GENIC HETEROGENEITY AT TWO ALCOHOL DEHYDROGENASE LOCI IN *DROSOPHILA PSEUDOOBSCURA* AND *DROSOPHILA PERSIMILIS*

JERRY A. COYNE AND ALEXANDER A. FELTON

Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138

Manuscript received February 15, 1977
Revised copy received May 31, 1977

ABSTRACT

A sequential electrophoretic survey of the second chromosome loci, alcohol dehydrogenase-6 (*Adh-6*) and octanol dehydrogenase (*Odh*), was performed on 147 isochromosomal lines of *Drosophila pseudoobscura* and 60 lines of its sibling species, *D. persimilis*. Gels run with a variety of acrylamide concentrations and buffer pH's revealed the presence of 18 alleles of *Adh-6* in the two species, where only eight had been previously detected by conventional electrophoretic methods. Only two alleles were added with our techniques to the previous total of nine in both species at the largely monomorphic *Odh* locus. Both enzymes show a predominance of one allele, with the other variants being fairly rare. There was no evidence of increased genetic divergence between the two species, but we found a striking increase in differentiation of *Adh-6* alleles between the main body of *D. pseudoobscura* populations and the conspecific isolate from Bogotá, Colombia. These results are compared with our previous surveys of xanthine dehydrogenase in these species and discussed in reference to theories of genic polymorphism.

GENETICISTS have considered for some time that the "alleles" revealed by gel electrophoresis may really be heterogeneous collections of alleles (KING and OHTA 1975), but until recently the amount and nature of hidden genetic variation has remained speculative. Statistical examination of allele frequencies, attempts to measure selection in the laboratory, and geographic analysis of gene patterns yield results which are often ambiguous. These ambiguities may well be caused by incomplete and therefore misleading electrophoretic data (SINGH, LEWONTIN and FELTON 1976). Genetical theories of speciation, which presently rest largely on patterns of similarities and differences at allozyme loci (*e.g.*, AYALA 1975), are also subject to the same caveat: the "similarities" may be illusory. For these reasons it is important to obtain a detailed locus-by-locus picture of gene frequencies as uncovered by a variety of biochemical methods.

Studies using heat denaturation of enzymes (BERNSTEIN, THROCKMORTON and HUBBY 1973; SINGH, HUBBY and THROCKMORTON 1975; COCHRANE 1976), gel sieving (JOHNSON 1976), and isoelectric focusing (MILKMAN and KOEHLER 1976) initially provided promising evidence of hidden alleles. A somewhat different set of methods was used by SINGH, LEWONTIN and FELTON (1976) and

COYNE (1976) to reveal surprising allelic heterogeneity at the xanthine dehydrogenase (*Xdh*) locus in the sibling species *Drosophila pseudoobscura* and *D. persimilis*. Isogenic lines from several populations of these two species were subjected to sequential electrophoresis at a variety of gel concentrations and pH's. The eleven "alleles" of *Xdh* observed in the two species under standard conditions of electrophoresis (a 5% acrylamide gel of pH 8.9) were shown by the detailed analysis actually to be forty-seven alleles, and heat denaturation of XDH in *D. pseudoobscura* added at least ten more to this total. As there are undoubtedly alleles in nature not picked up in our sample of 206 lines, the work showed a potentially vast array of alleles in natural populations.

This analysis was interesting in several other respects. In both species one allele of *Xdh* was in extremely high frequency (although a different one in each species) and the remainder of the variants were rather rare. There were marked interpopulational differences in the frequencies of rare alleles, possibly an artifact of the small sample sizes. The population of *D. pseudoobscura* from Bogotá, Colombia is geographically and partially reproductively isolated from the rest of the species, yet under standard conditions of electrophoresis Bogotá had *Xdh* allele frequencies similar to those of the rest of the species. The sequential analysis revealed, however, that this population is actually polymorphic for a largely unique set of alleles, casting doubt on previous theories that the isolate is of recent origin (PRAKASH 1972). Finally, the allelic overlap between the two sibling species observed under standard conditions was found to be spurious; the two are almost completely distinct at the locus, as the shared "alleles" are really species specific.

One cannot assume, however, that xanthine dehydrogenase is a typical enzyme. With a subunit molecular weight of about 150,000 (SEYBOLD 1974), it is one of the largest enzymes in Drosophila. The theory of allelic neutrality predicts a proportionality between mutation rate and heterozygosity, so smaller proteins may be less polymorphic. In addition, even at the outset of our study, *Xdh* was known to be one of the most polymorphic loci in the two species. It is possible that *monomorphic* loci would not show such a dramatic increase in variation under intense examination and might, in fact, remain monomorphic. Such a situation would be difficult to explain with neutral theory (see SINGH, LEWONTIN and FELTON 1976). Xanthine dehydrogenase is a Group II or variable-substrate locus according to the classification of GILLESPIE and KOJIMA (1968), and certain selectionist theories predict that these loci will be more variable than others. Finally, the increased differentiation between the species that we found for *Xdh* may occur most frequently in loci which are already somewhat differentiated under standard conditions of electrophoresis (this could not always be the case, however, in view of the results for *Xdh* in the Bogotá population).

Obviously, the allelic diversity of *Xdh* cannot be assumed to be representative of all genes. It is important to extend our methods to other loci, particularly monomorphic ones, loci coding for smaller proteins, and polymorphic loci with *similar* frequencies in closely related species. In this study we report the results of a detailed study in the two species of two other loci on the second chromo-

some which differ from *Xdh* in degree of polymorphism, size, and interspecific similarity:

Alcohol dehydrogenase-6 (*Adh-6*), one of several *Adh* loci in the two species, was first surveyed by SINGH (1976) in *D. pseudoobscura* and later located by him on the second chromosome. The locus is highly polymorphic with a heterozygosity of about 0.5. Under standard electrophoretic conditions *D. pseudoobscura* has a total of seven electrophoretic alleles, one of which is most frequent in almost all populations. *D. persimilis* is also polymorphic for *Adh-6* with gene frequencies similar to those of its sibling. Both species have a rather high frequency of null alleles (around 10%) at the locus.

Octanol dehydrogenase (*Odh*) is a second chromosome locus coding for an enzyme which accepts as substrates alcohols larger than butanol. Surveys by PRAKASH, LEWONTIN and CRUMPACKER (cited in SINGH 1976) and PRAKASH (1969) demonstrate that *Odh* is almost identically monomorphic in both species: most populations have the common allele at a frequency above 95%. There are a few rare variants in both species and a low frequency of null alleles. ODH is dimeric and has a subunit weight of about 55,000 in *D. melanogaster* (SIEBER, FOX and URSPRUNG 1972). The molecule is thus much smaller than XDH.

These two loci were subjected to the same type of analysis as was *Xdh*, with the addition of yet one more set of electrophoretic conditions as well as a heat denaturation analysis of the *Odh* locus.

