FURTHER EVIDENCE FOR SELECTIVE DIFFERENCES BETWEEN ISOALLELES IN DROSOPHILA

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ABSTRACT

A number of separate strains of *Drosophila pseudoobscura* were inbred for 38 generations of brother-sister mating with forced heterozygosity for two alleles of either the octanol dehydrogenase or esterase-5 locus. Crosses were set up within each of these inbred lines such that simple mendelian ratios were expected, and eggs from these crosses were placed on media with additions of simple chemicals likely to interact with alleles of the two loci—octanol and ethanol for the ODH locus and tributyrin and triacetin for the E-5 locus. Similar crosses were set up involving parental flies with normally heterozygous genetic background as a control.—Significant deviations from mendelian expectation were observed in inbred E-5 flies grown on tributyrin, inbred ODH males grown on octanol, and inbred ODH females grown on ethanol. There was also a strong effect of octanol medium on males of one of the inbred E-5 lines, and a weak effect of tributyrin medium on ODH inbred females.—The probability that these results reflect interactions between these loci and the environment is assessed in the light of differences between the present results and those obtained at earlier stages of inbreeding.

ONE of the most difficult problems facing population geneticists is determining whether, and how, alternative forms of enzymes are maintained in natural populations by selection. Much effort has been expended on statistical surveys of allele frequencies in an effort to determine whether selection has been operating in the past. This has sometimes led to equivocal results (see for example YAMAZAKI and MARUYAMA 1974) but has for the most part tended to support the view that selection has played a role in maintaining *at least some* polymorphisms (convincing arguments are set forth in AYALA 1972). Just exactly which polymorphisms have been maintained as a result of selection and which have occurred as a result of mutation to a neutral allele and subsequent drift it may be impossible to determine from historical considerations (LEWONTIN and KRAKAUER 1973) but in our opinion the weight of evidence now lies on the side of selection playing an important role.

We are concerned in this paper with an attempt to find laboratory conditions that would permit the detection of selection operating at the present time. It is our hope eventually to link up such differences with differences in the kinetics of the isozymes involved, but at the moment we have only proceeded as far as

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the invention of some crude bioassays of selective effect. These assays were designed with the presumed functions of the enzymes in mind, since experiments which have claimed single-gene effects in the past (e.g., YARBOROUGH and KOJIMA 1967; RICHMOND and POWELL 1970) suffer from the flaw that the selection observed is probably not due to the alleles under observation, but to alleles of other genes closely linked to the marker locus that form a selected block. In outbreeding populations such blocks are probably short (WILLS, CRENSHAW and VITALE 1970; FRANKLIN and LEWONTIN 1970; KOJIMA, GILLESPIE and TOBARI 1970; MUKAI, METTLER and CHIGUSA 1971). Even so, it is improbable that the marker alleles which these workers just happened to have the capability of detecting are the ones responsible for the observed selective effect.

The data presented in this paper demonstrate the extreme difficulty of detecting selection at single polymorphic loci drawn at random from a given species. They are by no means unequivocally in favor of our having isolated the effects of single genes. The mere fact of this difficulty enables us to draw some conclusions about the general nature of the majority of selected polymorphisms. In this connection we will consider criticism levelled against results obtained at the 12th generation of inbreeding (WILLS and NICHOLS 1971) by YAMAZAKI (1972).

MATERIALS AND METHODS

The strains of *Drosophila pseudoobscura* used were originally trapped by one of us (C.W.) from Strawberry Canyon, Berkeley, California, in 1964, and maintained for five years in the laboratory of DR. SATYA PRAKASH. DR. PRAKASH kindly sent us a large number of lines that had been maintained separately during this time in order to retain as much as possible of the genetic variability in the founding population. The two enzymes used were those coded by the X-linked Esterase-5 (*E-5*) and the autosomal (not 3rd chromosome) octanol dehydrogenase (ODH) loci. In this population the two commonest alleles are 1.00 and 1.12 at the *E-5* locus and 1.00 and 1.22 at the ODH locus. For brevity, these will be referred to as *S* (for slow) and *F* (for fast) in each case. Pure lines of each of these alleles were established in such a way as to maintain a large amount of heterozygosity in the rest of the genome.

