STUDIES OF ESTERASE-6 IN *DROSOPHILA MELANOGASTER*. II. THE GENETICS AND FREQUENCY DISTRIBUTIONS OF NATURALLY OCCURRING VARIANTS STUDIED BY ELECTROPHORETIC AND HEAT STABILITY CRITERIA

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ABSTRACT

Measurements of the electrophoretic mobility and thermostability of esterase-6 allozymes have been used to determine the amount of allelic variation at the *esterase-6* locus in *Drosophila melanogaster*. We studied 398 homozygous lines obtained from four natural populations. Use of a spectrophotometric assay for esterase-6 activity has allowed precise quantitation of heat-stability variants. Using these methods, eight putative alleles were detected within the two most common electrophoretic classes. Analyses of F_1 and F_2 progeny show that the behavior of stability variants is consistent with the hypothesis that this variation is due to allelic variation at the *Est-6* locus. Analyses of the gene-frequency distributions within and between populations show (1) that observed allele-frequency distributions do not deviate significantly from those expected for neutral variants, and (2) that there is little evidence for an increase in apparent divergence of the different populations at the genotypic or phenotypic levels when the additional variation detected is considered. These findings suggest that gene-frequency analysis alone is unlikely to resolve the question of the selective significance of allozyme variation.

IT is now widely accepted that conventional electrophoretic techniques alone are incapable of detecting much of the variation in amino acid sequences of enzyme molecules that exist in natural populations. When additional characteristics of enzymes, such as heat stability (WRIGHT and MACINTYRE 1965; BERNSTEIN, THROCKMORTON and HUBBY 1974; SINGH, HUBBY and LEWONTIN 1974; SINGH, HUBBY and THROCKMORTON 1975; TRIPPA, LOVERRE and CATAMO 1976; TRIPPA *et al.* 1978; LUSIS and WEST 1978; MILKMAN 1976; THÖRIG, SCHOONE and SCHARLOO 1975; COCHRANE 1976; SAMPSELL 1977; BONHOMME and SELANDER 1978), enzyme activity (PRAKASH 1977), or mobility under varied electrophoretic conditions (JOHNSON 1976, 1977; COYNE 1976; SINGH, LEWONTIN and FELTON 1976; MCDOWELL and PRAKASH 1976; COYNE and FELTON 1977; COYNE, FELTON and LEWONTIN 1978), are studied on material from inbred lines that show identical mobilities of particular enzymes under standard conditions, a

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wealth of new variation is revealed. The application of more refined techniques for the detection of protein variants has elaborated data that come closer to matching the assumptions of theoretical models, perhaps allowing a test of the adaptive significance of allelic protein variants. In particular, the infinite-alleles model (KIMURA and CROW 1964; EWENS 1972) and the charge-state model (OHTA and KIMURA 1973; BROWN, MARSHALL and ALBRECHT 1975) make specific predictions as to the nature of expected frequency distributions of cryptic alleles in natural populations. The former assumes that all alleles are detected, while the latter attempts to account for the limited resolution of conventional electrophoresis.

The key problem in virtually every search for hidden variation is that, as the techniques of molecular or enzymatic analysis employed become more refined, verification that differences observed are due to variation at a particular structural locus becomes more difficult. Thermal stability variation and electrophoretic mobility differences can result from post-translational modification (SCHWARTZ *et al.* 1975; FREE and SCHIMKE 1978; COCHRANE and RICHMOND 1979). Variation in enzyme activity can be due to differences in gene regulation, particularly of tightly linked, *cis*-acting elements (CHOVNICK *et al.* 1976; LUSIS and PAIGEN 1975; THOMPSON, ASHBURNER and WOODRUFF 1977), or to more nonspecific effects of genetic background (WARD 1975; BECKENBACH and PRAKASH 1977). Finally, inadequate standardization of experimental conditions may give spurious indications of *in vivo* variation (ARNOLD and LUSH 1975).

Given these unavoidable problems, what are the desirable attributes of parameters to be used in detecting hidden variation? Ideally, the following conditions should be met: (1) The parameters must be quantifiable in some reproducible manner. As measurements become more subtle, maintenance of observational objectivity becomes more critical. (2) The parameters measured must be amenable to genetic analysis. It must be demonstrated that observed variation segregates as a single Mendelian trait that does not recombine with the structural locus in question. (3) There should be *a priori* reasons to suspect that the parameters to be measured are sensitive to most variation in the amino acid sequence of the enzyme in question.

In initiating our search for hidden variation at the esterase-6 (*Est-6*) locus of *Drosophila melanogaster*, we sought a practical criterion that could be applied in conjunction with conventional electrophoresis. We felt that heat stability fulfilled these conditions adequately. The stability of an enzyme, provided a reliable means of assaying activity exists, can be expressed as a rate constant or as a half-life of activity at a particular temperature (PAIGEN 1971). This constant is largely independent of variation in enzyme activity per molecule, in the number of enzyme molecules present among different individuals, or in the amount of total protein present (JOHNSON 1977).

Demonstration of segregation in F_1 and F_2 generations is somewhat more difficult, since determination of rate constants requires the use of a mass homogenate of genetically identical individuals. However, it has been possible to demonstrate qualitative differences in esterase-6 enzyme stability on starch gels,

using individual flies as a source of enzyme (see MATERIALS AND METHODS). Thus, data were gathered on the association of heat stability with the *Est-6* structural locus, at least as the latter is reflected by electrophoretic variants.

