GENETIC HETEROGENEITY WITHIN ELECTROPHORETIC "ALLELES" OF XANTHINE DEHYDROGENASE IN *DROSOPHILA PSEUDOOBSCURA*[1]

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ABSTRACT

An experimental plan for an exhaustive determination of genic variation at structural gene loci is presented. In the initial steps of this program, 146 isochromosomal lines from 12 geographic populations of *D. pseudoobscura* were examined for allelic variation of xanthine dehydrogenase by the serial use of 4 different electrophoretic conditions and a heat stability test. The 5 criteria revealed a total of 37 allelic classes out of the 146 genomes examined where only 6 had been previously revealed by the usual method of gel electrophoresis. This immense increase in genic variation also showed previously unsuspected population differences between the main part of the species distribution and the isolated population of Bogotá, Colombia, in conformity with the known partial reproductive isolation of the Bogotá population. The average heterozygosity at the *Xdh* locus is at least 72% in natural populations. This result, together with the very large number of alleles segregating and the pattern of allelic frequencies, has implications for theories of genetic polymorphism which are discussed.

SINCE the first demonstrations by HUBBY and LEWONTIN and HARRIS in 1966 that genetic polymorphism at structural gene loci can easily be detected by gel electrophoresis, there has been an immense explosion of information about genic polymorphism in natural populations. The past ten years of work by many research workers, investigating many organisms from *E. coli* to *Homo sapiens*, have demonstrated conclusively that the typical natural population of organisms is highly polymorphic for genes specifying the primary amino-acid sequence of enzymes and other proteins (for a recent documentation of the evidence, see POWELL 1975). This extraordinary genetic diversity within populations has demanded an explanation, and from the outset population geneticists have been divided between those who believe the polymorphism to be held in stable equilibrium by some form of balancing natural selection (the "selectionist school") and those who believe it to be the result of a stochastic equilibrium between the input of nonselected mutations and their loss by random genetic drift (the "neutralist school"). Because it is exceedingly difficult to measure directly selective forces of the order of those that might reasonably be expected to be operating

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on most loci, the failure to measure such selective differentials has not been taken as compelling evidence for the "neutralist school". On the other hand, occasional observations of strong selection for variants at a locus cannot be regarded as proof of the "selectionist" scheme, since it is virtually impossible to separate selection at a locus from the effect of selection of linked blocks of genes that may happen to contain a single selected locus (the "hitch-hiking" effect, OHTA and KIMURA 1971).

As a result of the difficulties of measuring selection directly, attempts to validate one theory or the other have depended upon observations of the statistical distribution of allelic frequencies in different populations of a species or of closely related species. These attempts make use of specific predictions of allelic frequency distributions implied by neutral theory, showing either that the observations of allelic frequencies in many species are in accord with, or are in disagreement with, the predictions. Examples of statistical relationships that have been used to test neutral as opposed to selective theories include: the expected heterozygosity in a population (KIMURA and CROW 1964), the relation between proportion of loci polymorphic and average heterozygosity (KIMURA and OHTA 1971), the relationship between the number of alleles and the variation in frequency from allele to allele (JOHNSON and FELDMAN 1973; EWENS 1972), the average amount of heterozygosity expected to be contributed by different allelic frequency classes (MARUYAMA 1972) and variation among loci in the degree of genetic divergence between populations and species (LEWONTIN and KRAKAUER 1973). The list is not exhaustive. Some of these predictions depend upon the actual values of population size, migration between populations and mutation rates, while others are parameter-free. All, however, use as their basic data the frequency distributions of alleles with populations. At present, the observed allelic frequency distributions, when substituted into the various predictions, lead to contradictory or ambiguous results. In some cases, as, for example, the relationship between proportion of loci polymorphic and the average heterozygosity, agreement with neutral theory is good (KIMURA and OHTA 1971). In other cases, for example the relationship between number of loci and evenness of allelic frequency distribution, the results are at variance with the prediction of neutral theory (JOHNSON and FELDMAN 1973). Taken all together, the data on genic polymorphisms detected by gel electrophoresis fit neither the selectionist nor neutralist hypothesis satisfactorily and are in serious contradiction with these hypotheses in one way or another (LEWONTIN 1974).

One possibility for the contradictory situation in which we find ourselves is that the data are simply wrong. It was already recognized by HUBBY and LEWONTIN that the electrophoretic method used by them would underestimate the actual amount of polymorphism because many gene substitutions would not differ from each other in charge. A more serious effect of an undetected lumping of alleles into allelic classes is the serious distortion of the frequency distribution of alleles, so that the various statistical distribution predicted theoretically cannot validly be compared with the observations. This difficulty has been noted, as, for example, by KING and OHTA (1975), who refer to the classes observed in electrophoresis

as "electro-morphs", calling attention to the fact that they are *phenotypes*, and may confound an unknown number of allelic forms that are indistinguishable. By and large, however, the electromorphic classes have been taken seriously as measuring allelic frequencies, in the absence of any clear evidence to the contrary.

There are two other generalities that also depend centrally on the assumption that electromorphic classes are genetically homogeneous. One is the remarkable similarity of allelic frequency distributions between widely separated populations of some species (PRAKASH, LEWONTIN and HUBBY 1969; PRAKASH *et al.* 1976) or even between closely related species (AYALA and POWELL 1972). This similarity has been offered as a strong evidence of the selective control of these polymorphisms. What would the meaning of such evidence be if it turned out that the "allele" present in equal frequency in two populations was really a different allele in each? Second, there is the remarkable lack of allelic differentiation between clearly distinct species even in their monomorphic genes. It appears that in Drosophila, at least, speciation has not been accompanied by large scale allelic substitutions at structural gene loci (see LEWONTIN 1974, chapter 4 for a review of the evidence). But this allelic similarity between species may be entirely illusory because the methods used fail to pick up the amino acid substitutions that have taken place (see BERNSTEIN, THROCKMORTON and HUBBY 1973 and the paper by COYNE in the present issue of Genetics.)

For all these reasons, it is critically important that we attempt to determine exhaustively the actual allelic variation present in populations by any and all methods that will detect gene substitutions. In fact, we regard this as the most important task of experimental population genetics at the present time.

