ESTERASES OF DROSOPHILA
II. BIOCHEMICAL STUDIES OF ESTERASE-5 in *D. pseudoobscura*

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ABSTRACT

In vitro enzyme hybridization was carried out with combinations of six allozymic variants of Esterase-5 from *Drosophila pseudoobscura*. Studies on heat stability and specific activity changes accompanying hybridization were done to examine the possible expression of overdominance at the biochemical level. In 11 of 15 combinations no significant change in specific activity was found following hybridization. In two cases hybridization resulted in a decrease in activity in the mixture, while in two cases esterase activity was elevated. Heat stability studies, in several cases, revealed reduced rates of inactivation in *in vitro* and *in vivo* heterozygotes compared with homozygotes. From these and other data a model for the molecular mechanism of heterosis is presented.

ELECTROPHORETIC studies of natural populations of many species have revealed the existence of ubiquitous polymorphism for a host of gene-enzyme systems (e.g., LEWONTIN and HUBBY 1966; PRAKASH, LEWONTIN and HUBBY 1969; SELANDER, HUNT and YANG 1969; BERGER 1971, 1973; AYALA and POWELL 1972). The fundamental issue presently at hand among population biologists is whether the maintenance of this variation can be attributed primarily to selective forces (KOJIMA and YARBROUGH 1967; MILKMAN 1967; SVED, REED and BODMER 1967; CLARKE 1970; RICHMOND 1970; WILLS 1973) or to random genetic drift (KIMURA 1968; KING and JUKES 1969; KIMURA and OHTA 1971). With essentially identical data on allele frequency distributions, reasonable arguments favoring both viewpoints have been proposed. However, very little direct experimental evidence has been provided at the molecular level to demonstrate whether or not fundamental and important differences exist between allozyme variants.

The purpose of this study is to examine a single polymorphism biochemically and determine whether any important kinetic differences can be detected among allelic forms using several criteria. It was of especial interest to make comparisons between heterozygotes and homozygotes, since one popular notion is that overdominance (heterosis) may be an important selective force in nature (MILKMAN 1967; RICHMOND and POWELL 1970; MANWELL and BAKER 1970; WILLS and NICHOLS 1971; WILLS 1973).

The gene-enzyme system chosen for analysis is the Esterase-5 of *Drosophila pseudoobscura*. Briefly, Esterase-5 is an X-linked enzyme, and at least 8 electro-

phoretically distinguished alleles are known in nature (LEWONTIN and HUBBY 1966; PRAKASH, LEWONTIN and HUBBY 1969). The enzyme is a dimer of molecular weight 110,000 (NARISE and HUBBY 1966) and is the predominant naphthyl esterase of adults (PASTEUR and KASTRITSIS 1971; BERGER and CANTER 1973). The enzyme is most active on short chain acyl esters (NARISE and HUBBY 1966), although its natural substrate is unknown, and its activity can be inhibited by eserine or organophosphates but not by heavy metals or chelating agents (NARISE and HUBBY 1966). HUBBY and NARISE (1967) have shown that under mild physiological conditions mixtures containing extracts from two different homozygous lines will produce a "hybrid" enzyme which is indistinguishable from the intermediate heterodimer seen in zymograms of genetic heterozygotes. In addition *D. pseudoobscura* Esterase-5 was found to hybridize with a homologous enzyme from *D. persimilis* but not with any of the esterases from either *D. virilis* or *D. melanogaster*, two more distantly related species. HUBBY and NARISE (1967) speculate that the ease with which hybrid enzyme forms suggests a dynamic equilibrium between free pools of inactive monomers and activer dimers. In the work which follows, we have utilized this simple technique in an attempt to compare certain biochemical features of homozygotes with *in vitro* heterozygotes. Additional comparisons were made using genetic heterozygotes.

MATERIALS AND METHODS

Drosophila stocks—Six strains of *D. pseudoobscura* homozygous for different *Est-5* alleles were kindly provided by DRS. R. C. LEWONTIN and J. L. HUBBY. The strains were cultured at 25° on a banana-agar-yeast medium and adults or larvae were routinely collected and kept frozen at −20°.

Enzyme hybridization—According to the method of HUBBY and NARISE (1967) Drosophila were homogenized in 0.1 M Tris-HCl, pH 7.2 and centrifuged for 20 minutes at 4° at 15,000 RPM in a Sorvall RC-2B centrifuge and the postmitochondrial supernatants recovered. To promote hybrid enzyme formation between allelic variants aliquots of two different extracts, diluted to approximately equivalent enzyme activities, were mixed and incubated at 25° for up to 2 hours. The appearance of hybrid enzyme was monitored by acrylamide gel electrophoresis following the procedure of HUBBY and LEWONTIN (1966). It is important to note that normal subunit dissociating procedures (urea, free-thawing in salt) are not necessary for hybridization, which occurs spontaneously. For quantitative estimates of hybrid enzyme formation gels stained for esterase were scanned densitometrically in a Canalco instrument (Model G) with the green filter. The scans were then subjected to curve resolution (Dupont Curve Resolver) which permits the relative estimation of staining intensity among overlapping bands by first separating scans into their Gaussian components, and then measuring the percent of the total scan contributed by each component. In virtually all cases overlapping banding patterns could adequately be superimposed by three Gaussian curves.