The utility of differences in the thermal stabilities of enzymes as a technique for detecting differences in their amino acid sequences has been moderately well established. LANGRIDGE (1968a), using a series of mutations at known sites on the β-galactosidase gene of *Escherichia coli*, found that when a serine residue was inserted at each of these sites, over half of these changes caused detectable changes in stability when compared to a standard wild-type strain. In contrast, such changes were reflected in changes in Michaelis constants in only 4% of the cases (LANGRIDGE 1968b), although differences in maximal enzyme velocities also occur in most mutants. However, measurements made using impure enzyme preparations may give variable estimates of kinetic parameters (*e.g.*, MCDONALD *et al.* 1977). A similar situation has been reported for mutations of tryptophan synthetase (YUTANI *et al.* 1977). In this case, 11 of 12 of the single-site mutations examined showed altered stabilities, and those stabilities were both greater than or less than that of wild-type enzyme. While these findings do not suggest that stability determination by itself is more sensitive than other methods of analysis, they do suggest that if applied in conjunction with electrophoretic separation, considerable resolution of hidden variation will be possible.

With these considerations in mind, we initiated a study of heat stability variation of esterase-6 in *Drosophila melanogaster*. Earlier work (COCHRANE 1976) showed that there are in fact stability variants existing in natural populations. These results were based strictly on visual scoring of starch gels, and suffered therefore from a lack of sensitivity and quantification. We report here a substantial refinement of these techniques and consider the genetic basis of stability variants more rigorously. In particular, our aims were to try to detect as many hidden variants as possible among 398 isogenic third chromosome lines extracted from four populations, confirm the differences seen on starch gels by means of spectrophotometric assay, examine the segregation of thermal stability variation in F_1 and F_2 progeny for evidence of nonallelic effects, and, based on this large sample, try to reach some conclusions as to how well the observed frequency distributions agree with those predicted by the hypothesis of selective neutrality.

The choice of *Est-6* as an object of study was, to a considerable extent, suggested by these objectives. Its genetics are well characterized, the structural locus having been mapped to 3–36.8 on the standard genetic map (WRIGHT 1963) and to 69A1–5 on the cytological map (AKAM *et al.* 1978). It can be readily assayed in both gel and spectrophotometric systems (see MATERIALS AND METHODS). Most important, however, it is *a priori* a good candidate for a test of the neutral hypothesis. At least six electrophoretic classes are known to occur naturally (GIRARD and PALABOST 1976). Furthermore, the two most frequent classes have been subdivided into a total of six different thermal stability classes, with two showing a relative mobility of 1.00 and four showing one of 1.10 (WRIGHT and MACINTYRE 1965; COCHRANE 1976). Moreover, the homologous locus in *D. pseudoobscura*, *Est-5*, is one of the most variable known in that species (COBBS

1976; McDOWELL and PRAKASH 1977; COYNE, FELTON and LEWONTIN 1978). This wealth of apparent allelic variation, combined with the observation that flies homozygous for null alleles of *Est-6* are viable and fertile (JOHNSON, WALLIS and DENNISTON 1966; BELL, MACINTYRE and OLIVIERI 1972), suggests that this locus may be capable of being highly variable without affecting individual or population fitnesses. Indeed, YAMAZAKI (1971) has shown that *Est-5* genotype has no detectable effects on various fitness components in population cage experiments.

MATERIALS AND METHODS

Natural populations of *D. melanogaster* were sampled by collection from fermenting banana baits (for locations and dates of collections, see Table 6). Wild males and F_1 sons of wild females were crossed to virgin females heterozygous for the balancer chromosome, *TM3* (LINDSLEY and GRELL 1968), and homozygous third chromosome lines were obtained by the standard series of crosses (*e.g.*, WALLACE 1968). Chromosome samples from Cedar Rapids, Iowa, were isolated by the same method from isofemale lines from that area, kindly provided us by ROGER MILKMAN and BONNIE SAMPSELL. One chromosome was isolated from each line. All isogenic lines were maintained with the balancer present (*i.e.*, as *TM3*/+), so that chromosomes carrying recessive lethal or sterility alleles would not be lost.

The starch gel techniques of COCHRANE (1976) were used to survey isogenic lines for thermal stability variants. At least two separate assays were performed on each line. Many of the isogenic lines carried recessive lethal alleles and thus could not be made homozygous. In these cases, if the chromosome in question carried an *Est-6*$^{1.10}$ allele, the stability of esterase-6 was determined in *TM3*/+ heterozygotes, since *TM3* carries *Est-6*$^{1.00}$. If, on the other hand, the isolated chromosome was found to carry *Est-6*$^{1.00}$, then *TM3*/+ males were crossed to females homozygous for *Est-6*$^{1.10}$. Stability of the *Est-6*$^{1.00}$ band was then determined in wild-type F_1 progeny that were genotypically *Est-6*$^{1.00}$/*Est-6*$^{1.10}$. Control crosses with nonlethal chromosomes carrying known stability variants showed the stability of the allozyme in question to be unaffected by being heterozygous for a second electrophoretic allele.

For the analysis of single flies from crosses, individuals were homogenized in 40 μl of 0.1 M sodium phosphate buffer, pH 6.5. Half of each homogenate was then drawn into a capillary tube, which was inserted in a sealing block. This block was then placed in a waterbath at 59.5° for five min, after which the tubes were placed in an ice bath. The unheated portion of each homogenate was absorbed onto filter paper wicks, as were the heated portions in the capillary tubes. These were then inserted into a starch gel, electrophoresed, and stained for enzyme activity. Stability was scored as the presence or absence of a band after heating, relative to that present in the unheated aliquot.

