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Polymorphism at the G6PD and 6PGD loci in *Drosophila melanogaster*

II. Evidence for interaction in fitness

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SUMMARY

The influence of sodium octanoate on the polymorphism at the loci G6PD and 6PGD in *Drosophila melanogaster* was investigated by studying its effect on egg hatchability, larval-to-adult survival and adult survival. The results demonstrate the existence of differences in fitness between the different genotypes of the two loci both for larval-to-adult survival and for adult survival. Furthermore, changes in enzyme activity of both enzymes, brought about by the sodium octanoate treatment, were observed. This makes it highly probable that the observed differences in fitness can be ascribed to the loci themselves and not to linked fitness genes. Finally, this study demonstrates the existence of strong interaction between the two loci with respect to fitness. Epistasis was demonstrated both in the larval-to-adult survival experiment and in the adult survival experiment.

1. INTRODUCTION

Since the discovery of the high frequency of protein polymorphisms in natural populations of *Drosophila* and many other species, the question to be solved is whether this genetic variation is mainly selectively neutral and maintained by random processes, or is mainly maintained by some kind of balancing selection. In the latter case the question arises whether the selective forces act on single genes, or on groups of genes (Lewontin, 1974). For this reason there has been increasing interest in multiple-locus genetic systems.

Theoretical work on two- and multiple-locus systems clearly showed the invalidity of the single locus theory in the presence of higher-order interactions between loci, caused by epistasis and linkage (Karlin, 1975; Franklin & Lewontin, 1970; Slatkin, 1972). The models clearly showed that, in the presence of linkage and epistasis, selection acts on groups of genes rather than on single genes, and that, in order to understand the forces which maintain polymorphisms in natural populations, experimental data are needed on the existence of epistasis and linkage disequilibrium.

The purpose of this study was to investigate whether epistasis could be detected between two polymorphic loci in *D. melanogaster*. Epistasis is used in the sense of the definition by Karlin (1975): 'Epistasis means that the fitness

of a genotype cannot be partitioned into independent contributions due to each locus separately. The two loci studied code for the enzymes-glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49) and 6-phospho-gluconate dehydrogenase (6PGD, E.C. 1.1.1.44). In *D. melanogaster* both loci are *X*-linked with two alleles each, a fast (*F*) allele and a slow (*S*) allele, and the genes are located at 63 for G6PD and 0·9 for 6PGD, so in females there is 50 % recombination between the loci (Young, Porter & Childs, 1964; Young, 1966). Genetical and biochemical aspects of the two enzymes are discussed in detail by Dickinson & Sullivan (1975). Though the two genes behave as genetically unlinked genes in females, there is a strong biochemical link between them. Both enzymes are involved in the pentose phosphate pathway. In the first step of this pathway glucose-6-phosphate is converted by G6PD into 6-phospho-gluconolactone and this is rapidly hydrolysed, spontaneously or by the enzyme lactonase, to 6-phospho-gluconate. This in turn is the substrate for 6PGD and is converted into a pentose, ribulose-5-phosphate. The rationale for choosing these two enzymes is that epistatic interactions are more likely to occur between loci coding for enzymes having a functional relationship. This idea has been successfully utilized in the search for linkage disequilibrium and epistasis by, among others, Zouros & Krimbas (1973), Mitton & Koehn (1973) and Zouros & Johnson (1976).

As reported previously (Bijlsma & Van Delden, 1977), the polymorphism at both loci is subject to selection. The *F*-allele of both loci appeared to be favoured in a variety of different environments. Especially in connexion with the search for a possible interaction between the loci, the results found in an extinction experiment on food containing sodium octanoate were interesting. There appeared to be differences in fitness between the different genotypes of both loci.

Furthermore it is known that fatty acids interact with the biochemical function of the two enzymes. Fatty acids seem to change the levels of both enzymes in *D. melanogaster* (Geer *et al.* 1976), and also have an inhibitory effect *in vitro* on G6PD and 6PGD isolated from rat liver cells (Weber *et al.* 1967). In this paper, therefore, investigations are reported on the influence of sodium octanoate on egg hatchability, larval to adult survival and adult survival, and on the change in enzyme activity in larvae and adults caused by sodium octanoate.