On the assumption that in outbred flies there are many polymorphisms segregating that contribute to fitness under a given environmental stress (WILLS 1973), the flies were inbred by brother-sister mating with forced heterozygosity at the loci in question. Three separate lines of ODH flies and two of *E-5* (three were begun but one was lost partway through the experiment) were carried for 38 generations of brother-sister mating, the result of which was to make the great majority of the genome homozygous and leave only a small segment of genes surrounding the ODH or *E-5* loci heterozygous. The number of genes we cannot detect which are still heterozygous after this procedure is unlikely, from a variety of considerations (see DISCUSSION), to be very large. There are strong indications from the results that such genes are continuing to have an effect on egg-to-adult viability even after 38 generations of inbreeding.

For the first 30 generations, electrophoresis of individual parents of successful single-pair matings in order to determine their genotypes was carried out by the vertical acrylamide methods set forth by HUBBY and LEWONTIN (1966); after generation 30 the horizontal starch method of SMITHIES (1955) was employed with Poulik gel buffer at pH 8.6 and borate tray buffer at pH 8.1. 1400 units of heparin were added after cooking to each 350-ml starch gel which was to be developed for *E-5*. Staining was carried out as follows. For *E-5*, 20 mg. of α -naphthyl acetate and 50 mg. of fast red were added to each 100 ml of developing buffer, 0.1M K-PO₄, pH 6.5. For ODH, 20 mg. of NAD⁺, 20 mg. of nitro blue tetrazolium, and 6 mg. of phenazine methosulfate were added to each 100 ml of developing buffer, 0.2M Tris-HCl, pH 8.0. The *E-5* gels were developed at room temperature; the ODH gels were developed at 30° in the dark.

At various times during the inbreeding procedure, pure lines of *F* and *S* flies were derived *within each of the inbred lines*. When ODH *F* and *S* flies from a particular inbred line were crossed together, the great majority of their genome remained homozygous in the offspring, but all the progeny of such a cross were *F/S* and also heterozygous for the small number of residual genes still closely linked to the ODH locus. These flies were in turn allowed to mate *inter se*. Eggs from this cross were washed in Herb's solution (D. LINDSLEY, personal communication) and collected on sterile discs of Whatman #2 filter paper placed in a Millipore filter apparatus. The washed eggs, still on the paper, were then placed directly on a variety of stress media. (The stress media were in some cases so severe in their effects that the parental flies could not survive long enough to lay eggs directly on them.) Flies eclosing from these stress bottles could be frozen in small plastic vials at -76° for several months without noticeable deterioration in enzyme activity. These flies were electrophoresed to determine whether there was any deviation from the expected 1:2:1 ratio of *F/F* to *F/S* to *S/S* as a result of growth on the particular stress medium.

The same crosses and procedures were carried out on ODH P_1 , *E-5* P_1 and *E-5* I_{38} (inbred for 38 generations) flies. The *F* and *S* parental lines have been carried throughout the experiment in mass culture. Because the *E-5* gene is sex-linked, crosses had to be performed in a slightly different way than for ODH. Between the *F* and *S* parental lines, and within each of the two inbred lines after pure *F* and *S* lines had been established, the following crosses were performed:

$F/F \text{ } \varnothing \varnothing \times S \delta \delta \longrightarrow F/S \text{ } \varnothing \varnothing \times F \delta \delta \text{ inter se}$	Eggs placed on stress medium	Expected ratios
		1 <i>F/S</i> : 1 <i>F/F</i> $\varnothing \varnothing$ 1 <i>F</i> : 1 <i>S</i> $\delta \delta$
$S/S \text{ } \varnothing \varnothing \times F \delta \delta \longrightarrow F/S \text{ } \varnothing \varnothing \times S \delta \delta \text{ inter se}$	Eggs placed on stress medium	Expected ratios
		1 <i>F/S</i> : 1 <i>S/S</i> $\varnothing \varnothing$ 1 <i>F</i> : 1 <i>S</i> $\delta \delta$

One difficulty of these crosses is that the relative survival of the two homozygotes and the heterozygote could not be compared in the same cross on a particular stress medium. Male hemizygotes could be compared with each other, however; in all crosses a 1:1 ratio is expected.