MATERIALS AND METHODS

The lines of Drosophila flies used in this experiment are the same as those described in the papers of SINGH, LEWONTIN and FELTON (1976, *D. pseudoobscura*) and COYNE (1976, *D. persimilis*); all are isogenic for the entire second chromosome. The populations of *D. pseudoobscura* are from Santa Cruz Island, Cal. (SZ); Saratoga Spring, Cal. (SS); Corn Spring, Cal. (CN); Strawberry Canyon, Berkeley, Cal. (SC); Wild Rose, Cal. (WR); Charleston Mts., Nevada (CH); Cerbats Mt., Nevada (CE); Mesa Verde, Col. (MV); Hardin Ranch, Col. (HR); Austin, Texas (AU); Guatemala City, Guatemala (GU); and Bogotá, Colombia (BO). *D. persimilis* are from Fish Creek, Miranda, Cal. (FC); Mather, Cal. (MA); and Sisters, Oregon (SI). The 147 lines of *D. pseudoobscura* and 60 lines of *D. persimilis* were maintained at 17° on cornmeal food.

The method of sequential electrophoretic analysis is basically the same as that described in our previous papers. Several flies from each line were ground together in buffer solution and placed in adjacent pairs of pockets on the vertical acrylamide gel. All lines were subjected to five sets of gel conditions in the following order: the standard electrophoretic conditions of 5% acrylamide, pH 8.9 (gels run at 100 mA for 3.75 hr); 6.3% acrylamide, pH 8.9 (100 mA for 6 hr); 8% acrylamide, pH 8.9 (100 mA for 9 hr); 5% acrylamide, pH 7.5 (100 mA for 7 hr); and 6.3% acrylamide, pH 7.5 (100 mA for 8.5 hr). Gels were stained 2.5 hr with the standard ODH stain described by PRAKASH (1969), except that 1 ml of n-propanol and 1 ml of hexanol were used as substrates (at the last two sets of conditions only hexanol was used). In this way both loci could be visualized on the same gel.

All lines of each species were surveyed and classified under standard conditions. Then, all lines that appeared to have the same mobility at this condition were subjected to the next condition, re-classified, and so on through all five sets of conditions. Alleles with apparently different mobilities were run several times and only classified as different if the results were always repeatable.

Heat denaturation tests were carried out on the *Odh* alleles in every line of *D. pseudoobscura*

and ten lines of *D. persimilis*. (*Adh-6* bands are too faint to permit the densitometric scanning of individual flies which would be necessary to confirm the genetic nature of any heat variants.) A mass sample of 7 male flies from each line was ground in 105 microliters of buffered 5% sucrose, centrifuged, and aliquots of the supernatant were subjected to a temperature of 50° in an oil bath for 0 (control), 5, and 10 min. Samples were then loaded on the gel, electrophoresed for 2.5 hr under standard conditions, and the gel was stained for 2.5 hr with 1 ml of octanol as substrate. Gels were washed and scanned with an EC densitometer. Maximum peak height of each sample subjected to heat was compared to that of the control sample. Four lines were tested together on each gel. Dilution studies of the enzyme prior to densitometry confirmed that the loss of staining intensity was linear over a ten-fold range of dilution.

Molecular weight: The method of HEDRICK and SMITH (1968) was used to determine the molecular weights of ADH-6 and ODH by running them on gels with standards of known molecular weight. Standard lines of *D. pseudoobscura* were run on gels of four acrylamide concentrations (4, 5, 6.5, and 8%) along with the standard proteins horse heart myoglobin (m.w. 16,890), ovalbumin (45,000), human hemoglobin (64,500), the trimer of bovine serum albumin (205,500), and xanthine dehydrogenase from *D. pseudoobscura* (about 300,000). Gels were first stained with hexanol and n-propanol until the ODH and ADH-6 bands were visible, and then they were washed and restained with either Coomassie Blue (for protein) or xanthine dehydrogenase stain. On each gel the mobilities of the standards and ADH-6 were measured relative to that of the ODH band. Each relative mobility was measured three times on each gel and on three gels at each acrylamide concentration. The natural logarithm of the relative mobility was plotted *vs.* gel concentration for each protein and for ADH-6. This relationship is theoretically linear. Proteins larger than ODH exhibit a negative slope on this plot, those smaller have positive slope. Slopes were determined by least-squares regression, and then this slope for each protein was plotted on another graph *vs.* its known molecular weight. A least-squares fit to *these* points was then obtained, and the weights of ODH and ADH-6 could be determined by their location on this plot.

Genetics: The variants of *Adh-6* and *Odh* detected under standard conditions of electrophoresis are known from previous work to be allelic (PRAKASH 1969; SINGH, personal communication). However, genetic studies were done on all of the new alleles revealed by adding conditions of electrophoresis. The particular crosses used in these genetic tests are described in the RESULTS section.

RESULTS

Alcohol dehydrogenase-6

Under standard conditions of electrophoresis both species together have a total of 8 alleles at this locus, designated *1-*, *2-*, . . . , *7-*, and *null* in Table 1. The species have a strikingly similar pattern of alleles, with allele *4-* having the highest frequency in both, followed by allele 5- and then the null alleles. The remainder of the variants are rare. Allele *6-* is not found in *D. pseudoobscura*, nor is *7-* found in *D. persimilis*. Figure 1 shows the appearance of all the variants except the null alleles under standard conditions. Differences between the alleles are obviously small, but they are consistent when the running time is sufficiently long. These differences would often be impossible to score in flies not isogenic for the locus, for heterozygotes between alleles with close mobilities appear as only one slightly wider band. The observation of *three* bands in heterozygotes between alleles with widely disparate mobilities is taken to mean that *Adh-6* is a dimeric enzyme, although this need not necessarily be the case (D. WALLACE, personal communication).

As with xanthine dehydrogenase, the sequential alteration of conditions reveals

TABLE 1

Distribution of alleles of Adh-6 *in populations of* D. pseudoobscura *and* D. persimilis *based on all five electrophoretic criteria*

Allele	*D. pseudoobscura* populations													*D. persimilis* populations			
	MV	GU	CN	AU	SC	CS	SS	HR	SZ	WR	CB	BO	Total	FC	MA	SI	Total
10000												2	2				—
200w—									1				1				—
20000						1				1			2		1		1
30000						1					2		3	2	1		3
31000	1		1		1	1				2	2		8				—
40000						1							1				—
410w—												1	1	1			1
41000w			1	1						2			4				—
41000	5	4	5	5	5	10	12	8	9	6	8		77	14	16	10	41
42000													—	1			1
50000						1				1			2			1	1
50100												1	1				—
50110w												4	4				—
50110	7		1		5		3	2	1	2	4	3	28	4	1	3	8
50200	1												1				—
60000													—	1			1
70000							1						1				—
null			1		2		1	2	2	1	1	1	11	2	1	1	4
Total	14	4	9	6	13	15	17	12	13	15	17	12	147	24	21	15	60
Alleles	4	1	5	2	4	6	4	3	4	7	5	6	16	6	6	4	9
Unique alleles	1	0	0	0	0	1	1	0	1	0	0	4	—	2	2	0	—
Heterozyg.	0.61	0	0.64	0.28	0.68	0.53	0.47	0.49	0.50	0.77	0.69	0.78	0.584	0.62	0.41	0.50	0.517

The table gives the number of isochromosomal lines possessing each allele. The order of the integers in the allele name represents mobility at 5% gel, pH 8.9/6.3% gel, pH 8.9/8% gel, pH 8.9/5% gel, pH 7.5/6.3% gel, pH 7.5.

