Temperature-dependent Kinetic Variation among Phosphoglucose Isomerase Allozymes from the Wing-polymorphic Water Strider, *Limnoporus canaliculatus*

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Phosphoglucose isomerase (PGI) allozymes were isolated from the wing-polymorphic water strider, *Limnoporus canaliculatus*, and were characterized biochemically with respect to temperature-dependent kinetic and thermostability properties. At higher temperatures, the allozymes exhibited significant differences in Michaelis constant ($K_m$) values for substrates of both the forward and reverse reaction directions. Results were consistent with expectations of adaptive kinetic differentiation based on the latitudinal variation of PGI allele frequencies. PGI genotypes also differed with regard to maximal velocity ($V_{max}$)/$K_m$ ratios at higher temperatures. These differences were due primarily, if not exclusively, to allozyme-dependent variation in $K_m$ values. The allozymes also exhibited dramatic differences in thermostability. However, no thermostability differences were observed when the substrate analogue 6-phosphogluconate was present in the incubation medium. The data from this study, together with data from *Mytilus edulis* and *Metridium senile* on temperature-dependent kinetic variation among PGI allozymes, form a consistent picture of natural selection influencing the clinal variation of alleles at this locus in these three phylogenetically distant organisms. More definitive support of this hypothesis, however, must await additional studies on the physiological effects of the allozymic variation as well as direct measurements of fitness differences among the enzyme genotypes.

**Introduction**

A major goal of population genetics during the past 20 years has been to identify the forces acting on allozyme variation (Lewontin 1974; Nei 1975; Ayala 1976; Nei and Koehn 1983). Research on this topic has essentially attempted to determine the relative importance of selection versus mutation-drift in controlling the frequencies of allozyme variants in natural populations. An adequate demonstration that a specific enzyme polymorphism is influenced by selection is a difficult task requiring information on the biochemical properties of allozymes, the physiological consequences of allozymic variation, and the mechanisms whereby the physiological variation is translated into fitness differences (Clarke 1975; Koehn 1978). Biochemical studies of the kinetic and physical properties of allozymes are a key component in these multilevel investigations (Koehn et al. 1983; Zera et al. 1985). Biologically meaningful differences must be

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1. Key words: phosphoglucose isomerase (E.C. 5.3.1.9), *Limnoporus canaliculatus*, allozymes, temperature, enzyme kinetics, thermostability.

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266
demonstrated among allozymes before selection can justifiably be invoked as influencing an enzyme polymorphism in natural populations.

Both the reliability and the biological relevance of any observed biochemical variations among allozymes are contingent on a number of factors, including the kinetic parameters chosen for study, the conditions under which these parameters are estimated, and the interpretations drawn from the primary enzymological data. Although a large number of biochemical characterizations of allozymes have been reported during the past 15 years, many of these studies have shortcomings in one or more of the aspects mentioned above (Koehn et al. 1983; Zera et al. 1985). Indeed, there is a paucity of reliable, biologically meaningful data on the physical and kinetic properties of allozymes. Obtaining such data remains one of the most important tasks of current allozyme research (Zera et al. 1985).

For a variety of reasons, phosphoglucone isomerase (PGI) polymorphisms are useful experimental systems for addressing questions of allozymic adaptation. First, there is considerable background information on the structure, kinetic properties, and metabolic roles of PGIs from a variety of organisms (for review, see Noltman 1972). Second, the enzyme is highly polymorphic in natural populations of many plant and animal species, and in many cases gene-frequency clines associated with latitude have been reported (Williams et al. 1973; Koehn et al. 1976; Corbin 1977; Powers and Place 1978; Hoffman 1981b). These data suggest that PGI polymorphisms appear to be influenced by natural selection and also allow specific a priori predictions to be made regarding adaptive differences among allozymes in temperature-dependent kinetic and physical properties. Last, in recent years, both biochemical and population-genetic data have been reported for PGI polymorphisms in several species (Watt 1977, 1983; Hoffman 1981a, 1981b, 1983; Hall 1983, 1985b). Thus, additional studies of PGI polymorphisms afford the opportunity to investigate the comparative biochemical population genetics of an enzyme locus to a degree not possible with allozymes at most other loci.

In an earlier study, PGI alleles in the wing-polymorphic water strider, *Limnoporus canaliculatus*, were found to exhibit a steep latitudinal cline (Zera 1984; also see below), thus making this a convenient experimental system for studying the thermal adaptation of PGI allozymes. In addition, Pgi allele frequencies differed substantially between winged and wingless morphs throughout the entire range of *L. canaliculatus* (Zera 1984). This suggested that PGI allozymes might be differentially selected in the two morphs (analogous to differential selection between the sexes), possibly as a consequence of kinetic differences related to flight biochemistry. Thus, the PGI polymorphism in *L. canaliculatus* was also viewed as a useful experimental system for investigating the functional adaptation of allozymes to different metabolic contexts.

The goal of the present study was to estimate various steady-state kinetic and thermostability parameters of PGI allozymes under physiologically realistic conditions and under a range of biologically relevant temperatures. Correlation between temperature-dependent variation in enzymatic characteristics among the allozymes and latitudinal allele frequency variation would be the first step in implicating selection as a factor affecting the PGI polymorphism in *L. canaliculatus*. Comparison of these data to biochemical data on other clinally varying PGI allozymes would potentially identify general patterns of allozymic adaptation at this locus. Results of a companion study of the differential inhibition of PGI allozymes by pentose-shunt metabolites and its role in the association between wing polymorphism and PGI polymorphism will be reported elsewhere (A. J. Zera, accepted).
Material and Methods
Background on the PGI Polymorphism

