TARGETED SELECTION EXPERIMENTS AND ENZYME POLYMORPHISM: NEGATIVE EVIDENCE FOR OCTANOATE SELECTION AT THE *G6PD* LOCUS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

Published studies have reported significant selection with respect to the *G6pd* locus for *Drosophila melanogaster* reared on Na-octanoate food. We have reexamined the selective effects of Na-octanoate on egg to adult viability with respect to the *G6pd* polymorphism using specially constructed *X* chromosomes. Four experiments were carried out using different *6Pgd* backgrounds in two recombinant sets of chromosomes segregating for the *G6pd* locus but constructed so as to minimize variation over most of the *X* chromosome. In addition, two measures of viability were used, and the size of the experiments and their associated degrees of freedom are approximately double those reported in the former studies. Our results find no evidence for differential selection on *G6pd* genotypes (males and females) by Na-octanoate and, therefore, do not corroborate the positive results of selection reported by other investigators. The reasons for our different results are discussed.

MANY investigators have attempted direct measurements of fitness components in Drosophila for enzyme polymorphisms under laboratory conditions (see for example, GIBSON 1970; MARINKOVIC and AYALA 1975a,b; VAN DELDEN, BOERMA and KAMPLING 1978). This approach has regularly revealed significant fitness-related differences between allozyme genotypes, although these positive examples are often considered equivocal because of the confounding effects of potential nonrandom association between alleles at the locus of interest and closely linked loci (KIMURA and OHTA 1971; LEWONTIN 1974). To counter this criticism, experimentalists have coupled this direct fitness measurement with environmental conditions *targeted* directly at the presumed function of the study enzyme (CLARKE 1975). The best known examples are the use of ethanol as a selective environment for alleles at the *Adh* locus (GIBSON 1970; VAN DELDEN, KAMPING and VAN DIJK 1975) and the use of different carbohydrate sources in tests on the amylase locus (DE JONG and SCHARLOO 1976; HICKEY 1977). This coupled approach, with information on the functional biochemical phenotypes, has made a compelling case for the potential of selection on these polymorphisms. Finally, a series of informative studies using targeted selection experiments on allozymes in *E. coli* in chemo-

stats have seemingly taken this idea to its technical limit (DYKHUIZEN and HARTL 1980, 1983; HARTL and DYKHUIZEN 1981).

This approach has recently been applied to the glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44) polymorphisms in *D. melanogaster* in a series of studies using the fatty acid octanoate as a selective environment (BIJLSMA and VAN DELDEN 1977; BIJLSMA 1978; BIJLSMA and KERVER 1983). The rationale for this treatment arises from the fact that the pentose shunt generates about 40% of the NADPH generation for fatty acid synthesis in *D. melanogaster* (GEER, LINDEL and LINDEL 1979) and the observation that G6PD levels are reduced about 10% in larvae reared on a Na-octanoate diet. All three studies reported large selective differences between *G6pd* genotypes in Na-octanoate treatments. The implications of this observation are of particular importance and significance because of the increased involvement of this locus in population genetic and evolutionary problems (see for example, WILLIAMSON and BENTLEY 1984; COCHRANE, LUCCHESI and LAURIE-AHLBERG 1983; MIYASHITA and LAURIE-AHLBERG 1984; EANES 1984; OAKESHOTT *et al.* 1983; CAVENER and CLEGG 1981).

In view of this significance, we have undertaken this study to reexamine the selective effects reported for Na-octanoate on the *G6pd* polymorphism. We were concerned about the rationale of using fatty acids as a selective environment for pentose shunt polymorphisms, as well as the potential complications imposed by linked loci under their design. The experimental protocol utilized here is designed to specifically minimize the effects of *X*-linked loci. This particular approach has been recently used to examine the presence of viability differences of the common *G6pd* alleles in different *6Pgd* backgrounds in order to study *in vivo* flux differences (EANES 1984). We report that under this design no evidence for strong viability differences in octanoate environments can be detected.