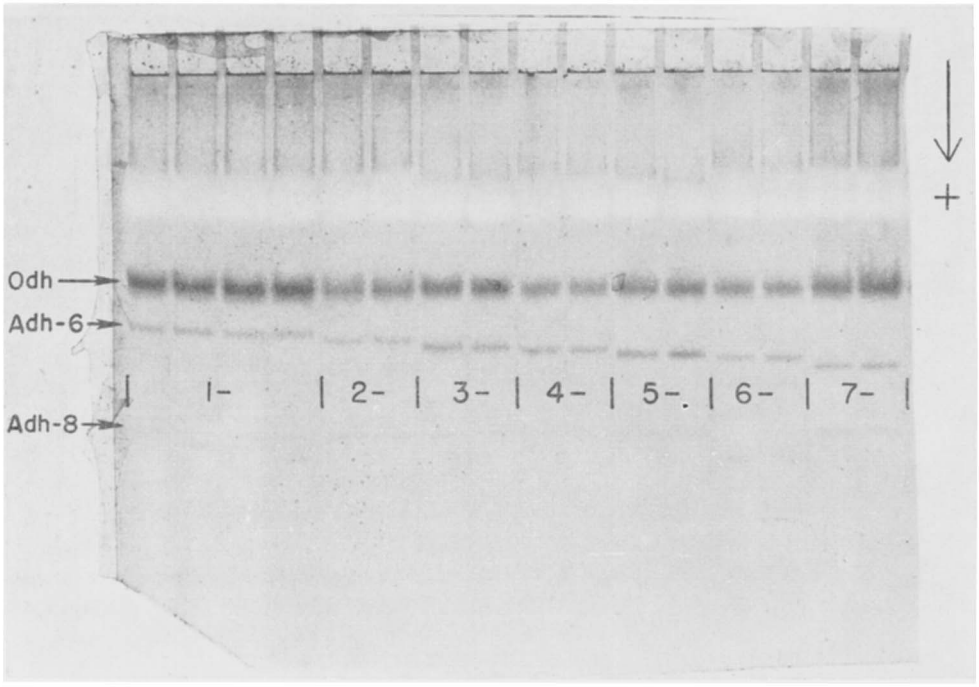

FIGURE 1.—All seven visible electrophoretic variants of *Adh-6* separated under standard conditions of electrophoresis (a 5% acrylamide gel of pH 8.9). Each adjacent pair of pockets contains the mass homogenate of 5 females from one line.

many new alleles of *Adh-6*. Table 1 gives the results of the total electrophoretic survey of both species. Here, alleles are named according to the method of SINGH, LEWONTIN and FELTON (1976), which uses a group of sequential integers. Each integer represents the rank of relative mobility of each class of an "allele" that shared common mobilities within the previous class (smallest integers represent slowest mobilities). The actual measured relative mobilities of the alleles as used by COYNE (1976) are given in the APPENDIX.

We also distinguished alleles by observing differences in staining intensities of bands which had the same mobilities under standard conditions. In this manner four variants were found: *200w—*, *410w—*, *41000w*, and *50110w*. All of these alleles stain perceptibly more faintly than standard alleles with identical mobilities ("w" stands for "weak"). The first two alleles are extremely faint under standard conditions, have the same mobility as members of their class under the first three electrophoretic conditions, and are too faint to be seen at all at the last two conditions. The latter two alleles have the same mobilities as members of their class at all conditions, but are much weaker at these conditions. Variant *410w—* is always fainter than *41000w*, which is in turn perceptibly weaker than the normal *41000* allele; and this distinction between these two weak alleles is confirmed by their different behavior in dimerization tests (see below). The appearance of the two weak subclasses of class *4-* is shown in Figure 2.

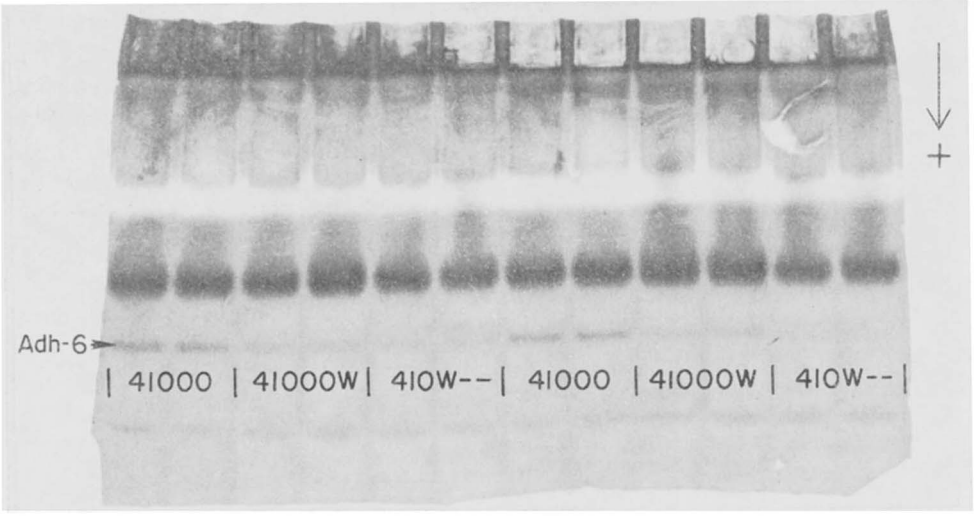

FIGURE 2.—Allele *41000* of *Adh-6* and the two weakly staining variants of class *4-* as seen under standard conditions of electrophoresis (see text for further explanation). Each adjacent pair of pockets contains the mass homogenate of 7 females from one line.

Table 1 shows that the eight "alleles" seen in both species under standard conditions have become a total of eighteen at the end of the survey. In *D. pseudoobscura* the number jumps from 7 to 16, but only from 6 to 9 in *D. persimilis*. Three additional alleles were found when the gel concentration was raised from 5% to 6.3% at pH 8.9, two more were found when it was raised again to 8% at the same pH, another was found at a pH of 7.5 in a 5% gel, and none were seen at the last electrophoretic condition. The four staining variants described above were found in eleven lines in the two species.

A doubling of the number of alleles has resulted from the more intensive analysis at this locus, then, but this is nowhere near the fivefold increase uncovered at the *Xdh* locus in the two species. Furthermore, all the new alleles of *Adh-6* are rare; this is reflected in the number of allelic classes in both species present in only one line. The most even split of an originally homogeneous class was that of allele *3-*, which under standard conditions comprised fourteen lines in both species (eleven of these in *D. pseudoobscura*). This class split into subclass *30-*, containing 3 lines of each sibling species, and class *31-*, with the remaining 8 lines of *D. pseudoobscura*. As was the case for *Xdh*, one allele of *Adh-6* predominates in each species, though in this case it is the same allele in each. This ubiquitous variant is allele *41000*, which comprises 77 lines (52%) of *D. pseudoobscura* and 41 lines (68%) of *D. persimilis*. Allele *50110* is the next most common in both species, and the remainder of the alleles have a frequency of less than eight percent each.

With a few exceptions, the total allelic distribution of the species is mirrored in that of the individual populations. All but three populations of both species have *41000* as their most common allele. The Strawberry Canyon and Mesa

Verde populations of *D. pseudoobscura* differ somewhat in their high frequency of *50110*, and these were the same two populations which had aberrant allelic frequencies at the *Xdh* locus (SINGH, LEWONTIN and FELTON 1976). One population of *D. pseudoobscura*—that from Bogotá—becomes radically different from the others when examined under the five sets of conditions. Under standard conditions Bogotá differed somewhat from the rest of the populations in its high frequency of *50110*, but ten of its twelve lines still had alleles found elsewhere in the species (including one line in the common class *4-*). At the end of the analysis, eight of the twelve lines from Bogotá are seen to have four alleles found nowhere else in the species; and no lines have allele *41000*, which is most common elsewhere. This polymorphism for unique alleles in Bogotá is similar to what was found by SINGH, LEWONTIN and FELTON (1976) at the *Xdh* locus.

