PROTEIN DIFFERENCES IN DROSOPHILA. III. ALLELIC DIFFERENCES AND SPECIES DIFFERENCES IN *IN VITRO* HYBRID ENZYME FORMATION¹

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THE study of possible structural differences between homologous proteins in different species is a tedious and arduous task. Tactical methods that might allow limited areas of the protein to be probed for differences would be quite useful. One such method is available if the protein consists of subunits, namely, the study of *in vitro* hybrids of these subunits from different species. The formation of such hybrid molecules clearly depends on specific complementary surfaces between the combining subunits. The ability to form *in vitro* hybrids between subunits of diverse species indicates the retention during evolution of at least a sufficient part of the binding sites to allow subunit interaction and multimer formation. The loss of ability implies change in sequence and/or conformation between the homologous molecules and thus may provide a measure of the rate or amount of change in these sites, if homologous molecules of many related species are studied.

Retention of complementary sites between molecules obtained from organisms widely separated through evolution is evidenced by the ability of lactate dehydrogenases from many species to form *in vitro* stable binomial hybrids (MARKERT 1964) and by the formation *in vitro* of hybrids between hemoglobins obtained from man and dog (ITANO and ROBINSON 1959). Such widespread ability to form *in vitro* hybrids between diverse forms is not universal. For example, Bullfrog heart lactate dehydrogenase will hybridize with the Leopard frog heart enzyme but will not with hagfish muscle lactate dehydrogenase (SALTHER, CHILSON and KAPLAN 1965). But it is not possible at present to say from these data that the ability to hybridize is necessarily based on degree of relationship.

This technique seemed useful enough to explore further; and so when, in the course of analyzing proteins from *Drosophila*, two enzymes were found which seemed ideal for such exploration, the present work was initiated. The first enzyme found was an esterase for which six electrophoretic variants (each allelic) were discovered in *D. pseudoobscura* (HUBBY and LEWONTIN 1966). This enzyme was purified and characterized for substrate specificity and for specific inhibitors (NARISE and HUBBY 1966). It could for this reason be distinguished operationally from other esterases in *Drosophila*. The second enzyme was malate

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dehydrogenase for which three electrophoretic forms (also allelic) were found in this species (HUBBY and LEWONTIN 1966). There is a single kind of molecule of this enzyme present in all *Drosophila* studied. Both of these enzymes form *in vivo* hybrids in individuals heterozygous for two different alleles, and it was possible to produce *in vitro* hybrids with each of these enzymes.

This ability to form *in vitro* hybrids and the series of alleles which were available for study gave us the opportunity to ask the following questions: (1) Is each form of an enzyme obtained from the same species equally capable of forming an *in vitro* hybrid with homologous enzymes from other species? (2) Are the esterase and the malate dehydrogenase equally capable of producing *in vitro* hybrids with the same spectrum of other species? The results indicate that different forms of the esterase from the same species do differ in their ability to form *in vitro* hybrids with enzymes from closely related species and that this enzyme, in general, is not able to form hybrids with distantly related species. In contrast, each form of malate dehydrogenase of *D. pseudoobscura* is capable of forming hybrid enzyme with every species of the genus *Drosophila* tested. This method may prove to be of some usefulness in evolutionary studies.

MATERIALS AND METHODS

Species stocks: Five stocks of *D. pseudoobscura* each homozygous for a different allele of esterase-5 were obtained by inbreeding pairs of flies from the natural populations studied previously (HUBBY and LEWONTIN 1966). Three stocks of *D. pseudoobscura* each homozygous for alternative electrophoretic forms of malate dehydrogenase were obtained similarly. The nomenclature of the structural genes for these enzymes and for the enzymes themselves is as described (1966). Other species stocks were obtained from the Genetics Foundation at the University of Texas and from DR. D. D. MILLER at the University of Nebraska. Those stocks listed in Table 1

TABLE 1

Relative mobility of Esterase-5 in D. pseudoobscura and other members of the obscura group