The stress media employed were suggested by our gradually increasing understanding of the substrate affinities of the two enzymes. Since ODH oxidizes long-chain alcohols, it seemed likely that octanol would be a suitable stress. In early experiments 3 mM octanol was added directly to normal cornmeal-molasses-agar medium to make a stress medium (WILLS and NICHOLS 1971, 1972); in the present experiments an equal volume of ethanol was added to bring about more smooth mixing with the medium. High concentrations of ethanol (6%, v/v) were initially used as a control, but were also found to be a stress medium affecting the inbred ODH flies; upon investigation it was found that faint ODH bands would develop in gels to which 4% or more ethanol (without octanol) was added to the developing solution. Preliminary data indicate that this effect is not due to a contaminating molecule with a lower boiling point in the ethanol; 4% of ethanol which had been distilled to 1/10 of its former volume was still required before the faint bands appeared.

The function of the *E-5* esterase in the fly's metabolism is even less clear. *E-5* will hydrolyze tributyrin but not the longer-chain triglycerides triolein and tripalmitin. It can be demonstrated in two ways that it is this esterase and not one of the other esterases of the fly that is primarily responsible for the hydrolysis.

1) Vertical acrylamide gel electrophoresis in which the buffer was made with the addition of 0.5% tributyrin, (v/v) polydispersed by 30 seconds of sonication, allowed visualization of faint cleared bands after electrophoresis of one or two flies per slot. These bands corresponded in position to the *E-5* genotypes of the flies being electrophoresed, a fact which could be checked by subsequent staining with α -naphthyl acetate and fast red. This produced bands superimposed on the cleared zones. No such zones could be observed in similar gels made up with triolein or tripalmitin.

2) The clearing of a sonicated solution of 0.05% (v/v) tributyrin upon addition of supernatant from a crude fly homogenate could be followed in 0.05 M $K-PO_4$ buffer, pH 6.5 at 535 m μ and gave linear kinetics. The best results were obtained at 37° . The greatest hydrolytic activity per unit protein was observed from *F/S* heterozygotes followed by *F/F* homozygotes.

Outbred and inbred flies showed the same absolute activities per unit protein and correlation of genotype with activity (E. SHELDON, in preparation). No hydrolytic activity could be observed in the spectrophotometer when triolein or tripalmitin were substituted for tributyrin.

We have some much weaker evidence that triacetin, which is water-soluble, may also be acted upon by the *E-5* enzyme. The appearance of the acid π bond can be followed at 215 nm, and there appeared to be differences in the rate of hydrolysis that are *E-5*-genotype related. A coupled assay employing the NAD-dependent acetaldehyde oxidase to follow the hydrolytic production of acetate is being developed; this should give more precise results.

As a result of these studies, stress media were used containing 5% (v/v) tributyrin or triacetin. In all media the stressing chemical was added after cooking. The tributyrin was polydispersed in the medium by 10-sec mixing in a 1-gal Waring Blender at low speed.

RESULTS

Effect of the stress media

One of the bases for YAMAZAKI's (1972) criticism of our earlier results was that the effect seen may have been due to a generalized stress and not to a specific substance added to the medium. The slight excess of heterozygotes seen on octanol medium among the inbred flies (an excess which is no longer apparent by the 38th generation) was due in his view to the fact that these flies were *relatively* more heterozygous (in comparison to the inbred homozygotes) than the outbred heterozygotes were in comparison to the outbred homozygotes. Any sufficiently severe regime, then, should produce an excess of heterozygotes after inbreeding. He supported this claim with a small experiment involving *D. melanogaster* (not *D. pseudobscura*) grown on 3 mM octanol and on 0.25 M KCl. This latter medium was used at the start of the present experiment as a stress medium unlikely to be

TABLE 1

Mean number of flies surviving per bottle on normal medium and the four stress media, classified by genotype

