

Direct Measurement of *in Vivo* Flux Differences Between Electrophoretic Variants of G6PD from *Drosophila melanogaster*

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ABSTRACT

Demonstrating that naturally occurring enzyme polymorphisms significantly impact metabolic pathway flux is a fundamental step in examining the possible adaptive significance of such polymorphisms. In earlier studies of the glucose-6-phosphate dehydrogenase (G6PD) polymorphism in *Drosophila melanogaster*, we used two different methods, exploiting both genotype-dependent interactions with the *6Pgd* locus, and conventional steady-state kinetics to examine activity differences between the two common allozymes. In this report we use $1\text{-}^{14}\text{C}$ - and $6\text{-}^{14}\text{C}$ -labeled glucose to estimate directly genotype-dependent flux differences through the pentose shunt. Our results show the *G6pd^A* genotype possesses statistically lower pentose shunt flux than *G6pd^B* at 25° . We estimate this to be about a 32% reduction, which is consistent with the two former studies. These results reflect a significant responsiveness of pentose shunt flux to activity variation at the G6PD-catalyzed step, and predict that the G6PD allozymes generate a polymorphism for pentose shunt flux.

THE enzyme and polymorphism for glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) in *Drosophila melanogaster* have been a focus of study in a number of investigations (YOUNG, PORTER and CHILDS 1964). This laboratory has examined the polymorphism in natural populations, focusing on 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* (EANES 1984; EANES and HEY 1986) and *in vitro* function (EANES, KATONA and LONGTINE 1990), and restriction map variation in the *G6pd* locus region (EANES *et al.* 1989). 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).

G6PD catalyzes the first step in the oxidative pentose phosphate cycle, or pentose shunt. The pathway's role is involved in synthesis of NADPH for lipid synthesis and detoxification, pentose phosphate for nucleic acid synthesis, and triose phosphate for glycogen synthesis. Using radiolabelled glucose and adult *D. melanogaster* deficient for G6PD activity, GEER, BOWMAN and SIMMONS (1974) confirmed that the pentose shunt is blocked. Subsequent studies by GEER, LINDEL and LINDEL (1979) estimated that about 40% of reduced NADP is contributed by this pathway, and the rate of lipidogenesis is tightly correlated with pentose shunt enzymatic activities, which can be induced under varying carbohydrate and lipid conditions. GEER *et al.* (1981) have reviewed the extensive

nutritional studies on the pentose shunt that have been carried out in *D. melanogaster*.

We have used two different methods to examine the hypothesis that activity differences exist between the two polymorphic allozyme variants of G6PD. Studies by other investigators suggested that the fast and slow electrophoretic alleles, designated here as *A* and *B*, possess different *in vitro* activities (STEELE, YOUNG and CHILDS 1968; BIJLSMA and VAN DER MEULEN-BRUIJNS 1979; HORI and TANDA 1980, 1981; WILLIAMSON and BENTLEY 1983). This was supported by EANES (1984) where dramatic differences were seen in the ability of the *G6pd^A* and *G6pd^B* genotypes to suppress the lethality associated with a 6-phosphogluconate dehydrogenase (6PGD) mutation low in activity, designated *6Pgd¹⁰¹*. In a *G6pd^B* background this mutation has low viability, while it is nearly normal in the *G6pd^A* background. This difference probably reflects a lower *in vivo* activity associated with the *G6pd^A* genotype, resulting in a reduced accumulation of 6-phosphogluconate, a potent inhibitor of glycolysis. This observation was repeated in EANES and HEY (1986) for a series of rare *G6pd* variants. We proposed that allele-specific differences in suppression reflected two clusters of *in vivo* activity, depending on whether the rare variant was derived from the *A* or *B* common allele. Finally, we have examined, by steady-state kinetics, the *in vitro* activity of the highly purified G6PD variants (EANES, KATONA and LONGTINE 1990). Our results predict that at 25° the *G6pd^A* genotype possesses about 40% lower activity than *G6pd^B*. While these studies both supported the hypothesis of activity differences, neither directly measured flux.