The PGI polymorphism in *Limnoporus canaliculatus* consists of two common and several rare (frequency < .01) allozymes. The two common PGI allozymes are encoded by codominant alleles at a single locus in linkage group 1 (Zera 1984, and accepted). PGI allele frequencies exhibit an interesting pattern of latitudinal variation (figs. 1, 2A). The $Pgi^F$ allele increases in frequency from a low of ~0.3 in central Maine, the northernmost population sampled, to a high of ~0.8 in southern Georgia-northern Florida. South of this point, the cline reverses and the frequency of $Pgi^F$ declines linearly to 0.3 in southern Florida. The reversal of the $Pgi$ cline occurs at 30° north latitude, precisely at the north-Florida suture zone (Remington 1968). This is

![Fig. 1.—Location of populations of *Limnoporus canaliculatus* sampled in the present study.](image-url)
Kinetics of PGI Allozymes from *L. canaliculatus* 269

North Florida Suture Zone (Remington, 1968)

![Graphs showing latitudinal variation of allele frequencies](image)

**FIG. 2.—** Latitudinal variation of (A) *Pgi*<sup>F</sup> allele frequencies, (B) *Idh*<sup>B</sup> allele frequencies, and (C) *Pgi* allele-X wing-morph associations in fall samples of *Limnoporus canaliculatus*. Data points represent population samples (*N* = 50–400 individuals per sample) taken in 1979 or 1981 (∙) or pooled samples from both years (○). □ = The weighted mean *Pgi*<sup>F</sup> allele frequency for samples in which *Pgi*<sup>F</sup> differed significantly between long-winged and wingless morphs. * = A significant difference (*P* < 0.05 and usually <0.005) in *Pgi*<sup>F</sup> allele frequency between long-winged and wingless morphs within individual population samples as determined by χ<sup>2</sup> contingency analyses. Lines represent results of regression analyses of untransformed allele frequency on latitude and are significant (*P* < 0.001) in each case, except for *Idh*<sup>B</sup> above 30° north latitude. Regressions of arcsine-transformed allele frequencies were also significant (*P* < 0.001) in each case. Because of the sharp discontinuity of allele frequencies at 30° north latitude, regression analyses were performed separately for samples north and south of this point. See text for additional details.

a narrow belt of hybridization of many plant and animal species, one presumably due to the contact of previously isolated peninsular Floridian populations or species and their continental near relatives. Besides the sharp reversal of the *Pgi* allele-frequency cline, two other characters in *L. canaliculatus* also change abruptly in this area: latitudinal variation of allele frequencies at the unlinked (Zera 1984) *Idh* locus (fig. 2B) and *Pgi* allele-X wing-morph associations (fig. 2C). The abrupt change in each of these
three characters indicates that populations of \textit{L. canaliculatus} north of 30° north latitude are strongly differentiated from populations south of this point, at least at some loci.

A sufficient study of the biochemical mechanisms underlying the entire pattern of latitudinal variation of the \textit{Pgi} locus in \textit{L. canaliculatus} requires isolation and characterization of PGI allozymes from both Floridian and continental populations. This is necessary because one or more of the PGI allozymes in Floridian populations, while electrophoretically indistinguishable from its counterpart in continental populations, could nevertheless be functionally differentiated (i.e., a cryptic variant), resulting in the reversal of the PGI allele-frequency cline. The present study is more limited in scope and focuses exclusively on the portion of the PGI cline north of the north-Florida suture zone, where the frequency of \textit{PgiF} is negatively correlated with latitude. Thus, in the present study, PGI-FF is considered the hypothetically warm-adapted enzyme while PGI-SS is considered the hypothetically cold-adapted enzyme.

Chemicals

Proteins, enzymes, substrates, coenzymes, and buffers used in this study were obtained from Sigma Chemical Company, with the exception of fructose-6-phosphate (F-6-P), which was purchased from Calbiochem-Behring (lot 1183258). F-6-P from Sigma contained an unknown substance that strongly and differentially inhibited PGI allozymes from \textit{L. canaliculatus} in a manner similar to that recently reported for PGI allozymes from \textit{E. coli} (Dykhuizen and Hartl 1983). All other organic and inorganic chemicals were reagent grade and were obtained from Fisher Chemical Company.

Animals

\textit{Limnoporus canaliculatus} collected from the Nissequogue River State Park, Long Island, New York, were used as the source of PGI allozymes. Stocks of the three \textit{Pgi} genotypes—\textit{PgiFF}, \textit{PgiFs}, and \textit{Pgis}—were constructed, and individuals of known \textit{Pgi} genotype were stored frozen at −60°C until homogenization for enzyme purification. Laboratory rearing conditions were as previously described (Zera 1981).

Enzyme Preparation

PGI allozyme preparations used in this study were purified by a combination of ion exchange, gel sieving, and hydrophobic interaction chromatography, the details of which are reported elsewhere (Zera 1984, and accepted). Although the preparations were highly purified (>800 fold), and PGI was the major protein present, the enzymes could not be purified to homogeneity. This was mainly due to the limited amount of tissue available for enzyme extraction and to the instability of the enzyme during the final ultrafiltration step.

Enzyme Assay

Initial reaction velocities were measured by following the change in absorbance at 340 nm using a Gilford model 2400-2 UV/VIS recording spectrophotometer equipped with a ThermoSet temperature-control device (±0.4 degrees C). The spectrophotometer was interfaced with a Motorola M6800 microcomputer that recorded the optical density readings and computed initial rates.