General Methodological Considerations

We cannot state exhaustively all the possible ways of detecting amino acid substitutions in polypeptides. Our own laboratory program includes the following:

1. Varying conditions of electrophoresis
   a. pH variation
   b. different gel materials
   c. different gel concentrations. JOHNSON (1976) has shown this method to be successful in butterflies.
   d. gradient gels
   e. iso-electric focussing
2. Denaturation
   a. heat *in vitro* and *in vivo* (BERNSTEIN, THROCKMORTON and HUBBY 1973 and SINGH, HUBBY and THROCKMORTON 1975)
   b. urea
3. Dimerization potential of different alleles
   a. *in vitro* (HUBBY and NARISE 1967)
   b. *in vivo* (COBBS 1976; PRAKASH, personal communication)
4. Immunological methods including gel diffusion and micro-complement fixation

5. Enzyme kinetics
6. Peptide mapping and fingerprinting.

These methods cannot, in general, be carried out on single flies from nature as has been possible with standard gel-electrophoresis. Methods 4, 5 and 6 obviously require purified protein, and even methods 1, 2 and 3 demand repeated trials for each suspected allelic type. For that reason, isogenic lines must be established and maintained which restrict experimental organisms to those that have breeding systems and/or genetic markers that will allow isogenizations to be carried out. Drosophila is obviously ideal.

A second, and equally important, requirement is the necessity of carrying out crosses to prove that observed differences do indeed segregate as alleles at a locus. Genetic tests have always been essential in studies of genic polymorphism, but early work soon established that electrophoretic differences observed were indeed allelic differences at single Mendelian loci. As more subtle methods of detection are used, however, especially methods 1, 2, 3 and 5, the possibilities for effects due to interaction between the protein studied and other molecules become very great. One cannot assume that enzyme activity or heat sensitivity or dimerization potential differences between isogenic lines result from amino acid substitutions in the enzyme being investigated. They may easily be post-translational modifications. Especially since isogenic lines must be used, tests of genetic hypothess are essential.

Ideally, the whole gamut of methods should be applied to the same loci so that we can build up a general picture of how much added information can be gathered by each method. An exhaustive study of a half dozen enzymes might easily show that peptide fingerprinting is unnecessary since virtually every amino acid substitution has already been detected by, say, micro-complement fixation. This may be impractical in some cases. Thus our study of xanthine dehydrogenase cannot include fingerprinting since Drosophila Xdh has a subunit molecular weight of 140,000.

Finally, the loci chosen should be representative in several respects. They ought to include loci previously thought to be monomorphic as well as polymorphic. An important question is whether there really is a major class of monomorphic loci, or whether those presently classed as monomorphic have amino acid substitutions that are, by chance, undetected by the usual method of electrophoresis. Second, except for method 3, both monomeric and dimeric enzymes should be included. Some theories of selective polymorphism make quite different predictions for these two classes, and the necessity to form polymers puts restrictions on possible amino acid substitutions. Third, a range of molecular weights must be included. If the number of polymorphic amino acid substitutions turns out to be very high, it will be very important to know whether this corresponds to a large proportion of all sites, and there is some evidence already that heterozygosity may be related to molecular size (R. KOEHN, personal communication), although this is disputed (H. HARRIS, personal communication). The results we report here on Xdh are for a very large molecule and may not apply to smaller enzymes.

In this paper we report the results of the first step in the program outlined above. We have made 146 lines isogenic for chromosome *II* from 12 different populations of *Drosophila pseudoobscura*. The populations, broadly covering the geographic distribution of the species, are: Santa Cruz Island, California (SZ); Saratoga Spring, California (SS); Corn Spring, California (CN); Strawberry Canyon, Berkeley, California (SC); Wild Rose, California (WR); Charleston Mountains, Nevada (CH); Cerbats Mountain, Nevada (CE); Mesa Verde, Colorado (MV); Hardin Ranch, Colorado (HR); Austin, Texas (AU); Guatemala City (GU) and Bogotá, Columbia (BO). These lines are being analyzed for their allelic compositions at the xanthine dehydrogenase locus and we report here the results of varying the conditions of electrophoresis (methods 1a and 1c) and *in vitro* heat sensitivity (method 2a). A similar study of three populations of the sibling species, *D. persimilis* is reported by J. COYNE (1976) in the accompanying paper.

MATERIALS AND METHODS

Electrophoresis on acrylamide slab gels was carried out by the standard method as described in PRAKASH, LEWONTIN and HUBBY (1969), and SINGH, HUBBY and LEWONTIN (1974), except that both 5% and 7% gels were used and, for each concentration, two different buffer pH's (8.9 and 7.1) were employed. In general, pooled adults of the same sex were ground together, except in backcross tests where, of course, single flies must be scored. Times of electrophoresis were adjusted for optimum separation at each pH and gel concentration. At pH 7.1 in 7% gels, runs were generally 7 hours as compared to the usual 4 hours at pH 8.9 in 5% gels. All gels were run at about 75 ma with voltages being adjusted during runs to maintain this current. When differences in mobility were detected between two lines in a run, replicates were made alternating lines between adjacent pockets on the slab to be sure of repeatability.

Staining procedures were as described in PRAKASH, LEWONTIN and HUBBY (1969) and SINGH, HUBBY and LEWONTIN (1974). For heat denaturation tests, staining was carried out for 16 hours.

Heat denaturation was carried out in serological baths filled with mineral oil at 66° ± .1°. Six flies were ground together, the tube placed in the bath, and aliquots drawn at 0, 5, 10 and 15 minutes, when they were loaded into the pockets of a chilled gel. The gels were 5% and the buffer at pH 8.9 for these tests. After staining for the prescribed period, gels were scanned in an EC gel densitometer. We tested several methods of measuring the area under the absorption curves including electronic integration and planimetry, but found that at pH 8.9 in 5% gels, bands were so well defined that maximum peak height was as reliable as any, and was less sensitive to the arbitrary background level of absorption than was area.

A dilution test was made to determine whether stain density fell linearly with enzyme concentration. There was no indication of nonlinearity over a 10-fold dilution. This is not true for all enzyme stains in gels and must be tested in each new case.

All lines were raised on Carpenter's medium at 18°.

