MEASUREMENT OF FITNESS AT THE ESTERASE-5 LOCUS IN *DROSOPHILA PSEUDOOBSCURA*[1]

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KNOWLEDGE of the amount of genic variation in natural populations is the first step towards understanding how such variation is maintained. The first systematic studies of the amount of genic variation in natural populations of *Drosophila pseudoobscura* were reported by HUBBY, LEWONTIN, and co-workers (HUBBY and LEWONTIN 1966; LEWONTIN and HUBBY 1966; PRAKASH, LEWONTIN and HUBBY 1969) who estimated that 40% of the loci are polymorphic and that on the average 12% of loci per individual are heterozygous in North American populations of the species. Similar estimates of heterozygosity have now been made for several organisms, including mice (SELANDER and YANG 1969) and humans (HARRIS 1969; LEWONTIN 1967). All these estimates indicate that the proportion of polymorphic loci in natural populations may be much greater than was previously supposed.

Were these polymorphisms maintained primarily by heterosis operating independently at each locus, populations probably would not be able to tolerate the huge genetic load involved (see LEWONTIN and HUBBY 1966). SVED, REED and BODMER (1967) have attempted to solve this dilemma by postulating that there is a limit to maximum fitness because of gene interaction. Owing to the rarity of the maximally fit genotypes, the amount of reduction in the selective advantage of heterozygote over homozygote at individual loci is extremely small. KING (1967) proposed a different threshold model in which he assumed that a certain proportion of the population with the "worst combination of genes, environment, and luck" will be eliminated by natural selection. The effect of KING's model is similar to that of SVED, REED and BODMER (1967) both in the sense that maximal fitness is approached asymptotically as heterozygosity increases, and that there are thousands of different genotypes with essentially the same fitness. According to these models a large number of polymorphic loci can be maintained without increasing genetic load to an excess. However, both hypotheses fail to explain the relatively small loss of fitness found when natural populations are inbred. The hypothesis of neutrality or very small selection coefficients of allozymes as proposed by KIMURA (1968) may be adequate to explain the maintenance of large amounts of polymorphism by assuming a rather high mutation rate, migration rate, and population size, but it does not appear to be an adequate explanation of

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the clinal variations or constancy of gene frequency in different populations as reported by PRAKASH, LEWONTIN and HUBBY (1969).

KOJIMA and his associates have reported evidence of gene frequency-dependent selection at the esterase-6 and alcohol dehydrogenase loci of *Drosophila melanogaster* (YARBROUGH and KOJIMA 1967; KOJIMA and YARBROUGH 1967; KOJIMA and TOBARI 1969), and have proposed that most protein polymorphisms may be maintained by this mechanism, whereby genes are advantageous when rare, but neutral when equilibrium is reached. This mechanism is attractive since it eliminates the problem of genetic load when gene frequency is at equilibrium.

In the experiments to be described, I have tried to test directly whether there are any differences in fitness or any indication of gene frequency-dependent selection of electrophoretic variants at the sex-linked esterase-5 locus, which is highly polymorphic in natural populations of *Drosophila pseudoobscura*. Four different experiments were conducted: (1) population cage (perturbation) experiments, (2) viability and developmental time experiments using the sex ratio, (3) frequency-dependent selection experiments (premating type), (4) viability, fecundity, and developmental time experiments (premating type). From the results of those experiments it will be shown that selective differences among genotypes at the esterase-5 locus of *D. pseudoobscura* must be very small.

MATERIALS AND METHODS

The flies used in all experiments were extracted from an established population cage of *Drosophila pseudoobscura*, which had been kept at 18°C with standard cornmeal food. This cage was established by Dr. R. C. LEWONTIN approximately 15 years ago by mixing 12 isofemale strains collected from Mather, California. Two alleles, Fast (F) and Slow (S), were found to be segregating in the cage at frequencies of approximately 45% and 55%, respectively, at the time I sampled them. These F and S alleles are equivalent to the *1.12* and *1.00* alleles in the notation used by HUBBY and LEWONTIN (1966).

Virgin females and males were collected from the cage and mated in pairs. After eggs had been laid, the parents were killed and their genotypes at the esterase-5 locus determined by acrylamide gel electrophoresis. If the female and the male were homozygous and hemizygous, respectively, for the same allele, the progeny flies were kept as a line. Therefore each line is monomorphic for the allele in question. These lines will hereafter be referred to as homozygous lines. In this way 22 homozygous lines of each allele were established. All 22 lines of each allele were made independently: none of the 22 lines came from the same egg samples. A large number of homozygous lines were used to reduce the effects of linkage disequilibrium and to adequately represent the cage backgrounds. Each homozygous line originated from three independently sampled X chromosomes (two from the female, and one from the male), since the esterase-5 locus is on the X chromosome (HUBBY and LEWONTIN 1966). The method for electrophoresis of the esterase was essentially the same as that described by HUBBY (1963) and by HUBBY and LEWONTIN (1966).

EXPERIMENTS

Experiment 1: population cage experiment: Twelve population cages were established at two different times, with two different foods, and at two different temperatures. All the flies used in founding the cages were from the 44 homozygous lines described above. The minimum number of flies used in starting population cages was 880 flies and the gene frequencies were the same in both sexes. The starting gene frequency of the S allele was 0.909 in Cages 1, 2, 5, 6, 9, and 10,

and 0.091 in Cages 3, 4, 7, 8, 11, and 12. These frequencies were used because they diverge considerably from the observed frequency (which probably represents an equilibrium) in the original stock population cage from which the lines were derived. Cages 1–4 were started on June 29, 1968, with SPASSKY's food (SPASSKY 1943) at 25°C. This food is nutritionally very poor and was used with the expectation that the competition for food would be severe. All 22 homozygous lines of each allele contributed equally to the cages. Cages 5–12 were established on January 4, 1969, with cornmeal food at two different temperatures (Cages 5, 6, 7, and 8 at 25°C; Cages 9, 10, 11, and 12 at 18°C), using 20 homozygous lines of each allele.

RESULTS

The history of gene frequency in the cages is shown in Figures 1a (Cages 1, 2, 3, 4), 1b (Cages 5, 6, 7, 8), and 1c (Cages 9, 10, 11, 12). Cages 2, 3, and 4 with SPASSKY's food were terminated after 9 months when most flies were killed because of a drastic temperature change resulting from incubator failure. Cage 1 was maintained for a longer period of time in spite of a drastic reduction in number of flies to about 20 survivors at the time of the accident. This cage showed a clear decrease in the frequency of the *S* allele which began just after the reduction of number, as shown in Figure 1a. This change in gene frequency may have been caused by linkage disequilibria established due to the small number of flies surviving. The remaining 11 cages, which were maintained with two kinds of food at two different temperatures, did not show any time trend in gene frequency. Deviations from Hardy-Weinberg proportions can be used to test the existence of viability differences among different female genotypes, because gene frequencies in the cage populations did not change during the course of the experiment (see LEWONTIN and COCKERHAM 1959). In applying x^2 tests, the data from two replicate cages (Cages 1 & 2, 3 & 4, 5 & 6, etc.) were pooled in order to increase the sample size. There is one degree of freedom in each test as the expected number was obtained from the observed frequency. Adult flies were sampled in Cages 1, 2, 3, and 4. In other cages egg samples were collected and transferred to a bottle, and the emerging adult flies examined. This difference in sampling technique did not appear to affect the results. The proportion of female genotypes which were collected from cages was not different from that predicted by a Hardy-Weinberg equilibrium (Table 1); x^2 values of Cages 1 & 2, 3 & 4, 5 & 6, 7 & 8, 9 & 10, and 11 & 12 were 0.33, 1.56, 1.92, 0.33, 0.73, 0.11, respectively. No difference in gene frequency between the two sexes was observed (see Table 1).

Therefore, it can be concluded from these three results—no change in gene frequency with time, very good fit with Hardy-Weinberg proportions, and no difference between the sexes in gene frequency—that there are no detectable differences in the fitnesses of the different genotypes at the esterase-5 locus under the conditions of the these experiments.

Experiment 2: viability and developmental time test using sex ratio: In this experiment two components of fitness, viability and developmental time, were measured using sex ratio. As this method does not involve electrophoresis, the scale of the experiment can be increased by a factor of 100 for a given amount of labor compared to experiments utilizing electrophoretic examination of protein. These experiments are also much more rapid than population cage experiments, and may also detect a lower intensity of selection. The experimental conditions in the present experiments are necessarily different from those found in cages.