The rare alleles of *Adh-6* differ from population to population. Seven populations of both species have unique alleles, with Bogotá having four and the Fish Creek and Mather populations of the sibling species having two each. One should not make too much of this phenomenon, as it may be an artifact of small sample size. Most populations are also rather polymorphic. Bogotá has six alleles in its twelve lines, Wild Rose has seven in fifteen lines, and Corn Spring displays five alleles in only nine lines. Heterozygosities calculated on the assumption of Hardy-Weinberg equilibrium approach eighty percent in the Bogotá and Wild Rose populations. The average weighted populational heterozygosity is 0.58 for *D. pseudoobscura* and 0.52 for *D. persimilis*.

Our interspecific analysis reveals that the originally similar distribution of allele frequencies remains so after the complete analysis. Nine rare alleles are unique to the larger sample of *D. pseudoobscura* and one to *D. persimilis*. Only in class *3-*, as we mentioned above, did there later prove to be evidence of interspecific differentiation.

Molecular weight: Figure 3 gives the plot of the slope of relative (to ODH) mobility curves *versus* molecular weights of the standards and unknown enzymes. The line is remarkably straight and has the regression equation shown on the graph. ADH-6 had a slope of -0.1708 ± 0.0018 which, when substituted is a dimer, the molecular weight of the monomer is about 143,000. ADH-6 is, then, a large enzyme about the size of XDH.

Genetics: At least one line from each of the six new allelic classes discovered by their differential mobilities was subjected to genetic analysis. This involved crossing it to both the slow allele *10000* and the null alleles and examining the heterozygotes at the electrophoretic conditions under which the alleles became visibly different. No heterodimers were ever seen in crosses to the null alleles, and homodimers of the variant alleles in these heterozygotes displayed mobilities identical to their constituent homozygotes. This was also true of the homodimers in crosses to the slow allele. Our new electrophoretic alleles are, then, either variants at the structural locus or closely linked, *cis*-dominant modifiers of mobility (the latter possibility can never be excluded in this type of genetic analysis). More important than these tests, however, is an investigation of the

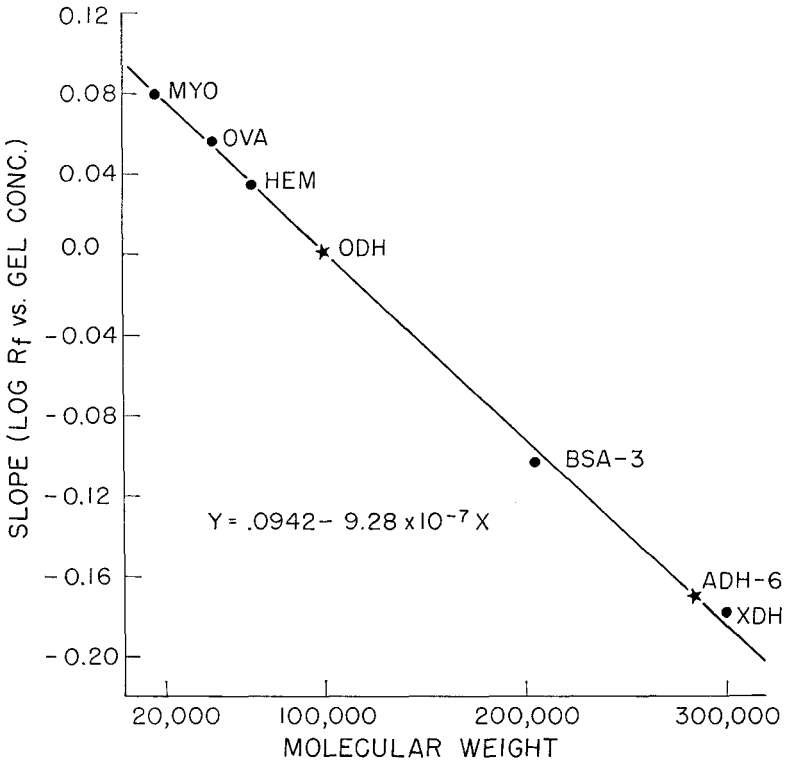

FIGURE 3.—Plot for molecular weight determination of ODH and ADH-6. The positions of these two enzymes are interpolated on the graph, as indicated by the stars.

genetics of the null alleles and especially the alleles detected by their weak staining activity. It is possible that this weakness is due to some other locus or to a regulatory element, especially since it appears in three distinct electrophoretic classes under standard conditions. Crosses were made of all weakly staining alleles to the slow line *10000* and staining intensity of the homodimers of weak alleles was examined in comparison to homodimers of standard alleles when both were in heterozygotes. Figure 4a shows some of these results. Here allele *410w*—, which gives an extremely faint band under standard conditions, cannot be seen at all in the heterozygote, nor does it appear to form active heterodimers with allele *10000*. Allele *41000w* does form obvious heterodimers with the slow allele, but the *41000w* homodimers are still visibly weaker than homodimers of standard allele *41000* when both are in heterozygotes with *10000*. This difference in visible dimerization ability of the two weak alleles, which also holds up in the F_2 generation, confirms our separation of them as distinct genetic forms.

Allele *200w*—, found in one line, also forms no visible heterodimers with *10000*. The Bogotá population has four lines containing allele *50110w*, which has about half the staining intensity of *50110*. Figure 4b shows both of these alleles as dimers in the cross with *10000*. The weak lines from Bogotá do form hetero-

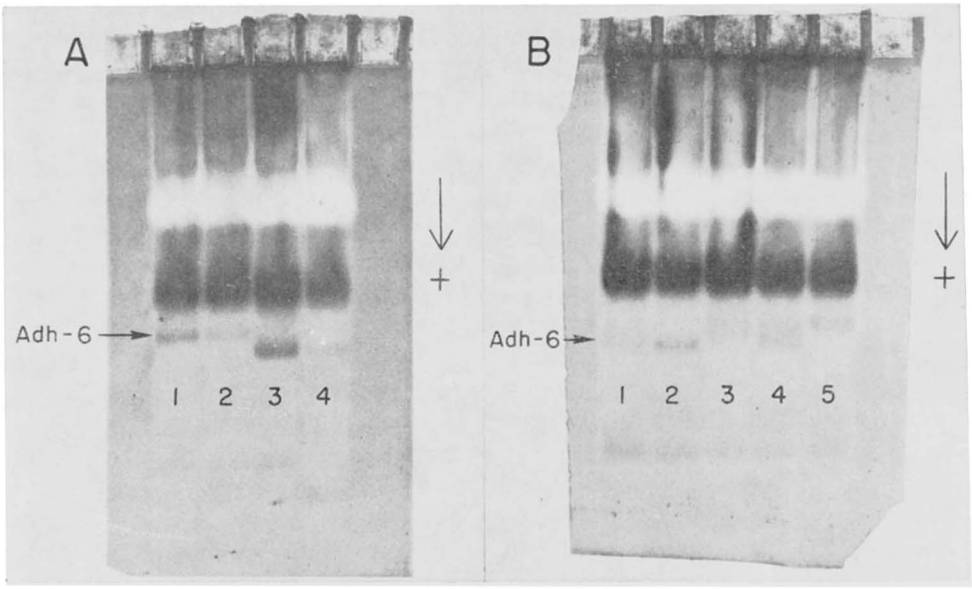

FIGURE 4.—Genetic tests of weakly staining variants of *Adh-6*. Each pocket contains the mass homogenate of 7 females from one line or cross. (A, left), Pocket 1: Standard slow variant *10000*; pocket 2: heterozygote *10000/410w*— (note absence of heterodimer and *410w*— homodimer); pocket 3: standard allele *41000*; pocket 4: allele *410w*—. (B, right), Pocket 1: Allele *50110w* (from Bogotá); pocket 2: standard allele *50110*; pocket 3: heterozygote *10000/50110w* (note weakly staining *50110w* homodimer); pocket 4: heterozygote *10000/50110*; pocket 5: standard slow variant *10000*.