Species	Relative mobility	Sensitivity to NaCl
<i>D. pseudoobscura</i> E-5 ^{.95}	.95	..
<i>D. pseudoobscura</i> E-5 ^{1.00}	1.00	+
<i>D. pseudoobscura</i> E-5 ^{1.03}	1.03	+
<i>D. pseudoobscura</i> E-5 ^{1.07}	1.07	+
<i>D. pseudoobscura</i> E-5 ^{1.12}	1.12	..
<i>D. persimilis</i> (2529.6)	1.17	..
<i>D. miranda</i> (Big Basin)	1.07	+
<i>D. miranda</i> (Boise, Idaho)	1.11	..
<i>D. affinis</i> (2069.2)	1.17	..
<i>D. affinis</i> (2528.3)	1.22	..
<i>D. azteca</i>	1.05	+
<i>D. athabasca</i>	1.05	+
<i>D. algonquin</i> (2528.5)	1.14	..
<i>D. subobscura</i> (Norway)	1.18	+++
<i>D. ambigua</i> (2529.7)	1.09	+++
<i>D. bifasciata</i>	1.02	+++

+ Slight loss of activity.

+++ Complete loss of activity.

with accession numbers are from the University of Texas Collection; the remainder are from MILLER.

Enzyme detection: Both the parental and the hybrid enzyme were detected after electrophoresis in acrylamide gels exactly as described in HUBBY and LEWONTIN (1966).

Formation of *in vitro* hybrids: (1) Esterase-5. The first attempts were made on purified extracts obtained by the method of NARISE and HUBBY (1966). Treatments of mixtures of different electrophoretic forms of this enzyme from *D. pseudoobscura* with urea (2 to 6 M) failed to produce detectable hybrids. However, freezing and thawing such mixtures in buffered 1 M NaCl in a manner described by MARKERT (1963) produced hybrid enzyme activity indistinguishable from natural hybrids. Hybrids were also formed when two flies were ground together in buffered 1 M NaCl, frozen and thawed. And later in this study it was found that simply incubating the extract of two flies at room temperature in 0.1 M Tris buffer pH 7.2 for 15 minutes was just as successful as any other type of treatment. (2) Malate dehydrogenase. It was necessary to freeze and thaw mixtures of extracts in 1 M NaCl before malate dehydrogenase *in vitro* hybrids were produced.

Sample placement: The array of treatments and controls routinely used in the electrophoretic analysis of hybrid enzyme formation was arranged in the pockets on a single gel in the following manner: (1) species A, fresh extract; (2) species A, extract frozen overnight in 1 M NaCl; (3) species A and B, fresh extract; (4) species A and B, frozen overnight in 1 M NaCl; (5) species B, frozen overnight in 1 M NaCl; (6) species B, fresh extract. In the case of Esterase-5, the incubation method was also performed using a similar array. This array allows detection of any loss of activity caused by the treatment and allows ease of detecting any hybrid formation.

RESULTS

Members of the obscura group available for study are listed in Table 1 together with the electrophoretic mobilities of the esterase, which has the following characteristics: (1) greater activity toward β -naphthyl acetate than α -naphthyl acetate, (2) low or no activity towards substrates with acyl chains greater than 4 carbon atoms, and (3) strongly inhibited by 10^{-4} M diisopropylfluorophosphate. These are the characteristics which delimit Esterase-5 in *D. pseudoobscura* (NARISE and HUBBY 1966). Thus, if an enzyme in another species fulfilled these criteria, it was assumed to be homologous with the Esterase-5 from *D. pseudoobscura*. In all early experiments, freezing in buffered 1 M NaCl was the method of hybrid enzyme formation. Such a procedure reduced or eliminated activity of some of the esterases studied. The sensitivity of the different enzymes to the treatment is also recorded in Table 1. In later experiments the analyses were performed in each case with the simpler and less drastic incubation method of forming enzyme hybrids. Only the Old World members of the subobscura group were sensitive to this treatment.

Five of the electrophoretic forms of Esterase-5 from *D. pseudoobscura* were tested for hybrid enzyme formation with a single form of the enzyme from *D. persimilis*, and in each case a hybrid enzyme was formed. In contrast, when all five forms of the enzyme from *D. pseudoobscura* were tested with the supposedly homologous enzyme from each of two members of the melanogaster, virilis, or repleta group, there was no indication in any case of hybrid enzyme formation (Figure 2). Data for the ability of different electrophoretic forms of Esterase-5 from *D. pseudoobscura* to form *in vitro* hybrids with other members of the Obscura Group is recorded in Table 2, and examples of *in vitro* hybridization of Esterase-5 is shown in Figure 1. Such tests were only possible when there was

