EXAMINATION OF ALLELIC VARIATION AT THE HEXOKINASE LOCI OF *DROSOPHILA PSEUDOOBSCURA* AND *D. PERSIMILIS* BY DIFFERENT METHODS

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ABSTRACT

Recently a number of electrophoretic techniques have been applied to reveal the presence of additional genetic variation among the electrophoretic mobility classes of the highly polymorphic xanthine dehydrogenase (*XDH*) and esterase-5 (*est-5*) loci. We examined the hexokinase loci of *Drosophila pseudoobscura* and *D. persimilis* using a variety of techniques to determine whether further allelic variation could be revealed for these much less polymorphic loci and to analyze the nature of the known variation at the hexokinase-1 (*hex-1*) locus. The following studies were conducted: 135 strains of the two species from six localities were examined with buffer pH ranging from 5.5 to 10.0; 40 strains of *D. pseudoobscura* and 9 strains of *D. persimilis* from Mather were studied using starch gel concentrations ranging from 8.5 to 15.5% and were examined for differences in heat stability and reactivity to the thiol reagent *p*CMSA; strains were also tested for susceptibility to urea denaturation and differences in relative activities. Major findings of the work are: (1) No additional allelic variation could be detected at any of the hexokinase loci by applying these techniques. The finding of abundant hidden genetic variation in *XDH* and *est-5* does not extend to all enzyme loci. (2) Evidence from studies using *p*CMSA indicates that the *hex-1* alleles *0.6*, *0.8*, *1.0* and *1.2* of the two species form a series of unit charge steps. Since the *0.94* allele of *D. persimilis* has mobility intermediate between *0.8* and *1.0*, it is argued that routine electrophoretic techniques are sensitive to at least some conservative amino acid substitutions. (3) Strong correlations were found at the *hex-1* locus between low allelic frequency, reduced relative activity and reduced stability to heat and urea denaturation. Since the three sibling species, *D. pseudoobscura*, *D. persimilis* and *D. miranda*, all appear to share a common high frequency allele (*1.0*) at that locus, these findings are taken as evidence that the observed allelic frequencies are a result of directional selection and mutation, rather than any form of balancing selection.

CONSIDERABLE attention has been directed toward obtaining a more refined answer to the question of how much genetic variation exists in natural populations. Recently, by employing a large number of techniques coupled with electrophoresis, several studies have revealed a considerable amount of allelic variation that could not be unambiguously distinguished by using routine techniques of electrophoresis (usually 5% acrylamide gels at pH 9.0). For example, COBBS (1976), MCDOWELL and PRAKASH (1976) and COBBS and

PRAKASH (1977a) have expanded the number of alleles known for the esterase-5 (*est-5*) locus of *Drosophila pseudoobscura* from about 12 to over 20 simply by employing different gel concentrations and buffer systems. Other authors have found up to six times as many alleles of xanthine dehydrogenase (*XDH*) as were previously known in Drosophila species by examining thermostability (BERNSTEIN, THROCKMORTON and HUBBY 1973; SINGH, LEWONTIN and FELTON 1976) or activity differences relative to a common standard (PRAKASH 1977c), or by employing different gel concentrations and buffer systems (COYNE 1976; SINGH, LEWONTIN and FELTON 1976). Similarly, on the basis of thermostability, SINGH, HUBBY and THROCKMORTON (1975) have suggested that there may be 2.6 times as many alleles of octanol dehydrogenase-1 in the species of the *D. virilis* group as had been previously recognized. Of these well-studied loci, *est-5* and *XDH* were already known to be very polymorphic. It is of particular interest to know whether these findings extend to the less polymorphic enzymes. That is, can similar amounts of hidden variation be revealed among the less polymorphic loci by applying the same techniques?

Hexokinases (E.C. 2.7.1.1) were chosen for the study for two reasons. First, because of their role in phosphorylating glucose to glucose-6-phosphate, they may serve as regulatory enzymes in glycolysis. Thus their structure and activity might be regulated quite closely by natural selection. Second, a number of hexokinase isozymes that show differing amounts of electrophoretic variation occur in most organisms. Hexokinase-1 (*hex-1*, chromosome 3) is polymorphic in *D. pseudoobscura* and *D. persimilis* with heterozygosities of 0.077 and 0.155, respectively (PRAKASH 1977a). Nomenclature of the electromorphs of *hex-1* is taken from PRAKASH (1977a) and reflects mobility differences that are observed on agar gels. Hexokinase-2 (*hex-2*, *X* chromosome) is polymorphic in *D. pseudoobscura* (PRAKASH 1977b), but hexokinase-6 (*hex-6*) and hexokinase-7 (*hex-7*) are monomorphic in both species (PRAKASH 1977a). Since electrophoretic mobility variants at *hex-1* and *hex-2* do not affect the mobilities of the *hex-6* or *hex-7* enzyme bands, we assume that the latter represent an additional genetic locus or loci.

The purpose of this study was to apply those techniques known to reveal hidden allelic variation in other enzyme systems to the hexokinase loci of *D. pseudoobscura* and *D. persimilis*. This work is presented according to the following plan. The first part examines both species for developmental changes in the hexokinase isozymes and the effects of age on the relative activities of the different mobility alleles of *hex-1*. This study provides necessary information preliminary to activity and stability comparisons of the allozymes. Next, a range of buffer pH and gel concentrations were used during electrophoresis to search for heterogeneity within each mobility class. In the next part, *hex-1* is examined for heterogeneity of activities both within and between electromorph classes. All strains were then examined for differences in sensitivity to heat and urea denaturation. Then the strains were tested for differences in reaction with the thiol reagent, *p*-chloromercuriphenylsulfonic acid. This technique has only limited scope for detecting hidden variation, but lends some insight into the

nature of known allelic differences, as it gives evidence of the effect a unit charge change has on mobility. Finally, the molecular weights of hexokinase-1 and hexokinase-7 were estimated using the method of FERGUSON plots (FERGUSON 1964; COBBS 1976). This analysis is directed at the question of whether differences in heterozygosity among hexokinase isozymes or between hexokinase and other enzyme systems simply reflect molecular weight differences.

MATERIALS AND METHODS

The *hex-1* locus is located on the third chromosome of *D. pseudoobscura* and its sibling species (PRAKASH 1977b). *Hex-2* is *X* linked, but the other isozymes are apparently monomorphic and therefore are unmapped. The strains used in this study are either isogenic or at least inbred lines homokaryotypic for the third chromosome. Eight homokaryotypic isofemale lines of *D. persimilis* from Mather, California, were maintained by sib-mating for many generations before being examined. One strain was found to be segregating for $hex\text{-}1^{0.6}$ and $hex\text{-}1^{1.0}$. It was split into two strains isogenic for those alleles by extracting with a line homozygous for $hex\text{-}1^{0.8}$. Both Whitney and Mendocino chromosomal arrangements were included. Forty strains of *D. pseudoobscura* from Mather, California, isogenic for the third chromosome were studied. These strains included five chromosomal arrangements: Standard (8 lines), Arrowhead (6), Chiricahua (6), Pikes Peak (10), and Treeline (10). In addition, isogenic strains of *D. pseudoobscura* for the third chromosome were examined from the following localities: Pinon Flats, California (26 lines), Mexico (13), Ruidoso, N.M. (13), and Black Canyon, Colorado (12). Sixteen strains from Bogotá, Colombia, were studied.