MATERIALS AND METHODS

The *X* chromosomes used in this study were recovered using an approach described in detail in EANES (1984). This approach uses the genetic scheme illustrated in Figure 1. Select *X* chromosomes are recovered where recombination has occurred between the two visible markers (*car* and *sw*.) closely flanking the *G6pd* locus. This region spans about 2.3 cM (EANES 1983) and an estimated 34 bands of 1012 on the *X* chromosome. Crossing over is prevented on the distal half of the chromosome by the inversion *In(1)d1-49*. The *6Pgd* locus is located at the distal tip at 0.64. Recombinant chromosomes recovered in F_1 males (carrying *car*) carry either the $G6pd^A$ or $G6pd^B$ electrophoretic alleles, depending on whether recombination has occurred in the right or left flanking regions. Independently recovered chromosomes represent replicates which differ only in the exact site of recombination in these well-defined, and limited, genetic regions. Chromosomes recovered in this fashion are on the average identical-by-descent for 98.3% of the *X*-linked loci (a minimum of 97%). F_1 males are mated with $FM6/N^{264\text{-}84}$ females and the chromosomes either balanced over *FM6* or made isochromosomal.

In this study, two independently derived sets of recombinant chromosomes were first produced and are designated sets *F* and *S*. Set *F* was generated using a $6Pgd^F$ *car sw* chromosome and, consequently bears the $6Pgd^F$ allele, and set *S* carries the $6Pgd^S$ allele. Therefore, tests with these two independent sets represent tests of *G6pd* genotypes in different *6Pgd* backgrounds. Set *F* is

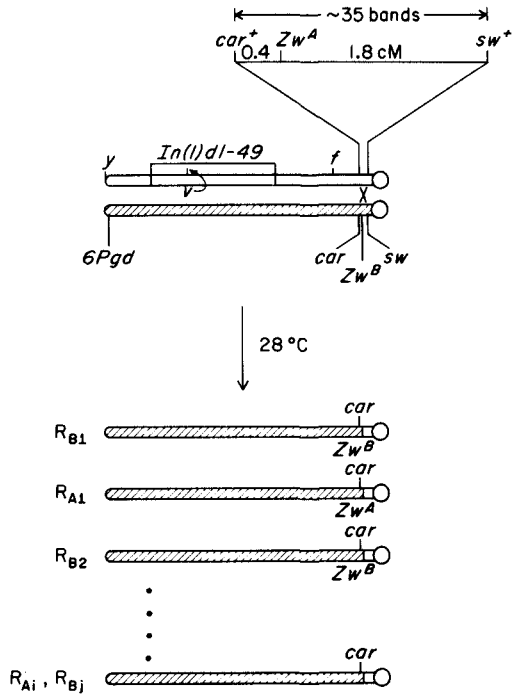

FIGURE 1.—The genetic scheme used to create sets of recombinant test chromosomes. These chromosomes are identical for the distal portion of chromosome *car sw* and the proximal section of *In(1)d1-49, yvf*. The position of *G6pd(=Zw)* relative to its flanking markers is shown.

comprised of five independently derived recombinant *A* chromosomes and six *B*-bearing chromosomes, set *S* of seven *A* bearing recombinant chromosomes and six *B* chromosomes.

In total, four experiments were carried out to study the effect of Na-octanoate on egg to adult viability. Each experiment consisted of a control (normal food) and treatment (0.15% Na-octanoate). In each experiment, male and female viabilities were determined separately.

The design for experiments 1 and 2 was similar to those of BIJLSMA (1978) and BIJLSMA and KERVER (1983). From isochromosomal lines for each of the test chromosomes in sets *F* and *S*, appropriate crosses were set to generate hemizygous male and both homozygous and heterozygous female genotypes. For instance, to generate homozygous female genotypes, crosses always involved combining independently derived recombinant chromosomes bearing the same allele (*e.g.*, $A_1 \times A_4A_4$). In each type of chromosomal cross approximately 50 virgin females were mass crossed with 50 males of the appropriate genotype and permitted to lay eggs for 12 hr on standard yeasted food. Eggs were then collected, washed with Ringer's solution and transferred on 1-cm^2 filter paper squares to 8-dram vials in batches of approximately 100 eggs/vial. In this fashion, five to seven replicates of each type of chromosomal cross were established. The "absolute" viability for each genotype was estimated as the proportion of emerging adults for that sex out of the number of eggs initially placed in each vial. Emerging adults were counted on days 15, 17 and 19, and each vial served as a single viability observation.