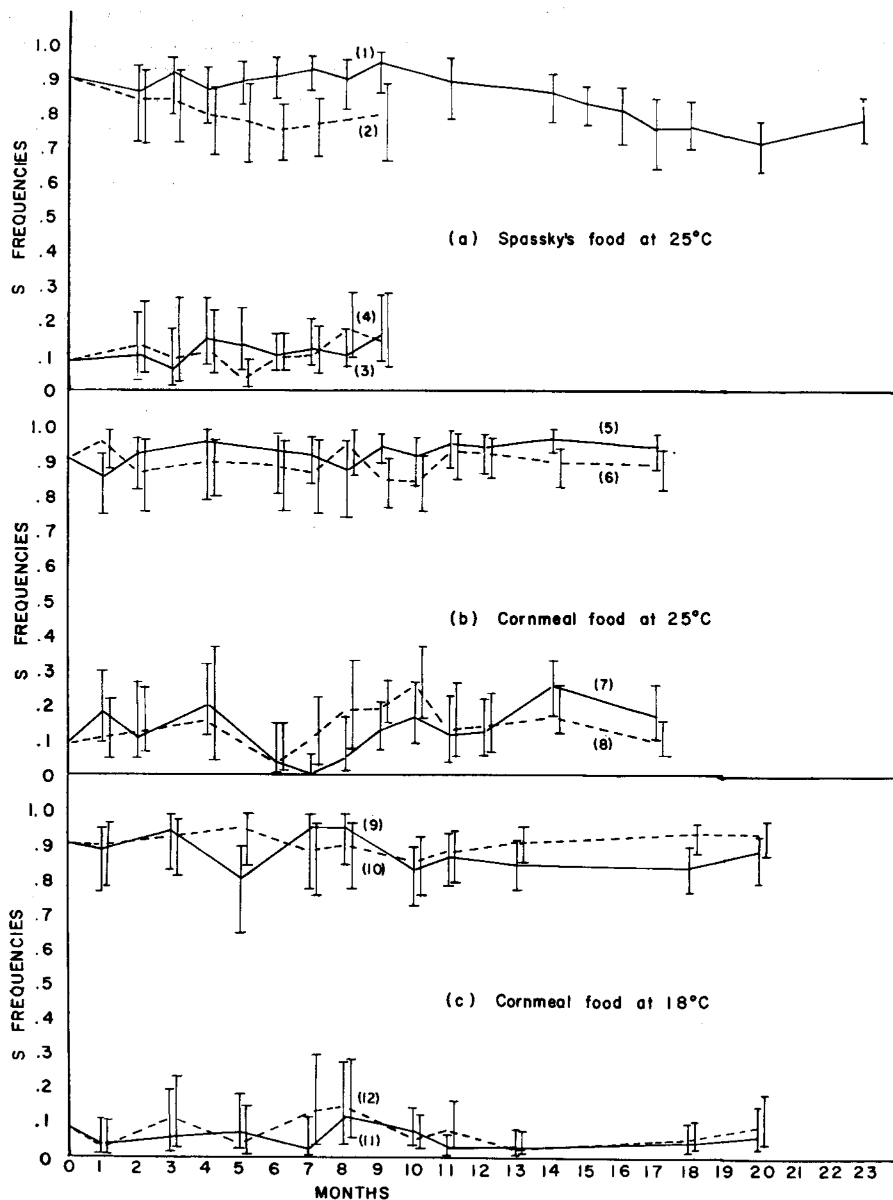

FIGURE 1.—Gene frequency changes in population cages at two different temperatures (18°C and 25°C) with two different foods (SPASSKY's and cornmeal). Vertical lines represent 95% confidence intervals.

Since no suitably marked chromosome with inversions (which would suppress recombination) is available on the *X* chromosome of *Drosophila pseudoobscura*, a mating scheme which takes advantage of sex ratio was used in order to test the viability and developmental time of the three genotypes (*S/S*, *S/F*, *F/F*) in females and of the two genotypes (*S*, *F*) in males. Figure 2 shows the four different matings used for estimating viability and developmental time of the five

TABLE 1

Average gene frequency and test of deviation from Hardy-Weinberg proportion of flies sampled from population cages

Cage Number		♀			♂		
		S/S	S/F	F/F	S	F	
1 and 2	Observed	310	96	7	307	56	♀ freq. = 0.867
	Expected	(305.45)	(99.45)	(8.09)			♂ freq. = 0.846
			χ^2 = 0.33				Total freq. = 0.860
3 and 4	Observed	9	90	337	36	318	♀ freq. = 0.124
	Expected	(5.97)	(90.07)	(339.94)			♂ freq. = 0.102
			χ^2 = 1.56				Total freq. = 0.117
5 and 6	Observed	754	127	9	395	39	♀ freq. = 0.919
	Expected	(748.39)	(135.48)	(6.13)			♂ freq. = 0.910
			χ^2 = 1.92				Total freq. = 0.917
7 and 8	Observed	20	218	626	74	352	♀ freq. = 0.149
	Expected	(20.49)	(225.13)	(618.38)			♂ freq. = 0.174
			χ^2 = 0.33				Total freq. = 0.154
9 and 10	Observed	477	118	9	307	30	♀ freq. = 0.887
	Expected	(481.66)	(115.43)	(6.92)			♂ freq. = 0.911
			χ^2 = 0.73				Total freq. = 0.893
11 and 12	Observed	2	74	579	19	351	♀ freq. = 0.060
	Expected	(2.20)	(71.57)	(581.22)			♂ freq. = 0.051
			χ^2 = 0.11				Total freq. = 0.058

Expected number was obtained from total frequency.

genotypes. The line number is indicated by j ($j = 1, \ldots 22$). From mating $(1,j)$ male offspring hemizygous for the S allele (S_{j+1}) and female offspring homozygous for the S allele (S_j/S_{j+1}) will appear at equal frequencies if there are no viability differences between males and females, and if there is no aberrant segregation. Similarly, S males (S_{j+1}) and heterozygous females (S_{j+1}/F_j) will appear at equal frequencies from mating $(2,j)$. Since the genotypes of the male progeny in both matings are the same, the genotypes of the female progeny (S homozygotes and heterozygotes) can be compared using the S_{j+1} male offspring as standard. In the same way, in matings $(3,j)$ and $(4,j)$ heterozygous progeny (S_{j+1}/F_j) and F homozygotes (F_j/F_{j+1}) are compared using F_j males as standard. In addition, the two male genotypes (S, F) can be compared using heterozygous females (S_{j+1}/F_j) as standard [matings $(2,j)$ and $(3,j)$]. In the above comparisons it is assumed that interaction among genotypes with respect to fitness are negligible and that the relative fitness of genotypes is not influenced by the competing genotypes. It is known that those assumptions are sometimes not correct (e.g., LEWONTIN and MATSUO 1963).

According to this mating scheme, heterozygosity of genetic background is the same between homozygote and heterozygote on the average with respect to the esterase-5 locus, assuming that the differences in genetic background between lines of the same allele are the same as between lines of different alleles. This condition is satisfied, as each of the 44 homozygous lines was established independently.

Five-day-old virgin male and female flies from all the lines were mated using an aspirating tube to avoid etherization, according to the mating scheme in Figure 2. The five pairs of flies used in each cross were transferred to a new vial five days later. They were allowed to lay eggs for another five days and then discarded. The flies emerging from these two vials were pooled and treated as one observation. Viability was tested at two different temperatures (25°C and 12.5°C)

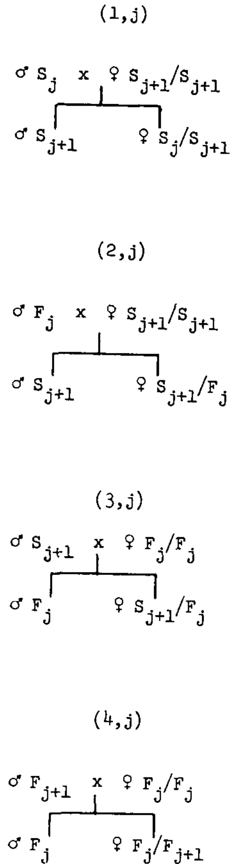

FIGURE 2.—Mating scheme used for Experiments 2, 3, and 4. In Experiment 4 the progeny in this figure were used as parents ($j = 1, \ldots 22$).

in order to examine the effect of temperature on viability. Developmental time was tested only at 25°C.