All assays were done using Na-MOPS buffer at 0.1 ionic strength (I). Initial rates were determined in the gluconeogenic direction (F-6-P → G-6-P) using an assay mixture containing Na-MOPS buffer at various pHs, 8 mM MgCl₂, 1 mM NADP⁺, various
F-6-P concentrations, and 0.5–2.0 units of glucose-6-phosphate dehydrogenase (G-6-PDH), depending on the temperature, in a total volume of 0.5 ml. Initial rates were determined in the glycolytic direction (G-6-P → F-6-P) using a modified version of the multiply coupled enzyme assay of Tilly et al. (1974). The assay mixture consisted of Na-MOPS buffer, 8 mM MgCl₂, 1 mM NH₄Cl, 0.15 mM NADH, 0.2 mM ATP, 0.1% β-mercaptoethanol, various G-6-P concentrations, and enough of the coupling enzymes (phosphofructokinase [PFK], α-glycerophosphate dehydrogenase [α-GPDH], triosephosphate isomerase [TPI], and aldolase [ALD]) to ensure that the coupling enzymes were not rate limiting (e.g., 1 unit of PFK, 5 units of TPI, and 0.5 units each of α-GPDH and ALD when rates were measured at 20 C). The standard assay used in the routine measurement of PGI activity (e.g., during thermostability studies, specific activity determinations, etc.) consisted of 0.1 l Na-MOPS, pH 7.1, at 20 C, 1 mM F-6-P, 1 unit of G-6-P, 8 mM MgCl₂, and 1 mM NADP⁺.

Assays in both directions were initiated by the addition of 10 µl or 20 µl of suitably diluted PGI stock solution to the assay cocktail, which had been preincubated at the assay temperature for a few minutes. In all cases <5% of the substrate was consumed during initial rate measurements in order to operate within the restrictions of steady-state kinetics (Fromm 1975). Initial G-6-P + F-6-P and F-6-P → G-6-P rates near maximal velocity (Vₘₐₓ) were estimated by unweighted linear least-squares analysis of velocity measurements. Since the Michaelis constant (Kₘ) for F-6-P was low (~40 µM), it was not possible to both accurately estimate initial velocities using a linear least-squares method and adhere to the restrictions of steady-state kinetics (consumption of <5% of the substrate) when the concentration of F-6-P was near or lower than the Kₘ. Consequently, the progress-curve analysis of Waley (1981; also see Hall and Koehn 1983) was used to measure initial F-6-P → G-6-P rates under subsaturating conditions. Stock solutions of substrates and cofactors were prepared according to the method of Lowry and Passonneau (1972) and were stored frozen at -70 C until use. Prior to kinetic studies all substrate concentrations were checked spectrophotometrically.

Kinetic Parameters Estimated

Kₘ and Vₘₐₓ values were estimated for the PGI allozymes at four temperatures—10, 20, 30, and 35 C—that bracketed the temperature range likely to be encountered by *Limnoporus canaliculatus* in natural populations. *Limnoporus canaliculatus* has a wide seasonal range and can be found continuously on ponds in New England from April until October (Zera 1980). In the spring and fall *Limnoporus canaliculatus* are commonly observed on the water surface at air temperatures >10 C—but uncommonly below this temperature (A. J. Zera, unpublished observations). Thirty-five degrees centigrade was considered to be within the upper limit in temperature experienced by populations in midsummer, especially in southern populations. The Vₘₐₓ and Kₘ data were subsequently used to estimate Vₘₐₓ/Kₘ ratios (see below).

Temperature-dependent kinetic studies were done using a variable pH protocol; that is, pH of the assay buffer was adjusted to vary at different temperatures in accordance with the pKₐ of imidazole. This procedure mimics the normal intracellular variation of pH with temperature and thus represents a more physiologically realistic experimental protocol than assaying enzyme activity at a constant pH at different temperatures (Somero 1978, 1981).
Experimental Design of Kinetic Studies and Data Analysis

Because the procedures used in the collection and analysis of kinetic data in this study are not widely known, they will be briefly outlined (see Hall and Koehn 1983 for a more detailed discussion and references). The experimental design used here was based on the design recently advocated by Duggleby (1979) and Endrenyi and Chan (1981). This method consists of measuring a large number of rates (7–12 in this study) at each of several substrate concentrations. The number of substrate concentrations at which the rates are measured is equal to the number of kinetic parameters to be estimated (e.g., estimates of $K_m$ and $V_{max}$ require rates to be measured at two substrate concentrations). The optimal experimental design for $K_m$ and $V_{max}$ estimates consists of measuring rates at (1) a high substrate concentration, as close to $V_{max}$ as possible without encountering substrate inhibition, and (2) a low substrate concentration, as close to the $K_m$ as possible.

Although this method results in a more accurate estimate of the kinetic parameters than the traditional method of measuring a smaller number of rates at each of five to seven substrate concentrations, it cannot be used to validate or reject a particular kinetic model (e.g., to determine whether an enzyme obeys Michaelis-Menten hyperbolic saturation kinetics). Since the statistical analysis of data obtained using this method required prior knowledge of the kinetic model of the reaction, preliminary kinetic studies were done to verify that PGI from L. canaliculatus exhibits hyperbolic saturation kinetics. It should be noted that PGI isolated from a variety of sources invariably exhibits simple Michaelis-Menten kinetics (see references in Noltman 1972). In preliminary studies of PGI from L. canaliculatus, initial rates were measured in the traditional manner in both directions at seven different substrate concentrations, ranging from 0.5 to ~10 times the $K_m$. Double reciprocal plots were constructed, and the lines were examined visually for deviations from linearity.

Enzyme kinetic data were analyzed using the biweight regression technique of Cornish-Bowden and Endrenyi (1981). This is a robust regression technique that requires minimal assumptions about the error structure of the data and mitigates the effects of outliers. The data were analyzed by a computer program written by A. Cornish-Bowden (personal communication) that estimates the various kinetic parameters, their SEs, and df's. As a consequence of the weighting procedure utilized in this method, sample sizes and their resulting df's are nonintegers (e.g., see table 1).

Statistical testing of kinetic parameters was done using analysis of variance (ANOVA) or (for samples containing heterogeneous variances) approximate tests of the equality of means with heterogeneous variances (Sokal and Rohlf 1981). These tests were done after reconstituting the variances from the output data. ANOVA of the kinetic parameters for the three genotypes at a particular temperature was followed by an a priori comparison of the means of the two homozygotes.

Protein Determination

Protein concentrations were determined using the Bio-Rad microassay procedure, which is based on the Coomassie blue G-250 dye-binding assay of Bradford (1976). Bovine serum albumin (BSA) was used as the standard.