		No. of bottles	Female	Male
Normal (ODH inbred only)		8	47.63	58.50
6% EtOH	ODH P_1	12	10.00	10.83
	I_{38}	36	7.14	7.58
	E-5 P_1	24	7.71	12.00
	I_{38}	48	5.77	9.77
3 mM Octanol (plus equal amt. of EtOH)	ODH P_1	12	15.00	20.25
	I_{38}	36	19.08	21.14
	E-5 P_1	24	17.96	18.58
	I_{38}	48	17.71	24.48
5% Tributyrin	ODH P_1	12	8.42	14.00
	I_{38}	36	5.92	9.36
	E-5 P_1	24	4.33	10.00
	I_{38}	48	4.23	11.73
5% Triacetin	ODH P_1	12	15.92	16.75
	I_{38}	36	11.58	12.58
	E-5 P_1	24	10.00	10.92
	I_{38}	48	10.08	14.02

related either to ODH or *E-5*, and before we discovered some of the functions of the *E-5* enzyme. Since *D. melanogaster* showed greater survival on KCl than ODH medium, he concluded that the excess of heterozygotes seen in inbred flies could have been due to generalized stress. In order to examine the possibility of a similar interpretation in the present experiment we recorded the survival of flies on normal medium and the four stress media that were employed (Table 1). From the averages of the numbers of flies that emerged, it appears that octanol is the least severe stress medium, followed by triacetin, and that ethanol and tributyrin are the most severe. In most but not all cases the *E-5* inbred males were the least likely to survive. The effect on the sex ratio varied from medium to medium, and was genotype-related. It may also be noted that our method of collecting eggs on filter paper meant that very few flies eclosed even from bottles

TABLE 2

Observed (heavy type) and expected (light type) numbers of genotypes of flies grown on 6% EtOH medium

	S/S	Female S/F	F/F	S/S	Male S/F	F/F
ODH P ₁	30	54	36	31	51	38
	30	60	30	30	60	30
I ₃₈ A	8	34	16	21	40	24
	14.5	29	14.5	21.25	42.5	21.25
B	10	27	20	10	20	10
	14.25	28.5	14.25	10	20	10
C	24	74	40	27	66	36
	34.5	69	34.5	32.25	64.5	32.25
I ₃₈ Total	42	135	76	58	126	70
	63.25	126.5	63.25**	63.5	127	63.5

	Hets	Homs	S	F
E-5 P ₁ SxS/F	58	59	121	90
	58.5	58.5	105.5	105.5*
FxS/F	62	57		
	59.5	59.5		
I ₃₈ A SxS/F	31	42	47	45
	36.5	36.5	46	46
FxS/F	53	38		
	45.5	45.5		
I ₃₈ B SxS/F	44	40	59	43
	42	42	51	51
FxS/F	52	52		
	52	52		
I ₃₈ Total SxS/F	75	82	106	88
	78.5	78.5	97	97
FxS/F	105	90		
	97.5	97.5		

The E-5 males in each line were totalled, since in all cases a 1:1 ratio of S to F hemizygotes would be expected.

* Significant at 5% level.

** Significant at 1% level.

containing normal medium. The effects of stress set out below cannot therefore be attributed to overcrowding.

Tables 2 through 5 set out the observed and expected numbers of flies of different genotypes surviving on the four stress media. Significant deviations from the expected ratios as measured by χ^2 are noted by asterisks. A pattern of complex genotype-environment interactions emerges. It is, however, consistent with the notion that the survival of flies segregating at a particular locus will be most affected by media chemically related to the function of that locus.

The medium having the least effect on relative survival was triacetin. Only one comparison was significant at the 0.05 level, and this is likely due to chance. With the benefits of hindsight it may be reasoned that even if there are differences in the utilization of triacetin by flies carrying different *E-5* genotypes, the end-products of the reaction are glycerol and acetate, both easily-utilizable metabolites. Thus, triacetin, while undoubtedly a survival stress, may not be a critical one for the *E-5* locus.

TABLE 3

Observed and expected numbers of genotypes of flies grown on 3mM octanol medium

	S/S	Female S/F	F/F	S/S	Male S/F	F/F
ODH P ₁	21	66	33	23	66	30
	30	60	30	29.75	59.5	29.75
I ₃₈ A	20	68	30	54	89	32
	29.5	59	29.5	43.75	87.5	43.75
B	22	27	9	41	53	24
	14.5	29	14.5	29.5	59	29.5*
C	16	32	10	26	45	19
	14.5	29	14.5	22.5	45	22.5
I ₃₈ Total	58	127	49	121	187	75
	58.5	117	58.5	95.75	191.5	95.75**
	Hets	Homs	S		F	
E-5 P ₁ SxS/F	63	57	121	119		
	60	60	120	120		
FxS/F	63	57				
	60	60				
I ₃₈ A SxS/F	52	43	124	115		
	47.5	47.5	119.5	119.5		
FxS/F	56	64				
	60	60				
I ₃₈ B SxS/F	71	49	146	93		
	60	60	119.5	119.5**		
FxS/F	46	61				
	53.5	53.5				
I ₃₈ Total SxS/F	123	92	270	208		
	107.5	107.5	239	239**		
FxS/F	102	125				
	113.5	113.5				

Significance levels are as in Table 2.