Spectrophotometric assay—The α naphthyl esterase activity was assayed spectrophotometrically in a Guilford 2400-S recording spectrophotometer according to the method of KATZENELLENBOGEN and KAFATOS (1971) using α naphthyl acetate (Sigma Biochemicals) as the enzyme substrate and Fast Blue RR salt (Sigma) as the dye coupler. Activity was measured over 10 minutes and was expressed either as the change of optical density at 625 nm per minute, or as units of enzyme per ml, where 1 unit of enzyme is equal to a change in optical density per minute per ml of 1.0. For specific activity determinations protein concentration were measured by the method of LOWRY *et al.* (1951) using 3x recrystallized bovine serum albumen as the standard Thermal denaturation studies were carried out in a Haake water bath at either 45° or 50°.

RESULTS

Development and allozymes of Esterase-5

The developmental profile of α naphthyl esterase isozymes has been described previously (BERGER and CANTER 1973) and is summarized in Figure 1. There are at least 10 isozymes detectable on acrylamide gels during development. Bands 1–4 are minor components of pupae and adults, while bands 6–10 are primarily found in early larval and pupal stages. Esterase-5 is present throughout development, being the major isozyme in adults. Enzymes 6–10 are not internally localized in pupae or adults but are rather found in the exuvial space of pupae (that region between the adult cuticle and pupal case), and are not resorbed by the adult but rather persist for some time as exuvial fluid contaminants on the cuticular surface. These enzymes are easily removed from the flies' surface by washing. Because Est-5 is by far the major adult α naphthyl esterase, most of the subsequent kinetic studies were carried out using the postmitochondrial supernatant of adult homogenates.

Eight major allozyme variants of *Esterase-5* are known to exist in natural populations (LEWONTIN and HUBBY 1966). In contrast to the rather uniform distribution of allele frequencies at most loci, among geographically distinct populations, *Est-5* allozyme frequencies vary from locale to locale (PRAKASH, LEWONTIN and HUBBY 1969). Figure 2 illustrates the electrophoretic patterns of the six variants used in this study. Since the active enzyme is a dimer, and the

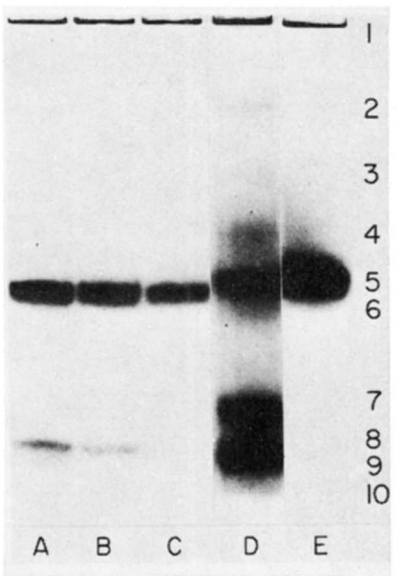

FIGURE 1.—Developmental profile of α naphthyl esterase isozymes in *Drosophila pseudoobscura* (strain Est-$5^{1,12}$). Stages shown are (A) first instar larvae, (B) second instar larvae, (C) third instar larvae, (D) pre-emergent pupa, (E) adult. Approximately 1 mg of tissue was homogenized from each stage. Isozymes are numbered consecutively from the origin; migration is toward the anode (bottom).

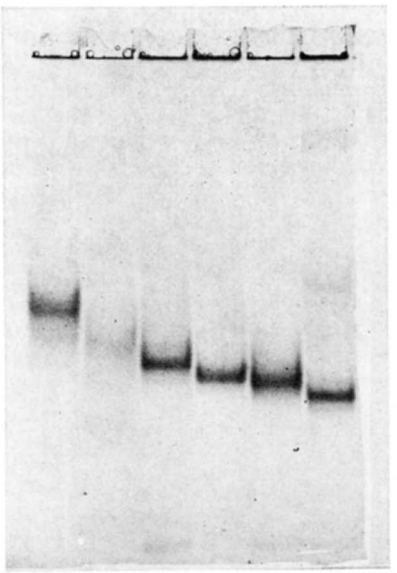

FIGURE 2.—Electrophoretic pattern of Est-5 allozymes from adult *D. pseudoobscura*. The variants from left to right are Est-$5^{.85}$, Est-$5^{.95}$, Est-$5^{1.0}$, Est-$5^{1.03}$, Est-$5^{1.07}$ and Est-$5^{1.12}$. Migration is anodal toward the bottom.

locus is *X*-linked, heterozygous females will show, on gels, a three-banded pattern (Figure 3f and 8) while homozygous females and hemizygous males will show only a single band. These single-band patterns when scanned in the densitometer produce patterns which can be fitted quite well to a single Gaussian distribution.