dimers but, as the photograph indicates, the performance of the homodimers is weak compared to the controls.

All of the null alleles of *Adh-6* were also crossed to *10000*, and no heterodimers were ever seen. These results are similar to the crosses of allele *10000* with alleles *200w*— and *410w*––, but the latter lines can be distinguished from nulls by their visible staining activity at every stage of the life cycle. Null alleles display no activity in early or late larva, pupa, or adult.

Although we cannot discount closely linked, *cis*-acting modifiers of activity and mobility as responsible for our results, we assume that all our alleles are variants at the structural locus. The fact that two of the four differentially staining alleles showed no heterodimerization does argue against any regulatory nature of the variants. Other inferential evidence on this point comes from a previous study of alcohol dehydrogenase in *D. melanogaster*. SCHWARTZ and SOFER (1976) demonstrated with a variety of biochemical methods that the majority of *null* homozygotes for *Adh* in this species contain inactive enzyme from the locus.

Octanol dehydrogenase

Table 2 presents the results of the identical study using the *Odh* locus of both species. In our populations under standard conditions the locus is nearly mono-

TABLE 2

Distribution of alleles of Odh in populations of D. pseudoobscura *and* D. persimilis based on all five electrophoretic criteria

	D. pseudoobscura populations													*D. persimilis* populations			
Allele	MV	GU	CN	AU	SC	CS	SS	HR	SZ	WR	CB	BO	Total	FC	MA	SI	Total
10000													—		1		1
20000								1					1				—
30000	13	4	9	6	11	13	16	11	12	15	17	12	139	23	19	15	57
30010									1				1				—
30001													—	1			1
40000													—		1		1
50000							1						1				—
60000					1								1				—
70000	1												1				—
80000					1								1				—
null						2							2				—
Total	14	4	9	6	13	15	17	12	13	15	17	12	147	24	21	15	60
Alleles	2	1	1	1	3	2	2	2	2	1	1	1	8	2	3	1	4
Unique alleles	1	0	0	0	2	1	1	1	1	0	0	0	—	1	2	0	—
Heterozyg.	0.13	0	0	0	0.27	0.23	0.11	0.15	0.15	0	0	0	0.098	0.08	0.18	0	0.095

The table gives the number of isochromosomal lines possessing each allele. The order of the integers represents mobility at 5% gel, pH 8.9/6.3% gel, pH 8.9/8% gel, pH 8.9/5% gel, pH 7.5/6.3% gel, pH 7.5.

morphic, with allele *3-* present in 198 of the 207 lines in both species. Eight other rare alleles of *Odh* are also seen under these conditions, with seven of them appearing only once. Two of the unique alleles are found in *D. persimilis* and the remainder, which are all different from these, are seen in the sibling species. The only variant found more than once is the null allele present in two lines of the Charleston population of *D. pseudoobscura*.

When the identical sequence of five electrophoretic conditions is used for analysis of this locus, almost no new variation is found. One more unique allele (*30010*) appears in a line of *D. pseudoobscura* in a 5% gel of pH 7.5, and a different unique one (*30001*) shows up in one population of *D. persimilis* in a 6.3% gel of pH 7.5. The weighted heterozygosity hardly changes in the course of the investigation, attaining a final value of 0.098 for *D. pseudoobscura* and 0.095 for *D. persimilis*.

This locus shows again the predominance of one allele and the extreme rarity of the others. Interpopulational differences in frequencies of rare alleles are again evident, but may again be ascribed to small sample sizes. No increased differentiation between the species was found by the intensive analysis.

Heat denaturation: Every line of *D. pseudoobscura* and ten lines of *D. persimilis* were examined at least once on a gel with three other lines in search of variation in response to heat treatment. Again, not one anomalous line was uncovered by this method. All lines appear to denature with almost identical loss of activity on the gel, and none of the erratic variation observed for XDH by SINGH, LEWONTIN and FELTON (1976) was found in our study. We have no explanation for the increased repeatability of heat tests on ODH. A few ODH samples appeared slightly different on initial gels, but these differences always disappeared in subsequent tests. The lines of *D. persimilis* did not differ from lines of *D. pseudoobscura* in their heat sensitivity. SINGH, HUBBY and LEWONTIN (1974) reported several heat-labile alleles of *Odh* in *D. pseudoobscura*, but we have seen none of these in our investigations.

Molecular weight: As ODH was used as the standard to which all other mobilities were compared, it has a relative slope to itself of one at all gel concentrations; and the logarithm of the slope is zero. The point of zero slope on the regression line of Figure 3 corresponds to a molecular weight of about 101,500, which is close to a previously determined value of 109,000 in *D. melanogaster* (SIEBER, FOX and URSPRUNG 1972). If one assumes it is a dimer, the molecular weight of the monomer is about 50,000, making it about one third the size of XDH and ADH-6.

Genetics: Alleles of *Odh* distinguishable under standard conditions of electrophoresis are known to behave as allelic variants (PRAKASH, LEWONTIN and HUBBY 1969). The two new alleles found by altered conditions in this study were crossed to a null allele of *Odh*. No heterodimer was formed, and the homodimers in the heterozygotes showed the same altered mobilities. The variant of *Odh* found in *D. pseudoobscura* was also crossed to the fast *Odh* allele *80000*, and a similar alteration of the homodimer mobility was observed. We assume that both variants represent structural mutants.

Finally, both of the null alleles of *Odh* in *D. pseudoobscura* were crossed to

the variant *80000*. No heterodimers were found. Gels run with flies in a variety of stages of the life cycle demonstrated that null alleles show no *Odh* activity at any time, whereas standard alleles are expressed at all stages.

DISCUSSION

Our work on these two alcohol dehydrogenase loci demonstrates that xanthine dehydrogenase is indeed unusual in its dramatic increase in polymorphism uncovered by added conditions of electrophoresis. Table 3 summarizes, for these three loci, the increase in number of alleles, effective number of alleles (the reciprocal of the homozygosity), and the weighted average heterozygosity made possible by the new methods. Xanthine dehydrogenase shows by far the largest increase in all three categories, *Adh-6* is generally intermediate, and *Odh* shows very little change. For *Adh-6*, like *Xdh*, the known number of alleles was more than doubled by the intensive analysis, but the lower increase in heterozygosity at the former locus reflects the fact that almost all of its new alleles were rare or unique. The lack of proportional increases in these parameters for the three loci is an important justification for the use of the new methods. SINGH, LEWONTIN and FELTON (1976) note that if this type of investigation revealed a proportional increase in polymorphism at all loci, no progress would be made toward resolving the question of how the genetic diversity is maintained. Table 3 shows that this is not the case, and we may expect some qualitatively different conclusions when these methods come into wider use.