General electrophoretic techniques: Electrophoresis was carried out on horizontal starch slab gels at 9.1 V/cm. The buffer systems employed are listed in Table 1. Ten μl of sample was layered on 6 × 9 mm rectangles of filter paper and electrophoresed for 8 to 24 hrs, depending on the buffer system and gel concentrations used. Migration of hexokinase-1 was usually greater than 5 cm and that of hexokinase-7 usually more than 10 cm. During extended runs, there is a marked tendency for the pH of the electrode buffer to change. Thus a system was devised allowing the buffer from both electrode chambers to drain continuously into a common container to be mixed and pumped back into the electrode baths. The cycled buffer pH was found to remain constant throughout the run. The bath buffer was cycled in all the work reported here.

All strains were compared repeatedly to one another with all the techniques. If a difference was observed among any strains, they were re-examined by repeated alternating pocket comparisons to a common tester strain.

The anodal slice of each gel was stained at room temperature in the dark for four hours in a solution of 50 ml 0.1 M tris HCl buffer (pH 7.5), 1.5 ml 0.1 M $MgCl_2$, 70 mg D-glucose, 20 mg ATP (Sigma grade), 10 mg NADP (Sigma grade), 10 mg NBT (grade III), 20 units G6PD (Type XI) and 3 drops of 2% PMS solution. These reagents were obtained from Sigma Chemical Company. Both anodal and cathodal slices were stained for gels run with gly-HCL, pH 5.5, since this pH is apparently very close to the isoelectric point for the proteins. Gels of

TABLE 1

Buffer systems

	Gel buffer	Bath buffer	pH
A	0.05 M Glycine-HCl	0.05 M Glycine-HCl	5.5
B	6 mM Tris, 3 mM Citric Acid	135 mM Tris, 45 mM Citric Acid	6.5
C	80 mM Tris, 12 mM Boric Acid, 1.3 mM EDTA	80 mM Tris, 12 mM Boric Acid, 1.3 mM EDTA	9.2
D	0.05 M Glycine-NaOH	0.05 M Glycine-NaOH	10.0

extreme pH were rinsed briefly in tris HCl buffer (pH 7.5) prior to staining. The hexokinases were also tested for fructokinase activity by staining a second slice of some gels by the method of JELNES (1971). All loci showed both glucokinase and fructokinase activity. In the work reported here, only glucokinase activity was routinely tested.

Studies of pH comparisons, activities, stabilities and the effects of thiol reagents on mobility were done using 10 to 12% Sigma starch. However, the cooking time of Sigma starch is strongly dependent on gel concentration. The molecular weight determinations and comparison of strains using different gel concentrations were carried out with electrostarch (Electrostarch Company) to make possible a single cooking procedure for all gel concentrations. The electrostarch suspension was heated to 92° while being constantly shaken. The suspension was then degassed and cooled to 85° before pouring the gel.

Developmental studies: The changing isozyme patterns of both species were studied to establish a standard, repeatable technique for all subsequent studies. The flies were raised at 25°. Ten stages of each species homozygous at *hex-1* were examined: third instar larvae, early pupae, late pupae (with wing bud showing through the cuticle), newly eclosed adults and flies aged one, two, three, four, eight or ten days. The adults were obtained by clearing actively eclosing vials and collecting the newly eclosed flies one hour later. The flies were then isolated and allowed to age. Six stages of adults of each species, heterozygous at *hex-1*, were examined to compare the development of activity of alleles at that locus. Electrophoresis was carried out using Sigma starch and buffer system C (Table 1). After staining, the bands from *hex-1* heterozygotes were scanned on a Beckman model R-112 densitometer at 520 nm and the relative areas under each curve, using a common baseline, were determined with a DuPont curve analyzer.

Electrophoretic conditions: Adults in this and all subsequent studies were aged for 7 to 10 days before electrophoresis. All strains were tested at least twice using 10% Sigma starch and all buffer systems in Table 1. All Mather strains of both species were tested at four concentrations of electrostarch gels (8.5, 11, 13, 15.5%) using buffer system C (Table 1). This series essentially spans the usable range of electrostarch concentrations.

Activity comparisons at the hex-1 *locus*: Crosses were made among the Mather strains from both species to obtain heterozygotes for the different mobility alleles. Two isogenic strains of *D. pseudoobscura* bearing the low frequency *1.2* allele were crossed to nine isogenic strains of this species carrying the *1.0* allele. The Mather strains of *D. persimilis* included a single isogenic strain with the *0.6* mobility allele, two isogenic strains of the *0.8* mobility class and six of the *1.0* mobility class. All possible crosses were made among these strains. The adults were collected and aged for 7 to 10 days. Electrophoresis was performed on 10% Sigma starch in buffer system C for 16 hr. The gels were then stained, scanned and analyzed as described for the developmental comparisons, above. The F_2 generation for all crosses was obtained, and the F_2 heterozygotes were scanned and analyzed for relative activity.

Stability comparisons at the hex-1 *locus*: Heat stability tests were first run on heterozygotes generated for the activity comparisons, so that the rare allele could serve as an internal control for comparisons among the *1.0* alleles. However, the low frequency alleles of both species proved less stable than any of the *1.0* alleles and were therefore not usable. Homozygotes of all Mather strains were examined and the band of the apparently monomorphic *hex-7* locus was used as the control. The flies were raised at 25° and aged for 7 days before testing. Four females were ground and centrifuged in 80 μl of distilled water, and 15 μl of supernatant was transferred to each of four 0.1 ml vials. One vial was left on ice while the other three were placed in a water bath at 50 ± 0.5°. Vials were returned to the ice bath after 2, 5 and 10 minutes of treatment, respectively. The rare alleles were tested at both 47° and 50°. The samples were then electrophoresed on 10% Sigma starch in buffer system C (Table 1) and scanned on the Beckman densitometer. The resulting curves were then examined for differences in the way the activity of *hex-1* relative to *hex-7* changed with time of treatment.

Urea denaturation was examined by grinding aged females heterozygous for *hex-1* mobility alleles individually in 20 μl of solution containing 0, 2, 3 or 4 M urea. The samples were then electrophoresed on 10% Sigma starch (buffer system C), stained and examined visually for differences in sensitivity. In each case, the alternative allele of *hex-1* was used for comparison,

since too many uncontrolled variables exist for valid comparisons to be made between different samples.

Reaction of hexokinases with a thiol reagent: The reagent, *p*-chloromercuriphenylsulfonic acid (*p*CMSA), is known to react with free -SH groups, adding a single negative charge to each (SINHA and HOPKINSON 1969). All Mather strains of both species were tested for reaction with this compound. Each strain was tested by placing single females into vials containing 20 μl of the following test solutions: distilled water (control), 10^{-4} M *p*CMSA or 3×10^{-4} M *p*CMSA. The flies were then ground, centrifuged and electrophoresed on 12% Sigma starch (buffer system C). After staining, the gels were examined for differences in activity and mobility.