Viability test: Offspring were counted for 30 days (in 25°C test) and 70 days (in 12.5°C test) from the day of mating. Almost all flies had eclosed by the end of the counting period. Offspring were generally counted on five occasions (16, 18, 20, 25, and 30 days), but occasionally on only two. In the 25°C experiment, 14 out of 17 observations were counted five times since both viability and developmental time were examined. Counting was done only twice in the 12.5°C experiment as developmental time was not tested.

As shown in Figure 2, there are four different types of matings ($i = 1, \ldots 4$) and 22 lines of each allele ($j = 1, \ldots 22$). In most cases 17 replicates were set up in each mating type of each line. The viability of female flies of one genotype was estimated as the proportion of female flies in each observation. The estimates of male viability were obtained in the same way as female viability, except that proportion of male flies in each observation of only matings (2,j) and (3,j) were used as viability index. The values in Table 2 are the average for all lines (the value for each line is the average of 17 observations). These values were converted into relative fitnesses by dividing the value for each genotype by the heterozygote viability with females, and by the S male viability with males.

Developmental time: The flies used in the developmental time experiment were exactly the

same as those used in the viability test. All the cultures were counted five times: 16, 18, 20, 25, and 30 days from the date of mating. The number of replicates was usually 14. In order to compare the developmental times of different female genotypes, relative developmental time, measured relative to a standard genotype, was used as follows:

Relative developmental time (female) = absolute developmental time (male)— absolute developmental time (female).

Relative developmental time (male) = — relative developmental time (female). As mentioned above, the genotypes of the male progeny are the same in matings $(1,j)$ and $(2,j)$, and in matings $(3,j)$ and $(4,j)$. Therefore the developmental times of the two female genotypes can be compared using relative developmental time as index. When the developmental time of male genotypes were compared, the heterozygous female genotype was used as standard [from matings $(2,j)$ and $(3,j)$]. A larger value of relative developmental time index indicates a faster developmental time of the corresponding genotypes. Developmental time was expressed in terms of average developmental time and was not standardized (Experiment 3, Experiment 4), or in terms of differences in developmental time among genotypes (Experiment 2).

RESULTS

A total of 1463 observations (two vials in an observation) was made in the experiment conducted at 25°C, of which 1232 observations were used for both viability and developmental time. All 1463 observations were used for the estimates of viability. In the 12.5°C experiment, all 562 observations were used only for viability estimation.

Viability test at 25°C: The mean viability indices were 0.5473 and 0.6436 in *S/S* and *S/F*; 0.5536 and 0.5547 in *S/F* and *F/F*; and 0.4564 and 0.4464 in *S* and *F* (Table 2). Pairwise comparisons were performed using the average values in each cross to test the viability differences between genotypes at 25°C. The results showed that there were no significant differences between any two genotypes compared (*S/S:S/F*, *S/F:F/F*, and *S:F*); t_{21} values were less than one in females, and 1.41 in males. The error variances $\left(S^2_{\bar{d}}\right)$ used in this t test measure the size of the differences in the relative fitness of the two genotypes concerned among different pairs. Accordingly, this variance was tested against the variance among replicates within crosses; there were 17 observations within each cross. The variance ratio was highly significant in every comparison ($F_{21,684} = 3.00$ for *S/S:S/F;* $F_{21,691} = 2.80$ for *S/F:F/F;* and $F_{21,685} = 4.67$ for *S:F*). This means that there is a large amount of genetic variability in the background (or linked genes) of *S* and *F* genes. It does not necessarily mean that there are physiological interactions between the esterase-5 locus and background genes. It probably means that the effects of linked loci are large relative to those of the locus in question when the number of chromosomes involved is small. These results emphasize the importance of using large numbers of chromosomes in this type of experiment in order to avoid as far as possible confounding the effect of the gene in question (as measured on the background characteristic of the base population) with the effects of linked genes.

Viability test at 12.5°C: At this low temperature it took approximately 40 days for flies to grow from egg to adult. Fifteen lines were selected at random for this experiment. These results are essentially the same as those obtained at 25°C (see

TABLE 2

Summary data of Experiment 2

Components of Fitness Tested	♀ Genotype			♂ Genotype	
	S/S	S/F	F/F	S	F
		A			
Viability at 25°C	0.5473	0.5436			
	D = +0.0037 ± 0.0059				
		0.5536	0.5547		
		D = -0.0011 ± 0.0058			
				0.4564	0.4464
				D = +0.0100 ± 0.0071	
Relative Fitness	1.0068	1	1.0020	1	0.9781
		B			
Viability at 12.5°C	0.5303	0.5247			
	D = +0.0056 ± 0.0088				
		0.5332	0.5179		
		D = +0.0153 ± 0.0088			
				0.4753	0.4668
				D = +0.0085 ± 0.0096	
Relative Fitness	1.0107	1	0.9723	1	0.9821
		C			
Developmental Time at 25°C	0.08	0.20			
	D = -0.12 ± 0.0689				
		0.01	0.00		
		D = +0.01 ± 0.0588			
				-0.20	-0.01
				D = -0.19 ± 0.0831*	

* Significant at 5% level.

Table 2). The mean viability indices were 0.5303 and 0.5247 for *S/S* and *S/F*; 0.5332 and 0.5179 for *S/F* and *F/F*; and 0.4753 and 0.4668 for *S* and *F*. There were no significant genotype effects in any of these comparisons ($t_{14} = 0.64$ for *S/S:S/F*; $t_{14} = 1.74$ for *S/F:F/F*; and $t_{14} = 0.88$ for *S:F*). The error variances were significantly greater than the variances among replicates ($F_{14,281} = 1.92$ for *S/S:S/F;* $F_{14,250} = 2.00$ for *S/F:F/F;* and $F_{14,257} = 2.57$ for *S:F*). Since the results are essentially the same at both temperatures (25°C and 12.5°C), it can be concluded that there is no evidence of temperature effect on viability at the esterase-5 locus in these experiments.

Developmental time: The average absolute developmental time was approximately 22 days. The mean developmental time indices were 0.08 and 0.20 for

S/S and *S/F*; 0.01 and 0.00 for *S/F* and *F/F*; and − 0.20 and − 0.01 for *S* and *F* males (Table 2). This indicates that the developmental time of *S/F* females is 0.01 days faster than *F/F* and 0.12 days faster than *S/S*, while that of *F* males is 0.19 days faster than that of *S* males. The differences were not significant among female genotypes ($t_{21} = 1.74$ for *S/S:S/F*; and $t_{21} = 0.12$ for *S/F:F/F*) but were significant between male genotypes at the 5% level ($t_{21} = 2.33$). The error variance was not significantly greater than the variance among replicates in the comparison between *S/F* and *F/F* females ($F_{21,572} = 1.54$), but highly significant in other comparisons ($F_{21,572} = 2.01$ for *S/S:S/F*; and $F_{21,572} = 2.75$ for *S:F*).

The results of all these experiments on viability and developmental time indicate that there are no differences in fitnesses among genotypes except for the difference in developmental times between male genotypes. Differences in viability were no greater than a few percent: the ratios of *S/S* : *S/F* : *F/F* were 1.0068 : 1 : 1.0020 at 25°C and 1.0107 : 1 : 0.9723 at 12.5°C; while those of *S:F* were 1 : 0.9781 at 25°C and 1 : 0.9821 at 12.5°C. The only significant difference was obtained for developmental time of male genotypes: D = 0.19 ± 0.081 (Table 2). That is, *F* males develop 0.19 day earlier than *S* males on the average. It is, however, unlikely that the esterase-5 locus is itself responsible for this difference as there are highly significant error variances. It is also possible that one significant difference out of nine different comparisons has arisen because of mere chance. In addition, it is difficult to use developmental time to predict changes in gene frequency in population cages (or in nature) as the genetic theory is poorly developed for overlapping generations and because we have limited information on the ecology of Drosophila.

