

## Comparison of *in Vitro* and *in Vivo* Activities Associated With the G6PD Allozyme Polymorphism in *Drosophila melanogaster*

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### ABSTRACT

Earlier studies of the *A* and *B* allozymes at the *G6pd* locus show a differential ability of the genotypes to suppress the loss of viability associated with a low activity 6-phosphogluconate dehydrogenase mutation, *6Pgd<sup>low</sup>*. This observation indicates a relatively lower activity for the *A* allozyme genotype, but it is not known if this level of suppression required a large difference in *in vivo* activity. To clarify this difference an analysis of the biochemical properties of the purified allozymes was carried out, as well as an analysis of the activity level associated with an original low activity *P* element-derived allele which had partially reverted and lost its suppression ability. G6PD activity and protein level were studied in 47 *X* chromosome lines from North America. The *A* genotype averages a 9% lower  $V_{\max}$ . From analysis of the correlation between G6PD activity and protein level it remains unclear whether the allozyme  $V_{\max}$  difference results from dissimilarity in protein level or  $k_{\text{cat}}$ . At 25° and physiological pH, comparative studies of the steady-state kinetics show the two purified allozyme variants differ significantly in their  $K_M$  values for glucose-6-phosphate and NADP, and the  $K_I$  for NADPH. In aggregate these parameters predict the *A* genotype possesses a 20% lower *in vitro* catalytic efficiency. A partial revertant of a *P* element-derived low activity *B* variant, was shown to lose the ability to suppress *6Pgd<sup>low</sup>* low viability after acquiring only 60% of normal *B* activity. This last comparison shows the *A* genotype activity must be reduced *in vivo* by at least 40%.

**A**N implicit assumption among advocates of selection on allozyme polymorphism is that enzyme variants possess *in vivo* properties sufficiently different to modulate pathway flux, and that these differences translate into selection coefficients sufficiently large to influence the fate of the polymorphism in the face of stochastic noise in natural populations. Since *in vivo* function is technically difficult to measure for many polymorphisms, one approach in evaluating the potential for selection operating on protein polymorphisms has been to examine the *in vitro* properties of variant molecules via measuring steady-state kinetics (e.g. HALL 1985; PLACE and POWERS 1979; WATT 1983; WHITE, MANE and RICHMOND 1988; KOEHN, NEWELL and IMMERMANN 1980; McDONALD, ANDERSON and SANTOS 1980). It is assumed that *in vitro* measures of function are tenable predictors of *in vivo* differences. The objective of this study is to examine this question with respect to the G6PD allozyme polymorphism.

In experimental population genetics of *Drosophila melanogaster*, the pentose shunt enzyme, glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and its allozyme polymorphism in natural populations have been the focus of many papers since its discovery in

1964 as the first sex-linked electrophoretic polymorphism (YOUNG, PORTER and CHILDS 1964). Recently, the locus has been cloned, sequenced, and shown to have 65% amino acid sequence homology with the human enzyme (GANGULY, GANGULY and MANNING 1985; HORI *et al.* 1985; FOUTS *et al.* 1988). This laboratory has focused on the polymorphism in natural populations, especially with respect to the geographic variation (OAKESHOTT *et al.* 1983; EANES and HEY 1986), the potential to respond to targeted selection (EANES *et al.* 1985), assessing potential differences in *in vivo* function (EANES 1984; EANES and HEY 1986), and restriction map variation in the *G6pd* locus region (EANES *et al.* 1989). While several studies have examined biochemical properties associated with the allozymes (STEELE, YOUNG and CHILDS 1968; BIJLSMA and VAN DER MEULEN-BRUIJNS 1979; CAVENER and CLEGG 1981; HORI and TANDA 1980, 1981; WILLIAMSON and BENTLEY 1983), no clear consensus has emerged as to whether activity differences exist between genotypes, or what the causal source of such differences might be. The first objective of this study is to compare variants by *in vitro* analysis using highly purified enzyme, under steady-state kinetic conditions judged to approximate the physiological environment (SOMERO 1981; HALL and KOEHN 1983).

In EANES (1984) dramatic differences were seen in the ability of the *G6pd<sup>A</sup>* and *G6pd<sup>B</sup>* genotypes to

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suppress the lethality associated with a 6-phosphogluconate dehydrogenase (6PGD) low activity mutation, designated *6Pgd<sup>lo1</sup>*. In a *G6pd<sup>B</sup>* background this mutation has low viability, while it is nearly normal in the *G6pd<sup>A</sup>* background. It was proposed that this difference reflected a lower *in vivo* activity associated with the *G6pd<sup>A</sup>* genotype, resulting in a reduced accumulation of 6-phosphogluconate, which is known as a potent inhibitor of glycolysis. This observation was repeated in EANES and HEY (1986) by examining a number of rare electrophoretic and activity variants from natural populations. Together these studies show unequivocally that differences in *in vivo* activity must exist between the genotypes. The second objective of this study was to use *P* element revertants from an *P* element-associated low activity *G6pd<sup>B</sup>* allele to determine the G6PD activity level where suppression of low activity 6PGD-mediated lethality appears. This will place an upper limit estimate on the amount of *in vivo* activity associated with the *A* variant, and allow us to contrast the predictions based on the *in vitro* study.

