THE GENETIC CONTROL OF ALCOHOL DEHYDROGENASE AND OCTANOL DEHYDROGENASE ISOZYMES IN DROSOPHILA

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The detection, by agar gel electrophoresis, of as many as ten alcohol dehydrogenase isozymes in Drosophila melanogaster (URSPRUNG and LEONE 1965) presents a problem in the interpretation of genetic and epigenetic control of synthesis and function of multiple forms of enzymes. The observation that the three slowest cathodally migrating isozymes show a more intensive formazan staining when primary octanol rather than ethanol is used as substrate suggested that these isozymes belong to a separate enzyme system.

Support for this assumption comes from the finding that there are D. melanogaster stocks which are different with regard to electrophoretic mobility of those isozymes that stain with a wide spectrum of alcohols (ADH), but are equal with regard to the mobility of the hexanol, heptanol, and octanol-specific enzyme (ODH) or vice versa (COURTRIGHT 1966a). Strong support for the distinction between ADH and ODH comes from the map locations. The locus for ODH is on the third chromosome, in contrast to the locus for ADH which according to GReLL, JACOBSON and MURPHY (1965) maps on the second chromosome. Finally, the two isozyme systems, ADH and ODH, elute differentially from diethylaminoethyl cellulose (DEAE-cellulose) columns.

MATERIALS AND METHODS

Flies: The Oregon-R (Ore-R), Oregon-R(CH), (Ore-R(CH)), ru h th st cu sr e4 ca, and the Cy/Pm, Sh/Ub (Curly/Plum; Stubble/Ultrabithorax) strains were obtained from stocks kept in this Department. The p6 cu stock was obtained from the Philadelphia stock center; ru lxd by ry4, from Dr. E. GLASSMAN. Third chromosome markers used were ru = rough (0.0); h = hairy (26.5); th = thread (43.2); st = scarlet (44.0); cu = curled (50.0); sr = striped (62); e4 = ebony-sooty (70.7); ca = claret (100.7); by = blistry (48.7); p6 = pink peach (48.0) (all values after BRIDGES and BREHME 1944); ry = rosy (52.35) (CHOVNICK et al. 1964); lxd = low xanthine dehydrogenase (33 ±) (KELLER and GLASSMAN 1964).

 Cultures: All strains were maintained on cornmeal-agar food seeded with the Y2 yeast strain isolated from cactus by Wagner (1949). Single-fly matings were done in 25 ml vials, mass matings, in half pint bottles. Progeny were collected 3 to 6 days after hatching, by light etherization.

Electrophoresis: For mass extracts flies were homogenized in Ten Broeck homogenizers at a concentration of 400 mg flies per ml of medium. Distilled water or a 1/20 dilution of the following stock buffer ("EBT") pH 8.7 was used for homogenization: 7.4 g versene; 30.9 g boric acid; 109.1 g Tris (Tris(hydroxymethyl)aminomethane) distilled H2O to 1,000 ml. Homogenization may equally be performed in 0.1 n Tris-HCl at pH 8.0. Extracts were centrifuged in a Servall Refrigerated Centrifuge for 20 minutes at 30,000 × g or in an International "Hematocrit" centri-
fuge for 3 min at 11,000 \times g. The supernatant was applied to 0.9% agar gels as described by Ursprung and Leone (1965) and electrophoresed for 30 to 40 min in the apparatus described by Wiele (1964) at temperatures between 5° and 15°C. Single flies were squashed directly on 3 mm wide strips of Whatman #3MM filter paper, the strips inserted into the agar gel and moistened with a drop of 1/20 EBT buffer. Before the electrophoresis was started, the strips were removed from the gels in order to prevent streaking.

