POLYMORPHISM FOR DIMERIZING ABILITY AT THE ESTERASE-5 LOCUS IN *DROSOPHILA PSEUDOOBSCURA*

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ABSTRACT

Three new alleles are reported at the esterase-5 locus of *Drosophila pseudoobscura*. All three of these alleles are different from those previously reported in their ability to dimerize. One allele will not form heterodimers or homodimers and exists only as a monomer. A second allele does form heterodimers but will not form homodimers. The third allele forms both hetero- and homodimers as well as forming monomers. Estimates of the frequency of these alleles in a natural population are given. The existence of these is discussed with respect to recently proposed models for a molecular mechanism for heterosis.

AS a result of all allozyme systems studied in both plant and animal species, one generalization that has emerged is that esterases are characteristically more variable than other enzymes or protein systems (JOHNSON 1974; SELANDER and KAUFMAN 1973). In most of the esterase systems studied, there is a broad range of substrates which can be hydrolyzed, although the actual physiological substrate is usually unknown. This is, in fact, the case with esterase-5 (*est-5*) of *Drosophila pseudoobscura* (NARISE and HUBBY 1966). It has been proposed by JOHNSON (1974) that esterases such as *est-5* in *D. pseudoobscura* most likely function in breaking down secondary compounds in plants; however, this is yet to be demonstrated. KOJIMA, GILLESPIE and TOBARI (1970) have proposed that the large number of alleles found at loci such as esterases are an adaptation to the large number of different substrates on which the enzyme operates in nature. Also, JOHNSON (1974) has suggested that differential behavior in metabolic regulation might be a selective mechanism for maintaining the polymorphism at loci such as the one studied here.

The *est-5* locus in *D. pseudoobscura* has been studied during the past decade by a number of investigators. The locus was initially described as being sex-linked and possessing six electrophoretically distinguishable alleles (HUBBY and LEWONTIN 1966). PRAKASH, LEWONTIN and HUBBY (1969) surveyed larger samples from natural populations and increased the observed number of electrophoretic alleles to twelve. PRAKASH and MERRITT (1972) obtained a null allele from a population at Mesa Verde, Colorado, thus extending the total to thirteen naturally occurring alleles at this locus.

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Females heterozygous for these *est-5* alleles have generally been found to possess a three-banded phenotype when examined by gel electrophoresis (HUBBY and LEWONTIN 1966). In their initial description of the locus, they also observed some heterozygous females which contained only two bands. They did not designate this unusual phenotype to any particular alleles, and apparently did not observe it in their samples from natural populations (LEWONTIN and HUBBY 1966). PRAKASH (1969) examined *est-5* phenotypes in the sibling species *D. persimilis* and found that some had a two-banded phenotype. The two-banded phenotype observed by PRAKASH was restricted to heterozygous females containing the allele *est-5 1.33* and hence is likely a property of this allele. In all the above reports homozygous females and hemizygous males had a single-banded phenotype.

The three-banded phenotype of heterozygous females has been attributed to the dimeric structure of the *est-5* enzyme (HUBBY and LEWONTIN 1966). The intermediate mobility is a heterodimer and the two extreme mobilities are the two types of homodimers. Males and homozygous females hence contain only a single type of homodimer. The dimeric structure of this enzyme is thought to be in a dynamic equilibrium with the monomeric state (HUBBY and NARISE 1967). Apparently the equilibrium concentration of the two forms is such that the monomers are very much less abundant than the dimers. This would account for the fact that one usually does not observe monomer bands on the gels. The dimer of *est-5* is thought to be considerably less stable than those of other enzymes such as malate dehydrogenase, as one can form *in vitro* heterodimers in a matter of minutes under very mild conditions.

HUBBY and NARISE (1967) have studied the difference in ability of the various *est-5* alleles to form heterodimers. They report some instances where there seems to be a propensity to form homodimers rather than heterodimers. They also found that the alleles of *D. pseudoobscura* would form heterodimers much more readily with esterases from closely related species than with those from more distantly related species. They interpret the tendency to form heterodimers as a measure of the similarity of the alleles. This then can be used to infer evolutionary relationships.

I report here the observation of three more alleles at the *est-5* locus in *D. pseudoobscura*. All three of these alleles are different from the previously reported alleles in that they form unusual phenotypes when heterozygous. Evidence will be presented that these alleles make products which exist as monomers under the conditions of the experiment. One of the alleles exists only as a monomer while the other two exist both as monomers and dimers.