CAVENER and CLEGG (1981) compared the relative fluxes between the putative high and low activity dilocus allozyme genotypes at the *G6pd* and *6Pgd* loci in *D. melanogaster* using the method of WOOD, KATZ and LANDAU (1963). This method was originally proposed for assessing the apportionment of carbohydrate metabolism between the Embden-Meyerhof and pentose shunt pathways. It is based on the rationale that the first carbon of glucose (1-C) is lost as CO₂ as it passes through the pentose cycle, while the number six carbon (6-C) is returned to the EM pathway. By using D-[1-¹⁴C]glucose and D-[6-¹⁴C]glucose separately as substrates in parallel experiments, increasing pentose shunt activity is measured by 1-C/6-C derived ¹⁴C count ratios that are less than one. They observed statistically significantly greater pentose shunt flux associated with the proposed higher activity *G6pd^B*-*6Pgd^S* dilocus genotype, but the experiment was not specifically designed to partition the contribution of the genotypes at the *G6pd* and *6Pgd* loci separately. In this report we extend the study of functional differences to this third independent method and focus on the *G6pd* genotypes. We estimate that the low activity *G6pd^A* allele possesses about 32% lower pentose shunt flux than the *G6pd^B* allele.

MATERIALS AND METHODS

Wild-type experimental lines: To generate lines that were either *G6pd^A* or *G6pd^B*, fixed for *6Pgd* genotype, and randomized for the wild autosomal background, isofemale lines were collected from Davis Peach Farm, Mt. Sinai, New York, in 1989, full-sib mated and electrophoresed to determine mated genotypes at the *G6pd* and *6Pgd* loci. Ten independent lines homozygous for *G6pd^A*-*6Pgd^F* and 23 homozygous lines for *G6pd^B*-*6Pgd^F* were isolated and pooled within genotype. Adults from these experimental composite populations provided the larvae for the flux experiments.

Flux studies: Larvae were raised in axenic culture at 25° for the duration of each experiment. All axenic stage transfers were carried out in a laminar flow hood. Adults from the mass lines were allowed to lay eggs for four hours on standard corn meal media covered with a dead yeast paste. Batches of several hundred eggs were collected from the surface by rinsing the eggs into a 20-ml beaker, and dechlorinating by two washes in 10 ml of 2.5% sodium hypochlorite, with 0.1% Triton X-100. Next, eggs were transferred into a plastic syringe, where they were surface sterilized with 0.1% benzyltrimethyl-*n*-hexadecylammonium chloride (ROBERTS 1986), followed by 80% ethanol, and final rinses with sterile water and sterile 0.7% NaCl. Dechlorinated sterile eggs were transferred to the surface of 2% agar plates. Newly hatched larvae (less than 20–22 hr old) were transferred into eight dram vials (50 larvae each) containing 5 ml of axenic Sang's medium C (see ASHBURNER 1989) and 290 mM sucrose to induce pentose shunt activity (CAVENER and CLEGG 1981). After 84 hr, samples of 10 larvae were transferred with wooden applicator sticks to 2-ml vials with 1.5 ml of identical medium, and 1 μCi of either D-[1-¹⁴C]glucose or D-[6-¹⁴C]glucose (Amersham). After 48 hr larvae were collected with forceps and ground in samples of 10 larvae each in 300 liters of 0.05 M phosphate buffer (pH 7.4). Lipid and protein were extracted separately as de-

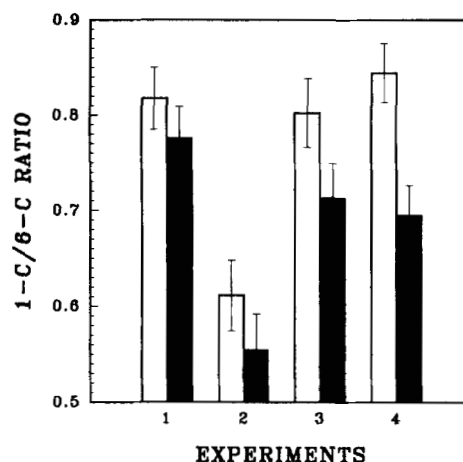


FIGURE 1.—Ratio of 1-C- and 6-C-derived ¹⁴C counts for the *G6pd^A* (open bars) and *G6pd^B* (solid bars) genotypes for each of four independent experiments. The vertical lines associated with each bar represent one standard error.

scribed in GEER and DOWNING (1972), and scintillation counts were carried out using ScintiVerse II (Fisher) in a Wallac 1410 Scintillation Counter (Pharmacia). Protein assays were done using the BRADFORD (1976) protein assay and bovine serum albumin as a standard.

It was recognized from our initial variance estimates and the data of CAVENER and CLEGG (1981) that for each individual experiment only a very large difference in flux would be statistically detectable with any reasonable type II error. Thus, testing the null hypothesis of genotype-dependent flux requires an experimental design where genotypic differences can be examined across experiments treated as random blocks. Because of the labor intensive nature of axenic culture, extraction of lipids and protein, assay of soluble protein, and scintillation counting, four separate experiments (treated statistically as random blocks) were carried out. Each consisted of five replicates of *G6pd^A* and *G6pd^B* genotypes reared separately on either D-[1-¹⁴C]glucose or D-[6-¹⁴C]glucose (a total of 20 vials per experiment).