One possible result of this type might be an increasing dichotomy between monomorphic and polymorphic loci. Octanol dehydrogenase remains monomorphic under six new conditions of investigation, while *Adh-6* and *Xdh* become rather more polymorphic. Preliminary results in our laboratory also demonstrate that the monomorphic locus, malic dehydrogenase, maintains its monomorphism under a variety of electrophoretic conditions. Combined with our previous work, this investigation indicates that in our sample of four loci the

TABLE 3

Change in genetic parameters of three loci engendered by new electrophoretic techniques

	Number of alleles			Heterozygosity			Effective no. of alleles		
	Standard conditions	All conditions	Increase	Standard conditions	All conditions	Increase	Standard conditions	All conditions	Increase
Xdh									
D. pseudoobscura	8	27	238%	0.436	0.628	44%	1.8	2.7	50%
D. persimilis	6	23	283%	0.406	0.676	67%	1.7	3.1	82%
Adh-6									
D. pseudoobscura	7	16	129%	0.499	0.584	17%	2.0	2.4	20%
D. persimilis	6	9	50%	0.442	0.517	17%	1.8	2.1	16%
Odh									
D. pseudoobscura	7	8	14%	0.082	0.098	19%	1.09	1.11	2%
D. persimilis	3	4	33%	0.063	0.095	33%	1.07	1.10	3%

Data for *Xdh* are taken from SINGH, LEWONTIN and FELTON (1976) and COYNE (1976).

polymorphic ones become more polymorphic, but the monomorphic ones remain monomorphic.

All genes tested so far do, however, share one similarity in their allelic pattern: a single main allele predominates at each locus, and all others are much lower in frequency. This phenomenon is typical of electrophoretic surveys in many organisms (SELANDER *et al.* 1971; MILKMAN 1973; AYALA *et al.* 1974) and must be taken into account by any theory purporting to explain the polymorphism (see MILKMAN 1973; COYNE 1976). In addition, the rare variants always show differences in frequency from population to population within a species. Before this can be ascribed to lack of migration between populations, it must be confirmed in samples much larger than ours.

The Bogotá population of *D. pseudoobscura* has shown remarkably similar patterns at both polymorphic loci we have examined. For *Xdh* the isolate appeared similar to the rest of the species under standard conditions, but at the end of the survey of SINGH, LEWONTIN and FELTON (1976) it was shown to be polymorphic for largely unique alleles. Nine of the twelve lines from Bogotá carried *Xdh* alleles unique to that population. *Adh-6* shows an initial quantitative difference in allele frequency between Bogotá and the rest of the species under standard conditions, but this difference is greatly magnified with the use of the additional methods. Eight of the twelve Bogotá lines have *Adh-6* alleles unique to the population. A computation of NEI's (1972) genetic identity for the locus shows that the *I* value for *Adh-6* between Bogotá and the rest of the species drops from 0.40 at the beginning of the investigation to 0.17 at the end, making the Bogotá population much more different from *D. pseudoobscura* than the two sibling species are from each other. The unique polymorphism of Bogotá at two out of the three loci examined so far is difficult to reconcile with previous theories invoking the action of genetic drift or founder effect in a recent isolate (PRAKASH 1972). The Bogotá population is likely to be much older than previously assumed, and its partial reproductive isolation need not have developed quickly. DOBZHANSKY (1974) also proposed that the Bogotá population was very old, based on his genetic analysis of sterility in Bogotá-U.S.A. hybrids.

Another difference between the present study and our previous work on *Xdh* is the lack of any increased differences between the species at the two alcohol dehydrogenase loci. In fact, *D. pseudoobscura* and *D. persimilis* become slightly more similar at *Odh* and *Adh-6* when new methods are used, because of an increase in the number of (rare) shared alleles detected with the four added conditions. The interspecific *I* value increases from 0.853 to 0.867 for *Adh-6* when all alleles are taken into account, and from 0.991 to 0.995 for *Odh*. In contrast, COBBS and PRAKASH (personal communication) have demonstrated a complete disjunction of allele frequencies in these two species at esterase-5, a locus at which the two species are largely disjunct under standard conditions. Perhaps loci interspecifically distinct under standard conditions will become more so with thorough investigation, while those originally similar will remain similar. This possible result is of some theoretical interest, and has been discussed by LI (1976) with reference to the theory of allelic neutrality.

Setting aside the question of interspecific differences, we still require an explanation for the allelic patterns and increased polymorphism found in our studies. Three theories have been proposed to account for allozyme polymorphism in natural populations. The neutral hypothesis assumes no selective differences between electrophoretic alleles. There are two selective hypotheses: balancing selection (including overdominance, frequency-dependent selection, and selection based on environmental or temporal heterogeneity) and purifying selection (KING and OHTA 1975; OHTA 1976), which posits one wild-type allele and a number of other slightly deleterious ones held in balance by mutation and selection. It is likely that no one theory will ever fully account for all molecular polymorphism, but it is useful to examine our findings so far in light of all three theories.

The only hypothesis which can be tested statistically against our allelic patterns is that of neutrality. EWENS' (1972) test measures departures from neutrality as deviations from the evenness of allelic frequencies predicted by a neutral, infinite-alleles model. Frequencies too even implicate purifying selection. In previous tests, *Xdh* showed significant departures from neutrality towards unevenness in lumped species data for both *D. pseudoobscura* and *D. persimilis*, and in the latter species all three individual populations were significantly deviant in the direction of unevenness (see our *Corrigendum* in GENETICS **85:** March, 1977). The data for *Adh-6* also show suggestive deviations from neutrality in this direction. EWENS' test yields a negative (uneven) L statistic and an F value of 0.5308 (df = 55.7, 17.0) for the lumped data for *D. pseudoobscura*, which gives an associated probability of 0.08. The combined data for the same locus in *D. persimilis* also give a negative L and an F of 0.3939 (df = 30.3, 9.8), which is barely significant with a probability of 0.046. As SINGH, LEWONTIN and FELTON (1976) point out, however, this unevenness might result from the pooling of populations with the same predominant allele, but different rare ones. Indeed, the data for the individual populations themselves show no consistent deviations from neutrality. Four of the populations of *D. pseudoobscura* are too even, with none significantly so; and seven are uneven, with one of these significantly so. All three populations of *D. persimilis* have uneven allele frequencies for *Adh-6*, but only one of these (Mather) is significant.

The situation is different for the octanol dehydrogenase locus. In the lumped population data for each species, there is significant departure from neutrality in the direction of unevenness (*D. pseudoobscura*: $L = -3.81$, $F = 0.0982$, $df = 16.2$, 8.7, $p < 0.001$; *D. persimilis*: $L = -2.33$, $F = 0.1428$, df = 7.3, 4.6, $p = 0.012$). Moreover, this unevenness cannot be due solely to the lumping of data, since all eight populations in both species with more than one allele of *Odh* have negative L values, in the direction of unevenness (populations with only one allele cannot be tested). The sign test gives a probability of 0.004 to the consistency of these deviations. As EWENS' test does not depend on mutation rate at the locus or specific population structure, these results implicate the action of purifying selection against the existing variants of *Odh*.