MATERIALS AND METHODS

Collections—All collections were made at the biological control citrus orchard on the campus of the University of California at Riverside. The collecting traps were baited with cut citrus fruit. Samples were taken in January, 1971, March, 1972, June, 1972, and May, 1973.

Electrophoresis—Polyacrylamide vertical slab gel electrophoresis was performed according to the methods of HUBBY and LEWONTIN (1966). Gels were developed for *est-5* using α-naphthyl

acetate as a substrate and fast red TRN as an indicator dye. The step of immersion of the gel in 0.5 M boric acid was omitted. The gels were made and run in plexiglass electrophoresis boxes purchased from Aardvark Instrument Company, 14176 Rochdale Circle, Lombard, Illinois. Gels were made with 6% acrylamide, unless otherwise stated.

Molecular weight determinations—The molecular weights of the enzymes were measured according to the methods of HEDRICK and SMITH (1968), with some modifications. The method involves studying the relative mobilities of the molecules in question at a number of different concentrations of acrylamide gel. Increasing the concentration of the gel lowers the mobility of all molecules, although not in equal proportions. The mobility of larger molecules is reduced proportionally more than those of smaller molecules. Several investigations have shown this to be a reliable method of determining molecular weight of enzymes (HEDRICK and SMITH 1968; PARISH and MARCHALONIS 1970). The results in all cases have shown a linear relation between log R_m and the concentration of acrylamide, and that the slope of this relation has a linear relation to molecular weight. This was also found in the present study.

Gels were run at four concentrations of acrylamide: 4%, 6%, 8% and 10%. Three enzymes of known molecular weight were included on the gels as well as the unknown sample. These enzymes and their molecular weights were as follows: (1) bovine pancreas α-chymotrypsin, MW = 22,500 (LASKOWSKI 1955); (2) pig heart malate dehydrogenase, MW = 70,000 (NOYES *et al.* 1974); (3) *D. pseudoobscura est-5* dimer, MW = 105,000 (NARISE and HUBBY 1966). The R_m values were calculated using the unknown sample as the reference rather than a dye molecule. The relation between log R_m and concentration of gel was found to be linear for all enzymes tested. The slope of this relation for each enzyme was plotted against its molecular weight, and this was also found to be linear. The point of zero slope was taken to be the molecular weight of the unknown molecule.

In vitro hybridization—*In vitro* hybridization of the *est-5 enzyme* was accomplished according to the method of HUBBY and NARISE (1967). The procedure is simply to incubate the two crude extracts together in 0.1 M tris-borate EDTA buffer for approximately one-half hour at room temperature.

Genetic extraction technique—Progeny from wild-caught females which showed unusual phenotypes were inbred by recurrent sib mating until homozygous lines were obtained.

RESULTS

The majority of wild-caught heterozygous females showed three bands with the intermediate mobility midway between the proximal and distal mobilities. Representative phenotypes of this type are shown in Figure 1, pockets E and F. There is a variety of unusual phenotypes which occurred in the samples. One such phenotype was a double-banded female in which the distal band usually had a mobility close to the *est-5 1.12* allele (Figure 1, pocket A). Another phenotype was a triple-banded female in which the intermediate mobility was not midway between the proximal and distal mobilities. This phenotype always involved a distal mobility of about 1.17 and a proximal mobility of *est-5 .85* or *est-5 .95*. The intermediate band was always closer to the proximal band than the distal band as shown in Figure 1, pocket C. A third phenotype was an unclean double-banded female. The area between the two bands was often a continuum of staining that was usually less dense than the bands. This phenotype is shown in Figure 1, pocket H.