TABLE 4

Observed and expected numbers of genotypes of flies grown on 5% tributyrin medium

	S/S	Female S/F	F/F	S/S	Male S/F	F/F
ODH P ₁	19	68	33	16	54	28
	30	60	30	24.5	49	24.5
I ₃₈ A	28	63	28	27	63	25
	29.75	59.5	29.75	28.75	57.5	28.75
B	21	53	14	12	28	9
	22	44	22	12.25	24.5	12.25
C	26	53	16	10	19	19
	23.75	47.5	23.75	12	24	12
I ₃₈ Total	75	169	58	49	110	53
	75.5	151	75.5*	53	106	53

	Hets	Homs	S	F
E-5 P ₁ SxS/F	54	66	44	58
	60	60	51	51
FxS/F	55	60		
	57.5	57.5		
I ₃₈ A SxS/F	49	71	56	40
	60	60*	48	48
FxS/F	63	74		
	68.5	68.5		
I ₃₈ B SxS/F	46	77	65	40
	61.5	61.5	52.5	52.5
FxS/F	60	58		
	59	59		
I ₃₈ Total SxS/F	95	148	121	80
	121.5	121.5	100.5	100.5
FxS/F	123	132		
	127.5	127.5		

Significance levels are as in Table 2.

Inbred *E-5* males and females were pronouncedly affected by growth on tributyrin medium. One of the products of hydrolysis of this substrate is butyrate. Since it is one step further removed from acetyl CoA than is acetate, an excess of butyrate may be more likely to disturb the flies' metabolism. In the males, an excess of *S* over *F* hemizygotes was seen; in the females an excess of *S/S* homozygotes over heterozygotes. Heterozygotes and *F/F* homozygotes survived equally well. The effect seen is therefore not conditional heterosis *on this particular medium*; other media may yet be discovered in which the heterozygotes have an advantage.

On this same medium, a slight excess of heterozygotes at the expense of *F/F* homozygotes was observed in the pooled data from the ODH inbred females. This was only significant at the .05 level. No effect was seen in the males.

Pronounced sex-limited effects are observed in the results on octanol and ethanol. While ODH inbred females segregated perfectly regularly on octanol

TABLE 5

Observed and expected numbers of genotypes of flies grown on 5% triacetin medium

	S/S	Female S/F	F/F	S/S	Male S/F	F/F
ODH P ₁	27	73	20	29	58	33
	30	60	30	30	60	30
I ₃₈ A	34	54	24	24	42	21
	28	54	28	21.75	43.5	21.75
B	33	61	26	39	46	33
	30	60	30	29.5	59	29.5*
C	25	63	32	31	56	30
	30	60	30	29.25	58.5	29.25
I ₃₈ Total	92	178	82	94	144	84
	88	176	88	80.5	161	80.5

	Hets	Homs	S	F
E-5 P ₁ SxS/F	62	56	113	123
	59	59	118	118
FxS/F	60	49		
	54.5	54.5		
I ₃₈ A SxS/F	66	54	103	137
	60	60	120	120
FxS/F	61	59		
	60	60		
I ₃₈ B SxS/F	66	54	93	80
	60	60	86.5	86.5
FxS/F	67	43		
	55	55		
I ₃₈ Total SxS/F	132	108	196	217
	120	120	206.5	206.5
FxS/F	128	102		
	115	115		

Significance levels are as in Table 2.

medium, the ODH inbred males showed a strongly significant deficiency of *F/F* homozygotes and an excess of *S/S* homozygotes. This is very different from our results (WILLS and NICHOLS 1971, 1972) from less inbred flies tested on the same medium, and may reflect further reduction in heterozygosity elsewhere in the genome. Since our laboratory was moved at generation I₃₀, it may also reflect unavoidable differences in media composition and culture conditions. In complete contrast to the octanol data, the ODH inbred *females* showed an effect on 6% ethanol medium while the relative survival of the male remained undisturbed. In this case the fitness order of the genotypes was reversed from that on octanol medium, with an excess of *F/F* homozygotes and a deficiency of *S/S* homozygotes.