Some support for the neutral theory is given by the relationship between

polymorphism of the low and molecular weight of their enzymes. *Xdh* and *Adh-6*, the very polymorphic loci, produce very large enzymes, while ODH (and MDH) are much smaller (according to O'BRIEN 1973, the weight of the MDH monomer is about 30,000). There should be a proportionality between the number of amino acid residues in a protein and the mutation rate of its locus, leading a neutralist to expect more polymorphism in larger enzymes. There is already some evidence for this in Drosophila, (KOEHN and EANES, personal communication), but not in man (HARRIS, HOPKINSON and EDWARDS 1977); and such a relationship cannot explain the degree of polymorphism of all loci in fruit flies (see, for example, SINGH, LEWONTIN and FELTON's (1976) discussion of esterases).

A result which cannot be explained by strict neutral theory, if found to be a general phenomenon, is the discovery of two disparate classes of loci—very polymorphic and nearly monomorphic. Such a finding would definitely indicate the working of some type of selection, which could be either heterotic selection on the polymorphic loci with the monomorphic ones being neutral, purifying selection on the monomorphic loci with the polymorphic ones being neutral, or, leaving out neutrality altogether, purifying selection on the monomorphic loci and heterotic selection on the polymorphic. Which of these seems more reasonable depends on analyses of more loci in more species, reliable studies of mutation rates and effective population sizes, and possible biochemical correlates of genetic variation. Neutral theory is, then, in accordance with some but not all of our results.

Even more problems are encountered, however, when one attempts to explain the data by invoking heterotic balancing selection. If one accepts neutrality as the null hypothesis, the only statistical departure from it at any of our loci is in the direction of purifying and not heterotic selection.

It is difficult to compare the number of alleles or the form of their distributions in our studies with those predicted by balancing selection, for no such theory exists to yield testable predictions. Proponents of heterosis as the prime maintainer of variation have been notoriously lax in producing theories to account for the number and frequencies of alleles present in natural populations. LEWONTIN and GINSBURG (personal communication) have recently investigated this theoretical problem, and they have found that very stringent assumptions about fitness distributions of heterozygotes and homozygotes are necessary to account for the many alleles we have found. Even these assumptions, however, cannot explain the form of our allelic distributions. Their results predict a predominant allele in much lower frequency than those in our loci. Undoubtedly heterotic models can be produced to explain our data, but they will certainly require a multitude of rigorous assumptions. The same is true for models based on fluctuating, frequency-dependent, or environmental selection.

Finally, it is difficult indeed to explain the rather high frequency of null alleles of *Adh-6* by *any* type of balancing selection. Nulls show no activity at any stage of the life cycle, and it would be a strange *ad hoc* hypothesis that could explain

the selective superiority of a heterozygote between an active and a nonactive allele.

This leaves us with the final theory, the mutation-selection model of KING and OHTA (1975). This is the only one which can explain the significant deviation of the *Odh* data from the pattern predicted by neutrality, and certainly the three loci tested to date show patterns consistent with purifying selection (all of the distributions of allele frequencies, with one main allele flanked symmetrically by rarer ones, also differ from neutrality in the direction of unevenness). Our tentative relationship between molecular weight and polymorphism could also be explained by this theory if one assumes that more weakly deleterious variants are possible in larger proteins. The higher frequency of null alleles than many other variants of *Adh-6* seems at first to violate the theory, since nulls would appear to be at the greatest selective disadvantage; but nulls may be produced by mutation more frequency than other alleles. There is some support for this assumption from mutation studies of other loci (MUKAI and COCKERHAM, personal communication).

As far as the interspecific comparison is concerned, we have found two cases of similarity between the two species and one of almost complete dissimilarity. COBBS and PRAKASH have, as mentioned above, found another case of complete dissimilarity in the esterase-5 locus. A possible result of future studies might be that closely related species are either completely similar or very different in their allozyme frequencies over all loci. This occurrence has already been discussed to some extent by AYALA *et al.* (1974). Unfortunately, it is not clear at present whether a U-shaped distribution of interspecific genetic identities at different loci provides evidence for balancing selection (AYALA and GILPIN 1974) or neutrality (NEI and TATENO 1975).

Obviously, our results are not conclusive since they are based on a limited sample of loci from one chromosome and preliminary data from another locus. There does seem to exist a heterogeneity of selective forces between loci, although it is not known whether this heterogeneity involves balancing or purifying selection. The statistical tests, at least, implicate some mixture of weak purifying selection and neutrality. Neutralists have always admitted the presence of purifying selection, but only recently has it been suggested as a force which might allow for molecular polymorphism. As selective coefficients on allelic variants must be small to account for much polymorphism in a balance between mutation and purifying selection, the demonstration that this type of selection exists would suggest that the variants are of potential importance in evolution. A small negative selection coefficient may easily become a small positive one with environmental change, and in the large populations necessary for mutation-selection balance this could lead to significant changes in gene frequencies.

We would like to thank DICK LEWONTIN and DON WALLACE for advice and assistance in the course of the surveys, and WYATT ANDERSON, FRED COHAN, ANAND GUPTA, PETER HAAS, DON HICKEY, RUSS LANDE, DICK LEWONTIN, ROGER MILKMAN, and ROLLIN RICHMOND for discussion and review of the manuscript. Other help was provided by LYNDAL ANDREWS, GARY

COBBS, A. C. COLEMAN, DOREEN LEWIS, LASZLO MESZOLY, and RAMA SINGH, as well as by the authors of the unpublished manuscripts who allowed us to mention their results. The work was supported by an NSF predoctoral fellowship to J.A.C. and by Public Health Service GM 21179 to R. C. LEWONTIN.

NOTE ADDED IN PROOF

It was recently pointed out to us by DR. CHARLES LANGLEY that ADH-6 appears to act also as an aldehyde oxidase. DR. RAMA SINGH and ourselves have found that ADH-6 indeed seems to be identical to acetaldehyde oxidase-2 (AO-2), an allozyme which is stained with benzaldehyde as substrate. AO-2 was previously reported by PRAKASH, LEWONTIN and HUBBY (1969) as a largely monomorphic locus in *D. pseudoobscura*. Preliminary investigations staining for the *Adh-6* locus with benzaldehyde instead of n-propanol show that the two substrates reveal identical mobility differences but that there are some differences between the two in their ability to detect activity differences. This leads us to the following observations: (1) The same allozyme may be unwittingly surveyed as the product of more than once "locus" when more than one stain is used. This may be especially troublesome for monomorphic loci. (2) Allozymes previously reported as monomorphic may actually be quite polymorphic, even under standard conditions of electrophoresis. (3) It may be difficult to generalize about the natural substrate of enzymes which can attack such disparate substances as propanol and benzaldehyde. (4) The use of different substrates to stain for the same allozyme may be a useful way of detecting genetic variation, particularly the type revealed by activity or thermostability differences (see YUTANI *et al.* 1977).

LITERATURE CITED

AYALA, F. J., 1975 Genetic differentiation during the speciation process. Evol. Biol. **8**: 1–78.

AYALA, F. J. and M. E. GILPIN, 1974 Gene frequency comparisons between taxa: support for the natural selection of protein polymorphisms. Proc. Natl. Acad. Sci. U.S. **71**: 4847–4849.