Hybrid enzyme formation

Preliminary studies were carried out to determine the time required for enzyme hybrid formation *in vitro*. For example mixtures of Est-$5^{.95}$ and Est-$5^{1.12}$ homogenates were incubated at 25° for varying time periods. Aliquots of the incubation at different times after mixing were electrophoresed and stained for α naphthyl esterase activity, and the gels were scanned. As seen in Figure 3, density profiles show the gradual appearance and accumulation of an intermediate, hybrid zone, which presumably represents heterodimeric enzyme (also see HUBBY and NARISE 1967). After 60 minutes the density profile of the *in vitro* mixture strongly resembles the pattern of the genetic heterozygote. Curve resolution studies (Figure 4) allowed the separation of the Gaussian components of each profile and the determination of the proportion of total staining localized in the "hybrid" middle band. In genetic heterozygotes the middle band accounts for about 41% of the total staining, while in the 60-minute mixture the middle band contained about 36% of the total density. It is important to note that peak height and relative area are not always correlated in these analyses. This is clear in Figure 3, where the hybrid band peak in 3E accounts for less of the total area than the hybrid peak in 3F. Incubations up to 2 hours did not appreciably change the *in vitro* pattern. The high value for zero time most likely reflects hybridiza-

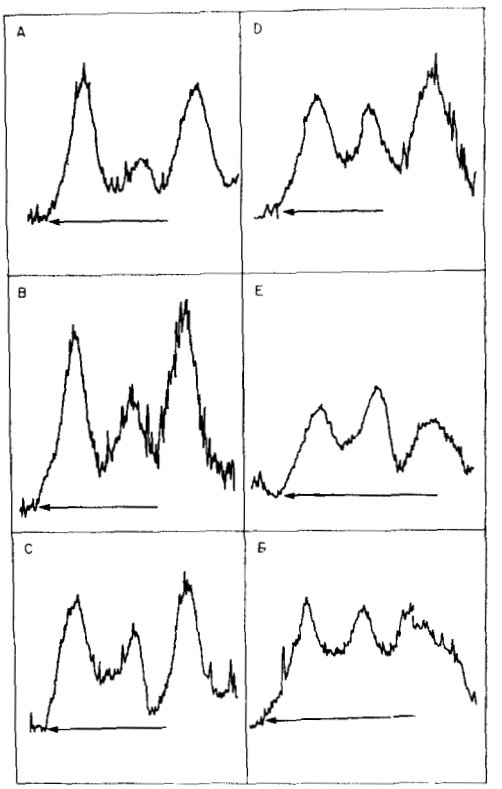

FIGURE 3.—Densitometry scans of Est-$5^{.95}$ + Est-$5^{1.12}$ *in vitro* hybrids after incubation of mixtures for (A) 0 min, (B) 5 min, (C) 20 min, (D) 30 min, (E) 60 min, at 25°. A scan of an Est-$5^{.95}$/Est-$5^{1.12}$ genetic heterozygote is seen in (F). Arrows indicate baseline. Migration is anodal to the left.

tion occurring during the early period of electrophoresis. We have found that hybridization will occur over a pH range of 6.6 to 9.0 and that incubation at 4° significantly reduces the rate of hybrid enzyme formation in a 60-minute incubation.

Enzyme hybridization was found to occur between all the allele combinations for which it was possible to distinguish an intermediate band, with equilibrium established by one hour (also see HUBBY and NARISE 1967). pH optima studies were not carried out for these mixtures. As a result of these studies we chose the 60-minute incubation as a standard time for later experiments.

It should be noted that significant variation was observed between duplicate experiments in Figure 4. We attribute this variation to several factors, among which are: (1) variations in activity between different preparations of the same genotype; this becomes an important consideration because the amount of hybrid enzyme formed very likely depends on the relative concentrations of the two allozyme species in the mixture, (2) errors introduced by staining differences in different experiments, by densitometry and by curve resolution, and (3) some