Spectrophotometric measurement of esterase activity was accomplished by means of a modification of the assay methods of VAN ASPEREN (1962). In a typical stability experiment, ten males of a particular *Est-6* genotype were homogenized in 1.0 ml of 0.1 M sodium phosphate buffer, pH 6.5. This homogenate was centrifuged at 16,500 g for five min, and the supernatant was divided into six aliquots in 6 × 50 mm test tubes. These tubes were then placed in a waterbath equipped with a Haake Model E52 circulator, which was maintained at 54.0 ± 0.1°, and heated for zero, three, six, nine, 12 and 15 min. Following heat treatment, two 50 μl samples of each aliquot were placed in a tube containing 5 ml of 0.04 M sodium phosphate buffer, pH 6.5. which was 3×10^{-4} M β-naphthyl acetate and 1% acetone. These tubes were then incubated at 27° for 30 min, after which 1 ml of a 5:2 solution of 5% sodium dodecyl sulfate and 1% tetrazotized *o*-dianisidine (diazo blue B, Sigma Chemicals) was added to quench the reaction. The red color that developed, the result of reaction between the dye and β-naphthol, was then measured at 555 nm in a Turner Model 330 spectrophotometer. Previous studies (RICHMOND and COCHRANE, in preparation) have shown that, under these conditions, the assay is linear with reaction time, protein concentration and amount of β-naphthol produced.

RESULTS

Detection of stability variation: A total of 398 isogenic lines from four natural populations were assayed for electrophoretic phenotype and for stability phenotype by means of the visual scoring system on starch gels (for examples of typical gels, see Figure 1 of COCHRANE 1976). This approach, which depends on differences in electrophoretic mobility and heat stability, allowed the detection of seven classes of variants; three possessed mobility of 1.00, while four showed mobility of 1.10. These classes are described in Table 1.

Of these seven classes, only *Est-6*$^{1.00(5)}$ was not detected in previous work (COCHRANE 1976). In addition, *Est-6*$^{1.10(1)}$ was considered to be a separate electrophoretic class by COCHRANE (1976), and was designated *Est-6*$^{1.08}$. Subsequent work (COCHRANE and RICHMOND 1979) has shown that this mobility difference is due to the presence of a rare recessive allele of a locus controlling a post-translational modification of esterase-6. In all of the lines surveyed here, this allele of the modifier locus was not present, so that the 1.08 mobility phenotype was not seen. Hence, this esterase-6 allozyme could be detected only by means of heat stability, and thus could not be considered an electrophoretic class.

While the visual scoring of zymograms following heat treatment of crude homogenates provides a rapid and reproducible means of assaying large numbers of isogenic lines, it does not provide a means of rigorously quantitating the observed heat stability differences, since apparent stability differences may in fact be due to differences in enzyme activity (BEWLEY 1978). In order to do this, a spectrophotometric assay system was developed to measure the rate of loss of esterase-6 activity with time of heating. The complicating factor here is that there are several enzymes in *Drosophila melanogaster* showing esterase activity (BECKMAN and JOHNSON 1964), so that it is potentially difficult to assay esterase-6 activity alone.

This problem was circumvented as follows. We had observed in starch gels that, after five minutes of heat treatment at 57.0°, the esterase-6 band was the only one apparent when the gel was stained for esterase activity (COCHRANE

TABLE 1

*Behavior of different heat stability classes on electrophoretic assay**

Allele	Time heated, min 0	5	10	15	20
1.00(1)	++++	++++	++++	+++	+++
1.00(2)	++++	+++	++	+	0
1.00(3)	++++	++	+	0	0
1.10(1)	++++	++++	+++	+++	++
1.10(2)	++++	+++	++	+	0
1.10(3)	++++	++	+	0	0
1.10(4)	++++	+	0	0	0

* Number of plus signs indicates staining intensity relative to that of unheated control (set at ++++). *1.00* lines were heated at 59.5°; *1.10* lines were heated at 57.0°. Zeroes indicate absence of detectable activity.

1976; Figure 1A). The availability of a stock line (*car*, available from the Mid-America Drosophila Stock Center, Bowling Green, Ohio) which was reported by JOHNSON, WALLIS and DENNISTON (1966) to be homozygous for a null allele of esterase-6, allowed us to confirm this fact. Figure 1 shows the results of treatment at 54.0° of homogenates from lines homozygous for *Est-6*$^{1.00(1)}$ and *Est-6*$^{1.10(4)}$, and of the *car* (*Est-6*^{null}) stock. Clearly, after three minutes, the preparation from the null stock shows no discernable esterase activity. Thus, we conclude that in those lines with active *Est-6* alleles, only the enzyme produced by that locus retains activity after this treatment.