The most anomalous result is a very strong effect of octanol medium on the males of one of the inbred *E-5* lines, resulting in an excess of *S* hemizygotes. The fact that this was not observed in the other inbred line suggests that genes other

than *E-5* may be responsible. Alternatively, the effects of octanol dehydrogenase and esterase-5 may not be as independent as a casual inspection of their functions might indicate. If ODH catalyzes the first step in the conversion of octanol to its acyl-CoA, then one of the products in the repeated series of beta-oxidations that shorten the acyl chain will be butyryl-CoA. A high concentration of this compound might in turn effect the equilibrium of substrate and product for esterase-5, since the specificity for simple lipids that we have discovered for this enzyme indicates that it is involved in the production of short-chain acyl-CoA compounds. The effect of octanol on some *E-5* flies and of tributyrin on some ODH flies may not therefore be due to the genetic background at all.

Only one other significant deviation at the 5% level was observed: an excess of *S* hemizygotes among parental males grown on ethanol medium. Since none of the other 19 parental comparisons on any of the four media were significantly different from mendelian expectation, this may be due to chance.

Generally, when a highly significant effect was observed in inbred flies, similar though not always significant deviations were found in each of the inbred lines of that genotype on that medium. In none of these groups were the data numerous enough or the deviations marked enough for heterogeneity χ^2 to be significant. Even the anomalous results obtained with the *E-5* inbred males on octanol medium were consistent in that both lines showed an excess of *S* hemizygotes. The B line deviation was highly significant, while that of the A line was not. Heterogeneity χ^2 was 1.87, not significant for one degree of freedom.

DISCUSSION

With some exceptions noted above, the results conform generally to the expectation that:

a) Outbred flies should show little or no effect on the various stress media, since they are presumably segregating for a number of other loci that affect relative survival on the stress. This is in accordance with the genotype-environment interaction model of the segregational genetic load. This model (see WILLS 1973 for review) posits that the magnitude and direction of selection coefficients are dependent on the environment, the rest of the organism's genotype, and the genotypes of the organisms with which it is competing. All three of these factors are to some extent controlled in the present experiments; it is our contention that such controls are necessary in order to pick up selective differences between isoalleles at the *average* polymorphic locus.

b) The greatest effect on inbred flies should be observed on media likely to interact with the loci for which they are segregating. This should be (and, in the experiments reported here, is) unrelated to the general severity of the stress medium.

Several large puzzles remain, however. Firstly, in contrast to our earlier results, no heterozygote advantage was observed except for the marginally significant case of ODH inbred females on tributyrin. If conditional heterosis is a factor in maintaining these polymorphisms, it is not apparent from our latest

laboratory results. This may be because it is in fact not important at these loci, or because we have simply not discovered the environment or environments in which it is expressed. Alternatively, the polymorphisms examined here may be held in the population by WALLACE's (1968) "marginal overdominance" or something very close to it. In WALLACE's formulation, a homozygous genotype disadvantageous in one environment may be neutral in another, while another homozygote at the same locus may be disadvantageous in the second environment and neutral in the first. As the environment changes over space and time, it would appear as if the heterozygote had an advantage. We would only add to this that a homozygote disadvantageous in one environment may be advantageous in another—at least judging by our present results.

(An analysis of *Drosophila* viability data (WILLS, in preparation) indicates that marginal overdominance or something very akin to it is the chief source of heterozygote advantage in *Drosophila* populations. Conditional heterosis seems to play a smaller role.)