From one of the double-banded females homozygous lines were obtained which when crossed would produce all double-banded females. In fact, three types of homozygous lines from the female's descendants were obtained which bred true

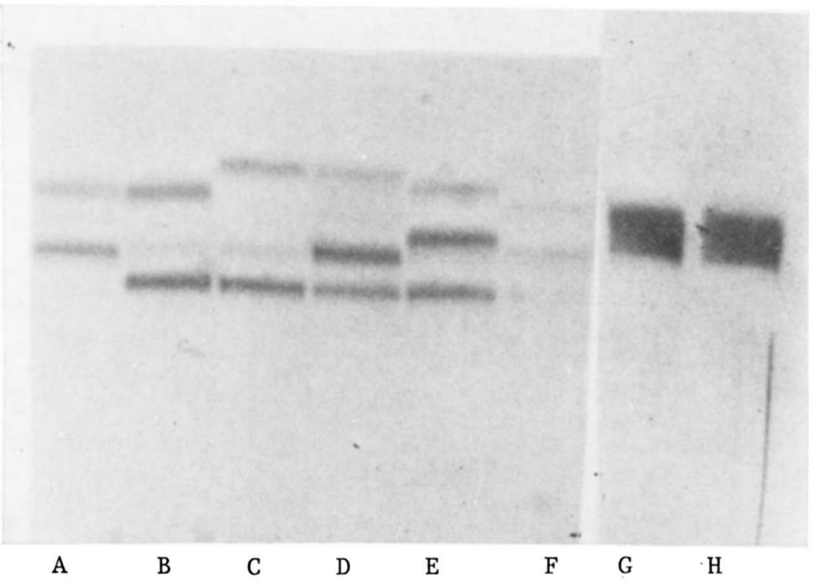

FIGURE 1.—Electrophoretic patterns of the various phenotypes discussed in the text. Origin is at bottom of picture.

for mobilities of *1.00*, *1.07*, and *1.13*. However, the double-banded phenotype was observed in the female offspring from crosses involving the *est-5 1.13* line only. The *est-5 1.00* and *est-5 1.07* lines when crossed produced the usual three-banded phenotype. Thus it seemed that the double-banded phenotype was a property of this particular *est-5 1.13* allele. During the inbreeding of this line all flies were examined and all observations were compatible with the hypothesis of the double-banded female phenotype being a property of the *est-5 1.13* allele. This homozygous *est-5 1.13* line was then crossed to a variety of *est-5* alleles which were known to produce the typical three-banded phenotype in heterozygous females. The alleles used were *est-5 .85*, *.95*, *1.00*, *1.02*, *1.03*, *1.07*, *1.09*, *1.12*, and *1.16*. In all cases the heterozygous females involving *est-5 1.13* showed a double-banded phenotype In heterozygotes involving alleles with mobilities very close to *est-5 1.13*, such as *1.09* and *1.12*, I could not be certain of the absence of an intermediate band. Females heterozygous for *est-5 .85* and *1.13* did produce a very faint intermediate band when stained very intensely. This phenotype is shown in Figure 1, pocket B. Reciprocal crosses were made to some of these lines and exactly the same result was observed regardless of which strain the males and females were derived from.

In four of the crosses mentioned above the F_1 females were backcrossed to the heterodimer-producing lines in order to study the segregation patterns of the alleles involved. The crosses used for this were: (1) *1.13* × *.85/.85*, (2) *.85* × *1.13/1.13*, (3) *1.13* × *1.00/1.00*, and (4) *1.00* × *1.13/1.13*. F_1 virgin females

from these crosses were mated to *est-5 1.00* or *est-5 .85* males. The F_2 resulting from each of these crosses was in complete agreement with the hypothesis of the *est-5 1.13* allele causing the double-banded phenotype. None of the results deviate significantly from the expected ratio of 1:1, and if the data are combined, there is still no significant deviation. The mobility of the *est-5 1.13* band in heterozygous females is exactly the same as that in homozygous *est-5 1.13/1.13* females and hemizygous *est-5 1.13* males. All this data supports the hypothesis that the double-banded phenotype is caused by the failure of the *est-5 1.13* gene product to form heterodimers when present in heterozygous females. An alternate hypothesis of a linked dominate modifier locus seems unlikely since no exceptions were found in 62 F_2 heterozygotes for *est-5 1.13*, indicating that the two-banded effect follows the *1.13* allele.

The question remains as to whether or not the *est-5 1.13* allele can form homodimers. This can be determined by measuring the MW of the *est-5 1.13* band. I employed the methods of HEDRICK and SMITH (1968) to measure the MW of the *est-5 1.13* bands in the *est-5 1.13* homozygotes and heterozygotes. The mobility of the *est-5 1.13* band relative to the other *est-5* bands changed dramatically when the concentration of the gel was changed. At lower gel concentrations the *est-5 1.13* allele migrated relatively much slower than the other *est-5* alleles, and at higher concentrations it was relatively much faster. These facts suggest that the MW of the molecules of *est-5 1.13* is much smaller than that of the other alleles.