Activity readings obtained after heat treatment were transformed into natural logarithms to determine if heat inactivation of esterase-6 followed first-order rate kinetics. The results of this transformation are plotted for the seven stability classes in Figure 2. In every case, when the activity measurements obtained after three minutes or more of heat treatment were examined, an excellent fit was obtained, with a least-squares linear fit routinely explaining over 95% of the observed variance. Accordingly, the slope of the regression can be considered to

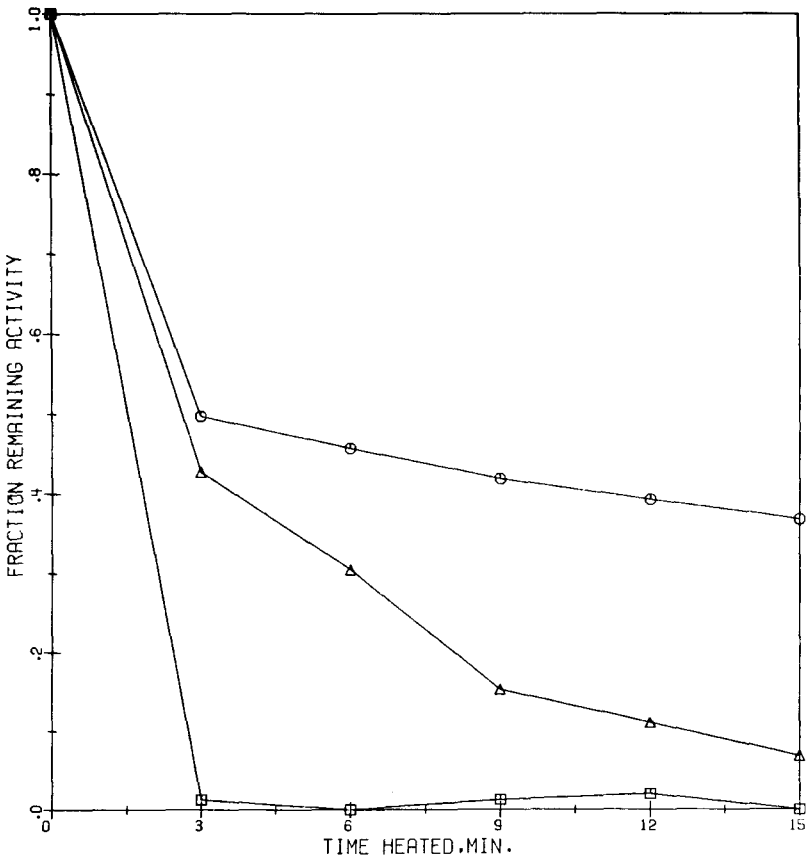

FIGURE 1.—Esterase activity remaining after heating for indicated times at 54.0°. □---□ *Est-6*^{null} allele. ○---○ *Est-6*$^{1.00(1)}$. △---△ *Est-6*$^{1.10(4)}$.

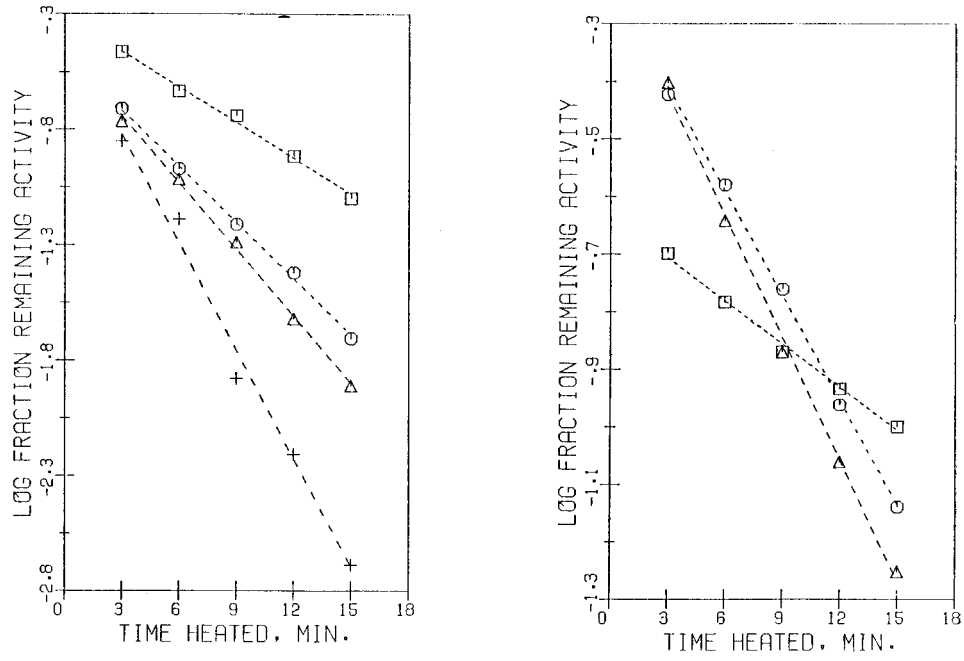

FIGURE 2.—Relation between log percent remaining esterase-6 activity and time heated at 54.0°. Left Panel —*Est-6*$^{1.10}$ alleles: □---□ *1.10(1)*; ○---○ *1.10(2)*; △---△ *1.10(3)*; +---+ *1.10(4)*. Right Panel—*Est-6*$^{1.00}$ alleles: □---□ *1·00(1)*; ○---○ *1.00(2)*; △---△ *1.00(3)*.

be a rate constant for the inactivation process, and the product of its reciprocal and the natural logarithm of 2 to be the half-life of enzyme activity.

