biochemical data is similarly unable to explain the high frequency of the Pgm-2-100 allele. Recently, Smouse (1986) has pointed out that the most common allele at multi-allelic overdominant loci should produce the fittest homozygote. The Pgm -2-100/100 genotype displayed kinetic properties that were generally intermediate with respect to the other homozygotes. Although the parameter levels expressed by the Pgm-2-100/100 homozygote may in fact be best suited for in vivo function, its great similarity to the Pgm-2-96 allele and the limited differentiation of the two other allozymes weakens the argument that kinetic intermediacy at this locus produces the fittest

genotype. The Pgm-2-100/100 genotype did exhibit superiority over the other homozygotes through its significantly lower thermal denaturation constant. The role played by differences between allozymes in their temperature stabilities in maintaining enzyme polymorphisms is unclear because in several instances the observed clinal variation in allele frequencies is exactly opposite to that predicted (e.g. Oakeshott et al. 1981, 1982; Place and Powers 1984a). The inability of these biochemical differences to clearly account for the maintenance of these alleles in a balanced polymorphism is a dilemma that has been reached in previous studies of this nature (e.g. Merritt 1972; Hoffman 1981; Hall 1985; Zera 1987).

An important limitation of the conclusions reached by studies of this nature concern the undetected presence of functional differences between Pgm-2 genotypes in enzymic parameters that were not examined. One particularly important property that could not be measured was substrate turnover number (kcat). Differences in kcat and kcat/Km ratios between these genotypes could easily have gone unnoticed by the substitution of Vmax for kcat, because Vmax is a composite term of the product of kcat and enzyme concentration. However, since the Vmax estimates were standardized to a common protein concentration and compared together under identical conditions, the actual kcat/Km ratios must remain similar to those presented in Figures 4 and 5; only the relative positions of the curves for different genotypes would be changed. Although it is

possible that the kcat/Km ratios for the Pgm-2-92/92, 96/96 and 104/104 homozygotes may all be shifted to a similar extent relative to the Pgm-2-100/100 genotype, this seems unlikely and the problem of accounting for the marginal overdominance of the three most common heterozygotes remains.

Another important property of phosphoglucomutase not examined in this study concerns its nonspecificity: PGM is capable of catalyzing the intermolecular transfer of a phosphate group between a variety of 5 and 6 carbon sugars (e.g. Lowry and Passonneau 1969). Therefore, differences could exist between Pgm -2 genotypes in their affinities for different substrates, as demonstrated for the 3 human PGM isozymes by Quick, Fisher and Harris (1974). The relative importance of this function for oyster PGM is probably limited because of the predominant role the primary reaction plays in the seasonal cycle of glycogen synthesis and degradation (e.g. Gabbott 1975).

In summary, the biochemical differences observed between the 7 Pgm-2 genotypes examined in this study do not seem capable of explaining the overdominance reported at this locus by Fujio (1982). As shown by Turelli and Ginzburg (1983), heterozygote superiority is expected to be manifested at all polymorphic loci subject to some form of balancing selection. Although the interpretation of fitness differences arising from in vitro kinetic properties is extremely complex, the biochemical data provide little insight into how this allozymic variation could

be actively maintained by selection in natural populations. The next chapter will examine the potential contribution of Pgm-2 specific activity variation in the expression of overdominance at this locus.

CHAPTER 3

ENVIRONMENTAL AND GENOTYPIC EFFECTS ON PGM ACTIVITY

INTRODUCTION

Many studies comparing the biochemical properties of genotypes at polymorphic enzyme loci have detected differences in specific enzyme activity (e.g. Vigue and Johnson 1973; Hay and Armstrong 1976; Hickey 1977; Danforth and Beardmore 1979). In several cases, the cause of this activity variation when examined by immunoelectrophoretic procedures has been found to result from differences in <u>in vivo</u> enzyme concentrations (e.g. Lai and Scandalios 1980; King and McDonald 1983; Estelle and Hodgetts 1984). Although tissue enzyme concentrations are affected by both environmental and genetic factors, a growing number of studies have demonstrated that these differences are produced by polymorphisms at "regulatory" gene loci (cf. Paigen 1979), i.e., genetic elements that control the developmental timing and/or levels of expression of structural enzyme loci (reviewed by Scandalios and Baum 1982; Laurie-Ahlberg 1985; Paigen 1986). Variation at these two levels of gene organization has presented a dilemma for the interpretation of the selective importance of allozymic variation. Do regulatory polymorphisms that cause differences in steady-state enzyme activity levels override the kinetic and/or structural differences that may also exist between allozymes (e.g. Wilson, Carlson and White 1977)?

Or are both functionally significant and, if so, what is the relative importance of each?

The answers to these questions are still largely unknown, even for the intensively studied alcohol dehydrogenase (Adh) and alpha-amylase (Amy) polymorphisms in Drosophila melanogaster (see discussion in Zera, Koehn and Hall 1985). Marked differences in specific activity exist between genotypes at both of these loci that are attributable to both linked and unlinked regulatory polymorphisms (Laurie-Ahlberg 1985). Therefore, the kinetic differences observed between these enzyme variants may be of little consequence to the maintenance of these polymorphisms in natural populations. However, the high activity Adh-F/F and Amy-4,6 homozygotes also display larger Michaelis constants for their respective substrates (e.g. McDonald, Anderson and Santos 1980 for Adh; Hoorn and Scharloo 1978 for Amy), which in combination with their increased activity levels may provide an additional advantage for functioning under high substrate conditions (cf. Atkinson 1977). These patterns suggest that an important interaction may exist between regulatory and structural locus polymorphisms. From these considerations, attempts to assess the selective importance of a specific enzyme polymorphism must simultaneously examine the existence and potential interaction between these different types of genetic variation.

Regulatory polymorphisms have been most extensively studied

in D. melanogaster (reviewed by Laurie-Ahlberg 1985). In this species, genetic variation affecting enzyme activity levels appears to be the rule rather than the exception. For example, all 23 enzymes studied by Laurie-Ahlberg et al. (1982) in isogenic lines established from 48 second and 48 third chromosomes extracted from natural populations exhibited a significant genetic component to their observed variation in activity levels. In marine invertebrates, no regulatory polymorphisms have yet been characterized, but this is not surprising in light of our limited knowledge of their genetic structure and organization. However, that regulatory variation analogous to that observed in Drosophila exists is suggested by variation in the specific activities of structural locus genotypes at the Pgi (Hoffman 1981) and Odh (Walsh 1981) loci in the anemone, Metridium senile, the Gpt locus in the copepod, Tigriopus californicus (Burton and Feldman 1983), the Pgi locus in the oyster, Crassostrea virginica (Martin 1979), and the aminopeptidase-1 locus in the mussel, Mytilus edulis (Koehn and Immerman 1981). The action of cis- or trans-acting regulatory variants affecting rates of enzyme synthesis or degradation can be excluded as an explanation for these activity differences for only the aminopeptidase-1 locus in M. edulis. Here, the observed variation in enzyme activities were shown by Koehn and Siebenaller (1981) to arise from differences between the allozymes in their substrate turnover numbers (kcat values), since the measured concentration of aminopeptidase-1 enzyme was similar in all genotypes studied.

In this chapter, the patterns of variation in specific activity between genotypes at the polymorphic Pgm-2 locus in the Pacific oyster, Crassostrea gigas are described. In marine bivalves, the examination of specific activity differences at polymorphic loci must simultaneously consider their potential interaction with seasonal changes in metabolism that reflect prevailing environmental conditions (i.e. food availability, temperature, salinity, etc.) and their annual reproductive cycle (Livingstone 1981; Gabbott 1983). In response to one or more of these factors the activities of a number of enzymes have been observed to fluctuate on a seasonal basis (e.g. Chambers et al. 1975; Livingstone 1976; Gabbott and Head 1980; Livingstone and Clarke 1983). One of the dominant annual cycles in marine bivalves involves the synthesis and degradation of glycogen, their primary energy storage compound (Gabbott 1975). In British Columbia populations of C. gigas, glycogen is synthesized in the fall and spring, stored in the mantle and digestive gland and degraded in the early summer prior to spawning (Quayle 1969; Whyte and Englar 1982). Since phosphoglucomutase functions in glycogen metabolism, its activity level may vary seasonally and exhibit differential responses between tissues or microenvironmental conditions. The potential influence of these environmental and physiological factors on PGM activity could produce important genotype-by-environment interactions relevant to the functional significance of this polymorphism, as found for the aminopeptidase-1 locus in M. edulis (Koehn, Newell and

Immerman 1980; Koehn and Immerman 1981). Therefore, the specific activities of 7 Pgm-2 genotypes were measured in two tissues (mantle and adductor muscle), at two intertidal heights (low and high water) in each of three seasons (summer, fall and winter).

The objective of this chapter was to determine if differences in specific activities, expressed between genotypes at the Pgm-2 locus in C. gigas, are capable of explaining the overdominance for adult body weight described at this locus by Fujio (1982). In the preceding chapter, I concluded that the kinetic and structural differences observed between the four most common Pgm-2 allozymes were insufficient to confer a net advantage of heterozygotes over homozygotes via the marginal overdominance mechanism (cf. Wallace 1959) that is required for a monomeric enzyme like PGM. This conclusion discounted the role played by functional differences at the structural enzyme locus, but left open the potential effects of regulatory gene variation that produce different steady-state levels of PGM activity in different genotypes. A number of theoretical models have been developed that are capable of producing a heterozygote advantage through spatial or temporal environmental heterogeneity (reviewed by Hedrick, Ginevan and Ewing 1976; Felsenstein 1976), some dealing explicitly with enzyme activity variation (e.g. Gillespie and Langley 1974; Latter 1975; Gillespie 1977). The applicability of these models to the results presented in this chapter is limited, however, because they are based on intermediate heterozygote behavior; some Pgm-2 heterozygotes

reported here display the unusual feature of overdominance for specific activity.

MATERIALS AND METHODS

Animals. Oysters were collected in the summer (late June), fall (early November), and winter (early March) from the Nanoose Bay, B.C. study site described in Chapter 2. Samples consisted of 150-250 mature oysters, ranging in size from 5-20 cm shell length, from each of two sampling stations located in the intertidal zone (designated as "low" and "high" water). Animals were transported back to the laboratory on ice in coolers where, immediately upon arrival, they were excised from their shells, blotted dry, and weighed. After a small section of mantle was dissected for electrophoresis the oysters were frozen at -40°C.

Electrophoresis. Starch gel electrophoresis was performed as outlined in Chapter 2. For each sample, "standard" running conditions were used initially to identify the Pgm-2 genotype of all oysters. The genotypes of oysters scored as Pgm-2-92/92, 96/96 and 104/104 homozygotes were checked a second time under "catalytic" running conditions prior to the determination of their specific activities.

Specific Activity Measurements. Oysters from each seasonal sample were divided into four arbitrarily assigned body weight classes (12.0-23.9 g; 24.0-35.9 g; 36.0-47.9 g; +48.0 g).

Specific activities of the four most common Pgm-2 genotypes (Pgm -2-100/100, 92/100, 96/100 and 100/104) were determined on a subsample of 3-6 individuals randomly selected from each of these weight classes. Owing to their rarity, all homozygotes for the Pgm-2-92, 96 and 104 alleles (and heterozygotes for these alleles examined in the fall sample) were included for study, irrespective of body weight.

PGM specific activities were determined in the mantle and posterior adductor muscle of these selected genotypes. Unless specified, all steps were carried out on ice or at 4°C. From the mantle approximately 1 g of tissue was dissected from the most posterior region of the left mantle lobe, blotted, and weighed to the nearest milligram. Extreme care was taken to ensure that exactly the same region of the mantle was used in all oysters. The tissue was homogenized by hand in a 10 ml Wheaton tissue grinder in 5 ml of ice-cold extraction buffer (10 mM Tris, 10mM maleic acid, 1 mM MgCl2, 1 mM EDTA, pH 7.4). Similarly, approximately 0.5 g of the "quick" portion of the adductor muscle was dissected, weighed, and homogenized in 3.5 ml of buffer. The crude homogenates were centrifuged at 12,000 x g for 20 min and a 1 ml aliquot of the supernatant was removed for the measurement of PGM activity and soluble protein.

Phosphoglucomutase activity was measured at 15°C in the forward reaction direction at 340 nm on a Pye Unicam SP 1800 UV/visible spectrophotometer as described in Chapter 2. The

reaction medium contained 50 mM imidazole-HCl, 3 mM MgCl2, 2 mM glucose-1-phosphate, 16 μM glucose-1,6-diphosphate, 0.4 mM NADP, 1 unit glucose-6-phosphate dehydrogenase, pH 7.0 (20°C) in a final volume of 1 ml. One unit of activity is defined as the quantity of enzyme required to convert 1 μmole of glucose-1-phosphate to glucose-6-phosphate per minute under these conditions. Assays were performed in triplicate using 20 μl of the crude homogenate from the mantle and 10 μl from the adductor muscle. After completion of these enzyme assays the samples were frozen at -70°C prior to the determination of soluble protein. General protein was measured in triplicate at room temperature by the method of Bradford (1976) on a Pye Unicam SP8-400 UV/visible spectrophotometer using gamma gobulin as a standard. Specific activity was expressed in units of PGM activity per mg protein in these crude homogenates. PGM activity/g wet tissue weight and soluble protein extracted/g wet tissue weight were calculated by assuming an intracellular water content of 75% in the mantle and 50% in the adductor muscle tissue (Walsh, McDonald and Booth 1984).

For each seasonal collection, the determination of specific PGM activity was completed within a 4 month period. To minimize the day-to-day variability in extraction and/or assay techniques and eliminate any bias in the results due to the spontaneous loss of PGM activity over time in the frozen oysters, several precautions were taken. First, of the 12 individuals studied on a typical day equal numbers were selected from each tidal

height. Second, a minimum of four different genotypes were chosen for study each day, thus ensuring a maximum representation of 3 individuals of the same genotype. Third, the order in which these genotypes were assayed for PGM activity and soluble protein was randomized on any given day. These precautions guaranteed that the specific activity measurements for all genotypes were carried out over the same length of time and suitably randomized across days so that no systematic errors would be present in the results.

Statistical Analyses. The specific activity data was analyzed by factorial analysis of variance (ANOVA) as described in Sokal and Rohlf (1981). Homogeneity of variance tests on the raw data revealed that only the soluble protein extracted/g wet tissue weight data required transformation to the log scale to normalize variances. Means were compared statistically using a posteriori Bonferroni multiple range tests.

RESULTS

The specific activity measurements were originally analyzed for each season by a three-factor ANOVA with tidal height, Pgm-2 genotype and body weight class as independent variables. Body weight was found to explain a significant portion of the variation in PGM specific activity only in the adductor muscle for the fall sample ($F(3,144) = 2.71$, $P < .05$). Here, oysters in the three largest weight classes (over 24.0 g) exhibited greater

specific activities than those in the lightest class (12.0-23.9 g), although multiple range tests were unable to detect any significant differences between the groups. Due to the negligible effect of body weight on PGM activity, it was eliminated as a factor in the overall analysis which is summarized in Table IV. For both mantle and adductor muscle, season and genotype exerted highly significant effects on PGM specific activity. In each tissue a highly significant season-by-tidal height interaction term was also observed; however, no interactions occurred between these environmental factors and Pgm-2 genotype. The overall analyses for mantle and adductor muscle tissue were remarkably similar. The only difference found between these tissues was the marginally significant effect of tidal position alone for the adductor muscle, an effect that was absent in the mantle.

The significant effects of these factors on specific activity could be mediated through differences in the activity of PGM extracted from these tissues, soluble protein levels, or some combination of the two. To determine the relative contribution of each, analyses were performed on PGM activity and soluble protein separately by expressing both on a per gram wet tissue weight basis. Table IV shows that the significant effects of season, genotype, and the tidal height-by-season interaction term appear largely to result from their influences on the activity level of phosphoglucomutase. Although soluble protein levels in these tissues differed significantly between

Table IV. F-ratios from analyses of variance on PGM specific activity, PGM activity/g tissue, and soluble protein extracted/g tissue in the mantle and adductor muscle tissues.

Source of Variation	df	Mantle			Adductor Muscle		
		Specific Activity	PGM Activity	Soluble Protein	Specific Activity	PGM Activity	Soluble Protein
Season	2	39.3***	81.7***	77.9***	271.3***	167.1***	23.0***
Tidal Height	1	2.52	13.3***	5.48*	5.56*	6.75**	3.46
Genotype	6	11.9***	9.60***	2.62*	21.9***	12.8***	1.40
Genotype x Season	12	1.34	0.83	0.54	1.09	0.53	0.50
Genotype x Tidal Height	6	0.54	1.29	0.24	0.84	1.06	0.40
Season x Tidal Height	2	23.8***	17.8***	0.35	13.7***	5.12**	3.73*
Genotype x Tidal Height x Season	12	0.74	0.80	1.14	1.20	0.52	0.37
Error	456						

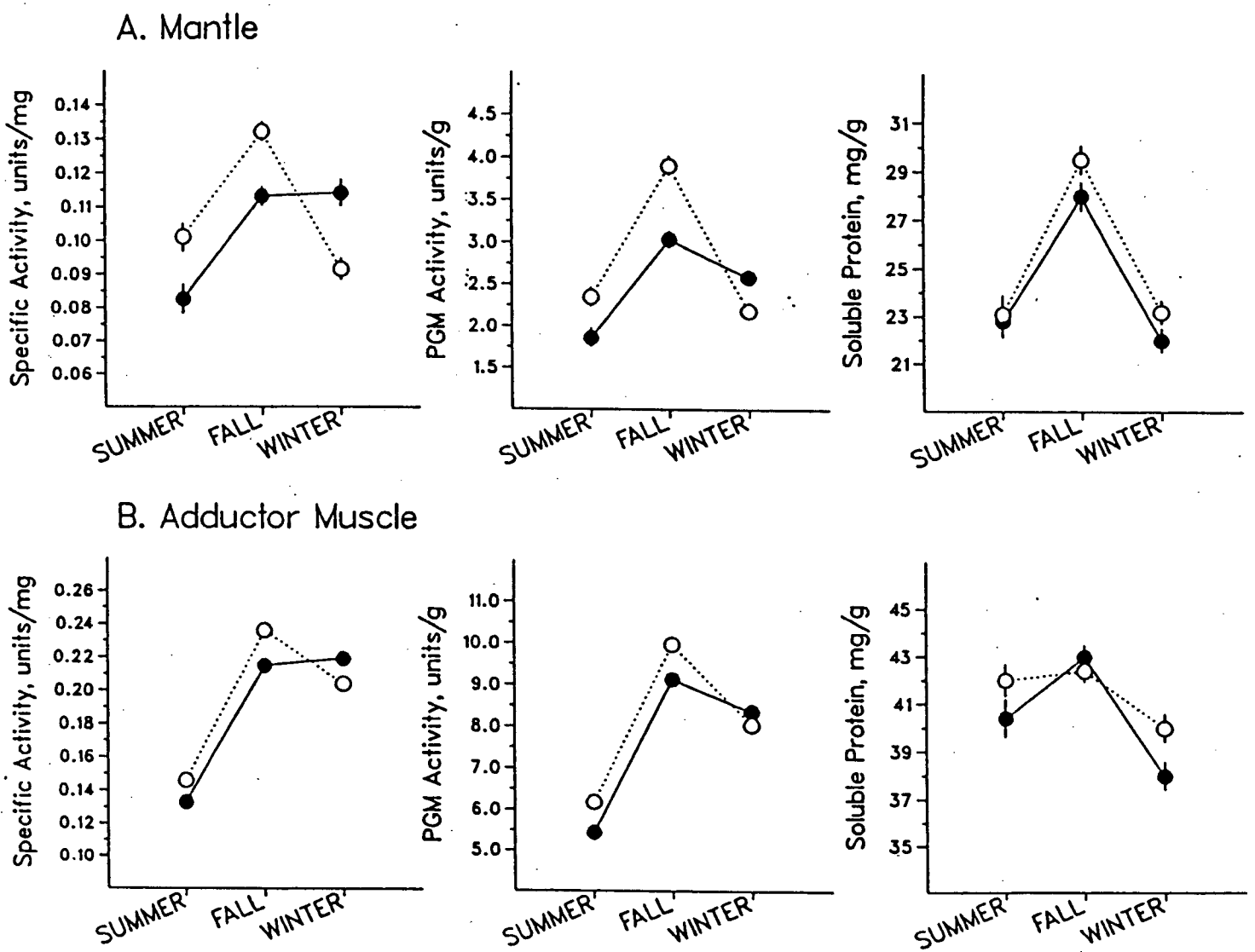
* P < .05
** P < .01
*** P < .001

seasons, relatively minor effects were apparent for the other factors and their respective interaction terms. The source of the significant effects of these factors will now be examined in detail.

EFFECTS OF SEASON AND INTERTIDAL POSITION

The influence of season and tidal position on the specific activity of phosphoglucomutase and soluble protein in the mantle and adductor muscle of C. gigas are presented graphically in Figure 12. At all times of the year, the adductor muscle was found to display significantly higher levels of PGM activity and soluble protein than the mantle. However, the seasonal variation in PGM activity, expressed on a soluble protein or wet tissue weight basis, was similar in both tissues, exhibiting a rank order of fall > winter > summer. Although soluble protein levels were also maximal in the fall, several important differences occurred between the two tissues. First, seasonal fluctuations in soluble protein levels in the mantle were more pronounced than in the adductor muscle and showed a significantly greater association with the observed PGM activity per gram tissue weight ($r = 0.65$) than seen in the adductor muscle ($r = 0.43$; statistical comparison of these Z transformed coefficients yields $t = 3.56$, $P < .001$, Sokal and Rohlf 1981, p. 589). Second, adductor muscle protein concentration in the summer significantly exceeded its level in the winter but in the mantle there were no differences between these seasons. Third, oysters

Figure 12. Effect of season and intertidal position on specific activity (units/mg protein), PGM activity (units/g tissue), and soluble protein (mg/g tissue) in the mantle and adductor muscle tissues. Open circles=low water; closed circles=high water. Low water sample sizes: summer n=64, fall n=96, winter n=89. High water sample sizes: summer n=57, fall n=100, winter n=92. Bars represent ±1 standard error where visible or fall within the plotted symbol.