TABLE 2

Hybrid enzyme formation of Esterase-5 between pseudoobscura and other obscura group species

<i>pseudoobscura</i> allele	New World						Old World		
	Obscura subgroup		Affinis subgroup				Subobscura group		
	<i>persimilis</i>	<i>miranda</i>	<i>affinis</i>	<i>azteca</i>	<i>athabasca</i>	<i>algonquin</i>	<i>subobscura</i>	<i>ambigua</i>	<i>bifasciata</i>
.95	+	+	+	+	+	+	+	*	*
1.00	+	+	+	+	TC	+	*	*	*
1.03	+	TC	+	—	TC	+	*	*	*
1.07	+	TC	+	TC	+	—	*	*	*
1.12	+	TC	+	—	—	TC	*	*	*

TC=Mobility of enzymes too close to test.

* Enzyme unstable to hybridizing treatment.

sufficient electrophoretic separation between the two tested enzymes. There was positive hybrid identification between *D. pseudoobscura* and the New World forms of the obscura subgroup. Of the New World forms of the affinis subgroup, only *D. affinis* was capable of formation with each form of the enzyme from *D. pseudoobscura*.

Esterase-5^{.95} and Esterase-5^{1.00} from *D. pseudoobscura* were able to form hybrids with all four of the affinis subgroup species tested. However, Esterase-5^{1.03} formed no detectable hybrid with *D. azteca*, Esterase-5^{1.07} formed no detectable hybrid with *D. algonquin*, and Esterase-5^{1.12} formed no detectable hybrid with either *D. azteca* or *D. athabasca*.

FIGURE 1.—*In vitro* hybridization of Esterase-5. From left to right: Esterase-5^{.95} *D. pseudoobscura*; fresh mixture of Esterase-5^{.95} and Esterase-5^{1.12}; 5 min incubation of the mixture; 10 min incubation of the mixture; 15 min incubation.

FIGURE 2.—Failure of *in vitro* hybridization of Esterase-5. From left to right: *D. virilis*; fresh *D. virilis* and *D. pseudoobscura*; incubated *D. virilis* and *D. pseudoobscura*; fresh *D. pseudoobscura*.

TABLE 3

Hybrid enzyme formation of Esterase-5 between species of the obscura group

	<i>miranda</i>	<i>affinis</i>	<i>azteca</i>	<i>athabasca</i>	<i>algonquin</i>
<i>persimilis</i>	+	TC	+	TC	TC
<i>miranda</i>	..	—	TC	TC	TC
<i>affinis</i>	..	..	—	+	+
<i>azteca</i>	..	..	..	—	TC
<i>athabasca</i>	..	..	..	..	+

TC = Mobility of enzymes too close to test.

Of the Old World obscura group species only *D. subobscura* was able to be tested successfully, and this species was only reliably used with Esterase-5⁹⁵ from *pseudoobscura*. The species in this subgroup all share a common feature in that their putative Esterase-5 is totally inactivated by treatment with 1 M NaCl and almost completely inactivated by brief incubation in buffer.

Only half of the possible interspecific tests between the New World forms of the obscura group were possible because of the similarity of the electrophoretic migration of this enzyme. Of those tested only five were positive. These results are shown in Table 3.

In contrast to the limited ability of Esterase-5 to form *in vitro* hybrids, each electrophoretic form of malate dehydrogenase was capable of forming a detectable and strong hybrid with all tested species within the genus (Figure 3). These one-way tests involved *D. pseudoobscura* and the following members of the genus *Drosophila*: *virilis*, *flavomontana*, *melanogaster*, *yukuba*, *repleta*, *hydei*, *immigrans*, *pallidipennis*, *tripunctata*, *saltans* and the members of the obscura group listed in Table 1.

