

## INHIBITION OF GLUCOSEPHOSPHATE ISOMERASE ALLOZYMES OF THE MOSQUITOFISH, *GAMBUSIA HOLBROOKI*, BY MERCURY

VINCENT J. KRAMER\* and MICHAEL C. NEWMAN

University of Georgia, Savannah River Ecology Laboratory, Aiken, South Carolina 29801

(Received 30 June 1992; Accepted 20 April 1993)

**Abstract**—Frequencies of allozyme genotypes are being used as population-level indicators of environmental heavy-metal contamination. A genotype of glucose phosphate isomerase, *Gpi-2*<sup>38/38</sup>, of mosquitofish (*Gambusia holbrooki*) has been identified as "mercury-sensitive" in an acute toxicity assay. Partially purified preparations of GPI-2 38/38 and GPI-2 100/100 were assayed to determine differences in maximum gluconeogenic reaction velocity at seven mercury (added as HgCl<sub>2</sub>) concentrations, 15 to 960 nM Hg. Log-Probit analysis of the inhibition curves indicated that the log(IC<sub>50</sub>) (log<sub>10</sub> of the Hg concentration causing a 50% reduction in reaction velocity) for GPI-2 100/100 was significantly lower than that for GPI-2 38/38, even though GPI-2 100/100 initial uninhibited reaction velocity was greater than that of GPI-2 38/38. Although the mechanism of inhibition was not experimentally determined, under the assumption of noncompetitive interaction between Hg and GPI-2, the inhibitor dissociation constants (95% asymptotic C.I.) for GPI-2 100/100 and GPI-2 38/38 were estimated from the log (IC<sub>50</sub>) as 204 nM Hg (155–269 nM Hg) and 479 nM Hg (363–617 nM Hg), respectively. These results suggested that Hg susceptibility related to the *Gpi-2*<sup>38/38</sup> genotype in acute toxicity assays was likely not due to enhanced Hg inhibition of GPI-2 38/38.

**Keywords**—Heavy metal Pocciliidae Allele Enzyme inhibitor Stress

### INTRODUCTION

An allozyme is an enzyme displaying allelic variation in a population. Frequencies of allozyme genotypes may be useful population-level indicators of environmental contamination by heavy metals [1–3]. However, a mechanistic explanation of shifted allozyme genotype frequencies in exposed vs. reference populations is lacking in most studies. Excessive pollution-induced mortality may cause shifts in allozyme genotype frequencies by increasing the probability of rare allele loss [4]. Differential inhibition of allozymes by heavy metals has been suggested as a cause for observed shifts in allozyme genotype frequencies in naturally exposed populations of shrimp [1] and minnows [2]. Diamond et al. [3] suggested that a similar mechanism may explain the differential sensitivity of a glucose phosphate isomerase (GPI, E.C. 5.3.1.9) allozyme genotype of mosquitofish (*Gambusia holbrooki*) in an acute toxicity assay of HgCl<sub>2</sub>. Specifically, fish expressing the *Gpi-2*<sup>100/100</sup> genotype were less Hg-sensitive than *Gpi-2*<sup>38/38</sup> fish, as measured by time-to-death.

This study tested the hypothesis that the allozyme GPI-2 38/38 (the enzyme product of the genotype *Gpi-2*<sup>38/38</sup>) was not affected by Hg differently from the allozyme GPI-2 100/100. In a previous study, Kramer et al. [5] showed that mosquitofish expressing the *Gpi-2*<sup>38/38</sup> genotype exhibited muscle tissue concentrations of lactate, fructose 6-phosphate, and glucose 6-phosphate (F6P and G6P are substrates of GPI) after exposure to HgCl<sub>2</sub> that were the same as unexposed fish but higher than other Hg-exposed *Gpi-2* geno-

types. A physiologically-based difference in the response to Hg by *Gpi-2*<sup>38/38</sup> mosquitofish was suggested, but the possibility of direct inhibition or activation of the GPI-2 allozyme could not be rejected.

This study tested the response of two partially purified GPI-2 allozyme preparations (GPI-2 38/38 and GPI-2 100/100) to Hg (added as HgCl<sub>2</sub>). Maximum initial reaction velocities in the gluconeogenic direction (F6P → G6P) were compared at seven Hg concentrations (15–960 nM Hg). The Hg concentrations were chosen to cover the range from minimum to maximum enzyme inhibition.