After completion of the electrophoresis, gels were stained in a mixture containing 50 mg NAD (nicotinamide adenine dinucleotide) (Sigma), 6.25 mg Nitro Blue Tetrazolium (Dajac), 0.5 mg phenazine methosulphate (Sigma); 21 ml of 0.2 M Tris-HCl, pH 9, and 0.5 ml primary octanol (Fisher). Final pH of this staining mixture was 9.0. At 37°C, staining is complete in 45 to 60 min. Gels can be fixed in a mixture of ethanol, glacial acetic acid, and water (15:1:5).

a. **Genetic mapping:** Mass homogenates of *Drosophila melanogaster* laboratory strains revealed three types of octanol dehydrogenase zymograms (Figure 1). The reaction of these three isozyme types is specific for the six, seven, and eight carbon primary alcohols, and to date no reaction has been observed with any other substrate. The possibly related glycerol phosphate, isocitric acid, malic acid, glucose-6-phosphate, histidinol, 2-octanol, nonyl alcohol, decyl alcohol, and 1, 2-decandiol were tested, but found not to serve as substrates for the reaction. Electrophoresis of single flies from strains possessing three zones of activity yielded either a single slower moving isozyme (type I); a single faster moving isozyme (type II); or three zones of activity (type III); but never type I and II.

Type I individuals are considered to be homozygous for the *ODH*\(^I\) (slow) allele, type II for the *ODH*\(^{II}\) (fast) allele; whereas type III individuals are heterozygotes, *ODH*\(^I\)/*ODH*\(^{II}\). The intermediate band observed in type III individuals represents the hybrid enzyme.

By selecting single flies, a derived Oregon-R stock was obtained which possessed only type II activity. Reciprocal crosses of I females \(\times\) II males and II females \(\times\) I males established that the genetic determinant is not sex-linked.
To determine which linkage group contained the ODH locus, the derived Oregon-R (type II, fast) was crossed to the balanced lethal dominant marker strain Cy/Pm; Sb/Ubx (type I, slow). The F₁ progeny were crossed among themselves. Examination of F₂ progeny showed that the fast allele (ODH⁺) derived from the Oregon-R strain segregates from the dominant 3rd chromosome markers Sb or Ubx but is assorted at random with respect to the 2nd chromosome markers.

**TABLE 1**

Results of crosses of Oregon-R (II,fast) × Cy/Pm; Sb/Ubx (I,slow)

<table>
<thead>
<tr>
<th>Generation</th>
<th>Chromosomal marker</th>
<th>Number of flies of ODH type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I(slow)</td>
</tr>
<tr>
<td>P</td>
<td>+/+; +/+</td>
<td>0</td>
</tr>
<tr>
<td>P</td>
<td>Cy/Pm; Sb/Ubx</td>
<td>10</td>
</tr>
<tr>
<td>F₁</td>
<td>Cy/+; Sb/+ or Pm/+; Ubx/+</td>
<td>0</td>
</tr>
<tr>
<td>F₂</td>
<td>Cy/+; Sb/+ or Pm/+; Ubx/+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+/+; +/+ or +/+; Ubx/+</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+/+; +/+</td>
<td>0</td>
</tr>
</tbody>
</table>

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**TABLE 2**

Crosses of Oregon-R(II,fast) × pP cu (I,slow)

<table>
<thead>
<tr>
<th>Generation</th>
<th>Genotype</th>
<th>Number of flies with ODH type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I (slow)</td>
</tr>
<tr>
<td>F₁</td>
<td>pP (ODH¹) cu</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>+ (ODH¹) +</td>
<td></td>
</tr>
<tr>
<td>Backcross</td>
<td>pP (ODH¹) +</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>pP (ODH¹) cu</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pP (ODH¹) +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pP (ODH¹) cu</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ (ODH¹) cu</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>pP (ODH¹) cu</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ (ODH¹) cu</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>pP (ODH¹) cu</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pP (ODH¹) cu*</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>pP (ODH¹) cu</td>
<td></td>
</tr>
</tbody>
</table>

* This noncrossover class is presented here in order to exclude the possibility that the ODH locus is not within the pP cu region.
Cy or Pm. Thus Sb or Ubx flies were always heterozygotes, \(ODH^1/ODH^II\) (type III) with three isozymes, whereas Cy or Pm \(F_2\) flies were fast (type II) like the Oregon-R parental strain (Table 1). It should be recalled at this point that the locus responsible for variants of ADH has been assigned to the 2nd chromosome (Grell et al. 1965). Our own experiments involving ADH mutants, not reported in this paper, have confirmed the finding of these authors.