Experiment 3: frequency-dependent selection experiment: The experiments described in this section are an attempt to test whether the relative fitnesses of different genotypes at the esterase-5 locus change as the gene frequency changes in a manner similar to that described by KOJIMA and YARBROUGH (1967). The design of these experiments is almost identical to that of KOJIMA and YARBROUGH. The mating scheme was the same as that used in Experiment 2 (described above).

This experiment differs from the sex-ratio experiment in that it requires electrophoresis for estimating fitness, since five genetically different offspring emerge from each bottle. As the size of the experiment is limited by this procedure, differences of a few percent in relative fitness will not be detected. If frequency-dependent selection is operating at the esterase-5 locus of *D. pseudoobscura* with respect to viability or fecundity to an extent as great as that reported by KOJIMA and YARBROUGH (1967) for *D. melanogaster*, these experiments should be sufficient to detect differences in selective forces. The following two kinds of experiments were carried out.

Adult-to-adult experiment: Four days after crossing according to the mating scheme shown in Figure 2, the four types of fertilized females ($S/S \times S$, $S/S \times F$, $F/F \times S$, $F/F \times F$) were separated from the males. Two days were allowed for recovery from the effects of etherization. All the females of the same mating from every line were then randomized by putting them together in a bottle.

One hundred fertilized females from four different matings were mixed in the desired proportions in a bottle and were allowed to lay eggs for two days. The proportions were chosen so that in three different experiments the expected gene frequency of the *S* allele in the progeny would be 0.7, 0.5, or 0.3, respectively, in the absence of selective differences, and the expected frequencies of female progeny were in the Hardy-Weinberg proportions in all cases (see Table 4). The numbers of the four types of female flies ($S/S \times S$, $S/S \times F$, $F/F \times S$, $F/F \times F$) were there-

TABLE 3

The mating scheme and model of the viability estimation in frequency-dependent selection experiment
(Experiment 3)

Mating types	Input frequency	Progeny genotype S/S	S/F	F/F	S	F
		Viability				
♀ ♂		V_1	1	V_2	1	$V♂$
$S/S \times S$	f_1	f_1V_1		..	f_1	..
$S/S \times F$	f_2	..	f_2	..	f_2	..
$F/F \times S$	f_2	..	f_2			$f_2V♂$
$F/F \times F$	f_3	..		f_3V_3	..	$f_3V♂$
	Output frequency	p_1	p_2	p_3	p_4	p_5

where $p_1 + p_2 + p_3 = p_4 + p_5 = f_1 + f_2 + f_2 + f_3 = 1$

fore 49, 21, 21, and 9, respectively, when the expected frequency of the *S* allele was 70%; 25, 25, 25, and 25, respectively, when the expected frequency of the *S* allele was 50%; and 9, 21, 21, and 49, respectively, when the expected frequency of the *S* allele was 30%.

Progeny were counted daily until all the F_1 flies had emerged. The output frequency of each genotype with regard to the esterase-5 locus was tested by acrylamide gel electrophoresis. In this experiment two components of fitness, female fecundity and egg-to-adult viability, are confounded since the input is fertilized adults and the output is adult offspring.

Egg-to-adult experiment: Egg samples were collected from the females of each mating type used for the adult-to-adult experiment and two hundred eggs were mixed in Hardy-Weinberg proportions for frequencies of the *S* allele in three different experiments equal to 0.7, 0.5, 0.3, as in the adult-to-adult experiment. In this experiment, viability from egg to adult is measured. Apart from this, the experiment was conducted in the same way as the adult-to-adult experiment.

Viability was estimated in both egg-to-adult and adult-to-adult experiments. When viability was estimated from the adult-to-adult experiment, genotypic differences in female fecundity were confounded with viability.

The mating scheme is summarized in terms of input (f_1,f_2,f_3) and output (p_1,p_2,p_3,p_4,p_5) frequency and viability $(V_1, V_2, V_♂)$ in Table 3. The output frequency is expressed in terms of of input frequency and viability from Table 3, as follows:

$$p_1 = \frac{f_1V_1}{N} \qquad p_4 = \frac{f_1 + f_2}{N_♂}$$

$$p_2 = \frac{2f_2}{N} \qquad p_5 = \frac{(f_2 + f_3)V_♂}{N_♂}$$

$$p_3 = \frac{f_3V_2}{N}$$

where $N = f_1V_1 + 2f_2 + f_3V_2$ and $N_♂ = f_1 + f_2 + (f_2 + f_3)V_♂$

The estimate of viability for each genotype and its variance is expressed in terms of input and output frequencies, and the number of flies tested, as shown below:

The method of derivation of these equations is described in more detail in the next section (Experiment 4).

$$V_1 = \frac{2f_2}{f_1} \cdot \frac{p_1}{p_2} \qquad \sigma^2_{V_1} = \frac{1}{TN}\left(\frac{2f_2}{f_1}\right)^2\left(\frac{p_1(p_1+p_2)}{p_2^3}\right)$$

$$V_2 = \frac{2f_2}{f_3} \cdot \frac{p_3}{p_2} \qquad \sigma^2_{V_2} = \frac{1}{TN}\left(\frac{2f_2}{f_3}\right)^2 \left(\frac{p_3(p_2+p_3)}{p_2^3}\right)$$

$$V_{\male} = \frac{f_1 + f_2}{f_2 + f_3} \cdot \frac{p_5}{p_4} \qquad \sigma^2_{V\male} = \frac{1}{TN}\left(\frac{f_1+f_2}{f_2+f_3}\right)^2 \cdot \frac{p_5}{p_4}$$

where TN = number of female flies tested
and $TN_{\male}$ = number of male flies tested

The developmental time of each genotype was also estimated in a manner similar to that of the previous experiment. Since all five genotypes emerge from one bottle in this experiment, all the genotypes can be compared directly.

RESULTS

Viability: The results of the adult-to-adult experiment are shown in Table 4. Expected porportions (= input proportion of premated female flies), observed proportions after selection, and total number of flies tested of each sex are shown in the first two rows of each input frequency. The third row shows the relative viabilities of each genotype with their standard errors as calculated by the method given above. In female viability, only one case (*F/F* homozygote) showed a significant difference ($P < 0.05$) from standard (heterozygote viability) at an input frequency of 30% *S*. All other estimates of female viability were not significantly different from the heterozygote viability. The viabilities of *F* males

TABLE 4

The results of viability test from adult-to-adult frequency-dependent selection experiment (Experiment 3)

Input Frequency of S Allele		Output ♀			No. of Flies Tested	Output ♂		No. of Flies Tested
		S/S	S/F	F/F		S	F	
70 per cent	Expected proportion	0.49	0.42	0.09	567	0.7	0.3	397
	Observed proportion	0.48501	0.42857	0.08642		0.74055	0.25945	
	Relative fitness	0.9700±0.0854	1	0.9410±0.1473		1	0.8175±0.0693**	
50 per cent	Expected proportion	0.25	0.50	0.25	564	0.50	0.50	397
	Observed proportion	0.28723	0.49113	0.22163		0.56423	0.43577	
	Relative fitness	1.1697±0.1158	1	0.9025±0.0975		1	0.7723±0.0436**	
30 per cent	Expected proportion	0.09	0.42	0.49	565	0.30	0.70	375
	Observed proportion	0.10442	0.45133	0.44425		0.35467	0.64533	
	Relative fitness	1.0797±0.1559	1	0.8437±0.0748*		1	0.7798±0.0300**	

* Significant at 5% level.
** Significant at 1% level.

TABLE 5

The results of viability test from egg-to-adult frequency-dependent selection experiment (Experiment 3)

Input Frequency of S Allele		Output ♀			No. of Flies Tested	Output ♂		No. of Flies Tested
		S/S	S/F	F/F		S	F	
70 per cent	Expected proportion	0.49	0.42	0.09	298	0.7	0.3	310
	Observed proportion	0.46644	0.43624	0.09732		0.71613	0.28387	
	Relative fitness	0.9165±0.1118	1	1.0411± 0.2138		1	0.9249±0.0837	
50 per cent	Expected proportion	0.25	0.50	0.25	321	0.50	0.50	335
	Observed proportion	0.25382	0.47706	0.26911		0.51343	0.48657	
	Relative fitness	1.0641±0.1446	1	1.1282±0.1503		1	0.9477±0.0529	
30 per cent	Expected proportion	0.09	0.42	0.49	348	0.30	0.70	308
	Observed proportion	0.09483	0.41954	0.48563		0.30195	0.69805	
	Relative fitness	1.0548±0.2032	1	0.9922±0.1122		1	0.9908±0.0374	

were significantly lower than *S* males irrespective of input frequency. In these experiments, flies bearing the *S* allele, especially males, seem to be more viable than flies with the *F* allele.