Accurate measurement of MW can be made by comparing the mobility with those of known standards at various concentrations of gel. The results of such an experiment are shown in Figure 2. In this experiment *est-5 .85*, *est-5 1.00*, and *est-5 1.16* alleles, all of which exist primarily as dimers, were used, and no differences between them were detected. If we assume that MW of the *est-5* dimer is 105,000 as reported by NARISE and HUBBY (1966), and treat it as a standard, the estimate of the MW of the *est-5 1.13* product is 56,000. If we do not treat the *est-5* dimer as a standard but as a second unknown, then the estimates of MW are 54,900 for *est-5 1.13* allele and 100,300 for the *est-5* dimer. This latter value is in approximate agreement with the value of 105,000 reported by NARISE and HUBBY (1966).

My data can also be compared to that reported by PARISH and MARCHALONIS (1970). Their analysis is ostensibly different from that employed here, but is, in fact, fundamentally the same method. They found a linear relation between a parameter called a frictional ratio and MW. Since both their method and my method presume a linear relation between some parameter and MW, they can be compared by linear interpolation. According to PARISH and MARCHALONIS, molecules with a MW of 22,500 and 70,000 have frictional ratios of 1.600 and 1.95, respectively. These would be the frictional ratios of α-chymotrypsin and malate dehydrogenase. Using my data and linear interpolation we find the frictional ratios of the *est-5 1.13* product and *est-5* dimer to be 1.838 and 2.174, respectively. This corresponds to a MW for *est-5 1.13* of about 55,000 and for the *est-5* dimer about 101,000.

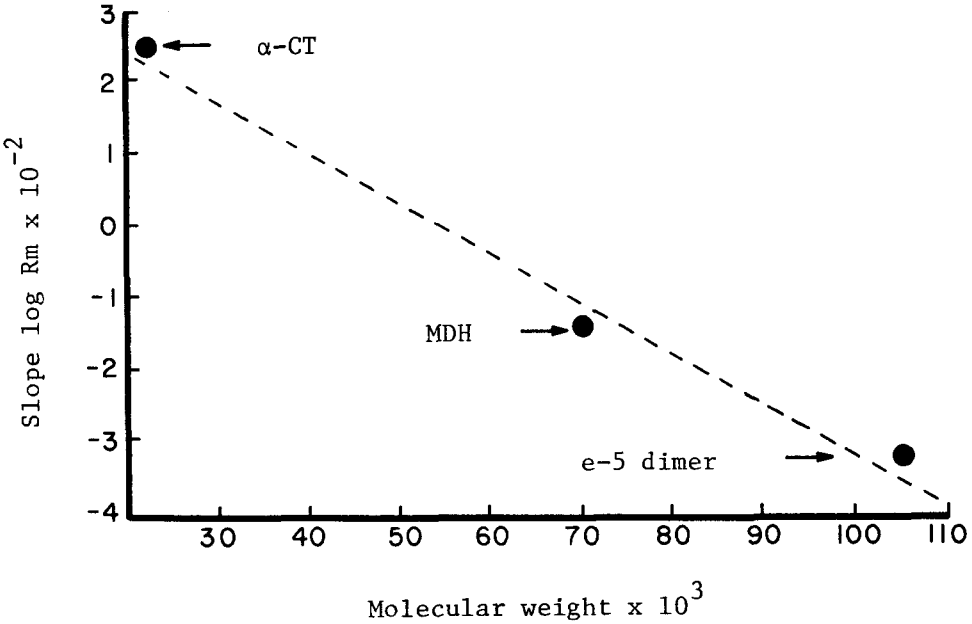

FIGURE 2.—Relation between slope of log *Rm* and molecular weight. *Rm* was measured with respect to the suspected esterase-5 monomer. (α-CT = α-chymotrypsin, MDH = malate dehydrogenase, *est-5* = esterase-5).

The good agreement between my MW measurement for *est-5* dimer with that of NARISE and HUBBY (1966) and between my linear relation and that of PARISH and MARCHALONIS (1970) lend credence to my estimate of the MW of the *est-5 1.13* product. The MW of the *est-5 1.13* product is close to being half the MW of the *est-5* dimer. This, in combination with the lack of heterodimer formation in *est-5 1.13* heterozygotes, leads me to postulate that the *est-5 1.13* product exists primarily as a monomer.