### MATERIALS AND METHODS

#### Chemicals

All buffers and ammonium sulfate were enzyme grade. Sepharose® CL6B-DEAE (anion exchange resin), Sephadex® G150, F6P, and NADP were purchased from Sigma Chemical Co. (St. Louis, MO). Glucose 6-phosphate dehydrogenase (G6PDH, E.C. 1.1.1.49, *Leuconostoc mesenteroides*) was obtained from U.S. Biochemical Co. (Cleveland, OH). A 4.985-mM Hg (as HgCl<sub>2</sub>) reference solution was purchased from Fisher Scientific (Pittsburgh, PA).

#### Collection of fish

Eastern mosquitofish (Girard 1859) were dip-netted from a 1.1-ha abandoned farm pond on the U.S. Department of Energy's Savannah River Site (Barnwell County, SC). The pond has no known history of Hg contamination. Two experiments unrelated to this study provided a supply of healthy control (unexposed to Hg) mosquitofish. All tissues of male and female fish, excluding the abdominal portion (viscera and embryos), were stored at –70°C for no more than 13 months before use.

\*To whom correspondence may be addressed.

The current address of V.J. Kramer is Department of Fisheries and Wildlife, 163 Natural Resources Bldg., Michigan State University, East Lansing, MI 48824-1222.



### Genotype

The mosquitofish used in this study express two loci for GPI: an invariant locus, *Gpi-1*, and a variable locus, *Gpi-2*. Loci were resolved using horizontal starch gel electrophoresis, as described in Diamond et al. [3]. Fish expressing *Gpi-2*<sup>100/100</sup> and *Gpi-2*<sup>38/38</sup> genotypes were used as sources of their respective allozymes, referred to as GPI-2 100/100 and GPI-2 38/38.

### Purification of allozymes

The purification procedure was derived from that described by Hall [6]. Approximately 8 g wet weight of fish tissue (from 10 fish) was suspended in 32 ml buffer A (75 mM Tris, 2 mM EDTA, pH 8.0) and homogenized at 0 to 4°C for 5 min using a mechanical tissue grinder (Tekmar [Cincinnati, OH] model SDT1810, 20% power setting). After centrifugation of the homogenate (20,000 g, 2–4°C, 30 min), the yellow-orange supernatant was brought to 40% (w/v) ammonium sulfate saturation (0.282 g solid per milliliter at 0–4°C) and stirred for 1 h. After a similar centrifugation (discarding the pellet), the clear supernatant was brought to 70% (w/v) ammonium sulfate saturation with an additional 0.212 g solid per milliliter at 0–4°C and stirred for 1 h. After another similar centrifugation, the pellet was resuspended in 20 ml buffer B (50 mM Tris, 1 mM EDTA, pH 8.0). To remove residual ammonium sulfate, the solution was filtered

through an Amicon (Beverly, MA) YM-10 membrane filter (molecular weight cutoff, 10 kDa) to 3 ml, diluted to 20 ml, and filtered again to 3 ml. The final volume of the filtered solution was about 6 ml after washing the membrane and apparatus. The filtered solution was loaded onto Sepharose CL6B-DEAE (2.5 × 35.4 cm) equilibrated with buffer B at 24°C and eluted at a linear flow rate of 6 cm/h (0.5 ml/min). The GPI-2 allozyme eluted isocratically just after the void volume. The GPI-1 allozyme eluted in a 100- to 400-mM-NaCl step gradient (Fig. 1). The identity of the peaks was confirmed by starch-gel electrophoresis (data not shown). The combined peak fractions of GPI-2 were concentrated to 6 ml, using ultrafiltration as before. This solution, brought to 5% glycerol, was loaded onto Sephadex G150 (2.5 × 61.6 cm) equilibrated with buffer C (25 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.1) at 5°C and eluted at a linear flow rate of 6 cm/h. The peak fractions containing the partially purified allozyme were stored at 5°C with negligible loss of activity for one month during the course of the experiment.