Although the values in Table 4 do not seem to give evidence of minority advantages of the type reported by KOJIMA *et al.*, frequency-dependent selection was tested in another way using only gene frequency (not with genotype frequency) since there were five genotypes to compare and the number of flies tested was small. Using the difference between observed and expected gene frequency in each replicate at each frequency, an analysis of variance was conducted to see if frequency-dependent selection was operating. The F value was less than one and there is therefore no evidence of frequency-dependent selection. Average differences from expected gene frequencies of the *S* allele were 0.0174 ± 0.0188, 0.0373 ± 0.0231, and 0.0323 ± 0.0199 at the input *S* allele frequencies of 0.7, 0.5, and 0.3, respectively. Since there was no evidence of frequency-dependent selection, the input frequencies were ignored and the differences of the observed from expected were pooled. The pooled difference showed that the excess of *S* allele over expected of 0.0290 was significant at the 5% level ($t = 2.5$, $df = 25$).

The results of the egg-to-adult experiment are shown in Table 5. in which the data are arranged as in Table 4 (adult-to-adult experiment). None of the genotypes has a viability which is significantly different from standard, and a test of frequency-dependent selection using gene frequency was also negative. The average differences of observed from expected frequency were -0.0046 ± 0.0146,

0.0005 ± 0.0260, and -0.0003 ± 0.0109 at input frequencies of 0.7, 0.5, and 0.3, respectively.

When viability was estimated from the adult-to-adult experiment it was assumed that fecundity differences between genotypes (F/F and S/S females) were negligible. Since both fecundity and viability are involved in the adult-to-adult experiment, viability estimates are correct only when there is no difference in fecundity between two genotypes.

KOJIMA and YARBROUGH attempted to avoid this complication by estimating the fecundity of two female genotypes in separate experiments, and adjusting the expected frequencies according to these fecundity estimates. In the present experiment, I have tried to estimate fecundity in an adult-to-adult experiment using the viability estimates obtained from an egg-to-adult experiment. Unfortunately it was not possible to apply the results of one experiment to the other in order to obtain the estimates of both viability and fecundity since the intensity of competition was very different in the two experiments. In the egg-to-adult experiment 68% of eggs eclosed as adult flies. On the other hand only 15% of eggs emerged as adults in the adult-to-adult experiment. Fecundity estimates are therefore not strictly comparable between the two experiments, since it is well known that fitness may be dependent on density (LEWONTIN 1955; SOKAL and HUBER 1963). If there is no differential selection in fecundity between the two female genotypes, it is possible to estimate the viability of all five genotypes as in the egg-to-adult experiment. If there are differences in fecundity between two genotypes, or if the fecundity is incorrectly estimated in the preliminary experiment, the estimate of viability is biased. The bias is apparent since five different genotypes emerge as progeny while only two different females are used as parents. Let the viabilities of S/S, S/F, F/F females, and of S or F males be V_1, 1, V_2, and 1 or $V_{♂}$, respectively. Let female fecundity of S/S and F/F be 1 and E, respectively. What we are actually estimating in adult-to-adult experiment when viability is estimated by ignoring fecundity as in Table 3, is expressed algebraically as follows: $S/S : S/F : F/F = \frac{2V_1}{1+E} : 1 : \frac{2EV_2}{1+E}$ and $S : F = 1 : EV_{♂}$. In this case the only unbiased estimates are the "total fitness" (product of fecundity and viability) of two homozygous females: $S/S : F/F = \frac{2V_1}{1+E} : \frac{2EV_2}{1+E} = V_1 : EV_2$.

Sometimes the ratio of Observed to Expected is used as a fitness index (see KOJIMA and YARBROUGH 1967). However this index is not frequency independent. If there is any difference in fitness among genotypes, the (Observed/Expected) ratio changes when the expected proportion changes. For example, in the present mating scheme if the viabilities of S/S, S/F, F/F are 1, 1, 0.7, the fitness index (Observed/Expected) of S/S female is 1.03 at 70% input frequency, 1.08 at 50%, and 1.17 at 30%. The relative fitness, however, is obtained from the Observed/Expected ratio since the ratio of fitness index among genotypes is the same among different input frequency. To avoid the misinterpretation, in this paper only the standardized relative fitness was used.

TABLE 6

The results of developmental time test from adult-to-adult frequency-dependent selection experiment
(Experiment 3)

Input frequency (percent)	Average developmental time by day ♀			♂	
	S/S	*S/F*	*F/F*	*S*	*F*
70	21.56 ± 0.24	20.93 ± 0.25	21.71 ± 0.56	20.84 ± 0.19	21.31 ± 0.32
50	22.94 ± 0.35	22.48 ± 0.27	23.19 ± 0.40	21.79 ± 0.26	21.73 ± 0.29
30	23.42 ± 0.46*	21.65 ± 0.22	22.22 ± 0.22	21.68 ± 0.27	22.18 ± 0.20

* *S/S* developmental time is different from both *S/F* and *F/F* at 5% level.

Developmental time: The average developmental time of each genotype with standard error at each frequency is shown in Table 6 (from adult to adult) and Table 7 (from egg to adult). Analyses of variance were conducted to see if there were any significant differences in developmental time among genotypes at each frequency of both adult-to-adult and egg-to-adult experiments. In the adult-to-adult experiment, female developmental times at 30% input frequency were significantly ($F_{2,562} = 6.37$; $P < 0.01$) different. Five other comparisons were not significantly different. None of the analyses of variance (six analyses in both sexes) showed significant results in the egg-to-adult experiments. Average developmental times in the adult-to-adult experiments were 3–4 days longer than in the egg-to-adult experiment. In the egg-to-adult experiment almost all flies emerged in a few days, while the eclosion of flies in the adult-to-adult experiment extended over more than 15 days. This is mostly due to the difference in the intensities of competition between experiments. Moreover, the developmental time in the adult-to-adult experiment includes not only the egg-to-adult developmental time but also the egg-laying time (maximum 2 days) of fertilized parental flies. However, the increase in variance in developmental time from the latter cause is not substantial because the 2-day egg-laying difference is small compared with the 15-day eclosion period. It is also possible that differences in the time of egg laying were intensified by the interaction between flies.

TABLE 7

The results of developmental time test from egg-to-adult frequency-dependent selection experiment
(Experiment 3)

Input frequency (percent)	Average developmental time by day ♀			♂	
	S/S	*S/F*	*F/F*	*S*	*F*
70	17.06 ± 0.10	17.08 ± 0.10	17.24 ± 0.21	17.62 ± 0.07	17.77 ± 0.11
50	17.33 ± 0.16	17.47 ± 0.11	17.61 ± 0.15	17.76 ± 0.10	18.01 ± 0.11
30	17.09 ± 0.26	17.60 ± 0.12	17.47 ± 0.11	18.20 ± 0.17	18.14 ± 0.11

TABLE 8

Mating schemes and model to estimate viability and fecundity simultaneously in Experiment 4

Mating type ♀ ♂	Input frequency	Female fecundity	Frequency after fecundity selection ♀ S/S	S/F	F/F	♂ S	F
			Mating I				
$S/F \times S$	f_1	1	$\frac{1}{2}f_1$	$\frac{1}{2}f_1$	..	$\frac{1}{2}f_1$	$\frac{1}{2}f_1$
$F/F \times F$	f_2	E_2	..	..	f_2E_2	..	f_2E_2
		Viability	V_1	1	V_2	1	$V♂$
			Mating II				
$S/S \times S$	k_1	E_1	k_1E_1	..	..	k_1E_1	..
$S/F \times F$	k_2	1	..	$\frac{1}{2}k_2$	$\frac{1}{2}k_2$	$\frac{1}{2}k_2$	$\frac{1}{2}k_2$
		Viability	V_1	1	V_2	1	$V♂$

The direction of the difference in developmental time among genotypes (although not significant) was not the same as that of Experiment 2 (sex-ratio experiment): the flies bearing an F allele seem to develop slower than those with an S allele in the frequency-dependent selection experiments (see Tables 6 and 7), while the reverse seems to be true in the sex-ratio experiment (see Table 2). This suggests that the small differences observed may not be ascribable to the effects of the locus in question.