#### MATERIALS AND METHODS

**Wild and mutant lines:** The 47 *X* chromosome lines used in this study were derived from isofemale lines collected at Davis Peach Farm, Mt. Sinai, New York, in 1985, and Watsonville, California, in 1985. Lines isogenic for the *X* chromosome were created by crossing wild males from these lines to virgin females of genotype *FM6/N<sup>264-84</sup>* and extracting with the *FM6* balancer chromosome (see EANES, HEY and HOULE 1985). Electrophoretic genotypes were determined as in EANES and HEY (1986).

**Enzyme purification:** Using affinity chromatography (LEE, LANGLEY and BURKHART 1978) G6PD was purified from the alleles present on the *G6PD<sup>A</sup>* and *G6pd<sup>B</sup>* recombinant chromosomes described in EANES *et al.* (1985). Both G6PD variants were purified to greater than 98% homogeneity as determined by ultrasensitive silver staining on 7% acrylamide native and SDS slab gels. The purification protocol is as follows. Twenty to thirty grams of frozen flies were ground in 100 ml ice cold buffer A (0.01 M sodium phosphate, pH 6.8, 5 mM 2-mercaptoethanol, 0.1 mM PMSF, 1 mM EDTA, 0.1 M NaCl) with 50  $\mu$ M NADP. The homogenate was centrifuged, the supernatant collected, filtered, and proteins precipitated sequentially, first with 40% and then 55% ammonium sulfate. The centrifuged pellet was again suspended in 50 ml of buffer A and applied to a 10-ml column of 8-(6-aminohexyl)-amino-2',5'-ADP-agarose (Sigma) previously equilibrated with buffer A. The column was then washed with 100 ml of buffer A and 200 ml of buffer B (0.05 M sodium phosphate, pH 8.0, 5 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 M NaCl). The enzyme was eluted in 5-ml fractions from the column with a NADP gradient of 0 to 200  $\mu$ M NADP in buffer B. Peak G6PD fractions were pooled, diluted to 350 ml with buffer A, and reappplied to the same ADP-agarose column (after washing the column with 0.1 M NaCl and 8 M urea) reequilibrated with buffer A. The same washes and NADP gradient were applied as above, and the peak fractions collected in 0.5 mM NADP. These preparations were free of any detectable 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase

or malic enzyme, and represents a nearly 1300-fold purification. Purified enzyme was stored at  $-80^{\circ}$  in 70% glycerol and 0.5 mM NADP.

**Line activity and assay measures:** G6PD line activity was measured by homogenizing 20 adult male flies (aged 5 days post eclosion at  $25^{\circ}$ ) in 100  $\mu$ l of 0.11 I (= ionic concentration, 0.05 M MOPS, 0.0875 M NaCl) Na-MOPS, pH 7.15 buffer with 0.32 mM NADP, immediately centrifuging for 5 min (at  $12,400 \times g$ ) and assaying spectrophotometrically at  $25^{\circ}$  for 3–5 min. The assay buffer was 0.051 Na-MOPS (pH 7.15), 3.2 mM glucose-6-phosphate, 0.32 mM NADP. One unit of G6PD activity will convert 1  $\mu$ mol of NADP to NADPH  $\text{min}^{-1}$  under these assay conditions. Preparations and assays were carried out in batches of six samples over 30-min periods to minimize the time interval between preparation and assay, although enzyme activity was stable over at least 2 hr under these buffer conditions. Total soluble protein was measured by the method of BRADFORD (1976) using bovine serum albumin (BSA) standards, and is treated as a covariate in the analysis of variance. Four samples of 20 flies were assayed per line; each line was reared in two bottles, and two samples (blocks) were collected per bottle. Assays were carried out over 4 days, with one set of samples (each block) assayed per day. Each sample preparation was assayed twice, and the mean used as a single variate in the analysis. The analysis involves partitioning six sources of variation; total protein, allozyme genotype, haplotype based on the six-cutter restriction analysis, *X* chromosome line, replicate vial, and pairs of samples from each vial which were assayed on different days (blocks). Statistical analyses were carried out using the SAS Statistical Program Package (SAS Institute, Inc.).

**Radial immunodiffusion:** The purified B enzyme was used to generate polyclonal antibody in rabbits for the quantitative measure of G6PD protein levels in different lines by radial immunodiffusion (MANCIGI, CARBONARA and HEREMANS 1965). Radial immunodiffusion was carried out at  $4^{\circ}$  on glass plates in 1% agarose (BRL Ultrapure) in Tris-acetate (0.0875 M Tris, 0.0185 M acetic acid, pH 8.6) buffer containing 9.5% antisera, 3.3% PEG, and 0.1 mM NADP. Plates were stained for G6PD activity as in EANES and HEY (1986). The diffusion area (designated here as AUE) is linear with amount of G6PD protein over the range of variation studied. The four supernatants prepared per line in the activity assays were used to estimate enzyme protein level (7  $\mu$ l per well, and 16 samples per RID plate). To standardize concentrations across many plates, a fourfold dilution series of a standard ammonium sulfate protein preparation of the *B* allele was also loaded on each plate. Individual sample measurements were expressed relative to a dilution of 4, 3, 2, and 1 AUE (arbitrary units of enzyme). From analysis of purified enzyme it is estimated that 1 AUE  $\approx 1.4 \times 10^{-5}$  mg G6PD protein.