Statistical analysis: Raw counts from total lipid and protein were first regressed (within each experiment) against total soluble protein as a covariate and then transformed to residuals from the fitted linear regression. A mixed model ANOVA was assumed, with counts as variates, genotype, and label-type (D-[1-¹⁴C]glucose or D-[6-¹⁴C]glucose) handled as fixed treatment effects and experiments as random effects in a general linear model (GLM) using SAS and pooling appropriate sums of squares when lower levels are nonsignificant. An approximate *F* statistic and degrees of freedom, as suggested by SATTERTHWAITTE (1946), were used to test label-type. Outliers were initially identified by SAS and tested for significance using the method of DIXON (1950) at a 1% significance level (one highly significant outlier, more than ten standard deviations from its group mean, was removed from the subsequent analysis).

RESULTS

In Figure 1, the results are presented as ratios of 1-¹⁴C- and 6-¹⁴C-derived counts with standard errors for each of the four independent experiments. The factorial ANOVA using ¹⁴C derived counts as variates is presented in Table 1. The first-order *G* × *E* interaction and second-order (*G* × *E* × *L*) interaction are

TABLE 1
ANOVA of 1-¹⁴C-, and 6-¹⁴C-derived counts for the *G6pd^A* and *G6pd^B* genotypes

Source	d.f.	SS	MS	F
Genotype (<i>G</i>)	1	20,334,598	20,334,598	6.41*
Experiment (<i>E</i>)	3	640,445,152	213,481,718	68.16***
Label-type (<i>L</i>)	1	242,837,558	242,837,559	9.08*
<i>G</i> × <i>L</i>	1	21,141,222	21,141,223	6.75*
<i>E</i> × <i>L</i>	3	73,694,099	24,564,700	7.77***
<i>G</i> × <i>E</i>	3	12,231,467	4,077,156	1.30 NS
<i>G</i> × <i>E</i> × <i>L</i>	3	7,569,665	2,523,222	0.80 NS
Error	63	199,164,643	3,161,344	

* $P < 0.05$; *** $P < 0.001$.

statistically nonsignificant permitting pooling of these interaction *SS* and their associated degrees of freedom with the error *SS* in subsequent tests of fixed effects and their interactions. The ANOVA shows statistically significant label-type, experiment and experiment-by-label interactions. These are expected simply because pentose-shunt function *per se* results in differential accumulation by label-type, and we expect its magnitude to vary with induction of the pentose shunt. In all four experiments the *G6pd^A* genotype shows lower flux (higher ratios). There is a significant G6PD genotype by label-type interaction, and this indicates differential flux by genotype. Therefore, we reject the null hypothesis of equal pentose shunt flux for the *G6pd* genotypes.

Assuming that zero flux will result in a 1-¹⁴C/6-¹⁴C count ratio of 1.0 (standardized for input 1-¹⁴C and 6-¹⁴C activities), we estimate from the mean of all four experiments that the *A* genotype possesses about a 32% lower flux (with 95% confidence limits of about 14.3%) than the *B* genotype.

DISCUSSION

The model of genetic dominance proposed by KACSER and BURNS (1981), and observed high frequencies of null activity variants for electrophoretically polymorphic enzymes of central metabolism in natural populations (VOELKER *et al.* 1980; LANGLEY *et al.* 1981; ALLENDORF, KNUDSEN and BLAKE 1982), challenge the notion that allozyme variants generate sufficient pathway flux variation to translate into fitness differences (EANES 1987). The KACSER and BURNS model predicts that only large activity differences between alleles will perturb pathway flux sufficiently to translate into fitness variation. Consistent with this prediction, the high frequency of null activity variants at many allozyme encoding loci indicates that marked reduction of enzyme activity (presumably halved in heterozygotes) either has little effect on associated pathway flux, or that changes in flux have little effect on fitness. Unfortunately, the direct experimental assessment of flux is a difficult technical problem for

most metabolic pathways. Thus with the exception of a handful of studies (CAVENER and CLEGG, 1981; MIDDLETON and KACSER 1983; ZAMER and HOFFMAN 1989; SILVA *et al.* 1989; FRERIKSEN *et al.* 1991), the issue remains poorly documented for most allozyme polymorphisms.