2. MATERIALS AND METHODS

(*a*) *Culture method.* Flies were raised in half pint bottles at 25 °C and 50–70 % R.H. on standard food consisting of 1000 ml water, 19 g agar, 32 g dead yeast, 54 g sugar and 13 ml nipagine solution (10 g nipagin in 100 ml ethanol 96 %). For the experiments on sodium octanoate the necessary amount of sodium octanoate was added to the food.

(*b*) *Notation.* Throughout the paper the following notation is used for the genotypes. The two first letters indicate the alleles of the G6PD locus and the following two letters refer to the 6PGD locus. So $\frac{FF}{FS}$ is homozygous *F* for G6PD and heterozygous for 6PGD.

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(*c*) *Stocks.* The flies used in the experiments originated from two homozygous strains (Tuscaloosa and Oregon-R) which were combined in a population cage for approximately two years. At that time 15 lines of each possible homozygous genotype ($\frac{FF}{FF}$; $\frac{FS}{FS}$; $\frac{SF}{SF}$; $\frac{SS}{SS}$) including hemizygous males were isolated in such a way that each line contained one single X-chromosome from the population. These are the lines of the second reisolation from the 50×50 I cage population as described by Bijlsma & Van Delden (1977). For the experiments all 15 lines of a given genotype were mixed and maintained as mixed populations for two generations before they were used for the experiments.

(*d*) *Egg hatchability.* Eggs were collected, 0–4 h old, from each of the four homozygous genotypes. Twenty-five eggs of a given genotype were transferred to a vial and after 36 h the number of hatched eggs was counted. This was done on three different food media with ten replicates of each genotype on each food medium. The food media were as follows.

(1) Normal food.

(2) Normal food supplemented with 0·10 % sodium octanoate.

(3) Normal food supplemented with 0·15 % sodium octanoate.

(*e*) *Larval to adult survival.* Virgin females and males of each of the four homozygous genotypes were collected, and all 16 possible crosses were made. The females were then allowed to lay eggs on 2 % agar gels for 6 h, and freshly hatched larvae from these eggs were collected and cultured in vials, 100 larvae per vial. Because both loci are sex-linked the genotype of the female progeny differs from that of the male progeny in each vial. Ten replicates of each of the ten female genotypes were therefore established in such a way as to give 25 replicates of each of the four male genotypes. This experiment was performed on standard food and standard food supplemented with 0·10 % sodium octanoate.

(*f*) *Adult survival.* For each of the possible genotypes, twenty vials with twenty females or males (7 days old) were established and the number of dead individuals in each vial was determined at successive time intervals. The food was supplemented with 0·15 % sodium octanoate.

(*g*) *Determination of enzyme activity.* A homogenate of whole flies or larvae (normally 20 flies or larvae in 0·5 ml buffer) was centrifuged for 5 min and 0·1 ml of the supernatant was added to 0·9 ml reaction mixture. This reaction mixture consisted of a 0·1 M Tris-HCl buffer, pH 7·5 at 21 °C, with a final concentration of 0·01 M $MgCl_2$, 1 mM $NADP^+$ and 1 mM substrate (glucose-6-phosphate or 6-phospho-gluconic acid). The increase in absorbance at 334 nm in the reaction mixture was followed in a spectrophotometer (1 cm light path, 25 °C). Activity is then given as the initial change in absorbance per mg fresh weight per minute.

3. RESULTS

(*a*) *Egg hatchability.* Table 1 gives the mean hatchability of the four homozygous genotypes on the three food media. It is clear that sodium octanoate, at the levels tested, has no effect on the egg hatchability. Furthermore it can be seen from the averages over the three media that differences in hatchability between the

genotypes do exist. The *FF* genotype has the lowest and the *SS* has the highest hatchability. An analysis of variance on the combined data showed that significant differences were due to both loci, leading to the conclusion that eggs which were homozygous *S* for each locus hatched better than homozygous *F* eggs.