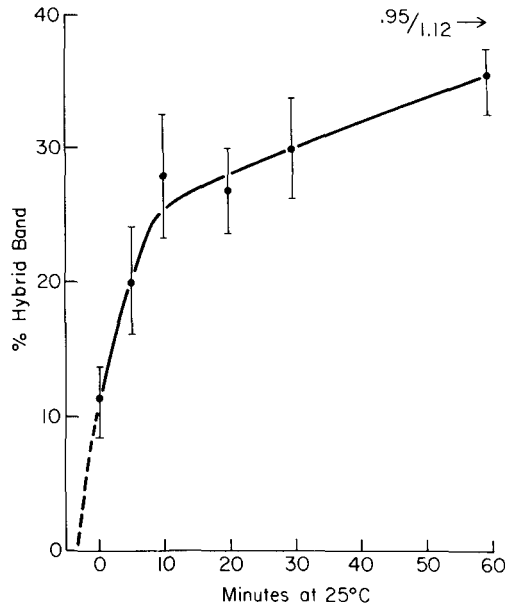

FIGURE 4.—The percent of Est-5 staining in the "hybrid" band following *in vitro* hybridization of Est-$5^{.95}$ + Est-$5^{1,12}$ for varying incubation times. The average and range of three experiments analyzed by curve resolution are presented. The arrow indicates the percent of Est-5 hybrid found in genetic heterozygotes.

contamination with Esterases 6–10 which would contribute to the specific activity. Nevertheless, as a first approximation for judging the time course of the hybridization reaction, the technique appears sufficiently sensitive.

Esterase activity during hybridization

Samples containing each homozygote extract alone were incubated at 25° along with all possible mixture combinations. At zero time and after 60 minutes aliquots of each tube were assayed spectrophotometrically. Comparisons between time zero and 60 minutes for each homozygote revealed, on the average, no change of activity. Comparisons between 60-minute mixtures, and the average of the 2 appropriate 60-minute homozygotes, revealed no significant differences for most combinations (Table 1); but in four cases the activity observed in mixtures differed significantly from the predicted value determined by averaging homozygote activities. Combinations Est-$5^{.85}$ with Est-$5^{1.03}$, and Est-$5^{1.03}$ plus Est-$5^{1.07}$ showed somewhat reduced activities. Combinations Est-$5^{.85}$ plus Est-$5^{1.12}$, and Est-$5^{.95}$ plus Est-$5^{1.07}$ showed, in contrast, increased activities following hybridization. We cannot adequately account for the magnitude of our standard deviations in many cases. The variability is not a function of the assay, for replicate assays from a single mixing experiment give deviations on the order of ±10%. The difficulty apparently stems from variations between duplicate experiments using different homogenates. In fact the largest variations in the data

TABLE 1

The average percent of expected esterase activity in mixtures of various genotypes following in vitro *hybridization, at 25° for one hour*

Mixture	$\frac{\text{Obs. act.}}{\text{Exp. act.}} \times 100$	Standard deviation	Number of experiments
0.85 + 0.95	95.1	8.2	6
+ 1.00	98.6	10.6	9
+ 1.03	86.0	5.0	2
+ 1.07	103.0	3.0	2
+ 1.12	132.8	10.4	13
0.95 + 1.00	103.4	3.8	5
+ 1.03	97.5	5.5	2
+ 1.07	115.0	3.0	2
+ 1.12	101.2	4.8	6
1.00 + 1.03	96.0	12.0	2
+ 1.07	98.5	2.5	2
+ 1.12	99.7	5.6	7
1.03 + 1.07	95.0	1.0	2
+ 1.12	101.0	3.0	2
1.07 + 1.12	105.0	7.0	2

usually appear in cases where many repetitions of the mixing experiment were carried out.

The Est-$5^{.85}$ plus Est-$5^{1.12}$ combination, however, consistently showed elevated activity following hybridization and this combination was studied in further detail. A time course analysis (Figure 5) revealed that the rate at which esterase activity was rising roughly paralleled the rate at which hybrid enzyme was forming (Figure 4). Both plateaued after about 60 minutes, with a steady increase from time zero. The apparent loss of activity in the mixture after 60 minutes, and the slight increase in the Est-$5^{1.12}$ preparation seen in Figure 5, were not reproducible. Generally each homozygote extract maintained the same activity for over 2 hours at 25°, and the activity elevation in the mixture remained stable. A second experiment was done in which the relative ratio of Est-$5^{.85}$ and Est-$5^{1.12}$ was varied in mixtures. As seen in Table 2, the greatest elevation in activity occurred when the ratio of input activity was about 1:1.

Temperature stability studies

We next investigated the thermal stability properties of esterase activity following hybridization. From preliminary work it was found that at 45° or 50° one could follow thermal inactivation over a reasonable time scale. Five combinations were selected for analysis and the results are presented in Figure 6. The major observations are, firstly, that Est-$5^{.95}$ appears to be far more heat stable than the other variants. The remaining variants appear to have stabilities in the order $1.07 > (1.12 \geqslant 1.00 \geqslant 1.03) > .85$. *In vitro* hybrid enzyme stability after 40