RESULTS

It immediately became clear that altering the pH and gel concentration would reveal reliable differences between alleles that were formerly regarded as identical or as marginally and unreliably different. Indeed, as the results will show, the standard conditions of pH 8.9 and 5% gels are the *poorest* conditions for allelic discrimination among those we tried. This fact, for gel concentration, had already

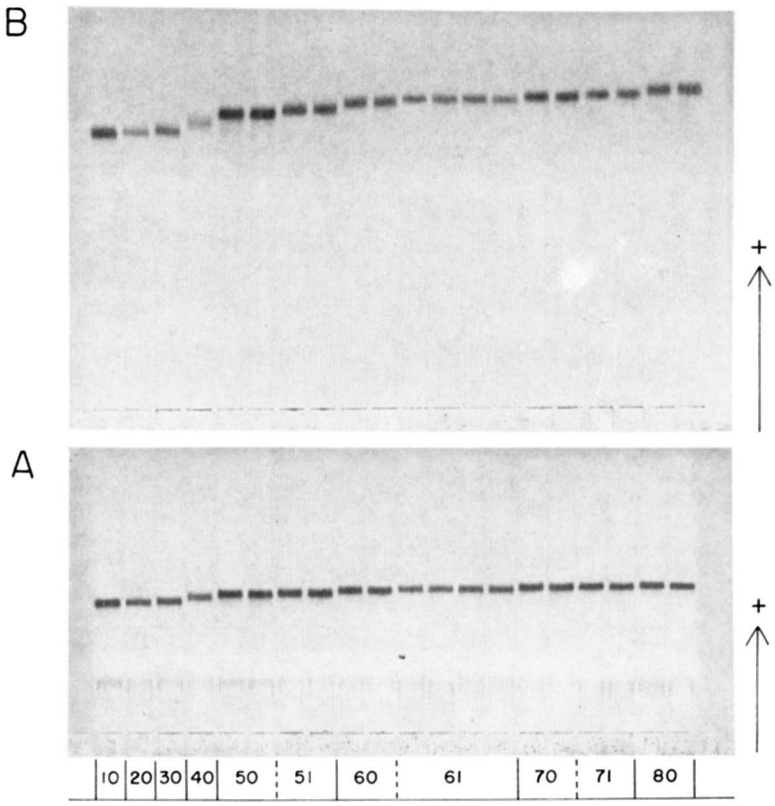

FIGURE 1.—Two gels showing the effect of varying gel concentration on electrophoretic mobility. In this Figure, there are 11 electrophoretic alleles of which only 8 can be reliably detected at 5% gel and pH 8.9 (A). The members of the 3 allelic pairs, 50 and 51, 60 and 61, 70 and 71, are indistinguishable in their mobilities at 5% gel but become different at 7% gel (B).

been noted by JOHNSON (1976) who, in his comparison of butterfly species over a range of gel concentrations, found many mobility curves to cross in the region of 5% acrylamide concentration.

Figure 1 shows the effect of changing gel concentration on a selected sample of lines for illustrative purposes. At 5%, there are 8 different "alleles", designated *1*–, *2*–, . . . *8*–, although even on this gel, classes *1*– and *2*– cannot be seen as different, class *3*– is barely discernible as different from class *2*– and class *8*– is minimimally different from class *7*–. These differences can, however, be reliably detected on replicate gels. A change to a 7% gel concentration (Figure 1B) considerably expands the previous differences, but also reveals 3 new classes within alleles 5–, 6– and 7–, designated *50,51*; *60,61* and *70,71*. In the light of the knowledge this second gel provides, one can go back to the first and see very small differences which would have been passed over before. But this is after the fact.

The possibility of detecting at 7% the difference between homozygotes and

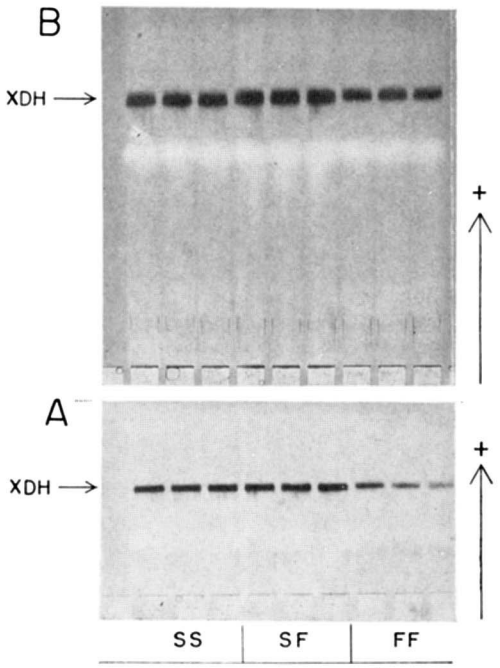

FIGURE 2.—Two gels showing the effect of varying gel concentration on electrophoretic mobilities of two parents (SS and FF) and their F_1's (SF). A = 5% gel, pH 8.9; B = 7% gel, pH 8.9.

heterozygotes for alleles that are indistinguishable on 5% gels is shown in Figure 2. The two homozygotes SS and FF and their heterozygote, SF, are of apparently identical mobility at 5%, but can be differentiated at 7%. On the 7% gel, the slow homozygote lines up with the trailing edge of the heterozygote, while the fast homozygote lines up with the leading edge. Because of the small mobility difference, we still do not see three clear bands in the heterozygote representing the two homodimers and the heterodimer.

The effect of varying pH's on the electrophoretic mobility is shown in Figure 3. The alleles S_1, S_2, and S_3 have the same electrophoretic mobility at pH 8.9 but become different at pH 7.1. The heterozygotes are in between the two respective homozygotes. Similarly, alleles S_4 and S_5 have identical mobility at pH 8.9 but become different at pH 7.1. The differences between S_4 and S_5 is the largest we have observed between any two alleles having identical mobilities at pH 8.9, but different at pH 7.1. Alleles S_1, S_2 and S_3 are examples of smallest differences observed. It should not be supposed, however, that pH 7.1 always exaggerates the mobility differences as in Figure 3; in some cases relative mobilities are reversed or reduced.

Survey of the lines by electrophoresis: The 146 isogenic lines were surveyed in the following manner. The lines were first characterized under the standard conditions of 5% gel, pH 8.9 (criterion 1). After all the lines had been grouped