Experiment 1 was carried out using the S-lines ($6Pgd^S$). In total, 20 different interchromosomal crosses were set up to generate independent replicates of the three female and two male *G6pd* genotypes from the recombinant chromosomes just described. For experiment 1 these interchro-

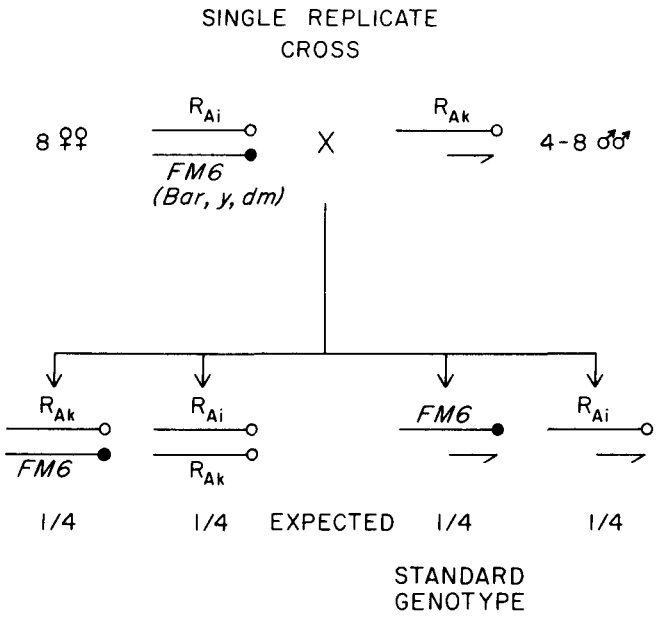

FIGURE 2.—The genetic cross used to measure relative viability for a single replicate in experiments 3 and 4. An example for the *G6pd-A* and *G6pd-AA* genotypes is shown.

mosomal crosses generated six A_iA_j, seven A_iB_k and seven B_kB_l genotypes, as well as their respective male genotypes (eight A_i and 12 B_k). Subscripts here refer to the specific recombinant chromosome line numbers. Experiment 2 used the F-lines ($6Pgd^F$) and 24 crosses were set up (six A_iA_j, ten A_iB_k and eight B_kB_l female chromosomal crosses, 11 A_i and 13 B_k hemizygous crosses).

Experiments 3 and 4 were carried out to measure viability relative to *FM6* males as a standard genotype (EANES 1984), as shown in Figure 2 for an A_iA_j cross. Relative viability was used because larger experiments could be quickly set up and results could be compared with former studies (EANES 1984). Under this scheme virgin females homozygous for each chromosome are crossed to *FM6* males and the F_1 male and female progeny used to set up crosses as in the former experiments. Each cross was established by crossing 50 females with 50 males of the appropriate genotype and again collecting eggs to be placed on control and Na-octanoate foods (adults do not readily oviposit on Na-octanoate food). Approximately 200 eggs were transferred per vial. The relative viability of each genotype is computed as the number of emerging adults of that genotype divided by the sum of that number and the number of *FM6* males plus one, as in MUKAI (1964).

Experiment 3 used the S-lines and is represented by 24 crosses (eight A_iA_j, eight A_iB_k and eight B_kB_l; 11 A_i and 13 B_k hemizygous crosses). In experiment 4, using F-lines, 22 interchromosomal crosses were set up (six A_iA_j, eight A_iB_k and eight B_kB_l female crosses; eight A_i and 14 B_k hemizygous crosses). Ten replicate vials per cross were generated in each experiment, and emerging flies were counted on days 12, 15 and 19.