**Specific activity estimation:** Comparisons of specific activity (units activity  $\text{mg}^{-1}$  purified enzyme) were made using two independent approaches. The first was to immediately assay G6PD activity in the fraction showing peak G6PD activity as it eluted from the ADP-agarose column. Samples of this peak fraction were frozen for protein determination by the BRADFORD (1976) method. Relative specific activity was also estimated using the observed correlation of *X* chromosome line activity against line G6PD protein level as determined by the radial immunodiffusion study of the 24 *G6pd<sup>A</sup>* and 23 *G6pd<sup>B</sup>* lines. We assume that within G6PD allele variation in G6PD activity reflects variation in protein level, and if there are no specific activity differences the slopes of the major axes of the bivariate plots will be equal.

**Estimation of kinetic parameters:** Virtually all eukary-

TABLE 1

ANOVA of G6PD activity variation among 45 lines collected from Davis Peach Farm, New York, and Watsonville, California

Source	d.f.	SS	MS	F	P
Total protein	1	0.6426	0.6426	110.94	0.0001
A/B genotype	1	0.4656	0.4656	7.18	0.016
Haplotype	16	1.0346	0.0647	1.01	NS
Line	27	1.7332	0.0641	11.08	0.001
Vial	1	0.0122	0.0122	2.11	NS
Block (days)	1	0.0089	0.0089	1.55	NS
Error	135	0.7819	0.0058		

NS = not significant.

otic G6PDs follow a bisubstrate ordered sequential binding model (LEVY 1979), where NADP binds first followed by glucose-6-phosphate, with the sequential release of the product D-glucose- $\delta$ -6-phosphate followed by NADPH. In aqueous solution D-glucose- $\delta$ -6-phosphate is unstable, so inhibition by it is insignificant. The rate Equation 1 describing initial velocity for this reaction is,

$$v = \frac{V_{\max}[A][B]}{K_{iA}K_B + K_A[B] + K_B[A] + [A][B] + Q(K_{iA}K_B + K_A[B])/K_{iQ}} \quad (1)$$

(KANJI, TOEWS and CARPER 1976; THOMPSON, SPIVEY and KATZ 1976), where A = NADP, B = G6P and Q = NADPH. Initial reaction rates were measured spectrophotometrically in a Gilford Response spectrophotometer with Thermostat temperature control. Reaction rates were determined in parallel for both variants, and individual cuvette use was randomized with respect to genotype every series of reactions. All assays were carried out in 0.11 Na-MOPS buffer at pH 7.15, as an approximation of physiologically buffered pH (SOMERO 1978). To estimate the apparent  $K_M$  values for both NADP and glucose-6-phosphate, initial estimates were obtained at 25° using eight substrate concentrations from 0.5  $K_M$  to 20  $K_M$  (initial guesses were from the literature; LEVY 1979). Over this range, fits of initial rate versus substrate concentration did not significantly deviate from expectation under Michaelis-Menton kinetics. From these initial rate estimates, an optimal design was determined where for each variant a  $K_M$  estimate was determined from 10–20 replicate initial velocity estimates made at each of two substrate concentrations approximating 0.5  $K_M$  and  $V_{\max}$  conditions (DUGGLEBY 1979). Enzyme concentrations were also adjusted so that both variants showed effectively identical  $V_{\max}$  values during  $K_M$  and  $K_i$  estimation at each temperature.  $K_M$  estimation under this design is robust if preliminary estimates of  $K_M$  are close to the true values (WEISS and DARVY 1981). From the initial rate data final  $K_M$  and  $K_i$  values were estimated by nonlinear regression (CORNISH-BOWDEN and ENDRENYI 1981). Cuvette enzyme concentrations were held such that less than 5% of the substrate was consumed during a 10–25-min reaction event, except for the estimates of  $K_M$  and  $K_i$  for NADP and NADPH, where, at low substrate concentrations, initial velocities were estimated by the fitting of chords to reaction trajectories of varying duration, and solving for  $t = 0$ . All reactions were carried out in 0.09 mg ml<sup>-1</sup> BSA. Substrate concentrations were determined enzymatically (LOWRY and PASSONNEAU 1972) and checked daily.

**Generation of  $G6pd^B$  P element revertants and viability measurement:** The lines used in the revertant study (4B and 2Blo1) were two of the paired recombinant test chromo-

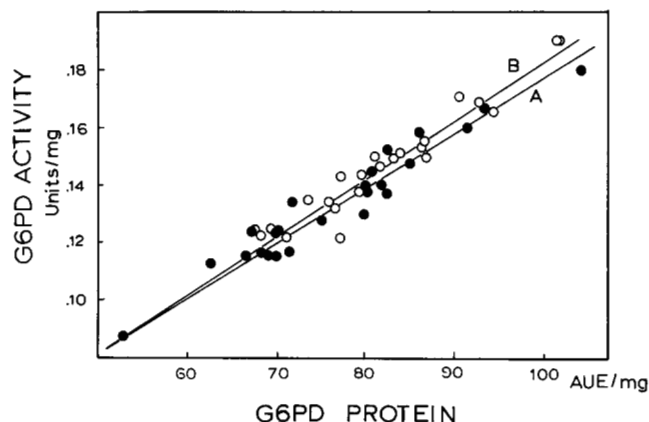


FIGURE 1.—Distribution of 47 bivariate line means for G6PD activity and G6PD protein level. The filled and open circles denote A and B allele line means respectively, and the axes values are in units mg<sup>-1</sup> total soluble protein and AUE mg<sup>-1</sup> total soluble protein (see text for definition of AUE). The slopes of the major axes for the A and B correlations are 0.00183 and 0.00203 units AUE<sup>-1</sup>. The major axes are not statistically different.