Total protein was measured using the Bio Rad (Richmond, CA) semimicro dye-binding assay modified to a 3-ml final volume. Enzyme activity was measured by adding 50 μl of GPI-containing sample to 2,700 μl buffer D (0.4 M triethanolamine HCl, 10 mM MgCl<sub>2</sub>, 1 mM EDTA, pH 7.6), plus 100 μl each of 50 mM F6P and 50 mM NADP, and 50 μl 35 U/ml G6PDH. The rate of change of absorbance at 340 nm was used to monitor production of NADPH (extinction co-

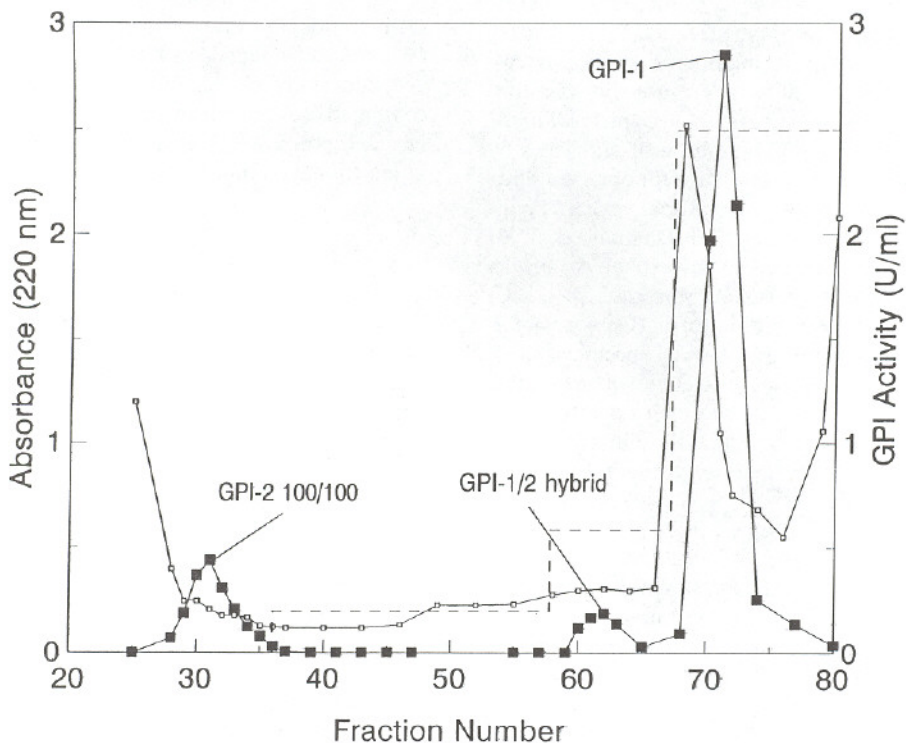


Fig. 1. Separation of GPI-2 100/100 from other GPI isozymes (isolated from mosquitofish) by DEAE ion-exchange chromatography, conditions as described in text (5.5 ml per fraction): (■) GPI activity; (□) absorbance at 220 nm; (---) NaCl step gradient; step 1, 0 to 40 mM NaCl; step 2, 40 to 100 mM NaCl; step 3, 100 to 400 mM NaCl in buffer B.



efficient,  $6.3 \text{ mM}^{-1}$  in a 1-cm cuvette). One unit of GPI activity was defined as that quantity of enzyme converting  $1 \mu\text{mol}$  F6P to  $1 \mu\text{mol}$  G6P per minute at  $25^\circ\text{C}$  and pH 7.6.

#### Hg inhibition of GPI-2 allozymes

Inhibition by Hg was measured as the reduction in the maximum reaction velocity in the gluconeogenic direction (F6P  $\rightarrow$  G6P) for equal activities (0.014 U GPI-2 activity at  $25^\circ\text{C}$ ) of GPI-2 100/100 and GPI-2 38/38. The assay conditions were selected to approximate resting muscle tissue pH [7], ionic strength [6], and temperature similar to the conditions under which mosquitofish were assayed for Hg toxicity by Diamond et al. [3]. In the presence of Hg, the GPI reaction was not monitored directly using G6PDH, because the Hg inhibited G6PDH as well as GPI. Therefore, the product of the reaction, G6P, was measured by a rapid-step technique.