All crosses were raised on food essentially the same as described by BIJLSMA (1978) with the exception that propionic acid was used as a fungicide (0.7% v/v). Control or normal food consisted of 1000 ml of water, 19 g of agar, 32 g of dead yeast and 54 g of sucrose. For treated food, octanoate (Sigma) was added at 0.15% (v/v) and the solution was adjusted to pH 7.0 with 2 N NaOH. All experiments were carried out at 25°.

As the sample sizes across genotypes in all of the analyses were unequal, two-way analysis of variance (ANOVA) by conventional analysis is impractical. We used a nonorthogonal ANOVA method developed by HEMMERLE (1974). The program for this iterative procedure using balanced residuals was written by CLIFFORD L. PELLETIER and obtained from F. J. ROHLF.

RESULTS

In combination, these large experiments resulted in total counts of more than 170,000 adult flies from more than 1400 vials. The results of experiments 1 and 2 are summarized and statistically analyzed in Tables 1 and 2 for males and females, respectively. In these results it is possible to partition the viability variation in relevant pairs of experiments into three effects or sources as shown in the ANOVAs. The first source addresses whether there are regular viability differences between *G6pd* genotypes irrespective of treatment. The second measurable source (treatment) simply addresses whether Na-octanoate changes viability irrespective of *G6pd* genotypes. Na-octanoate clearly reduces viability, but this is of little interest or importance here. The cause is not known. The third and major effect of interest addresses the question of whether different *G6pd* genotypes exhibit differential viability responses in control and Na-octanoate environments (the treatment by genotype interaction). It should be emphasized that comparisons between experiments 1 and 2 are not appropriate because these separate sets of chromosomes (*F* and *S*) differ not only for their *6Pgd* alleles but also for large portions of the *X* chromosomes. Although in these experiments we do not test the effect of *6Pgd* genotypes, or any potential dilocus interaction, the experiments by BIJLSMA and KERVER (1983) demonstrated a consistent significant difference involving the *G6pd* locus, and *6Pgd* never showed a significant contribution.

In experiment 2 there are statistically significant ($P < 0.01$) differences between *G6pd* genotypes in males only. Otherwise, the first and third effects are not statistically significant in any of the other six tests. The general direction of all four experiments in both sexes suggests that $G6pd^B$-bearing chromosomes confer slightly greater viability over $G6pd^A$, but these experiments are not strictly independent. These results indicate no *differential* absolute viabilities induced by Na-octanoate treatment for the *G6pd* genotypes.

Experiments 3 and 4 (Tables 3 and 4) are again tests of the same hypotheses as before, but they measure viability relative to the standard *FM6* genotype. The results are consistent with the former experiments. None of the four interaction tests is statistically significant, again indicating that, within the limits of the statistical power of the tests, Na-octanoate has no compelling effect on *G6pd* viabilities.

DISCUSSION

The impetus for this study arose from several concerns. First, we were concerned that the basic logic of using fatty acids as an informative target environment for pentose shunt polymorphisms was weak. In addition, we felt that linked loci perhaps responding to Na-octanoate selection were contributing to the observed outcome. We cannot prove that linked loci have generated the positive outcomes of former studies but rather show only that, by tightening the conditions under which linkage effects can contribute, a negative result is observed. Given these reservations the specific purpose of our study was to determine whether octanoate generated differential viability selection

TABLE 1

Viability of G6pd *genotypes in males in control and Na-octanoate-supplemented food*

Experiment	Treatment	G6pd genotype *A*	G6pd genotype *B*	Source	d.f.	Mean squares	*F*
1: S-lines (*6 Pgd^s*)	Control	45.5	42.4	Genotype	1	53.6	1.0
	Na-octanoate	38.6	39.6	Treatment	1	1225.9	22.2***
				Interaction	1	212.0	3.8
				Error	211	55.3	
2: F-lines (*6Pgd^F*)	Control	31.4	34.5	Genotype	1	596.3	6.8**
	Na-octanoate	25.0	28.3	Treatment	1	2017.7	25.2***
				Interaction	1	0.1	0.1
				Error	205	80.1	

**$P < 0.01$.
***$P < 0.001$.

TABLE 2

Viability of G6pd *genotypes in females in control and Na-octanoate supplemented food*