(*b*) *Larval to adult survival.* Table 2(*a*) and (*b*) show the results of the larval survival experiment. As mentioned earlier, the genotype of the female progeny in

Table 1. *Mean egg hatchability* (*mean* ± S.E. *in angles*) *of the four homozygous genotypes on three food media*

	Sodium octanoate concentration			
Genotype	0	0·10%	0·15%	Average
FF	61·5 ± 2·3	64·3 ± 2·0	63·0 ± 1·9	63·0 + 1·2
FS	69·8 ± 2·1	65·1 ± 2·0	68·3 ± 2·3	67·7 ± 1·2
SF	69·6 ± 1·1	67·7 ± 1·4	70·6 ± 2·8	69·3 ± 1·1
SS	75·6 ± 2·7	70·6 ± 2·1	67·9 ± 1·8	71·4 ± 1·4
Average	69·1 ± 1·3	67·0 ± 1·0	67·4 ± 1·1	

Table 2. (*a*) *Mean larval survival of females on normal food and on food supplemented with 0·10 % sodium octanoate together with the analysis of variance of these data* (* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$)

Normal food

	G6PD						
	F/F	*F/S*	*S/S*	Source	D.F.	Mean square	*F* value
6PGD *F/F*	27·6	37·1	34·8	G6PD	2	3·9	< 1
6PGD *F/S*	26·5†	34·8	39·8	6PGD	2	24·1	< 1
6PGD *S/S*	35·7	34·6	34·0	G6PD × 6PGD	4	54·8	1·20
				Error	80	45·5	

0·10 % sodium octanoate

	G6PD						
	F/F	*F/S*	*S/S*	Source	D.F.	Mean square	*F* value
6PGD *F/F*	27·6	32·1	19·1	G6PD	2	297·2	5·52**
6PGD *F/S*	26·5	33·8	28·6	6PGD	2	58·2	1·58
6PGD *S/S*	24·7	29·0	30·6	G6PD × 6PGD	4	186·5	3·46*
				Error	81	53·9	

† Nine replicates

each vial differs from that of the male progeny. Therefore the actual numbers of emerging females and males were analysed separately. By comparing the total survival on sodium octanoate with normal food it is apparent that sodium octanoate decreases the survival of larvae.

Table 2(*a*) shows the survival of females in both environments. The experimental design results in the generation of two different double heterozygous genotypes, namely a coupling and a repulsion heterozygote ($\frac{FF}{SS}$ and $\frac{FS}{SF}$), with ten replicates each. As a Student's *t* test showed that the survival of these two genotypes did not

differ significantly, these two genotypes can be treated as one. For the statistical analysis presented in Table 2(*a*) the first five replicates of these two genotypes were pooled to form one double heterozygous class. On normal food there are only slight differences in survival between the different genotypes, which are not significant. On sodium octanoate, however, there are significant differences between the

Table 2. (*b*) *Mean larval survival of males on normal food and food supplemented with 0·10 % sodium octanoate together with the analysis of variance of these data* (* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$)

Normal food

		G6PD *F*	G6PD *S*	Source	D.F.	Mean square	*F* value
6PGD	*F*	40·3†	35·7	G6PD	1	69·3	1·64
				6PGD	1	55·9	1·32
	S	35·8†	37·1	G6PD × 6PGD	1	216·1	5·11*
				Error	94	42·3	

0·10 % sodium octanoate

		G6PD *F*	G6PD *S*	Source	D.F.	Mean square	*F* value
6PGD	*F*	30·4	24·6	G6PD	1	39·7	< 1
				6PGD	1	28·1	< 1
	S	27·0	30·2	G6PD × 6PGD	1	506·3	7·14**
				Error	96	70·9	

† Twenty-four replicates.