It can be concluded that, as only one out of 12 tests (three tests in either sex of each experiment) showed a significant difference in developmental time, there are no detectable differences in developmental time among genotypes. For viability, there were no significant differences among genotypes in the egg-to-adult experiment. In the adult-to-adult experiment, males bearing the S allele are clearly more fit than those bearing the F allele. There was no evidence of frequency-dependent selection in either experiment.

Experiment 4: viability and fecundity experiment: In the present experiment, I have tried to estimate the two components of fitness (viability and fecundity) simultaneously. In order to estimate five unknowns (two estimates of female viability, one of male viability, two of female fecundity), two different mating schemes are necessary as there are two degrees of freedom in the female, and one in the male in each experiment. This is only possible because the locus is sex linked. Male viability can be estimated in two ways since there are two degrees of freedom and only one unknown. It is assumed from the previous results that frequency-dependent selection is negligible in the present experiments. The model by which both components of fitness can be estimated is set forth in Table 8.

In mating I, let the input frequency of heterozygous females (S/F) fertilized by S males and F/F homozygous females fertilized by F males be f_1 and f_2, respectively. Similarly, in mating II let the input frequency of S/S homozygotes fertilized by S males and heterozygous females (S/F) mated with F males be k_1 and k_2, respectively. Let the output frequency of S/S homozygote, heterozygote, F/F homozygote, S male, and F male be p_1, p_2, p_3, p_4, and p_5 after normalization in mating I, and q_1, q_2, q_3, q_4, and q_5 in mating scheme II. These output frequencies can be expressed in terms of input frequencies (f_1, f_2 in mating scheme I; and k_1, k_2 in mating scheme II), viabilities (V_1, V_2, $V_♂$), and fecundities (E_1, E_2) as follows.

From Mating Scheme I

$$p_1 = \frac{1}{N_1}\left(\frac{f_1V_1}{2}\right)$$

$$p_2 = \frac{1}{N_1}\left(\frac{f_1}{2}\right)$$

$$p_2 = \frac{1}{N_1}(f_2E_2V_2)$$

$$p_4 = \frac{1}{N_{1_{\text{♂}}}}\left(\frac{f_1}{2}\right)$$

$$p_5 = \frac{1}{N_{1_{\text{♂}}}}\left(\frac{f_1V_{\text{♂}}}{2} + f_2E_2V_{\text{♂}}\right)$$

where $N_1 = \frac{f_1V_1}{2} + \frac{f_1}{2} + f_1E_2V_2$

$$N_{1_{\text{♂}}} = \frac{f_1}{2} + \frac{f_1V_{\text{♂}}}{2} + f_2E_2V_{\text{♂}}$$

From Mating Scheme II

$$q_1 = \frac{1}{N_2}(k_1E_1V_1)$$

$$q_2 = \frac{1}{N_2}\left(\frac{k_2}{2}\right)$$

$$q_3 = \frac{1}{N_2}\left(\frac{k_2V_2}{2}\right)$$

$$q_4 = \frac{1}{N_{2_{\text{♂}}}}\left(k_1E_1 + \frac{k_2}{2}\right)$$

$$q_5 = \frac{1}{N_{2_{\text{♂}}}}\left(\frac{k_2V{\text{♂}}}{2}\right)$$

$$N_2 = k_1E_1V_1 + \frac{k_2}{2} + \frac{k_2V_2}{2}$$

$$N_{1_{\text{♂}}} = k_1E_1 + \frac{k_2}{2} + \frac{k_2V{\text{♂}}}{2}$$

Since $p_1 + p_2 + p_3 = p_4 + p_5 = q_1 + q_2 + q_3 = q_4 + q_5 = 1$, the above 10 equations are reduced to six by dividing p_1 and p_3 by p_2; p_4 *by* p_5; q_1 and q_3 by q_2; and q_4 by q_5 as shown below:

From Mating Scheme I

$$\frac{p_1}{p_2} = \frac{\left(\frac{f_1V_1}{2}\right)}{\left(\frac{f_1}{2}\right)}$$

$$\frac{p_3}{p_2} = \frac{f_2E_2V_2}{\left(\frac{f_1}{2}\right)}$$

$$\frac{p_4}{p_5} = \frac{\left(\frac{f_1}{2}\right)}{\left(\frac{f_1V{\text{♂}}}{2} + f_2E_2V{\text{♂}}\right)}$$

From Mating Scheme II

$$\frac{q_1}{q_2} = \frac{k_1E_1V_1}{\left(\frac{k_2}{2}\right)}$$

$$\frac{q_3}{q_2} = \frac{\left(\frac{k_2V_2}{2}\right)}{\left(\frac{k_2}{2}\right)}$$

$$\frac{q_4}{q_5} = \frac{k_1E_1 + \frac{k_2}{2}}{\frac{k_2V_{\text{♂}}}{2}}$$

From these six equations viability and fecundity can be expressed in terms of input and output frequency as follows:

$$V_1{\text{♂}} = \frac{p_5p_2q_3}{p_4(p_2q_3 + p_3q_2)} \qquad V_1 = \frac{p_1}{p_2}$$

$$V_{2_{\text{♂}}} = \frac{q_5(p_1q_2 + q_2q_1)}{q_4p_1q_2} \qquad V_2 = \frac{q_3}{q_2}$$

$$V_1E_1 = \frac{k_2q_1}{2k_1q_2} \qquad E_1 = \frac{k_2p_2q_1}{2k_1p_1q_2}$$

$$V_2E_2 = \frac{f_1p_3}{2f_2p_2} \qquad E_2 = \frac{p_3q_2f_1}{2p_2q_3f_2}$$

The variances of the fitness estimates were computed using the approximation of the Taylor expansion by the following equation:

$$\sigma^2_X = \sigma^2_{p_1}\left(\frac{\partial X}{\partial p_1}\right)^2 + \sigma^2_{p_2}\left(\frac{\partial X}{\partial p_2}\right)^2 + \sigma^2_{q_1}\left(\frac{\partial X}{\partial q_1}\right)^2 + \sigma^2_{q_2}\left(\frac{\partial X}{\partial q_2}\right)^2 +$$

$$2\left\{\sigma_{p_1p_2}\left(\frac{\partial X}{\partial p_1}\right)\left(\frac{\partial X}{\partial p_2}\right) + \sigma_{q_1q_2}\left(\frac{\partial X}{\partial q_1}\right)\left(\frac{\partial X}{\partial q_2}\right)\right\} + \sigma^2_{p_4}\left(\frac{\partial X}{\partial p_4}\right)^2 + \sigma^2_{q_4}\left(\frac{\partial X}{\partial q_4}\right)^2$$

where X : the estimate in question
σ^2_X : the variance of estimate X

The variances of the fitness estimates are then as follows:

$$\sigma^2_{V_1} = \frac{p_1(p_1+p_2)}{TN_1\,p^3_2} \qquad \sigma^2_{E_1V_1} = \left(\frac{k^2_2}{4k^2_1}\right)\left(\frac{q_1(q_1+q_2)}{TN_2\,q^3_2}\right)$$

$$\sigma^2_{V_2} = \frac{q_3(q_2+q_3)}{TN_2\,q^3_2} \qquad \sigma^2_{E_2V_2} = \left(\frac{f^2_1}{4f^2_2}\right)\left(\frac{p_3(p_2+p_3)}{TN_1\,p^3_2}\right)$$

$$\sigma^2_{E_1} = \left(\frac{k^2_2}{4k^2_1}\right)\left(\frac{q^2_1p_2(p_1+p_2)}{TN_1\,q^2_2p^3_1} + \frac{p^2_2\,q_1(q_1+q_2)}{TN_2\,p^2_1q^3_2}\right)$$

$$\sigma^2_{E_2} = \left(\frac{f^2_1}{4f^2_2}\right)\left(\frac{q^2_2p_3(p_2+p_3)}{TN_1\,q^2_3p^3_2} + \frac{p^2_3\,q_2(q_2+q_3)}{TN_2\,p^2_2q^3_3}\right)$$

where TN_1 = the number of female flies tested in Mating I
TN_2 = the number of female flies tested in Mating II

In order to reduce the labor involved the variances of male viability estimates were not expressed algebraically, but were computed numerically. Before the actual experiments were conducted, the input frequency giving the estimates of fitness with minimum variances was sought numerically using several different fitness sets. For example, when f_1, f_2, k_1 and k_2 in Table 8 were 0.7, 0.3, 0.3, and 0.7, respectively, the variance of the estimates of fitnesses, V_1, V_2, E_1, E_2, $V_{1♂}$, $V_{2♂}$, E_1V_1, and E_2V_2 were 0.0037, 0.0037, 0.0079, 0.0079, 0.0099, 0.0044, 0.0042, and 0.0042, respectively, assuming $V_1 = V_2 = E_1 = E_2 = V_{♂} = 1$, and $TN_1 = TN_2 = TN_{1♂} = TN_{2♂} =$ 1500. This input frequency generally gave the minimum variance of the estimates under several different fitness sets, and for that reason the actual experiment was carried out with these input frequencies of f and k. The input frequency and the expected output frequency of each genotype under the assumption of no selection are shown in Table 9.