Neither the *in vivo* nor the *in vitro* sites of enzyme activity of Esterase-5 appear to follow a 1:2:1 ratio of parental A:hybrid:parental B expected of random forma-

FIGURE 3.—*In vitro* hybridization of malate dehydrogenase in *D. pseudoobscura*. From left to right: Frozen and thawed sample of malate dehydrogenase^{1.00} and malate dehydrogenase^{1.20}; a separate sample of the same; fresh malate dehydrogenase^{1.00}; fresh malate dehydrogenase^{1.20}.

tion of dimer (or higher aggregates) from monomers. In all cases investigated the hybrid form of the enzyme appears to be deficient so that the results appeared visually as a 1:1:1 relation. The amount of hybrid enzyme produced between species was not subject to a great deal of variation; but whenever there was a departure from the usual 1:1:1 relationship, it was always at the expense of the hybrid. This was not true in the case of malate dehydrogenase where both the *in vivo* and the *in vitro* activities followed a strictly binomial form. The validity of these conclusions, however, must be substantiated by more than visual estimates.

DISCUSSION

Esterase-5 differs from proteins such as lactate dehydrogenase and hemoglobin studied by others previously and from malate dehydrogenase studied in this report. First, the ability of this enzyme to form interspecific hybrids is limited to closely related species. Second, different alleles from the same species produce enzymes which differ among themselves in their ability to form *in vitro* hybrids with other species. It is probable that these two attributes have a common cause, i.e., the number and kind of binding sites involved in the association of the monomers. It has been possible to distinguish experimentally several alternative kinds of behavior of multimeric macromolecules. With some proteins high or low pH, high ionic strength, and/or freezing and thawing are necessary treatments before *in vitro* hybrids can be detected. For example, it is necessary to freeze and thaw certain lactate dehydrogenases under high ionic conditions before *in vitro* hybrids are formed, and the same is true of malate dehydrogenase in *Drosophila*. Thus the multimer forms of these proteins are quite stable and extreme treatments are necessary to disrupt the molecules and to form monomers. In other proteins the monomers appear to be in a state of dynamic equilibrium with the multimeric state. This appears to be the case for most vertebrate hemoglobins, although the equilibrium under physiological conditions is greatly in favor of the tetramer (GUIDOTTI, KONIGSBERG, and CRAIG 1963). There is the additional factor that some associations of particular monomers appear to be much less stable than others. This has been suggested as the reason for not detecting hybrid human hemoglobin molecules of the type $\alpha_2^A\beta^A\beta^S$ (GUIDOTTI *et al.* 1963; BENESCH, BENESCH and TYUMA 1966). Also, it has been demonstrated that some subunits of alkaline phosphatase from *E. coli* combine with greater affinities than others (FAN *et al.* 1966). This instability may also explain the deviations from random assortment encountered in some *in vitro* experiments with lactate dehydrogenase (SALTHER, CHILSON and KAPLAN 1965).

Regardless of the stability of the multimers, it is widely held that the bonds between the subunits involve ionic, hydrogen and/or polar-polar interaction and are not covalent bonds. The number and kind of such linkages and the arrangement of the interacting subunits is known with a degree of precision only in the case of hemoglobin (PERUTZ 1965).

It is clear that subunits of Esterase-5 are present under physiological conditions in all forms of the enzyme studied. This is indicated by our ability to detect *in*

vitro hybrid molecules by incubation procedures with each species enzyme. Therefore, the inability to detect *in vitro* hybrids must reside either in the inability of the hybrid structure to form or in its ability to be maintained. The differences encountered in *in vitro* formation lie most probably in those residues that bind the subunits together and *not* in the general conformation of the subunits since there is in each instance *in vitro* hybrid formation between at least one pair of the array of enzymes tested.

The limited ability of Esterase-5 to form or maintain *in vitro* hybrids, in contrast to malate dehydrogenase, may lie in two factors: there has been a greater number of changes (amino acid substitutions, etc.) in the esterase than in malate dehydrogenase per evolutionary time period, or the number of bonds holding the subunits of the esterase together is far fewer than in malate dehydrogenase. Several items of information suggest that the latter is more consistent with the present data. The mild conditions and the ease of hybridization (equilibrium is reached in a matter of minutes) suggest that the esterase dimer is relatively unstable, i.e., it may have weaker intermolecular bonds; whereas with malate dehydrogenase no hybrid molecules are formed without freezing and thawing in high salt concentration. Even in heterozygotes of two electrophoretic forms of Esterase-5 there is a lack of a 1:2:1 ratio as judged visually. This suggests that even a limited degree of difference between monomers favors homopolymer combinations. This is also consistent with there being few intermolecular bonds. The fact that different forms of the enzyme within the same species differ in their ability to form *in vitro* hybrids is also consistent with this hypothesis. The most reasonable assumption is that in this allelic series, the molecules differ by only a few amino acid substitutions. Therefore, the ability of Esterase-5^{1.00} from *pseudoobscura* to hybridize with the enzyme from *D. azteca* in contrast to its allelic form, Esterase-5^{1.03}, suggests that single substitutions in the molecule may determine whether a dimer molecule can be formed or maintained. However, it could also be argued that sufficient changes had already occurred between the enzymes from *D. pseudoobscura* and *D. azteca* so that a single additional change was critical.