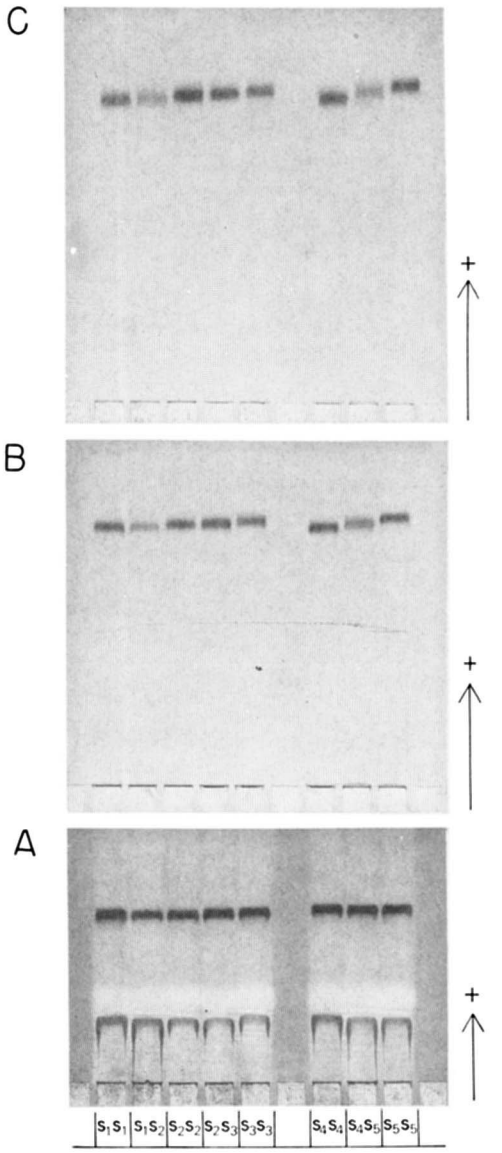

FIGURE 3.—Three gels showing the effect of varying pH on electrophoretic mobility of parents (S_1S_1, S_2S_2, S_3S_3, S_4S_4, and S_5S_5) and their F'$_1$s (S_1S_2, S_2S_3 and S_4S_5). $A = 5\%$ gel, pH 7.1 (7 hours); $B = 5\%$ gel, pH 7.1 (5 hours); $C = 5\%$ gel, pH 8.9 (2.5 hours). The electrophoresis was started at 300 volts and about 100 ma, with periodical adjustment of ma to about 100.

into electrophoretic classes by this criterion, the lines within each of these classes were subsequently examined at the next criterion, broken down into new classes, which were in turn examined under the third criterion, and so on. Thus, the original lines were grouped by a hierarchical procedure, each criterion successively breaking down previously homogeneous classes. The order of the criteria

were: 5% gel, pH 8.9 (criterion 1); 7% gel, pH 8.9 (criterion 2); 5% gel, pH 7.1 (criterion 3); 7% gel, pH 7.1 (criterion 4).

With this sequential process, we have adopted a new method of allelic designation, which is sufficiently flexible to allow an arbitrary number of new criteria to be added in the future. Each allele is identified by a string of digits, each digit position in order representing a successive criterion applied *within* the previous criterion.

The numerical value at a digit position is the allelic designation for that criterion and for electrophoresis the numbers represent increasing electrophoretic mobility. Thus the allele *6310* had relative mobility order *6* under criterion *1* and within all alleles with that mobility under criterion 1, it had relative mobility order *3* under criterion 2, and within all those alleles it had relative mobility order *1* under criterion 3 and so on. We have chosen this ordering procedure rather than the more usual method of assigning relative measured mobilities because most alleles at the *Xdh* locus described here are so close that their relative mobility differences are too small to be of use in identification, especially between laboratories. Moreover, when we change pH from 8.9 to 7.1, some alleles reverse their relative mobilities.

Table 1 is a summary over all populations, while Table 2 gives the results for the four electrophoretic criteria for each population. The results are remarkable. Using criterion 1, the standard condition of gel electrophoresis, eight allelic classes appear, with 103/146 = 71% falling in the main class. These classes correspond to the "alleles" previous reported (PRAKASH, LEWONTIN and HUBBY 1969), as follows: *1 = 0.90*, *2 = 0.92*, *5 = 0.99* and *6 = 1.00*. Classes *3* and *4* do not correspond to any previously reported; they are faster than 0.92 but slower than 0.97. Similarly, classes *7* and *8* are faster than *1.00* but slower than the allele *1.02* found in *Drosophila persimilis*. When criterion 2 was applied, the 19 lines in class 5 broke up into two equal classes and the 103 lines in the main class now produced 4 distinguishable classes. The original 8 allelic classes are now increased to 13 and the most frequent class now accounts for 88/146 = 60% of alleles. With the addition of criterion 3, a dramatic increase in allele number occurs, adding 11 new classes for a total of 24. The most frequent class, *631*, now represents only 54% of all lines. Finally, with the addition of the fourth criterion, another three classes appear, making a total of 27 alleles detectable by the four electrophoretic criteria. The distribution of allele frequencies is extremely J-shaped. A single allelic class, *6311*, is represented by 68/146 = .466 of the lines. The next most frequent allele, *6310*, has a frequency of .075, followed by *5010* at a frequency of .055, and then 24 other alleles with frequencies between .007 and .04.

When we turn to the detailed allelic structure of different populations, as given in Table 2, there are some regularities and some surprises. First, each population sample has a very large number of allelic classes given the very small number of lines examined from each population. Thus, out of 13 lines examined in SZ, there were 9 different alleles, of which 3 were unique to the SZ sample, In MV, out of 13 lines tested, there were 8 different alleles, 3 of which were

TABLE 1

Allelic classes distinguished by successive criteria and the number of isogenic lines falling in each class

Criteria used in sequence*				
(1)	(1+2)	(1+2+3)	(1+2+3+4)	Allelic code
1	1	1	1	*1000*
1	1	1	1	*2000*
5	5	5	5	*3000*
4	4	3	3	*4000*
		1	1	*4010*
19	9	1	1	*5000*
		8	8	*5010*
	10	1	1	*5100*
		7	2	*5110*
			5	*5111*
		2	2	*5120*
103	4	1	1	*6000*
		3	3	*6010*
	3	3	3	*6100*
	8	6	6	*6200*
		2	2	*6210*
	88	6	6	*6300*
		79	11	*6310*
			68	*6311*
		3	3	*6320*
7	6	1	1	*7000*
		1	1	*7010*
		4	1	*7020*
			3	*7021*
	1	1	1	*7100*
6	6	3	3	*8000*
		3	3	*8010*
No. of total alleles detected:				
8	13	24	27	
No. of new alleles added:				
–	5	11	3	

* Criterion 1 = 5% gel, pH 8.9; No. alleles detected independently = 8.
Criterion 2 = 7% gel, pH 8.9; No. alleles detected independently = 13.
Criterion 3 = 5% gel, pH 7.1; No. alleles detected independently = 17.
Criterion 4 = 7% gel, pH 7.1; No. alleles detected independently = ?.

unique to that population. In AU there were 4 alleles in only 6 lines and so on. The *least* variable population samples were SS and CE, each of which had 4 alleles out of 17 lines tested. With such small numbers of lines in each population, too much cannot be made of the unique alleles with the exception of a few cases. In the MV population, the unique class *6010* appears in frequency 3/13, while

TABLE 2

Number of lines in each population falling into each allelic class based on criteria 1–4