All inhibition assays were conducted in 1.5-ml microcentrifuge tubes equilibrated to  $20^\circ\text{C}$  by suspension in a water-circulating bath. In a final volume of 1.5 ml buffer C, aliquots of GPI-2 allozyme solution (88  $\mu\text{l}$  GPI-2 38/38 preparation, 100  $\mu\text{l}$  GPI-2 100/100 preparation) and of the  $\text{HgCl}_2$  solution were incubated for 5 min. Nominal Hg concentrations were 960, 480, 240, 180, 120, 60, 30, 15 nM Hg ( $N=4$  at each concentration), and 0 nM Hg ( $N=8$ ). Buffer C was used to avoid complexation of free  $\text{Hg}^{2+}$  by organic ligands such as EDTA and triethanolamine found in buffer D [8]. Phosphate forms soluble complexes with Hg but requires hours to reach equilibrium [9]. GPI concentration, assuming a molecular weight of 124 kDa [10], was approximately 0.55 nM GPI-2. Total protein in the GPI-2 100/100 aliquot was 0.41  $\mu\text{g}$ , whereas that in the GPI-2 38/38 aliquot was 0.096  $\mu\text{g}$ . Therefore, a third experiment was conducted in which 0.4  $\mu\text{g}$  bovine serum albumin (BSA) was added to the GPI-2 38/38 reaction mixture to mimic the potential for complexation of Hg by the excess protein in the GPI-2 100/100 preparation.

The reaction was initiated by the addition of 50  $\mu\text{l}$  of 50 mM F6P (final F6P concentration, 1,700  $\mu\text{M}$ ). The F6P concentration exceeded by approximately 50-fold the reported

$K_m$  for GPI [6], providing nearly maximal enzyme reaction rate. Portions (200  $\mu\text{l}$ ) of the reaction solution were removed at 2 and 4 min after initiation of the reaction. The reaction in the aliquots was immediately halted by mixing with an equal volume of 6 M HCl. The acidified solution was neutralized with 3 M  $\text{Na}_2\text{CO}_3$ . The entire neutralized solution was assayed for G6P by measuring the total change in absorbance at 340 nm of a solution containing the following: 1,000  $\mu\text{l}$  buffer D, 10  $\mu\text{l}$  100-U/ml G6PDH, 50  $\mu\text{l}$  50-mM NADP, and 600  $\mu\text{l}$  neutralized sample. The estimate of the initial velocity of the reaction was calculated by dividing the concentration of G6P by the elapsed time. The reaction was approximately linear over 4 min. Therefore, Waley's progress curve analysis was not used to make estimates of the reaction velocity [11]. Rather, velocity estimates at 2 and 4 min were averaged.

#### RESULTS

The final allozyme solutions had unequal specific activities (Table 1); therefore, reaction velocities were not normalized to protein concentration. Horizontal starch-gel electrophoresis of aliquots of the GPI-2 preparations revealed single bands with no detectable amounts of GPI-1 or of the inter-locus hybrid molecule GPI-1,2. Detection limit for electrophoresis was 0.015 U GPI activity per milliliter of sample.

Log-Probit analysis [12] of the inhibition of GPI-2 reaction velocity by Hg revealed significant differences in  $\log(\text{IC}_{50})$ , ( $\text{IC}_{50}$  is the 50% inhibitory concentration) between GPI-2 100/100 and GPI-2 38/38 (Fig. 2, Table 2). Specifically, the  $\log(\text{IC}_{50})$  for GPI-2 100/100 was lower than that for GPI-2 38/38, indicating greater susceptibility to inhibition by Hg for GPI-2 100/100 over GPI-2 38/38. Addition of BSA to GPI-2 38/38 did not significantly affect  $\log(\text{IC}_{50})$ . The model estimated that the uninhibited initial reaction velocities,  $V_0$ , were significantly different in the three GPI-2 preparations. That  $V_0$  for the two GPI-2 38/38 experiments was less than that for GPI-2 100/100 indicated that although aliquots of the allozyme solutions had equivalent activity under the buffer D assay conditions (the standard assay for measuring GPI activity in this laboratory), buffer C assay conditions favored GPI-2 100/100 over GPI-2 38/38. The es-