Molecular weight determinations: The technique described by COBBS (1976) was applied to obtain an estimate of the molecular weight of hexokinase-1 and hexokinase-7. One strain of each *hex-1* mobility class of each species was examined, using four complete series of gel concentrations with two replicates of each strain per series. Eight electrostarch gel concentrations (8.5 to 15.5%, in 1% steps) were used with buffer system C. Each fly was ground in a 10^{-4} M solution of one of the following proteins of known molecular weight: ovalbumin (OVA, MW = 43,000), bovine hemoglobin (68,000), bovine serum albumin (BSA, 134,000) or horse spleen ferritin (450,000). After electrophoresis, the top slice was stained for general protein with 0.01% Coomassie blue in a solution of water, methanol and acetic acid in a ratio of 5:5:1. The bottom slice was stained for hexokinase activity. The mobility of each protein (including hexokinase-1) was measured relative to hexokinase-7. Measurements were corrected for differences in shrinkage of the two slices by comparing their total lengths.

RESULTS

Developmental patterns: Since it is well known that the isozyme patterns of hexokinases change during development in Drosophila (MADHAVEN, FOX and URSPRUNG 1972), a number of problems may arise in their study. If different ages of adults are sampled in a haphazard survey, changing isozyme patterns or epigenetic alterations in the enzyme may be misinterpreted as polymorphism. Activities of any isozyme will also be sensitive to such changes, and even heat denaturation results appear to be age-dependent (SINGH, LEWONTIN and FELTON 1976). Therefore a preliminary study of these changes was undertaken to allow minimization of these developmental variables in all subsequent work.

Figure 1 chronicles the changes which occur in the two species studied. Labelling of the isozymes along the margin of the figure identifies the bands for the present study. All loci except *hex-1* and *hex-2* are apparently monomorphic, so their genetic basis is not known. The pre-adult stages do not show any activity at either the *hex-1* or *hex-7* loci and attain full activity only after the third day from eclosion. The most prominent band in the larval and pupal stages is hexokinase-2, which often lingers on into the adult stages. The intermediate bands, hexokinase-3 to hexokinase-6, were not consistently scorable in the systems used in this study. None appears to be species-specific. Thus most of the work presented in this paper is restricted to hexokinase-1 and hexokinase-7, assayed in adults aged four or more days after eclosion.

Table 2 gives the developmental changes in relative activity in heterozygotes at the *hex-1* locus in *D. persimilis*. The rare allele (*0.8*) appears a little slower in developing, having only 50% of the activity of the *1.0* allele during the first two days. Its activity stabilizes at a higher relative level 2.5 days after eclosion. A similar pattern of development of activity of the *1.2* allele of *D. pseudoobscura*

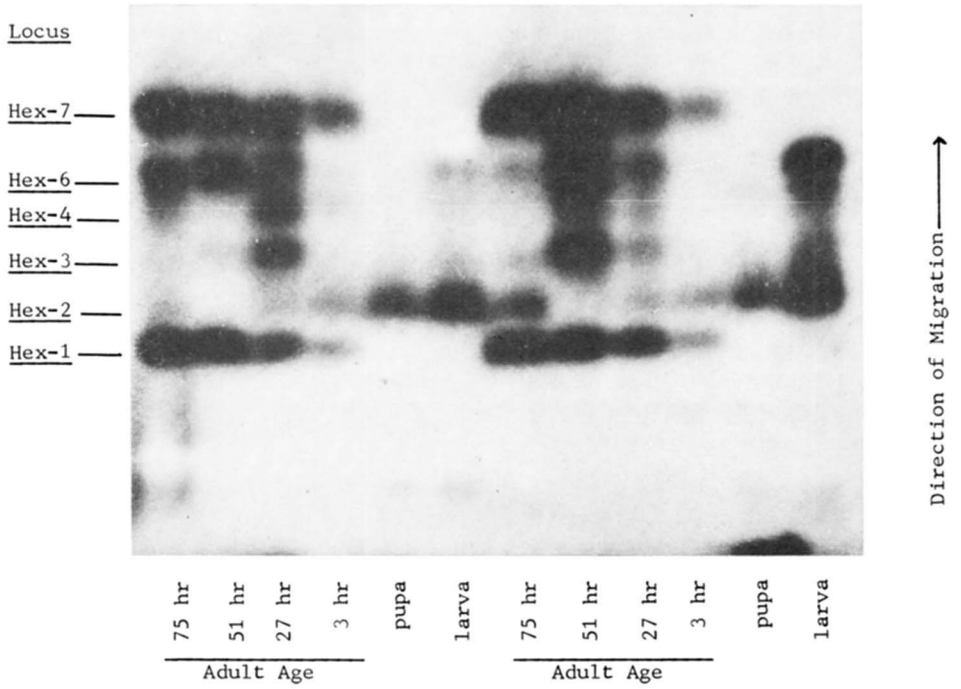

FIGURE 1.—Developmental changes in the isozyme patterns of hexokinase. The six pockets on the left are *Drosophila persimilis*; those on the right are *D. pseudoobscura*. The larvae are third instar.

was observed, but the proximity of the hexokinase-2 band prevented densitometric analysis of the faint bands in the early stages of development for this species.

Examination of strains under varied electrophoretic conditions: Examination of all strains using buffers with pH 5.5, 6.8, 9.2 and 10.0 failed to reveal any additional variation among the electromorphs. Extended runs of up to 24 hr also failed to separate allelic differences at either the *hex-1* or *hex-7* loci. *Hex-1* and *hex-7* of all Mather strains of both species were studied using a range of electro-

TABLE 2

Developmental changes in relative activity for the 0.8 *and* 1.0 *alleles of* hex-1 *in* D. persimilis

Time from eclosion (hrs)	Number of samples examined	Relative activity *0.8/1.0*	S.E.
4	6	0.49	0.08
13	3	0.73	0.12
26	9	0.42	0.07
36	4	0.53	0.09
48	7	0.54	0.07
59	4	0.86	0.08
78	9	0.92	0.11
7 days	27	0.80	0.04

starch gel concentrations (8.5 to 15.5%). Retardation coefficients relative to ovalbumin (K_r) were calculated for both loci in each strain (FERGUSON 1964). No differences in K_r could be detected and no differences in mobility at any concentration could be detected. Pooling the values for all strains gave K_r (mean ± S.E.) of −0.00083 ± 0.00014 for hexokinase-7 and 0.00022 ± 0.00035 for hexokinase-1. Free mobility (M_o) was calculated for each strain and both loci, but is subject to massive extrapolation errors when analyzed on starch gels. No obvious differences in M_o were observed, but this result is not particularly meaningful because of the errors. The difficulty is less apparent with acrylamide gels, but efforts to resolve hexokinases on 5 to 7% acrylamide gels in varying pH buffers were unsuccessful.

Activity at the hex-1 *locus*: Differences at the structural or regulatory regions of an enzyme locus could be reflected in differences in activity. It is clear, however, that a large number of other variables might also give rise to activity differences. (1) Differences in genetic background have been shown to affect alcohol dehydrogenase activity in *D. melanogaster* (WARD 1975). (2) Activity may be strongly dependent on developmental stage (see Figure 1). (3) Differences in environmental conditions during development could give rise to considerable activity variation when comparing strains. (4) Differences in body size and protein content could affect comparisons between strains. (5) Random variation may be an additional source of activity differences.

These difficulties are compounded by problems that arise from the use of a densitometer for measuring activity. (6) Densitometric measurement of high stain concentrations has been found to be nonlinear (MARKERT and MASUI 1969) so that care must be taken not to overstain the gels. (7) The densitometer automatically sets a new baseline and sensitivity for each scan, based on the absorbance at the starting point. Thus, strictly speaking, separate scans cannot be compared directly.