With this quantitative assay of stability available, the reliability of the gel assay system could be confirmed. It would have been most desirable to examine all of the lines using this assay. However, all lethal-bearing lines could not be assayed, and in addition, many of the lines analyzed earlier had been lost. Thus, for each stability class (as determined by the gel assay), two isogenic lines were selected at random and assayed spectrophotometrically. Two replicate assays of each line were performed. Decay constants and half-lives were then obtained, (shown in Table 2). The data summarized in Table 2 were analyzed by analysis of covariance methods (SOKAL and ROHLF 1969). The following questions were asked: (1) Are there significant differences between replicate determinations of K for a particular isogenic line? Use of t-tests to compare two regression coefficients indicated no heterogeneity of slope within any line. (2) Is there variation between lines that had been categorized as having identical stability by means of the gel assay? To test this, results from the two replicate assays for each line were pooled to give a single regression for each line, and pairwise comparisons were made between lines believed to show identical esterase-6 stability and mobility. In six of the seven cases, no significant differences were found. However, in the case of *Est-6*$^{1.10(3)}$, the difference between the two lines was highly significant ($p<0.01$), indicating that the spectrophotometric assay system is more sensitive

TABLE 2

Average rate constant of thermal denaturation for each esterase-6 class detected on gel assay

Allele	K	*s.e.*
1.10(1)	—0.0458	0.005
1.10(2)	—0.0811	0.001
*1.10(3a)**	—0.0959	0.005
*1.10(3b)**	—0.123	0.003
1.10(4)	—0.158	0.005
1.00(1)	—0.0245	0.002
1.00(2)	—0.0615	0.001
1.00(3)	—0.0727	0.002

* Separate rate constants for lines initially scored as *1.10(3)* on gel assay, but found to be different on spectrophotometric assays (see text).

than the gel assay. (3) Do the lines categorized as having different stabilities in the gel assay show different decay rates? In order to test this, single regressions were obtained from the data for all lines having a particular stability; the rate constants thus obtained are those reported in Table 2. These were then divided into electrophoretic classes, and the *a posteriori* test of differences among regression coefficients given by SOKAL and ROHLF (1969, page 457) was used to test all pairwise comparisons of stability classes within electrophoretic classes. All such comparisons except for that between *Est-6*$^{1.10(2)}$ and *Est-6*$^{1.10(3a)}$ were significant at or beyond the 0.05 level, indicating that the majority of differences observed on gels are not artifacts of the qualitative scoring method employed. It was found in the one case of the two classes that showed no significant difference that if the temperature of heat treatment was increased to 58.0°, the rate constants obtained did indeed show a highly significant difference (—0.193 for *Est-6*$^{1.10(2)}$ and —0.294 for *Est-6*$^{1.10(3a)}$).

The analysis of covariance of the regression data also allows an assessment of the potential number of variants that can be detected by the techniques employed. A formula given by SOKAL and ROHLF (1969, page 455) for a test of the significance of the difference between two regression coefficients allows us to determine the minimum difference that would be significant, based on the error mean square for the pooled regressions. For the five 1.10 classes, this minimum significant difference was found to be 0.016. The range of K for these variants is 0.113, indicating that approximately seven (0.113/0.016) thermal variants having 1.10 mobility might have been detected. A similar calculation for the 1.00 variants indicates that six thermal variants could have been found. These calculations are likely to provide only a minimum estimate of the number of potentially detectable thermal variants within an electrophoretic class, since they assume that the variants found provide an approximation to the total range of K values likely to exist. Nevertheless, if each of the six electrophoretic classes of esterase-6 could be fractionated into only five thermal variants per class, this procedure could potentially reveal at least 30 variants at this locus.

Genetic analysis of stability variation: Thus far, we have shown that there is extensive, genetically determined variation in heat stability of esterase-6 in the lines examined. What has not been shown is whether the variation is in fact due to allelic variation at the *Est-6* locus, or whether it is due to the effects of other loci on the conformation of the protein molecule. In order to determine if the variation is allelic, we sought to demonstrate two conditions. First co-dominance for heat stability should be seen in F_1 progeny of crosses of lines of different stability categories. Second, stability should not be separable from mobility by recombination in F_2 progeny of similar crosses.

With respect to the co-dominance of stability variants, the methods used for examining alleles on lethal-bearing chromosomes suggests that this is indeed the case. All variants were examined in both homozygous viable lines and in heterozygotes resulting from crosses to lines bearing a different electrophoretic allele. In no case was the apparent stability, as visualized on starch gels, different when the two were compared. Therefore, at least when in combination with the electrophoretic allele carried by the chromosome to which these lines were crossed, co-dominance was observed.

As an alternative means of demonstrating co-dominant inheritance of stability in F_1's, we employed the simplified triple-testcross design of FULKER (1972). Briefly, this procedure involves crossing isogenic lines that differ for some measurable phenotype to two lines that show extreme values of that phenotype, and measuring the phenotype in the progeny of each cross and in each parental line. The results of such a design, when fully replicated, can be subjected to an analysis of variance, in which the variance is partitioned into additive, dominance, and epistatic components. In the case of enzyme stability, if co-dominant alleles of a single locus are the sole source of genetic variation, we would predict that only the additive component would be significant.

Table 3 presents the results of such a cross of homozygous lines of five different stability classes. Each cross was repeated and replicate assays were performed on each. As a result of the exponential nature of the decay curve, the expected midparental value of the rate constant of decay in a heterozygote is the geometric mean of the parental rate constants. Therefore, in performing the analysis of variance, rate constants were transformed into their natural logarithms. As predicted, the additive component is highly significant. However, there is also a significant dominance component. This finding is not consistent with the single-locus co-dominant alleles hypothesis, but has been found in other systems (WATT 1977).

We explain this discrepancy as follows. In other work (RICHMOND and COCHRANE, in preparation), we have found large strain-specific differences in esterase-6 activity that appear to be due to factors other than the *Est-6* genotype. Since we measure stability by means of enzyme activity, if the two alleles present in particular F_1's make unequal contributions to the total enzyme activity pool present, the apparent stability of the enzyme will be different from the midparental value, in the direction of that of the parental allele making the larger contribution.