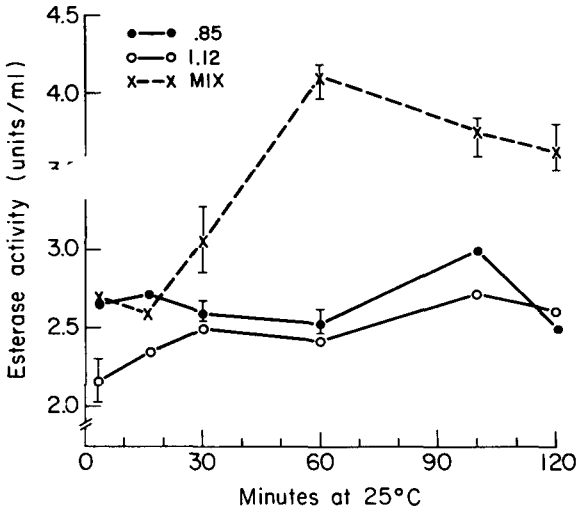

FIGURE 5.—Esterase activity in homogenates of Est-5$^{.85}$, Est-5$^{1.12}$, and the mixture after incubation at 25° for various periods of time. The average and range of activities are shown. Initial activities were adjusted to 2.2 units/ml for Est-5$^{1.12}$, 2.7 units/ml for Est-5$^{.85}$, and the mixture contained an equal volume of each homozygote extract. Twenty microliter aliquots were assayed at the appropriate times.

minutes appears to be somewhat greater than expected in four of the five combinations, although in only two cases (.85+1.07, .85+1.12) is the difference markedly apparent. *In vivo* hybrids for two combinations (Figure 7) also show greater stability than would be predicted on the basis of parental enzyme stability.

Genetic heterozygotes

In vitro hybridization studies of Est-5$^{.85}$ and Est-5$^{1.12}$ revealed an elevation of activity accompanying hybrid enzyme formation. One prediction of this experiment was that genetic heterozygotes would have a higher specific activity than homozygotes. I measured specific esterase activity in males and females in

TABLE 2

The extent of activation in mixtures as a function of the relative proportion of esterase-5$^{.85}$ and esterase-5$^{1.12}$ after a 60-minute incubation at 25°

Volume ratio of .85/1.12	Esterase activity (units/ml)	Percent of expected activity
10:0	2.15	—
9:1	2.45	111
7:3	3.00	130
5:5	3.55	148
3:7	2.95	120
1:9	2.85	110
0:10	2.60	—

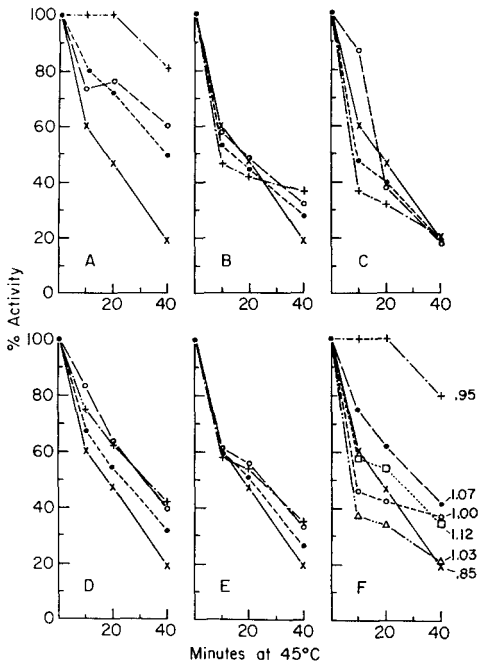

FIGURE 6.—Heat stability at 45° of esterase activity in homogenates of several Est-5 variants, and *in vitro* hybrid mixtures, all first incubated for 1 hour at 25°. The combinations studied were (A) Est-5$^{.85}$ + Est-5$^{.95}$, (B) Est-5$^{.85}$ + Est-5$^{1.0}$, (C) Est-5$^{.85}$ + Est-5$^{1.03}$, (D) Est-5$^{.85}$ + Est-5$^{1.07}$, (E) Est-5$^{.85}$ + Est-5$^{1.12}$, (F) the Est-5 homozygotes alone. The observed activity in "hybrid" mixtures is shown in open circles, while the expected activity obtained by averaging homozygotes is shown in filled circles.

both parental strains, and in the F_1 (Table 3). Since *Est-5* is *X*-linked the F_1 male is hemizygous, in this case for the *Est-5*$^{1.12}$ allele; it was convenient to express the results, then, as a ratio of specific activities in females and males, and thus control somewhat for differences in culture conditions or autosomal background. In short, it was found that the ratio of activity in the F_1 is no greater than that predicted by averaging the ratios of its parents. These results also indicate dosage compensation.

It was suggested that the basis for these results could simply be that F_1 females contained less of a more active enzyme. Such a result would not be unexpected if Esterase-5 activity were somehow regulated by the genetic background and by environmental conditions (e.g., substrate availability). To investigate this latter point the Est-5$^{1.12}$ strain was cultured on medium containing a range of α naphthyl acetate concentrations, and specific esterase activity was measured in third instar larvae and adults. As is seen in Table 4, no significant differences in activity could be detected among adults over the substrate concentration used, but in third instar larvae (Table 5) there was a substantially greater level of enzyme activity when the naphthyl ester was present in the food.