We are interested in examining the average effect of *G6pd* genotype on pentose shunt flux. The line construction, identical to that used by CAVENER and CLEGG (1981), assumes randomization (with respect to the G6PD genotypes) of genetic variation affecting pentose shunt flux. There are both X-linked and autosomal modifiers of G6PD activity (LAURIE-AHLBERG *et al.* 1980; MIYASHITA *et al.* 1986; EANES, KATONA and LONGTINE 1990), as well as a significant contribution by G6PD allozyme genotype (MIYASHITA *et al.* 1986; EANES, KATONA and LONGTINE 1990; MIYASHITA 1990). We have, by design, fixed the chromosomes for the *6Pgd^F* genotype, which is the common *6Pgd* allele in the Davis Peach Farm population, and the autosomal background is a random sample of the Davis Peach Farm population.

The results of this investigation corroborate the findings of our two earlier studies. The study of G6PD protein levels and steady-state kinetics by EANES, KATONA and LONGTINE (1990) predicted that at 25° the *A* genotype possesses about 28% lower activity than *B*, and this is derived largely from that variant's relatively lower affinity (higher K_M) for glucose 6-phosphate. In EANES (1984), EANES and HEY (1986) and EANES, KATONA and LONGTINE (1990), clear differences were seen in the ability of the *G6pd^A* and *G6pd^B* genotypes to suppress the lethality associated with a 6-phosphogluconate (6PGD) low activity mutation. This reflects a lower *in vivo* activity associated with the *G6pd^A* genotype, resulting in a reduced accumulation of 6-phosphogluconate. In EANES and HEY (1986) this was extended to a series of rare G6PD variants. We observed two clusters of different *in vivo* activity, presumably separating rare variants into two sets that were derived from the *A* and *B* common allozyme alleles respectively. Our recent analysis of

the amino acid sequences of those eleven alleles is, without exception, consistent with that prediction (W. F. EANES, unpublished). Finally, in EANES, KATONA and LONGTINE (1990) we used revertants of a *P* element associated low activity *B* allele to scale viability differences, and concluded that the *A* allele bearing genotypes possess an *in vivo* activity that is 40% lower than the *B* genotypes. Our results using direct flux measures with radiolabeled glucose are consistent with that magnitude of difference.

The evidence from these four studies supports the proposition that the G6PD allozyme polymorphism is associated with, and likely responsible for, a polymorphism for pentose shunt flux at 25°. The *G6pd^A* allele, which is the consequence of a single amino acid polymorphism (proline to leucine substitution at residue 382; W. F. EANES, unpublished), increases in frequency in temperate regions. The frequency is less than 5% in sub-Saharan Africa, but increases to about 90% in Europe, where an additional low activity allele, *G6pd^{AF1}* (EANES and HEY 1986), is found at frequencies of 5 to 15%. The *G6pd^A* polymorphism is reciprocally clinal with latitude in both North America and Australia (OAKESHOTT *et al.* 1983), increasing with more temperate climate. From these observations we propose a working hypothesis where an increase of the low activity *G6pd^A* allele has paralleled the spread of Afrotropical *D. melanogaster* into the temperate climates of the world. The observed lower levels of restriction site polymorphism associated with the *A* allele (EANES *et al.* 1989; MIYASHITA 1990) are consistent with this proposal.

The model and data presented by KACSER and BURNS (1981) to explain the general phenomenon of genetic dominance argue that the response of overall pathway flux to changes in activity at individual enzyme steps is low. The theory assumes flux is the consequence of many catalytically linked enzyme steps, each reversible and following Michaelis-Menten kinetics. Overall flux becomes a systematic feature of such a pathway, and a property of this system is that the relationship of flux to activity variation at any one step is nonlinear, the nonlinearity arising from the kinetic interactions between successive steps in the pathway.

In contrast to this expectation, the sensitivity of pentose shunt flux to activity variation at G6PD is high. Assuming the catalytic analysis is accurate, a reduction of G6PD enzyme activity of about 28% reduces pentose shunt flux by about 32%, implying high sensitivity. The mechanistic key to understanding the relatively high sensitivity associated with G6PD probably involves the fact that it sits at the branch point between glycolysis and the pentose shunt, and it is an effectively irreversible step. D-Glucolactone-6-phosphate, which is the product of the oxidation of D-

glucose-6-phosphate by G6PD, is unstable and spontaneously converts to 6-phosphogluconate, which does not inhibit G6PD. This is probably the mechanism behind the G6PD-6PGD activity viability interaction seen in our laboratory experiments (EANES 1984; EANES and HEY 1986; EANES, KATONA and LONGTINE 1990) and even among wild-type chromosomes (CLARK 1989). It is this regulatory potential that G6PD exerts on the pentose shunt that may make it a suitable target for adaptive substitution and polymorphism.

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