Specific Activities

Specific activities were determined for each of the PGI genotypes by means of the standard enzyme assay. Specific activities were measured in each sex and morph
Kinetics of PGI Allozymes from *L. canaliculatus*

Table 1

<table>
<thead>
<tr>
<th>ENZYME AND TEMPERATURE</th>
<th>K_m (µM) OF ALLOZYME</th>
<th>RESULTS OF SIGNIFICANCE TESTS *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>FS</td>
</tr>
<tr>
<td></td>
<td>(°C)</td>
<td></td>
</tr>
<tr>
<td>G-6-P:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>246 (4), 11.8</td>
<td>274 (19), 11.3</td>
</tr>
<tr>
<td>30</td>
<td>212 (10), 14.1</td>
<td>220 (6), 11.9</td>
</tr>
<tr>
<td>20</td>
<td>136 (4), 12.3</td>
<td>138 (6), 14.8</td>
</tr>
<tr>
<td>10</td>
<td>139 (5), 19.7</td>
<td>ND</td>
</tr>
<tr>
<td>F-6-P:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>81.2 (4.5), 11.7</td>
<td>75.9 (3.1), 15.8</td>
</tr>
<tr>
<td>30</td>
<td>63.2 (2.6), 15.8</td>
<td>69.2 (3.3), 19.2</td>
</tr>
<tr>
<td>20</td>
<td>38.3 (1.2), 14.0</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>40.3 (1.3), 22.4</td>
<td>41.6 (1.8), 14.4</td>
</tr>
</tbody>
</table>

Note.—NS = Not significant; ND = no data.

*See Material and Methods for details of statistical tests of differences among K_m values.

Fractional values are the consequence of the biweighting procedure (see Material and Methods).

Type (long winged and wingless) to eliminate potential confounding influences of these variables on variation in activity among *Pgi* genotypes.

Thermal Denaturation Study

The thermal denaturation experiment consisted of incubating 200 µl of each allozyme solution at various temperatures for 5 min in a parafilm-sealed test tube. After the incubation period, the tubes were placed in an ice bath and were assayed for PGI activity, in triplicate, using the standard assay. Percent loss in enzyme activity was determined by comparing the PGI activity of solutions incubated at various temperatures with that of a control solution kept on ice. There was no detectable loss in activity of any of the PGI allozymes over several days when diluted in the buffer mentioned above and kept on ice. Prior to these studies, appropriately diluted allozyme solutions were dialyzed against two changes of 200 x vol NaMOPS, 0.1 I, pH 7.1 at 20 °C, containing 0.1% β-mercaptoethanol. This was done to remove F-6-P and G-6-P from the allozyme stock solutions and to thus eliminate possible spurious thermostability differences due to differential stabilization of allozymes by the substrates in the incubation solutions. The G-6-P and F-6-P in the allozyme stock solutions were due to the substrate-DEAE ion-exchange chromatographic step, the last step in the purification of the allozymes. All allozyme solutions were dialyzed in the same vessel. Postdialysis enzyme solutions were diluted 1:1 with dialysis buffer containing 1 mg BSA/ml.

The thermostability of PGI allozymes was also monitored in the presence of the substrate analogue and competitive inhibitor, 6-phosphogluconate (6-PG). Assays with inhibitor were done at a concentration of 436 µM 6-PG. The *K_1* for 6-PG is ~30 µM at 30 °C (Zera 1984, and accepted).

Computation and Comparison of Relative V_max/K_m Ratios

Differences among the allozymes' V_max values observed in this study were the consequence of both biologically relevant factors (e.g., variation in k_cat and/or enzyme
concentration) and biologically irrelevant factors (e.g., differential loss of enzyme activity during purification, differences in yields of the various allozyme purifications, etc.). The following method was used to standardize $V_{\text{max}}$ values among the allozymes to eliminate the biologically irrelevant factors in the variation of $V_{\text{max}}$ among the allozymes. This would then allow biologically meaningful comparisons of $V_{\text{max}}/K_m$ ratios to be made among the allozymes. $V_{\text{max}}/K_m$ ratios were not computed for PGI-FS since, at several temperatures, kinetic constants were not obtained for this enzyme.

Field-collected animals were assayed for genotype-dependent PGI activity by means of the standard assay (at substrate concentrations $>20 \times K_m$) in order to measure velocities essentially at $V_{\text{max}}$ (velocity $> 0.95 V_{\text{max}}$ at this substrate concentration). No differences in $V_{\text{max}}$ were observed among the $Pgi$ genotypes (table 2). Consequently, the $V_{\text{max}}$ values for the genotypes obtained in the kinetic studies could be standardized to a common value at the temperature at which the field-collected animals had been acclimated. However, the precise temperature to which the field-collected gerrids had been acclimated was not known. Consequently, two different sets of relative $V_{\text{max}}/K_m$ values were computed, one in which the $V_{\text{max}}$ values were standardized at 10°C and another in which the $V_{\text{max}}$ values were standardized at 20°C. These were considered reasonable values with which to bracket the range in temperatures at which the fall (September 26, 1983)-collected $L. canaliculatus$ would have been acclimated. Standardization at either value gave virtually identical results. It should be noted that these estimates of $V_{\text{max}}/K_m$ do not give SEs, thus precluding statistical tests of differences among the allozymes.