somes reported in the study of the  $G6pd^{Blo1}$  activity allele described in EANES and HEY (1986). The recombinant chromosomes are genetically identical for over 98% of the X chromosomes, and have similar autosomal backgrounds. The original  $Blo1$  allele possesses B allozyme mobility but only 20–30% of the crude activity. Because of its low activity, it has nearly normal viability in association with the  $6Pgd^{lo1}$  allele. Examination of this line by *in situ* hybridization to its polytene chromosomes with the P element bearing clone p $\pi$ 25.1 (O'HARE and RUBIN 1983) showed the presence of a P element at 18F, a minor polytene band and the site of the G6PD locus. This allele was restriction mapped as described in EANES *et al.* (1989), and carries a 2.96-kb insertion in the first intron.

To recover P element revertant mutations from this allele, males carrying the replicate recombinant  $car G6pd^{2Blo1} 6Pgd^{lo1}$  chromosome, designated 2Blo1, were crossed with an M cytotype line C(1)DX to produce P-M dysgenic male offspring. From this cross 100 males were individually mated with two  $FM6/N^{264-84}$  virgin females each and placed in separate vials. From each cross ten  $FM6/car G6pd^{lo1} 6Pgd^{lo1}$  females were mated with their  $FM6$  brothers and placed individually in vials containing 54 g D-glucose, 5 g yeast, 16 g agar, and 7 ml propionic acid per 1000 ml at 25°. The  $G6pd^B 6Pgd^{lo1}$  genotype shows very depressed viability on standard test media (see EANES and HEY 1986), and becomes effectively lethal on this food. All X chromosomes indicating the acquisition of a lethal or semilethal chromosome (consistently few or no  $car$  males) were saved balanced over  $FM6$ .  $FM6/+$  females from each line were screened electrophoretically (the  $FM6$  chromosome carries the A allele) for noticeable increase in B electromorph activity, and crossed with  $w^+Y/FM6$  males. The  $w^+Y$  translocation carries a small region of the X chromosome including the  $6Pgd^+$  allele. Recovery of both G6PD activity and full viability in  $w^+Y$  males from this cross implicates the lethal phenotype with the former interaction between loci. Male viability was estimated as the proportion of males emerging ( $car$  and  $FM6$ ).

**mRNA quantification:** Polyadenylated mRNAs from frozen male adult flies were prepared as described in BINGHAM and ZACHAR (1985) and analyzed by Northern transfer (MANIATIS, FRITSCH and SAMBROOK 1982) to Gene Screen Plus nylon filters. Relative levels of G6PD mRNA were

TABLE 2

ANOVA of G6PD protein level variation among 43 lines from Davis Peach Farm, New York, and Watsonville, California

Source	d.f.	SS	MS	F	P
Total protein	1	3.272	3.272	32.31	0.0001
A/B genotype	1	2.365	2.365	3.19	NS
Haplotype	13	9.631	0.741	0.89	NS
Line	25	20.76	0.831	8.20	0.0001
Vial	1	0.136	0.136	1.35	NS
Block (days)	1	0.642	0.642	6.34	0.0132
Error	116	11.74	0.101		

NS = not significant.

determined by autoradiography after hybridizing with a 3-kb  $^{32}\text{P}$ -labeled *SalI-SalI* fragment (donated by S. HORI) corresponding to the front of the *G6pd* gene. To standardize for differences during preparation the same filters were stripped and rehybridized with the *λsc112* probe (CAMPUZANO *et al.* 1985) to the *yellow* gene region. All test chromosomes are identical for this region, and therefore should show similar levels of *yellow* transcript. Absolute amounts of both mRNAs were determined by densitometry and G6PD levels then expressed relative to *yellow* transcript level.

**Restriction map analysis:** The restriction maps of *2Blo1* and *2Blo1RV* were determined as described in EANES *et al.* (1989).

## RESULTS

**Interline and allozyme variation:** Table 1 lists the ANOVA for G6PD activity variation among the 45 lines (some lines did not have their haplotypes determined), which is partitioned into restriction map haplotype (EANES *et al.* 1989), allozyme genotype, line, vial, block and error. Total soluble protein was used as a covariate. The lines were not partitioned by locality since both locality samples carry similar haplotypes (EANES *et al.* 1989). Significant G6PD activity variation is attributed to both G6PD allozyme and X chromosome line. The A genotype averages about 9% lower activity than the B genotype. The line effects may be attributed largely to the X chromosome because all lines possess different X chromosomes, but share common autosome backgrounds because of the extraction crosses. There is no significant contribution of haplotypes as defined by the six-cutter restriction maps of the *G6pd* region. However, it is worth noting that the line with the lowest activity (only 60% of the mean) carries a 10-kb *B104* transposable element (SCHERER *et al.* 1982) in the first intervening sequence (EANES *et al.* 1989).