For mapping the \(ODH\) locus on the third chromosome, Oregon-R (II, fast) was crossed to \(ru\ h\ th\ st\ cu\ sr\ e^+\ ca\) (I, slow). The \(F_1\) females were backcrossed to \(ru\ h\ th\ st\ cu\ sr\ e^+\ ca\) and the progeny were analyzed without regard to sex. The results of these crosses confirmed the third chromosome as the linkage group and indicated a map position at 49.4 ±. Use was then made of the third chromosome markers, \(ru\ lxd\ by\ ry^{e}\) (II, fast), in order to determine more precisely the location of the \(ODH\) gene. The values obtained from these crosses indicate a map position of 49.2. Finally, the \(ODH\) gene was also mapped using the markers \(p^o\ cu\) (I, slow). The results obtained in these crosses are listed in Table 2 and place the \(ODH\) gene at 49.1.

There is evidence, thus, that ODH and ADH systems are genetically separate isozyme systems controlled by loci on two different chromosomes.

b. Chromatography: We have also evidence that the two isozyme systems are different proteins on the basis of their purification properties.

Mass homogenates were prepared in 0.1 M Tris-HCl, pH 8, as described in Methods, except that 10 mg Bovine Serum Albumin (Sigma) were added to each milliliter of homogenizing medium. 30,000 × g supernatants were passed through a 2.5 × 40 cm column of Sephadex G-25 to remove eye pigments. The ratio \(OD_{280}:OD_{260}\) was determined in each fraction eluted from the

![Figure 2](image_url)

**Figure 2.—Separation of octanol dehydrogenase from alcohol dehydrogenase. Zymograms of aliquots from each of the fractions obtained on elution from a DEAE-cellulose column. The gel was stained for both ADH and ODH activity by supplying ethanol and n-octanol to the staining mixture. Also indicated are \(OD_{280}\) profile (curve) and salt gradient (straight line). Origin near the top.**
column; all those fractions in which this ratio indicated 60% protein or more (Warburg and Christianson 1941) were pooled. A saturated solution of protamine sulfate (Nutritional Biochemical Co.) was added slowly with continuous stirring to a final concentration of 8% (v/v) in order to remove nucleic acids from solution. The resulting solution was centrifuged (20 min, 30,000 \( \times g \)), and the pale yellow supernatant brought to 40% saturation with saturated ammonium sulphate, previously adjusted to pH 8.0 with concentrated ammonium hydroxide. The resulting precipitate did not contain ODH activity when it was dissolved in dilute buffer, electrophoresed, and the gel stained for ODH. The supernatant was adjusted to 60% saturation with neutralized, saturated ammonium sulfate, the resulting precipitate collected by centrifugation, redissolved in distilled water, and dialyzed for 3 hours against 0.01 M Tris-HCl pH 8.5, which was also 0.001 molar with respect to EDTA. This fraction was then applied to a 3.3 \( \times 10 \) cm column of DEAE cellulose (Whatman, 1.0 milliequivalents/gram) previously equilibrated with 0.01 M Tris, 0.0005 M EDTA pH 8.5. The sample was eluted with a linear NaCl gradient ranging in concentration from 0.0 to 0.3 M and collected in 11 ml fractions. Aliquots were electrophoresed in agar gels and stained.

As seen from Figure 2, separation of ADH from ODH is obtained by this procedure.