Results

Michaelis-Menten Kinetics of PGI

Traditional Lineweaver-Burke plots of the $1/v$ versus $1/s$ data for both the forward and reverse reactions were constructed (figs. 3, 4) in order to obtain background in-

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Mean Specific Activities for PGI Genotypes in Winged and Wingless, Male and Female, <em>Limnoporus canaliculatus</em></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SPECIFIC ACTIVITY (SE) OF PGI GENOTYPE</td>
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<tr>
<td></td>
<td>(µmol/min/mg protein $\times 10^4$)</td>
</tr>
<tr>
<td>MORPH AND SEX</td>
<td>FF</td>
</tr>
<tr>
<td>Winged:</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2,173 (149)</td>
</tr>
<tr>
<td></td>
<td>$N=6$</td>
</tr>
<tr>
<td>Female</td>
<td>2,080 (156)</td>
</tr>
<tr>
<td></td>
<td>$N=5$</td>
</tr>
<tr>
<td>Wingless:</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2,325 (120)</td>
</tr>
<tr>
<td></td>
<td>$N=7$</td>
</tr>
<tr>
<td>Female</td>
<td>2,060 (0)</td>
</tr>
<tr>
<td></td>
<td>$N=1$</td>
</tr>
</tbody>
</table>

NOTE.—Results of the three-way ANOVA of specific activity data: variation among morphs, $F(1, 101) = 3.99$, $P < 0.05$; variation among PGI genotypes, sexes, and all two- and three-way interactions were nonsignificant.
FIG. 3.—Lineweaver-Burke double-reciprocal plot of PGI-SS from *Limnoporus canaliculatus*. The substrate was G-6-P (see Material and Methods); ♦ = Two superimposed points.

*V_0* × 10⁻¹ (μmole/min/ml⁻¹)

```
-30
-20
-10
0
10
20
30
```

```
2 4 6 8 10 12 14 16 18 20
```

\( [G-6-P]^{-1} \) (mM)⁻¹

FIG. 4.—Lineweaver-Burke double-reciprocal plot of PGI-SS from *Limnoporus canaliculatus*. The substrate was F-6-P (see Material and Methods); ♦ = Two superimposed points.

*V_0* × 10⁻¹ (μmole/min/ml⁻¹)

```
1.0
2.0
3.0
4.0
```

```
10 20 30 40 50
```

\( [F-6-P]^{-1} \) (mM)⁻¹

Fig. 3. Lineweaver-Burke double-reciprocal plot of PGI-SS from *Limnoporus canaliculatus*. The substrate was G-6-P (see Material and Methods); ♦ = Two superimposed points.

Fig. 4. Lineweaver-Burke double-reciprocal plot of PGI-SS from *Limnoporus canaliculatus*. The substrate was F-6-P (see Material and Methods); ♦ = Two superimposed points.
formation on the reaction mechanism of PGI from *Limnopus canaliculatus*. This is a prerequisite for using the kinetic-parameter estimation procedure of Duggleby (1979) and the computer program of Cornish-Bowden. As can be seen, the 1/v versus 1/s data fit well a straight line for both reaction directions. These data thus provide no evidence that PGI from *L. canaliculatus* differs from other PGIs that invariably exhibit simple hyperbolic saturation kinetics (Noltman 1972). All subsequent kinetic analyses were done under the assumption that PGI from *L. canaliculatus* exhibits simple hyperbolic saturation kinetics.

**Kₘ Values**

The **Kₘ** values when G-6-P was used as the substrate ranged from a low of ~130 μM at 10 C to a high of 250–275 μM at 35 C. The **Kₘ** values for G-6-P exhibited by the PGI allozymes were virtually identical at 10 and 20 C but increasingly diverged at 30 and 35 C (table 1). The **Kₘ** values of the PGI-FS enzyme were not intermediate between the **Kₘ** values of PGI-FF and PGI-SS but was slightly higher than that of PGI-SS at all temperatures. The **Kₘ** values for F-6-P ranged from a low of 40 μM at 10 C to a high of 80 μM at 35 C. Similar allozymic differences were observed for the F-6-P **Kₘ** values as were observed for the G-6-P **Kₘ** values (table 1). Except at 20 C, the PGI-FS enzyme exhibited a lower **Kₘ** than did PGI-SS. As did the PGI-FS enzyme, the **Kₘ** values of the PGI-FS enzyme were not intermediate between the **Kₘ** values of PGI-FF and PGI-SS but was slightly higher than that of PGI-SS at all temperatures.

**Maximal Velocities**

Maximal velocities for the forward and reverse directions exhibited by the three PGI allozymes at 10–35 C are given in table 3. Arrhenius plots of these data (fig. 5) indicate similar temperature-**Vₘₐₓ** profiles for the allozymes in both G-6-P → F-6-P and F-6-P → G-6-P.

**Reliability of the Kinetic Data**

An important advantage in studying the steady-state kinetics of a reversible reaction is that, if **Vₘₐₓ** and **Kₘ** values can be measured in both directions, the reliability of these kinetic parameters can be ascertained by substitution of the **Vₘₐₓ** and **Kₘ** values into the Haldane relation:

\[
K_{eq} = \frac{V_{max}(f) \cdot K_m(r)}{V_{max}(r) \cdot K_m(f)}.
\]

This yields a value for the equilibrium constant (**Kₘ**<sub>eq</sub>) of the reaction that can then be compared to an independently determined value for **Kₘ**<sub>eq</sub>. Substitution of the experimentally determined **Vₘₐₓ** and **Kₘ** values for the forward and reverse directions obtained in this study into the Haldane relation (eq. [1]) yielded **Kₘ**<sub>eq</sub> values that closely approximated independently estimated **Kₘ**<sub>eq</sub> values (table 4). In all cases except
Table 3
V\textsubscript{max} Values for G-6-P → F-6-P and F-6-P → G-6-P Reactions as Exhibited by PGI Allozymes from \textit{Limnoporus canaliculatus}

<table>
<thead>
<tr>
<th>REACTION AND TEMPERATURE (°C)</th>
<th>V\textsubscript{max} (SE), \textsuperscript{a} df\textsuperscript{b} OF ALLOZYME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
</tr>
<tr>
<td></td>
<td>FS</td>
</tr>
<tr>
<td></td>
<td>FF</td>
</tr>
<tr>
<td><strong>G-6-P → F-6-P:</strong></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>1424 (7), 10.8</td>
</tr>
<tr>
<td>30</td>
<td>947 (11), 14.1</td>
</tr>
<tr>
<td>20</td>
<td>470 (4), 12.3</td>
</tr>
<tr>
<td>10</td>
<td>271 (4), 19.7</td>
</tr>
<tr>
<td><strong>F-6-P → G-6-P:</strong></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>1339 (26), 11.7</td>
</tr>
<tr>
<td>30</td>
<td>967 (12), 15.8</td>
</tr>
<tr>
<td>20</td>
<td>568 (4), 14.0</td>
</tr>
<tr>
<td>10</td>
<td>350 (3), 22.4</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Expressed as μmole/min/ml × 10\textsuperscript{2}. Values are lower for FS because rates were measured using a more dilute stock solution.