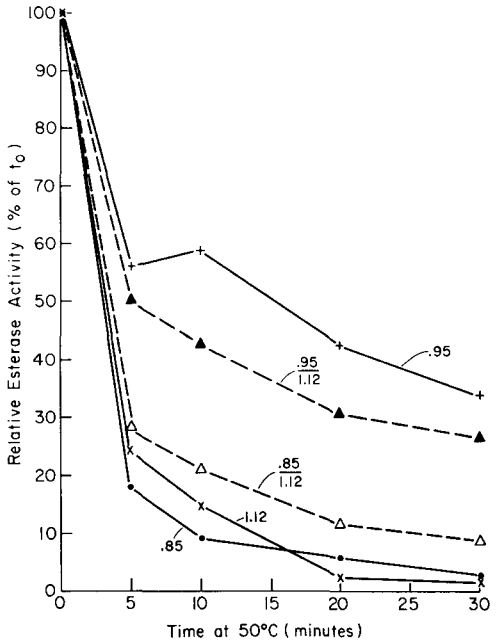

FIGURE 7.—Heat stability at 50° of esterase activity in homogenates of several Est-5 variants and their F_1 heterozygotes.

In an effort to confirm the possibility that the Est-$5^{.85}$/Est-$5^{1,12}$ heterozygotes contained less of a more active enzyme, an attempt was made to directly measure the amount of Est-5 enzyme. Esterase-5 can be inhibited by diisopropylfluorophosphate, and from the literature (KARLSON 1966) it is known that inactivation involves the formation of a covalent bond between the inhibitor and the hydroxyl group of the serine in the enzyme's active center. Since the reaction at 100% inhibition is stoichiometric, each Est-5 polypeptide should bind exactly one molecule of DFP. By using a tritiated isotope of DFP to inhibit activity, and then

TABLE 3

Specific esterase activity in strains of D. pseudoobscura *carrying various Est-5 genotypes*

Esterase-5 genotype	Sex	Esterase activity	Protein conc.	Specific activity	F/M ratio
0.85/0.85	F	1.80 ± .03	.796	2.26	1.08
0.85/ Y	M	1.35 ± .12	.650	2.09	
0.95/0.95	F	1.04 ± .11	.666	1.60	.99
0.95/ Y	M	.72 ± .06	.448	1.61	
1.12/1.12	F	.83 ± .11	.285	2.86	1.00
1.12/ Y	M	.86 ± .11	.300	2.87	
0.85/1.12	F	.77 ± .01	.365	2.20	.91
1.12/ Y	M	.93 ± .09	.385	2.41	
0.95/1.12	F	.86 ± .04	.500	1.72	1.04
1.12/ Y	M	.61 ± .05	.366	1.66	

TABLE 4

Specific esterase activity in Est-5$^{1.12}$ adults cultured in the presence or absence of alpha naphthyl acetate

Sex	a-n.a. conc. (M)	Esterase activity	Protein conc.	Specific activity	Average sp. act.
F	0	.562 ± .032	.224	2.50	2.35
M		.682 ± .042	.300	2.21	
F	2×10^{-6}	.670 ± .006	.304	2.24	2.13
M		.394 ± .012	.196	2.01	
F	2×10^{-5}	.470 ± .062	.210	2.24	2.33
M		.630 ± .122	.260	2.42	
F	2×10^{-4}	.814 ± .076	.270	3.01	2.57
M		.532 ± .012	.250	2.13	

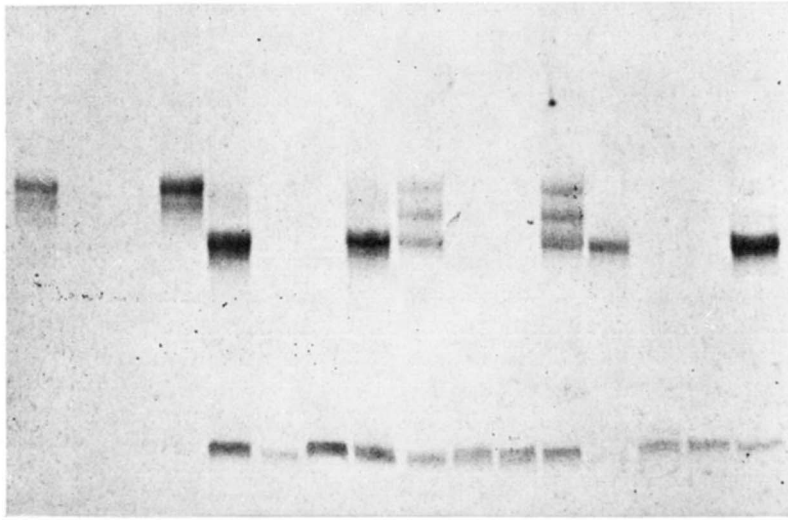

FIGURE 8.—Electropherogram showing inactivation of Est-5 activity by 10^{-3} M DFP, but resistence to inhibition of Est-8. Wells 1–4, beginning from the left, contain Est-5$^{.85}$ homogenates; wells 5–8 and 13–16 contain Est-5$^{1.12}$ extracts, wells 9–12 contain Est-5$^{.85}$/Est-5$^{1.12}$ heterozygotes. In all cases the center two wells of each group are DFP-inactivated while the outer two wells have not been inactivated.