genotypes of the G6PD locus. Summarized over the three 6PGD genotypes the heterozygote for G6PD has a survival of 31·6 while the homozygous *F* and homozygous *S* have a survival of respectively 26·9 and 26·1. This shows apparent overdominance for larval survival at the G6PD locus, which is statistically significant. Furthermore the analysis of variance shows a significant interaction between the two loci. By applying Tukey's test for non-additivity (Sokal & Rohlf, 1969) it was shown that this interaction was not due to multiplicative effects but was caused by higher-order interaction. This leads to the conclusion that the interaction found is due to epistasis between the loci. For the males (Table 2*b*) the results are somewhat different. On normal food there is already a significant interaction between G6PD and 6PGD, which seems to be intensified by sodium octanoate. This interaction is caused by the higher survival of *FF* and *SS* males than of the other two types.

Thus sodium octanoate induces differential survival between the different genotypes. This raises the question whether these differences are due to the loci under study or to linked genes. For this reason the enzyme activities were measured of larvae which were grown for 3 days (from young larvae just up to pupation) on normal food or on 0·10 % sodium octanoate. The results are summarized in Table 3. Sodium octanoate caused a significant decrease in the activity of G6PD

in homozygous genotypes but had no effect on 6PGD activity. So sodium octanoate also interferes with the enzyme activity of G6PD.

Noteworthy also is the observed difference in enzyme activity of the two homozygous genotypes of each locus. For both G6PD and 6PGD homozygous *S* flies have a higher enzyme activity than the homozygous *F*. This is true for larvae (Table 3) and adults (Table 4).

Table 3. *Mean enzyme activities* ($\Delta E \times 10^3$*/min/mg live weight*) *and standard error of homozygous larvae fed for 3 days on normal food and on food supplemented with 0·10% sodium octanoate* (*means based on 12 replicates*)

	G6PD		6PGD	
	Normal food	0·10% octanoate	Normal food	0·10% octanoate
F	13·44 ± 0·53	12·05 ± 0·35*	13·81 ± 0·43	13·61 ± 0·66
S	15·97 ± 0·37	14·56 ± 0·46*	16·59 ± 0·38	16·93 ± 0·55
S/*F* ratio	1·19**	1·21**	1·20**	1·24**

* Significantly different from normal food at $P = 0{\cdot}05$.
** Significant differences between *F* and *S* at $P = 0{\cdot}015$)

Table 4. *Enzyme activity* ($\Delta E \times 10^3$*/min/mg live weight*) *and standard error of seven days old homozygous adults kept on normal food* (*means based on 12 replicates*)

	G6PD		6PGD	
	Females	Males	Females	Males
F	16·20 ± 0·54	28·29 ± 0·79	10·82 ± 0·36	17·90 ± 0·62
S	18·59 ± 0·53	32·17 ± 1·16	14·13 ± 0·53	24·59 ± 1·08
S/*F* ratio	1·15*	1·14*	1·31*	1·37*

* Significant difference between *F* and *S* at $P = 0{\cdot}05$).

(*c*) *Adult survival.* Sodium octanoate at a concentration of 0·15%, which is approximately twice the concentration used in the extinction experiment by Bijlsma & Van Delden (1977), causes mortality of adult flies. The percentage of flies that died at successive time intervals is shown in Fig. 1. The first flies die within 48 h and the mortality has reached its maximum (100% for males and approximately 80% for females) within 80 h. It should be noted that in the same time interval hardly any flies died on normal food.

Fig. 1 also shows clearly the difference in death rate between the different genotypes. Flies homozygous *S* for both loci are more resistant than the other homozygous genotypes (solid lines). The relative difference in mortality between the different homozygous female genotypes is the same as between the corresponding hemizygous male genotypes. Furthermore it can be seen that the genotypes heterozygous for one locus or for both loci, except $\frac{FF}{SF}$, are intermediate between the homozygous $\frac{SS}{SS}$ and the other three homozygous genotypes.