Preliminary studies by rocket immunoelectrophoresis repeatedly showed 25% higher levels of enzyme protein for the *G6PD<sup>A</sup>* genotype, as reported by WILLIAMSON and BENTLEY (1983). However, comparisons with purified enzyme yielded the same 25% higher level with equivalent amounts of each variant. This result might be expected since, under electrophoresis, the dimeric A variant migrates faster than the tetra-

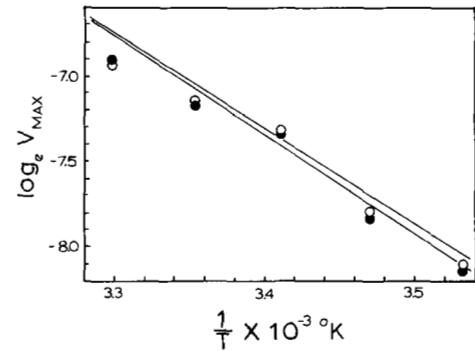


FIGURE 2.—The Arrhenius plots for the two variants. The ordinate is  $\ln V_{\max}$  and the abscissa is the reciprocal of temperature in degrees Kelvin. The regression equations are  $Y = -26.30 + 0.063X$  and  $Y = -25.32 + 0.060X$  for the A (filled circles) and B (open circles) allozymes respectively, and the slopes are not statistically different.

meric B variant, and this could differentially effect the formation of the enzyme-antibody precipitate. Conversely, radial immunodiffusion carried out for 7–9 days at 4° yielded statistically identical diffusion areas for equal amounts of purified A and B enzyme.

Figure 1 shows that G6PD line activity (as units  $\text{mg}^{-1}$  total soluble protein) and line G6PD protein levels (as AUE units  $\text{mg}^{-1}$ ) are highly correlated ( $r = 0.97$ ,  $P < 0.01$ ). Table 2 provides a summary of the ANOVA for G6PD protein level as determined by radial immunodiffusion. There is a highly significant line contribution to G6PD protein variation, but the allozyme genotype and haplotype contributions are not statistically significant.

**Estimation of specific activities and G6PD protein level:** From G6PD activity measurements made immediately on the affinity-purified enzyme preparations specific activities were estimated at 171.6 and 189.1 units  $\text{mg}^{-1}$  G6PD protein for the A and B variants respectively. By examining the correlation of G6PD line activity and G6PD line protein level for the two genotypes separately (Figure 1), we may use the slope estimates of the major axes to estimate relative specific activities. The slopes of the major axes are  $0.00183 \pm 0.00091$  and  $0.00202 \pm 0.00011$  units AUE $^{-1}$  for the A and B genotypes respectively. This is consistent in direction with the relative difference above, but is not a statistically significant difference.

The Arrhenius plots of  $\ln V_{\max}$  vs. the reciprocal of temperature ( $^{\circ}\text{K}$ ) are given in Figure 2. The slopes of the two allozymes are not statistically different. This indicates that the relative  $k_{\text{cat}}$  values remain the same across the temperature range from 10° to 30°.

**Estimates of apparent  $K_M$  for glucose-6-phosphate and NADP, and  $K_I$  for NADPH:** Estimates of the kinetic parameters are summarized in Table 3. The low  $K_M$  for NADP ( $< 10 \mu\text{M}$ ) required measuring very low reaction rates to obtain accurate estimates of initial velocities at the  $K_M$  concentrations. This prac-

TABLE 3

Summary of the kinetic properties of the G6PD A and B variants at 25°

Property	G6PD-A	G6PD-B
$K_M[\mu\text{M G6P}]$	$34.12 \pm 0.51$	$30.65 \pm 0.61$
$K_M[\mu\text{M NADP}]$	$6.28 \pm 0.12$	$7.06 \pm 0.16$
$K_I[\mu\text{M NADPH}]$	$10.05 \pm 0.37$	$11.67 \pm 0.38$
Units $\text{mg}^{-1}$ G6PD	171.1	187.7
Units $\text{AUE}^{-1}$ G6PD $\times 10^3$	$1.83 \pm 0.091$	$2.02 \pm 0.11$
Units $\text{mg}^{-1}$ soluble protein	$0.134 \pm 0.004$	$0.1474 \pm 0.001$

tical limit prevented the study of reaction rates where both G6P and NADP concentrations were varied simultaneously, and thus the estimation of the dissociation constant for the NADP-enzyme complex,  $K_{i\text{-NADP}}$  (Equation 1). The estimates of the apparent  $K_M$  for glucose-6-phosphate over a temperature range from 10° to 30° are shown in Figure 3. The B variant shows a significantly lower  $K_M$  over most of the upper range, the variants have virtually identical values at 15 and 20°, and there is a statistically significant reversal at 10°. The estimates of  $K_M$  for NADP across the temperature range from 10 to 30° are shown in Figure 4. The two variants show no significant difference over most of the temperature range, except at 25°. Overall the A variant has a statistically significant lower value (paired  $t$ -test,  $t = 3.13$ ,  $P < 0.05$ ).