A. Mantle
Specific Activity, units/mg
0.14
0.13
0.12
0.11
0.10
0.09
0.08
0.07
0.06
SUMMER
FALL
WINTER
PGM Activity, units/g
4.5
4.0
3.5
3.0
2.5
2.0
1.5
SUMMER
FALL
WINTER
Soluble Protein, mg/g
31
29
27
25
23
21
SUMMER
FALL
WINTER
B. Adductor Muscle
Specific Activity, units/mg
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
SUMMER
FALL
WINTER
PGM Activity, units/g
11.0
10.0
9.0
8.0
7.0
6.0
5.0
SUMMER
FALL
WINTER
Soluble Protein, mg/g
45
43
41
39
37
35
SUMMER
FALL
WINTER

from the low water site had greater soluble protein concentrations in their mantle tissues than those in the high intertidal area in all seasons, but only in the summer and winter for the adductor muscle tissue. Because of the greater tendency of PGM activity to covary with total protein concentration in the mantle, the seasonal variation in specific activity for this tissue was nearly 50% lower than observed in the adductor muscle.

Figure 12 shows that the significant effect of season on PGM specific activity was a consequence of its influence on *in vivo* enzyme activity, not on soluble protein levels. In both tissues the activity of PGM extracted per gram wet tissue weight fluctuated seasonally by 64%, whereas the amount of soluble protein varied by 27% in the mantle and only 10% in the adductor muscle. These changes in tissue protein concentrations between seasons tended to parallel the PGM activity/g tissue levels, and thus acted to eliminate rather than produce seasonal differences in specific activity. It is only because the relationship between these measures was not exactly compensatory that any seasonal variation in PGM specific activity was observed. Figure 12 also shows that the interaction noted between season and tidal position on specific activity was attributable to their effects on tissue PGM levels. In the summer and fall, oysters in the low intertidal zone possessed greater activities of PGM than animals situated higher in the intertidal area, but in the winter this pattern was reversed. The increased PGM activity of

oysters at the high water site in the winter, combined with their lower tissue protein levels, accentuated the differences in specific activity observed between tidal heights in this season. The significant season-by-tidal height interaction seen in Table IV for adductor muscle protein concentration also occurred because of a reversal of tidal ranking in the fall relative to the other two seasons. This can be seen to have no effect on the similar interaction between these environmental factors on PGM specific activity.

EFFECT OF PGM-2 GENOTYPE

The mantle and adductor muscle specific activities of seven Pgm-2 genotypes measured in the summer, fall, and winter are presented in Tables V and VI. Within each season the data from both tidal positions has been pooled owing to the rarity of the Pgm-2-92/92, 96/96 and 104/104 homozygotes. The clear trend evident in both tables was that the three Pgm-2 heterozygotes tended to exhibit greater specific activities than their respective homozygotes. This apparent overdominance for PGM specific activity was extremely consistent across season, tidal height and tissue. By chance alone the specific activity of a heterozygote was expected to exceed both homozygotes by a probability of 1/3. Using data from both tidal positions there were 36 comparisons and in 33 of the cases the specific activity of the heterozygote was greater than either homozygote (occurring 16 out of 18 times in the mantle, and 17 out of 18

Table V. Seasonal variation in the mantle specific activities (units/mg protein) of seven *Pgm*-2 genotypes.

Genotype	Season							
	N	Summer	N	Fall	N	Winter	N	Pooled
92/92	5	0.085±.015	8	0.128±.011	9	0.085±.010	22	0.101±.006
92/100	23	0.111±.007	41	0.129±.005	35	0.115±.005	99	0.120±.003
96/96	4	0.082±.016	7	0.113±.011	11	0.066±.009	22	0.084±.006
96/100	25	0.085±.006	42	0.129±.005	31	0.112±.005	98	0.112±.003
100/100	29	0.086±.006	42	0.109±.005	40	0.091±.005	111	0.097±.003
100/104	23	0.100±.007	40	0.128±.005	40	0.119±.005	103	0.118±.003
104/104	12	0.078±.009	16	0.109±.008	15	0.079±.008	43	0.090±.005
	F(6,77) = 2.42*		F(6,144) = 3.09*		F(6,132) = 8.23***		F(6,456) = 11.9***	

* P < .05
*** P < .001

Table VI. Seasonal variation in the adductor muscle specific activities (units/mg protein) of seven *Pgm*-2 genotypes.

		Season						
Genotype	N	Summer	N	Fall	N	Winter	N	Pooled
92/92	5	0.127±.013	8	0.219±.013	9	0.176±.010	22	0.181±.007
92/100	23	0.155±.006	41	0.233±.006	35	0.230±.005	99	0.214±.003
96/96	4	0.130±.014	7	0.219±.014	11	0.175±.009	22	0.181±.007
96/100	25	0.146±.006	42	0.236±.006	31	0.226±.006	98	0.210±.003
100/100	29	0.124±.005	42	0.206±.006	40	0.191±.005	111	0.179±.003
100/104	23	0.153±.006	40	0.238±.006	40	0.233±.005	103	0.217±.003
104/104	12	0.121±.008	16	0.204±.009	15	0.181±.008	43	0.173±.005
		F(6,77) = 2.42*		F(6,144) = 3.09*		F(6,132) = 8.23***		F(6,456) = 11.9***

* P < .05
*** P < .001

times in the adductor muscle). The probability of these results occurring by chance alone is approximately 4×10^{-13}.

Although Pgm-2 genotype explained a significant portion of the variation in specific activity observed in both tissues in all three seasons, the effect was sometimes weak and multiple range tests were often unable to differentiate between the genotypes. However, when the data for each genotype was combined across seasons and tidal positions the differences became highly significant. In the mantle, the three Pgm-2 heterozygotes behaved as a homogeneous group exhibiting specific activities that significantly exceeded all homozygotes except Pgm-2-92/92. Similar results were found for the adductor muscle except that the three heterozygotes were distinct from all four homozygotes. The exceptional behavior of the Pgm-2-92/92 homozygote's mantle activity was caused by its unusually high level in the fall when it was indistinguishable from any of the heterozygotes. The significance of this apparently anomalous result appears questionable because the adductor muscle activity of this homozygote at this time was not elevated and no similar tendencies were displayed in other seasons. Therefore, I believe that these heterozygous and homozygous genotypes represent nearly homogeneous groups, as found in the adductor muscle tissue. The magnitude of the difference in specific activity between homozygotes and heterozygotes was nearly identical in both tissues. The specific activities of Pgm-2 heterozygotes exceeded homozygotes by 24% in the mantle and 20% in the

adductor muscle.

The specific activities of these genotypes have been decomposed in Table VII to determine if the overdominance observed was attributable to differences in enzyme activity levels or soluble protein concentrations. As found for the effects of season and tidal position, the increased specific activities of Pgm-2 heterozygotes was a consequence of their larger in vivo PGM activity levels. In both tissues, heterozygotes possessed a greater PGM activity per gram of tissue relative to homozygotes that was similar in magnitude to the differences observed between these groups in their specific activities (29% and 24% larger in the mantle and adductor muscle, respectively). Surprisingly, Pgm-2 genotype also exerted a marginally significant effect on the amount of soluble protein extracted from the mantle but not the adductor muscle. Multiple range tests comparing the mantle protein concentrations of these genotypes detected a difference only between Pgm-2-100/104 and Pgm-2-104/104. However, it can be seen that in both tissues heterozygotes consistently displayed slightly higher levels of soluble protein than homozygotes. Since these differences would act in the direction of lowering rather than increasing the specific activities of heterozygotes relative to homozygotes, the overdominance observed for these measurements must result solely from the differences between genotypes in their PGM enzyme activities.

Table VII. Decomposition of Pgm-2 specific activities (units/mg protein) into enzyme activities and soluble protein levels expressed on a gram wet tissue weight basis.

		Mantle			Adductor Muscle		
Genotype	N	Specific[1] Activity	PGM[2] Activity	Soluble[3] Protein	Specific[1] Activity	PGM[2] Activity	Soluble[3] Protein
92/92	22	0.101±.006	2.46±.214	23.7±1.11	0.181±.007	7.40±.370	40.5±1.12
92/100	99	0.120±.003	3.09±.100	25.5±.517	0.214±.003	8.62±.173	41.0±.526
96/96	22	0.084±.006	2.07±.214	23.2±1.11	0.181±.007	7.21±.370	39.9±1.12
96/100	98	0.112±.003	2.90±.101	25.9±.526	0.210±.003	8.78±.175	42.1±.535
100/100	111	0.097±.003	2.47±.095	24.9±.489	0.179±.003	7.31±.164	40.1±.497
100/104	103	0.118±.003	3.07±.099	25.9±.510	0.217±.003	8.79±.171	41.2±.518
104/104	43	0.090±.005	2.06±.153	22.6±.790	0.173±.005	7.01±.264	40.6±.803
F(6,456)	=	11.9***	9.60***	2.62*	21.9***	12.8***	1.40

[1] units/mg protein
[2] units/g wet tissue
[3] mg protein/g wet tissue
* P < .05
*** P < .001

Due to the high frequency of the Pgm-2-100 allele in the study population, the Pgm-2-92/100, 96/100 and 100/104 genotypes represent the dominant class of heterozygotes at this locus. The next most frequent group of heterozygotes are formed between the three less frequent Pgm-2 alleles, i.e., Pgm-2-92/96, 92/104 and 96/104. To determine if these heterozygotes also displayed overdominance for specific activity, they were included for study in the fall sample. Table VIII summarizes the results from this season, pooled across tidal heights, for two classes of homozygotes and heterozygotes that have been grouped according to the presence or absence of the Pgm-2-100 allele. In contrast to heterozygotes possessing the Pgm-2-100 allele, heterozygotes lacking this allele did not exhibit overdominance for PGM specific activity in either the mantle or adductor muscle. In each tissue, the Pgm-2-92/96, 92/104 and 96/104 heterozygotes had specific activities that were indistinguishable from both homozygote classes and significantly lower than the level expressed in the other heterozygote group. The absence of an overdominant effect in these less frequent heterozygotes was a consequence of their reduced PGM activity per gram of tissue. No differences were observed between any of these genotypic classes in the amount of soluble protein extracted from their adductor muscles. However in the mantle, heterozygotes for the Pgm-2-100 allele had significantly larger amounts of soluble protein than both homozygotes and heterozygotes lacking this allele. Therefore, overdominance for specific activity does not appear to be ubiquitous at the Pgm-2 locus. Heterozygotes between the

Table VIII. Comparison of enzyme activities (units/mg protein and units/g tissue) and soluble protein levels (mg/g tissue) of homozygote and heterozygote classes possessing or lacking the Pgm-2-100 allele.

Genotypic Class	N	Mantle			Adductor Muscle		
		Specific[1] Activity	PGM[2] Activity	Soluble[3] Protein	Specific[1] Activity	PGM[2] Activity	Soluble[3] Protein
Homozygotes for 100 allele	42	0.109±.005	3.10±.163	28.4±.856	0.206±.006	8.83±.281	42.6±.745
Homozygotes without 100 allele	31	0.117±.005	3.00±.193	26.1±1.00	0.211±.007	8.90±.327	42.2±.863
Heterozygotes for 100 allele	123	0.129±.003	3.70±.095	29.2±.500	0.236±.003	9.97±.164	42.5±.435
Heterozygotes without 100 allele	33	0.105±.005	2.65±.187	24.9±.187	0.198±.006	8.53±.320	42.9±.850
F(3,197)	=	7.76***	8.81***	6.71***	12.9***	7.79***	0.84

[1] units/mg protein
[2] units/g wet tissue
[3] mg protein/g wet tissue
*** P < .001

Pgm-2-92, 96 and 104 alleles behaved in a manner analogous to homozygotes and showed a tendency to display underdominance rather than overdominance.

PREDICTED EFFECTS OF A PGM-2 NULL ALLELE

The overdominant specific activities of the Pgm-2-92/100, 96/100 and 100/104 heterozygotes could be caused by the undetected presence of a null allele at this locus. Null heterozygotes, misclassified as homozygotes and pooled unknowingly into the four homozygote classes, would have the effect of depressing their enzyme activities relative to heterozygotes with two functional alleles. This will produce what may be termed "null overdominance". A Pgm-2 null allele in homozygous condition could be lethal in C. gigas because of the central role played by glycogen in the energy metabolism of marine bivalves. In accordance with this prediction, no null homozygotes were observed at the Pgm-2 locus in the 1442 oysters examined electrophoretically. If null heterozygotes are fully viable, an apparent deficiency of heterozygotes will result at this locus because of their misclassification as homozygotes. The maximum frequency of this null allele cannot exceed the magnitude of this heterozygote deficiency as measured by D = (Ho - He)/He, where Ho and He are the observed and expected heterozygosities, respectively. Although D values fluctuated between sampling dates (see Chapter 2), their mean levels at the low and high intertidal stations in the study population were -

0.085 and -0.067, respectively. The frequency of a null allele required to generate these estimates of D are 0.044 for the high water site, and 0.034 for the low water site.

A null allele is expected to affect differentially the specific activities measured for the four homozygote classes. These predicted specific activities and PGM activities/g tissue of these homozygotes are presented in Table IX by making the following assumptions: 1) the activity of a normal homozygote equals the mean of the three overdominant heterozygotes, 2) null heterozygotes have 60% of the activity of a genotype with two functional alleles [as shown by Katoh and Foltz (1987) for Lap null heterozygotes in C. virginica], and 3) the frequency of the null allele is 0.044. Table IX shows that the magnitude of the predicted reductions for the Pgm-2-92/92, 96/96 and 104/104 homozygote classes are remarkably similar to their observed values, particularly in the adductor muscle. However, the Pgm-2-100/100 homozygote exhibits much lower activities than expected, and once more this is most apparent in the adductor muscle tissue.

The expected inequality between these homozygote classes is a consequence of the much higher frequency of the Pgm-2-100 allele compared to the three rarer alleles. Accordingly, it can be estimated that only 13% of the genotypes scored as Pgm-2-100/100 homozygotes are expected to be null heterozygotes, but in the three other homozygote groups the proportion of null

Table IX. Predicted reductions of enzyme activities (units/mg and units/g tissue) and soluble protein levels (mg/g tissue) in Pgm-2 homozygote classes assuming a null allele is present at a frequency of 0.044. Soluble protein concentrations were calculated by assuming PGM enzyme represents (a) 1% and (b) 5% of the total intracellular protein pool.

Genotype	Specific Activity[1] Observed	Specific Activity[1] Expected	PGM Activity[2] Observed	PGM Activity[2] Expected	Soluble Protein[3] Observed	Soluble Protein[3] Expected (a)	Soluble Protein[3] Expected (b)
A. Mantle							
Heterozygotes	0.117	0.117	3.02	3.02	25.8	25.8	25.8
100/100	0.0968	0.111	2.47	2.87	24.9	25.8	25.7
104/104	0.0849	0.0998	2.06	2.58	22.6	25.7	25.5
96/96	0.0839	0.0942	2.07	2.43	23.2	25.7	25.4
92/92	0.101	0.0973	2.46	2.51	23.7	25.7	25.5
B. Adductor Muscle							
Heterozygotes	0.214	0.214	8.73	8.73	41.4	41.4	41.4
100/100	0.179	0.203	7.31	8.28	40.1	41.4	41.2
104/104	0.173	0.183	7.01	7.47	40.6	41.3	41.0
96/96	0.181	0.172	7.21	7.02	39.9	41.3	40.8
92/92	0.181	0.178	7.40	7.26	40.5	41.3	40.9

[1] units/mg protein
[2] units/g wet tissue
[3] mg protein/g wet tissue

heterozygotes should be significantly larger (approximately 37%, 42%, and 49% for the Pgm-2-104/104, 92/92, and 96/96 classes, respectively). Therefore, genotypes scored as Pgm-2-100/100 homozygotes were expected to exhibit specific activities and PGM activities/g tissue that were similar to heterozygotes for the Pgm-2-100 allele and thus significantly larger than the other homozygote groups. The clear absence of this pattern, which should hold irrespective of the null allele's actual frequency, contradicts the predicted effects of a null allele.

Table IX also presents the reductions in soluble protein levels in Pgm-2 homozygotes expected by a null allele that produces no protein product. These were calculated by assuming PGM represents (a) 1% and (b) 5% of the total intracellular protein pool, percentages expected to bracket the true in vivo proportion (see Czok and Bucher 1960; Ottaway and Mowbray 1977). Virtually no changes in soluble protein levels are predicted if PGM accounts for 1% of the total pool and, even at the 5% level, the expected reductions are lower than observed. In fact, for a null allele producing no enzyme to explain these patterns, PGM must represent approximately 35% of the protein pool in the mantle and 20% of that present in the adductor muscle. The increased PGM activity/g tissue weight measurements observed in the overdominant heterozygotes are far too small to account for the higher protein levels expressed by these genotypes. The mean PGM activity level extracted per gram tissue in heterozygotes exceeded homozygotes by 29% and 24% in the mantle and adductor

muscle, respectively. If PGM represents 1% of the intracellular protein pool, the increased enzyme levels expressed in heterozygotes can explain only 4% of the differences observed in the mantle and 7% of that in the adductor muscle. The proportion of this additional protein attributable to PGM increases to 21% and 34% for the same tissues if the enzyme represents 5% of the protein pool, but this still leaves a sizable amount of the difference unexplained.

The presence of null heterozygotes in the four homozygote groups are also expected to produce two additional effects that should distinguish them from the overdominant heterozygotes. First, the variance of the specific activity and PGM activity/g tissue measurements in homozygotes should be larger than seen in heterozygotes. No evidence of this predicted inflation of variance was apparent in the analyses of the mantle or adductor muscle data. Second, normalized frequency distributions of these activity measurements should exhibit a bimodal pattern in the homozygotes, corresponding to null heterozygotes and normal homozygotes, and this is expected to be most clearly seen in homozygotes for the three less frequent Pgm-2 alleles. Visual inspection of these distributions detected no evidence of this expected bimodality. Therefore, the overdominance observed for PGM activity and protein levels in Pgm-2 heterozygotes does not match any of the predicted effects of a null allele.

DISCUSSION

Seasonal variation in the specific activities of many enzymes have been documented in a variety of marine bivalves, most notably Mytilus edulis (reviewed by Livingstone 1981; Gabbott 1983). These changes are directly related to the seasonal patterns of metabolism in these organisms that reflect the availability of food, prevailing abiotic conditions (i.e. temperature and salinity), and the state of their annual reproductive cycle (Widdows 1978; Zandee et al. 1980). Phosphoglucomutase activity has not been demonstrated to fluctuate seasonally in any species of marine mollusc. However, the general pattern observed in this study is consistent with that seen for a number of other enzymes in the American oyster, Crassostrea virginica, i.e., a reduction in specific activity during the reproductive period (summer) followed by an increase in the non-reproductive phase (fall and winter) (Chambers et al. 1975; Martin 1979). The lack of a relationship between PGM specific activity and body weight also agrees with the patterns exhibited for 10 out of the 13 enzymes studied in C. virginica by Chambers et al. (1975) and Martin (1979).

The influence of tidal position on enzyme activity levels in marine bivalves has rarely been examined. Martin (1979) found the specific activities of phosphoglucose isomerase (PGI) and phosphofructokinase (PFK) to vary by more than 2-fold between oysters located in low and high intertidal areas, but in

opposite directions; PGI activity increased but PFK activity decreased as a function of tidal height. The effect of tidal position on PGM activity in the present study was much less pronounced, but showed an interaction with season; the specific activities of oysters in the low intertidal area exceeded those in the high intertidal zone in both June and November, but not in March.

The causative factors responsible for the seasonal variation in PGM activity and the observed interaction between tidal position and season are difficult to interpret. On one hand, the patterns are consistent with compensatory changes in enzyme levels in response to different temperature conditions as documented for a variety of glycolytic enzymes (e.g. Hazel and Prosser 1974). However, the observed changes in PGM activity in both mantle and adductor muscle were also inversely related to the amount of glycogen present (presented in Chapter 4). Since the ability of oyster tissue to synthesize glycogen is inversely related to its existing glycogen store (L-Fando, Garcia-Fernandez and R-Candela 1972; Goromosova 1976), these fluctuations could simply reflect the annual cycle of glycogen synthesis and degradation. A further complication arises because phosphoglucomutase catalyzes a freely reversible reaction and thus functions _in vivo_ in both glycogen synthesis and glycogenolysis. Its relative importance in the latter process is still uncertain because an alternative degradative pathway from glycogen utilizing amyloglucosidase has been detected in _M._

edulis (Alemany and Rosell-Perez 1973; Zaba 1981). Therefore, it is possible that the reduced PGM activity observed in June, when glycogen is being degraded for gametogenic purposes, is a consequence of its diminished catabolic role instead of being a compensatory response to the warmer prevailing temperatures at this time of year. At present it does not appear possible to separate the relative importance and interaction between these factors in explaining the observed seasonal changes in PGM specific activity.

Although the effects of these environmental factors on PGM activity may be interpreted within the framework of the known seasonal metabolic changes in C. gigas, the overdominance observed in heterozygotes possessing the Pgm-2-100 allele is extremely unusual. When homozygotes for two different electrophoretic alleles have been found to differ in specific activity, heterozygote intermediacy is almost invariably observed (e.g. Gillespie and Langley 1974; Harris 1975), although dominance has sometimes been reported (e.g. Gibson et al. 1986; King and McDonald 1987). Overdominance for enzyme activity has rarely been described, and then only in exceptional circumstances. For example, Whaley (1952) reported an increased catalase activity in maize meristematic tissue in F1 hybrids relative to their parental inbred lines. Dickinson, Rowan and Brennen (1984) also observed a greater ADH activity in interspecific hybrids between D. melanogaster and D. simulans than exhibited by either parental species, although this effect

may have resulted solely from their increased size. In natural populations a clear-cut example of overdominance for enzyme activity has not been demonstrated. Heterozygotes have been shown to display greater activities than homozygotes at intermediate ranges of temperature at an esterase locus in both the freshwater sucker, Catastomus clarkii by Koehn (1969) and the sand shiner, Notropis stramineus by Koehn, Perez and Merritt (1971). Watt (1977, 1983) has reported overdominant Vmax/Km ratios for some phosphoglucose isomerase heterozygotes in Colias butterflies. In none of these examples was it shown that the overdominance resulted from increased enzyme activity levels in heterozygotes. The overdominance for specific activity at the Pgm-2 locus in C. gigas reported in the present study appears unique in its clarity of expression, reproducibility across different seasons, tidal positions and tissues, and its insensitivity to environmental factors.

Before examining the causes of this apparent overdominance, it is necessary to discount the possibility that it arose through uncontrolled factors or by systematic errors. One important factor not examined in this study was the potential influence of an individual's sex on PGM specific activity. In the mantle tissue of M. edulis, females have been found to possess significantly greater levels of soluble protein than males at all times of the year (Livingstone 1981; Livingstone and Clarke 1983) or only in the pre-reproductive period (Koehn and Immerman 1981). Therefore, if both sexes produce equal

quantities of a certain enzyme, males will exhibit greater specific activities than females solely because of these differences in soluble protein levels. Similar patterns may also exist between the sexes in C. gigas. However, these sex-related differences can account for the observed overdominance only if the sex of heterozygotes possessing the Pgm-2-100 allele is somehow biased in the direction of males. From what is known about the multiple-locus sex determination mechanisms of oysters (Haley 1978), and because of their ability to change sex depending upon environmental conditions (Hoagland 1978) and the size and the sex of adjacent individuals (Buroker 1983), this requirement appears unlikely to be met. Furthermore, the overdominance for mantle PGM specific activity was shown not to arise through differences in tissue protein concentrations, but rather from genotypic differences in in vivo enzyme activity levels. An identical pattern was also noted in the adductor muscle tissue where protein concentration has not been observed to differ between the two sexes.