Most of these problems can be circumvented by using heterozygotes for different mobility alleles, with one allele serving as an internal control for the other. Then the initial comparison is between alleles of a single individual, electrophoresed and scanned in a single pocket. Thus the alleles share a common genetic background and a common developmental and environmental history. Since variables (1) to (4) and the sensitivity problem of (7) above primarily involve proportionality constants, by examining the ratio of activities, these unknowns are simply divided out and the resulting ratios should be comparable between strains. This method has been applied by PRAKASH (1977c), who found activity differences within electromorphs for the enzyme xanthine dehydrogenase. The nonlinearity problem (6) was minimized in two ways. Examination of a serial dilution series indicated that the reaction was within the linear range if stained for four hr at 25°. In addition, crosses involving many different strains were electrophoresed and stained in each gel, while replicates of each cross were examined on separate gels, to reduce the chance of systematic differences between strains arising as artefacts of nonlinearity.

Tables 3 to 5 show the activities of the rare alleles relative to the *1.0* allele

for *hex-1*. In Table 5, activity of the *0.6* allele is expressed relative to the *0.8* allele in *0.6/0.8* heterozygotes. Analysis of variance failed to reveal any differences between strains of the *1.0* or the *1.2* mobilities of *D. pseudoobscura* or the *0.8* mobility of *D. persimilis* (Tables 3 and 4). A significant *F*-test demonstrated heterogeneity of activity among the *1.0* strains of *D. persimilis*. Pairwise *t*-tests showed two activity classes, but in the F_2 heterozygotes for these strains the differences disappeared, suggesting that they are not allelic.

One fact that is immediately obvious when examining the gels is the relatively low activity of the rare alleles (Figure 2). Since the activities in Tables 3 to 5 are expressed relative to the more common allele, we can simply test for deviation of the ratio from 1. All comparisons involving the *1.2* allele of *D. pseudoobscura* are significantly less than 1 (Table 3) and the relative activity and 95% confidence limits from 149 comparisons is 0.701 ± 0.022. Examination of 65 F_2 heterozygotes from these crosses gave a mean activity (with 95% confidence interval) of 0.668 ± 0.040. The differences between the F_1 and F_2 generations is not significant ($t_{212} = 0.231$). Thus the lower activity clearly segregates with the less frequent *1.2* mobility class (Figure 3).

The differences in activity of the *0.8* and *1.0* alleles of *D. persimilis* are not as clear; while all of the comparisons are less than 1, only 5 are significant (Table 4). If all comparisons (141 tests) are pooled, the relative activity (with 95%

TABLE 3

Relative activity at the* hex-1 *locus for the* Drosophila pseudoobscura *crosses*

	Strain bearing the *1.0* allele					
	ST1	ST2	CH2	CH6	PP3	Totals
Strain bearing the *1.2* allele						
TL3	0.658±0.075 (17)	0.744±0.091 (7)	0.674±0.092 (12)	0.642±0.084 (17)	0.709±0.082 (15)	0.677±0.082† (68)
AR1	0.731±0.045 (21)	0.703±0.058 (14)	0.731±0.066 (15)	0.705±0.081 (18)	0.737±0.090 (13)	0.721±0.028 (81)
Totals	0.698±0.041 (38)	0.717±0.045 (21)	0.706±0.052 (27)	0.674±0.056 (35)	0.722±0.057 (28)	0.701±0.022 (149)

* Activity was determined on aged females heterozygous for the *1.2* and *1.0* alleles. The numbers indicate mean avtivity and 95% confidence intervals expressed as a ratio of the activity of the *1.2* allele to that of the *1.0* allele. The number of females tested is given in parentheses.

† Four additional *1.0* strains, two each with TL and AR gene arrangements, were crossed to the TL3 strain (*1.2* allele) and gave relative activities of .656±0.076 (N=17).

	Analysis of variance			
	D.f.	S.S.	M.S.	F
1.2 mobility class	1	0.0726	0.0726	3.878 n.s.
1.0 mobility class	4	0.0435	0.0109	0.582 n.s.
Interactions	4	0.0475	0.0119	0.636 n.s.
Within subclasses	139	2.6025	0.0187	
Totals	148	2.7660		

TABLE 4

Relative activity at the hex-1 *locus for the* Drosophila persimilis *crosses: heterozygotes for the* 0.8 *and* 1.0 *alleles*

	Strain bearing the *1.0* allele						
	WT1	WT4	WT6	WT11.1	WT18	MD15	Totals
Strain bearing the *0.8* allele							
WT8	0.936±0.069 (15)	0.945±0.145 (10)	0.847±0.098 (18)	0.925±0.090 (4)	0.861±0.076 (20)	————	0.890±0.041 (67)
WT10	0.888±0.132 (13)	0.994±0.140 (16)	0.784±0.144 (14)	0.860±0.180 (5)	0.800±0.109 (13)	0.807±0.080 (13)	0.860±0.051 (74)
Totals	0.914±0.067 (28)	0.975±0.097 (26)	0.819±0.079 (32)	0.889±0.087 (9)	0.837±0.060 (33)	0.807±0.080 (13)	0.874±0.033 (141)

The numbers indicate mean activity and 95% confidence intervals expressed as a ratio of the activity of the *0.8* allele to that of the *1.0* allele. The number of females tested is given in parentheses.

Analysis of variance	D.f.	S.S.	M.S.	F
0.8 mobility class	1	0.0123	0.0123	0.3113, n.s.
1.0 mobility class	4	0.4474	0.1119	2.8310, $p<0.05$
Interactions	4	0.0895	0.0224	0.5670, n.s.
Within subclasses	118	4.6620	0.0395	
Totals	127	5.2112		

TABLE 5

Relative activity at the hex-1 *locus for the* Drosophila persimilis *crosses: heterozygotes with the* 0.6 *allele and* 1.0 *or* 0.8

	Strain with the *0.8* allele			Strain with the *1.0* allele			
	WT8	WT10	Total for *0.8* alleles	WT1	WT4	MD15	Total for *1.0* alleles
WT11.6	0.439±0.078 (16)	0.464±0.115 (8)	0.448±0.060 (24)	0.483±0.099 (6)	0.475±0.198 (6)	0.475±0.191 (2)	0.479±0.076 (14)

The numbers indicate mean activity and 95% confidence intervals expressed as a ratio of the activity of the *0.6* allele with the more common form of the enzyme. The number of females tested is given in parentheses.

