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Alcohol-Oxidizing Enzymes in 13 *Drosophila* Species

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Starch and polyacrylamide gel electrophoresis were used to ascertain the substrate specificities of alcohol-oxidizing enzymes in 13 Drosophila *species. The substrates used were a variety of long- and short-chain aliphatic alcohols, one aromatic alcohol, and benzaldehyde. Only one enzyme (product of a single-gene locus) showed significant NAD^+-dependent alcohol dehydrogenase activity with short-chain aliphatic alcohols. The 13 species, belonging to four different* Drosophila *groups, all showed a similar complement of alcohol-oxidizing enzymes, although differences in electrophoretic mobility and in levels of activity existed from species to species. These findings are relevant to the adaptation of* Drosophila *to alcohol environments.*

KEY WORDS: substrate specificity; alcohol dehydrogenase; octanol dehydrogenase; aldehyde oxidase; *Drosophila.*

INTRODUCTION

The *Adh* locus in *Drosophila melanogaster* (II, 50.1) and its gene product, alcohol dehydrogenase (ADH, E.C. 1.1.1.1), have been the subject of intensive study in recent years (e.g., Day *et al.*, 1974; Hewitt *et al.*, 1974; Vigue and Johnson, 1973; Ward, 1975). While it is known that tolerance to short-chain aliphatic alcohols correlates well with activity levels of the enzyme produced by the *Adh* locus (McDonald and Avise, 1976; McDonald *et al.*, 1977), it is also known that other enzymes, such as octanol dehydrogenase (ODH, E.C.

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1.1.1.73), are capable of catalyzing the oxidation of alcohols in *Drosophila* species. For example, in *D. pseudoobscura* Singh (1976) has demonstrated the presence of eight electrophoretic zones of activity (called by him "*Adh* loci") distinguishable on the basis of substrate specificities. Several of these proteins appear to be capable of oxidizing short-chain aliphatic alcohols as detected by tetrazolium deposition on polyacrylamide gels. These findings have prompted us to look for equivalent enzymes in the much-studied *D. melanogaster* as well as in other *Drosophila* species with the purposes of (1) determining homologies between species and (2) examining substrate specificities in order to identify the enzymes responsible for the various staining zones and hence to evaluate their roles in the metabolism of short-chain aliphatic alcohols.

MATERIALS AND METHODS

All flies examined come from laboratory stocks, established with wild-caught females, and maintained in our laboratory in standard cornmeal–molasses medium for times ranging from several months to several years. The 13 *Drosophila* species examined and the collection places and dates are as follows (the abbreviations given in parentheses are used in Figs. 2–4):

D. ananassae (*ana*), Mérida, Yucatán, Mexico, 1974.
D. birchii (*bir*), Wau, New Guinea, 1972.
D. equinoxialis (*equi*), Chetumal, Quintana Roo, Mexico, 1974.
D. immigrans (*immi*), Sonoma County, California, 1976.
D. insularis (*ins*), Monserrat, West Indies, 1963.
D. mauritiana (*mau*), Mauritius Island, Indian Ocean, 1973.
D. melanogaster (*mel*), Napa County, California, 1976.
D. nebulosa (*neb*), Peto, Yucatán, Mexico, 1974.
D. persimilis (*per*), Napa County, California, 1976.
D. pseudoobscura (*pseu*), Napa County, California, 1976.
D. simulans (*sim*), Redwood City, California, 1976.
D. tropicalis (*trop*), Belém, Brazil, 1969.
D. willistoni (*will*), Chetumal, Quintana Roo, Mexico, 1974.

Drosophila melanogaster, D. simulans, and *D. mauritiana* (Tsacas and David, 1974) are sibling species; together with *D. ananassae* and *D. birchii,* they belong to the *melanogaster* species group. *Drosophila pseudoobscura* and *D. persimilis* are siblings belonging to the *obscura* group. *D. equinoxialis, D. insularis, D. tropicalis,* and *D. willistoni* are also sibling species that together with *D. nebulosa* belong to the *willistoni* species group. All these 12 species belong to the subgenus *Sophophora. D. immigrans* belongs to the *immigrans* species group of the subgenus *Drosophila.*

Electrophoresis was carried out using starch as well as polyacrylamide

gels. Starch gel electrophoresis was carried out according to the methods of Ayala *et al.* (1972) using starch from the Sigma Chemical Co. and the discontinuous buffer system of Poulik (1957). Polyacrylamide gels were run in an Ortec apparatus following the methods of Singh (1976).