Another complicating factor was the contribution of the more cathodal Pgm-1 locus to the measured activity of phosphoglucomutase in these crude homogenates. There are several reasons why the Pgm-1 locus could not have had a significant effect on these results. First, this locus remained unscorable in both tissues throughout the study due to its low level of activity compared to the Pgm-2 locus. Therefore, the vast majority of enzyme measured in these crude tissue homogenates

must have been produced by the Pgm-2 locus. Second, the Pgm-1 locus could explain these results only if 1) it existed in complete linkage disequilibrium with the Pgm-2 locus, 2) both Pgm loci were segregating for allelic variants that differed in specific activity, and 3) the alleles present at the two linked loci exhibited reversing dominance relationships. As an example, suppose that a high activity allele exhibiting dominance at the Pgm-1 locus was in disequilibrium with the three less frequent Pgm-2 alleles, which behaved as low activity recessives, and the opposite pattern existed for a Pgm-1 variant linked to the Pgm-2-100 allele. The pooling of these dominance effects at both loci could produce an apparently overdominant Pgm-2 heterozygote. However, to produce the 20-25% larger enzyme activities of Pgm-2 heterozygotes, substantial differences must exist between the specific activities of the separate Pgm-1 and Pgm-2 genotypes, and the Pgm-1 locus must account for a sizable (30% or more) proportion of the total PGM activity. There was absolutely no evidence for either requirement from the electrophoretic staining patterns of oyster PGM.

It is also difficult to account for this overdominance as a consequence of long term systematic errors in extraction and/or assay techniques. Day-to-day errors were minimized by randomizing the selection of Pgm-2 genotypes and the order in which they were assayed for both enzyme activity and soluble protein. The spontaneous loss of PGM activity should also have been similar for all genotypes because these measurements were

completed within identical periods of time. If differences in the rates of *in vivo* degradation have influenced these results, heterozygotes would have been expected to display intermediate rather than overdominant specific activities, based on the *in vitro* stabilities of these allozymes observed in Chapter 2. In addition, because of the marked thermolabilities of the *Pgm*-2-92 and 96 alleles, both homozygotes and heterozygotes for these alleles may be expected to have lost a greater proportion of their activities than genotypes containing the more stable *Pgm*-2-100 and 104 alleles. The lack of a clear distinction in the specific activities of these genotypic classes suggests that the spontaneous loss of enzyme activity has had little impact on the results. Hence, the observed overdominance is unlikely to have been caused by uncontrolled factors or systematic errors.

The existence of a null allele at the *Pgm*-2 locus has the potential to provide a simple yet powerful explanation for the overdominant enzyme activities of the *Pgm*-2-92/100, 96/100 and 100/104 heterozygotes. Zouros, Singh and Miles (1980) originally discounted the possibility that the overdominance for growth rate observed in *C. virginica* was caused by null alleles, exclusively on theoretical grounds. However, Foltz (1986a, 1986b) has recently detected null alleles segregating at several loci in *C. virginica*, including one locus (*Lap*-2) previously scored by Singh and Zouros (1978) and Zouros, Singh and Miles (1980). The predicted effects of a null allele on the enzyme activities and soluble protein levels of the four *Pgm*-2

homozygote classes shown in Table IX are incompatible with the patterns expressed by these genotypes. Because of the large frequency differences between these allozymes in the study population, a marked dichotomy is expected between the reductions in enzyme activities caused by the null allele between the Pgm-2-100/100 and the Pgm-2-92/92, 96/96 and 104/104 homozygote groups. There was no evidence for this distinction between homozygote classes. Furthermore, the slight overdominance for soluble protein levels in heterozygotes was far greater than could be accounted for by 1) the reductions expected in homozygotes from a null allele producing even no protein product, or 2) the increased levels of PGM activity exhibited by the overdominant heterozygotes. These discrepancies directly contradict the influence of a null allele and further suggest that the overdominance may involve a number of unidentified enzyme loci in addition to the Pgm-2 locus.

Perhaps the strongest evidence against the null allele explanation is provided by the relative performance of the two heterozygote groups examined in the fall (Table VIII). Here, heterozygotes between the Pgm-2-92, 96 and 104 alleles did not exhibit overdominance for specific activity, PGM activity expressed on a tissue weight basis, or soluble protein contents in either the mantle or adductor muscle. If the null allele explanation is correct, these heterozygotes, possessing two functional alleles, are expected to display PGM activities and soluble protein levels that were identical with the three

heterozygotes for the Pgm-2-100 allele. In fact, they were indistinguishable from homozygotes for these three rarer Pgm-2 alleles. Therefore, as well as contradicting a key prediction of the effects of a null allele, the results show that heterozygosity per se (cf. Lerner 1954) is not sufficient for the expression of overdominance for enzyme activity at the Pgm-2 locus in C. gigas. Instead, a particular allelic configuration is required; the Pgm-2-100 allele must be paired with either the Pgm-2-92, 96, or 104 alleles before the overdominant effects are manifested.

An alternative hypothesis, capable of providing a better explanation for these results, is that tightly linked to or associated with the Pgm-2 structural locus, is an overdominant regulatory locus that produces greater steady-state levels of enzyme in heterozygotes relative to homozygotes. The mode of action of this putative regulatory locus is unknown, but conceivably it could act at any of the known levels of regulatory control (i.e. transcription, mRNA processing, translation, or degradation). If a regulatory element is involved, its behavior is unlike any previously characterized example. The general pattern that has emerged from studies on regulatory polymorphisms in eukaryotes is that both trans-acting (e.g. Scandalios et al. 1980; Doane et al. 1983; Lusis et al. 1983) and cis-acting (e.g. Dickinson 1975; Shaffer and Bewley 1983) variants affecting rates of transcription produce intermediate enzyme activities in heterozygotes, while those

acting post-translationally tend to be inherited in a dominant/recessive fashion (e.g. Rechcigl and Heston 1967; Lai and Scandalios 1980; King and McDonald 1983, 1987; Gibson et al. 1986).

An overdominant regulatory locus could theoretically exert its effects at the level of enzyme synthesis or degradation. The increased soluble protein levels observed in the tissues of heterozygotes for the Pgm-2-100 allele, above that explainable by phosphoglucomutase enzyme alone, suggests that the level of regulatory control is transcriptional and that pleiotropic effects are exerted on other unidentified enzyme loci. However, the negative association between multiple-locus heterozygosity and protein turnover rates recently reported by Hawkins, Bayne and Day (1986) offers the possibility that the putative regulatory locus in heterozygous condition leads to decreased rates of PGM turnover, thus resulting in greater steady-state levels of enzyme in heterozygotes relative to homozygotes. Since it is unlikely that such a protease will have act specifically on PGM alone, the in vivo levels of its other enzyme substrates should also increase.

This explanation is intuitively appealing because it can provide a general explanation for the increased "metabolic efficiency" of heterozygotes (through the reduced expenditure of ATP used for polypeptide synthesis) suggested by Berger (1976) and supported by a number of recent studies (e.g. Koehn and

Shumway 1982; Garton 1984; Garton et al. 1984). Extrapolating from previously characterized regulatory polymorphisms, this overdominant locus in C. gigas must be trans-acting and somehow display unique properties in heterozygous condition. These requirements could be met if the diffusible gene product is a multimer. If the heteromultimeric gene product produced by a heterozygote has altered functional properties (producing an enhancement of transcription rates in the case of a transcription factor or diminished proteolytic function if it is a protease), then overdominance for PGM activity would result. The existence of this hypothetical regulatory locus must await further study.

The interpretation of these results as a case of genuine overdominance has several advantages over the null allele explanation. First, it is capable of providing an explanation for the the maintenance of a stable polymorphic equilibrium at the Pgm-2 structural locus by collapsing the multi-allelic system into a two allele regulatory polymorphism. Based on the results summarized in Table VIII it is possible to group the 10 Pgm-2 genotypes into three phenotypic classes according to their hypothesized genotype at this regulatory locus (see Table X). According to this model, regulatory variant "A" exists in complete disequilibrium with the Pgm-2-100 structural allele. Associated with the Pgm-2-92, 96 and 104 alleles is a different allele designated as "B". Heterozygotes for the A and B alleles produce an overdominant phenotype manifested in the Pgm-2-

Table X. Collapse of the multi-allelic Pgm-2 structural locus polymorphism by a hypothetical tightly-linked regulatory locus segregating for two alleles.

Phenotypic Class	Regulatory locus Genotype	Structural locus Genotype(s)		
1	"A/A"	100/100		
2	"A/B"	92/100	96/100	100/104
3	"B/B"	92/92	96/96	104/104
		92/96	92/104	96/104

92/100, 96/100 and 100/104 genotypes. Homozygotes for the B allele produce six phenotypically equivalent Pgm-2 genotypes comprising both homozygotes and heterozygotes for the Pgm-2-92, 96 and 104 alleles. This model can explain the similarities observed between the different structural locus genotypes and eliminates the difficulties associated with maintaining stable multi-allelic polymorphisms by overdominance (e.g. Lewontin, Ginzburg and Tuljapurkar 1978). For the null allele explanation, it is difficult to see how a Pgm-2 null heterozygote with a reduced enzyme activity can enjoy a selective advantage over genotypes with two functional alleles (required to maintain the presence of a null allele in populations of this species).

The persistence of this hypothesized disequilibrium presents a problem in interpretation, however, because it could be easily broken down by intragenic recombination as detected, for example, between the fast and slow alleles at the Adh locus in D. melanogaster by Aquadro et al. (1986). Similar crossovers are expected within the Pgm-2 locus of C. gigas. This becomes even more probable in light of the potentially great age of this polymorphism that is suggested by the similarity of allele frequency distributions between congeneric Crassostrea species and closely related genera (cf. Buroker, Hershberger and Chew 1979a, 1979b), some of which are known to have been isolated for at least 30 million years (Stenzel 1971). Perhaps the only way to account for this disequilibrium is for an inversion to exist within the chromosomal region encompassing the Pgm-2 structural

locus that includes either the Pgm-2-100 allele or the three less frequent alleles.

The proposed overdominant model also predicts that the individual frequencies of the Pgm-2-92, 96 and 104 alleles are of little consequence to the equilibrium state achieved. Instead, it is the combined frequency of these less frequent alleles and their resulting fitnesses in homozygous and heterozygous state relative to the Pgm-2-100/100 genotype that control the dynamics of the polymorphism. This interpretation is consistent with the observed frequencies of these Pgm-2 alleles in natural and cultured populations of the Pacific oyster. For example, in 23 population samples of C. gigas from 5 geographic areas of Japan, Ozaki and Fujio (1985) observed that the frequency of the most common Pgm-2 allele fell within a limited range, exhibiting a mean of 0.596 and a coefficient of variation of only 5.4%. In contrast, the frequencies of alleles in adjacent mobility classes fluctuated rather dramatically, each by more than a factor of 3, giving rise to coefficients of variation ranging from 29%-49%. The extent of this variation appears far too large to be caused by sampling error alone. The erratic patterns displayed by the frequencies of these rarer alleles is difficult to reconcile with the maintenance of a stable multi-allelic polymorphism by overdominance, or any other type of balancing selection, unless they are functionally equivalent as predicted by the model. Since the frequency of the Pgm-2-100 allele in populations of C. gigas is always greater

than 0.5 (Buroker, Hershberger and Chew 1975, 1979a; Ozaki and Fujio 1985), the fitness of the Pgm-2-100/100 genotype must exceed that of homozygotes and heterozygotes possessing these less frequent alleles. If fitness is correlated with enzyme activity at this locus, this prediction is supported in the mantle tissue where the Pgm-2-100/100 homozygote displays PGM activities and soluble protein levels on a tissue weight basis that are greater than the mean of these other genotypes by 14% and 8%, respectively.

The interpretation of these results by an overdominant model is also supported by the biochemical data of Chapter 2, in which it was concluded that the kinetic and structural differences detected between these Pgm-2 allozymes were not sufficient to account for the phenotypic effects of heterozygosity at this locus reported by Fujio (1982). The overdominant specific activities of heterozygotes possessing the Pgm-2-100 allele strongly suggest that it is through these differences in enzyme activity levels that the selective effects of this locus are expressed; the allelic differences observed at the structural enzyme locus may be largely, if not entirely, neutral. The increased enzyme activities of the Pgm-2-92/100, 96/100 and 100/104 heterozygotes automatically imparts upon these genotypes larger Vmax/Km ratios and, hence, greater flux capacities than the other Pgm-2 genotypes listed in Table X. This in turn may provide the biochemical basis for the demonstration of the selective importance of this enzyme

polymorphism through its influence on glycogen metabolism and subsequent impact on fitness related traits.

These results have several important implications to the growing number of studies documenting associations between multiple-locus heterozygosity and a variety of phenotypic-level traits (reviewed by Mitton and Grant 1984; Zouros and Foltz 1987). As reported for the Pgi locus in Colias butterflies by Watt (1977, 1983), it is one of the few cases in which overdominance has been detected at a polymorphic enzyme locus, and it is the first time it has involved one previously implicated with a relationship with multiple-locus heterozygosity. Therefore, for at least one locus, the present study has been successful in distinguishing between the various alternative hypotheses put forward to explain these associations. Both the inbreeding and associative overdominance explanations are untenable: overdominance was manifested at the Pgm-2 locus in C. gigas even though it may have been caused by a tightly linked gene, or block of genes, in complete disequilibrium with the structural locus.

Another important ramification of these results arises from the distinctive properties of different Pgm-2 heterozygotes. In most studies examining the phenotypic effects of multiple-locus heterozygosity, it has been common practise to pool all homozygotes and heterozygotes together, thus implicitly assuming that all genotypes combined within these groups are equivalent.

The absence of an overdominant effect in heterozygotes between the Pgm-2-92, 96 and 104 alleles found in this study suggests that a sizable amount of error may be introduced by the assumption of heterozygote equivalency, since these genotypes represent approximately 24% of all heterozygotes at this locus. Furthermore, in several species of marine bivalves, laboratory crosses between limited numbers of parents have occasionally resulted in the elimination of an association between multiple-locus heterozygosity and growth rate that was observed in progeny collected from natural populations of the same species (discussed by Zouros and Foltz 1987). Although these discrepancies have been explained by the requirement for a large number of differing parental genomes (e.g. Gaffney and Scott 1984), the results from the present study suggest that an additional explanation is possible: the overdominant effects at some loci may depend on particular heterozygous states. The potentially confounding effects of disparate heterozygous groups at an enzyme locus scored in these types of studies provides an additional reason for adopting the "adaptive distance" model of Smouse (1986) in interpreting more accurately the causes of these correlations involving multiple-locus heterozygosity.

In summary, the present study has demonstrated that overdominance for enzyme activity is expressed by the three most common heterozygotes at the Pgm-2 locus in Crassostrea gigas. The magnitude of this overdominant effect was similar in the mantle and adductor muscle tissues, and was unaffected by

environmental factors. Systematic errors, unexamined variables, or an undetected Pgm-2 null allele are unable to account for these findings. The best explanation for this overdominance is via a regulatory locus, tightly linked to the Pgm-2 structural locus, that produces greater steady-state levels of PGM activity in heterozygotes relative to homozygotes. This interpretation can explain the similarities between an array of different structural locus genotypes, account for the frequencies of these alleles in natural populations, and provide an explanation for the maintenance of the Pgm-2 polymorphism in a balanced state by collapsing the multi-allelic system into a two allele polymorphism at this regulatory locus. Definitive evidence for the selective importance of the observed overdominance must involve a demonstration of its impact on glycogen metabolism, the metabolic pathway in which PGM functions. Examination of the physiological effects of PGM activity variation on tissue glycogen concentrations is the subject of Chapter 4.

CHAPTER 4

PHYSIOLOGICAL EFFECTS OF THE PGM-2 LOCUS ON GLYCOGEN METABOLISM

INTRODUCTION

A large body of studies have documented the existence of differences in structural and functional properties between allozymes at polymorphic enzyme loci. A single comprehensive review of this literature has not been done, but some of the better characterized polymorphisms have been discussed by McDonald (1983), Koehn, Zera and Hall (1983), Zera, Koehn and Hall (1985), and Watt (1985b). The detection of biochemical differences between allozymes provides only the foundation upon which the demonstration of the functional, and ultimately the selective, importance of this type of genetic variation in natural populations must be based. As pointed out by Clarke (1975) and Koehn (1978), it is essential to show that this allozymic variation imparts significant effects on relevant physiological processes that differentially affect the fitnesses of the enzyme genotypes. The vast majority of biochemical studies on allozymes fall short of achieving this end and, as a consequence, have only been able to speculate on the adaptive significance of the kinetic data (e.g. Merritt 1972; Hoffman 1981; Hall 1985).

Studies that attempt to demonstrate the physiological

consequences of enzyme variation encounter a number of formidable difficulties. Zera, Hall and Koehn (1985), and Eanes (1984) have addressed some of the problems that arise from extrapolating the significance of *in vitro* kinetic differences to *in vivo* function. Such limitations are unavoidable in these studies, but may be minimized through the use of highly purified allozyme preparations, physiologically realistic assay conditions, and robust kinetic experimental designs and analytical methodologies. Sufficient background knowledge is also required on the catalytic properties and metabolic function(s) of the enzyme selected for study. This information is essential for identifying the catalytic and/or regulatory parameters that might be of selective importance (Watt 1983, 1985a). Dykhuizen, Dean and Hartl (1987) have discussed additional obstacles faced in these studies that arise from the inherent complexity of metabolic phenotypes, the small magnitude of the kinetic differentiation usually present between allozymes, and the unknown effects of loci tightly linked to the one under study. Finally, enough must be known about the study organism's ecology to allow reasonable assumptions to be made concerning the potential selective agent(s) that could be acting on the polymorphism under natural conditions.

The ability to measure the physiological effects of allozymic variation has also met with some interesting theoretical difficulties. A large proportion of the enzymes studied electrophoretically do not function independently, but

catalyze reactions that are embedded within complex biochemical pathways. Hence, the physiological impact of this enzyme variation must be manifested at the level of the entire pathway in which they function before being visible to selection. Extending the basic premises of metabolic control theory (cf. Kacser and Burns 1973, 1979, 1981), Hartl, Dykhuizen and Dean (1985) have predicted the existence of a concave relationship between enzymic flux and activity for many enzymes of intermediary metabolism. They have argued that through the action of natural selection, the activities of many enzymes have been pushed well onto the plateau of this relationship, at which point substantial changes in catalytic activity would result in negligible changes in flux. Therefore, this saturation theory of Hartl, Dykhuizen and Dean (1985) predicts that the majority of electrophoretic variants, which usually exhibit minor kinetic differences, are selectively neutral.

Control theory has stimulated considerable discussion because of its important implications for the physiological relevance of allozymic variation in particular, and biochemical adaptation in general (e.g. Koehn, Zera and Hall 1983; Watt 1985b; Burton and Place 1987; Pogson 1988). Experimental work has contradicted its predictions in some cases (e.g. Cavener and Clegg 1981; DiMichele and Powers 1982a, 1982b; Hilbish, Deaton and Koehn 1983; Burton and Feldman 1983), but not in others (e.g. Middleton and Kacser 1983; Barnes and Laurie-Ahlberg 1987). The elegant series of competition experiments by Hartl

and co-workers utilizing E. coli strains that differ by single allelic substitutions at different electrophoretic loci have largely supported control theory. No fitness differences were detected between strains possessing different alleles at the gnd (on standard genetic backgrounds) (Dykhuizen and Hartl 1980; Hartl and Dykhuizen 1981), pgi (Dykhuizen and Hartl 1983), zwf (Dykhuizen, de Framond and Hartl 1984), or lacZ loci (Dean, Dykhuizen and Hartl 1986; Dykhuizen, Dean and Hartl 1987). The only locus at which allelic variation exerted significant effects on fitness was lacY (Dykhuizen, Dean and Hartl 1987). However, all of these chemostat experiments measured the effects of these polymorphic alleles on fitness (i.e., growth rates), and therefore it is not known in any of these experiments if allelic variation affected metabolic fluxes. More empirical studies are clearly needed to assess both the physiological consequences of allozymic variation and the general validity of metabolic control theory.

The expression of overdominant enzyme activities by the three most common heterozygotes at the Pgm-2 locus in C. gigas has provided the potential for demonstrating the adaptive significance of this polymorphism. To be physiologically relevant, this variation in enzyme activity must now be shown to influence the metabolism of glycogen. Since PGM is not known to possess any regulatory properties (Ray and Peck 1972), its function is believed to be strictly catalytic. As argued for the phosphoglucose isomerase locus by Watt (1977, 1983), the

physiological effects of Pgm-2 genotypes are most likely to be expressed by differences in their Vmax/Km ratios. These ratios differ significantly between Pgm-2 genotypes for reaction directions because of variation in their specific enzyme activities (Chapter 3); Michaelis constants measured for glucose-1-phosphate and estimated for glucose-6-phosphate exhibited only minor differentiation between allozymes (Chapter 2). Although kcat differences could exist between allozymes that may be of selective importance, Vmax (as determined by specific activity) is probably the more important in vivo parameter because its composite nature (Vmax = kcat[enzyme]) ensures that differences that may exist between genotypes in steady-state enzyme activity levels are simultaneously incorporated (Hoffman 1981).

Examination of the physiological effects of the Pgm-2 locus on glycogen metabolism in the Pacific oyster has a number of advantages over previous studies of this nature. The synthetic pathway from glucose to glycogen is short, involving only four enzymic reactions. Glycogen is the major energy storage compound of oysters, at times representing up to 25% of their total body weights (Quayle 1969). Demonstrable effects of this locus on glycogen metabolism are thus likely to be physiologically important because of the central role played by glycogen in the energy metabolism of oysters. Because glycogen is an easily quantified end product, the effect of variation in PGM activity on pathway function may also be more readily measurable than if

it catalyzed a reaction in a more complex energy-producing pathway (such as glycolysis). Seasonal patterns of glycogen synthesis and degradation are well documented in marine bivalves (Gabbott 1975; Bayne 1976). Since glycogen levels change slowly (over periods of weeks and months), Pgm-2 genotypes have the capacity to exert cumulative effects on tissue glycogen concentrations which may be more easily observed than if the cycle operated on a much shorter time period.