AYALA, F. J., M. L. TRACEY, L. G. BARR, J. F. MCDONALD and S. PEREZ-SALAS, 1974 Genetic variation in natural populations of five Drosophila species and the hypothesis of the selective neutrality of protein polymorphisms. Genetics **77**: 343–384.

BERNSTEIN, S. C., L. H. THROCKMORTON and J. L. HUBBY, 1973 Still more genetic variability in natural populations. Proc. Natl. Acad. Sci. U.S. **70**: 3928–3931.

COCHRANE, B. J., 1976 Heat stability variants of esterase-6 in *Drosophila melanogaster*. Nature **261**: 131–132.

COYNE, J. A., 1976 Lack of genic similarity between two sibling species as revealed by varied techniques. Genetics **84**: 593–607.

DOBZHANSKY, TH., 1974 Genetic analysis of hybrid sterility within the species *Drosophila pseudoobscura*. Hereditas **77**: 81–88.

EWENS, W. J., 1972 The sampling theory of selectively neutral alleles. Theor. Pop. Biol. **3**: 87–112.

GILLESPIE, J. and K. KOJIMA, 1968 The degree of polymorphism in enzymes involved in energy production compared to that in nonspecific enzymes in two *Drosophila ananassae* populations. Proc. Natl. Acad. Sci. U.S. **61**: 582–585.

HARRIS, H., D. A. HOPKINSON and Y. H. EDWARDS, 1977 Polymorphism and the subunit structure of enzymes: A contribution to the neutralist-selectionist controversy. Proc. Natl. Acad. Sci. U.S. **74**: 698–701.

HEDRICK, J. L. and A. J. SMITH, 1968 Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. Arch. Biochem. Biophys. **126**: 155–164.

JOHNSON, G. B., 1976 Hidden alleles at the alpha-glycerophosphate dehydrogenase locus in Colias butterflies. Genetics **83:** 149–167.

KING, J. L. and T. OHTA, 1975 Polyallelic mutational equilibria. Genetics **79:** 681–691.

LI, W.-H., 1976 Electrophoretic identity of proteins in a finite population and genetic distance between taxa. Genet. Res. **28:** 119–127.

MILKMAN, R., 1973 Electrophoretic variation in *Escherichia coli* from natural sources. Science **182:** 1024–1026.

MILKMAN, R. and R. KOEHLER, 1976 Isoelectric focusing of MDH and 6-PGDH from *Escherichia coli* of diverse natural origins. Biochem. Genet. **14:** 517–522.

NEI, M., 1972 Genetic distance between populations. Am. Naturalist **106:** 283–292.

NEI, M. and Y. TATENO, 1975 Interlocus variation of genetic distance and the neutral mutation theory. Proc. Natl. Acad. Sci. U.S. **72:** 2758–2760.

O'BRIEN, S. J., 1973 Comparative analysis of malate dehydrogenases of *Drosophila melanogaster*. Biochem. Genet. **10:** 191–205.

OHTA, T., 1976 Role of very slightly deleterious mutations in molecular evolution and polymorphism. Theor. Pop. Biol. **10:** 254–276.

PRAKASH, S., 1969 Genic variation in a natural population of *Drosophila persimilis*. Proc. Natl. Acad. Sci. U.S. **62:** 778–784. ——, 1972 Origin of reproductive isolation in the absence of apparent genic differentiation in a geographic isolate of *Drosophila pseudoobscura*. Genetics **72:** 143–155.

PRAKASH, S., R. C. LEWONTIN and J. L. HUBBY, 1969 A molecular approach to the study of genic heterozygosity in natural populations. IV. Patterns of genic variation in central, marginal, and isolated populations of *Drosophila pseudoobscura*. Genetics **61:** 841–858.

SCHWARTZ, M. and W. SOFER, 1976 Alcohol dehydrogenase-negative mutants in Drosophila: Defects at the structural locus? Genetics **83:** 125–136.

SELANDER, R. K., M. H. SMITH, S. Y. YANG, W. E. JOHNSON and J. B. GENTRY, 1971 IV. Biochemical polymorphism and systematics in the genus *Peromyscus*. I. Variation in the old-field mouse (*Peromyscus polionotus*). Univ. Tex. Publ. **7103:** 49–90.

SEYBOLD, W. D., 1974 Purification and partial characterization of xanthine dehydrogenase from *Drosophila melanogaster*. Biochim. Biophys. Acta **334:** 266–271.

SIEBER, F., D. J. FOX and H. URSPRUNG, 1972 Properties of octanol dehydrogenase from Drosophila. FEBS Letters **26:** 274–276.

SINGH, R. S., 1976 Substrate-specific enzyme variation in natural populations of *Drosophila pseudoobscura*. Genetics **82:** 507–526.

SINGH, R. S., J. L. HUBBY and R. C. LEWONTIN, 1974 Molecular heterosis for heat-sensitive enzyme alleles. Proc. Natl. Acad. Sci. U.S. **71:** 1808–1810.

SINGH, R. S., J. L. HUBBY and L. H. THROCKMORTON, 1975 The study of genic variation by electrophoretic and heat denaturation techniques at the octanol dehydrogenase locus in members of the *Drosophila virilis* group. Genetics **80:** 637–650.

SINGH, R. S., R. C. LEWONTIN and A. A. FELTON, 1976 Genetic heterogeneity within electrophoretic "alleles" of xanthine dehydrogenase in *Drosophila pseudoobscura*. Genetics **84:** 609–629.

YUTANI, K., K. OGASAHARA, Y. SUGINO and A. MATSUSHIRO, 1977 Effect of a single amino acid substitution on stability of conformation of a protein. Nature **267:** 274.

Corresponding editor: W. W. ANDERSON

APPENDIX

Relative mobilities of Adh-6 *alleles* [Nomenclature explained in text. See also COYNE (1976).]

Allele	Mobility
10000	0.939/1.000/1.000/1.000/1.000
20000	0.962/1.000/1.000/1.000/1.000
200w—	0.962/1.000/1.000/——
30000	0.985/0.985/1.000/1.000/1.000
31000	0.985/1.000/1.000/1.000/1.000
40000	1.000/0.989/1.000/1.000/1.000
41000	1.000/1.000/1.000/1.000/1.000
4100w	"
410w—	1.000/1.000/1.000/——
42000	1.000/1.010/1.000/1.000/1.000
50000	1.019/1.000/0.986/1.000/1.000
50100	1.019/1.000/1.000/0.985/1.000
50110	1.019/1.000/1.000/1.000/1.000
50110w	"
50200	1.019/1.000/1.010/1.000/1.000
60000	1.034/1.000/1.000/1.000/1.000
70000	1.071/1.000/1.000/1.000/1.000
null	————

Relative mobilities of Odh *alleles*

Allele	Mobility
10000	0.863/1.000/1.000/1.000/1.000
20000	0.886/1.000/1.000/1.000/1.000
30000	1.000/1.000/1.000/1.000/1.000
30010	1.000/1.000/1.000/1.018/1.000
30001	1.000/1.000/1.000/1.000/1.022
40000	1.081/1.000/1.000/1.000/1.000
50000	1.094/1.000/1.000/1.000/1.000
60000	1.110/1.000/1.000/1.000/1.000
70000	1.170/1.000/1.000/1.000/1.000
80000	1.258/1.000/1.000/1.000/1.000
null	————