TABLE 3

Results of the triple-testcross analysis

Female parental allele	Replicate	Male parental allele *1.00(1)*	*1.00(2)*	*1.00(3)*	*1.10(1)*	*1.10(4)*
1.00(1)	A	0.029	0.051	0.044	0.027	0.057
	B	0.025	0.048	0.046	0.033	0.045
	Expected*	0.027	0.042	0.044	0.032	0.062
1.10(4)	A	0.069	0.070	0.090	0.059	0.123
	B	0.067	0.081	0.078	0.077	0.156
	Expected*	0.062	0.095	0.100	0.073	0.140
Male	A	0.029	0.066	0.071	0.038	0.123
Parental†	B	0.025	0.063	0.072	0.039	0.156

Analysis of Variance

Source	*d.f.*	*MS*	*F*	Significance
Additive	4	0.2980	26.14	0.001
Dominance	4	0.0560	4.91	0.025
Epistasis	4	0.0097	0.85	n.s.
Error	13	0.0114		

Values given are absolute values of rate constants of inactivation at 54.0° of esterase-6 of the various genotypes.

Crosses were replicated twice, and stability was assayed twice on each replicate. Thus, all rate constants given are the average of two determinations.

* Expectations in heterozygotes are the geometric means of the average rate constants of the two parental lines in question. Those given for *1.00(1)* and *1.00(4)* homozygotes are the average of all determinations on such homozygotes.

† Results of assays of lines homozygous for indicated alleles, raised in conditions identical to those of crosses.

Data presented in Table 4 support this hypothesis. Esterase-6 activity was determined by multiplying the *Y*-intercept of the linear portion of the log percent remaining activity *vs.* time heated regression (the percent of the total esterase in the unheated sample that is esterase-6) by the observed activity in the unheated sample. In this experiment, two isogenic lines, which were previously known to exhibit differences in both stability and activity, were crossed, and the two

TABLE 4

Determination of the relative contribution of alleles with different levels of esterase-6 activity to the apparent thermal stability of esterase-6 in F_1 progeny

Est-6 genotype	$K \pm$ s.e.	Half-life	*Est-6* activity*
1.00(2)/1.00(2)	—0.0664 ± 0.006	10.44	0.948
1.10(1)/1.10(1)	—0.0451 ± 0.002	15.37	0.444
1.00(2)/1.10(1) (F_1)	—0.0625 ± 0.002	11.09	0.890
1.00(2)/1.00(2)† *+1.10(1)/1.10(1)*	—0.0592 ± 0.002	11.71	0.740
1.00(2)/1.00(2) +1.10(1)/1.10(1)‡	—0.0518 ± 0.002	13.38	0.843

* O.D. units per individual.

† Mixture of equal number of flies of two genotypes.

‡ Mixture of equal activity from homogenates of two homozygous lines.

parental lines, the F_1 progeny, and mixtures of (1) equal numbers of parental flies, and (2) amounts of parental homogenates that have equal activity, were assayed. The decay constant of the F_1 enzyme is significantly different from both the predicted midparental value of −0.0547 and that of the mixture of equal amounts of activity, but is not significantly different from the mixture of equal numbers of parental flies. This suggests that the two lines do in fact have different amounts of esterase-6 activity, and this difference persists in F_1 progeny. Therefore, at least in this case, the apparent dominance for stability can be explained as an artifact of the assay procedure.

The degree of genetic linkage between the loci determining electrophoretic mobility and thermal stability of esterase-6 was determined by using the single-fly gel assay outlined in MATERIALS AND METHODS. Flies of different mobility phenotypes and with the most extreme possible stability difference—*Est-6*$^{1.00(1)}$ and *Est-6*$^{1.10(4)}$—were crossed, the resulting F_1 crossed, and F_2 progeny were collected. When testing parental individuals in the single-fly system, we found that, after treating for five minutes at 59.5°, all *Est-6*$^{1.10(4)}$ activity was lost, while substantial *Est-6*$^{1.00(1)}$ activity remained. A representative gel is shown in Figure 3. Three hundred nine F_2 males were treated in this manner, and the results are presented in Table 5. No recombinants between stability and mobility were found. If one such recombinant had been seen, this would correspond to a map distance of 0.32 map units, or using the estimate of GELBART, MCCARRON and CHOVNICK (1976) of 580,000 bases of DNA per map unit in Drosophila, to less than 185,600 bases. Thus, while these data do not rule out the possibility of a second, tightly linked gene being responsible for determining the enzyme's stability, they strongly suggest that this variation is due to allelic variation at the *Est-6* structural locus.

Analysis of allele frequencies: Having determined that the detected variation of esterase-6 is best explained by the hypothesis of allelic variation at the *Est-6* structural locus, we wished to analyze the frequency distributions of these putative alleles within and between the four populations sampled. Allele frequencies are given in Table 6. As noted above, it was impossible to assay all the lines spectrophotometrically; thus, the frequencies given are based upon the visual

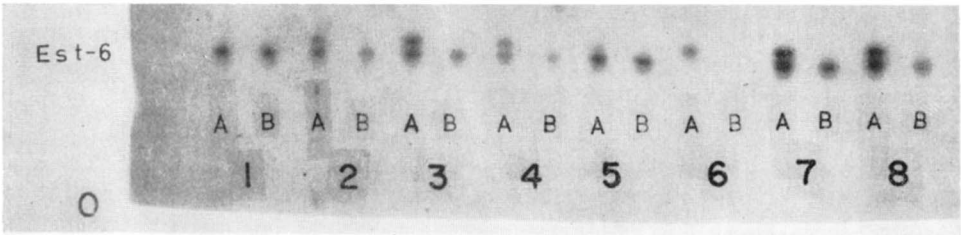

FIGURE 3.—Zymogram showing the results of heat treatment of single fly homogenates for five minutes at 59.5°. Slots labelled A are unheated portions; those labelled B are heated.
Slot 1 : *Est-6*$^{1.00(1)}$ homozygote
Slot 6 : *Est6*$^{1.10(4)}$ homozygote
Slots 2, 3, 4, 5, 7, 8: *Est-6*$^{1.00(1)}$/*Est-6*$^{1.10(4)}$ heterozygotes