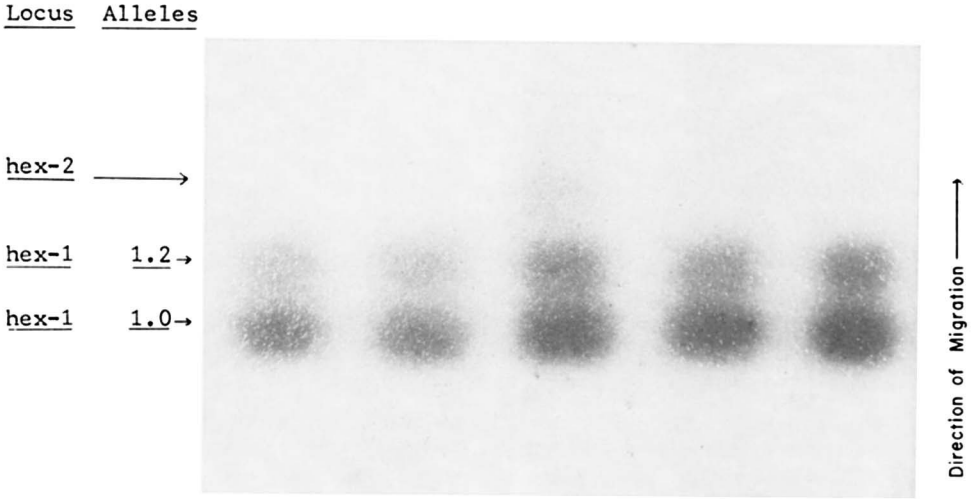

FIGURE 2.—Heterozygotes for *hex-1*$^{1.0}$ and *hex-1*$^{1.2}$ of *Drosophila pseudoobscura*, showing consistently lower staining intensity of the low frequency allele, *hex-1*$^{1.2}$.

confidence interval) is 0.874 ± 0.033. Examination of 22 F_2 heterozygotes confirms that this difference segregates with the mobility difference. The low frequency *0.6* allele of *D. persimilis* had less than 50% of the activity of the *0.8* and *1.0* alleles of this species (0.448 ± 0.060 and 0.479 ± 0.076, respectively). Thus there is a correlation between the frequency of the alleles and their activity relative to the *1.0* allele.

Thermostability: In a number of recent studies electromorphs have been sub-

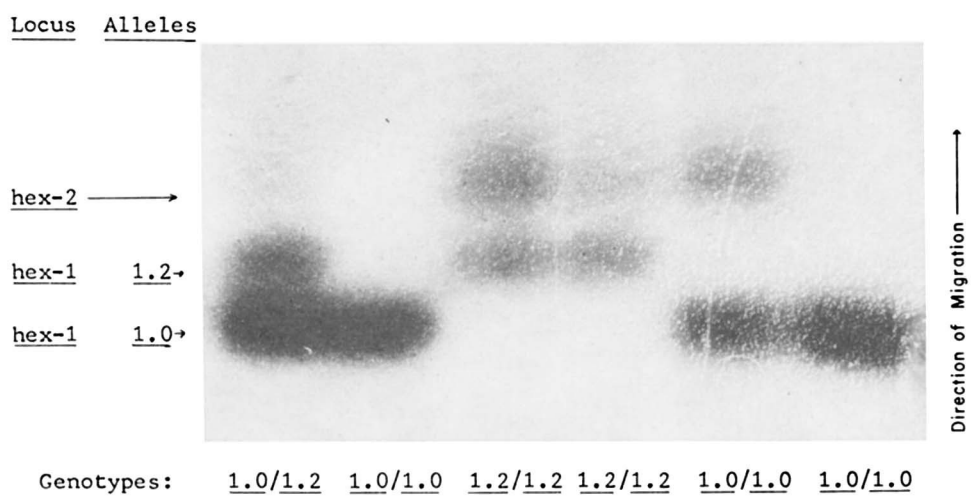

FIGURE 3.—F_2 generation of crosses between strains bearing the low frequency *hex-1*$^{1.2}$ allele and those with the common *hex-1*$^{1.0}$ allele. The lower staining intensity segregates with the *hex-1*$^{1.2}$ allele.

divided on the basis of heat stability (*e.g.*, BERNSTEIN, THROCKMORTON and HUBBY 1973; SINGH, LEWONTIN and FELTON 1976; MILKMAN 1976). SINGH, LEWONTIN and FELTON (1976) showed in heterozygotes for different xanthine dehydrogenase (*XDH*) mobility alleles that at least some of the stability differences are associated with the structural locus. However, it was not clear whether all strains differing in thermostability necessarily bear different structural genes at that locus. During preliminary examination, the Mather strains showed considerable heterogeneity of thermostabilities for their hexokinases, but even though the hexokinase isozymes appear to be monomers, all loci in each strain had the same thermostability properties. An example of this result is given in Figure 4. In replicate runs, ST2 had much greater sensitivity than CH6 to denaturation when incubated for five minutes at 50°, but this difference was not confined to one locus. Neither strain was discernably affected by treatment at 47°. These observations, in conjunction with that of SINGH, LEWONTIN and FELTON (1976) that age-specific differences in heat sensitivity occur in *XDH*, demonstrate the importance of factors not associated with the structural locus. Thus it is necessary to have some internal control in thermostability analyses or to do segregation analysis on any observed differences.

All low frequency *hex-1* alleles, when examined in heterozygotes, were found to be less stable to heat denaturation than the *1.0* electromorphs. The *0.6* allele of *D. persimilis* and the *1.2* alleles of *D. pseudoobscura* lost all activity in two to five minutes at 48°. The *0.8* allele of *D. persimilis* was stable at 48°, but when treated at 50°, their activity relative to the *1.0* alleles declined from 0.75 ± 0.048 to 0.53 ± 0.015 in five minutes.

Ideally, a control should be more stable than the proteins to be tested so that

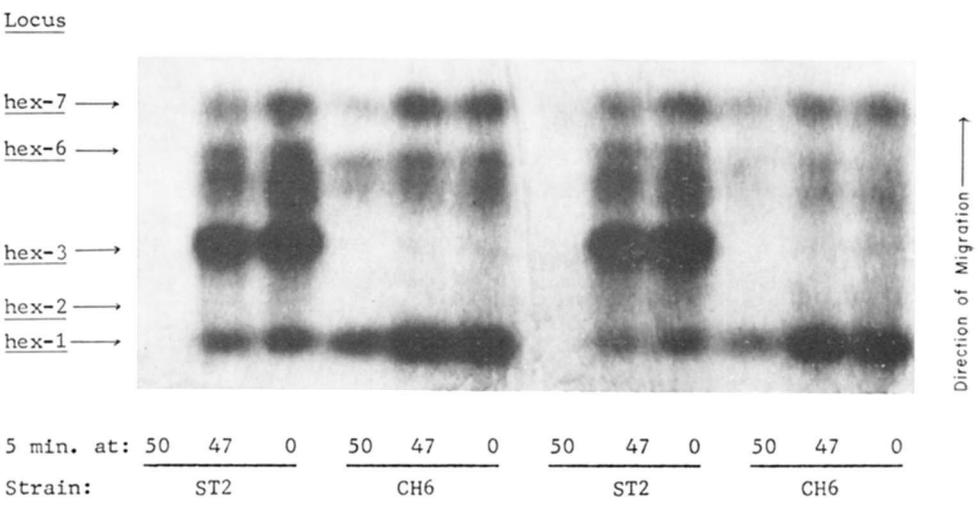

FIGURE 4.—Effect of heat denaturation on hexokinase. In replicate samples of the two strains, repeatable differences occur in the sensitivity of hexokinase to denaturation for five minutes at 50°. However, the difference is *strain* specific, not locus specific. Some factor other than structural differences at the hexokinase loci must account for these sensitivity differences.

substantial control activity remains at the critical treatment levels of the experimental proteins. Since the low frequency *hex-1* alleles do not meet this criterion, the stability of *hex-1* in all *1.0* strains was compared to that of the *hex-7* locus. Strains were examined for differences in the relative sensitivity of the two proteins to heat denaturation. Most of the strain-specific variation was eliminated by this comparison and none of the strains could be unambiguously distinguished by thermostability properties.