There is an additional factor that should be considered. The manner in which the multimer is formed from the subunits has interesting evolutionary consequences in itself. There are clearly three general types of such interactions. (1) isologous $\frac{A}{Z} \frac{Z}{A}$; (2) heterologous $\frac{A}{H} \frac{G}{Z}$; and (3) homologous $\frac{A}{A} \frac{Z}{Z}$. It is clear that nonpolar bonds are mainly involved in homologous associations of subunits, whereas both polar and nonpolar bonds are possible in the other cases. Selection for dimerization (or more complex associations) would be strong for those cases where the subunits are inactive or less effective than the multimer, or not subject to effective regulation. Therefore, intraspecific selection would tend to retain strongly those complementary areas of the molecule responsible for aggregation. With all bonds there would be severe selection against change if elimination of a single bond altered the desired criteria for stability (or lability). However, if there were an excess of bonding sites, these regions might be more plastic during evolution. For stability to remain the same with replacement of

one kind of binding situation with another, both isologous and heterologous associations would, in general, require the simultaneous substitution of two different amino acid residues in the molecule. However, in homologous associations, a new bond could result from a single amino acid substitution. Thus the possibility exists that the restricted ability of Esterase-5 to form hybrids between different species might be due to the fact that it is a homologically paired multimer in which substitutions have occurred more often within the binding sites of the subunits during evolution.

Do these findings have anything to bear on the usefulness of *in vitro* hybridization studies for determining phyletic distances between organisms? The differences in the ability of the several forms of Esterase-5 to form *in vitro* hybrids clearly contains some phyletic information. First, it delineates the obscura group from other species groups within the genus *Drosophila*. Second, it discriminates between certain New World members of this group. In order to evaluate this discrimination we must determine whether this within group delineation is consistent with the present concept of evolution within the group. Let us consider any formation of hybrid enzyme as an indication of species similarity, and then evaluate the similarities and differences among the species studied. By such a criterion two clusters of species can be obtained: *pseudoobscura*, *persimilis*, *miranda*, *azteca*; and *affinis*, *algonquin*, *athabasca*. By considering allelic differences in hybridization as discriminatory, the following phenogram can be derived.

There is additional possible phyletic information from these results based on the sensitivity of all of the Old World obscura group species to brief incubation and their great sensitivity to the salt treatment.

Such a scheme is completely consistent with the known evolutionary relationships of this group (STURTEVANT 1940; PATTERSON and STONE 1952). Such consistency must be viewed with caution at present. Recall that only a single form of the enzyme was tested in species other than *D. pseudoobscura*. If polymorphism of this enzyme is as great in the other species as it is in *D. pseudoobscura* (LEWONTIN and HUBBY 1966), not all forms of the enzyme were available for study. Additional forms might be found which alter the relationship. For example, if Esterase-5⁹⁵ of *pseudoobscura* had not been studied, the relationship indicated would not have been found. In sum, more enzymes like Esterase-5 will have to

be investigated before the general utility of *in vitro* hybridization can be fully evaluated as a tool for evolutionary study.

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SUMMARY

Two enzymes, an esterase and malate dehydrogenase, each form *in vitro* hybrids under suitable conditions. Five alleles of the esterase and three alleles of malate dehydrogenase were available for study from *D. pseudoobscura*. Each form of these two enzymes were tested for their ability to form *in vitro* hybrids with the homologous enzyme from other members of the obscura group and from selected species of other species groups of the genus *Drosophila*. All forms of malate dehydrogenase can form *in vitro* hybrids with all of the species tested. In contrast, *in vitro* hybrids of the esterase were only formed between *D. pseudoobscura* and members of the obscura group. Furthermore, the enzymes produced by different alleles in *D. pseudoobscura* differed in their ability to form *in vitro* hybrids with these obscura group species.

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