The mating scheme used for this experiment was essentially the same as that used for the previous experiments. Since heterozygous females were used in this experiment, one more generation was necessary: the offspring in Figure 2 were used as parents. The matings were always done between the offspring from different matings and from different lines; the matings, $S/S \times S$, $F/F \times F$, $S/F \times S$, $S/F \times F$ were made by crossing the offspring of matings $(1,j)$ with $(2,j+1)$, $(4,j+1)$ with $(3,j)$, a mixture of $(2,j)$ and $(3,j)$ with $(1,j+2)$, and a mixture of $(2,j)$ and $(3,j)$ with $(4,j+2)$, respectively ($j = 1, \ldots 22$). This procedure was followed in order to avoid the inbreeding effects of homozygotes by incorporating the background genes of one allele into that

of the other. It is necessary only if the inbreeding coefficient is higher among different lines of the same allele than among lines of different alleles.

The developmental times of female genotypes were tested in a similar way as in the previous experiments. In this experiment only *S/S* and *S/F* female progeny are produced by the same parent in mating I, and *S/F* and *F/F* in mating II. Therefore only genotypes from the same parents were compared.

RESULTS

The results of the experiments are shown in Tables 9 and 10. For this experiment 2240 female and 1334 male flies were examined by electrophoresis. In neither experiment (mating I or mating II in Table 8) was the deviation of observed number from expected number statistically significant. From these data there is no evidence of selective differences among genotypes. Viability, fecundity, and the product of viability and fecundity (Table 9) were estimated by the methods described above. Both the viability and fecundity of heterozygotes were

TABLE 9

Results of fecundity and viability test
(Experiment 4)

	♀			♂	
	S/S	S/F	F/F	S	F
	Mating I S/F x S (70 per cent); F/F x F (30 per cent)				
	Total number of flies examined: ♀ 888, ♂ 545				
Expected proportion	0.35	0.35	0.30	0.35	0.65
Observed proportion	0.3382	0.3301	0.3316	0.3249	0.6751
		x^2 = 4.47, D.F. = 2		x^2 = 1.53, D.F. = 1	
	Mating II S/S x S (30 per cent); S/F x F (70 per cent)				
	Total number of flies examined: ♀ 1352, ♂ 789				
Expected proportion	0.30	0.35	0.35	0.65	0.35
Observed proportion	0.3227	0.3326	0.3447	0.6685	0.3315
		x^2 = 3.60, D.F. = 2		x^2 = 0.82, D.F. = 1	
	Fitness Estimates				
Viability	1.0245±0.1682	1	1.0364±0.1371	1	1.0551±0.2565
Fecundity	1.1048±0.2345	1	1.1308±0.2392		0.9655±0.1855
Product (VxE)	1.1319±0.1520	1	1.1720±0.1934		average = 1.010

TABLE 10

Results of developmental time test from Experiment 4

	Average developmental time by day			
	S/S	*S/F*	*F/F*	
From mating I	19.67 ± 0.14	20.00 ± 0.14	....	t = 1.69
	D = —0.33 ± 0.20			df = 591
From mating II	....	19.76 ± 0.12	19.43 ± 0.12	t = 1.95
		D = +0.33 ± 0.17		df = 914

inferior to those of both homozygotes: in viability by a few percent, in fecundity by more than 10%. However, none of the estimates was significantly different from one since the standard errors were very large. The average developmental time of female genotypes was obtained in a similar way to that used in the frequency-dependent selection experiment. Heterozygous females were slower in development by 0.33 days than either homozygote (Table 10). The difference between *S/F* and *F/F* was not significant, but its associated probability was just over 5% (t = 1.956, df = 914).

In all three fitnesses estimated (viability, fecundity, and developmental time), heterozygous females were more or less inferior to both homozygotes, although none of the comparisons was statistically significant. Heterozygote inferiority was not observed in previous experiments. Increased heterozygosity in the background genes of homozygotes might be responsible for the decreased relative fitness of heterozygous flies.

DISCUSSION

In this paper the fitnesses of those five genotypes at the esterase-5 locus commonly found in natural populations of *D. pseudoobscura* were estimated to examine whether selection is likely to be responsible for this polymorphism, and by extension, for protein polymorphisms in general. It is important to consider methodological difficulties which may affect my estimations of fitness.

Four experiments were carried out in the work described in this paper. One of them consisted of population cage experiments. In most population cage experiments, initial gene frequencies are chosen so as to be some distance from the equilibrium frequency in the original population, and fitnesses are estimated from the changes in the frequency of genotypes in successive generations. However, in estimating fitnesses from experimental populations, several difficulties are encountered.

The first of these arises from heterogeneity of the material to be tested. For example, if one allele originates from one inbred population and the other allele from a different inbred population, genes affecting fitness and closely linked to the alleles under investigation may differ. If each allele is linked to deleterious genes, heterozygotes at the locus in question may show spurious overdominance because homozygotes at the locus are also homozygous for linked deleterious gens, particularly during early generations when linkage equilibrium has not been

established by recombination (see FRYDENBERG 1963). Similar problems may be encountered even if the two chromosomes are sampled from the same population since populations are often heterogeneous enough to cause this type of situation. It is difficult to estimate how many generations of random mating will be required before the linked genes reach linkage equilibrium. Although the problem of linkage described above is well known, it is often neglected when experiments are carried out, partly because of the unavailability of suitable material.

Another problem in the estimation of fitness arises from the assumptions about the modes of selection (such as random mating and sex-independent fitness) usually made for the sake of convenience, even though it is generally known that such assumptions cannot be justified. The consequence is to obtain spurious fitness estimates.

Further difficulties arise because the enumeration of genotypes in each generation is usually carried out in the middle of the selection process. PROUT (1965) has demonstrated that "net fitnesses cannot be estimated from two generations of a population when the population is counted at partially selected stages." If genotypes are counted at partially selected stages, it is necessary to measure two components of fitness, pre-counting selection and post-counting selection (which usually correspond to viability and fertility, respectively) in order to estimate total fitness. If a more realistic model such as nonrandom mating or sex-dependent selection is considered, estimating problems will be much more complicated, and more information will be needed in order for fitness to be estimated. PROUT (1969) cautioned that apparent frequency-dependent selection can arise in two cases: if the assumptions about the mode of selection are not justified, or if we assume that genotypes are counted after all selection has taken place when counting was actually done before or during selection. In addition, it must be kept in mind in estimating fitness that demographic changes may be accompanied by gene frequency changes; gene frequency may change in response to changes in rate of population growth if selective differences among genotypes vary with age: e.g., if one genotype starts reproducing earlier, it will make a greater contribution to the gene pool when population size is increasing than when it is stationary or declining (CHARLESWORTH and GIESEL 1971).

In the cage experiments reported in this paper the gene frequency did not change and genotype frequency of adult flies was not different from proportions expected from a binomial distribution. Therefore, none of the problems associated with mode or timing of selection has arisen in estimating fitness.