Resistance to urea denaturation: Clear differences between the stability of the mobility classes were observed, but no variation could be detected within any mobility class. The activity of the *hex-1*$^{0.6}$ allele of *D. persimilis* and the *hex-1*$^{1.2}$ alleles of *D. pseudoobscura* was eliminated when heterozygous females were ground in 20 μl of a 2 M urea solution. Substantial activity of the *hex-1*$^{1.0}$ alleles and the *hex-7* locus was retained. The *hex-1*$^{0.8}$ and *hex-1*$^{1.0}$ alleles were denatured in 3 M urea, while some activity was sometimes present at the *hex-7* locus even in a 4 M solution. This pattern of resistance to urea denaturation correlates well with relative activities of untreated proteins (see above) and may be simply a property of the amount of enzyme present. If production of a band on the gel is a threshold effect of enzyme quantity, as serial dilution studies would indicate (KLEBE 1975), then these results would be obtained if a constant proportion of each enzyme were denatured at each treatment level.

Reaction with pCMSA: All electromorphs of *hex-1* reacted with *p*CMSA. When 20 μl of 10^{-4} M *p*CMSA was used as grinding solution, a portion of the band migrated faster than the main band (Figure 5). Thus it appears that these proteins have at least one free -SH group that is not essential for activity in the staining reaction. At slightly higher *p*CMSA concentrations, when the mobility of the entire hexokinase-1 band had been increased, occasionally a second unit increment of mobility was observed, suggesting that two free -SH groups with slightly different binding affinities are present. No intermediate mobility bands were ever observed. None of the other hexokinase loci was affected. With even higher concentrations of *p*CMSA (10^{-3} M or higher), activity of hexokinase was eliminated. No differences in reactivity could be detected within the electromorph classes of any of the Mather strains.

This technique can be used to give direct evidence on the nature of the differences between electromorphs. Alternating pocket comparisons of treated extract of each mobility class of *hex-1* were made with untreated extract of the next faster mobility allele. In each case, the band representing the altered protein was found to migrate with the next faster allele (Figure 5). As will be shown below, the mobility differences of the *0.6*, *0.8*, *1.0* and *1.2* electromorphs are caused by charge differences alone. Thus it is apparent that these mobility differences are produced by a single unit charge difference. PRAKASH (1977a) has reported an allele of *D. persimilis* with relative mobility (on agar gels) of 0.94. This allele must represent a difference of less than a unit charge from either the *0.8* or the *1.0* alleles, or both. The *0.94* allele was not available for study, but must be a conservative amino acid substitution from one of those electromorphs.

Molecular weight determination: Polymorphism of an enzyme depends in

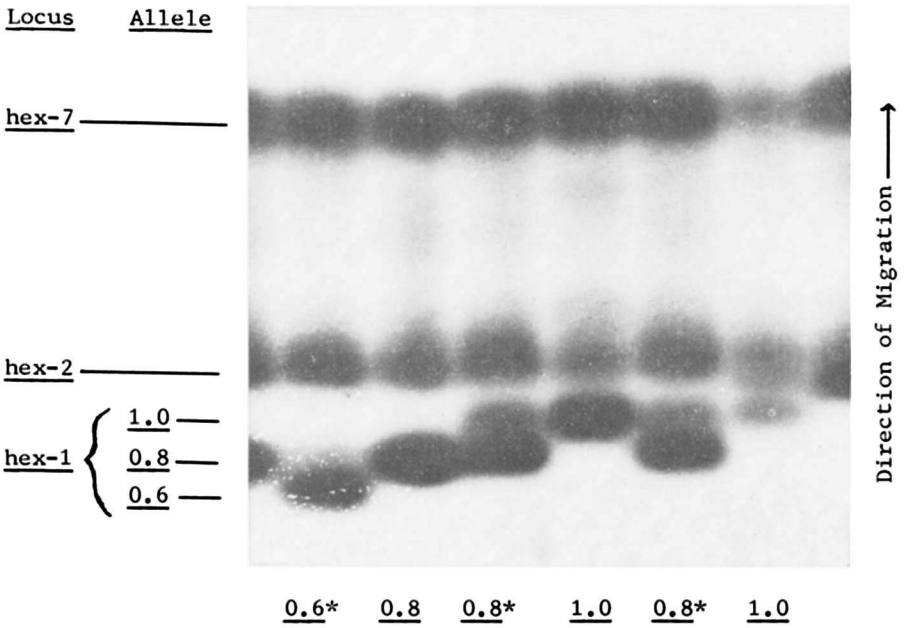

FIGURE 5.—Effect of a unit charge change on the mobility of the *hex-1* mobility classes. In those marked with an asterisk (*), 20 μl of 10^{-4} M *p*CMSA was used as the grinding solution. In each case a portion of the hexokinase-1 band has migrated with the next faster mobility class of an adjacent pocket. This effect is most noticable in pockets marked *0.8**.

part on the number of amino acid sites available for substitution. Thus as a first approximation, we might expect polymorphism to be related to the size of the molecule. The highly polymorphic enzymes are, in fact, quite large: *XDH* has a molecular weight of about 140,000 (CANDIDO, BAILLIE and CHOVNICK 1974) and the molecular weight of the *cst-5* subunit is about 52,000 (NARISE and HUBBY 1966). Thus estimates of the molecular weight of hexokinase-1 and hexokinase-7 were obtained using the method of COBBS (1976).

Efforts to run hexokinases on acrylamide were not successful. Therefore a caveat must be offered at the outset: the use of starch gels in constructing FERGUSON plots (FERGUSON 1964) is not nearly as precise as is possible with acrylamide. The usable range of pore sizes is much more restricted. Finally, the degree of polymerization does not appear to be as easily controlled. An assumption common to all techniques that rely on retardation coefficients to estimate molecular weight, and therefore applicable here, is that the molecule is globular in shape. Thus the estimates obtained here must be regarded as approximations.

The relative mobilities of OVA, BSA, bovine hemoglobin and ferritin were obtained using hexokinase-7 as a standard. The least squares regressions of log R_m to starch concentration was calculated for each protein and the resulting slopes plotted against molecular weight. This relationship was found to be linear and the point of 0 slope on the regression line was calculated and taken as the