To measure a single paired  $K_I$  comparison with the same statistical power as a single  $K_M$  estimate requires was about fourfold greater effort. For this reason, and because we are primarily interested in comparing the *in vitro* and *in vivo* estimates, the  $K_I$  for NADPH was determined only at 25°, the same temperature that the *in vivo* experiments of EANES (1984) and EANES and HEY (1986) were carried out. The  $K_I$  values for NADPH were 10.05 and 11.67  $\mu\text{M}$  NADPH for the A and B variants, respectively, and this is a statistically significant difference ( $t = 3.043$ ,  $P < 0.01$ ).

***G6pd*<sup>Blo1</sup> revertant activity and viability:** A total of 1218 *car* X chromosomes were recovered from the P-M dysgenic crosses. Among these, 48 chromosomes were hemizygous lethal, but only one line (designated as *G6pd*<sup>Blo1RV</sup>) showed an increase in G6PD activity after electrophoresis and could be saved by the presence of the *w*<sup>+</sup>Y translocation. The approximate restriction map of the original *2Blo1* allele with the position of its estimated 2.96-kb insertion and the partial revertant, *2Blo1RV*, are depicted in Figure 5. There has been a drop in size of about 1 kb with loss of the *SalI* site in the revertant.

Table 4 summarizes the G6PD activity, relative mRNA, and viability measures for this putative revertant chromosome, the original nonrevertant *2Blo1* chromosome, and its recombinant *G6pd*<sup>B</sup> homolog (*4B*) from EANES and HEY (1986). The G6PD activity

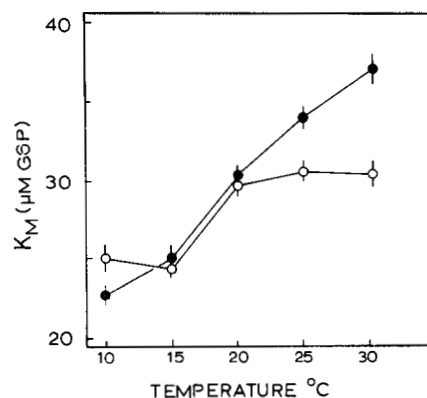


FIGURE 3.—Plot of the apparent  $K_M$  for glucose-6-phosphate against temperature for the A (closed circles) and B (open circles) variants. Confidence intervals are one standard error.

has doubled in the revertant allele from 29% to 58% of *4B* variant activity, and this is associated with increased mRNA level. As required by the screen, the relative viability on the test media has dropped sharply from about 90% to less than 50% of wild type.

## DISCUSSION

The objective of this study was to characterize the *in vitro* biochemical phenotypes associated with the two common allozymes of G6PD in *Drosophila melanogaster*, and then independently validate predicted differences using an *in vivo* assay. The initial focus was on the properties that could be directly determined by quantifying genotype-specific activity and protein variation, and estimating relevant catalytic parameters by steady-state kinetics. The second focus was on utilizing partial activity revertants recovered from a *P* element-derived low activity mutation to examine the sensitivity of the earlier described suppression scheme to G6PD activity level. This could be used to validate the prediction of the *in vitro* analysis.

The comparative assessment of *in vitro* catalytic efficiencies from steady-state kinetic data is a complicated issue, and much has been written about this question (see critique by HALL and KOEHN 1983). Direct measurements of intracellular pH indicate maintenance across a narrow range spanning neutrality (ROOS and BORON 1981), yet studies often fail to approximate physiological pH values and ionic strength. Experimental designs used in parameter estimation are often inappropriate and provide biased estimates of parameters. Finally many studies fail to measure all the parameters indicative of catalytic efficiency, concentrating on assays of activity under saturating substrate levels. This study attempts to recognize and comply with these issues.

Several studies have reported large activity differences between G6PD allozyme genotypes. However, confidence in these differences is compromised by the

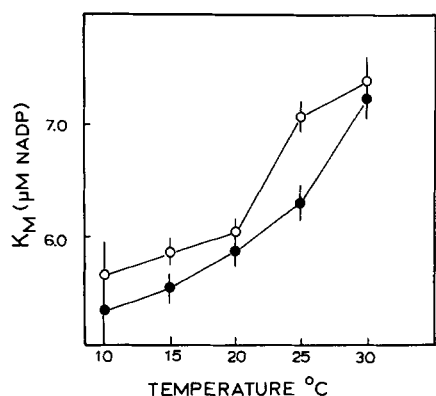


FIGURE 4.—Plot of the apparent  $K_M$  for NADP against temperature for the A (closed circles) and B (open circles) variants. Confidence intervals are one standard error.

instability of the A variant in Tris-HCl buffers (CAVENER and CLEGG 1981). The studies by WILLIAMSON and BENTLEY (1983), HORI and TANDA (1980, 1981), STEELE, YOUNG and CHILDS (1968) and BIJLSMA and VAN DELDEN (1977) used Tris-HCl buffers and reported the A genotype to have from 19 to 35% lower activity. Conversely, using phosphate buffers MIYASHITA *et al.* (1986) saw a relative difference of about 16% (estimated from Figure 3 in that paper), while CAVENER and CLEGG (1981) reported no significant difference between allozymes. Based on our 95% confidence limits and the error variance observed in those studies, this study is not significantly different from either study. With the exception of the last two studies, it appears earlier studies have overestimated the difference in crude activity between the allozyme genotypes.