In the other three experiments (Experiments 2, 3, and 4), components of fitness were directly estimated under specific mating schemes rather than from the change in genotype frequencies under the assumption of random mating. Since special mating schemes were used, neither an assumption of random mating nor of the absence of differential selection between sexes was made in estimating components of fitness. In all three experiments, both viability and developmental time were estimated in each sex separately, partly because the esterase-5 locus is sex linked. Fertility was estimated only in females (female fecundity) in Experiment 4. Male fertility (male mating ability) was not measured because of

limitations of time and labor, and also because no differences in other components of fitness were found.

The only problem involved in estimating fitness in those experiments was therefore that of linkage of the esterase-5 locus with other genes affecting fitness. Throughout the experiments great care was taken to minimize this problem; all the materials were sampled from an established population cage which had been kept in the laboratory for 15 years, so that an equilibrium is likely to have been attained; twenty-two independently derived homozygous lines of each allele (66 independently derived chromosomes) were used throughout the experiments.

The results and conclusion of fitness estimation from the four experiments described above are as follows. A few estimates of components of fitness show significant differences among genotypes. However, none of the trends was consistent from experiment to experiment, and most of them were not significant. The results of Experiment 2 are probably most reliable as more than 300 thousand flies were examined in this experiment, compared to under four thousand in experiments requiring electrophoresis. The estimated difference in viability among genotypes from Experiment 2 was generally less than 2%, and the difference in developmental time was less than 0.2 day. In experiments requiring electrophoresis, estimates of differences in fitness were greater than in Experiment 2 (sometimes more than 10 percent). Although many of these variations in the magnitude of the fitness differences from experiment to experiment may be ascribed to differences in the scale of the experiments, there was also a considerable difference in experimental conditions between the sex-ratio experiment and the premating experiments. In the former, a genotype competes only with a standard genotype. If the different genotypes interact ecologically with each other to a substantial degree, this method will give biased results. In the latter experiment all five genotypes compete with each other. These conditions are closer to those found in population cages or in nature. The experiments involving electrophoresis were, however, conducted on a much smaller scale than the sex-ratio experiment, so that it is difficult to tell how much of the differences in results between the two types of experiments is due to interaction among genotypes and how much is due to sampling errors.

The results of the population cage experiments clearly showed that overall fitness differences among genotypes are fairly small. As there were 12 cages in the population cage experiments, and as all cages (except for one in which linkage disequilibria were probably established) showed consistent results, it can be stated with relatively little doubt that selective differences among genotypes are small, although the population cage technique is not very efficient for detecting selection. All four experiments described in this paper showed similar results; this is strong evidence in favor of the hypothesis of weak selection or selective neutrality of protein polymorphisms.

There are several reports which indicate neutrality or weak selection of protein polymorphism. MUKAI (1968, 1969) has suggested neutrality or near-neutrality of protein polymorphisms of *D. melanogaster*, since existing genetic load in the second chromosomes can be explained by only about 10 heterozygous loci on the

basis of simulation using the genetic parameters estimated by himself. MacIntyre and Wright (1966) reported that in population cages the esterase-6 locus of *D. melanogaster* showed little change in gene frequency in a homozygous background, while rather rapid change was observed when the background was heterozygous. The results reported by Ohba (1969) seem to show a similar conclusion; there were no changes in gene frequency after 35 generation serial transfer by Pearl's method at two esterase loci (esterase-2, esterase-7) in experimental populations of *D. virilis*. There was some slight evidence of a change in gene frequency at the esterase-9 locus, but this does not appear to be significant.

Acrylamide gels show many bands when stained for esterase activity. Possibly there are several loci which have the same function as the esterase-5 locus. This redundancy in esterase activity may mean that a neutral or even a detrimental enzyme may not be eliminated so rapidly at less redundant loci. Sasaki and Ohba (1969) reported that all the esterase bands active on alpha-naphthylacetate are immunologically indistinguishable. The same was true for these bands acting on beta-naphthylacetate.

The evidence against selective neutrality of protein polymorphism comes mainly from the analysis of natural populations, except for the case of frequency-dependent selection reported by Kojima and his associates (whose results may be affected by linkage disequilibrium). Prakash, Lewontin and Hubby (1969) reported that different alleles at the esterase-5 locus were different in different populations and that there were gradual changes in allelic frequencies from western population to eastern populations. Such a cline in allelic frequency and consistency of the frequency of rare alleles from population to population at the esterase-5 locus (on which a part of the balancing selection hypothesis proposed by them was based) seems to be inconsistent with a hypothesis of selective neutrality. Recently other evidence of clinal variation in frequency of alcohol dehydrogenase alleles with climate has been found in *D. melanogaster* (Grossman, Koreneva and Ulitskaya 1970). However, Kimura and Ohta (1971) have reported that gene frequency clines and similarity of gene frequencies over populations are not necessarily inconsistent with the hypothesis of neutrality; the cline of gene frequency can be observed when isolation is rather complete between populations and marked local differentiation of gene frequency is possible only when the product of effective population size and migration rate is smaller than one.

One other important phenomenon to be considered is the effect of linkage disequilibrium. In this paper I have tried to avoid the complication of linkage in estimating fitness by randomizing the background so that only the average effects of different genotypes were estimated. There is little experimental data on the magnitude of linkage disequilibrium (Lewontin and White 1960; Prakash and Lewontin 1967; Mukai, Mettler and Chigusa 1970; Kojima, Gillespie and Tobari 1970) although there have been several theoretical works on conditions for linkage disequilibrium (Kimura 1956; Lewontin and Kojima 1960; Karlin and Feldman 1969). More data about the amount of permanent linkage disequilibrium using allozyme variants in natural populations may be needed

for better understanding the mechanism of protein polymorphism. However, even neutral genes may be maintained in a population by linkage disequilibrium if those genes are located sufficiently close to the overdominant genes (KIMURA 1956). It is rather unlikely that most allozyme polymorphisms are maintained by this mechanism because it requires the existence of a very large number of overdominant loci.

KIMURA (1968) proposed a hypothesis of selectively neutral protein polymorphisms based on a consideration of the rate of nucleotide substitution during the evolution of organisms. The esterase-5 locus of *D. pseudoobscura* is one of the most polymorphic loci known in this species. According to the definition of KIMURA and CROW (1964), the effective number of alleles at the esterase-5 locus is 3.39 (data of PRAKASH, LEWONTIN and HUBBY 1969). Assuming the neutrality of those alleles and a mutation rate of 1.5×10^{-5} (see KIMURA 1968), the effective population size necessary to keep 3.39 alleles in the population is 48,000 (using the relevant formula from KIMURA and CROW 1964). A population size of the order of 10^4 may be reasonable in natural populations of *Drosophila pseudoobscura*, although there are some data which suggest much smaller population sizes (WRIGHT, DOBZHANSKY and HOVANITZ 1942; DOBZHANSKY and WRIGHT 1943). However, the ecology of *D. pseudoobscura* is not well understood and there is considerable uncertainty on this point. As far as the number of alleles is concerned, the neutrality hypothesis is therefore consistent with the available data.

The data presented in this paper suggest with relatively little doubt that the selective difference among genotypes at the esterase-5 locus in *Drosophila pseudoobscura* is very small, and this is consistent with the hypothesis of allozyme neutrality with respect to fitness.

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SUMMARY

The fitnesses of different genotypes at the *X*-linked esterase-5 locus of *Drosophila pseudoobscura* were estimated in order to obtain experimental evidence about the maintenance of the polymorphism at this locus in nature. Two different alleles, *S* and *F*, which are among the most common alleles in natural populations, were segregating at approximately 55% and 45%, respectively, in a 15-year-old population cage. Twenty-two lines descended from 22 different single pairs homozygous for each allele at this locus were used throughout the experiments in order to minimize any biases due to genes linked to the esterase-5 locus. Four different types of experiments were conducted: (1) population cage experiments, (2) viability and developmental time experiments using the sex ratio, (3) frequency-dependent selection experiments, and (4) viability and fecundity experiments. No significant changes in gene frequency were observed in 12 popula-

tion cages after examination for 1–2 years. There were no differences in components of fitness among genotypes from the other experiments. Although some of the tests showed significant results, these differences could be ascribed to the effect of background genes, considering the inconsistency among different genetic backgrounds in Experiment 2. No evidence of frequency-dependent selection was obtained. These results are consistent either with selective neutrality of protein polymorphisms or with the hypothesis that most polymorphisms are maintained by selection too weak to be experimentally detectable, but the results rule out the possibility of strong selection at this locus.

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