Samples from each species were prepared by grinding 100 individuals at 4 C in 1.5 ml of 0.1 M tris-borate-EDTA buffer, *p*H 8.9, containing 10% (w/v) sucrose. Homogenates were centrifuged in order to remove insoluble material. For polyacrylamide gels, 10-μl samples from this solution were used; filter paper wicks (Whatman No. 1) moistened in the solution were employed for starch gels.

Staining mixtures consisted of 100 ml of 55.5 mM tris-HCl buffer, *p*H 8.6, containing 20 mg nitro blue tetrazolium, 1 mg phenazine methosulfate, 1 ml of the test substrate (either an alcohol or aldehyde), and 25 mg NAD^+ where indicated. Gels were incubated in the staining solution at 37 C for 1–2 hr, at which point staining regions were scored; the gels were then incubated for an additional 2–3 hr (occasionally longer if necessary to show the presence of weak-staining bands) and scored again. After staining, the gels were fixed in an acetic acid–methanol mixture (Ayala *et al.*, 1972).

In the case of starch gels, three slices were usually obtained from each gel; these were stained as follows:

Slice 1: test alcohol plus NAD^+.
Slice 2: isopropanol plus octanol plus NAD^+.
Slice 3: test alcohol without NAD^+.

Slice 2 was used as a control since it would be expected to exhibit all bands shown in the various tests.

In the case of polyacrylamide gels, three identical sets of six samples each were placed on each gel, which after electrophoresis was cut into three pieces stained as for the three starch gel slices.

RESULTS

Comparison of *Drosophila melanogaster* and *D. persimilis* with *D. pseudoobscura*

Starch gel electrophoresis was used first to examine staining regions detectable in third instar larvae and in 5- to 10-day-old adults of *D. melanogaster, D. persimilis,* and *D. pseudoobscura*, using as substrate a variety of alcohols and benzaldehyde. The substrate specificities are shown in Table I, where the bands are numbered as in Fig. 1. This figure represents the patterns observed in starch "control" gels (i.e., those using isopropanol plus octanol plus NAD^+ as substrates).

Table I. Substrate Specificities of Electrophoretic Bands on Starch Gels, Using the Indicated Substrates in the Presence or Absence of NAD^+[a]

		D. melanogaster				*D. pseudoobscura*				*D. persimilis*			
Substrate	NAD^+	1	2	3	4	1	2	3	4	1	2	3	4
Ethanol	Present	+	+	+	−	+	+	+	−	+	+	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	−	−
Isopropanol	Present	+	+	+	−	+	+	+	−	+	+	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
n-Propanol	Present	+	+	+	−	+	+	+	−	+	+	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Butanol	Present	+	+	+	−	+	+	+	−	+	+	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Allyl alcohol	Present	+	+	+	−	+	+	+	+	+	+	+	+
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Octanol	Present	±	−	+	+	−	−	+	+	−	−	+	+
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Heptyl alcohol	Present	+	+	+	+	±	−	+	+	±	−	+	+
	Absent	−	−	+	−	−	−	+	−	−	−	−	−
Decyl alcohol	Present	−	−	+	−	−	−	+	−	−	−	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Decyl alcohol and acetone	Present	+	+	+	−	+	±	+	−	+	±	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Benzyl alcohol	Present	±	−	+	−	−	−	+	−	−	−	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Benzaldehyde	Present	−	−	+	−	−	−	+	−	−	−	+	−
	Absent	−	−	+	−	−	−	+	−	−	−	+	−
Isopropanol and octanol	Present	+	+	+	+	+	+	+	+	+	+	+	+

[a] Bands 1, 2, 3, and 4 are as in Fig. 1. + indicates a well-expressed band, − indicates absence of band, ± indicates a faint band.