A second kind of unusual phenotype was analyzed. In this case, progeny from two females, both of which had triple-banded phenotypes (Figure 1, pocket C) with the intermediate band not in the middle, were sib crossed for several generations and homozygous lines were obtained. Several different alleles were made homozygous that produced single-banded males and females. In crossing these lines it was found that the displaced intermediate band phenotype resulted only when a line homozygous for a *1.17* mobility was involved. The other lines produced only the usual three-banded phenotype with the intermediate band exactly halfway between the other two. Numerous sib crosses were made with the progeny from the two original females for several generations and the phenotypes of the flies were examined. In all cases there was complete agreement with the hypothesis that the *est-5 1.17* band segregated as an allele which produced displaced middle bands when heterozygous. An alternative hypothesis of a dominant modifier locus seems highly unlikely.

The homozygous *est-5 1.17* lines were then crossed to a variety of lines homo-

zygous for the usual *est-5* alleles which form heterodimers midway between the two homodimers. In all cases there was a displaced intermediate band. In some of these heterozygous females the bands were too close to determine whether or not there was an intermediate band, but in these cases there was always a very thick band which appeared to be two very close bands. These ambiguous cases were seen in heterozygotes with the *est-5 1.00*, *1.02*, *1.03*, *1.07*, and *1.12* alleles.

Molecular weight measurements on the gene products of *est-5 1.17* homozygotes, heterozygotes, and hemizygotes showed that the *est-5 1.17* band in all cases had a MW indistinguishable from the *est-5 1.13* allele discussed previously. Also, the intermediate band had a MW indistinguishable from the standard *est-5* dimer. Hence I propose that the *est-5 1.17* gene product will form heterodimers with all the standard alleles tested in this study, but will not form homodimers.

Finally, a third kind of unusual phenotype was examined. Here progeny from two other females were sib mated until homozygous. One of these females was scored as an *est-5 1.07* homozygote with a noticeable band near a *1.12* mobility and a continuum of stain between these two bands. The other female had a displaced intermediate band between *est-5 .95* and *est-5 1.17* with a noticeable smearing of the *1.17* band toward *est-5 1.07*. Some of the male progeny as well as female progeny had the double-banded phenotype with a lightly stained continuum between the bands. Homozygous lines were obtained which bred true for this phenotype in both males and females. Homozygous lines derived from each of the original females appeared identical. Both had bands at *1.07* and *1.17* with a continuum of activity between the bands (Figure 1, pockets G and H). Sometimes these lines gave a clear double-banded phenotype. The amount of staining between the two bands seemed to be correlated with the running temperature of the gels. This will henceforth be referred to as the double-banded effect. One of these lines was then crossed to lines homozygous for the *est-5 .85*, *.95*, *1.00*, *1.02*, *1.03*, *1.07*, *1.09*, *1.12*, and *1.16* alleles. In all cases it was found that the double-banded effect remained and that heterodimers were formed. The heterodimer was midway between the normal allele used and the proximal band of the double-banded effect (Figure 1, pocket D).

Molecular weight measurements on the bands in these double-banded females and hemizygous males showed that the MW of the *est-5 1.17* band was equal to that of the postulated monomers previously described. The *est-5 1.07* band had a MW equal to that of the *est-5* dimer alleles. Hence I propose that these lines are homozygous for an allele whose product exists as both monomers and dimers. I will refer to this allele as *est-5 1.07MD*. The continuum of activity between the two bands can be explained as the result of an equilibrium of these two forms of the enzyme. A phenomenon such as this would give rise to precisely the pattern observed. A genetic analysis of this postulated *est-5 1.07MD* allele has shown it to behave as an allele of the *est-5* locus.

The phenotypes of all the heterozygous females for each of the monomer-generating alleles discussed above were also generated by *in vitro* enzyme hybridization. This suggests that the dimerizing properties seen in heterozygotes

of these alleles are properties of the protein molecules rather than a result of the mechanism of synthesis.