Allele	SZ	SS	CN	SC	WR	CH	CE	MV	HR	AU	GU	BO	Total lines
1000				1									1
2000								1					1
3000	1				1	2			1				5
4000		1					1	1					3
4010									1				1
5000							1						1
5010	1	2			1	1			1	2			8
5100	1												1
5110	2												2
5111	1			1	2			1					5
5120		1				1							2
6000									1				1
6010								3					3
6100				1								2	3
6200												6	6
6210												2	2
6300	1						2			2		1	6
6310				5		2		2	1	1			11
6311	4	13	7	4	8	6	13	3	7	1	2		68
6320			1			1		1					3
7000												1	1
7010	1												1
7020						1							1
7021				1	1	1							3
7100								1					1
8000	1		1		1								3
8010				1							2		3
N	13	17	9	14	14	15	17	13	12	6	4	12	146
Total alleles:	9	4	3	7	6	8	4	8	6	4	2	5	
Unique alleles:	3	0	0	1	0	1	1	3	2	0	0	3	
Heterozygosity:	.84	.39	.37	.77	.63	.78	.39	.84	.62	.72	.50	.68	
	$\overline{H} = .628$												

it is completely absent in all the remaining 133 lines tested. The most important case is the BO (Bogotá) population which had previously been reported by PRAKASH, LEWONTIN and HUBBY (1969) to be monomorphic for the allele most common in the rest of the species distribution (class *6*). Instead, Bogotá is highly polymorphic with 5 alleles out of 12 lines. Moreover, 9 out of the 12 lines carry alleles that are *unique to Bogotá*, and a further 2 lines have an allele that is found only once in the rest of the population. Thus Bogotá is virtually distinct genetically at this locus.

Some alleles are in repeated low frequency in many populations, such as *5010*, which is present in one or two lines from six populations, and *6310* which is in intermediate frequency in 5 populations. The most common allele in the sample, *6311*, is generally the most common allele in all populations, but not invariably. It is at intermediate frequency in SC and MV, rare in AU and totally absent in in Bogotá which, as we have already pointed out, has its own set of alleles.

The estimated heterozygosity at the *Xdh* locus, based on the four electrophoretic criteria, is shown in the last line of Table 2. These estimates are made by assuming that the population from which the lines were sampled are random mating units and that the lines represent random samples of haploid genomes from the populations. The individual values of heterozygosity cannot be taken too seriously since each is based on so few lines, but the weighted average $\bar{H} = .628$ is much more accurate, being based on 146 lines. It is impossible to say whether the variations in estimated heterozygosity represent real differences or not.

Heat sensitivity: The fifth criterion used to separate lines was heat sensitivity of the total *Xdh* activity on the gels. Before electrophoresis, whole fly extracts were screened for 0, 5, 10 and 15 minutes at 66° and the gel bands after staining were measured with a densitometer. In general, no further changes in activity occurred after 10 minutes of heat treatment, so the 15-minute figures are omitted from our results. There was a striking effect of age of flies on staining intensities. Young flies were much more heat sensitive than older ones and, in addition, had less activity even in controls. We do not understand the phenomenon of greater heat sensitivity of younger flies, but have used only flies 7 days old or older to produce more consistent results. It is a peculiarity of *Xdh* since it does not occur, for example, in octanol dehydrogenase in *D. pseudoobscura* in our experiments.

In our hands, heat treatment of *Xdh* does not give consistent results for all strains, so we have been forced to be quite conservative in our use of the data. Some lines are absolutely consistent and we are able to make out unambiguous phenotypic classes using these. Other lines, however, vary enough between replicates so that we are not certain to which of two classes to assign them. J. L. HUBBY (personal communication) finds highly repeatable results with *Xdh* in *Drosophila virilis* using the same technique and temperature as we report here. Whether the difference lies in the species or the investigators is not clear. Table 3 shows a typical experimental result. Four lines were tested on the same gel, with two replicate pockets for each treatment. The entire experiment was then

TABLE 3

Activities of enzyme from different isogenic lines, after heating for 5 and 10 minutes at 66°, expressed as proportion of control activity. Two replicates, a *and* b, *are given in each experiment*

Line		Experiment 1 5 min	Experiment 1 10 min	Experiment 2 5 min	Experiment 2 10 min	Experiment 3 5 min	Experiment 3 10 min	Mean 5 min	Mean 10 min
WR-B-50	*a*	.570	.502	.480	.377	.574	.502	.552	.469
	b	.559	.532	.566	.421	.566	.482		
SZ-B-39	*a*	.348	.178	.323	.181	.316	.161	.328	.177
	b	.334	.181	.338	.182	.307	.175		
CH 55	*a*	.459	.415	.423	.254	.357	.238	.411	.295
	b	.464	.394	.425	.277	.335	.190		
SS 23	*a*	.563	.474	.575	.515	.456	.451	.527	.493
	b	.553	.471	.564	.544	.453	.505		

repeated twice more. The values in the table are the ratios of activity of each trial to the untreated control of that line on the same gel. There are three clearly distinguishable heat-sensitivity classes with WR-B-50 and SS-23 the least sensitive, CH-55 the next, and SZ-B-39 the most sensitive. On the basis of single experiments, we might be led to distinguish WR-B-50 and SS-23. So, for example, in experiment 2, WR-B-50 seems clearly more sensitive at 10 minutes than is SS-23, but in experiment 3, WR-B-50 is clearly *less* sensitive at both times. Obviously there is no consistency over experiments for these two lines. The four lines belong to three different electrophoretic classes, but two that are identical electrophoretically, CH-55 and SS-23 (*5120*) have now been distinguished by the heat-sensitivity criterion and thus are designated *51200* and *51201* respectively.

Lack of consistency from experiment to experiment applies to lines of intermediate sensitivity, while lines of extreme sensitivity or stability to heat are consistent. Thus, line CH-51 retains 85–90% of its activity at 5 minutes and 75–80% at 10 minutes in replicated experiments, while line CE 45 has only 10% activity after 5 minutes and no detectable activity after 10 minutes. Both of these belong to electrophoretic class *6311*, the main class, but have been designated *63114* and *63110* on the basis of their heat sensitivity.

Because some lines of intermediate sensitivity are inconsistent from experiment to experiment, we are unable to assign each line to an allelic class and to give the complete allelic distribution for the 146 lines. We are, however, able to distinguish a minimum number of classes by criterion 5 within each of the electrophoretic classes, based on those cases in which there are unambiguous differences in replicated experiments. The number of heat sensitivity classes established within each electrophoretic class is shown in Table 4. Counting all five criteria, then, we have detected a minimum of 37 distinguishable classes at the xanthine dehydrogenase locus in 146 genomes sampled from nature.