It is hard to make a quantitative statement on the purification that was achieved in this procedure, since staining intensity of ODH bands on the gel was the only criterion used for activity. In our gel assay, we routinely apply aliquots of 3 µl to the origin of the gel. It takes about 50 times less protein from the ODH peak than of a crude homogenate to produce a similar staining intensity, indicating that approximately 50-fold purification was achieved in the steps described above.

c. Electrophoresis: In order to investigate further the possible interdependence of the two isozyme clusters, ADH and ODH, it was important to know whether the observed mutations affected the electrophoretic mobility of ADH and ODH coordinately. As is evident from Figure 3, this is not the case however. Fast ADH variants can be fast or slow with respect to ODH, and vice versa.

The ODH bands observed on our gels migrate to a position very similar to aldehyde oxidase bands observed in the course of a related investigation (Courtwright 1966b). It appeared possible, therefore, that the aldehyde produced by ADH activity during the staining reaction could serve as substrate for aldehyde oxidase bands. ODH bands accordingly would be artifacts. In fact when acetaldehyde, benzaldehyde, or pyridoxal are supplied as substrates, a formazan band is observed at or near the position of the ODH type I. However, we have several lines of evidence that these two reactions are not due to only one enzyme present in the gel. First, flies of both ODH types III or II, respectively, have but a single oxidase band, with an electrophoretic mobility different from either intermediate or fast ODH (Figure 4). Second, ma-I and lxd flies, which have no, or little oxidase activity, respectively, show ODH staining equal to that of wild-type. Third, Drosophila simulans strains with aldehyde oxidases of different electrophoretic mobility, have ODH of equal mobility (Figure 4b).

d. ODH and ADH in flies of different biogeographic origins: The habitats of South American drosophilids have been investigated by Pipkin (1965), and Pipkin, Rodríguez and León (1966). It was of interest in our analysis to determine whether the activities of ADH and/or ODH were correlated with the feed-
ing habits of the flies. We are indebted to Dr. S. B. Pipkin in this department for kindly supplying the following preliminary qualitative data, obtained by visual inspection of formazan bands formed on agar gels after ODH or ADH staining. The following species of nearctic or palaeartic origin showed strong ADH and ODH activity: *D. melanogaster*, *D. simulans*, *D. immigrans*, *D. pseudoobscura*, *D. lebanonensis*, *D. castelei*, *D. patternsoni* and *D. busckii*. In contrast, only three species of neotropical origin, *D. thoracis* and an undescribed member of the annulimana and peruviana species groups respectively show strong ADH and ODH activity. However, 27 neotropical species belonging to ten different species groups showed ODH activity but virtually no ADH activity. These included the following species: Ground feeders (class A, Pipkin 1965) *D. albirostris*, *D. mediostriata*, *D. crocina*, *D. metcii*, *D. pellewae*, *D. trifiloides*, *D. johnstonae*, *D. blumelae*, *D. greerae*, *D. calloptera*, and four undescribed members of the tripunctata species group; Ground feeders (class B, Pipkin 1965) *D. capricorni*; Ground feeders (class C, Pipkin 1965) *D. neomorpha*, *D. guaraja*, *D. briegeri*, *D. carmargoi*, *D. paranaensis* and an undescribed member of the dreyfusi species group; Oligophagous flower feeders (Pipkin, Rodriguez, and Leon 1966) *D. sticta*, and three undescribed members of the flavopilosa species group, Monophagous flower feeders (Pipkin, Rodriguez, and Leon 1966) *D. mcclintockae*. Two sibling species, *D. unipunctata* and *D. roerhae*, of the tripunctata species group consistently failed to show activity for either ADH or ODH. Thus no correlation is apparent between types of alcohol dehydrogenase and feeding habits. It is also clear however that a correlation does exist between biogeographic origin and type of alcohol dehydrogenase.

**DISCUSSION**

The original observation of as many as ten “alcohol dehydrogenases” in crude extracts of *Drosophila melanogaster* (Ursprung and Leone 1965) poses difficult questions with regard to the genetic control and enzymological meaning of isozymes.

In this paper we present evidence that the ten isozymes can be grouped into two clusters, one including seven isozymes termed ADH, the other, three isozymes termed ODH. The two isozyme clusters, ADH and ODH, differ in substrate specificity, chromatographic behavior, and chromosome linkage. Mutations altering the mobility of ADH do not necessarily affect the migration of ODH. Also, the two clusters clearly migrate as separate groups in electrophoresis.