MATERIALS AND METHODS

Chemicals. Buffers, substrates, coupling enzymes and standards used for the glycogen assays were obtained from Sigma. Amyloglucosidase was provided by Boehringer Mannheim and the electrostarch for electrophoresis from Connaught Laboratories.

Animals. The seasonal collections, transportation and storage of oysters was identical to that described in Chapter 3.

Electrophoresis. Pgm-2 genotypes were identified by starch gel electrophoresis. Details of the electrophoretic procedure can be found in Chapter 2.

Glycogen Assays. Glycogen concentrations were determined in the mantle and adductor muscle tissues of all oysters selected for specific activity measurements described in Chapter 3. The experimental design involved the comparison of glycogen levels

in animals from four body weight classes (12.0-23.9 g; 24.0-35.9 g; 36.0-47.9 g; 48.0 g+), from two tidal heights (low and high), in three seasons (summer, fall and winter) comprising 7 Pgm-2 genotypic classes. Glycogen could not be measured in some smaller individuals because of the limited amount of tissue present. Hence, the sample sizes were slightly lower than the specific activity measurements presented in Chapter 3.

Approximately 0.4 g of tissue was dissected from both the mantle and posterior adductor muscle, blotted, and weighed to the nearest milligram. The section of mantle removed was directly adjacent to that taken for the measurement of PGM activity, thus ensuring that a comparable region was studied in all oysters. Similarly, the adductor muscle dissected represented one half of the tissue chosen for enzymatic study. Tissues were added to 2 ml of ice-cold 0.6 N perchloric acid (PCA) and homogenized for 30 s with an Ultra-Turrax tissue grinder. A 1 ml aliquot was transferred to an Eppendorf microcentrifugation tube and frozen at -70°C for up to 3 months prior to the determination of glycogen.

Glycogen was assayed by the amyloglucosidase method modified slightly from Keppler and Decker (1974). Duplicate 100 µl samples of the PCA homogenates from both tissues were added to 1 ml of 200 mM sodium acetate, 50 mM potassium hydrogen carbonate buffer, pH 4.8 containing approximately 6 units of amyloglucosidase (lyophilized from Aspergillus niger) in 10 x 75

mm polycarbonate culture tubes. The tubes were sealed with plastic caps and incubated for 2 h in a shaking water bath at 40°C. The hydrolysis of glycogen was stopped by the addition of 500 µl 0.6N PCA and the samples were spun for 5 min at top speed in a clinical centrifuge. A 500 µl sample of the supernatants were pipetted into Eppendorf microcentrifugation tubes and frozen at -70°C.

The liberated glucose was quantified enzymatically at room temperature by the hexokinase/glucose-6-phosphate dehydrogenase system at 340 nm on a Pye Unicam SP8-400 UV/visible spectrophotometer. The reaction medium contained 300 mM triethanolamine, 4 mM MgSO4, 1 mM ATP, 0.5 mM NADP, 0.8 units glucose-6-phosphate dehydrogenase, pH 7.5 in a final volume of 1 ml. Assays were performed in duplicate on 20 µl of the hydrosylates from both tissues. After sample addition, the cuvettes were mixed thoroughly and allowed to equilibrate for at least 10 min before an initial absorbance reading was taken. 1.5 units of hexokinase was then added, the cuvettes were again mixed and the reaction was allowed to proceed to completion (> 15 min) before a second absorbance reading taken. The difference between these absorbance readings was stoichiometrically equal to the amount of free glucose present in the hydrolyzed sample. Glycogen concentrations represented the mean of four assays per individual and were expressed as µmoles glucosyl units/g wet tissue weight by assuming an intracellular water concentration of 75% in the mantle and 50% in the adductor muscle tissue

(Walsh, McDonald and Booth 1984). Corrections were not made for the free glucose present in the original PCA homogenates because its concentration was found to be too low to affect the glycogen estimates. To assess the efficiency and repeatability of the enzymatic hydrolysis of glycogen, standards were prepared by dissolving 100 mg oyster glycogen (Type II) in 10 ml of 50 mM imidazole-HCl buffer, pH 7.0 (20°C). Fifty µl aliquots of this stock solution were hydrolyzed alongside the tissue samples from each season and assayed in duplicate for glucose as described previously.

The glycogen measurements were completed within six months of each seasonal collection. Although the spontaneous degradation of glycogen in the frozen oysters was not determined, several precautions were taken to minimize the influence of this factor on the results. As described in Chapter 3, the selection of genotypes was randomized each day such that the time taken to process them was similar. The selection of genotypes for the enzymatic hydrolysis of glycogen was also completely randomized, as was the order in which the glucose assays were carried out. Therefore, it was expected that day-to-day errors were kept to a minimum and the spontaneous loss of glycogen was similar for all genotypes.

Statistical Analyses. The effects of season, tidal position, and Pgm-2 genotype on the glycogen concentration of the two tissues studied was examined by analysis of variance (ANOVA) as outlined

in Sokal and Rohlf (1981). Means were compared by a posteriori Bonferroni multiple range tests.

RESULTS

Comparison of the hydrolysis of the glycogen standards with amyloglucosidase confirmed the consistency of the glycogen assays throughout the study. The efficiency of hydrolysis of standards included with the summer, fall, and winter samples averaged 85.9%, 84.8%, and 87.4%, respectively. However, statistical comparison of these angular transformed percentages by one-way ANOVA still produced a significant effect of season ($F(2,95) = 4.82$, $P < .05$), a difference that multiple range tests showed to be a consequence of the higher efficiency of hydrolysis in the winter relative to the fall. Tissue glycogen concentrations determined for the fall sample will therefore be underestimated by 3% compared to the winter, but this would have had a negligible effect on the results.

Similar to the analysis of the PGM specific activity data reported in Chapter 3, the glycogen data for both the mantle and adductor muscle tissues were originally analyzed in each season by a 3-factor ANOVA treating tidal position, body weight class, and Pgm-2 genotype as independent variables. Body weight was again found to have a minor effect on tissue glycogen levels, explaining a significant portion of the observed variation only in the adductor muscle tissue from the fall sample ($F(3,144)$ =

5.99, $P < .001$). Interestingly, this was also the only season and tissue in which body weight exerted a significant effect on PGM specific activity. An inverse relationship was observed between these variables and body size. For the glycogen data, oysters in the smallest weight class (12.0-23.9 g) possessed higher levels of this carbohydrate in their adductor muscles than measured in all animals above 24.0 g. Multiple range tests detected a significant difference between this group and the two weight classes above 36.0 g, but not from those in the 24.0-35.9 g range. Statistical comparison of the adductor muscle specific activities revealed similar differences between these four weight classes. However, the relationship between specific activity and body weight was opposite to that seen for the glycogen data; the smallest oysters exhibited lower enzyme activity levels than the other three groups. A similar association between body weight and glycogen was evident in the mantle, but as with the specific activities measured in this tissue the differences between weight classes were not statistically significant. Because of its minor importance, body weight was excluded as a factor in the overall analysis. This is presented in Table XI together with the results from the specific activity data from Chapter 3 for comparative purposes.

Season and tidal position exerted highly significant effects on glycogen levels measured in both the mantle and adductor muscle tissues of C. gigas. In combination, these factors produced highly significant season-by-tidal height

Table XI. F-ratios from analyses of variance on glycogen concentrations and PGM specific activities in the mantle and adductor muscle tissues.

Source of Variation	df	Glycogen Content		Specific Activity[1]	
		Mantle	Adductor Muscle	Mantle	Adductor Muscle
Season	2	128.2***	449.7***	39.3***	271.3***
Tidal Height	1	88.2***	166.2***	2.52	5.56*
Genotype	6	1.13	1.38	11.9***	21.9***
Genotype x Season	12	3.52***	1.70	1.34	1.09
Genotype x Tidal Height	6	1.55	1.15	0.54	0.84
Season x Tidal Height	2	17.4***	111.9***	23.8***	13.7***
Genotype x Tidal Height x Season	12	3.06***	1.44	0.73	1.20
Error		451	448	456	456

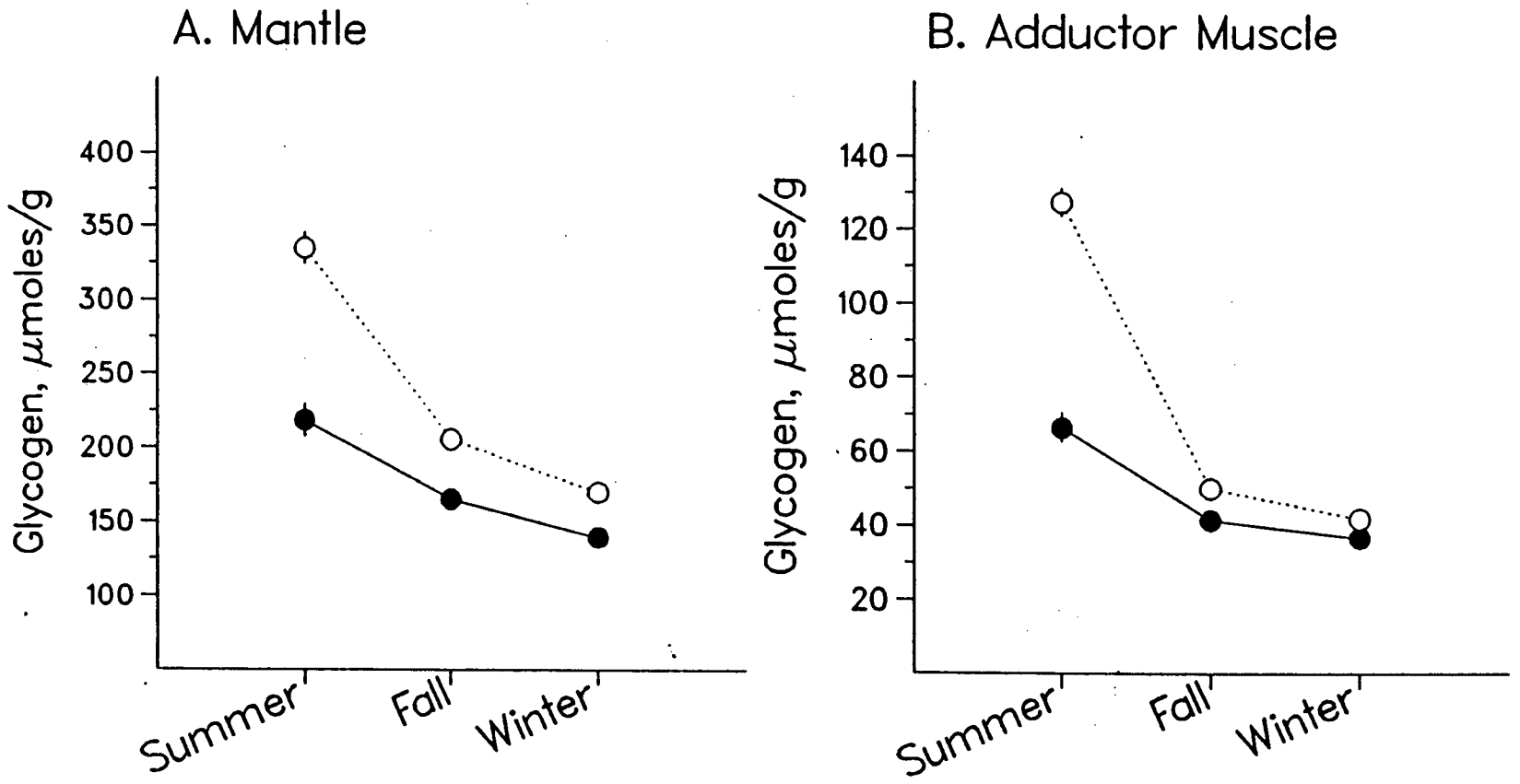
* P < .05
*** P < .001
[1] from Chapter 3

interaction terms, as observed for their effects on PGM specific activity. Despite these similarities, several important differences can be seen between the results of these analyses. First, intertidal position alone accounted for a highly significant amount of the variation in the glycogen concentrations, but not the enzyme activities, observed in in these tissues. Second, the highly significant effect of Pgm-2 genotype (as a separate factor) on specific activity was not repeated in the glycogen data. Third, highly significant genotype-by-season, and genotype-by-season-by-tidal height interaction terms were observed in the mantle glycogen results, but were absent from the specific activity data. Fourth, the two tissues produced nearly identical results for the specific activity but not the glycogen data: the genotype-by-environment interactions observed in the mantle were not present in the adductor muscle tissue.

EFFECTS OF SEASON AND INTERTIDAL POSITION

The effects of season and tidal position on the mantle and adductor muscle glycogen concentrations are presented graphically in Figure 13. In each season, mantle glycogen levels were consistently three- to four-fold greater than observed in the adductor muscle. Consequently, the seasonal patterns displayed in these tissues were similar, exhibiting rank orders of summer > fall > winter. Oysters lower in the intertidal zone possessed significantly greater quantities of glycogen than

Figure 13. Seasonal variation in the mantle and adductor muscle glycogen concentrations (μmoles glucosyl units/g tissue) at the two intertidal positions. Open circles=low water; closed circles=high water. Mantle sample sizes: summer, low=61 and high=57; fall, low=96 and high=100; winter, low=88 and high=91. Muscle sample sizes: summer, low=58 and high=57; fall and winter are the same as the mantle. Bars represent ±1 standard error where visible or fall within the plotted symbol.


A. Mantle
Glycogen, μmoles/g
400
350
300
250
200
150
100
Summer
Fall
Winter
B. Adductor Muscle
Glycogen, μmoles/g
140
120
100
80
60
40
20
Summer
Fall
Winter

animals situated in the more exposed, high intertidal area. This relationship held in both tissues in all three seasons, thus accounting for the significant effect of tidal position seen in Table XI. As evident from Figure 13, the highly significant season-by-tidal height interaction terms observed in Table XI were produced by the more pronounced difference in tissue glycogen levels between oysters at the two intertidal positions in the summer compared to the fall and winter. In the summer, oysters in the low intertidal had 53% more mantle glycogen than individuals sampled from the high intertidal zone, whereas comparable differences in the fall and winter were 25 and 22%, respectively. Even more dramatic differences were observed in the adductor muscle tissue. Here, oysters low in the intertidal region possessed 92, 21, and 14% more glycogen than those in the high intertidal area in the summer, fall, and winter, respectively.

The source of this interaction between season and tidal height on tissue glycogen concentrations differs from the similar interaction observed between these factors on PGM specific activity shown in Table XI and discussed in Chapter 3. For the specific activity data, the interaction was caused by shifts in the enzyme levels measured in oysters at the two tidal heights in different seasons. In the summer and fall, oysters at the low water station had significantly greater specific activities than individuals at the high water site, but in the winter this pattern was reversed. The nature of these

interactions between season and intertidal position in the glycogen and specific activity data offer insights into the causal relationship between these measures. Between the summer and fall, glycogen levels changed in a markedly non-additive fashion at the two intertidal positions. However, over the same period PGM specific activity increased in a strictly additive manner at these tidal heights. In addition, the reversals of the PGM activities exhibited by oysters at these tidal positions between the fall and winter were not reflected in the glycogen concentrations measured in these seasons. These discrepancies suggest that it is not variation in PGM activity *per se* that is responsible for the season-by-tidal height interactions seen in the glycogen concentrations of these tissues.

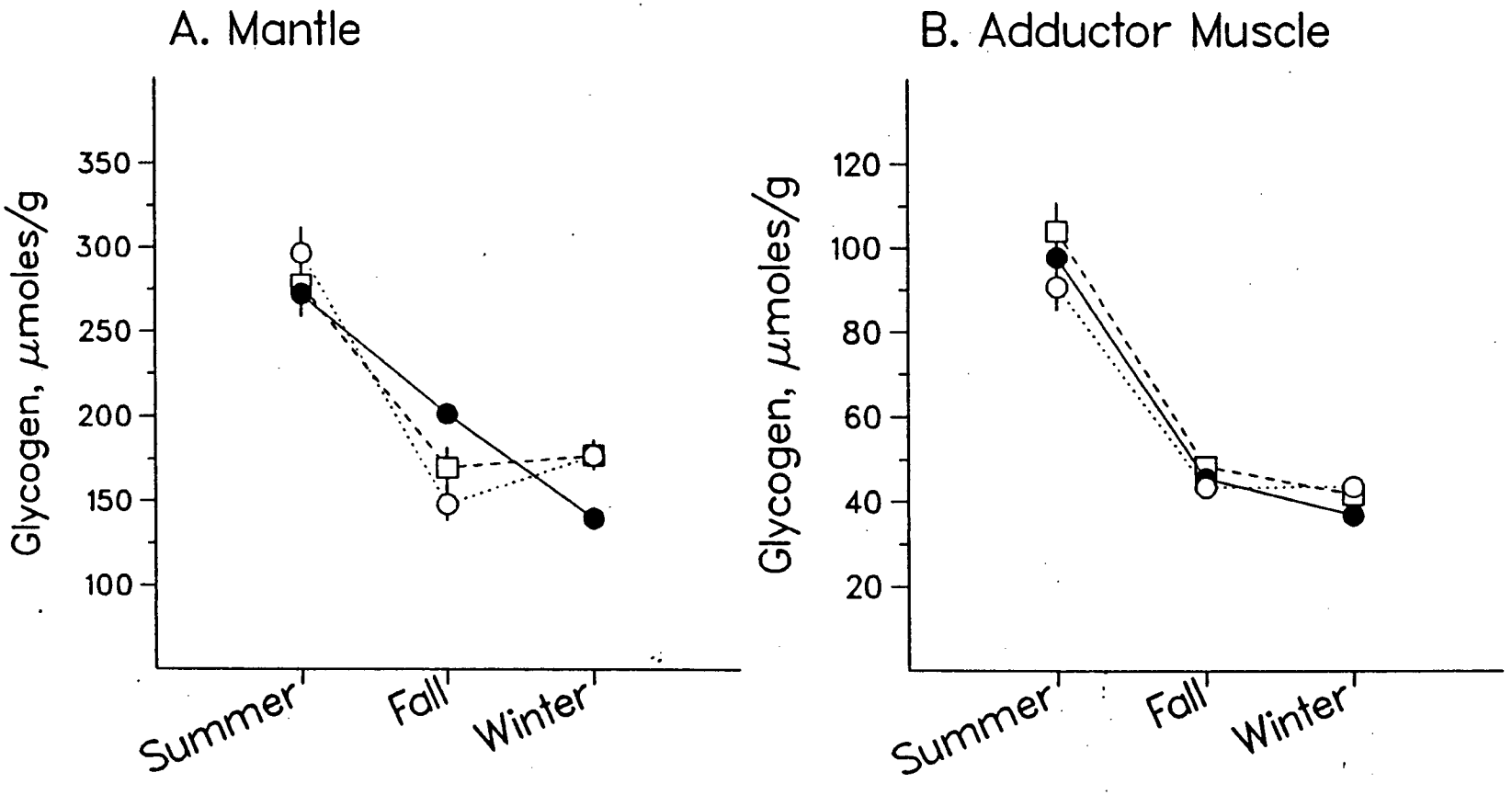
EFFECT OF *PGM*-2 GENOTYPE

The major finding of Chapter 3 was the expression of overdominance for enzyme activity at the *Pgm*-2 locus in *Crassostrea gigas*. The *Pgm*-2-92/100, 96/100, and 100/104 heterozygotes possessed greater specific activities than the *Pgm*-2-92/92, 96/96, 100/100, and 104/104 homozygotes. These homozygotes and heterozygotes behaved as homogeneous groups, exhibiting similar patterns in both tissues studied: enzyme activities in heterozygotes exceeded homozygotes by 24% and 20% in the mantle and adductor muscle, respectively. This knowledge allowed *a priori* comparisons of the associations between these differences in PGM activity and tissue glycogen concentrations.

Consequently, analyses were performed on 1) the 7 Pgm-2 genotypes separately, and 2) the same genotypes pooled into three genotypic groups (Pgm-2-100/100 homozygotes, heterozygotes for the Pgm-2-100 allele, and homozygotes for the three less frequent alleles). As will be seen, little information is lost in these analyses by pooling genotypes into these classes.

In the overall analysis, Pgm-2 genotype did not explain a significant amount of the variation in glycogen levels observed in either tissue. However in the mantle, highly significant interactions were detected between Pgm-2 genotype and season, and for the second order genotype-by-season-by-tidal height term. Both interactions were directly associated with the differences in enzyme activities expressed between Pgm-2 homozygote and heterozygote groups. The source of the genotype-by-season interaction is illustrated in Figure 14, which presents the mean glycogen concentrations of the Pgm-2 homozygote and heterozygote classes, pooled across tidal heights, in each of the three seasons. In the mantle (Figure 14A), summer glycogen levels did not differ significantly between allelic classes when averaged over the two tidal positions ($F(2,95) = 1.12$, $P > .30$). (However, a highly significant allelic class-by-tidal height interaction was present ($F(2,95) = 6.31$, $P < .01$) that will be discussed later.) In the fall, significant differences existed between allelic groups ($F(2,172) = 12.0$, $P < .001$). The Pgm-2 heterozygotes possessed higher glycogen levels than the two homozygote groups,

Figure 14. Seasonal variation in the mantle and adductor muscle glycogen concentrations (μmoles glucosyl units/g tissue) of Pgm-2 homozygote and heterozygote classes. Open circles= Pgm-2-100/100 homozygotes (summer n=29, fall n=42, winter n=38). Closed circles=heterozygotes for the Pgm-2-100 allele (summer n=71, fall n=123, winter n=108). Open squares=homozygotes for the Pgm-2-92, 96, and 104 alleles (summer n=18, fall n=31, winter n=33). Bars represent ±1 standard error where visible or fall within the plotted symbol.


A. Mantle
B. Adductor Muscle
Glycogen, μmoles/g
350
300
250
200
150
100
120
100
80
60
40
20
Summer
Fall
Winter

but were found by multiple range tests to differ significantly only from the Pgm-2-100/100 genotypes. The allelic classification also explained a significant amount of the variation in mantle glycogen concentrations in the winter ($F(2,155) = 10.2$, $P < .001$), but the situation was reversed from that seen in the fall. Both Pgm-2 homozygotes now displayed significantly more mantle glycogen than the three Pgm-2 heterozygotes.

Genotypes pooled into these allelic classes behaved similarly in both the fall and winter. Because genotype-by-tidal height interactions were absent in both analyses, six comparisons of the glycogen levels of Pgm-2 heterozygotes relative to their respective homozygotes were available in each season. In the fall, heterozygotes displayed higher glycogen concentrations in 5 of the 6 comparisons. In all six winter comparisons heterozygotes possessed lower quantities glycogen than their respective homozygotes. The unusual reversals in mantle glycogen levels observed between homozygotes and heterozygotes over the fall and winter was responsible for the significant genotype-by-season interaction term seen in Table XI.