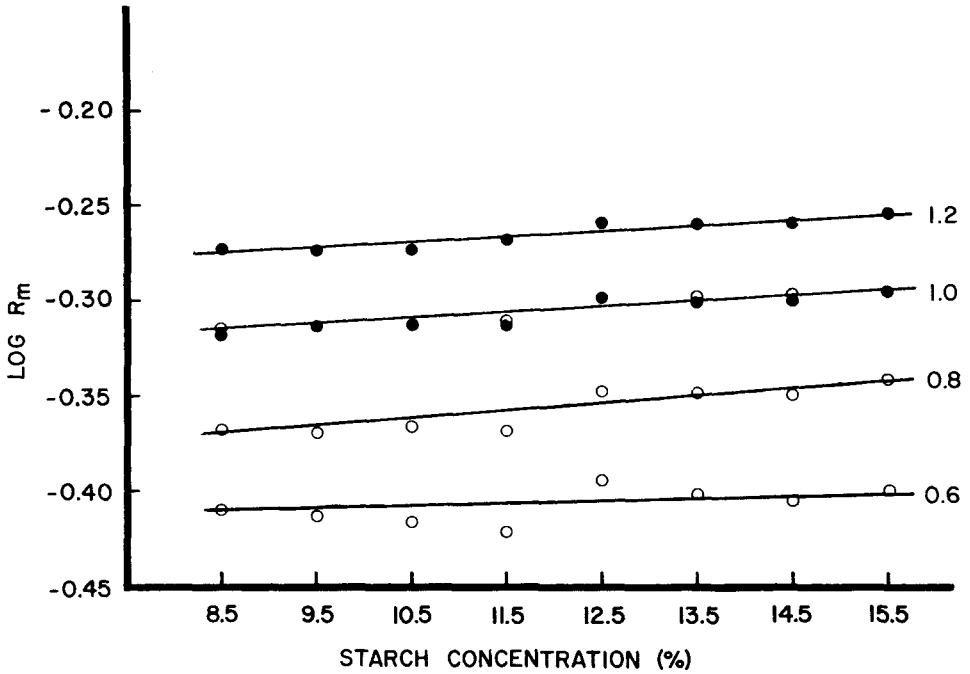

FIGURE 6.—Effect of gel concentration on the mobilities of the four allelic classes of *hex-1*. R_m was measured relative to hexokinase-7. The slopes (± S.E.) for the regression lines of the alleles are: *1.2*, 0.0031 ± 0.00064; *1.0*, 0.0031 ± 0.00075 (both species combined); *0.8*, 0.0039 ± 0.00104; and *0.6*, 0.0024 ± 0.00117. Identical slopes of the allelic classes indicate that they differ by charge alone, rather than by size or shape. The *1.0* alleles of *D. pseudoobscura* and *D. persimilis* are indistinguishable by this method.

molecular weight of hexokinase-7 (72,000). Thus hexokinase-7 appears to be somewhat larger than the highly polymorphic *est-5* subunit.

Comparison of the mobility classes of hexokinase-1 by the method of FERGUSON plots (Figure 6) gives identical slopes for all alleles. This result provides evidence that they differ by charge alone, not by size or shape. Two replicates of all allelic classes (including *1.0* alleles of both species) were run on each series of gels. Within each series the retardation coefficients for each allele was identical, but analysis of variance revealed a significant variance between series. Thus, to determine the molecular weight of hexokinase-1, retardation coefficients obtained in the survey of all strains were included to take advantage of the much larger sample size. The resulting estimate of the molecular weight of hexokinase-1 is 45,000.

DISCUSSION

The evidence presented in this paper falls into two categories: (1) evidence that no further subdivision of the electromorphs of *hex-1* or *hex-7* could be made among the strains of *D. pseudoobscura* or *D. persimilis* tested, using a wide variety of techniques, and (2) evidence concerning the nature of the differences

between the electrophoretically distinguishable alleles. Each of these lines of inquiry is of significance when viewed in the perspective of the current state of our knowledge of electrophoretic variation.

A growing number of studies have revealed extensive hidden variation among several enzymes which had previously exhibited high electrophoretic heterogeneity (*e.g.*, *XDH* and *est-5*). These studies have strengthened the notion that routine electrophoresis (usually 5% acrylamide, pH 9.0) greatly underestimates the total variability at these loci and opens the question of whether the occurrence of monomorphic loci (as *hex-7*) or those with low heterozygosities (as *hex-1*) may be artefacts of the limitations of the techniques. Even when it is shown that routine techniques can detect differences of much less than a unit charge difference (COBBS and PRAKASH 1977b), more refined analyses involving different gel concentrations or pH can produce further subdivision of the electromorph classes. Can these findings be generalized to all loci? Apparently, the answer is no. Examination of 135 strains of two species of Drosophila from six localities with a range of buffer pH failed to show any heterogeneity among the electromorphs of *hex-1* or *hex-7*. Examination of 40 strains of *D. pseudoobscura* and 9 strains of *D. persimilis* from Mather with a range of gel concentrations, and for differences in stability to heat denaturation or for differences in reactivity to thiol reagents also failed to reveal further heterogeneity. Study of a more restricted series of strains of these species failed to reveal any variation within electromorph classes for either activity or susceptibility to urea denaturation. Many of these techniques have been used successfully to subdivide electromorphs of *XDH* and *est-5*, and in this study, the *0.6* allele of *D. persimilis* and the *1.2* allele of *D. pseudoobscura* could have been distinguished from the *1.0* alleles of each species by any of the techniques except reaction to *p*CMSA. The *0.8* allele of *D. persimilis* could have been distinguished from the *1.0* alleles of each species by any of the techniques except susceptibility to urea denaturation and reaction to *p*CMSA. While we cannot claim that no additional variation exists, even among the strains tested, clearly in the case of the hexokinase loci, low electrophoretic variability reflects low total variability for these loci. We believe that this qualitative observation will stand.

Very few of the strains of the low frequency *hex-1* alleles were examined in this study (one strain of the *0.6* allele and two of the *0.8* allele of *D. persimilis* and two strains of the *1.2* allele of *D. pseudoobscura*). Thus, the chance of finding differences between strains of a low frequency electromorph was remote. However, as NEI and CHAKRABORTY (1977) have shown, most concealed (neutral) variation should occur in the most frequent electromorph class, since on the average it has been present in the population longer and at higher frequencies than the rarer forms. This point is particularly evident for *hex-1*$^{1.0}$, since that mobility class is the only one shared by *D. persimilis* and *D. pseudoobscura* and it is fixed in the sibling species, *D. miranda* (PRAKASH 1977a).

The nature of the electrophoretic variation at the hex-1 *locus*: We have presented evidence that mobility differences among the electrophoretic alleles reflect

stepwise unit charge differences. Reaction of crude extract with *p*CMSA, a reagent which is known to attach a negative charge to free -SH groups, can increase the mobility of a portion of any *hex-1* allele one step: if we designate the altered allozyme due to reaction with *p*CMSA with an asterisk, *0.6** migrates with *0.8*, *0.8** migrates with *1.0* and *1.0** migrates with the *1.2* allele. The presence of the *hex-2* locus prevents examination of the altered *1.2* allele of *hex-1*. We must ask the question: if only about 27% of all amino acid substitutions cause a unit charge change (MARSHALL and BROWN 1975), what has happened to all the changes which do not alter the charge? One such allele may be the *0.94* allele of *D. persimilis* reported by PRAKASH (1977a). If our analysis of the effect of a unit charge change is correct, this allele must carry less than a unit charge difference from either the *0.8* or the *1.0* alleles (or perhaps both). Unfortunately, strains with this allele were not available for study. Other allelic differences may exist which are not distinguishable by the techniques employed, or are too rare to be represented among the strains examined.