TABLE 4

Minimum number of heat-sensitivity alleles within each electrophoretic class

Electrophoretic class	No. of heat classes	Electrophoretic class	No. of heat classes
1000	1	*6200*	2
2000	1	*6210*	1
3000	1	*6300*	2
4000	1	*6310*	2
4010	1	*6311*	5
5000	1	*6320*	2
5010	1	*7010*	1
5100	1	*7010*	1
5110	1	*7020*	1
5111	2	*7021*	1
5120	2	*7100*	1
6000	1	*8000*	1
6010	1	*8010*	1
6100	1	Total	37

Genetic analysis: The use of isogenic lines raises the possibility that the mobility and heat-sensitivity differences do not reside at the *Xdh* locus, but are the result of other loci that modify the mobility of the enzyme, or its heat sensitivity. It was already shown by HUBBY and LEWONTIN (1966) and PRAKASH, LEWONTIN and HUBBY (1969) that mobility differences detected by criterion 1 segregate as single alleles at a locus. We have chosen representative lines of the classes appearing under the new criteria and made F_1's and backcrosses. For example, the cross WR-B-52 (70000) × BO-8 (70210) was backcrossed to both parents and single backcross progeny assayed under criteria 3 and 4, by which these two lines differ. The backcross progeny showed that expected segregations in each backcross. Of course, such tests do not prove that all substitutions are at the *Xdh* structural locus and not at some other closely linked locus. However, GELBART, MCCARRON and CHOVNICK (1976) have shown by fine-structure mapping that all electrophoretic variants for *Xdh* in *D. melanogaster* map within the limits of the structural gene, and there is no reason to suppose the situation would be different for *D. pseudoobscura*.

Given that the electrophoretic variants are allelic, we have used a simple test for the allelism of the heat-sensitivity differences. Using a standard heat-sensitive allele of a slow mobility from strain MV-81 (20000), crosses were made to strains of faster mobility with different heat sensitivities. F_1 individuals, when heat treated, showed three bands corresponding to the standard allele homodimer, the heterodimer and the homodimer of the allele being tested. In every case the heat sensitivity accompanied the parental mobility (see Figure 4). That is, if the standard MV-81 was crossed with a heat-insensitive strain, for example, then in F_1 individuals the homodimer with the mobility of the tested strain was heat insensitive, while the homodimer of the standard mobility retained its characteristic heat sensitivity. Thus, heat sensitivity must either be the result of altera-

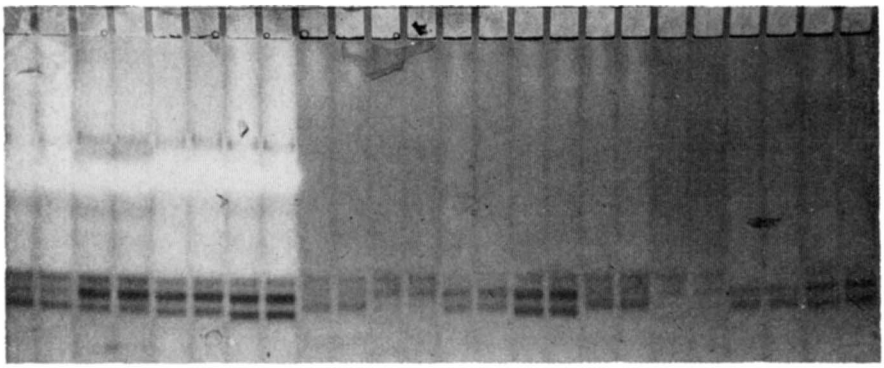

FIGURE 4.—Heat treatment of heterozygotes between the standard slow migrating, heat-sensitive allele (*20000*), stain MV-81, with several fast-moving allele of varying heat stability. Pockets 1–8, controls; 9–16, 5 minutes; 17–24, 10 minutes.

tion at the structural gene locus, or else a a linked *cis*-acting allele-specific modifier. It is important to note that lines with ambiguous heat sensitivity in homozygous tests also showed this ambiguity as homodimers in the heterozygote tests. It would appear that the variability of heat sensitivity of these lines is a property of the protein itself rather than of some general metabolic state of the isogenic lines.

In making these crosses, it became evident that there was a difference in activity among tested alleles even in non-heat-treated controls. In general, the homodimer of the standard allele (20000) was less active than those being tested against it. When the relative activity in controls was compared with heat sensitivity of alleles, a negative relationship was found. This is shown in Table 5, which compares the activity of homodimers, relative to the standard tester allele, of a number of lines of the group *6311* belonging to different heat sensitivity allele classes. Several experiments are averaged in each figure. We have no explanation for this inverse relationship between control activity and heat sensitivity.

TABLE 5

Comparison of the untreated homodimer activity of several isogenic lines with their heat sensitivity. Activities given as proportion of standard strain MV-81

Line	Allelic designation	Control activity
CE 45	*63110*	.889
AU 10	*63111*	.794
CE 36A	*63112*	.824
MV 66	*63112*	.825
HR-B 1	*63112*	.814
MV 103	*63112*	.684
SC-H 3	*63113*	.563
SC-H 5	*63113*	.579
SC-H 11	*63113*	.506
CH 51	*63114*	.585

Finally, we must caution against the indiscriminate use of heat stability as a means of discriminating alleles at the *Xdh* locus, or for any protein that does not give absolutely reproducible results. Single flies cannot be used either in homozygous or heterozygous state, and no difference can be counted on as real, unless several independent replications on separate gels have been made.

Is Xanthine dehydrogenase typical?

We have found 37 alleles of *Xdh* among 146 genomes from nature. In the accompanying paper, COYNE (1976) finds 23 alleles among only 60 genomes sampled in *D. persimilis*, using only the four electrophoretic criteria. Moreover, only 3 of the alleles in *D. persimilis* are indistinguishable from those in *D. pseudoobscura*. Thus, in the two sibling species together, there are 57 alleles segregating in natural populations, based on a sample of only 206 genomes. If this situation were typical of structural gene loci, the amount of genetic heterogeneity in natural populations would be vastly greater than has yet been supposed. There is reason to think, however, that *Xdh* may be exceptionally variable. R. K. KOEHN and W. F. EANES (personal communication) have found a positive correlation between molecular weight and amount of heterozygosity in Drosophila species, although H. HARRIS (personal communication) has found no such relationship in man. *Xdh* in Drosophila is an unusually large molecule with a subunit molecular weight of 140,000, corresponding to about 1300 amino acids (CANDIDO, BAILLIE and CHOVNICK 1974). A theory that polymorphism is the result of selectively neutral mutations predicts that the number of alleles would be proportional to the total mutation rate at a locus which, in turn, should be proportional to the number of codons. We already have some preliminary evidence that octanol dehydrogenase is not nearly so polymorphic when subject to the same methods as *Xdh* (COYNE, personal communication). On the other hand, the Esterase-5 locus already has 16 alleles known using criterion 1 only, and we have found 3 unambiguous heat-sensitivity alleles within the main electrophoretic class out of only 12 lines tested. Esterase-5 has a subunit molecular weight of only 52,000 (NARISE and HUBBY 1966). Finally, it must be remembered that there still remain the other criteria of allelic differentiation to be explored. Unfortunately, *Xdh* is too large to attempt the ultimate criterion, protein fingerprinting.