TABLE 5

Results of electrophoretic and stability analysis of F_2 progeny from the cross Est-$6^{1.00(1)}$/Est-$6^{1.00(1)}$ × Est-$6^{1.10(4)}$/Est-$6^{1.10(4)}$

Electrophoretic phenotype, unheated	Possible bands remaining after heating	Number
1.00	1.00	46
	none	0
1.00/1.10	1.00	195
	1.10	0
	1.00/1.10	0
	none	0
1.10	1.10	0
	none	68
Total		309

scoring system. In at least one case (*Est-*$6^{1.10(3)}$), the "allele" frequencies given are now known to be the sum of the frequencies of at least two different alleles. Therefore, any consideration of these data must be made with the recognition that not all alleles have been detected.

When the electrophoretic mobility criterion alone is applied, the 1.00 allele is most frequent in all populations. This picture does not change when the stability criterion is also applied; *Est-*$6^{1.00(2)}$ is most common, with a frequency of over 0.5 in all populations. However, the remainder of the distribution does become more complex, although no variant is unique to a particular population.

In order to further analyze the within-population distributions with respect to the question of the selective significance of the polymorphism, we applied the test of selective neutrality proposed by EWENS (1972), first to the frequency

TABLE 6

Allele frequencies based on electrophoretic mobility and the distribution of stability alleles within electrophoretic classes

Location	Collection date	N†	Electrophoretic class frequencies 1.00	1.10	1.15	2-band*
Bloomington, IN	9/76	138	0.557	0.429	0.000	0.014
McDougall, NY	7/76	103	0.602	0.379	0.019	0.000
Lake Higgins, MI	8/76	52	0.730	0.270	0.000	0.000
Cedar Rapids, IA	6/75	105	0.648	0.352	0.000	0.000

	N†	Thermal class frequencies within electrophoretic classes 1.00(1)	1.00(2)	1.00(3)	1.10(1)	1.10(2)	1.10(3)	1.10(4)
Bloomington, IN	138	0.007	0.507	0.043	0.116	0.152	0.065	0.094
McDougall, NY	103	0.058	0.534	0.010	0.165	0.078	0.029	0.107
Lake Higgins, MI	52	0.115	0.577	0.038	0.058	0.135	0.000	0.077
Cedar Rapids, IA	105	0.057	0.562	0.029	0.229	0.057	0.010	0.057

* This refers to an allele that shows two very closely spaced bands in homozygotes. See COSTA, DANIELI and MORBINI (1977).

† Number of lines examined.

distribution of electrophoretic variants and subsequently to that observed when the stability criterion was added. Our intent was to determine if, with more refined detection of variation, the results obtained from the test were altered significantly. We found that in no case was there a significant deviation from the hypothesis of selective neutrality. This test, however, suffers from some weaknesses that are reviewed below (see DISCUSSION). The alternative testing procedure of WEIR, BROWN and MARSHALL (1976), designed to test the charge-state model, is not applicable here, since it is appropriate only in the analysis of electrophoretic allele distributions and does not address the question of the distribution of electrophoretically silent alleles.

Lack of divergence of allele frequencies among different populations might be taken as evidence that selective forces are operating to maintain this variation. In order to compare the frequency distributions of the different populations, we employed the measure of minimum genetic distance between populations suggested by NEI and ROYCHOUDHURY (1974):

$$D_m = \left[\sum_{i=1}^{k} p_{i1}^2 + \sum_{i=1}^{k} p_{i2}^2 \right] /2 - \sum_{i=1}^{k} p_{i1}p_{i2}$$

where p_{ij} is the frequency of the ith allele in the jth population (j=1,2) and k is the number of different alleles. This measure has the advantage that the null hypothesis that D_m equals zero can be tested by means of the χ^2 statistic:

$$\chi^2 = n_1 n_2 \sum_{i=1}^{k} \left[\frac{(p_{i1} - p_{i2})^2}{p_{i1}n_1 + p_{i2}n_2} \right]$$

where n_j is the sample size (number of alleles) drawn from the jth population. Our intent was to use this measure to determine if the observed similarity of electromorph frequencies, suggestive of selective maintenance of the polymor-

TABLE 7

Values of interpopulational minimum genetic distance (D_m) *determined by methods of* NEI *and* ROYCHOUDHURY *(1974)*

Population 1	Population 2	D_m	Electrophoretic frequencies χ^2	d.f.	Signif.
Bloomington	McDougall	0.0023	0.66	3	n.s.
Bloomington	Lake Higgins	0.0277	5.08	2	n.s.
Bloomington	Cedar Rapids	0.0072	3.19	2	n.s.
McDougall	Lake Higgins	0.0143	3.06	2	n.s.
McDougall	Cedar Rapids	0.0016	2.27	2	n.s.
Lake Higgins	Cedar Rapids	0.0067	1.07	1	n.s.
		Electrophoretic and Thermal Frequencies			
Bloomington	McDougall	0.0072	17.05	8	< 0.05
Bloomington	Lake Higgins	0.0125	17.80	7	< 0.05
Bloomington	Cedar Rapids	0.0161	22.23	7	< 0.005
McDougall	Lake Higgins	0.0113	10.10	7	n.s.
McDougall	Cedar Rapids	0.0045	7.01	7	n.s.
Lake Higgins	Cedar Rapids	0.0197	10.83	6	n.s.

phism, could simply be a result of the limited resolution of electrophoresis. If so, divergence, manifested as genetic distances significantly different from zero, should be found when additional alleles are resolved.