The findings of Dr. S. B. Pipkin, that various species of *Drosophila* may either contain ADH and ODH activity, or primarily ODH, or neither ADH nor ODH

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**Figure 3.**—Independent migration of octanol dehydrogenase and alcohol dehydrogenase. Single fly zymograms. The following stocks were used to obtain the various ODH and ADH types: ODH 1; ADH II (Bethylie); ODH II; ADH II (derived Oregon-R); ODH II; ADH I (ru xyz by ryz), and ODH I; ADH I (Oregon-R (CH)). In order to show the difference in staining intensity between ODH and ADH, only octanol was supplied as substrate. Origin near the top. Bands near the origin: ODH. Bands near the cathode: ADH.
supports the assumption of two separate loci for the two enzyme systems and furthermore suggests that the genetic control of the two enzymes is remarkably similar throughout Drosophila species.

These findings reduce the problem considerably, because we can now legitimately regard the two clusters as independent systems. The formation of seven isozymes of ADH has been interpreted earlier, by Grell et al. (1965) and Ursprung and Leone (1965) on the assumption that each homozygous mutant synthesized three dimers composed of different subunits. In the heterozygote, seven isozymes would then be expected, some of the parental type, some representing hybrid enzymes. The proposed dimer model is being challenged by new evidence which questions the involvement of different protein subunits in the formation of ADH isozymes (Ursprung and Carlin 1967.) But nevertheless the independent genetic control of ADH remains valid.

The three ODH isozymes are more easily explained than multiple forms of ADH. Most likely, the three ODH forms represent the three possible dimers formed by interactions of two subunits, each controlled by its ODH allele. Thus, the fast ODH (I) would be composed of two subunits, I/I; the slow variant, ODH
II, of two subunits II/II, whereas the hybrid band found in heterozygotes would be a dimer of the constitution I/II.

Summing up this genetic aspect, it appears sufficient to assume two alleles for ADH, and two for ODH. Polymerization of the subunits synthesized by the ADH alleles would then account, epigenetically, for the formation of seven ADH isozymes in a heterozygote. Polymerization of the subunits synthesized by the ODH alleles would lead to three more isozymes in a heterozygote. All total, ten isozymes would thus be formed in a double heterozygote.

The biological role of ODH is poorly understood. It may be functioning in the utilization of long chain alcohols that are found esterified in plant oils (Buchanan et al. 1965). The presence of esterases (Wright 1963) and aldehyde oxidases (COURTRIGHT 1966b) in D. melanogaster renders possible the hydrolysis and subsequent oxidation of these long chain alcohol esters. It should be pointed out however that ODH activity apparently is not indispensable in some Drosophila species of the tripunctate group which were found to lack it. Also, no correlation has thus far been found between feeding habits and ODH content.

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SUMMARY

Crude extracts of Drosophila melanogaster contain as many as ten electrophoretically separable alcohol dehydrogenases, which on agar gels migrate as two clusters, one including seven, the other, three isozymes.—Those isozymes belonging to the cluster of seven are particularly reactive with ethanol and are termed alcohol dehydrogenases (ADH). Those belonging to the cluster of three react with octanol and other long-chain alcohols; they are termed octanol dehydrogenase (ODH).—Genetic variants for the electrophoretic mobility of either ADH, ODH, or both have been found. The locus for ADH is 2-50.1 (Grell et al. 1965). The locus for ODH, as determined in this paper, is 3-49.2+. ADH and ODH thus are independent enzyme systems on genetic grounds.—ADH and ODH are different also in their chromatographic behavior; they were found to elute differentially from DEAE-cellulose columns.—A preliminary analysis of about 40 Drosophila species, carried out by Dr. S. B. Pipkin, revealed no correlation between feeding habits and ADH or ODH content, but such correlations were found with regard to biogeographic origin.

LITERATURE CITED