The meaning of the results

We began by pointing out that the statistical distribution of allelic frequencies in different populations of a species, and between species, has been the chief form of data on which theories of genetic variation have rested. Various tests of the neutralist-selectionist controversy have depended upon (1) the average heterozygosity, (2) the variation of heterozygosity between loci, (3) the relationship between the number of alleles and their frequency distribution, (4) differences and similarities between populations, and (5) species differences and similarities. What are the consequences of the new data for these issues?

The first five criteria in our general methodology have revealed 37 allelic forms at the *Xdh* locus where previously only 6 had been found. While most of these alleles are present only once in the sample, the increase in our estimate of genetic variation at this locus is very considerable, since only 146 genomes have been examined. In such a sample, even alleles present once cannot be relatively rare in nature. The average population heterozygosity at the *Xdh* locus in this sample was .439 when only the standard method of electrophoresis (criterion 1) was used, and this rose to .628 after all four electrophoretic criteria were applied. The estimated homozygosity of .378 arises chiefly from the single most common allele, *6311*, whose frequency is $68/146 = .466$, and which then contributes $(.466)^2 = .217$ homozygosity. We cannot estimate accurately how much further increase in heterozygosity has been contributed by criterion 5 because of ambiguous lines. We could, however, estimate the maximum heterozygosity that would be achieved with this criterion by making allelic assignments of all lines based on apparent consistencies in repeated experiments and pooling all populations into a single sample. When this is done, the heterozygosity rises to .73. Half of the remaining homozygosity arises from the single most common allele, *63111*, which has a frequency of $45/146 = .308$. This figure must not be taken too seriously, however, since some allelic assignments are ambiguous in the *6311* class.

A purely neutral theory of polymorphism predicts that the average heterozygosity in a population should be approximately

$$H = 1 - \frac{1}{4N\mu + 1} \tag{1}$$

where N is the effective population size and μ the mutation rate to new alleles on the assumption that a large number of allelic states are available from mutation. Taking the approximate heterozygosity of *Xdh* after 5 criteria as .73, this gives a value of $N\mu = .68$. As pointed out by LEWONTIN (1974), since μ is known only to an order of magnitude and N is not known at all, any product can be rationalized. If μ is of order 10^{-5}, then we need only assume that the typical population of *D. pseudoobscura* has an effective population size of about 50,000. More important, the shape of the function (1) is such that any value of H from .05 to .95 is compatible with only a small range of values of $N\mu$, between .01 and 10. The present range of average heterozygosities in different species is from .056 to .185 and this corresponds to an extremely narrow range of values of $N\mu$, between .015 and .057. This predicts, on the neutral theory, that all species have population sizes within a factor of 4 of each other, or just as unlikely, that mutation rates and population sizes have become mutually adjusted to each other in evolution so that one is proportional to the reciprocal of the other. This peculiarity would disappear if heterozygosities varied a great deal more among species or if the average heterozygosities were above .90 where $N\mu$ can vary over many orders of magnitude with little effect on H. Heterozygosity has a non-linear relationship to the number of alleles segregating. If a locus has a very low heterozygosity because nearly all genes are in a single allelic class, then the discovery of heterogeneity within that class will raise the estimate of heterozygosity by a large

factor. On the other hand, if heterozygosity is already, say, 20%, then newly discovered heterogeneity will not have a dramatic effect on *H*. For example, if a locus formerly thought to be monomorphic is discovered to have two distinguishable alleles in the frequencies of .75 and .25, the heterozygosity would rise from 0 to .375. If subsequently the most frequent class is found to be heterogeneous with two alleles in a 3 to 1 ratio, the total heterozygosity would only rise from .375 to .414. Thus it is unlikely that average heterozygosities over the whole genome will rise to levels where population sizes and mutation rates might take any unconstrained values, although *Xdh* is now near that range. On the other hand, if a great deal more variation were uncovered at some loci in some species, the variation in heterozygosities between species might increase and this would, in part, resolve the contradiction of neutral theory.

An important question is whether the major increase in genetic variation observed at the *Xdh* locus will be typical of all loci tested by extensive methods. One possibility is that the standard technique of electrophoresis detects about the same proportion of polymorphism at all loci. If that is the case, then monomorphic loci will remain pretty much monomorphic, although a few will turn out to have a small amount of polymorphism, and polymorphic loci will all show a proportional increase in their heterozygosity. In that case, there will simply be an increase in average polymorphis observed, and nothing important will have changed. On the other hand, "monomorphic" loci may prove to be quite polymorphic on finer examination so that the proportion of polymorphic structural gene loci will approach 100%, although heterozygosity per locus remains in an intermediate range. This is not to be expected under the neutral theory (see KIMURA and OHTA 1971 and LEWONTIN 1974, p. 210), and would strongly support the hypothesis that a reasonable fraction of polymorphisms are maintained by selection. A third possibility is that monomorphic loci will remain monomorphic, while loci already known to be polymorphic will all resemble *Xdh* in having a very high heterozygosity. This too would show a heterogeneity among loci with respect to selective forces, but it would not necessarily demonstrate balancing selection, since the monomorphic loci might tolerate no substitutions, while the polymorphic loci are more flexible to neutral substitutions. For all these reasons, it is critical to carry out the extensive program of search for variation among loci now considered monomorphic.

The third question to be considered is the observed frequency distribution of alleles. It would be difficult on any theory to explain 37 alleles segregating at a locus if all these alleles were present within a population. Because of the small samples, however, we have not observed more than 10 alleles (including criterion 5) in any population, based on sample sizes of between 4 and 17 genomes. Further sampling would undoubtedly reveal more alleles in each population. For example, out of 13 genomes, SZ has 10 alleles, 8 of which are present once, one present twice, and one present three times. Using the theory of allele sampling (LEWONTIN and PROUT 1956), we estimate that this population contains 20 effective alleles and a heterozygosity of .95. The actual distribution of allelic frequencies may also be used as a test of the neutral theory, employing EWENS'

(1972) theory of relationship between allele number and evenness of the frequency distribution. If x_i is the frequency of the i^{th} allele in a population, then the information measure

$$B = -\sum_i x_i \ln x_i$$

has an expectation under the neutral theory that is related to the number of alleles k. If there is heterotic selection, the distribution of allelic frequencies will be more even than under the neutral theory and B will be larger than expected. If one allele is favored by selection, the frequencies will be very uneven and B will be much smaller than expected for a given k. The test given by EWENS in a computer program form tests whether B is too large or too small by an F ratio, with degrees of freedom calculated from the data. This test has no power for the small numbers of lines in each of our populations separately. Testing the pooled lines of Table 1 gave an $F = .6603$ with 88 and 26 degrees of freedom in the numerator and denominator respectively, corresponding to a two-tailed probability of .20. Thus, the pooled sample is too uneven but not significantly so. Moreover, some of the unevenness comes from the pooling since, if different populations have the same main class (excluding Bogotá) but different rare alleles, the pooled distribution will have about the same B value as the average population, but a much larger k. We must conclude that there is no evidence of selection of any kind in the observed distribution of allelic frequencies.