Secondly, we cannot as yet account for the sex-limited nature of many of the effects. One speculation might be that the differential survival of the two sexes on a particular medium might explain the sex-limited expression of some of the effects. However, an inspection of Table 1 shows, for example, that the relative survival of ODH inbred females and males on ethanol and octanol medium is comparable. Yet the females showed an effect on ethanol medium and the males an effect on octanol medium. Inbred ODH males did very poorly on tributyrin medium in comparison to females, yet they showed no effect while the females showed a slight one. Our earlier data for the ODH locus on octanol medium (WILLS and NICHOLS 1971, 1972) showed an effect first in the males and then, to a limited extent, in the females. This effect was, as stated earlier, very different from that found in the results from the 38th generation. It was proposed at that time that inbreeding would first reveal an effect in the males since they were already hemizygous for the *X* chromosome. Any sex-linked polymorphisms that might also be important in survival on octanol medium would tend to mask the effect of the ODH polymorphism in females but not in males. The present effects seen at both the ODH and *E-5* loci, strongly sex-limited in nature and apparent both in males and females, argue against this hypothesis. We have no good explanation for this phenomenon, and it is painfully apparent that despite our strenuous efforts we have not succeeded in removing genetic background as a complicating factor.

YAMAZAKI (1972) criticized some of our earlier results on just these grounds. In order to bolster his argument he followed by computer simulation the decline in heterozygosity by brother-sister mating in a single chromosome (his Figure 1). This decline was very steep for homozygotes and far less so for heterozygotes over 100 generations. The difference was greatly enhanced by his using a logarithmic scale, so that heterozygosity dropped through almost four orders of magnitude. YAMAZAKI's unstated assumption, in drawing the graph, was that there were approximately 10,000 loci on that chromosome with the potential for heterotic selection.

This seemed to us unrealistically high, and it also seemed that plotting the decline in heterozygosity on a logarithmic scale would give an exaggerated impression of the difference between homozygotes and heterozygotes as inbreeding proceeded. We replotted the comparison between homozygotes and heterozygotes on an arithmetic scale. It was therefore necessary to estimate the number of selectively important loci that, on liberal assumptions, might reasonably be expected to be heterozygous on this chromosome in an outbred fly.

JUDD, SHEN and KAUFMAN (1972) and others suggest that there are about as many indispensable functions in the *Drosophila* genome as there are bands on the salivary chromosomes. This is about 5,000 for *D. melanogaster*, and probably does not differ greatly for *D. pseudoobscura*. If we assume that in the average fly about 10% of these are heterozygous and that the ODH autosome comprises about 1/5 of the genome, then a fly drawn at random from the general population should be heterozygous for about 100 indispensable functions on the ODH chromosome. In actuality, flies of our original founding population, which was maintained for many generations in the laboratory and was in addition mildly inbred to produce the pure-breeding *F* and *S* lines, are probably somewhat less heterozygous for indispensable functions.

It is unclear how many translated genes code for functions that are not indispensable (O'BRIEN 1973), and what the effect of polymorphism may be for regions of the genome that are transcribed but not translated. To be on the safe side, we multiplied the number above by 10, so that the average fly from our outbred population is heterozygous on this chromosome at 1,000 loci of some importance to its survival. Our simulations were carried out using this number. We also set the length of the ODH chromosome at 60 centimorgans, or 1/5 of the total map distance in *D. melanogaster*.

One of the consequences of the genotype-environment interaction model is that very large numbers of polymorphisms can be maintained without loss due to fixation (WILLS, CRENSHAW and VITALE 1970). There must be some upper limit to this capability, however, particularly if one of the characteristics of the polymorphism is that it is so strongly selected that it shows similar frequencies in different parts of the species range. This is the case with most of the polymorphisms examined in *D. pseudoobscura* (PRAKASH, LEWONTIN and HUBBY 1969). Though the two presently being considered show slight variations, the two alleles we investigated are common in most populations. Obviously our line of reasoning is open to question, but for the above reasons we consider 10,000 heterotically selectable loci per chromosome to be too high an estimate.

The results of our simulations are shown in Figure 1. We have, unlike YAMAZAKI, made two alternative assumptions about the nature of the heterozygosity in the starting flies. The first assumption (the results of which correspond numerically to his simulation so that we assume it is the one he made) is that the initial pair of flies is heterozygous for *different* alleles at each locus—four alleles in all. The decline in heterozygosity due to inbreeding will therefore be relatively slow. This we call the assumption of maximum heterozygosity. The second assumption is that at each locus in the starting pair of flies there are only

FIGURE 1.—Decline of heterozygosity in organisms initially heterozygous at 1,000 loci on a single chromosome and subjected to brother-sister mating. Each generation, heterozygosity was enforced for a marker locus in the middle of the chromosome. Crossing over was permitted in only one sex. Each line is an average of five simulations. Every five generations, organisms homozygous or heterozygous for the marker locus were constructed and the number of loci that remained heterozygous elsewhere on the chromosome was determined. The results of inbreeding differed considerably depending on the starting conditions (see text).

two alleles, and that only one of the flies (chosen at random) is heterozygous at a particular locus. The other is homozygous for one or the other allele. This is the minimum heterozygosity consistent with there being heterozygosity at each of the 1,000 loci at the outset. The minimum lines show a much more rapid decline in amount of heterozygosity and remain at a lower level throughout the simulation. The data from the flies themselves, if it were accessible and there actually were 1,000 loci heterozygous at the outset, might be expected to fall between these lines.