The differences in death rate are also demonstrated in Table 5 in which the averaged percentage dead flies per vial is shown at the moment at which approxi-

mately 50 % of the flies of a particular sex had died (61 h for females and 54 h for males). The analysis of variance clearly shows the highly significant effect of both G6PD and 6PGD on the death rate, and moreover a significant interaction between the loci. In the females this interaction was again due to higher order interaction. It is therefore concluded that also with respect to adult survival there is epistasis between the loci.

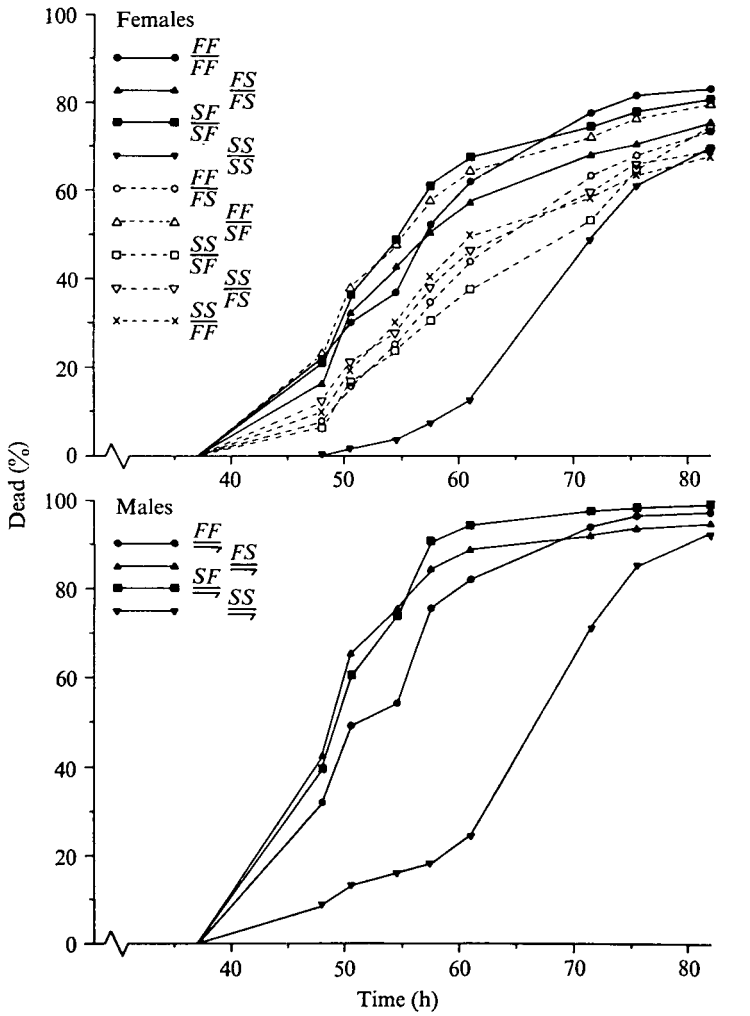

Fig. 1. The cumulative adult mortality on food supplemented with 0·15 % sodium octanoate. Females top figure and males bottom figure.

Again the influence of sodium octanoate on the enzyme activity of both enzymes was tested. The results are summarized in Table 6 and Fig. 2. Table 6 shows the percentage increase in enzyme activity of homozygous females which survived 7 days on 0·15 % sodium octanoate, compared to flies of the same age from normal food. There is a significant increase in all genotypes and for both enzymes. Fig. 2

gives the results of surviving adult males staying for 1, 2 and 3 days on sodium octanoate. The figure shows that the treatment of adult males tends to cause an increase in the activities of both enzymes by the third day of treatment, with the

Table 5. *Mean mortality per vial (angles) of adult females and males on 0·15 % sodium octanoate together with the analysis of variance of these data* (* $P < 0{\cdot}05$; ** $P < 0{\cdot}01$)

Females (61 hrs)