The fourth question is the differentiation among populations. In general, the populations have the same most frequent allele, *63111*, and the same most frequent electrophoretic class, *6311*. Each population then has a sprinkling of alleles in other classes, but no strikingly frequent unique allele. The exception to this role is Bogotá, which is polymorphic for unique alleles. PRAKASH, LEWONTIN and HUBBY (1969) had previously reported that Bogotá was monomorphic for the most common allele in the rest of the species (our class *6*). On this basis, they postulated that Bogotá was a recent colonization from a smaller group of founders and thus was particularly striking in view of the finding by PRAKASH (1972) that Bogotá flies were partly reproductively isolated from the rest of the species. This story must be completely revised now. Bogotá is both polymorphic and unique, suggesting an ancient origin for this isolated population and agreeing with the observation of partial reproductive isolation. The similarity of all the rest of the populations, including Guatemala, and the uniqueness of the totally isolate Bogotá, are completely compatible with a neutral theory of this polymorphism, assuming that the North and Central American populations exchange a small number of migrants over the continuous part of the range of the species.

The observation of the unique alleles in Bogotá brings us finally to the question of differences between closely related species. A strong argument for balancing selection operating on enzyme polymorphisms is made by AYALA and POWELL (1972) on the basis of striking similarities in allelic frequencies of polymorphic loci in four closely related species of the *D. willistoni* group. PRAKASH (1969) had found a like similarity between the siblings *D. pseudoobscura* and *D. persimilis*. In the accompanying paper to the present one, however, COYNE (1976) finds

that the similarity at the *Xdh* locus is illusory and, on the basis of our first four criteria, *D. pseudoobscura* and *D.persimilis* are essentially non-overlapping in allelic composition. This means that the claimed similarity between species cannot be relied upon in the absence of a thorough search for all amino-acid substitutions. The consequences of this differentiation between species for the theory of speciation are discussed by COYNE. We restrict ourselves to the observation that attempts to differentiate between selective and neutral hypotheses of polymorphism on the basis of population and species similarities are premature until the correct facts are known.

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LITERATURE CITED

AYALA, F. J. and J. R. POWELL, 1972 Allozymes as diagnostic characters of sibling species of *Drosophila*. Proc. Nat. Acad. Sci. U.S. **69**: 1094–96.

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

CANDIDO, E. P. M., D. L. BAILLIE and A. CHOVNICK, 1974 A rapid purification of xanthine dehydrogenase for genetic studies. Genetics **77**: S9 (abstract).

COBBS, G., 1976 Polymorphism for dimerizing ability at the Esterase-5 locus in *Drosophila pseudoobscura*. Genetics **82**: 53–62.

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

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

GELBART, W., M. MCCARRON and A. CHOVNICK, 1976 Extension of the limits of the *Xdh* structural element in *Drosophila melanogaster*. Genetics **84**: . . .–. . . .

HARRIS, H., 1966 Enzyme polymorphisms in man. Proc. Roy. Soc. Ser. B. **164**: 298–310.

HUBBY, J. L. and R. C. LEWONTIN, 1966 A molecular approach to the study of genic heterozygosity in natural populations. I. The number of alleles at different loci in *Drosophila pseudoobscura*. Genetics **54**: 577–594.

HUBBY, J. L. and S. NARISE, 1967 Protein differences in Drosophila. III. Allelic differences and species differences in *in vitro* hybrid enzyme formation. Genetics **57**: 291–300.

JOHNSON, G. B., 1976 Characterization of electrophoretically cryptic variation in the Alpine butterfly, *Colias meadii*. (Submitted for publication.)

JOHNSON, G. B. and M. W. FELDMAN, 1973 On the hypothesis that polymorphic enzyme alleles are selectively neutral. I. The evenness of allele frequency distribution. Theor. Pop. Biol. **4**: 209–221.

KIMURA, M. and J. CROW, 1964 The number of alleles that can be maintained in a finite population. Genetics **49**: 725–738.

KIMURA, M. and T. OHTA, 1971 *Theoretical Aspects of Population Genetics*. Princeton University Press, Princeton, N.J.

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

LEWONTIN, R. C., 1974 *The Genetic Basis of Evolutionary Change*. Columbia University Press, New York.

LEWONTIN, R. C. and J. L. HUBBY, 1966 A molecular approach to the study of genic heterozygosity in natural populations. II. Amount of variation and degree of heterozygosity in natural population of *Drosophila pseudoobscura*. Genetics **54**: 595–609.

LEWONTIN, R. C. and J. KRAKAUER, 1973 Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. Genetics **74**: 175–195.

LEWONTIN, R. C. and T. PROUT, 1956 Estimation of the number of different classes in a population. Biometrics **12**: 211–223.

MARUYAMA, T., 1972 Some invariant properties of a geographically structured finite population—distribution of heterozygotes under irreversible mutation. Genet. Res. **20**: 141–149.

NARISE, S. and J. L. HUBBY, 1966 Purification of Esterase-5 from *Drosophila pseudoobscura*. Biochemical et Biophysica Acta **122**: 281.

OHTA, T. and M. KIMURA, 1971 Behavior of neutral mutants influenced by associated overdominant loci in finite populations. Genetics **69**: 247–260.

POWELL, J., 1975 Protein variation in natural populations of animals. Evol. Biol. **8**: 79–119.

PRAKASH, S., 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., 1969 Genic variation in a natural population of *Drosophila persimilis*. Proc. Nat. Acad. Sci. U.S. **62**: 778–784.

PRAKASH, S., R. C. LEWONTIN, D. W. CRUMPACKER and J. S. JONES, 1976 A molecular approach to the study of genic heterozygosity. VI. The remarkable genetic similarity of geographical populations of *D. pseudoobscura*. In preparation.

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

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

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

YAMAZAKI, T. and T. MARUYAMA. Evidence for the neutral hypothesis of protein polymorphism. Science **178**: 56–58.

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