TABLE 5

Specific esterase activity in Est-5$^{1.12}$ third instar larvae cultured in the presence or absence of alpha naphthyl acetate

a-n.a. conc. (M)	Esterase activity	Protein conc.	Specific activity	Relative specific activity
0	.434 ± .021	.244	1.78	100
2×10^{-6}	.585 ± .045	.204	2.87	161
2×10^{-5}	.862 ± .048	.281	3.07	172
2×10^{-4}	.395 ± .035	.153	2.58	145

separating Est-5 on electrophoresis from other DFP binding enzymes (e.g., proteases), I hoped to be able to measure directly label localized in the Est-5 region of the gel. It was found that Esterase-5 showed complete inhibition after 60 minutes at 10^{-3} M DFP (Figure 8). Unfortunately, when the gel was sliced and counted several prominent peaks of radioactivity appeared in all extracts: none corresponded exactly to the mobility of uninhibited enzyme. Thus either the enzyme is at too low a concentration to appear labelled, or its mobility is altered by inactivation. In either case further investigations of this type will require the use of highly purified enzyme. Several attempts at enzyme purification using the method of NARISE and HUBBY (1966) have failed. Activity is never recovered from the ion-exchange chromatography step in our hands.

DISCUSSION

It is becoming increasingly clear that population surveys of allozyme frequencies by themselves offer very little hope of distinguishing whether allelic variation is in general maintained by natural selection or by neutral mutation and drift (SING, BREWER and THIRTLE 1973). Too many unknown factors (population size, migration rate, linkage) confound any interpretation of patterns of allele distribution. Clearly any strategy that hopes to distinguish selection from neutrality must have the power to single out the adaptive significance of a single gene polymorphism, independent of other genetic variables, just as electrophoresis permitted the simple analysis of discreet variation at single loci. Biochemical studies, coupled of course with ecological and physiological analyses, may provide the appropriate methodology. Indeed substantial progress in this direction has already been made in several laboratories (GIBSON 1970; KOEHN 1969; WILLS and NICHOLS 1971; HARPER and ARMSTRONG 1973; VIGUE and JOHNSON 1973; ROCKWOOD-SLUSS, JOHNSTON and HEED 1973).

The initial intent during this work was to determine whether any important biochemical differences could be found between various Esterase-5 allozymes, and more specifically whether one could find any indication of heterosis at the molecular level. The reason for choosing the Esterase-5 system is that it has been intensively studied at both the population level (HUBBY and LEWONTIN 1966; PRAKASH, LEWONTIN and HUBBY 1969; YAMAZAKI 1971) and at the biochemical level (NARISE and HUBBY 1966; HUBBY and NARISE 1967; PASTEUR and KASTRITSIS 1971; BERGER and CANTER 1973). The ability to construct *in vitro* enzyme hybrids easily was a major factor in the choice.

The rationale for singling out heterosis as a selective force comes from three sources. First, if one considers the fate of a newly arising mutation it becomes clear that if an allele is to be beneficial and spread through a population it must provide its benefit for many generations as a heterozygote. This requirement becomes apparent when one calculates the average number of generations preceding the first appearance of homozygotes in populations of varying size (Figure 9). Upon the segregation of homozygotes the new allele may go toward fixation if the new homozygote is superior, or reach an equilibrium frequency if the heterozygote is most fit.

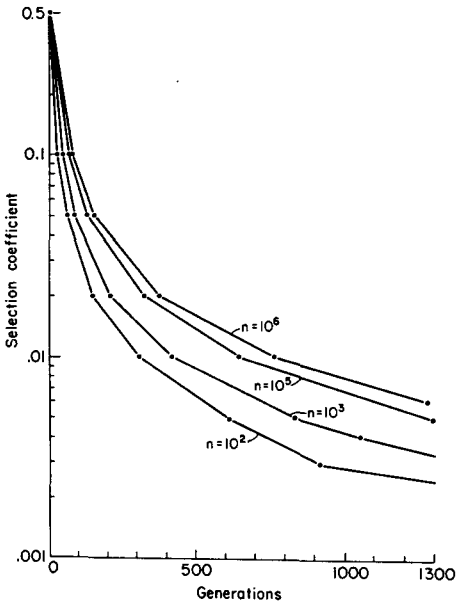

FIGURE 9.—The average number of generations required for a newly arisen mutant allele (a') to change from a frequency of $1/2n$ (its first appearance as heterozygotes aa') to $\sqrt{1/n}$ (its first appearance as a homozygote $a'a'$) where the selection coefficient (s) against the aa genotype is varied, in population of n individuals. The calculation is from LI (1955), page 256 formula 7[1] where:

$$sn = \frac{qo\text{-}qn}{qoqn} + \log_e \frac{qo(1\text{-}qn)}{qn(1\text{-}qo)} \text{ and } n = \text{number of generations}$$