Experiment	Treatment	G6pd genotype *AA*	*AB*	*BB*	Source	d.f.	Mean squares	*F*
1: S-lines (*6 Pgd*^{s})	Control	44.2	42.6	44.0	Genotype	2	147.9	2.1
	Na-octanoate	39.8	35.5	36.8	Treatment	1	2077.9	29.4***
					Interaction	2	45.6	0.6
					Error	209	70.6	
2: F-lines (*6Pgd*^{F})	Control	35.4	33.3	36.5	Genotype	2	83.9	1.1
	Na-octanoate	24.4	29.8	27.3	Treatment	1	3000.1	38.3***
					Interaction	2	207.5	2.7
					Error	203	79.5	

***$P < 0.001$.

TABLE 3

Viability (relative to FM6 *males) of* G6pd *genotypes in males in control and Na-octanoate supplemented food*

Experiment	Treatment	G6pd genotype		Source	d.f.	Mean squares	*F*
		A	*B*				
3: S-lines ($6\ Pgd^s$)	Control	58.4	59.7	Genotype	1	0.9	<0.1
	Na-octanoate	59.6	58.0	Treatment	1	10.4	0.2
				Interaction	1	201.1	3.4
				Error	446	59.7	
4: F-lines ($6Pgd^F$)	Control	59.4	59.0	Genotype	1	7.1	0.2
	Na-octanoate	55.4	56.4	Treatment	1	919.6	12.6***
				Interaction	1	35.2	0.5
				Error	355	73.2	

***$P < 0.001$.

TABLE 4

Viability (relative to FM6 *males) of* G6pd *genotypes in females in control and Na-octanoate supplemented food*

Experiment	Treatment	G6pd genotype			Source	d.f.	Mean squares	*F*
		AA	*AB*	*BB*				
3: S-lines (*6 Pgd*^{S})	Control	61.0	59.6	60.9	Genotype	2	20.4	0.4
	Na-octanoate	58.3	58.4	56.9	Treatment	1	748.6	13.0***
					Interaction	2	73.1	1.3
					Error	427	57.8	
4: F-lines (*6Pgd*^{F})	Control	61.5	62.3	62.5	Genotype	2	196.9	3.2*
	Na-octanoate	56.0	60.0	55.7	Treatment	1	2091.7	34.4***
					Interaction	2	165.9	2.7
					Error	361	60.9	

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

on *G6pd* genotypes as reported by BIJLSMA (1978) and BIJLSMA and KERVER (1983). The results of the four experiments using two different viability measures did not indicate any statistically significant differential responses of *G6pd* genotypes to Na-octanoate treatment in either males or females, although there were apparent small viability differences between *G6pd* genotypes for this set of chromosomes irrespective of treatment. It must be emphasized that the absence of statistically significant responses in our study is not the result of a reduced statistical power to detect viability differences. To the contrary, the error mean squares are comparable to the former experiments and the associated degrees of freedom per genotype are on the average twice as large. Our tests are, therefore, statistically more powerful.

The rationale for using external substrates, such as ethanol in the case of the *Adh* polymorphism and starch or maltose in the case of the *Amy* locus, is founded in the assumption that such substrates act directly on targeted locus function, thereby making the conditions for spurious effects by linked loci more restrictive. In conjunction with targeted selection experiments, there must be *a priori* expectations based on detailed knowledge of the molecular characteristics of the allozymes themselves. Enzymological data gathered for both the *Adh* and *Amy* loci indicate characteristic differences in allele function (see review by ZERA, KOEHN and HALL 1984), and these in turn can be used to make predictions of the effect of selective environments.

Unfortunately, the coupling of cause, effect and function are not clear in the case of Na-octanoate and the *G6pd* polymorphism. One recognized role of the pentose shunt is to generate NADPH for fatty acid synthesis (among other things), but it is impossible to predict *a priori* how the incorporation of specific fatty acids into larval diet would differentially select against *G6pd* genotypes. The suppression of G6PD activities by Na-octanoate (about 10%) is not noteworthy because a large number of dietary supplements, with less direct metabolic connections, also modify activity (see GEER, WOODWARD and MARSHALL 1978). Octanoate at these concentrations clearly reduces viability, but the mechanism or source of mortality is not known. Consequently, it would seem that the effects of Na-octanoate on the *G6pd* polymorphism are ambiguous in this case. The power of targeted selection experiments lies in their logical simplicity (CLARKE 1975), which appears to be lacking under these conditions.