\textsuperscript{b} Fractional values are the consequence of the biweighting procedure (see Material and Methods).

PGI-FF at 35°C, \( K_{eq} \) determined from the Haldane relationship was within 10%—and often within 5%—of the experimentally determined \( K_{eq} \). The most deviant \( K_{eq} \) value from the Haldane relationship—that for the PGI-FF allozyme at 35°C—still exhibited a good fit to the expected \( K_{eq} \) (table 3). This indicates that the experimentally determined kinetic constants are internally consistent and appear to be reliable estimates of the true kinetic constants.

![Fig. 5](image-url)

\textbf{Fig. 5.}—Arrhenius plots of \( V_{max} \) for PGI-SS (○) and PGI-FF (●) determined in (A) F-6-P → G-6-P and (B) G-6-P → F-6-P reactions. To facilitate comparisons between the allozymes, \( V_{max} \) values were standardized to a common value at 20°C. \( K = \) Degrees Kelvin.
Table 4

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$K_{eq}$ Value of Allozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
</tr>
<tr>
<td>35</td>
<td>0.34, 0.32</td>
</tr>
<tr>
<td>30</td>
<td>0.29, 0.30</td>
</tr>
<tr>
<td>20</td>
<td>0.23, 0.25</td>
</tr>
<tr>
<td>10</td>
<td>0.22, 0.20</td>
</tr>
</tbody>
</table>

Note.—For each entry, the first number is the value as determined by substitution of estimated parameters into the Haldane eq. and the second number is the experimentally determined value. Experimentally determined $K_m$'s are from Hall (1983), obtained under assay conditions identical to those used in the present study. Ellipses indicate that parameters were not measured in either direction.

Specific Activities

Specific activities for the $Pgi$ genotypes from each sex and morph type are presented in table 2. In general, within each sex and morph, the $Pgi^{SS}$ genotype tended to exhibit a slightly higher specific activity than did $Pgi^{FF}$, with the heterozygote exhibiting intermediate values. However, these differences were not significant as determined by a three-way ANOVA (table 2). A three-way ANOVA of the raw activity data also showed nonsignificant differences, as did separate analyses of genotype-dependent activity in each sex and morph.

$V_{max}/K_m$ Ratios

In both $F-6-P \rightarrow G-6-P$ and $G-6-P \rightarrow F-6-P$, $V_{max}/K_m$ values were very similar for PGI-FF and PGI-SS at low (10–20 °C) temperatures but increasingly diverged at higher temperatures (table 5). Similar results were obtained whether $V_{max}$ values were standardized at 10 or 20 °C (see Material and Methods), and only the values obtained when $V_{max}$ values were standardized at the latter temperature are reported here. At higher temperatures, PGI-FF in all cases exhibited higher $V_{max}/K_m$ values than did PGI-SS.

Thermostability

Results of the thermostability studies are given in figure 6. PGI from $L. canaliculatus$ is fairly labile at temperatures $>35$ °C, with a total loss in activity after incubation for 5 min at 50 °C in the absence of 6-PG and $>85\%$ loss in activity when incubated at the same temperature in the presence of a saturating concentration of 6-PG. 6-PG substantially increased the resistance of PGI to thermal denaturation in the temperature range of 35–48 °C; for example, PGI from each of the three genotypes lost $>93\%$ activity when heated at 45 °C for 5 min without 6-PG but only lost 9%–12% activity when heated for the same period of time in the presence of 6-PG.

The PGI allozymes exhibited substantial differences in thermostability in the absence of 6-PG (fig. 6). PGI-SS and PGI-FS were virtually indistinguishable in stability characteristics (data for PGI-FS not shown), while the PGI-FF allozyme proved to be much more labile at higher temperatures; for example, during the 5-min incubation period at 40.0 °C, PGI-FF lost 32% more activity than did PGI-SS. The difference in
loss of activity between these two allozymes was even greater at 42.5°C. However, in the presence of 6-PG, there were no significant thermostability differences among the allozymes (fig. 6).

The thermal lability of PGI at 35°C in the absence of 6-PG could potentially present problems in the kinetic studies, especially for the more thermally labile PGI-FF enzyme. Consequently, at 35°C rates were measured at saturating and subsaturating F-6-P concentrations at 0-2 as well as at 2-4 min after the addition of the enzyme. This was done in order to determine the extent of any thermal denaturation under conditions employed during the kinetic studies. No differences were observed in the rates for either PGI-FF or PGI-SS, indicating that there was no thermal denaturation during the initial velocity measurements. The absence of thermal denaturation at 35°C in the presence of F-6-P suggests that this compound also stabilizes the enzyme at higher temperatures, an effect similar to that observed with 6-PG.

Discussion

PGI allozymes from *Limnoporus canaliculatus* differ in several important enzymatic characteristics in a manner consistent with expectations of adaptive kinetic differentiation. The $K_m$ values for F-6-P exhibit increasing divergence among the allozymes with increasing temperature (table 1). Moreover, allozyme-dependent variation in the $K_m$ values is inversely related to habitat temperature; that is, the $K_m$ values of the hypothetically warm-adapted PGI-FF enzyme are significantly lower than those of the PGI-SS enzyme at higher temperatures. An identical pattern of allozyme-dependent variation for the $K_m$ values for G-6-P was also observed (table 1).