The two G6PD allozymes possess *in vitro* catalytic properties that could result in different *in vivo* catalytic efficiencies at 25° (Table 3). There is an overall 9% relative difference between the genotypes in activity per fly, which will reflect  $V_{max}$  differences. Since  $V_{max} = k_{cat}[E_0]$ , where  $k_{cat}$  is the "turnover number" per active site, and  $E_0$  is the concentration of active sites or enzyme concentration, this difference has two potential allele-specific causes, one catalytic and the other related to protein level. The best method to measure  $E_0$  is to titrate with dead end irreversible inhibitors, as used effectively in the case of esterase-6 (WHITE, MANE and RICHMOND 1988) and Adh (WINBERG, HOVIK and MCKINLEY-MCKEE 1985). Unfortunately, without the availability of a suitable inhibitors for G6PD, we have been forced to use more equivocal approaches. Analysis of line variance in G6PD protein levels, estimated by radial immunodiffusion, shows no statistically significant difference between the allozyme genotypes. Our direct estimates of specific activity from purified enzyme are similar to the relative estimates of  $195.3 \pm 11.9$  and  $203.5 \pm 8.0$  (based on four replicates) by HORI and TANDA (1981), and together they suggest differences in  $k_{cat}$ .

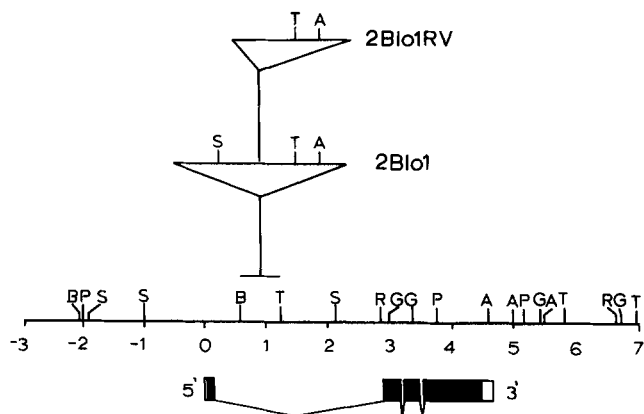


FIGURE 5.—Restriction map of the 10-kb *G6pd* region showing the position of the P element insertion in the *2Blo1* and partial revertant *2Blo1RV* alleles. The position and organization of the *G6pd* locus are taken from FOUTS *et al.* (1988). The map is from the original *Blo1* allele, and neither allele is mapped for *PstI* (P) and *EcoRI* (R). The other letters stand for the restriction enzymes *AvaI* (A), *BamHI* (B), *BglIII* (G), *SalI* (S) and *SstI* (T). Both alleles contain the large 12.5-kb insertion *i* (not shown) between 5.8 and 6.6, as depicted in EANES *et al.* (1989).

However, the difference in the slopes of the major axes of the allozyme-specific correlations between G6PD activity and G6PD protein (also indicative of  $k_{cat}$ ) are not statistically different, so it is equivocal whether the activity difference is the result of differences in  $k_{cat}$  or  $E_0$ . Both sources deviate in the direction consistent with involvement in  $V_{max}$  differences, but neither is statistically significant. Since there is only a 9% difference in allozyme-dependent activity it is difficult to statistically partition the causes.

If intracellular substrate concentrations are below saturating levels, as generally assumed (FERSHT 1977), then  $V_{max}$  alone is insufficient as a predictor of catalytic efficiency. The individual Michaelis and inhibition parameters become important and must be estimated. Because of the experimental difficulties inherent with estimating  $K_M$  values at increasing lower substrate concentrations, and the compounding of error variances in the estimation of  $K_I$ , our confidence in the estimates of these parameters may be given in the decreasing order as  $K_{M-G6P} > K_{M-NADP} > K_{I-NADPH}$ , which also reflects the increasing coefficients of variation. At 25°, the temperature where our *in vivo* suppression studies have been carried out, there are significant variant-specific differences in  $V_{max}$ , the  $K_M$  values for both glucose-6-phosphate and NADP, and the  $K_I$  for NADP.

We can use Equation 1 to predict for the two variants the relative difference in catalytic efficiency represented collectively by these parameters. We possess estimates of all parameters except  $K_{i-NADP}$ , which is generally about twice the value of the  $K_M$  for NADP in studies where this has been estimated (KANJI, TOEWS and CARPER 1976; THOMPSON, SPIVEY and KATZ 1976). We will assume G6P and NADP concen-

TABLE 4

Comparison of G6PD activity, mRNA level and viability for recombinant chromosomes bearing the *G6pd*<sup>4B</sup>, *G6pd*<sup>2Blo1RV</sup> and *G6pd*<sup>2Blo1</sup> alleles

Allele	G6PD activity	mRNA level	Viability
<i>G6pd</i> <sup>4B</sup>	7.70 ± 0.23	1.00	0.209 ± 0.030
<i>G6pd</i> <sup>2Blo1RV</sup>	4.50 ± 0.13	0.51	0.256 ± 0.019
<i>G6pd</i> <sup>2Blo1</sup>	2.27 ± 0.18	0.39	0.465 ± 0.037

trations equal to the average observed  $K_M$  values and will use the NADPH/NADP ratio of about 9.2 estimated by GEER, LINDEL and LINDEL (1981). The parameter estimates predict that at 25° the *G6pd*<sup>A</sup> genotype will possess about 78% of the activity of the *G6pd*<sup>B</sup> genotype. By randomly sampling 10 individual values from the distribution of 10–20 variant-specific estimates available for each parameter we estimate the lower and upper 95% confidence limits of this mean difference to be 66.5% and 89.5%, respectively.