		G6PD						
		F/F	*F/S*	*S/S*	Source	D.F.	Mean square	*F* value
6PGD	*F/F*	52·1	54·0	59·0	G6PD	2	2990·5	13·20**
	F/S	41·5	44·4	33·3	6PGD	2	6145·8	27·13**
	S/S	49·7	42·6	13·8	G6PD × 6PGD	4	2607·0	11·51**
					Error	190	226·5	

Males (54 hrs)

		G6PD					
	F	*S*	Source	D.F.	Mean square	*F* value
6PGD	*F*	48·0	62·5	G6PD	1	6406·1	8·88*
				6PGD	1	5867·4	8·13*
	S	63·3	12·9	G6PD × 6PGD	1	20995·9	29·09**
				Error	76	721·8	

Table 6. *Relative increase in enzyme activity of adult females surviving after seven days on 0·15 % sodium octanoate compared with females of the same age kept on normal food (based on 6 replicates)*

	$\frac{FF}{FF}$	$\frac{FS}{FS}$	$\frac{SF}{SF}$	$\frac{SS}{SS}$
G6PD	38·6%	36·2%	36·3%	45·3%
6PGD	25·7%	32·7%	30·5%	44·5%

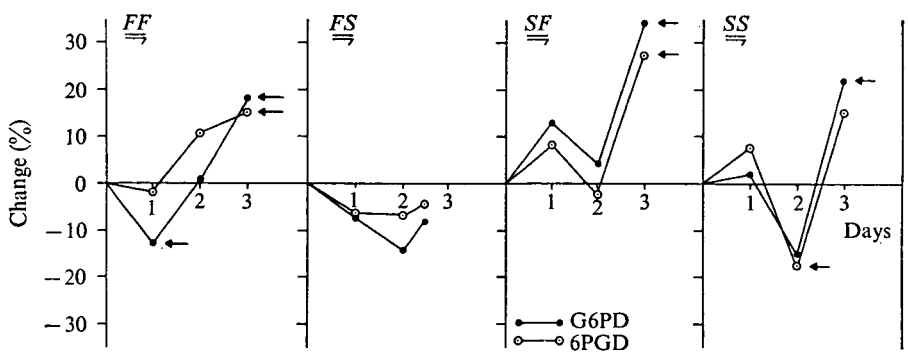

Fig. 2. The relative change in enzyme activity of the four hemizygous male genotypes staying 1, 2 and 3 days on 0·15% sodium octanoate compared to normal food. The arrows indicate the points representing a significant change (at the 5% probability level).

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possible exception of *FS* males. Altogether the results from Table 6 and Fig. 2 suggest that continual treatment of adults with sodium octanoate tends to increase the enzyme activity of both G6PD and 6PGD.

4. DISCUSSION

Changes in enzyme activity caused by sodium octanoate treatment are obvious from Tables 3 and 6 and from Fig. 2. In larvae there is a significant decrease in G6PD activity, after feeding on sodium octanoate, but no noticeable decrease in 6PGD activity. These results contradict to a certain extent the results of Geer, *et al.* (1976). When larvae of *D. melanogaster* were fed on a fat sucrose medium instead of a fat-free sucrose medium, they found a similar decrease in enzyme activity for a number of $NADP^+$-using enzymes including both G6PD and 6PGD. The differences between their results and the results presented in this paper may be due to a difference in culture method used by these authors or (and) in the kind of fat (phosphatidyl choline) added to the food. In adults the effect of sodium octanoate treatment on the enzyme activity is different. At the time the first flies are dying, the activities of both enzymes increase sharply (Fig. 2) and stay at a high level in the surviving flies (Table 6). It has not been determined whether this increase in enzyme activity is due to an increase in enzyme activity level in each individual fly or to the survival of flies with highest enzyme activity levels. The former possibility, however, seems the most probable one as in a few cases the sharp increase was already found when only a minor proportion of the flies had died.