The overall allozyme-dependent variation of $K_m$ with temperature observed in this study is similar to observed variation among $K_m$ values of the extensively studied lactate dehydrogenase enzymes from species inhabiting different thermal regimes (So-
mero 1978; Graves and Somero 1982). Somero and colleagues have interpreted the inverse relationship between $K_m$ and habitat temperature as the outcome of biochemical adaptation in which the $K_m$ values of the enzyme homologues are "adjusted" to substrate levels occurring at the respective habitat temperatures. This tracking of substrate levels by the $K_m$ values is hypothesized to provide catalytic and regulatory flexibility for enzyme homologues occurring in different thermal habitats. Alternatively, this pattern is consistent with expectations of adaptive catalytic-efficiency differences among the enzymes. The $K_m$ occurs in the denominator of catalytic-efficiency equations (both overall catalytic efficiency, $y^{-1}$, and $k_{cat}/K_m$, the most important component of catalytic efficiency; Albery and Knowles 1976; also see discussions in Hall and Koehn 1983; Hall 1985a, 1985b). Consequently, an inverse relationship between $K_m$ and habitat temperature will contribute to a positive association between habitat temperature and catalytic efficiency.

At this point a caveat must be given. Since PGI from *L. canaliculatus* could not be purified to homogeneity (see Material and Methods), $k_{cat}$ estimates could not be obtained for the allozymes. The kinetic contribution to the reaction rate under physiological conditions is in large part determined by $k_{cat}/K_m$. Thus, it is possible that $k_{cat}$ differences could occur in a direction opposite to and offsetting that portion of the variation in $k_{cat}/K_m$ that is due to allozyme-dependent differences in the $K_m$ values.

**Fig. 6.**—Loss of activity of PGI allozymes after incubation for 5 min at various temperatures in the presence (O = PGI-SS; △ = PGI-FF) or absence (● = PGI-SS; ▲ = PGI-FF) of 6-PG. Bars represent SEMs; for points without SEMs, symbols were larger than the SEM bars. Results of t-tests comparing the difference in the loss of activity between PGI allozymes at each temperature: in the absence of 6-PG, % loss in activity of PGI-FF > PGI-SS at 35-47.5°C (P < 0.05 and usually <0.001) and nonsignificant (P > 0.01) at 30 and 50°C; in the presence of 6-PG, results of all t-tests were nonsignificant.
In principal, this could result in the abolition or even rank-order reversal of the apparent temperature adaptation of the allozymes that has been inferred on the basis of the $K_m$ data alone. Ultimately, this issue can only be settled by future kinetic studies of PGI allozymes from \textit{L. canaliculatus}, studies in which $k_{cat}$ estimates are obtained on homogeneously purified allozymes.

Note that inferences of kinetic adaptation drawn from $K_m$ data alone often agree well with those drawn from $k_{cat}/K_m$ data. For example, studies by Place and Powers (1979), Graves and Somero (1982), and Hall (1985b) each reported a negative association between $K_m$ and habitat temperature—a finding similar to that of the present study—and a positive association between $k_{cat}/K_m$ and habitat temperature. In none of the aforementioned studies did variation in $k_{cat}$ abolish or even significantly diminish the contribution of $K_m$ to variation in $k_{cat}/K_m$. In all cases, variation in $K_m$ was either the major contributor to variation in $k_{cat}/K_m$ among the enzymes (Place and Powers 1979; Graves and Somero 1982) or variation in $k_{cat}$ contributed as much did variation in $K_m$ (Hall 1983). Thus, in each of these three cases, inferences of kinetic adaptation based solely on the $K_m$ values are identical to those based on $k_{cat}/K_m$ values. Similar results were also obtained for esterase-6 allozymes (Manc et al. 1983), in which variation in $k_{cat}/K_m$ (measured at one temperature) for $\beta$-naphthylpropionate was due almost exclusively to variation in $K_m$.

$V_{max}/K_m$ ratios also varied between \textit{Pgi} genotypes in a manner consistent with expectations of adaptive differentiation (table 5). At higher temperatures, the hypothetically warm-adapted PGI-FF enzyme was associated with a higher $V_{max}/K_m$ ratio than was the PGI-SS enzyme. An enzyme \textit{preparation} possessing a higher $V_{max}/K_m$ ratio under a specific set of conditions is catalytically more efficient than one possessing a lower $V_{max}/K_m$ ratio; however, unlike variation in $k_{cat}/K_m$—which, by definition, must be due to allozyme-dependent kinetic variation—it is often not possible to specify whether variation in $V_{max}/K_m$ is a consequence of allozyme-dependent kinetic variation or of allozyme-associated regulatory variation. This is so because $V_{max}$ is a composite parameter determined by two independent parameters, $k_{cat}$ and enzyme concentration. It is therefore possible that variation in $V_{max}/K_m$ ratios among allozymes could be due entirely to variation in enzyme concentration as a consequence of variation at a tightly linked regulatory locus. This important distinction has not been appreciated by several workers who have compared $V_{max}/K_m$ ratios of allozymes (Watt 1977, 1983; Hoffman 1981a). However, the ambiguity as to whether variation in $V_{max}/K_m$ is a consequence of kinetic or regulatory variation is only a problem when variation in $V_{max}$ is a major contributor to differences in $V_{max}/K_m$. In the case of PGI allozymes from \textit{L. canaliculatus}, $V_{max}$ values do not differ among PGI genotypes (table 2) and the PGI genotypes exhibit essentially identical $V_{max}$-temperature profiles (fig. 5). This means either that $k_{cat}$ values do not differ among the allozymes or that $k_{cat}$ values differ but are canceled out by reciprocal variation in enzyme concentration between the allozymes that results in identical $V_{max}$ values. For whatever reason, the absence of differences in $V_{max}$ among the allozymes indicates that the variation in $V_{max}/K_m$ ratios is due to allozyme-dependent kinetic variation (i.e., variation in $K_m$ values).