Seasonal changes in the adductor muscle glycogen concentrations of these allelic groups are shown in Figure 14B. The significant genotype-by-tidal height interaction in summer mantle levels was not repeated for the adductor muscle tissue,

and, in both the summer and fall, homozygotes and heterozygotes possessed identical quantities of muscle glycogen. However, significant differences were present between allelic classes in the winter ($F(2,155) = 8.13$, $P < .001$). As was seen for the mantle tissue in this season, both homozygote classes possessed more glycogen in their adductor muscles than heterozygotes, but the difference was significant only for the Pgm-2-100/100 genotypes. An examination of genotypic patterns at both tidal heights in the winter revealed that in 5 of the 6 possible comparisons, Pgm-2 heterozygotes displayed lower glycogen levels than their respective homozygotes. The differentiation between these allelic classes in the winter was marginally below that required to produce a significant genotype-by-season interaction for the adductor muscle glycogen data ($F(6,448) = 1.70$, $P < .10$).

The mantle glycogen concentrations of Pgm-2 homozygotes and heterozygotes are presented separately for both intertidal positions in Table XII to illustrate the source of the significant genotype-by-season-by-tidal height interaction. Significant differences were observed between allelic classes at both tidal heights in all seasons with the exception of the high intertidal site in the summer. In this season, homozygotes in the low intertidal zone displayed higher quantities of mantle glycogen than the heterozygotes. At the high water site however, heterozygotes possessed larger glycogen levels than both homozygote groups, but these differences were not significant

Table XII. Combined effects of season and intertidal position on the mantle glycogen concentrations (μmoles glucosyl units/g tissue) of Pgm-2 homozygote and heterozygote classes.

Season	Tidal Position	N	Glycogen Concentration[1] Homozygotes for 100	N	Heterozygotes for 100	N	Homozygotes without 100	
Summer	Low	15	396.9±19.5	38	301.4±12.3	8	375.4±26.8	F(2,49) = 8.85***
	High	14	189.1±24.3	33	236.5±15.3	10	199.3±27.6	F(2,47) = 1.62
Fall	Low	22	171.4±16.0	60	220.5±9.7	14	195.2±20.0	F(2,84) = 3.76*
	High	20	122.0±12.0	63	183.1±6.7	17	148.7±13.1	F(2,88) = 10.9***
Winter	Low	19	179.6±13.0	50	157.7±8.0	19	192.8±13.0	F(2,76) = 3.11*
	High	19	174.2±10.4	58	124.2±5.9	14	155.7±12.2	F(2,79) = 9.78***

[1] μmoles glucosyl units/g wet tissue
* P < .05
*** P < .001

($F(2,47) = 1.62$, $P > .20$). In the fall and winter the differences expressed between these genotypic classes were similar at both tidal positions, but in contrast to the summer, the difference between allelic classes was more extreme at the high intertidal.

Table XII shows that the season-by-tidal height component of this second-order interaction was a consequence of the greater seasonal fluctuations of glycogen concentrations in the four Pgm-2 homozygotes. Taking the average of the two homozygote groups, mantle levels at the two intertidal positions were found to differ by 101, 35, and 12% in the summer, fall, and winter, respectively. In contrast, mantle glycogen concentrations in heterozygotes at these tidal heights declined in a linear and parallel fashion, differing by only 27, 20, and 27% in the summer, fall, and winter, respectively. The genotypic component of this second-order interaction was caused entirely by the differences between these allelic classes. The differential patterns displayed by Pgm-2 homozygotes and heterozygotes between tidal positions in the summer, combined with the net reversal between these groups over the fall and winter, was thus responsible for the significant genotype-by-season-by-tidal height interaction. Despite these differences, the mantle glycogen concentrations of homozygotes and heterozygotes were nearly identical when averaged over all seasons and tidal positions.

Table XIII presents the seasonal changes in adductor muscle glycogen levels of Pgm-2 homozygotes and heterozygotes at the two intertidal locations. No significant differences were observed between genotypic classes at either tidal position in the summer or fall. In the winter, significant differences were present between allelic groups in the high intertidal area ($F(2,79) = 7.58$, $P < .001$), with the homozygotes possessing on average 23% more glycogen than heterozygotes. A similar trend was evident at the low water site but the magnitude of this difference was diminished (homozygotes exceeding heterozygotes by 8%) and thus was not statistically significant ($F(2,76) = 1.51$, $P > .20$). Because of these winter results, homozygotes exhibited slightly higher glycogen levels than heterozygotes when averaged over the three seasons, but since Pgm-2 genotypes were indistinguishable in the summer and fall, these differences were not significant ($F(6,448) = 1.38$, $P > 0.20$).

In Chapter 3 it was shown that overdominance for enzyme activity was expressed only in heterozygotes possessing the most common Pgm-2-100 allele. Heterozygotes between the less frequent Pgm-2-92, 96 and 104 alleles displayed specific activities that were significantly lower than the overdominant heterozygotes and indistinguishable from both homozygote classes. In Table XIV the mantle and adductor muscle glycogen concentrations of these same genotypic classes are presented, together with their Pgm-2 specific activities from Chapter 3. As found for the specific activity measurements, a clear distinction was observed between

Table XIII. Combined effects of season and intertidal position on the adductor muscle glycogen concentrations (μmoles glucosyl units/g tissue) of Pgm-2 homozygote and heterozygote classes.

Season	Tidal Position	N	Glycogen Concentration[1] Homozygotes for 100	N	Heterozygotes for 100	N	Homozygotes without 100	
Summer	Low	15	119.0±9.1	35	127.6±6.0	8	141.1±12.5	F(2,46) = 1.16
	High	14	60.6±6.1	33	66.3±3.8	10	74.3±6.9	F(2,47) = 0.95
Fall	Low	22	47.7±2.9	60	50.6±1.7	14	51.0±3.6	F(2,84) = 0.62
	High	20	39.0±2.4	63	40.8±1.4	17	46.4±2.6	F(2,88) = 2.41
Winter	Low	19	44.2±2.0	50	40.4±1.2	19	43.4±2.0	F(2,76) = 1.51
	High	19	43.3±2.2	58	34.0±1.2	14	39.9±2.6	F(2,79) = 7.58***

[1] μmoles glucosyl units/g wet tissue
*** P < .001

Table XIV. Glycogen concentrations (μmoles glucosyl units/g tissue) and specific activities (units/mg protein) of homozygotes and heterozygotes possessing or lacking the Pgm-2-100 allele.

Genotypic Class	N	Mantle		Adductor Muscle	
		Specific[1,2] Activity	Glycogen[3] Content	Specific[1,2] Activity	Glycogen[3] Content
Homozygotes for 100 allele	42	0.109±.005	147.9±10.0	0.206±.006	43.5±1.8
Homozygotes without 100 allele	31	0.117±.005	169.7±11.6	0.211±.007	48.5±2.1
Heterozygotes for 100 allele	123	0.129±.003	201.3±5.8	0.236±.003	45.6±1.1
Heterozygotes without 100 allele	33	0.105±.005	148.7±11.3	0.198±.006	45.1±2.1
F(3,197)	=	7.76***	10.5***	12.9***	1.21

[1] units/mg protein
[2] from Chapter 3
[3] μmoles glucosyl units/g wet tissue
*** P < .001

these heterozygote groups in their mantle glycogen levels. The Pgm-2-92/96, 92/104, and 96/104 heterozygotes exhibited glycogen concentrations that were again significantly lower than the other heterozygote class and not different from the two homozygote groups. In this tissue a strong positive relationship existed between the specific activities of these genotypic classes and their glycogen stores. Adductor muscle glycogen concentrations did not differ between these genotypic groups, despite showing differences in enzyme activities similar to those observed in the mantle.

DISCUSSION

The physiological relevance of naturally occurring enzyme polymorphisms can only be assessed through an examination of the effects of this variation on the output of the biochemical pathways in which these enzymes function. For the Pgm-2 locus in Crassostrea gigas, this requirement translates into a demonstration of the impact of this polymorphism on glycogen metabolism. In marine bivalves, seasonal variation in the synthesis and degradation of glycogen is functionally coupled to their annual reproductive cycle (Bayne 1976; Zandee et al. 1980; Gabbott 1983). One important component of this cycle involves long term changes in the activities of enzymes involved in the storage and subsequent mobilization of these glycogen reserves for gametogenesis (Livingstone 1981; Zaba 1981; Gabbott and Whittle 1986a). Because changes in glycogen levels take place

rather slowly, examination of the associations between the differing enzyme activities of Pgm-2 genotypes and their existing tissue glycogen concentrations enables an evaluation, albeit indirect, of the physiological effects of this polymorphism. The highly significant effects of season and intertidal position on glycogen levels and their interactions with Pgm-2 genotype observed in this study provide some important insights concerning the influence of allozymic variation on metabolic flux.

The seasonal changes in the glycogen content of the mantle and adductor muscle tissues of C. gigas found in the present study are in general agreement with those reported previously for British Columbia populations of this species (Quayle 1969; Whyte and Englar 1982). The marked difference in glycogen levels observed in the two tissues examined was expected since the mantle is one of the primary storage sites for this carbohydrate in marine bivalves (Eble 1969; Bayne et al. 1982). It was unexpected that fall glycogen levels would exceed those measured in the winter. However, the winter sample was taken when natural food levels in Nanoose Bay were unusually low (P. MacClelland, personal communication), and thus glycogen concentrations could have been below their mean levels for this time of year.

Intertidal position also exerted a highly significant effect on tissue glycogen levels: oysters from the low intertidal zone consistently displayed significantly larger

quantities of glycogen than animals sampled from the more exposed high intertidal area. Two factors are probably responsible for these observations. First, oysters in the high intertidal zone experience longer periods of aerial exposure compared to animals in the low intertidal. This would directly reduce their opportunities to feed and hence assimilate glycogen reserves. Second, during periods of aerial exposure oysters switch to anaerobic pathways of energy production (Hochachka and Mustafa 1972; Zandee, Holwerda and de Zwaan 1980). Hypoxic conditions would occur more frequently and persist for longer durations in individuals situated higher in the intertidal zone. Since glycogen is the primary fuel for anaerobic metabolism (de Zwaan 1983), the increased demands on these energy reserves in the high intertidal area would further limit the capability of oysters in this area to store glycogen. The interaction observed between season and tidal position could in part be explained by the accentuation of these effects caused by the increased availability of food in the early summer coupled with the more extreme tides at this time of year.

In Chapter 3, season, intertidal position, and Pgm-2 genotype were shown to exert highly significant effects on the specific activity of phosphoglucomutase measured in both the mantle and adductor muscle tissues. Examination of the effects of these same factors on tissue glycogen concentrations reported in the present chapter showed a number of important differences from the enzyme activity data. First, glycogen levels in oysters

sampled from the two intertidal heights shifted in a non-additive fashion between the summer and fall when the changes in PGM activity observed between these seasons was strictly additive. Second, PGM specific activity exhibited a reversal in net ranking between these tidal positions over the fall and winter that had no apparent effect on tissue glycogen concentrations. Third, analysis of the mantle glycogen data produced highly significant genotype-by-environment interactions that were not observed in the analysis of the specific activity data. These interactions were not repeated in the adductor muscle despite the great similarity of its enzyme activity data with the mantle. Together, these differences suggest that the observed variation in PGM activity cannot be directly responsible for the significant effects of these factors on tissue glycogen concentrations.

This conclusion is not entirely unexpected. Phosphoglucomutase is not known to possess any regulatory properties and catalyzes a freely reversible reaction that in marine bivalves, as in other organisms, appears to operate near equilibrium *in vivo* (e.g. Ebberink and de Zwaan 1980). The metabolic role attributed to near-equilibrium reactions like PGM is to transmit the flux rates through biochemical pathways dictated by enzymes with comparatively low flux capacities that catalyze reactions displaced far from equilibrium (Newsholme and Start 1973; Atkinson 1977). For the synthesis and degradation of glycogen, these pivotal reactions are catalyzed by glycogen

synthetase and glycogen phosphorylase, respectively. In mammalian tissues, the activities of these enzymes are coordinately regulated in an antagonistic fashion by reversible phosphorylation/dephosphorylation events catalyzed by specific kinases and phosphatases (reviewed in Madsen 1986; Roach 1986; Cohen 1986) in response to neuronal stimulation, hormones, and general physiological condition through a number of different effectors (see reviews by Cohen 1983; Hems and Whitton 1980; Stalmans 1976; Hers 1976). From what is known about the regulation of glycogen metabolism, glycogen synthetase and glycogen phosphorylase represent the classic "flux-generating" steps of glycogen synthesis and glycogenolysis, respectively.

Although the control of glycogen metabolism in marine bivalves has not been as extensively studied, glycogen synthetase and phosphorylase undoubtedly serve similar regulatory functions. In the mantle tissue of the blue mussel Mytilus edulis, the active dephosphorylated form of glycogen synthetase exhibits marked seasonal variation (Cook, Gabbott and Whittle 1979; Gabbott and Whittle 1986a), and its steady elevation in activity throughout the spring and early summer directly parallels glycogen deposition. For flux proceeding in the glycogenolytic direction, Zaba (1981) observed that the activities of both glycogen phosphorylase and amyloglucosidase increased over a period of time when glycogen was being rapidly degraded for gametogenesis. These observations, together with the near-equilibrium status of the phosphoglucomutase reaction,

would predict that the marginally greater enzyme activities of Pgm-2 heterozygotes relative to homozygotes should have little, if any, impact on tissue glycogen concentrations.

Surprisingly, the results of the present study contradict this prediction for the mantle but not the adductor muscle tissue. Classification of Pgm-2 genotypes as either homozygotes or heterozygotes explained a significant amount of the observed variation in mantle glycogen levels in seven of the eight comparisons shown in Table XII. In contrast, the association of these specific activity differences and glycogen concentrations in the adductor muscle was weak, accounting for a significant result in only one of the same eight comparisons (Table XIII). It seems reasonable to assume that the overall control of the annual cycle of synthesis and degradation is mediated through the differential regulation of the activities of glycogen synthetase and the combined phosphorylase/amyloglucosidase system. Despite the control exerted by these regulatory enzymes, however, PGM specific activity variation still appears to have influenced glycogen metabolism. This may be a consequence of the differential abilities of Pgm-2 genotypes to respond to the rates of flux dictated by these regulatory enzymes. The apparent impact of these activity differences was complex, exhibiting reversals between homozygotes and heterozygotes between seasons (i.e. fall and winter) and between intertidal positions within a seasonal season (summer). Since glycogen was measured at a limited number of stages of its annual cycle, it is not possible

to determine from the outset if the variation in PGM activity differentially affects glycogen synthesis and/or glycogenolysis, or represents a fortuitous association through a linkage effect. Of these alternatives, a direct influence of this variation in enzyme activity on the rate of glycogen synthesis is expected through a partitioning effect at the glucose-6-phosphate branch point.

Net flux through the PGM reaction in the direction of glycogen formation is determined by the opposing rates of the forward and reverse reaction directions by the following equation (Savageau 1976):

$$Vnet = \frac{Vmax(f)/Km(f)\ [G\text{-}6\text{-}P] \ - \ Vmax(r)/Km(r)\ [G\text{-}1\text{-}P]}{1 \ + \ \frac{[G\text{-}6\text{-}P]}{Km(f)} \ + \ \frac{[G\text{-}1\text{-}P]}{Km(r)}} \quad ..(4)$$

where Vmax(f) and Vmax(r), and Km(f) and Km(r) are the maximum velocities and Michaelis constants for the forward and reverse reaction directions, and [G-1-P] and [G-6-P] are the intracellular concentrations of glucose-1-phosphate and glucose-6-phosphate, respectively. Estimates of these kinetic parameters in the mantle tissue for the four homozygotes and the heterozygotes for the Pgm-2-100 allele are presented in Table XV. Maximum velocities for the reverse reaction represent the mean enzyme activities of these genotypic classes from Chapter

Table XV. Kinetic parameters in the mantle tissues of the four homozygotes and the three heterozygotes for the Pgm-2-100 allele.

Parameter	Heterozygotes	Homozygotes
Vmax(r)[1]	3.02	2.47
Km(r)[2]	21.2	21.5
Vmax(f)[1]	1.01	0.823
Km(f)[3]	121.9	123.2
Vmax(r)/Km(r)	0.142	0.115
Vmax(f)/Km(f)	0.00829	0.00668

[1] units/g wet tissue
[2] μmoles glucose-1-phosphate
[3] μmoles glucose-6-phosphate

3. Similarly, Michaelis constants for the reverse reaction represent the means expressed by these groups over the temperature range of 5 to 30° presented in Chapter 2. Kinetic parameters for the forward reaction direction were estimated from the Haldane equation, assuming that Vmax(r) exceeds Vmax(f) (as written above) by a factor of three (Ray and Roscelli 1964b; Chapter 2). Heterozygotes for the Pgm-2-100 allele are thus expected to possess Vmax/Km ratios for both reaction directions that are approximately 24% larger than the four homozygotes.

Since at equilibrium Vnet is zero, a flux towards glycogen must involve a displacement of one or both of the two substrates from their equilibrium concentrations (Newsholme and Crabtree 1976). It might be expected that the larger forward reaction rate in heterozygotes would be exactly counterbalanced by the simultaneously greater reverse rate under flux-generating conditions, thus negating any catalytic advantage. However, numerical solution of equation 4 with various concentrations of G-1-P and G-6-P producing a positive Vnet shows that the flux advantage of heterozygotes persists under these conditions irrespective of the magnitude of Vnet. For example, if [G-6-P] and [G-1-P] are 300 and 10 μM, respectively, the calculated Vnet for heterozygotes (0.271 μmoles/min/ml) is 24% larger than estimated for homozygotes (0.219 μmoles/min/ml). An identical relationship between these genotypic groups holds for flux proceeding in the glycogenolytic direction. Surprisingly, these results are unaffected by substrate concentrations, although the

realized Vnet is directly determined by the magnitude of their displacement from equilibrium.

The flux advantage of Pgm-2 heterozygotes might not be expressed in vivo if the enzyme catalyzed a reaction occupying an intermediate position in the pathway. However, PGM is responsible for catalyzing the first step in the synthesis of glycogen from the glucose-6-phosphate branch point. The synthesis of glycogen operates by a "pull" mechanism. Activation of glycogen synthetase in mammalian liver by the injection of glucose has been observed to cause significant reductions in the concentrations of the pathway intermediates glucose-6-phosphate and UDP-glucose (De Wulf and Hers 1967; Hue and Hers 1974). In addition to lowering the concentrations of pathway intermediates, this mechanism must also create displacements from equilibrium at both the PGM and UDP-glucose pyrophosphorylase reaction steps. When driven by exogenous glucose, this pull mechanism will create competition for the available G-6-P between PGM and other enzymes that utilize this metabolite. The predominant competitor at the G-6-P branch point (by net activity criteria) is phosphoglucose isomerase (PGI) which catalyzes the formation of fructose-6-phosphate from G-6-P for entry into glycolysis. Since basal metabolism must be maintained during periods of glycogen formation, some partitioning of G-6-P between glycolysis and glycogen synthesis is unavoidable. An estimate of the extent of this partitioning is provided by the flux studies on mantle tissue slices from M.

edulis by Zaba and Davies (1980, 1981) and Zaba, Gabbott and Davies (1981). These experiments found that 40-60% of the [14C]-glucose label was incorporated into glycogen; the remainder appeared in organic and amino acids.

The partitioning of flux at metabolic branch points has been studied theoretically by Kacser (1983) and empirically by LaPorte, Walsh and Koshland (1984). When two enzymes compete for a common substrate under steady-state conditions, an increased rate of flux through one will cause an identical decline in flux through the other. This antagonistic relationship between competing enzymes can produce negative control coefficients in the language of control theory (Kacser 1983), or ultra- or hyper-sensitivity to regulation depending on the kinetic parameters of the specific enzymes involved (LaPorte, Walsh and Koshland 1984). The division of flux at the glucose-6-phosphate branch point may be quantified by the partitioning coefficient, PC, defined by LaPorte, Walsh and Koshland (1984):

$$PC = \left[\frac{Vmax(2)\ (Km(1) + [G\text{-}6\text{-}P])}{Vmax(1)\ (Km(2) + [G\text{-}6\text{-}P])} + 1 \right]^{-1} \qquad ...(5)$$

where PC quantifies the proportion of flux directed towards glycogen, Vmax(1) and Km(1) represent the standard kinetic parameters for the forward reaction (to glycogen) of PGM, Vmax(2) and Km(2) are the same for PGI, and [G-6-P] is the intracellular concentration of glucose-6-phosphate. Inspection

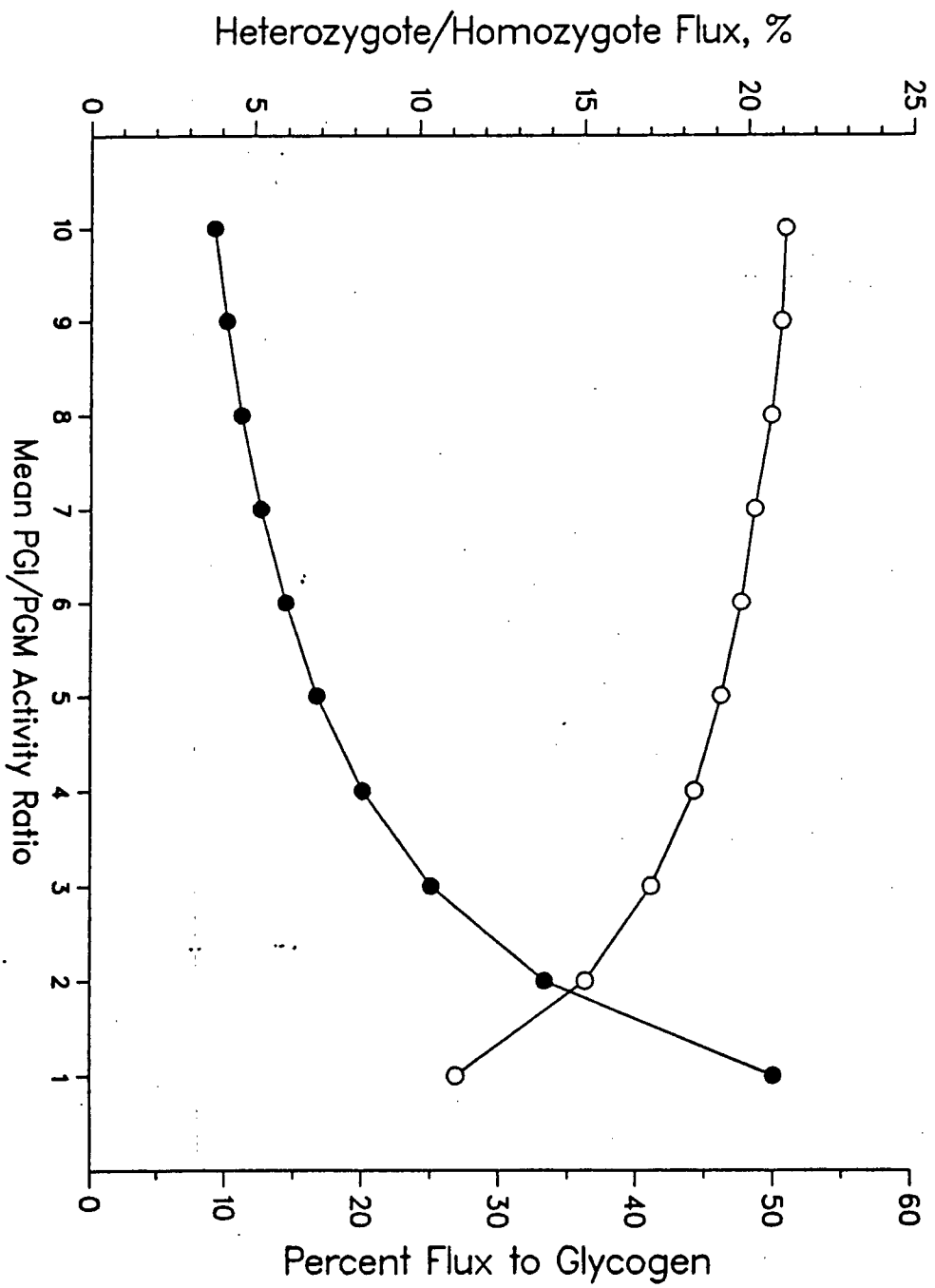
of equation 5 shows that the partitioning coefficient is unaffected by the concentration of glucose-6-phosphate. Furthermore, since the Km for G-6-P of PGI from several bivalve species is very similar to that estimated for C. gigas PGM in Chapter 2 (Martin 1979; Hall 1985), equation 5 may be approximated as:

$$PC = \left[\frac{\text{PGI Specific Activity}}{\text{PGM Specific Activity}} + 1 \right]^{-1} \qquad ...(6)$$

Therefore, the division of flux at the glucose-6-phosphate branch point when both glycolysis and glycogen synthesis are occurring is largely determined by the PGI/PGM specific activity ratio.