DISCUSSION

I have presented evidence for the isolation of three new alleles at the *est-5* locus in *D. pseudoobscura*. All of these alleles were shown to have unusual polymerization properties which give rise to unusual phenotypes when examined on electrophoretic gels. This information makes it possible to interpret some of the atypical phenotypes observed in wild-caught flies in this species. I feel that there are likely to be other alleles present in natural populations that give rise to other atypical phenotypes. In surveys of wild-caught flies several other unusual phenotypes were observed but were not recovered in the offspring due to limitations in effort. In many of these cases I cannot be certain of a genetic basis of the observations, as some of these mimic a gel with poor resolution. For example, flies containing the *est-5 1.07MD* allele were initially thought to be an artifact of the electrophoresis method. Isolation of homozygous lines and adequate genetic analysis are essential to the identification of these types of alleles.

So far nothing has been said about the frequency of these alleles in natural populations. A problem in detecting the presence of an allele such as *est-5 1.13* is that it is necessary to observe it in a heterozygous female in order to distinguish it from other alleles of similar mobility. Also, an *est-5 1.13* mobility could easily be misclassified as a *1.12* mobility, especially if the investigator was of the opinion that a *1.13* mobility did not exist. This is, in fact, how the gels were scored in this study.

One can estimate the frequency of alleles that cause double-banded females from the frequency of such females in a sample. Specifically, I will estimate the frequency of alleles with a mobility greater than *est-5 1.00* that form only two bands when heterozygous with an allele of mobility less than or equal to *est-5 1.00*. If p = frequency of alleles with mobility $\leq$ *1.00*, q = frequency of alleles with mobility $>$ *1.00* that form three bands in heterozygotes, and r = frequency of alleles with mobility $>$ *1.00* with properties previously mentioned, then the frequency of females with the double-banded phenotype will be equal to $r/(q+r)$. This method showed the frequency of this type of allele to range from 0.005 to 0.035 in these samples. Thus they are sometimes as frequent as some of the less frequent dimerizing alleles such as *est-5 .85* and *est-5 1.16*. The double-banded phenotype reported by PRAKASH (1969) in *D. persimilis* has also been shown to be due to a monomer allele similar to *est-5 1.13* discussed here and will be discussed in a future publication. The allele which appears responsible for this phenotype in *D. persimilis* has a frequency of 0.12.

These observations suggest that one must use extreme caution when using enzyme dimerization studies such as those reported by HUBBY and NARISE (1967) and by MACINTYRE (1971) to infer evolutionary relationships. The alleles reported here would give a totally misleading picture if the ability to dimerize was taken as a measure of evolutionary relationship. There is also the possibility that observations of dimerization of enzymes would allow one to detect allelic

variants which are not distinguishable on the basis of electrophoretic mobility. For example, the *est-5 1.13M* allele will slightly dimerize with the *est-5 .85* allele. Possibly within the other mobility classes there are alleles which will dimerize with *est-5 1.13M*. To test this, heterozygotes between *est-5 1.13M* and 25 independently extracted *est-5 1.00* lines from the UCR citrus orchard were examined. All phenotypes were identical in exhibiting no indication of a heterodimer band.

At the present time there is no conclusive information as to whether or not selection plays a role in maintaining these types of alleles in natural populations. These monomer alleles do not seem to make an unusually aberrant enzyme. Mammalian esterases which are normally dimers and tetramers can be separated into monomers which are still enzymatically active (KRISCH 1971). Thus it is likely that these alleles have arisen by point mutations which affect the binding sites on the protein molecule and leave the enzymatically active site relatively unaffected. The *est-5 1.17* allele and the monomer band of *est-5 1.07MD* have the same mobility (see Figure 1, pockets C and D); yet they are quite different in their dimerization properties. An amino acid substitution in the binding site which does not change the charge on the monomer molecule could be the cause of this observation. No significant differences in the intensity of staining were observed between the dimerizing alleles and the various monomer alleles. It would be interesting to know if this kind of variation is peculiar to esterase loci. These observations may mean that this enzyme can function even in the face of unusually large alterations of quaternary and possibly even tertiary structure. There also is the possibility that the patterns seen on the gels do not accurately reflect the state of the *est-5* enzyme in an undisturbed organism.

One possible mechanism for maintaining the polymorphism of dimerizing alleles is the increased fitness for heterozygotes due to the properties of the heterodimer. Evidence for this type of mechanism has been reported by SCHWARTZ and LAUGHNER (1969) for alcohol dehydrogenase in maize and by BERGER (1974) for esterase-5 in *D. pseudoobscura*. The observations presented here lead one to question whether such a mechanism is a significant factor in maintaining the polymorphism at the esterase-5 locus.

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