Table 1. Specific activity of GPI throughout purification

Step	Gpi-2 genotype	Vol. (ml)	Activity (GPI U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Homogenate	100/100	14.5	121	82.7	1.46	100	1
	38/38	28.1	171	122	1.40	100	1
40-70% $(\text{NH}_4)_2\text{SO}_4$	100/100	20.0	107	39.0	2.74	88	1.9
	38/38	20.0	157	53.4	2.94	92	2.1
Amicon I	100/100	6.00	101	35.4	2.85	83	2.0
	38/38	5.55	146	52.1	2.80	85	2.0
Sephacose®-DEAE	100/100	61.0	13.1	0.641	20.4	11	14
	38/38	21.6	18.5	0.560	33.0	11	24
Amicon II	100/100	7.70	11.0	0.493	22.3	9.1	15
	38/38	6.90	14.3	0.414	34.5	8.4	25
Sephadex® G150	100/100	62.1	9.44	0.254	37.2	7.8	25
	38/38	74.1	13.2	0.0859	154	7.7	110

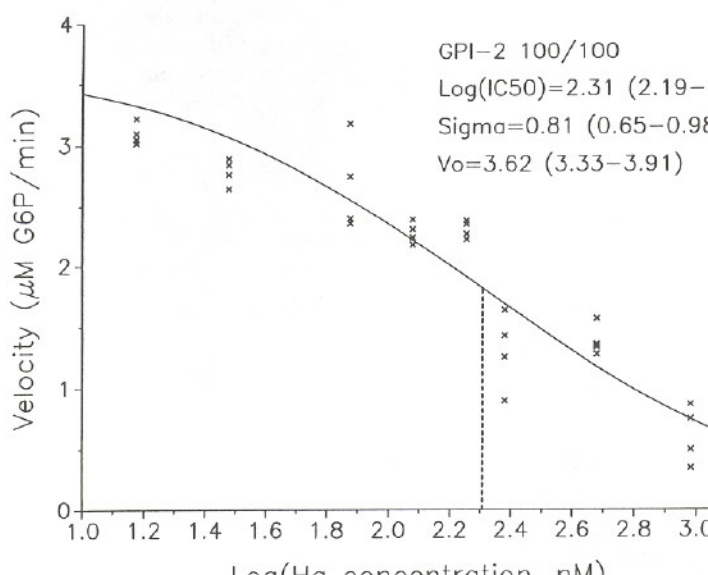
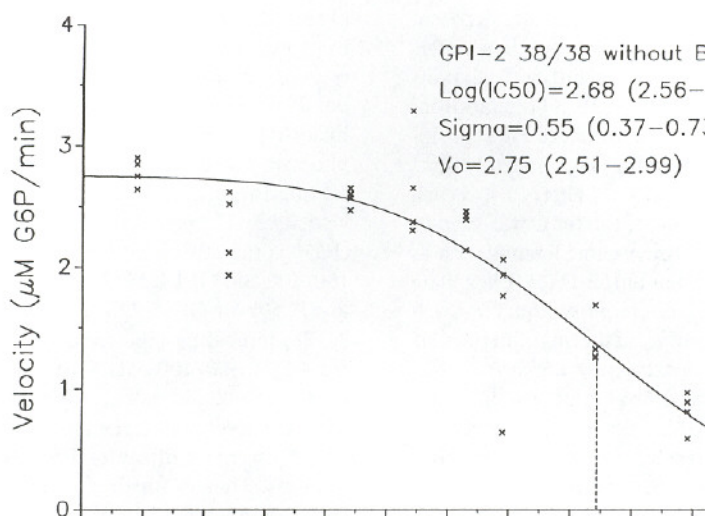
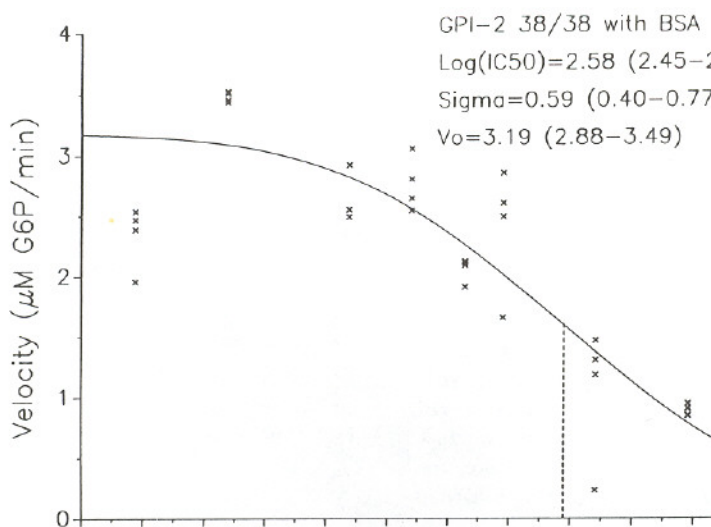