The expected flux towards glycogen and the resulting flux difference between Pgm-2 heterozygotes and homozygotes as a function of the PGI/PGM activity ratio is illustrated in Figure 15. At equal activities of these competing enzymes, 50% of the flux is directed towards glycogen, however, the flux for heterozygotes is only 11% larger than homozygotes despite their 24% larger Vmax/Km ratios. As the PGI/PGM activity ratio increases, the proportion of flux proceeding towards glycogen declines and the flux advantage of heterozygotes asymptotically approaches their net catalytic advantage. In freshly ground tissues from 12 oysters, the mean ratio of the specific activities of PGI to PGM was 7.4 and 8.4 in the mantle and

Figure 15. Predicted flux advantage of Pgm-2 heterozygotes and percent flux to glycogen as a function of the PGI/PGM activity ratio. Open circles=percent flux excess of heterozygotes over homozygotes; closed circles=percent flux to glycogen.


Heterozygote/Homozygote Flux, %
0
5
10
15
20
25
Mean PGI/PGM Activity Ratio
1
2
3
4
5
6
7
8
9
10
Percent Flux to Glycogen
0
10
20
30
40
50
60

adductor muscle, respectively. These ratios are similar to those reported in the adductor muscle tissues of the mussel, Mytilus edulis (Ebberink and de Zwaan 1980) and the bay scallop, Argopecten irradians concentricus (Chih and Ellington 1986). Therefore, Pgm-2 heterozygotes are expected to exhibit larger fluxes towards glycogen than homozygotes, similar in magnitude to their enzyme activity excess, because of this partitioning effect at the glucose-6-phosphate branch point. These patterns are expected to hold irrespective of the overall rate of glycogen synthesis dictated by glycogen synthetase.

When glycogen is synthesized from gluconeogenic precursors the flux advantage of heterozygotes could also be maintained. The overall rate of synthesis would now be determined by the availability of the specific precursor involved, the crucial bypass reactions catalyzed by pyruvate decarboxylase and fructose bisphosphatase in addition to glycogen synthetase. However, a displacement from equilibrium is still required at the PGM step and under these conditions the net flux in Pgm-2 heterozygotes is still expected to exceed homozygotes. The differences in mantle glycogen levels observed between homozygotes and heterozygotes could in part be explained by these differential rates of synthesis.

The impact of this PGM activity variation on glycogenolysis is less certain. Three factors suggesting a limited effect of PGM on this catabolic process are 1) the reaction mechanism of

glycogen phosphorylase, 2) regulatory aspects of glycogen mobilization, and 3) the existence of an alternative degradative pathway. The reaction catalyzed by glycogen phosphorylase differs from glycogen synthetase in being potentially reversible (Fletterick and Madsen 1980), yet is believed to proceed almost exclusively in the forward direction (i.e. glycogen breakdown) because of the high molar ratio of inorganic phosphate to glucose-1-phosphate (Stalmans 1976). This high molar ratio and the low relative activity of phosphorylase compared to PGM would ensure that the glucose-1-phosphate moieties liberated from glycogen would be rapidly converted by PGM into glucose-6-phosphate and fed into glycolysis. The large catalytic excess at the PGM reaction strongly favors the uni-directional flow of glucosyl units from glycogen and suggests that the minor variations in activity present between genotypes would not affect the degradative rate.

The elegant regulatory cascade controlling the activation of glycogen phosphorylase a is well known, and different cell types exhibit modifications of the cascade architecture in accordance with their requirements for the rapid mobilization of glycogen (Cohen 1978). Situated in an intermediate position between phosphorylase and additional regulatory reactions, PGM is expected to have a limited impact on glycogenolytic rate. Several experiments with marine bivalves have borne out this prediction. For example, Ebberink and de Zwaan (1980) monitored mass action ratios (MAR) for 14 glycolytic enzymes in the

posterior adductor muscle of M. edulis over a 24 h period of anaerobiosis induced by aerial exposure. They identified phosphofructokinase as the major control point during the first few hours of valve closure, but noted a shift in control to pyruvate kinase after 8 h of prolonged anoxia. The Keq/MAR ratio for the PGM reaction remained close to unity throughout the experiment despite large increases in the concentrations of both substrates. Since the regulation of glycolysis in oysters is probably similar to that in mussels, the differential abilities of Pgm-2 genotypes to catalyze the interconversion of G-1-P and G-6-P would not be expected to affect glycogenolytic rates under similar anoxic conditions.

In addition to the phosphorolytic pathway, glycogen may also be hydrolyzed directly by amyloglucosidase to produce free glucose. This in turn could be exported from the cell or converted into glucose-6-phosphate by hexokinase. The existence of this second degradative route in marine invertebrates has been recognized for many years (e.g. Alemany and Rosell-Perez 1973; Hino, Tazawa and Yasumasu 1978; Zaba 1981). However, the relative contributions of the phosphorolytic and hydrolytic pathways of glycogen mobilization in marine bivalves have yet to be determined. Knowledge of the proportion of glycosyl units channeled through both degradative routes is essential for assessing the potential effect of PGM activity variation on glycogenolysis. The observation of membrane-enclosed glycogen particles in the mantle vesicular cells of M. edulis by Bayne et

al. (1982) would suggest an important role for the hydrolytic mechanism. If the phosphorolytic pathway is used less frequently, PGM must exert a significant effect on glycogenolysis that is too great to be reconciled with the known function and regulation of glycogen phosphorylase.

Although phosphoglucomutase is not believed to influence glycogenolysis under standard conditions, situations have been described where it could exert some degree of control. For example, Chih and Ellington (1986) noted that after 80 contractions of the posterior adductor muscle of the bay scallop, Argopecten irradians concentricus the Keq/MAR ratio of glycogen phosphorylase fell from 175 to 27 while that for PGM increased from 3.5 to 25. Under these conditions PGM could exert a significant effect on the rate of glycogen degradation. Circumstantial evidence suggesting an effect of PGM on glycogen mobilization has also been reported in the field mouse, Apodemus sylvaticus (Leigh Brown 1977) and the rainbow trout, Salmo gairdneri (Allendorf, Leary and Knudsen 1983).

The extremely high concentrations of glycogen stored in oyster tissues suggests a mechanism by which PGM could affect glycogenolysis. In the mantle tissues of marine bivalves, glycogen is stored in vesicular cells that lie in close proximity to the digestive gland and developing gonad (Gabbott 1983). These cells contain large quantities of glycogen concentrated in the cytoplasm as small granules or large β-

particles (Eble 1969; Bayne et al. 1982). Unfortunately, the degradation of glycogen in bivalve vesicular cells has not been studied. It is conceivable that the high concentrations of glycogen phosphorylase associated with these B-particles (Eble 1969) could release large amounts of G-1-P that result in substantial displacements from equilibrium at the PGM reaction. If these displacements occur, differences in enzyme activities between Pgm-2 homozygotes and heterozygotes have the potential to influence rates of glycogen breakdown.

From these considerations, the genotype-by-environment interactions observed for the mantle glycogen data could be caused by the differential capabilities of Pgm-2 genotypes to respond to flux rates dictated by glycogen synthetase, and possibly glycogen phosphorylase. In Crassostrea gigas, mantle glycogen is synthesized rapidly in the fall after the completion of spawning and the necessary reorganization of tissue (Quayle 1969). In this season, a strong association was observed between PGM activity and mantle glycogen (Table XIV). Heterozygotes possessing the Pgm-2-100 allele had higher levels of mantle glycogen than homozygotes or heterozygotes for the less frequent Pgm-2-92, 96, or 104 alleles. These results are exactly as expected if this enzyme activity variation differentially affects the rates of glycogen synthesis between these genotypic classes.

When the summer sample was taken, considerable gonadal

development was observed and, therefore, glycogen levels were probably on the decline because of the strong relationship between the depletion of these reserves and gametogenesis (Gabbott 1975; Bayne 1976). In this season Pgm-2 homozygotes had higher concentrations of mantle glycogen than heterozygotes in the low intertidal area, but lower levels in the high intertidal region. A clear explanation for these results is not possible. These patterns could have arisen from differences in glycogen levels that existed before the onset of glycogenolysis, differential rates of degradation, or perhaps to the asynchronous timing of gonadal development in different genotypes. Neither of these possibilities are mutually exclusive and further studies on the metabolism of glycogen in different Pgm-2 genotypes are required to determine which, if any, is correct.

In the winter, Pgm-2 homozygotes had higher concentrations of mantle glycogen than heterozygotes at both intertidal locations. The same relationship between genotypes was evident in the adductor muscle tissue, but the differences were significant only in the high intertidal area. As stated previously, this sample was taken when natural food conditions were extremely poor, a factor that might explain the lower than expected levels of glycogen observed at this time. If the population at this time was experiencing negative "scope for growth" (cf. Bayne and Newell 1983), glycogen reserves might have been utilized to meet basal metabolic requirements, given

the dormant state of the reproductive cycle. One explanation for these results is that Pgm-2 heterozygotes were depleting these energy reserves at a faster rate than homozygotes. Rodhouse and Gaffney (1984) studied the relationship between multiple-locus heterozygosity and starvation-induced weight loss in the American oyster, C. virginica, over a 42 day period. These authors found that the rate of weight loss was lower in more heterozygous individuals, thus suggesting that heterozygotes are more viable over periods of nutritive stress than homozygotes.

If these conclusions are applicable to the Pacific oyster, heterozygotes would have been expected to exhibit higher, rather than lower, levels of mantle glycogen compared to homozygotes under these adverse environmental conditions. Comparison of these results are questionable however, since Rodhouse and Gaffney (1984) were able to control food availability, water temperature, and the starvation period. None of these factors were known or manipulated in this study. Furthermore, Rodhouse and Gaffney (1984) did not find a significant relationship between carbohydrate levels and heterozygosity either before or after the starvation period. It should be pointed out, however, that any effect contributed by the Pgm locus on glycogen metabolism in their study may have been obscured by the random assignment of Pgm homozygotes and heterozygotes across the different heterozygosity classes examined.

Marked differences were observed between the effects of Pgm

-2 genotype in the analyses of the mantle and adductor muscle glycogen data. These results were surprising in light of the great similarity shown by these tissues in their PGM specific activity measurements and the seasonal patterns of their glycogen levels. Apparently, enzyme activity variation at the Pgm-2 locus exerted an effect on glycogen metabolism in the mantle, but not the adductor muscle. In mammals, the regulation of glycogen metabolism differs between the liver and muscle in several important aspects. In contrast to the liver, the muscle form of glycogen synthetase phosphatase is inhibited directly by glycogen itself (Hers, De Wulf and Stalmans 1970). This inhibition prevents excessive deposition of glycogen in muscle fibres that could interfere with contractile function. Extrapolating these differences to molluscan systems is difficult because the homologous regulatory enzymes have not been characterized. However, if a similar mechanism exists in oyster adductor muscle, differences between Pgm-2 genotypes in their rates of glycogen synthesis may not be expressed. Deposition of glycogen in the adductor muscle may occur at different rates, but no net differences between Pgm-2 genotypes would be expected if further synthesis is prevented beyond this limit.

Fewer constraints may apply to the absolute quantity of glycogen stored in the mantle tissue. Upper limits obviously exist for the storage capacities of individual vesicular cells, but their mantle proportions are known to vary seasonally and

between different populations at the same time of year (Lowe, Moore and Bayne 1982). This may allow for the less restrictive accumulation of glycogen in the mantle in comparison with the adductor muscle tissue. Under these conditions, the effect of enzyme activity variation at the Pgm-2 locus on metabolic flux could be manifested, even though the overall rates would still be determined by regulatory enzymes like glycogen synthetase.

It should be stressed that these arguments rely heavily on the assumption that the effects of the Pgm-2 locus on glycogen metabolism were randomized with respect to the remainder of the genetic background. Non-random associations of this PGM activity variation with adjacent enzymic reactions of glycogen metabolism could lead to erroneous conclusions concerning the effects of the Pgm-2 locus. The next chapter tests the validity of this assumption by examining the activity relationships between the kinetically-linked reactions of the glycogen synthesis pathway.

CHAPTER 5

ACTIVITY STRUCTURE OF THE GLYCOGEN SYNTHESIS PATHWAY

INTRODUCTION

Attributing a physiological effect to allozymic variation at a single enzyme locus in a complex multi-enzyme pathway encounters difficulties beyond those concerned with its detection (e.g. Dykhuizen, Dean and Hartl 1987). In studying natural populations, a major concern involves effects of the genetic background. Marine bivalves such as C. gigas possess substantial levels of protein heterozygosity (Buroker, Hershberger and Chew 1979a; Ozaki and Fujio 1985), and thus the central energy-producing pathways are likely to be segregating for genetic variation at enzyme loci in addition to the one under study. Although yet unquantified in marine bivalves, regulatory gene variation (cf. Paigen 1979) might also be present that has the potential to exert pleiotropic effects on a number of different pathway enzymes, as suggested by studies on D. melanogaster (Laurie-Ahlberg et al. 1982; Wilton et al. 1982). Molecular studies of DNA sequence variation have also revealed that considerable linkage disequilibrium may exist between structural alleles and their 5' and 3' flanking regions that are sometimes associated with genotypic differences in in vitro specific activities (e.g. Aquadro et al. 1986; Langley et al. 1988). Each of these factors complicates the assignment of

physiological effects to allozymic differences at a specific enzyme locus.

Studies of this nature in marine bivalves are further confounded by the well documented seasonality of their metabolism of lipid, protein, and carbohydrate, and in the accumulation of anaerobic end-products (e.g. Gabbott 1975; Pieters et al. 1979; Zandee et al. 1980). Associated with these seasonal cycles are tissue-specific changes in specific activities (e.g. Livingstone 1981; Gabbott 1983), and in some instances the kinetic properties of enzymes (e.g. Livingstone 1975; Livingstone and Clarke 1983). Although PGM specific activity has been shown in Chapter 3 to vary on a seasonal basis, these changes do not necessarily have to involve the polymorphic locus studied to complicate the detection of its physiological consequences. Alterations in activity levels and/or kinetic properties of pathway enzymes have the potential to affect the architecture of the pathway's regulatory control that in turn could differentially affect the impact of the polymorphism between different seasons.

In the laboratory, genetic backgrounds may be standardized through the creation of isogenic lines (e.g. Laurie-Ahlberg et al. 1980, 1981) or by the use of genetic transduction to introduce single allelic substitutions (e.g. Hartl and Dykhuizen 1980; Dykhuizen and Hartl 1981). Neither of these techniques are amenable to the study of natural populations, and thus it is

necessary to monitor simultaneously the presence of genetic variation in the entire metabolic pathway studied to help alleviate problems contributed by genetic background effects. Furthermore, due to the annual cycles of metabolism in marine bivalves, the physiological effects of enzyme polymorphisms may have to be studied on a season- and tissue-specific basis if their full adaptive significance is to be understood.

In Chapter 4, mantle glycogen concentrations were shown to differ between Pgm-2 genotypes in a complex season-dependent fashion. Although the patterns observed were directly associated with the differences in PGM specific activity documented in Chapter 3, the contribution of additional loci, particularly those metabolically related with PGM, could not be discounted. To examine their potential effects, the existence of genetic variation and the activity levels of the remaining glycogen synthesis pathway enzymes were studied in different Pgm-2 genotypic classes in the fall sample. In this season, glycogen reserves are rapidly accumulated in the mantle tissue (Quayle 1969), thus allowing a more thorough examination of the effects of the Pgm-2 locus on glycogen synthesis without complications due to the concurrent operation of the glycogenolytic pathway.

MATERIALS AND METHODS

Chemicals. Buffers, substrates, cofactors, and coupling enzymes used for the electrophoresis and enzyme assays were obtained from Sigma. The electrostarch for electrophoresis was supplied by Connaught Laboratories.

Animals. Oysters were collected from the Nanoose Bay study population in the fall of 1985. The collection, transportation and storage of animals has been described in Chapters 2 and 3.

Electrophoresis. For the entire sample, Pgm-2 genotype was determined by starch gel electrophoresis as outlined in Chapter 2. In the subsample of individuals selected for the enzyme activity and glycogen measurements, electrophoretic analysis was also performed to determine genotypes at the hexokinase and UDP-glucose pyrophosphorylase loci. UDP-glucose pyrophosphorylase (UDPGP; E.C. 2.7.7.9) activity was resolved electrophoretically by the discontinuous Tris-citrate, Li-OH buffer system of Ridgway, Sherburne and Lewis (1970). UDPGP activity was stained visually by incubating a gel slice for approximately 30 min in 100 ml of 100 mM Tris-HCl buffer, pH 7.5 containing 120 mg magnesium chloride, 90 mg UDP-glucose, 65 mg sodium pyrophosphate, 25 mg NADP, 0.7 mg glucose-1,6-diphosphate, 80 units phosphoglucomutase, 40 units glucose-6-phosphate dehydrogenase, 2 mg MTT, and 1 mg PMS. Hexokinase (HK; E.C. 2.7.1.1) activity was examined electrophoretically by the

standard Tris-borate-EDTA pH 8.3 buffer system described in Chapter 2, and stained according to Shaw and Prasad (1970).

Enzyme Assays. The mantle activities of hexokinase, phosphoglucomutase, UDP-glucose pyrophosphorylase, and glycogen synthetase were determined in a subsample of 229 oysters (108 from the low intertidal site, 121 from the high intertidal site). These individuals represented seven Pgm-2 genotypic classes that were randomly picked from four arbitrarily assigned body weight classes described in Chapter 3. Preparations of the crude tissue homogenates were identical to that outlined in Chapter 3, except for the inclusion of 5 mM dithiothreitol (DTT) in the extraction buffer. Addition of DTT stabilized the activities of UDP-glucose pyrophosphorylase and glycogen synthetase, but was found to have no adverse effects on either hexokinase or phosphoglucomutase.

All enzyme activities were measured in triplicate at 340 nm on a Pye Unicam SP 1800 UV/visible spectrophotometer. Assay temperature was maintained at 15 C by a Lauda K-2/RD circulating water bath. Phosphoglucomutase activity was determined by the standard assay on 20 µl aliquots of the crude homogenates as described in Chapter 2. One unit of activity is defined as the quantity of enzyme required to convert 1 µmole of glucose-1-phosphate to glucose-6-phosphate per minute under these conditions.

Hexokinase was assayed according to Bergmeyer (1974) on 60 µl samples of the crude homogenates. The reaction medium contained 50 mM triethanolamine pH 7.5, 200 mM D-glucose, 5 mM MgCl2, 0.6 mM ATP, 0.4 mM NADP, and one unit of glucose-6-phosphate dehydrogenase (G6PDH) in a total volume of 1 ml. One unit of activity corresponds to the amount of enzyme needed to convert 1 µmole of glucose to glucose-6-phosphate per minute at 15 C. UDP-glucose pyrophosphorylase was also measured as outlined by Bergmeyer (1974) on 20 µl aliquots of the tissue extracts. The assay mixture contained 50 mM Tris-HCl pH 7.5, 5 mM MgCl2, 1.5 mM sodium pyrophosphate, 1 mM UDP-glucose, 0.4 mM NADP, 16 µM glucose-1,6-diphosphate, 5 units of PGM, and one unit of of G6PDH in a final volume of 1 ml. The unit definition for UDPGP is the amount of enzyme required to convert 1 µmole of UDP-glucose to glucose-1-phosphate per minute at 15 C.

Glycogen synthetase (GS; E.C. 2.4.1.11) activity was determined by the direct one-step assay of Passoneau and Rottenberg (1973) on 50 µl samples of the crude homogenates. The assay medium contained 50 mM Tris-HCl pH 7.5, 50 mM KCl, 10 mM hydrazine-HCl, 10 mM sodium amobarbital, 10 mM UDP-glucose, 10 mM oyster glycogen (Type II), 5 mM MgCl2, 1 mM Na-EDTA, 1 mM phospho(enol)pyruvate, 0.15 mM NADH, 20 units of pyruvate kinase, 10 units of lactate dehydrogenase, and 0.02% (w/v) bovine serum albumin in a final volume of 1 ml. Glycogen synthetase was not assayed in the presence of glucose-6-phosphate, thus only the activity of the dephosphorylated I form

was quantified. One unit of GS activity is defined as the quantity of enzyme required to transfer the addition 1 μmole of UDP-glucose onto the primer glycogen per minute under the above conditions. Because of the significant oxidation of NADH by the crude homogenates, even in the presence of the amobarbital, the activity measurements necessitated corrections by blank cuvettes that contained all of the above assay components except UDP-glucose. Similar corrections were not required for the other assays. Soluble protein levels in the crude homogenates were measured in triplicate as described in Chapters 2 and 3.