The effect of sodium octanoate on these two enzymes can be explained by its influence on the $NADPH/NADP^+$ ratio. It is known that the pentose shunt supplies the amount of energy (NADPH) necessary for the synthesis of fatty acids (Weber *et al.* 1966; Geer *et al.* 1976). If fatty acids are added to the food less NADPH is used and hence the $NADPH/NADP^+$ ratio increases. This ratio regulates all NADPH-producing systems and an increase in this ratio causes a decrease in the production of NADPH and therefore a decrease in the activity of the pentose shunt (Holten, Procsal & Chang, 1976). This could explain the decrease in enzyme activity in the larvae. However, the increase in enzyme activity in the adults cannot be explained in this way and is probably another, secondary, effect of sodium octanoate on the metabolism of adult flies. This may also hold for the lethal effect on adult survival which was also found by Keith (1966), who observed that adult flies died on a number of different fatty acids. The effect was most striking when C8 and C10 fatty acids were added to the food. It is possible that the differences in reaction to sodium octanoate treatment in larvae and adults are the result of differences in metabolism between these developmental stages.

As sodium octanoate changes the enzyme activity of both enzymes as well as the fitness of the genotypes, the question arises whether there is a relationship between enzyme activity and fitness. There is evidence that enzyme activity indeed is strongly correlated with fitness, especially with respect to 6PGD. Bewley &

Lucchesi (1975) found that *D. melanogaster* flies bearing a 'null'-allele of 6PGD or alleles with low enzyme activity have a poor viability. The same was found by Gvozdez *et al.* (1976, 1977). The latter authors also found a positive correlation between the decrease in viability and the extent of reduction of 6PGD activity. In contrast, G6PD mutants with low activity ($\pm 10\%$ of the normal activity) survive well except under special circumstances (Geer *et al.* 1974). Furthermore the 6PGD 'null'-mutant found by Bewley and Lucchesi also had a decreased G6PD activity. However, the mutants isolated by Gvozdez *et al.* (1976), which showed decreased 6PGD activity had normal levels of G6PD activity. But strikingly, the deleterious effect of these mutants on viability could be suppressed when also low activity of G6PD was induced. These authors suppose that accumulation of 6-phosphogluconate is harmful for the flies. When the activity of G6PD is also blocked the whole pentose shunt is blocked but the accumulation of 6-phosphogluconate is prevented.

The above-mentioned observations that genetically controlled changes in enzyme activity affect fitness point to the possibility that also changes in activity caused by sodium octanoate treatment can result in changes in fitness. It can be concluded therefore that the differences in fitness between the genotypes found on food supplemented with sodium octanoate are most probably caused by differences at the loci themselves and not by linked fitness genes. The fact that sodium octanoate has different effects in larvae and adults makes it difficult to give a sensible biochemical explanation for the differences in fitness. For the same reason it is difficult to explain on biochemical grounds the interaction in fitness found between the loci. Nevertheless it seems reasonable to assume that the interaction in fitness must be based on differences in biochemical properties of the different allozymes. This is corroborated by other experiments (R. Bijlsma, in preparation) showing that the enzyme activity of a particular genotype for one locus depends on the genotype of the other locus. The experiments of Zouros & Krimbas (1973), Mitton & Koehn (1973) and Zouros & Johnson (1976) also gave evidence of the existence of epistasis between functionally related allozyme loci. These findings and the results presented in this paper suggest that single-locus fitness estimates are far from realistic at least with respect to allozyme polymorphisms. In general it would therefore be desirable to examine groups of genetically and (or) biochemically linked loci.

It has already been demonstrated in an earlier paper (Bijlsma & Van Delden, 1977) that differences in fitness between the different genotypes for G6PD and 6PGD exist on normal food, leading to high frequencies of the *F*-allele at both loci. The results presented in this paper clearly show differences in fitness between these genotypes on food supplemented with sodium octanoate. Since only a few fitness components are investigated it is difficult to predict the changes in gene frequency that would occur on this food. By using the data from Tables 2(*a*), (*b*) and 5 as relative fitness estimates, a simulation showed that the frequencies of the *F*-allele of both loci are expected either to increase simultaneously or to decrease simultaneously depending on the initial allele frequencies.

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