Table 2. Tests of significant differences between inhibition curve parameters<sup>a</sup>

Null hypothesis	Parameter	Difference	$S_p^b$	Student's $t$	$d.f.$	Significance
GPI-2 100/100 = GPI-2 38/38, no BSA	Log(IC50)	-0.370	0.371	-4.46	78	***
	$V_0$	+0.870	0.826	+4.71		***
	$\sigma$	+0.267	0.535	+2.23		**
GPI-2 100/100 = GPI-2 38/38 with BSA	Log(IC50)	-0.270	0.386	-3.13	78	***
	$V_0$	+0.430	0.918	+2.09		**
	$\sigma$	+0.226	0.537	+1.88		*
GPI-2 38/38, no BSA = GPI-2 38/38 with BSA	Log(IC50)	+0.100	0.380	+1.18	78	NS <sup>c</sup>
	$V_0$	-0.440	0.853	-2.31		**
	$\sigma$	-0.041	0.566	-0.32		NS

<sup>a</sup>Log<sub>10</sub> of the 50% inhibitory concentration of Hg (nM), log(IC50); initial uninhibited reaction velocity,  $V_0$ ; slope function,  $\sigma$  (sigma); estimated by log-probit analysis of the reduction of GPI-2 reaction velocity (F6P → G6P) by Hg (0–960 nM) under three experimental conditions – GPI-2 100/100, 0.41 μg total protein per assay; GPI-2 38/38, 0.096 μg total protein per assay; GPI-2 38/38, 0.096 μg total protein per assay plus 0.4 μg BSA protein per assay,  $N = 40$  for each treatment.

<sup>b</sup>Pooled sample standard deviation.

<sup>c</sup>Not significant,  $P > 0.10$ .

\* $P < 0.10$ , two-tailed Student's  $t$  test.

\*\* $P < 0.05$ , two-tailed Student's  $t$  test.

\*\*\* $P < 0.01$ , two-tailed Student's  $t$  test.

estimated slope function,  $\sigma$  (sigma), indicated that GPI-2 38/38 reaction velocity was reduced less quickly by incremental increases in Hg than the reaction velocity of GPI-2 100/100.

## DISCUSSION

The inhibitor dissociation constant,  $K_1$ , is a characteristic parameter that describes the affinity of the protein-binding site for the inhibitor [13]. The IC50 can be used to calculate  $K_1$ , provided that the mechanism of inhibition is known [14]. The inhibition of GPI-2 by Hg most likely followed one of three mechanisms: competitive, noncompetitive, or a combination of both [15]. Experimental determination of the mechanism of inhibition of GPI-2 by Hg was beyond the limited scope of this study. However, examination of the estimated  $K_1$  under assumed mechanisms of inhibition provided insightful comparisons of the Hg susceptibility of the GPI-2 allozymes.

Competitive inhibitors reversibly bind to the active site of the enzyme, displacing the substrate by mimicking the three-dimensional structure and charge characteristics of the substrate [14]. The effects of a competitive inhibitor can be eliminated with excess substrate. F6P, the substrate of GPI in this experiment, is anionic and occupies a larger molecular volume than Hg, suggesting that the inhibitory mechanism may not have been competitive. However, another possibility was that Hg interacted with F6P to produce a competitive inhibitor of the form F6P-Hg. The formation of such a complex has not been reported [8,16].

Noncompetitive inhibitors bind to sites other than the substrate-binding site, altering enzyme structural conformation and inactivating the enzyme. Mercury binds to enzyme sulfhydryl groups with high affinity, log  $K$  approximating -42 [15], and therefore can exhibit noncompetitive inhibition at low concentrations. If noncompetitive inhibition was the dominant mode of inhibition, then IC50 (after back-transformation of log (IC50) and the 95% asymptotic confidence limits) provided a direct estimate of  $K_1$  from Hg [13]: GPI-2 100/100,  $K_1 = 204$  nM (155–269 nM); GPI-2 38/38, no BSA added,  $K_1 = 479$  nM (363–617 nM); GPI-2 38/38 with added BSA,  $K_1 = 380$  nM (282–501 nM). Inspection of the 95% C.I.s indicated that addition of BSA did not significantly affect  $K_1$  for GPI-2 38/38. However, both values of  $K_1$  for the GPI-2 38/38 preparations were significantly different from that of GPI-2 100/100. For comparative purposes, the competitive inhibition constant was also calculated from the IC50, using the equation