In summary, the kinetic data, taken together, indicate that the PGI-FF allozyme is kinetically superior to the PGI-SS allozyme at higher temperatures. However, the biochemical data only explain part of the PGI latitudinal variation—namely, why $Pgi^F$ should increase in southern latitudes. There is no biochemical explanation for the increase in the frequency of $Pgi^S$ in northern latitudes, since there was no case in
which the PGI-SS allozyme was kinetically superior to the PGI-FF allozyme at lower temperatures.

Significance of the allozyme-dependent differences in thermostability is more difficult to ascertain. Although the allozymes exhibited substantial differences in thermal stability at temperatures likely to be encountered by *L. canaliculatus* in the field (see Material and Methods), these differences were contingent on the absence of 6-PG in the incubation medium (fig. 6). The strong stabilizing influence of 6-PG (and probably other substrates and substrate analogues; see Results) means that the expression of allozymic differences in thermal stability in vivo will be determined by the relative proportion of unbound enzyme vis-à-vis that bound to substrates and substrate analogues. Consequently, the biological significance of the in vitro allozymic differences in thermal stability can only be determined by future in vivo studies of this characteristic.

If the allozymic differences in thermal stability are expressed in vivo, then this property appears to be maladaptive, at least in the portion of the cline north of Florida. In this area, PGI-FF increases in southern latitudes yet is more thermolabile than is PGI-SS. It is possible that thermostability is a more important enzymatic characteristic in the continuously warm subtropical habitats of southern Florida, which might account for the reversal of the PGI cline in this area.

One unusual feature of this study was the tendency for the heterozygote to be outside the range of the homozygotes in a variety of properties. This was generally observed for the $K_m$ values for both substrates (table 1), as well as for the inhibition constants ($K_i$ values) for 6-PG (Zera 1984, and accepted). Since the $K_m$ values and the $K_i$ values for the heterozygote were higher than those for the homozygotes, the overdominance is negative. Since negative overdominance resulting from heterozygote inferiority cannot lead to a stable polymorphism, the overdominance observed in this study cannot account for the persistence of the *Pgi*<sup>S</sup> allele in the absence of any observed kinetic advantage of the PGI-SS enzyme over the PGI-FF enzyme.

Although the majority of enzyme polymorphisms result in the intermediacy of kinetic properties of the heterozygote (Koehn et al. 1983; Zera et al. 1985), this is not universally observed (Martin 1979; McKetchie et al. 1981; Watt 1983). Presumably, these examples of overdominance are the result of nonadditive effects due to subunit interactions in the heterodimer. At present there are no studies that have isolated and kinetically characterized the heterodimer of a polymorphic enzyme.

Besides the present study, there are two other biochemical characterizations of clinally varying PGI allozymes (Hoffman 1981a, 1983; Hall 1985b). How do the results of these studies compare with each other, and do any general patterns emerge? Hall (1985b) estimated, at several temperatures and under physiological conditions, all the component parameters of catalytic efficiency for the two common latitudinally varying PGI allozymes from the bivalve mollusc, *Mytilus edulis*. He observed significant differences in overall catalytic efficiency ($y^{-1}$) and in $k_{cat}/K_m$ ratios among the PGI allozymes at higher (15–25 °C) but not at lower (5–10 °C) temperatures. The differences in both of these parameters were in accordance with expectations of adaptive kinetic differentiation based on the latitudinal *Pgi* gene-frequency cline.

In the other kinetic study of latitudinally varying PGI allozymes, this one in the sea anemone, *Metridium senile*, Hoffman (1981a) observed kinetic variation among the allozymes in the $K_m$ values for G-6-P and F-6-P. In this case, it is somewhat difficult to draw strong inferences of adaptation from the data. The rank order of
allozymic differences in $K_m$ values is reversed in the forward versus the reverse reaction directions, and the kinetic studies in the forward ($G-6-P \rightarrow F-6-P$) direction were performed at a nonphysiological pH (pH 8.5 at all temperatures). Nevertheless, the kinetic data suggest that the PGI-FF allozyme is catalytically superior to the PGI-SS allozyme at higher temperatures. In addition, the results are consistent with the gene-frequency data, in which the $Pgi^F$ allele increases in southern latitudes (Hoffman 1981b).

The correspondence, in each of these three species, between the kinetic properties of the PGI allozymes and the latitudinal variation of the PGI allele frequencies implicates selection as having some influence on these PGI polymorphisms. Additional studies on the physiological effects of the allozymic variation as well as direct measurements of fitness differences among the enzyme genotypes are required to substantiate the role of selection.

Although these data suggest that natural selection is at least partially responsible for the PGI clinal variation in the three aforementioned species, the data are equivocal as to whether the clines are stable or transient. As mentioned above, the work of Hoffman (1981a, 1983) and Hall (1985b), as well as the present study, have documented only that PGI from one genotype is catalytically superior to that from the other genotype(s), primarily or solely at higher temperatures. Hoffman (1981a) has reported preliminary evidence on the differential inhibition of PGI allozymes of *Metridium* by the pentose-shunt metabolite, 6-PG, at low temperatures. This difference could provide a selective advantage for the $Pgi^{SS}$ genotype at colder temperatures (see Discussion in Hoffman 1981a) and thus could provide the basis for the maintenance of the PGI cline in *Metridium* via balancing selection. On the other hand, detailed studies of the inhibition of PGI allozymes of *Mytilus* (Hall 1985b) and *Limnoporus* (Zera 1984, and accepted) by 6-PG and other pentose-shunt metabolites at several temperatures failed to document any differences among the allozymes. In short, at present there is no strong evidence supporting the existence of stable PGI clines maintained by balancing selection in *Metridium, Mytilus,* or *Limnoporus.* It is therefore possible that the PGI clines, while apparently influenced by selection, are nevertheless transient and that one allele will eventually reach fixation in each species.

**Acknowledgments**

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