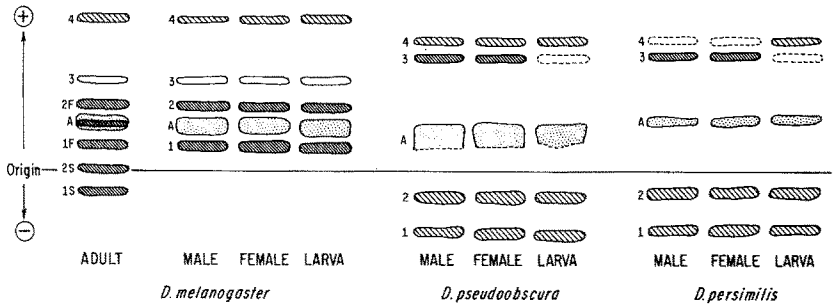

Fig. 1. Staining regions exhibited in starch gels by three *Drosophila* species, using a mixture of isopropanol, octanol, and NAD^+ as substrates. The larvae are third instar; all other samples are from adults 5–10 days old. The *D. melanogaster* flies at the extreme left are males and females heterozygous for the *F* and *S* alleles at the *Adh* locus; the other three *D. melanogaster* samples are from homozygotes for the *F* allele. Bands 1 and 2 are due to ADH, band 3 to AO, and band 4 to ODH. Band A is not an alcohol dehydrogenase.

All three species examined exhibit the same complement of staining regions and identical patterns of substrate specificities. In this experiment, band 3 was not detected in *D. persimilis* samples stained with ethyl or heptyl alcohol in the absence of NAD^+. We attribute this observation to a low enzyme concentration in these particular samples as this activity was subsequently detected in the species survey. The staining regions may be characterized as follows.

Bands 1 and 2. Bands 1 and 2 are attributable to the well-characterized alcohol dehydrogenase (ADH). The enzyme is relatively nonspecific in that it shows reaction with almost all alcohols tested (no reaction with decyl alcohol without acetone, and weak or no reaction with octanol or benzyl alcohol). It does, however, show a marked preference for short-chain aliphatic alcohols and an absolute requirement for NAD^+. The two bands behave as products of a single allele at a gene locus, which in *D. melanogaster* maps at 50.1 in chromosome II. We observed only two bands, but anywhere from one to five bands have been reported (Dickinson and Sullivan, 1975). The multibanded pattern of this single-gene product is believed to result from noncovalent binding of different amounts of a negatively charged NAD-carbonyl compound (Schwartz and Sofer, 1976); it has also been suggested that the bands are conformational isomers (Jacobson *et al.*, 1972; Knopp and Jacobson, 1972).

Band A. Band A exhibits a blue rather than the normal purple color associated with the deposition of insoluble tetrazolium. The band is present in gels incubated with any one of the alcohols tested (but is stronger with the more hydrophobic alcohols) in the presence or absence of NAD^+. For these

reasons, we do not consider it to be the product of a true *Adh* locus. Indeed, it seems likely that this staining region does not result from alcohol oxidation at all but rather from a direct interaction between the protein and nitro blue tetrazolium. This possibility was further investigated using polyacrylamide gels.

Band 3. Band 3 appears with any of the alcohols tested in the presence or absence of NAD^+, and is identical in mobility to the aldehyde oxidase band detected when benzaldehyde is employed as substrate. We infer, therefore, that band 3 is due to aldehyde oxidase (AO, E.C. 1.2.3.1, the product of the *Ao,* or *Aldox,* locus which in *D. melanogaster* maps at III, 56.6; see Dickinson and Sullivan, 1975).

Band 4. Under the test conditions, band 4 shows a preference for long-chain aliphatic alcohols, particularly octanol and a requirement for NAD^+. This allows identification of this band as an octanol dehydrogenase (ODH, E.C. 1.1.1.73; see Dickinson and Sullivan, 1975).

This experiment, which employs a limited range of test conditions on crude extracts purified only by electrophoresis, cannot be considered adequate to define fully the substrate specificities of the enzymes under study. However, the information obtained together with the electrophoretic mobilities of the bands permits unambiguous identification of the enzymes involved by reference to previously published work of our own and other laboratories (see Dickinson and Sullivan, 1975, and references therein).

The same three species—*D. melanogaster, D. persimilis,* and *D. pseudoobscura*—were examined using polyacrylamide gel electrophoresis to see whether any additional staining regions could be detected, and to be able to compare our results with those of Singh (1976). The patterns observed in a control gel are shown in Fig. 2. The same staining solutions were used as for

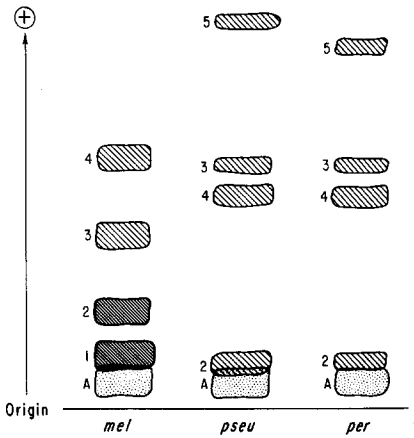

Fig. 2. Staining regions exhibited in polyacrylamide gels by three *Drosophila* species, using a mixture of isopropanol, octanol, and NAD^+ as substrates. All samples are from adult flies 5–10 days old; the species abbreviations are given in Materials and Methods. Bands A and 1–4 are the same as in Fig. 1; band 5 is a second ODH enzyme.

starch gels, except that the *n*-propanol was not tested, and additional gels were stained with nitro blue tetrazolium (with and without NAD^+) but no alcohol substrate, in order to test our hypothesis mentioned above concerning band A.