$$K_{1c} = \text{IC50}/(1 + (S/K_m))$$

where  $K_{1c}$  is the dissociation constant for the purely competitive inhibitor,  $S$  is the substrate concentration, and  $K_m$  is the Michaelis-Menten constant for the reaction F6P → G6P. Using a reported value of  $K_m$  approximating 30 μM [6] and a substrate concentration of 1,700 μM F6P,  $K_{1c}$  was calculated: GPI-2 100/100,  $K_{1c} = 3.5$  nM (2.7–4.7 nM); GPI-2 38/38, no BSA added,  $K_{1c} = 8.3$  μM (6.3–10.7 μM); GPI-2

Fig. 2. Inhibition by Hg (added as HgCl<sub>2</sub>) of reaction velocity (F6P → G6P) of GPI-2 allozymes isolated from mosquitofish. Log-Probit analysis (SAS® PROC NLIN, Gauss-Newton method [12,18]) provided estimates (95% asymptotic C.I.s) of log<sub>10</sub> of the 50% inhibitory concentration of Hg (nM), log(IC50); initial uninhibited reaction velocity,  $V_0$ ; and the slope function,  $\sigma$  (sigma); under three experimental conditions – GPI-2 100/100, 0.41 μg total protein per assay; GPI-2 38/38, 0.096 μg total protein per assay; GPI-2 38/38, 0.096 μg total protein per assay plus 0.4 μg BSA per assay. Each assay contained 0.014 U GPI-2 activity, as defined in the text.  $N = 4$  at each Hg concentration, except at Hg = 0,  $N = 8$  (data points at Hg = 0 not shown on log scale but included in model).



38/38 with added BSA,  $K_{ic} = 6.6$  nM (4.9–8.7 nM). The noncompetitive inhibition constant for the combined model was not calculated because an independent estimate of the competitive inhibition constant, in addition to the IC50, was required.

After Kramer et al. [5] showed that glycolysis metabolite concentrations remained at control levels in *Gpi-2*<sup>38/38</sup> mosquitofish whereas those in other genotypes decreased after HgCl<sub>2</sub> exposure, the question of whether Hg directly affected GPI-2 38/38 remained. This in vitro study has shown a significant difference in Hg inhibition between GPI-2 38/38 and GPI-2 100/100: GPI-2 100/100 activity was more susceptible to Hg inhibition than GPI-2 38/38 under both the noncompetitive and the competitive scenarios. This result suggests that GPI-2 38/38 allozyme inhibition was not directly related to the Hg sensitivity of *Gpi-2*<sup>38/38</sup> mosquitofish observed previously by Diamond et al. [3]. The glycolysis metabolites of *Gpi-2*<sup>38/38</sup> mosquitofish acutely exposed to Hg remained at control levels while other genotypes, including *Gpi-2*<sup>100/100</sup>, exhibited reduced glycolysis metabolite concentrations [5], indicating a greater resistance to Hg-related metabolic change by *Gpi-2*<sup>38/38</sup> than other genotypes. Consistent with the resistance to metabolic change was, in this study, the greater resistance to Hg inhibition of GPI-2 38/38 over GPI-2 100/100. However, the results of the present study should not be construed to support the hypothesis that Hg-related inhibition of GPI-2 allozymes was the sole source of mortality in the acute toxicity assay [3]. Mercury-related inhibition of GPI-2 alone would not be expected to cause significant changes to in vivo glycolysis metabolite concentrations because GPI has little regulatory metabolic activity [6] and because GPI-2 accounts for only 10 to 30% of the total GPI activity in mosquitofish (V. Kramer, unpublished data).

Another hypothesis concerning *Gpi-2*<sup>38/38</sup> susceptibility to Hg toxicity is that the *Gpi-2*<sup>38/38</sup> genotype acted as a genetic marker closely linked to a gene or suite of genes that conferred susceptibility to Hg-related stress. The demonstrated sensitivity of *Gpi-2*<sup>38/38</sup> mosquitofish to acute arsenate toxicity [17] further supports the view that the *Gpi-2*<sup>38/38</sup> genotype was a toxicant nonspecific marker for susceptibility to acute toxicity.