The results of all pairwise determinations of genetic distance, using first the mobility criterion alone and then both mobility and stability criteria, are presented in Table 7. There is no significant divergence between any pair of populations on the basis of electromorph frequencies. When the stability criterion was added, we found that, while the Bloomington populations showed significant divergence from each of the other three populations, no differences were found among the remaining three. Without analyzing additional populations, it is difficult to draw conclusions about the amount of divergence that has occurred at this locus.

DISCUSSION

The intent of this study was to determine the genetic basis of variation in heat stability of esterase-6 in *Drosophila melanogaster*, and to use this criterion in conjunction with electrophoretic techniques to determine the frequency distributions of such variants in different natural populations. It was hoped that such data would allow a less ambiguous test of the selective significance of allelic variation at the *Est-6* locus.

It is clear, for esterase-6, that heat stability is an excellent means of detecting genetic variation not resolved by conventional electrophoretic techniques. Its power lies in the fact that it is possible to confirm stability differences quantitatively and objectively, without reliance on subjective visual scoring. Furthermore, use of a spectrophotometric assay made it possible to verify that the enzymatic reaction in question is linear with respect to time, protein concentration and product production. Demonstration of these conditions is essential if an enzyme assay, either conventional or using gel scanning techniques, is to be valid.

It may be argued that we have not conclusively demonstrated that stability variation is in fact due to allelic variants of *Est-6*. If we accept a subunit molecular weight of 51,000 (SASAKI and NARISE 1978), the *Est-6* locus should be on the order of 1.5 kB of DNA, or 0.003 map units. In order to map variants to this range, over 30,000 individuals would have to be scored; in the absence of a selective screening system, this is clearly impractical. We must, therefore, accept this level of uncertainty and proceed on the assumption that the variants detected are in fact *Est-6* alleles.

The allele frequency distributions in themselves offer little hope of resolving the question of selection *vs.* neutrality. The Ewens test, as elsewhere noted (EWENS 1977), suffers from some weaknesses. First, it lacks power in detecting selection for a common allele, and indeed its power to detect balancing selection in a sample of less than 200 alleles is questionable (EWENS and FELDMAN 1976). Second, this lack of power will make it nearly impossible to distinguish between the null hypothesis that all alleles are selectively equivalent and a situation in which some alleles are affected by selection while others are not.

Conclusions regarding interpopulational divergence of gene frequencies are similarly unsatisfactory. While heat stability provides an excellent means of detecting amino acid sequence differences in protein molecules, we have no good idea of how sensitive it may be. It is evident that the frequency data reported do not reflect all the allelic variation present, since in one case ($Est\text{-}6^{1.10(8)}$) the gel assay did not resolve two variants. Therefore, the lack of detectable divergence may still be due to pooling of allele frequencies. On the other hand, even if all alleles were to be detected, low levels of interpopulational migration could account for the observed similarities (LATTER 1973). Thus, without accurate knowledge of the true allele frequency distributions, and without some knowledge of population structure, gene frequency analysis of this sort will almost certainly give ambiguous results (EWENS and FELDMAN 1976).

Analysis of electrophoretically silent alleles of homologous loci in the sibling species *D. pseudoobscura* and *D. persimilis* has provided evidence that greater genetic divergence has occurred between these species than had been believed previously (COYNE 1976; COYNE and FELTON 1977). The allele frequency data reported here do not provide conclusive evidence with respect to genetic divergence between the populations studied. It was hoped that, as an alternative to making allele frequency comparisons, the distributions of phenotypes in different populations might be informative. RICHARDSON and SMOUSE (1976) give mathematical methods for partitioning phenotypic variance into components due to different levels of taxonomic diversity—among populations, among species, etc. We have modified their methods slightly to estimate the fraction of phenotypic variance due to intra- and interpopulation variation. Variance components for these populations were calculated for two sets of phenotypic measures—relative mobilities of esterase-6 and relative mobility and rate constant of decay at 54.0°. In both cases, among-population variance accounted for 6% of the total observed variance. Hence, we conclude in this case that, as in the case of gene frequency analysis, consideration of phenotypic distribution is no more informative when a more sensitive measure is applied. Incidentally, this finding, if found to hold generally, supports the use of electrophoretic data in studies of genetic divergence among taxa, since the addition of a more refined phenotypic measure may not provide any more information concerning the amount of divergence that has taken place.

In summary, it would appear that gene frequency analysis, by itself, cannot be expected to shed light on the role of natural selection in maintaining enzyme polymorphisms. In order to make such inferences, it may first be necessary to gain understanding of the *in vivo* physiological function of the enzyme in question, and then to determine if there are functional differences between allozymes that result in fitness differences between individuals under laboratory conditions. Such information could make possible the experimental use of specific selective agents as a means of determining the influence of environmental factors on allele frequencies at the specific locus in question (CLARKE 1975). Only then will it be possible to make inferences about the role of selection in maintaining the observed polymorphism.

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