The enzymes of the glycogen synthesis pathway were assayed concurrently with the mantle and adductor muscle PGM specific activity measurements for the fall sample. As described in Chapter 3, the selection of Pgm-2 genotypes was randomized across experimental days thus ensuring that day-to-day variation in assay technique and systematic errors due to the spontaneous loss of enzyme activity would be minimized.

Glycogen Assays. Determination of the mantle glycogen concentrations of Pgm-2 genotypes in this sample have been previously described in Chapter 4.

Statistical Analyses. Activity relationships between pathway enzymes were examined by testing the significance of their product-moment correlation coefficients from random expectations. To examine the effects of Pgm-2 locus on pathway

activity structure, the sample was partitioned into the four Pgm -2 allelic classes described in Chapter 3 (i.e., homozygotes for the Pgm-2-100 allele, heterozygotes possessing the Pgm-2-100 allele, homozygotes for the less frequent Pgm-2-92, 96, and 104 alleles, and heterozygotes between these three less frequent alleles). Three-way analyses of variance were performed on the activities of HK, PGM, UDPGP, GS, and mantle glycogen levels treating intertidal position, body weight class, and Pgm-2 locus genotype as independent variables. Significant effects of Pgm-2 genotype class in the analyses of the HK, UDPGP, or GS data thus demonstrate an association of the Pgm-2 locus with the activities of these sequential enzymic reactions. The effects of variation in the activities of HK, PGM, UDPGP, and GS on existing mantle glycogen levels was examined by multiple linear regression analysis. The relative importance of each individual enzyme was assessed by testing the significance of the t-ratio of its partial regression coefficient to its standard error, a procedure analogous to partial F-tests on the explained sums of squares attributable to each factor in the multiple regression.

RESULTS

ELECTROPHORESIS

The discontinuous Ridgway buffer system produced excellent electrophoretic resolution of UDP-glucose pyrophosphorylase. Two distinct zones of activity were visible, suggesting the presence

of two distinct genetic loci. Examination of the 229 individuals selected for the enzyme activity and glycogen measurements showed that both presumptive loci were completely monomorphic. Using the Tris-borate-EDTA pH 8.3 buffer system, hexokinase also appeared to be encoded by two separate loci. The more cathodal locus produced a faint single band. The anodal region also stained faintly, but was characterized by a two-banded pattern. Because of the extremely low activity of HK, determination of the existence of genetic variation at either locus could not be determined for the entire sample. However in a sample of 62 individuals yielding good resolution, no heterozygosity was observed at either locus.

CORRELATIONS BETWEEN PATHWAY ENZYME ACTIVITIES

Product-moment correlation coefficients between the mantle activities of the glycogen synthesis pathway enzymes are presented in Table XVI. At both intertidal locations, highly significant positive correlations were observed between the activities of HK and UDPGP, and between PGM and UDPGP. Weak positive correlations were observed between UDPGP and GS, but no relationships were evident between the activities of HK and GS. Two notable differences were found between the correlation matrices of the two intertidal samples. First, HK was significantly correlated with PGM in oysters from the low intertidal but not in those situated in the high intertidal. Second, a weak positive relationship existed between the

Table XVI. Product-moment correlation coefficients between the activities of enzymes in the glycogen synthesis pathway. Values above the diagonal are for the low intertidal sample (n=108); those below for the high intertidal sample (n=121).

	Enzyme			
Enzyme	HK	PGM	UDPGP	GS
HK	1.000	0.316**	0.463***	0.084
PGM	0.135	1.000	0.512***	0.105
UDPGP	0.491***	0.419***	1.000	0.137
GS	0.006	-0.146	0.134	1.000

** P < .01
*** P < .001

activities of PGM and GS in the low intertidal, whereas in the high intertidal the correlation between these enzyme activities was negative. Statistical comparison of the Z-transformed coefficients of the HK-PGM and PGM-GS enzyme pairs between the high and low intertidal zones did not detect any significant differences.

PATHWAY ACTIVITIES OF PGM-2 GENOTYPIC GROUPS

The results of the three-factor ANOVA's on mantle glycogen levels and the activities of HK, PGM, UDPGP, and GS are summarized in Table XVII. Intertidal position explained a significant proportion of the variance observed for all five dependent variables. A consistent pattern was observed in all analyses: oysters in the low intertidal region possessed significantly larger quantities of glycogen and significantly greater activities of all four enzymes compared to those sampled from the more exposed high intertidal area. As evident from the magnitude of the F values, the most extreme difference was observed for glycogen synthetase: its mean activity at the low intertidal site exceeded that at the high intertidal site by 32%. In contrast, oysters in the low intertidal area displayed 22% more HK activity, 15% more PGM activity, 21% more UDPGP activity, and 24% more glycogen than individuals higher in the intertidal zone. No significant effects of body weight class as a separate factor were present in any of the analyses.

Table XVII. F-ratios from the analyses of variance on the mantle activities of the glycogen synthesis pathway enzymes and mantle glycogen concentrations.

Source of Variation	df	Dependent Variable HK	PGM	UDPGP	GS	Glycogen
Tidal Height	1	19.1***	15.0***	14.8***	67.7***	19.2***
Body Weight	3	0.79	1.88	1.14	1.51	2.06
Allelic Class	3	2.40	7.76***	1.70	3.25*	10.5***
Tidal Height x Body Weight	3	1.48	0.75	0.36	3.67*	1.21
Tidal Height x Allelic Class	3	0.94	0.52	2.27	4.90**	0.61
Body Weight x Allelic Class	9	0.59	0.22	0.31	1.83	0.78
Tidal Height x Body Weight x Allelic Class	9	1.95*	1.72	2.14*	0.67	1.06
Error	197					

* P < .05
** P < .01
*** P < .001

Highly significant differences existed between the PGM activities and glycogen concentrations of the four Pgm-2 allelic classes. The specific activities and mantle glycogen levels of these genotypic groups have been presented previously in Table XIV of Chapter 4. Heterozygotes for the Pgm-2-100 allele had significantly greater specific activities than the Pgm-2-100/100 homozygotes and the heterozygotes for the Pgm-2-92, 96, and 104 alleles, but did not differ significantly from the homozygotes for these less frequent alleles. Identical differences between these genotypic classes were observed in their mantle glycogen concentrations. Of particular interest in this study however, were differences that might exist between the HK, UDPGP, and GS activities of the Pgm-2 allelic classes. Table XVII shows that only glycogen synthetase activity differed significantly between Pgm-2 genotypes in the overall analysis. Homozygotes for the Pgm -2-92, 96, and 104 alleles had GS activities that were 14.4% larger than the Pgm-2-100/100 homozygotes (7.31 units/g vs. 6.39 units/g). The two heterozygous groups showed intermediate GS activities. A posteriori Bonferroni multiple range tests were unable, however, to detect any significant differences between the allelic groups.

The general absence of any relationships between Pgm-2 genotypic class and the activities of these other enzymes in the overall analysis is somewhat deceptive. Tables XVIII and XIX present the results from two-way ANOVA's carried out separately for the low and high intertidal samples, respectively. The

Table XVIII. Activities (units/g soluble protein) of HK, PGM, UDPGP, and GS of four Pgm-2 genotypic classes in the low intertidal zone.

Pgm-2 Allelic Class	N	Enzyme Activity[1] HK	PGM	UDPGP	GS	Glycogen[2]
Homozygotes for 100 Allele	22	6.06±.49	116.2±6.2	31.9±2.7	6.75±.31	171.4±16.0
Homozygotes without 100 Allele	14	6.17±.62	124.9±7.8	35.4±3.4	7.75±.39	195.2±20.0
Heterozygotes for 100 Allele	60	6.57±.30	139.2±3.8	35.3±1.6	7.93±.19	220.5±9.7
Heterozygotes without 100 Allele	12	6.90±.67	106.2±8.5	29.1±3.7	7.80±.43	154.6±21.8
F(3,92)	=	0.48	5.67**	0.86	3.68*	4.03**

* $P < .05$
** $P < .01$
[1] units/g soluble protein
[2] μmoles glucosyl units/g wet tissue

Table XIX. Activities (units/g soluble protein) of HK, PGM, UDPGP, and GS of four Pgm-2 genotypic classes in the high intertidal zone.

Pgm-2 Allelic Class	N	Enzyme Activity[1] HK	PGM	UDPGP	GS	Glycogen[2]
Homozygotes for 100 Allele	20	4.77±.44	101.6±6.4	27.8±2.2	6.00±.40	122.0±12.0
Homozygotes without 100 Allele	17	6.04±.49	106.7±7.0	34.8±2.4	6.94±.44	148.7±13.1
Heterozygotes for 100 Allele	63	4.93±.25	118.5±3.6	25.8±1.3	5.30±.23	183.1±6.8
Heterozygotes without 100 Allele	21	6.19±.44	104.7±6.3	29.7±2.2	6.24±.39	145.3±11.7
F(3,105)	=	3.35*	2.92*	3.25*	4.56**	7.93***

* $P < .05$
** $P < .01$
[1] units/g soluble protein
[2] μmoles glucosyl units/g wet tissue

analysis of the low intertidal sample produced results that were similar to that seen in Table XVII. No significant differences were observed between the HK and UDPGP activities of the four Pgm-2 allelic groups. Significant differences were again found between their GS activities that multiple range tests showed to be caused by the greater activities of heterozygotes for the Pgm -2-100 allele relative to the Pgm-2-100/100 homozygotes. The overdominance for PGM activity expressed by the Pgm-2-92/100, 96/100 and 100/104 heterozygotes was not manifested in the activities of these other enzymes. These heterozygotes exhibited UDPGP and GS activities that were indistinguishable from the homozygotes for the Pgm-2-92, 96, and 104 alleles. The HK activities of heterozygotes for the Pgm-2-100 allele were slightly higher than either homozygote group, but marginally below that measured in the other class of heterozygotes. Mantle glycogen concentrations differed significantly between Pgm-2 allelic groups ($F(3,92) = 4.03$, $P < .01$), in a pattern consistent with their activities of PGM, but not any of the other pathway enzymes.

In contrast to the overall analysis, significant differences were observed between Pgm-2 genotypic groups in their activities of all four enzymes in the high intertidal sample (Table XIX). However, the level of significance in most analyses was low and multiple range tests were able to detect significant differences between allelic groups only for their UDPGP and GS activities. For both enzymes, homozygotes for the

Pgm-2-92, 96, and 104 alleles had significantly greater activities than the Pgm-2-92/100, 96/100 and 100/104 heterozygotes. Homozygotes and heterozygotes for the three less frequent alleles also exhibited larger HK activities than either genotypic class possessing the Pgm-2-100 allele. In comparison to the other genotypic groups, heterozygotes for the Pgm-2-100 allele had HK, UDPGP, and GS activities that differed substantially from that observed in the low water sample. Although they still expressed overdominant PGM activities, these genotypes expressed slightly underdominant UDPGP and GS activities, and levels of HK similar to the Pgm-2-100/100 homozygotes. In the high intertidal region the Pgm-2-92/100, 96/100 and 100/104 heterozygotes thus displayed HK, UDPGP, and GS activities that resembled one homozygote (Pgm-2-100/100), but in the low intertidal area were more similar to the alternate homozygotes (Pgm-2-92/92, 96/96, and 104/104). Consequently, when pooled over the two tidal heights, heterozygotes for the Pgm-2-100 allele expressed overdominant PGM activities, but intermediate HK, UDPGP, and GS activities. As seen in the low intertidal sample, the glycogen concentrations of the genotypic groups showed a strong positive relationship with PGM activity, but not with any other pathway enzymes.

A number of significant interaction terms were present in the ANOVA results presented in Table XVII. The analyses for both HK and UDPGP produced significant second-order tidal height-by-body weight-by-allelic class interactions. For HK, the two-way

ANOVA for the high intertidal sample yielded a significant body weight-by-allelic class interaction term ($F(9,105) = 2.15$, $P < .05$). In this sample, the HK activity in heterozygotes for the Pgm-2-100 allele decreased with increasing body weight but showed the reverse trend in the other genotypic groups. In the low intertidal HK analysis the interaction between body weight and allelic class was non-significant ($F(3,92) = 0.59$, $P > .80$), but activities in heterozygotes for the Pgm-2-100 allele tended to increase as a function of body weight. No relationships were observed in the other genotypic groups. The second-order interaction observed for HK was thus largely caused by a reversal in the relationship between body weight and enzyme activity in heterozygotes for the Pgm-2-100 allele between the two tidal positions.

The similar second-order interaction term observed for UDPGP differed in origin from that seen for HK. At both intertidal positions, the body weight-by-allelic class interaction terms were non-significant. In the high intertidal sample, both homozygote groups showed a positive association between UDPGP activity and body weight. No relationships were evident in the two heterozygote groups. In oysters from the low intertidal site, heterozygotes for the Pgm-2-100 allele displayed a positive association between UDPGP activity and body weight, however no relationships were observed for the other genotypic groups. The differential responses of UDPGP activity and body weight in homozygous and heterozygous genotypes between

intertidal positions produced the significant second-order interaction observed for this enzyme in Table XVII.

Analysis of the glycogen synthetase data yielded significant interactions between tidal height and body weight, and between tidal height and allelic class. In the high intertidal sample, significant differences in GS activity existed between the four body weight groups ($F(3,105 = 3.98$, $P < .01$). Multiple range tests showed that oysters in the smallest weight class (12.0-23.9 g) had significantly lower enzyme activities than individuals above 48.0 g. No significant differences between the GS activities of these weight groups were present in the low intertidal sample. However, animals in the smallest weight class now expressed greater GS activities than measured in the largest individuals. These reversals in GS activity between the smallest and largest oysters over the two tidal positions were responsible for the significant body weight-by-allelic class interaction term. The significant tidal height-by-*Pgm*-2 allelic class interaction was a consequence of the change in ranked GS activity level first to last by the heterozygotes for the *Pgm*-2-100 allele between the two tidal heights as seen in Tables XVIII and XIX.

MULTIPLE REGRESSION ANALYSES

Simultaneous measures of the activities of HK, PGM, UDPGP, GS and existing glycogen levels allowed an assessment of the relative effects of each enzyme on the synthetic pathway by multiple regression analysis. For the low intertidal sample (Table XX), the overall regression was highly significant ($F(4,103) = 5.06$, $P < .001$), explaining 16.4% of the observed variation in mantle glycogen levels. Estimation of the t-ratios for each enzyme in the pathway showed that only PGM accounted for a significant proportion of the explained sums of squares of the multiple regression ($t = 2.35$, $P < .05$). The multiple regression on the high intertidal data (Table XXI) was also significant ($F(4,116) = 3.49$, $P < .01$), but explained less of the variation in glycogen than found for the low water sample ($r^2 = 10.8\%$). Once again, only the t-ratio for PGM was significant ($t = 3.52$, $P < .001$). In contrast to the regression equation obtained from the low water sample, the partial regression coefficients for UDPGP and GS in the high intertidal sample were negative. The data presented in Table 4 suggests that this may have been caused by the heterozygotes for the Pgm-2-100 allele. These genotypes expressed the lowest UDPGP and GS activities, yet the highest glycogen levels of the four genotypic groups.

Table XX. Results from the multiple regression analysis of the glycogen synthesis pathway enzyme activities on glycogen levels in the low intertidal sample.

Regression Equation:

Glycogen = 54.4 + 0.527HK + 0.626PGM + 1.27UDPGP + 2.42GS

Enzyme	Regression Coefficient	Standard Error	t-ratio
Hexokinase	0.527	3.61	0.15
Phosphoglucomutase	0.626	0.27	2.35*
UDP-Glucose Pyrophosphorylase	1.27	0.72	1.76
Glycogen Synthetase	2.42	4.73	0.51

* $P < .05$

Table XXI. Results from the multiple regression analysis of the glycogen synthesis pathway enzyme activities on glycogen levels in the high intertidal sample.

Regression Equation:

Glycogen = 94.1 + 1.76HK + 0.729PGM - 0.673UDPGP - 0.510GS

Enzyme	Regression Coefficient	Standard Error	t-ratio
Hexokinase	1.76	2.90	0.61
Phosphoglucomutase	0.729	0.207	3.52***
UDP-Glucose Pyrophosphorylase	-0.673	0.656	-1.03
Glycogen Synthetase	-0.510	2.89	-0.18

*** $P < .001$

DISCUSSION

The correlations between the activities of the glycogen synthesis pathway enzymes, their activity levels in different Pgm-2 genotypic groups, and the results of the multiple regression analyses all provide consistent evidence favoring a significant effect of the Pgm-2 locus on glycogen metabolism in C. gigas. In the glycogen synthesis pathway, both hexokinase and glycogen synthetase are known to catalyze non-equilibrium reactions (Newsholme and Start 1973), and thus are expected to exert significant effects on the overall flux of D-glucose units into glycogen. Significant positive correlations between the activity of PGM with either HK or GS could, therefore, seriously weaken effects previously attributable to genotype-dependent enzyme activity variation at the Pgm-2 locus on synthetic rates. An association between PGM and glycogen synthetase activity is of particular importance because of the strong relationship demonstrated between GS activity and rates of glycogen synthesis in mammalian liver (Stalmans 1976), and between the I form activity and mantle glycogen levels in M. edulis (Gabbott, Cook and Whittle 1979; Gabbott and Whittle 1986a, 1986b).

In comparing the correlation matrices obtained for the two intertidal samples shown in Table XVI, it is notable that the only observed differences involved the activity relationships of PGM with both HK and GS. Hexokinase exhibited a significant positive association with PGM in the low but not in the high

intertidal sample, and the correlation coefficients between PGM and GS reversed in sign between the two tidal positions. Despite these differences, the mantle glycogen concentrations of the four Pgm-2 genotypic groups exhibited identical patterns at both intertidal locations. Therefore, any influence of the Pgm-2 locus on glycogen metabolism in this fall sample has not appeared to occur through correlations of its activity with either the HK or GS reaction steps.

In a study of the activity relationships between 23 enzymes in 48 second and third chromosome isogenic substitution lines of Drosophila melanogaster, Wilton et al. (1982) observed a pattern of significant positive correlations between sequential enzymic reactions, particularly at the glucose-6-phosphate branch point. The significant relationships observed in this study between the activities of PGM and UDPGP are consistent with this general trend. In D. melanogaster however, highly significant correlations were observed between the activities of HK and PGM (0.58 in second chromosome lines, and 0.55 in third chromosome lines). In the Pacific oyster, hexokinase activity exhibited much lower correlations with PGM, yet was strongly associated with the activity of UDP-glucose pyrophosphorylase. This discrepancy could perhaps be explained by the marked differences in carbohydrate metabolism between these groups of organisms. In the mantle tissue of Mytilus edulis, hexokinase exhibits significant changes in specific activity and kinetic properties on a seasonal basis that coincides with the utilization of

endogenous versus exogenous supplies of glucose at different times of the year (Livingstone and Clarke 1983). Although PGM activity may have been expected to show a strong relationship with hexokinase in this seasonal sample (due to the active synthesis of glycogen from external glucose supplies), their activities could at times be effectively uncoupled via the seasonal induction of distinct anabolic or catabolic pathways (cf. Livingstone 1981).

The apparent impact of the Pgm-2 locus on glycogen synthesis is not complicated by presence of genetic variation at adjacent enzymic steps. Both presumed UDP-glucose pyrophosphorylase loci were monomorphic, and no allozymic variation was observed in the subsample of individuals yielding good resolution of hexokinase activity. Glycogen synthetase activity could not be examined electrophoretically, so the possibility of confounding effects due to genetic variants of this enzyme cannot be discounted.

The results presented in Tables XVIII and XIX demonstrate that enzyme activity variation at the Pgm-2 locus did not occur independently of that observed for the other glycogen synthesis pathway enzymes. Significant differences were detected between Pgm-2 genotypic classes in their HK and UDPGP activities in the high intertidal sample, and between their GS activities at both intertidal heights. An important finding was that these differences were not caused by the unusual behavior of Pgm-2-

92/100, 96/100, and 100/104 heterozygotes. In both intertidal samples, heterozygotes for the Pgm-2-100 allele expressed overdominance for their activities of PGM, but not in their levels of HK, UDPGP or GS. Therefore, the higher glycogen concentrations displayed by these heterozygotes was not an indirect consequence of the coordinated elevation of the entire synthetic pathway.

These non-random associations do not appear capable of explaining the differences in mantle glycogen concentrations observed between Pgm-2 genotypes. Homozygotes for the Pgm-2-92, 96 and 104 alleles had greater HK, UDPGP, and GS activities than Pgm-2-100/100 homozygotes at both tidal heights. Excluding their PGM activities, the Pgm-2-92/100, 96/100, and 100/104 heterozygotes approximated the Pgm-2-100/100 homozygotes in the high intertidal region, but shifted to resemble the alternate class of homozygotes in the low intertidal zone. Despite exhibiting HK, UDPGP, and GS activities in the low intertidal area that were similar to the heterozygotes possessing the Pgm-2-100 allele, the Pgm-2-92/92, 96/96, and 104/104 homozygotes displayed lower quantities of mantle glycogen than these overdominant heterozygotes. In the high intertidal zone, these same homozygotes had considerably larger activities of all pathway enzymes (with exception of PGM) than the heterozygotes for the Pgm-2-100 allele, yet still possessed significantly lower mantle glycogen levels. These results indicate that the different activity levels of HK, UDPGP, and GS expressed between

the Pgm-2 genotypic groups are poor predictors of their differing mantle glycogen concentrations.

In Chapter 3 it was hypothesized that a tightly-linked "regulatory" locus, in complete disequilibrium with the Pgm-2 structural locus, could be responsible for producing the different enzyme activities of Pgm-2 genotypes. It was suggested that this regulatory element was segregating for two alleles, one of which was associated with the Pgm-2-100 allele, the other shared by the Pgm-2-92, 96, and 104 alleles. The different activity levels of the glycogen synthesis pathway enzymes shown by the two homozygote groups appear to support this explanation, but also require that this regulatory element exert pleiotropic effects on these other pathway enzymes. These observations provide further evidence against a Pgm-2 null allele being responsible for the overdominant PGM activities of heterozygotes possessing the Pgm-2-100 allele. It seems highly improbable that an undetected Pgm-2 null allele would have any impact on the pathway activity structure other than depressing the PGM activities of the two homozygous groups.