**Acknowledgement**—Support for this research came from contract DE/AC09/76SR00/819 between the U.S. Department of Energy and the University of Georgia Savannah River Ecology Laboratory. Thanks are extended to M. Mulvey, P. Dixon, M.G. Heagler, A. Chazal, C. Lee, M. Davis, J. McCloskey, and M. Vella-Kramer for assistance in this and other research, and to B. Herbert for use of the Amicon filtration apparatus. Thanks are also extended to J. Giesy for the use of word processing equipment.

## REFERENCES

1. Nevo, E., R. Ben-Shlomo and B. Lavie. 1984. Mercury selection of allozymes in marine organisms: Prediction and verification in nature. *Proc. Natl. Acad. Sci. USA* **81**:1258–1259.
2. Gillespie, R.B. and S.I. Guttman. 1989. Effects of contaminants on the frequencies of allozymes in populations of the central stoneroller. *Environ. Toxicol. Chem.* **8**:309–317.
3. Diamond, S.A., M.C. Newman, M. Mulvey, P.M. Dixon and D. Martinson. 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute exposure to inorganic mercury. *Environ. Toxicol. Chem.* **8**:613–622.
4. Mulvey, M. and S.A. Diamond. 1991. Genetic factors and tolerance acquisition in populations exposed to metals and metalloids. In M.C. Newman and A.W. McIntosh, eds., *Metal Ecotoxicology: Concepts and Applications*. Lewis, Chelsea, MI, pp. 301–321.
5. Kramer, V.J., M.C. Newman, M. Mulvey and G.R. Ullsch. 1992. Glycolysis and Krebs cycle metabolites in mosquitofish, *Gambusia holbrooki* (Girard, 1859), exposed to mercuric chloride: Allozyme genotype effects. *Environ. Toxicol. Chem.* **11**:357–364.
6. Hall, J.G. 1985. Temperature related kinetic differentiation of glucosephosphate isomerase alloenzymes isolated from the blue mussel, *Mytilus edulis*. *Biochem. Genet.* **23**:705–728.
7. Parkhouse, W.S., G.P. Dobson and P.W. Hochachka. 1988. Control of glycogenolysis in rainbow trout muscle during exercise. *Can. J. Zool.* **66**:345–351.
8. Smith, R.M. and H.E. Martell. 1989. *Critical Stability Constants*, Vol. 6, Second Supplement. Plenum, New York, NY.
9. Quarfort-Dahlman, I. 1975. On some phosphate equilibria. 6. The systems Hg(I) and Hg(II) phosphoric acid in 3 M NaClO<sub>4</sub>. *Chem. Scripta* **8**:112–125.
10. Harris, H. and D.A. Hopkinson. 1976. *Handbook of Enzyme Electrophoresis in Human Genetics*. Elsevier/North Holland, Amsterdam, The Netherlands, p. 8.
11. Waley, S.G. 1981. An easy method for the determination of initial rates. *Biochem. J.* **193**:1009–1012.
12. Bruce, R.D. and D.J. Versteeg. 1992. A statistical procedure for modeling continuous toxicity data. *Environ. Toxicol. Chem.* **11**:1485–1494.
13. Zubay, G. 1988. *Biochemistry*, 2nd ed. Macmillan, New York, NY, pp. 272–276.
14. Cheng, Y.-C. and W.H. Prusoff. 1973. Relationship between the inhibition constant ( $K_i$ ) and the concentration of inhibitor which causes 50 percent inhibition (IC50) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108.
15. Webb, J.L. 1966. *Enzyme and Metabolic Inhibitors*, Vol. 2. Academic, New York, NY, pp. 729–986.
16. Perrin, D.D. 1979. *Stability Constants of Metal-Ion Complexes*, Part B—Organic Ligands. IUPAC Chemical Data Series 22. Pergamon, Oxford, UK.
17. Newman, M.C., S.A. Diamond, M. Mulvey and P. Dixon. 1989. Allozyme genotype and time to death of mosquitofish, *Gambusia affinis* (Baird and Girard) during acute toxicant exposure: A comparison of arsenate and inorganic mercury. *Aquat. Toxicol.* **15**:141–156.
18. SAS Institute. 1990. *SAS®/STAT Guide for Personal Computers*, 4th ed., Vol. 2. Cary, NC.