The inevitable problem which must be faced in electrophoretic analyses involves the forces responsible for the observed patterns of polymorphism. EWENS' (1972) test for goodness-of-fit to the infinite alleles model was run on the gene frequency data of PRAKASH (1977a). For *D. pseudoobscura* (two alleles) $F(v_1,v_2) = 0.554$ (0.79, 1.36) n.s. and for *D. persimilis* (four alleles) $F = 0.329$ (7.60, 8.54) $p \doteq 0.07$. Neither of these tests is significant, but as EWENS points out, the test is not very powerful: when the same allele predominates in several closely related species and alternative alleles always occur at low frequency, a subjective interpretation of selection favoring the common allele may be the best interpretation. The *hex-1*$^{1.0}$ allele appears at frequencies of 0.92 in *D. persimilis*, 0.96 in *D. pseudoobscura* and 1.00 in *D. miranda* (PRAKASH 1977a). OHTA and KIMURA (1975) and OHTA (1976) have extended the step model to include very slightly deleterious mutants in order to account for the occurrence in Drosophila of an excess of rare alleles over theoretical expectation. Hexokinases may provide examples of this selection-mutation balance.

TABLE 6

Summary of the differences between the electrophoretically detectable alleles of Drosophila pseudoobscura *and* D. persimilis

	D. persimilis				*D. pseudoobscura*	
	0.94	*0.6*	*0.8*	*1.0*	*1.0*	*1.2*
Frequency*:	0.004	0.002	0.078	0.916	0.96	0.04
Relative activity:	——	0.48	0.87	1.00	1.00	0.70
Relative stability						
Heat:	——	5 min at 48 C	5 min at 50 C	10 min at 50 C	10 min at 50 C	5 min at 48 C
Urea:	——	2 M	3 M	3 M	3 M	2 M

Stabilities refer to the treatment necessary to eliminate activity. The *0.94* allele of *D. persimilis* was not available for testing.

* From PRAKASH 1977a. N=350 in each species.

Table 6 summarizes the differences between electromorph classes found in this study. A correlation is evident between activity, stability and frequency for those alleles studied. It is obviously not possible to infer selective differences from such correlations, particularly for such qualities as electrophoretic mobility, urea denaturation and stability at temperatures far above the survival limits of the fly. However, activity differences between alleles of this important enzyme may be of significance to the organism. Thus, our subjective interpretation of EWENS' test and the correlations between the frequency of alleles and their activity provide evidence in favor of the selection-mutation model of OHTA (1976).

Molecular weight and polymorphism: Molecular weight determinations of hexokinase-7 (about 72,000) and hexokinase-1 (about 45,000) show that they are both moderately large proteins, similar in size to the subunit of the highly polymorphic *est-5* (about 52,000, NARISE and HUBBY 1966). Thus the differences in polymorphism between these hexokinase enzymes and the esterase-5 enzyme are not due to differences in the size of the molecules. This finding is not particularly novel. More interesting, perhaps, is the observation that the apparently monomorphic hexokinase-7 enzyme is larger than the polymorphic hexokinase-1 molecule. Thus even within a functional group of enzymes, selection can apparently place greater constraints on variation of one form than the other.

Conclusions. Previously work by COYNE (1976) and SINGH, LEWONTIN and FELTON (1976) studying *XDH* and MCDOWELL and PRAKASH (1976) and COBBS and PRAKASH (1977a) studying *est-5* has demonstrated considerable heterogeneity within electromorphs of these highly polymorphic enzymes. In this study, no additional variation could be demonstrated for the very conservative hexokinase enzymes. Thus we have broadened the gap between the extremes in the spectrum of enzyme polymorphism. The evidence gathered for the *est-5* locus suggests that most of the variation at that locus is neutral (COBBS and PRAKASH 1977a). The variation at the *hex-1* locus can be explained as a case of selection favoring a single allelic form. If balancing selection plays any role in maintaining enzyme polymorphisms in natural populations, enzyme systems of intermediate numbers of alleles and heterozygosity may offer the greatest opportunity for demonstrating such mechanisms.

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LITERATURE CITED

BERNSTEIN, S., L. H. THROCKMORTON and J. L. HUBBY, 1973 Still more genetic variability in natural populations. Proc. Natl. 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.

COBBS, G. and S. PRAKASH, 1977a A comparative study of the esterase-5 locus in *Drosophila pseudoobscura*, *D. persimilis* and *D. miranda*. Genetics **85**: 697–711. ——, 1977b An experimental investigation of the unit charge model of protein polymorphism and its rela-

tion to the esterase-5 locus of *Drosophila pseudoobscura*, *D. persimilis* and *D. miranda*. Genetics **87**: 717–742.

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.

FERGUSON, K. A., 1964 Starch-gel electrophoresis—Application to the classification of pituitary proteins and polypeptides. Metabolism **13**: 985–1002.

JELNES, J. E., 1971 Identification of hexokinases and localization of a fructokinase and a tetrazolium oxidase locus in *Drosophila melanogaster*. Hereditas **67**: 291–293.

KLEBE, R. J., 1975 A simple method for the quantitation of isozyme patterns. Biochem. Genet. **13**: 805–812.

MADHAVEN, K., D. J. FOX and H. URSPRUNG, 1972 Developmental genetics of hexokinase isozymes in *Drosophila melanogaster*. J. Insect Physiol. **18**: 1523–1530.

MARKERT, C. L. and Y. MASUI, 1969 Lactate dehydrogenase isozymes of the Penguin *Pygoscelis adeliae*. J. Exptl. Zool. **172**: 121–145.

MARSHALL, D. R. and A. H. D. BROWN, 1975 The charge-state model of protein polymorphism in natural populations. J. Molec. Evol. **6**: 149–163.

MCDOWELL, R. E. and S. PRAKASH, 1976 Allelic heterogeneity within allozymes separated by electrophoresis in *Drosophila pseudoobscura*. Proc. Natl. Acad. Sci. U.S. **73**: 4150–4153.

MILKMAN, R. D., 1976 Further evidence of thermostability variation within electrophoretic mobility classes of enzymes. Biochem. Genet. **14**: 383–387.

NEI, M. and R. CHAKRABORTY, 1977 Electrophoretically silent alleles in a finite population. J. Molec. Evol. (In press.)

NARISE, S. and J. L. HUBBY, 1966 Purification of esterase-5 from *Drosophila pseudoobscura*. Biochim. Biophys. Acta **122**: 281.

OHTA, T., 1976 Role of very slightly deleterious mutations in molecular evolution and polymorphism. Theor. Pop. Biol. **10**: 254–275.

OHTA, T. and M. KIMURA, 1975 Theoretical analysis of electrophoretically detectable polymorphisms: models of very slightly deleterious mutations. Am. Naturalist **109**: 137–145.

PRAKASH, S., 1977a Genetic divergence in closely related sibling species *Drosophila pseudoobscura*, *D. persimilis* and *D. miranda*. Evolution **31**: 14–23. ——, 1977b Further studies on gene polymorphism in the mainbody and geographically isolated populations of *Drosophila pseudoobscura*. Genetics **85**: 713–719. ——, 1977c Allelic variants at the xanthine dehydrogenase locus affecting enzyme activity in *Drosophila pseudoobscura*. Genetics **87**: 159–168.

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.

SINHA, K. P. and D. A. HOPKINSON, 1969 The investigation of reactive sulphydryls in enzymes and their variants by starch gel electrophoresis. Studies on the human red cell peptidase variant Pep A 5–1. Ann. Hum. Genet. **33**: 139–148.

WARD, R. D., 1975 Alcohol dehydrogenase activity in *Drosophila melanogaster*: a quantitative character. Genet. Res. **26**: 81–93.

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