Modifier loci having coordinate effects on different enzymes have been observed in Mus musculus (Womack, Yan and Potier 1980) and D. melanogaster (Bentley and Williamson 1979; Belote and Lucchesi 1980). If this putative regulatory element is responsible for these results in C. gigas, it displays three extremely unusual properties. First, in heterozygous condition

it gives rise to overdominant PGM activity levels. Second, the allele segregating with the three less frequent structural alleles produces greater HK, UDPGP, and GS activities when homozygous than the variant linked to the Pgm-2-100 allele. Third, it appears to cause reversals in the dominance relationships of regulatory genotypes between the two intertidal positions such that heterozygotes resemble the Pgm-2-100/100 homozygote in the high intertidal, but the Pgm-2-92/92, 96/96, and 104/104 homozygotes in the low intertidal. The results presented in Chapter 3 have shown that the first property is consistently observed in the mantle and adductor muscle tissues at both intertidal positions in three different seasons. The second and third effects suggested by the data presented in this section require further study to determine their repeatability in different seasons and tissues.

A specific prediction of the hypothesis of an overdominant regulatory locus is the equivalence of homozygotes and heterozygotes formed between the three less frequent Pgm-2 structural alleles, since both would be homozygous for the same regulatory element. Homozygotes and heterozygotes lacking the Pgm-2-100 allele possessed very similar HK activities in the high intertidal region, and GS activities in the low intertidal area. However, the UDPGP activities of the Pgm-2-92/96, 92/104, and 96/104 heterozygotes were considerably lower than measured in the homozygotes for these alleles. Further discrepancies from the predicted patterns were observed between their low water HK

activities and high water GS activities. Therefore, apart from their PGM activities, homozygotes and heterozygotes for these less frequent Pgm-2 alleles did not exhibit the strong similarities expected by the regulatory model. One feature of the model supported by these results however, was the disparity of the two heterozygous groups. These two allelic classes expressed substantially different PGM activities in the low intertidal sample, altered levels of all four enzymes in the high intertidal sample, and dissimilar glycogen concentrations at both tidal heights.

An alternative explanation for the different enzyme activities of Pgm-2 genotypic classes could be that they arose as an indirect consequence of the amount of glycogen present in their mantle tissues. An inverse relationship is predicted between glycogen concentration and enzyme activity levels simply because of the limited storage capacities of mantle vesicular cells. Some evidence supporting this relationship was seen in the high intertidal zone, where heterozygotes for the Pgm-2-100 allele exhibited the highest glycogen levels and depressed HK, UDPGP, and GS activities. Conversely, the two genotypic groups lacking the Pgm-2-100 allele possessed lower glycogen concentrations and higher activities of these three enzymes. The Pgm-2-100/100 homozygotes strongly contradicted this pattern however, by expressing low activities throughout the entire pathway, but glycogen levels that were 50% lower than the overdominant heterozygotes. Virtually no evidence favoring this

explanation was observed in the low intertidal sample. Therefore, even if glycogen concentrations had some effect on the pathway enzyme activities, some other factor(s) must also be involved.

The multiple regression analyses served to confirm what was already evident from Tables XVIII and XIX. Of the four pathway enzymes, only PGM explained a significant amount of the observed variation in mantle glycogen levels. Similar results were observed for both intertidal samples, but the t-ratio of PGM was more highly significant in the high intertidal area. The complete absence of effects by either hexokinase or glycogen synthetase in these analyses is rather surprising. The catalytic and regulatory properties of hexokinase have identified this enzyme as a major regulatory site in carbohydrate metabolism (Newsholme and Start 1973). Recently, Torres et al. (1986) estimated the glycolytic control coefficient of hexokinase in rat liver preparations to be 0.77, suggesting that this reaction may also exert a dominant role in supplying glucose-6-phosphate for the synthesis of glycogen. The non-significant effects of hexokinase activity variation on mantle glycogen levels in C. gigas could perhaps be a consequence of 1) the reaction operating below its catalytic potential because of the low haemolymph glucose concentrations in marine bivalves (e.g. Zaba 1981; Livingstone and Clarke 1983), 2) its control of flux being overridden by the glycogen synthetase reaction, or 3) that a large proportion of glycogen was synthesized in the fall from

gluconeogenic precursors.

The activity of glycogen synthetase in mammalian liver has been found to directly parallel glycogen synthesis rates (Hue and Hers 1974, Stalmans 1976). Evidence is accumulating that this enzyme exerts a similar degree of control in molluscan mantle tissue (see Figure 13 in Gabbott 1983). The significant effect of PGM activity variation on mantle glycogen, and the corresponding absence of any relationship with GS activity, may at first appear to contradict these previous studies. However, an important distinction must be made between a regulatory enzyme's dictation of overall pathway flux, and modulation of these flux rates by non-regulatory enzymes like PGM. Glycogen synthetase may well be largely responsible for controlling the net rate of glycogen synthesis. The higher glycogen concentrations measured in oysters from the low compared to the high intertidal area is most likely due to a combination of their greater "scope for growth" and higher GS, not PGM, activities. As shown in Chapter 4, PGM activity variation is expected to influence these flux rates through a partitioning effect at the glucose-6-phosphate branch point (cf. LaPorte, Walsh and Koshland 1984). In responding to flux rates determined by GS, the overdominant PGM activities of the Pgm-2-92/100, 96/100, and 100/104 heterozygotes should result in higher rates of synthesis than observed in the other genotypic classes. In a single population sample, the influence of glycogen synthetase would be obscured by effects of the Pgm-2 locus. However if Pgm-

2 genotype is ignored, a series of similar measurements taken over a period of active synthesis would demonstrate a strong correlation between GS activity and mantle glycogen levels, as reported in M. edulis by Gabbott, Cook and Whittle (1979), and Gabbott and Whittle (1986a).

The impact of the Pgm-2 locus on glycogen synthesis would be similar for different genotypes only if their activities of glycogen synthetase were equal. However in both intertidal samples, significant differences in these activities were detected between the four genotypic classes. In the low intertidal sample, the two heterozygous groups and the homozygotes lacking the Pgm-2-100 allele possessed on average 17% higher GS activities than the Pgm-2-100/100 homozygotes. The mean glycogen concentrations of these three genotypic classes were in turn 21% larger than measured in the Pgm-2-100/100 homozygotes. Although exhibiting similar levels of glycogen synthetase activity, substantial differences in glycogen levels were observed between these allelic classes that were directly correlated with their PGM activities. The overdominant class of heterozygotes had 11% higher PGM activities and 13% more glycogen than the Pgm-2-92/92, 96/96, and 104/104 homozygotes. Compared to the other heterozygote group, the overdominant heterozygotes expressed 31% more PGM activity and 43% higher glycogen levels. These patterns demonstrate that GS activity is a major factor in determining glycogen levels, but the realized flux rates appear strongly affected by Pgm-2 genotype-dependent

enzyme activity variation.

An interaction between PGM and GS activity in affecting mantle glycogen was also evident in the high intertidal sample, but was complicated by the unusual behavior of the Pgm-2-92/100, 96/100, and 100/104 heterozygotes. These genotypes exhibited the highest concentrations of glycogen and the lowest GS activities of the four allelic classes. This discrepancy brings to light an important assumption that significantly affects the conclusions of the multiple regression analyses, namely, that the activity relationships of the pathway enzymes have remained unchanged over the time period preceding the sampling date. Glycogen has been implicated as a negative regulator of its own synthesis by inhibiting glycogen synthetase phosphatase and thus lowering the proportion of the enzyme in the active (I) form (Danforth 1965; Watts and Malthus 1980). Therefore, it is possible that the GS levels in heterozygotes for the Pgm-2-100 allele were larger before the sample was collected but have been subsequently depressed through the inhibitory effect of their higher glycogen concentrations. Estimates of the total (I plus D) glycogen synthetase activity of Pgm-2 genotypic classes could have provided a test of this prediction, but these assays were not performed.

The expression of overdominant GS activities by the heterozygotes for the Pgm-2-100 allele in the high intertidal area prior to the sampling date cannot be discounted. However in

the high intertidal, the magnitude of the inhibition of GS activity by glycogen appears far too great to be rectified with its observed levels, since these were below those present at other tidal height. Another factor discounting the importance of this explanation is the fact that mantle glycogen concentrations can reach considerably higher levels than measured in this season (Chapter 4). Therefore, the extent of this potential inhibition by glycogen on GS activity in the fall is expected to have been negligible. The comparable HK and UDPGP activities of the heterozygotes possessing the Pgm-2-100 allele and the Pgm-2-100/100 homozygotes in the high intertidal sample suggest that the GS activities of these groups may have previously been at similar levels. The higher glycogen concentrations of the Pgm-2-92/100, 96/100, and 100/104 heterozygotes in the high intertidal sample could indicate that their pathway activity levels are more optimally suited to the synthesis of glycogen than expressed in the homozygotes and heterozygotes lacking the Pgm-2-100 allele.

In summary, the activity relationships between the glycogen synthesis pathway enzymes provides further evidence favoring an effect of the Pgm-2 locus on glycogen metabolism. There is some evidence suggesting additional effects of the putative regulatory element producing the overdominant activity levels of heterozygotes for the Pgm-2-100 allele on the activities of these other enzymes, but none that can account for the differing glycogen levels of these genotypic groups. These results

demonstrate a surprisingly strong effect of allozymic variation at the Pgm-2 locus on metabolic flux, consistent with that expected from a partitioning effect at the glucose-6-phosphate branch point discussed in Chapter 4.

CHAPTER 6

GENERAL DISCUSSION

Heterozygotes at five enzyme loci (including Pgm-2) have been shown by Fujio (1982) to exhibit greater adult body weights than homozygotes in 20 Japanese populations of Crassostrea gigas. Although similar relationships have not been demonstrated in the present study, there are historical reasons for believing that the same patterns would be present in British Columbia populations of this species. The colonization of British Columbia by C. gigas has not been accompanied by founder effects significantly reducing levels of genetic variability: heterozygosity estimates from both geographic regions are nearly identical (e.g. Buroker, Hershberger and Chew 1975, 1979a; Ozaki and Fujio 1985). Furthermore, allelic frequency distributions at these polymorphic enzyme loci have not diverged to any appreciable extent between the western and eastern Pacific. For example at the Pgm-2 locus, the frequency of the most common allele in the Nanoose Bay study population (0.595) was almost identical to its mean frequency in the 23 Japanese populations (0.596) studied by Ozaki and Fujio (1985). The close similarity of C. gigas populations between both Pacific coasts suggests that if a genetic mechanism(s) is responsible for these multiple-locus heterozygosity relationships, it should also exist in the B.C. population studied.

Fujio (1982) proposed that inbreeding was the most likely explanation for the greater sizes of heterozygotes at all five loci because a significant negative relationship was observed between the mean body weights of the population samples and the magnitude of their inbreeding coefficients. However, his data showed that the 20 populations could be equally partitioned into two distinct groups; ones yielding high F values, and others with inbreeding coefficients close to unity. In nearly all samples, heterozygous individuals were larger than homozygotes, irrespective of the extent of the apparent inbreeding. If the inbreeding hypothesis is correct, this pattern should have been observed only in the populations exhibiting high F values. The fact that it was present in all samples shows that inbreeding per se could not have been responsible for the larger body weights of heterozygous oysters.

My study has demonstrated that overdominance for enzyme activity is expressed by the three most common heterozygotes at the Pgm-2 locus in Crassostrea gigas. The magnitude of this overdominance was nearly identical in both the mantle and adductor muscle tissues, and was consistently observed at both intertidal locations sampled in three different seasons. Since allele frequencies at this locus show only limited inter-populational differentiation (confined to the three less frequent alleles), these same genotypes must have been responsible for the increased size of Pgm-2 heterozygotes reported in Fujio's study. These results are unique in providing

evidence favoring the overdominance explanation for one locus involved in a positive association between multiple-locus heterozygosity and growth rate. The enzyme activity levels expressed by different Pgm-2 homozygotes and heterozygotes were incompatible with the predicted effects of an undetected null allele, and the associative overdominance hypothesis, invoking unidentified tightly-linked loci segregating for deleterious recessives, may therefore be deemed unnecessary.

The underlying mechanism producing the overdominant PGM activities of heterozygotes for the Pgm-2-100 allele is unknown. Although entirely speculative, the overdominant polymorphic "regulatory" locus model discussed in Chapter 3 is intuitively appealing because it can simultaneously explain the enzyme activity levels of ten Pgm-2 genotypes, allelic frequency distributions in natural populations, and the maintenance of this genetic variation in a balanced state, by distilling the multi-allelic system into a two allele polymorphism exhibiting overdominance. However, associated with the overdominance observed at the Pgm-2 locus were pleiotropic effects on the soluble protein levels measured in both tissues and the mantle activities of the adjacent enzymes of the glycogen synthesis pathway. These results suggest that heterozygosity in the vicinity of loci scored in multiple-locus studies could exert a number of metabolic effects that may act to complicate the explanation(s) for the correlations observed.

Genotype-dependent enzyme activity variation at the Pgm-2 locus was shown in Chapter 4 to be directly associated with differing glycogen concentrations in the mantle, but not the adductor muscle tissue. Complex Pgm-2 genotype by environment interactions were observed in the analysis of the mantle glycogen data: the relative levels measured in homozygotes and heterozygotes reversed between the fall and winter, and over the two intertidal positions within the summer sample. In addition to demonstrating a physiological impact of this polymorphism, a comparison of the mantle glycogen concentrations of Pgm-2 genotypes allows an assessment of the relative importance of kinetic versus enzyme activity variation at this locus. For the specific activity data, heterozygotes for the Pgm-2-100 allele behaved as a homogeneous group in all population samples; the four homozygotes acted as another homogeneous specific activity group. Within both of these groups, genotypes possessing the Pgm -2-92 allozyme may have been expected to enjoy a catalytic advantage over the others due to the superior kinetic properties of this variant shown in Chapter 2. However, the glycogen concentrations of homozygotes and heterozygotes differed in a manner consistent only with their enzyme activity differences: genotypes having the Pgm-2-92 allele showed absolutely no tendency to outperform others as expected from their advantageous kinetic properties. These results, combined with low observed frequency of the Pgm-2-92 allele, argue that the allozymes themselves could be neutral markers of the genetic differences that give rise to the differences in specific

activity.

The adaptive significance of this overdominance would be realized only if it, in turn, affected the relative fitnesses of Pgm-2 genotypes. Through its effects on mantle glycogen levels, this polymorphism has the potential to influence two important fitness components: viability and fecundity. The differential abilities of Pgm-2 genotypes to synthesize glycogen reserves could directly affect viability, particularly under adverse environmental conditions when this carbohydrate is important as a fuel for supporting basal metabolism (de Zwaan 1983). This effect on viability should be more pronounced in immature oysters, which utilize a much higher proportion of their glycogen stores during the winter months (Holland and Hannant 1976). Since glycogen has been strongly implicated as the major energy source for gametogenesis (Gabbott 1983), heterozygotes for the Pgm-2-100 allele could also enjoy a net fecundity advantage. The allocation of energy between somatic growth and gamete production has been shown by Rodhouse (1978) to shift towards the latter as marine bivalves increase in age. The impact of this polymorphism on fecundity is thus expected to be more significant in older individuals. Interestingly, an effect of heterozygosity at the Pgm locus has been described for both fitness components; on viability to 2 and 3 years of age in Crassostrea virginica (Zouros et al. 1983), and on the fecundity of the largest size class in Mytilus edulis (Rodhouse et al. 1986).

In Crassostrea gigas, multiple-locus heterozygosity has been significantly correlated only with adult body weights. Since these results were obtained by pooling 20 populations of differing age structures, it cannot be determined if heterozygosity was associated with differential growth rates or viabilities. In other species of marine bivalves, multiple-locus heterozygosity has been correlated with a number of physiological parameters which suggest that heterozygous individuals are metabolically more efficient than homozygotes. This energetic advantage is presumably reflected in the negative relationships between heterozygosity and rates of oxygen consumption (Koehn and Shumway 1982; Garton, Koehn and Scott 1984; Diehl et al. 1985) and protein turnover (Hawkins, Bayne and Day 1986), lower rates of weight loss under nutritive stress (Rodhouse and Gaffney 1984), and the observation that in some instances growth rates are significantly correlated with heterozygosity only under more stressful environmental conditions (Green et al. 1983; Gentili and Beaumont 1988). Understanding the biochemical mechanism(s) responsible for the increased metabolic efficiency of enzyme heterozygotes may thus provide a foundation for the explanation of these relationships in bivalves and other species.

Recently, Koehn, Diehl and Scott (1988) examined the relative contribution of 15 loci to a positive correlation between multiple-locus heterozygosity and growth rate in a

single cohort of the coot clam, Mulinia lateralis. This study was unique in demonstrating a significant contribution by enzymes that functioned in glycolysis and protein catabolism, but not for those involved in maintaining cellular redox balance or the generation of reducing equivalents. To account for these results and to provide a general explanation for the effects of enzyme heterozygosity, these authors proposed that (p. 128):

> "... the cost of pathway maintenance (i.e., biosynthesis and degradation of constituent enzymes) is inversely related to the magnitude of catalytic variation among the sequence of reactions in a pathway."

This explanation predicts that more heterozygous biochemical pathways are maintained by lower expenditures of energy, thus resulting in a more efficient physiological phenotype. This metabolic advantage of allelic diversity is an extension of ideas first developed by East (1936), Lerner (1954) and Berger (1976).

The results from the present study may be interpreted in a manner consistent with the above premises, but differ in the expression of this overdominance. Koehn, Diehl and Scott (1988), acting on the well-founded basis of heterozygote intermediacy, suggested that the "advantage" of heterozygosis arises from the "averaging out" of the differing catalytic performances of the two homozygotes (i.e. marginal overdominance). The minor kinetic differentiation exhibited by the four most common Pgm-2 allozymes shown in Chapter 2 appears to preclude this mechanism

from operating at this locus. Instead, the greater specific activities of the Pgm-2-92/100, 96/100, and 100/104 heterozygotes has provided direct evidence for the expression of unconditional biochemical overdominance. Because this enzyme is a monomer, the larger enzyme activities of these heterozygotes are most easily envisaged as arising through increased levels of PGM enzyme. Steady-state enzyme concentrations are achieved through a balance between the opposing rates of synthesis and degradation (Goldberg and St. John 1976). If the overdominant enzyme activities result from increased synthetic rates, heterozygotes for the Pgm-2-100 allele would be expected to experience higher, rather than lower, energetic requirements. However, if synthetic rates are the same for all genotypes, but the heterozygous enzyme ensembles are degraded at lower rates, overdominant enzyme activity levels would result through the reduced expenditure of cellular energy reserves. The increased metabolic efficiency of these genotypes would in turn be reflected in their increased rates of growth and also in decreased rates of oxygen consumption.

These arguments suggest that the overdominance observed at the Pgm-2 locus may not be the cause of the increased "efficiency" of heterozygotes, but rather an effect of an altered feature of routine metabolism, such as protein turnover. The higher Vmax/Km ratios of the overdominant Pgm-2 heterozygotes could produce a more efficient phenotype only through 1) responding more effectively to the flux rates

dictated by regulatory enzymes like glycogen synthetase, or 2) simply catalyzing the interconversion of G1P and G6P more efficiently as suggested by the theoretical analysis of Watt (1986). The energetic advantage that would result from either mechanism is unknown, but probably much lower than reduced biosynthetic costs predicted by the alternative explanation.

If the metabolic advantage of heterozygosity at the Pgm-2 locus resulted solely from a decreased rate of protein turnover, no direct effect of this polymorphism is necessarily predicted on glycogen metabolism. Although the increased efficiency of these heterozygotes could indirectly result in higher mantle glycogen concentrations, this was not a consistent observation of Chapter 4. The strong relationship between PGM activity variation and glycogen levels in the fall argues for a major impact of this polymorphism on metabolic flux, as expected from the partitioning of substrate at the glucose-6-phosphate branch point. Therefore, a dual advantage might result from heterozygosity in the region of the Pgm-2 locus in Crassostrea gigas. First, an indirect effect from reduced rates of protein degradation (resulting in the expression of overdominant enzyme activities), and second, a direct impact on viability or fecundity through the effect of this variation in enzyme activity on glycogen metabolism.

The expression of overdominant enzyme activities by heterozygotes for the Pgm-2-100 allele is extremely unusual, and

thus unlikely to provide a general explanation for the associations between multiple-locus heterozygosity and fitness-related traits in marine bivalves. Heterozygosity at the PGI locus in Crassostrea virginica has been involved in positive correlations with both growth rate (Singh and Zouros 1978; Zouros, Singh and Miles 1980) and viability (Zouros et al. 1983). Martin (1979) examined the biochemical properties of these PGI allozymes and found significant differences between the three most common genotypes in their specific enzyme activities. However, heterozygotes exhibited strict intermediacy in their activity levels that were interpreted as being advantageous in the face of fluctuating environmental conditions. Given these genotypic relationships, heterozygosity at the PGI locus in C. virginica could fit into the general scheme proposed by Koehn, Diehl and Scott (1988).

A particularly well-studied polymorphism in M. edulis, at which heterozygosity has been implicated in relationships with growth rate (Koehn and Gaffney 1984), morphological variation (Mitton and Koehn 1985), viability (Diehl and Koehn 1985) and oxygen consumption (Diehl et al. 1985), involves the aminopeptidase-1 (Lap) locus. Genotypes possessing the Lap-94 allele exhibit the greatest enzyme activities in high salinity environments (Koehn and Immerman 1981) caused by the greater kcat of this allelic variant (Koehn and Siebenaller 1981). A physiological effect on osmoregulation of this genotype-dependent variation in activity has been documented (Hilbish,

Deaton and Koehn 1982) which can explain the adaptive significance of this polymorphism (reviewed by Koehn and Hilbish 1987). Hilbish and Koehn (1985) have demonstrated that dominance is expressed at this locus at both the biochemical and physiological levels: Lap genotypes form two homogeneous groups depending on the presence or absence of the 94 allele. This is a particularly important result, since the pooling of the Lap-94/94 genotype with the two low activity homozygotes in multiple-locus studies, would result in the apparent expression of overdominance by heterozygotes for the Lap-94 allele although the underlying mechanism is strict dominance. The prevalence of similar dominance relationships at other polymorphic loci involved in these correlations is unknown.

Foltz (1986a, 1986b) has recently detected null alleles segregating at the Lap locus in Crassostrea virginica. The resulting misclassification of null heterozygotes as homozygotes has the potential to simultaneously explain the heterozygote deficiencies and superior performance of heterozygotes for two functional alleles reported in earlier studies (Singh and Zouros 1978; Zouros, Singh and Miles 1980; Foltz et al. 1983). The results of the present study and the genotypic relationships at these three other loci strongly suggest that a single genetic mechanism may not be responsible for these correlations involving multiple-locus heterozygosity. Rather, a full explanation for these patterns may have to depend on separate locus-specific studies. Although delaying the ultimate goal of

explaining these stimulating observations, this approach will undoubtedly increase our understanding of the functional relevance of enzyme polymorphisms, the intricacies of the selective process, and the evolution of complex biochemical systems.

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