We attempted to minimize the effect of linked genetic variation segregating throughout the *X* chromosome by using the controlled genetic scheme outlined in MATERIALS AND METHODS. Traditional designs in laboratory studies often start with chromosomes sampled from artificial population cages or collections of chromosomes from isofemale lines. Crosses are set up to "randomize" genetic backgrounds, and it is argued generally, without objective criteria, that observed selective differences are due to the enzyme genotypes *per se*. Several studies have criticized this approach.

Recently, MUKAI and YAMAZAKI (1980) examined observational data from several population cage studies (VAN DELDEN, BOERMA and KAMPING 1978; AYALA and ANDERSON 1973; FONTDEVILA *et al.* 1975) and estimated selection coefficients by both analytical methods and computer simulation. Their results

suggested that allozyme-associated selection coefficients were of the order 0.10, which is considered large for single-locus selection. They argued that disequilibria are routinely generated during the establishment of artificial populations or lines, and results are often the measured effects of those disequilibria. More recently, YAMAZAKI *et al.* (1983) reexamined the observation of diversifying selection on allozyme polymorphisms in laboratory populations (see POWELL 1971; POWELL and WISTRAND 1978) using population cages derived from a population cage originated from 400 wild inseminated females and maintained at a population size of approximately 4000 for 6 yr. Using this material they found no evidence for diversifying selection in any of their experiments.

The chromosomes used by BIJLSMA and VAN DELDEN (1977), BIJLSMA (1978) and BIJLSMA and KERVER (1983) were derived from two cages. One cage was initiated 52 generations prior to the reported experiments by mixing two inbred homozygous lines (Oregon-R and Tuscaloosa); the other cage, designated Bogata, has been in the laboratory since 1965 (BIJLSMA and VAN DELDEN 1977). The authors argue that linkage disequilibrium in these cages has decayed to "insignificant" levels except for loci very close to the enzyme loci, and this appears an acceptable argument under simple assumptions. However, the authors do not address the potential disequilibria that are set up when chromosomes from these cages are reisolated prior to their experiments. This regenerated disequilibrium is potentially significant and moreover involves the *entire X* chromosome.

The average linkage disequilibrium generated by sampling can be computed using the estimators of HILL (1974). For the *G6pd* locus and any unobserved locus segregating for alternative alleles, the maximum linkage disequilibrium possible and the average sampling disequilibrium depend on the frequencies at both loci. Given that the frequencies at *G6pd* are $p = q = 0.50$, and a sampling of $n = 64$ chromosomes, the *average* linkage disequilibrium expected from sampling will be from 13 to 60% of the maximum disequilibrium as the heterozygosity at the second hypothetical locus varies from 50 to 1%. It is clear that significant portions of the *X* chromosomes will be in substantial nonrandom association with the *G6pd* locus after extraction from these cages even if the cages are in linkage equilibrium. Because the chromosomes are subsequently pooled and maintained as homozygous *G6pd* genotypes, there was no oppotunity for this sampling effect to decay in later generations, and studies of Na-octanoate selection will, to varying degrees, reflect genetic variation across the entire *X* chromosome. Inspection of the *G6pd* viabilities in separate experiments by BIJLSMA (1978) and BIJLSMA and KERVER (1983) show no consistent direction of selection for different *G6pd* genotypes, but rather reversals of relative viability. These separate studies were carried out using chromosomes from two different cages and suggest sampling effects rather than the influence of cage genetic background. In our study, genetic variability for differential viability in Na-octanoate must not only be present but, in addition, be segregating within a *very limited* section of the *X* chromosome. However, this is not an issue because we do not find evidence for selection under Na-octanoate.

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