Is this *in vitro* difference in catalytic efficiency indicative of the *in vivo* activity differences? We propose that in the *6Pgd*<sup>lo1</sup> background, 6-phosphogluconate is accumulating at the 6PGD step, and the level of that intermediate depends on the activity of G6PD. This predicts a negative functional relationship between viability and *in vivo* activity. Assuming this relationship the 6PGD lethal suppression studies (EANES 1984; EANES and HEY 1986) would predict that the A genotype has lower activity, but without knowledge of the shape of the function describing the relationship between suppression viability and *in vivo* activity we do not know whether this requires a large drop in activity. Examining the relationship between activity and viability using a number of independent mutant alleles, as in EANES and HEY (1986), is complicated because of the many different catalytic features (Equation 1) that are free to vary between the electrophoretic variants (which differ by amino acid substitutions). In that study we measured only  $V_{max}$  differences, which explains the unclear relationship between viability and activity seen in Figure 2 in EANES and HEY (1986). To study the relationship between activity and viability we require variants that differ only in enzyme level  $E_0$ , such as a series of transcriptional variants of the B allele.

One of the low activity B variants, *Blo1*, discovered in a natural population by EANES and HEY (1986) appears to be a P element-mutated allele possessing a 2.96-kb insertion within the 5' end of the first intron. It possesses low G6PD activity (only 20–30% of normal B), and is an excellent suppressor of the strongly depressed viability associated with the low activity *6Pgd*<sup>lo1</sup> mutant. P element-derived partial revertants and rearrangements have been used to study features transcriptional control for a number of genes (*e.g.*, VOELKER *et al.* 1984; TSUBOTA and SCHEDL 1986;

GEYER *et al.* 1988). A fraction (perhaps the majority) of P-M dysgenic excision events are partial excisions where an indeterminate amount of the element is retained (ENGELS 1989), and a spectrum of phenotypes are recovered. The *Blo1* allele therefore presented the opportunity to vary increasing amounts of B allele activity through partial reversion mutations. Only a single partial revertant was ultimately recovered but it proved very informative. This revertant, which had lost the ability to suppress *6Pgd*<sup>lo1</sup> low viability mutant on test media showed only 60% of the normal levels of B variant activity. It therefore appears that a substantial drop in G6PD activity is necessary to suppress the viability loss associated with the *6Pgd*<sup>lo1</sup> low activity mutant. In this genetic background the A allele shows repeatable viabilities that are 70–80% wildtype (EANES 1984; EANES and HEY 1986). Since the *G6pd*<sup>A</sup> allele suppresses the 6GPD associated lethality better than this partial revertant, we must conclude that it possesses less *in vivo* activity. This would indicate that the A variant possesses at most only 60% of the activity of a normal *G6pd*<sup>B</sup> variant.

From a qualitative standpoint the two assessments of relative activity give similar answers; the A genotype has lower activity. However, quantitatively the *in vivo* analysis predicts a relatively large activity difference between genotypes. The kinetic results show that the allozyme polymorphism for G6PD may involve dissimilarity in a number of parameters affecting catalytic efficiency, but in aggregate these do not predict the magnitude of the *in vivo* difference. One possibility is that the remaining unmeasured parameter,  $K_{i-NADP}$ , would explain the discrepancy. However, it is also possible that too much is being asked of the steady-state kinetic analysis. In such a kinetic comparison it is impossible, to sample the entire experimental variable space (*e.g.*, temperature and pH), the microscopic intracellular substrate concentrations are unknown, and the effect of every conceivable cofactor or inhibitor (such as ATP) can not be determined without yet greater increases in complexity. Enzyme kinetics developed as an experimental approach to understand the fundamental mechanisms of enzyme catalysis (FERSHT 1977; CLELAND 1977), and the jump to comparative studies of allozyme polymorphism involves a certain act of faith. Another explanation for the observed discrepancy is that viability as used here is not a good indicator of *in vivo* activity. The original argument was developed from the observation that null alleles of 6PGD, which were effectively lethal, could be rescued by null alleles of G6PD (HUGHES and LUCCHESI 1977; GVOZDEV *et al.* 1976, 1977). This lead to the prediction that variation in ability to rescue viability would depend on G6PD activity. If this is true, it is difficult to see how the A variant would have

higher activity than the revertant *Blo1* allele, yet better suppress the lethal effects of low 6PGD activity. Studies are underway to independently measure *in vivo* flux using radiolabeled D-glucose (CAVENER and CLEGG 1981).

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