The second impetus for examining molecular heterosis is indirect and comes from SCHLESSINGER and LEVINTHAL's work on intragenic complementation at the alkaline phosphatase locus of *E. coli* (SCHLESSINGER and LEVINTHAL 1963). Essentially they found that when subunits from two different and individually inactive dimeric enzymes were combined to form a hybrid enzyme unexpectedly high catalytic activity was often restored. Thus the chemical properties of heteromultimers are not necessarily predictable from knowledge of homomultimer properties. As FINCHAM (1966) has clearly pointed out, heterosis for multimeric enzymes could be mediated by the unique properties of heteromultimers.

The third and most direct reason for considering heterosis emerges from several theoretical (MILKMAN 1967; SVED, REED and BODMER 1967) and experimental (RICHMOND and POWELL 1970; WILLS and NICHOLS 1971; WILLS 1973; HEBERT, WARD and GIBSON 1972) studies suggesting the plausibility and apparent operation of overdominance at polymorphic enzyme loci.

Unfortunately the present studies have failed to reveal any general interaction between allozymes analogous to intragenic complementation, at least for those properties and under the conditions studied. It has been shown in one case that a highly significant increase in catalytic activity accompanies heterodimer formation, and in other cases the appearance of a more heat-stable enzyme was

noted. However, the significance of these findings is not readily apparent since the *Est-5*$^{.85}$/*Est-5*$^{1.12}$ heterozygote must be very rare in nature (PRAKASH, LEWONTIN and HUBBY 1969), and the temperatures used in enzyme activation are not physiological. A further problem would of course exist if the use of unpurified enzyme affected the kinetic or heat stability properties. There is simply no data available to evaluate this effect. Temperature has been implicated as a selective agent in at least two other systems (SCHOPF and GOOCH 1971; KOEHN 1969), one of them an esterase, but in both cases the differences observed between allozymes occurred within the range of environmental temperatures.

One further point concerning our activation data is appropriate. Experiments involving ratio mixing, and time course analysis indicate that the activity increase in the *Est-5*$^{.85}$+*Est-5*$^{1.12}$ mixture was correlated with hybrid enzyme formation. However, neither experiment could distinguish between activation resulting from a higher specific activity for heterodimers, or the formation of a heterodimer which is simply a more stable enzyme configuration, in which case heterodimer formation simply shifts the equilibrium between inactive monomer and active dimer toward the dimer. The general deviation from the 1:2:1 distribution in staining of heterozygotes (Figure 3F) does not appear to support the latter hypothesis. The results are further complicated by the observation that genetic heterozygotes for this pair of alleles have no more esterase activity than would be predicted by simply averaging the values of the parental strains. It is tantalizing to hypothesize that genetic heterozygotes have actually less of a more active enzyme, but direct attempts to measure the amount of Est-5 dimer or monomer by DFP binding have thus far failed to provide the needed specificity. We have found that the level of esterase activity in larvae can be changed by direct feeding of substrate, as was the case for alcohol dehydrogenase activity in *D. melanogaster* (GIBSON 1970) so that it is not unreasonable to suspect that enzyme levels may be in part regulated by the environment. If this were so then heterosis could operate simply by permitting heterozygotes to maintain the same optimal regulated level of activity as homozygotes, but require the synthesis and accumulation of less enzyme to achieve that level. Thus the benefit accrued by heterozygosity would be in the conservation of metabolic energy involved in the biosynthesis of proteins required for normal metabolic processes. This conserved energy could then be channelled into other "luxuriant" anabolic functions such as growth or gamete production. It is clear that proof of this argument at a molecular level requires exact measurements of enzyme concentration. Hopefully experiments like DFP binding with highly purified enzyme will permit us to test this hypothesis for Esterase 5, and may provide a general strategy for investigating overdominance in other gene-enzyme systems. One indirect but confirming bit of evidence for this model comes from HEBERT, WARD and GIBSON (1972) study of allozyme variation in natural populations of *Daphnia magna*, a species which has one sexual generation per year followed by about 20 apomictic parthenogenic generations. During the course of the parthenogenic cycle heterosis was observed at several loci. The process operating to increase the relative frequency of heterozygotes was shown to be increased fecundity. Although

this "energy balance" model is now based on scant and often indirect evidence, it has a general appeal in that it is testable, and does not require the discovery of a different specific set of environmental condition to explain the maintenance of each gene polymorphism observed.

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