In actuality, however, the approach to homozygosity is unlikely to follow a theoretical curve. If selection for heterozygotes is at all a factor, then the curves should drop less rapidly. In fact, one would expect that as the remaining heterozygous loci become more and more important in the survival of the organism, the approach to homozygosity should slow proportionately.

Assuming that one can rely on the curves, arithmetic plotting of the data is instructive, since it shows clearly that after 40 generations of brother-sister mating there are only small absolute differences in numbers of heterozygous loci

between the lines heterozygous and homozygous for the marker alleles. The difference (average of five separate runs) is 34 for the maximum lines and 25 for the minimum lines. We suspect that such a difference would be further obscured by random fluctuations in residual heterozygosity on the other chromosomes. While there undoubtedly are still effects of the background genotype influencing the results even after 38 generations, and indeed such effects may have been important in producing the apparent conditional heterosis reported at the ODH locus at generations 12 and 17, the current results are not such as can be explained by generalized heterosis. We appear to have entered a region in which single-gene effects, while not completely isolated, are of predominant importance to the relative survival of the flies.

The generalized heterosis suggested by YAMAZAKI to be an explanation for our results manifested itself more clearly earlier in the experiment. It was briefly reported (WILLS 1970) that heterotic effects were found under a variety of selective conditions using the *F* and *S* alleles at the *E-5* locus as markers after five generations of inbreeding. This generalized heterosis was a response to selective conditions of broad rather than narrow effect. These were treatments with cold, ether and CO₂, which are not likely to interact specifically with the loci in question.

In summary, it is extremely difficult to detect single-gene effects when the polymorphisms are fully functional and all genotypes can survive equally well in a non-stressed environment. Selection coefficients associated with any particular polymorphism in an outbred population must be very small except when the stress is strong. For example, in order to obtain consistent shifts in the frequencies of ADH alleles in outbred *D. melanogaster* laboratory populations, BIJLSMA-MEELES and VAN DELDEN (1974) were forced to use medium supplemented with 20% ethanol. Ordinary medium or mild stress is unlikely to yield unequivocal results. It is interesting to note in this connection that two alleles used in this study, *E-5 F* and *S*, are coincidentally the same as those employed in an exhaustive study by YAMAZAKI (1971) on a randomized genetic background using normal medium. Under these conditions he could detect no selective differential between these alleles.

The possibility exists that the "*F*" and "*S*" alleles used in this and other studies are not always the same. BERNSTEIN, THROCKMORTON and HUBBY (1973) have recently presented evidence that electrophoretic similarity may mask a collection of alleles with different temperature sensitivity. Unfortunately their data as presented do not rule out the possibility that the temperature sensitivity might segregate independently of the alleles examined. The fact that in most cases in the present data an effect, when it was detectable, was expressed similarly in all the independent inbred lines, suggests that if indeed our *F* or *S* alleles are heterogeneous they all have the same biochemical properties. If we do indeed have more than one allele in the *F* or *S* category, then these results are in line with the suggestion of BERNSTEIN, THROCKMORTON and HUBBY that detectably different selection coefficients are likely to be observable between alleles that are

electrophoretically or immunologically distinguishable, but may not be observable when alleles are more similar in their properties.

As our sophistication in picking up polymorphisms without obvious effect increases, the difficulty of detecting selective differences between them will likewise increase. Such a polymorphism is that found by MROSS, DOOLITTLE and ROBERTS (1970) in the fibrinopeptide B of gibbons. Of 12 fibrinopeptides sequenced, two had a serine at position 3 and the rest had a glycine. It would be very difficult indeed to devise a selective scheme for determining whether this polymorphism were indeed neutral!

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