The observed substrate specificities allow us to make the band assignments shown in Fig. 2. The correspondences of these staining regions with those described by Singh (1976) are as follows.

Bands 1 and 2 (ADH) correspond with Singh's *Adh-1* and *Adh-3*. Two bands are observable only in *D. melanogaster*; band 1 migrates cathodally in the other two species.

Band A seems the same as Singh's *Adh-2*. Band A looks more purple in polyacrylamide than in starch gels, but as predicted reacts with nitro blue tetrazolium in the absence of alcohol and in the absence (or presence) of NAD^+. Thus this band is not an alcohol dehydrogenase.

Band 3 (AO) corresponds to Singh's *Adh-6*; as pointed out above, this band is due to aldehyde oxidase.

Band 4 (ODH) corresponds to Singh's *Adh-4* (*Odh-1*).

Band 5 appears as a weak activity zone after overnight staining and only in *D. pseudoobscura* and *D. persimilis*, not in *D. melanogaster*; it corresponds to Singh's *Adh-8* (*Odh-3*) and has identical substrate specificity to band 4.

We have failed to detect Singh's bands *Adh-5* and *Adh-7*. *Adh-5* appeared as a very weakly staining band in only one of Singh's experiments. *Adh-7* was reported as a weak band showing only in old females.

Survey of 13 *Drosophila* Species

We have examined 13 species belonging to four different species groups of *Drosophila* to ascertain whether the same general patterns of zones of activity for alcohol dehydrogenase and aldehyde oxidase are common to all species, or whether distinctive differences exist that might be related to phylogenetic propinquity. Both gel techniques were used: Fig. 3 shows a control starch gel containing samples from nine different species, and Fig. 4 a polyacrylamide control gel with 11 species.

Figures 3 and 4 show that essentially the same complement of enzymes is present in all species, with the following two exceptions.

1. Band 5, the fastest ODH zone of activity, is not revealed in polyacrylamide gels for five species: *D. birchii, D. melanogaster, D. nebulosa, D. persimilis,* and *D. pseudoobscura.* However, as indicated above, band 5 appears, at least in *D. pseudoobscura* and *D. persimilis,* when staining is continued for many hours.

2. In starch gels, several aldehyde oxidase (band 3) zones of activity are revealed in some species with benzaldehyde staining, but not all these bands

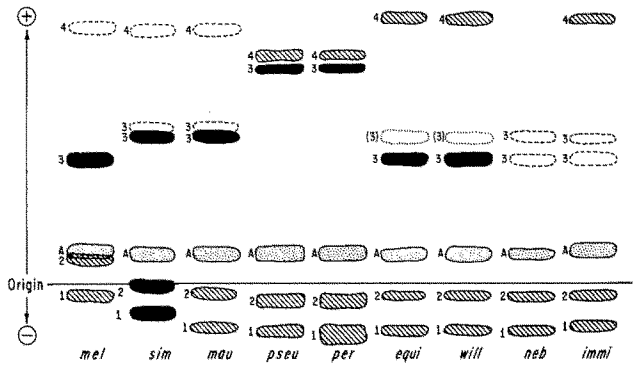

Fig. 3. Staining regions exhibited in starch gels by nine *Drosophila* species, using a mixture of isopropanol, octanol, and NAD^+ as substrates. All samples are from adult flies 5–10 days old; the species abbreviations are given in Materials and Methods. The bands are labeled as in Fig. 1. Bands marked (3) are aldehyde oxidase bands revealed only after staining with benzaldehyde as substrate. Note that the *D. melanogaster* strain used was homozygous for the *S* allele at the *Adh* locus.

appear when alcohol is employed as substrate. For example, in *D. immigrans* and *D. nebulosa* both AO bands are revealed by alcohol staining, with or without NAD^+; while in *D. willistoni* and *D. equinoxialis* only one of the two AO bands appears when alcohol is used, whether or not NAD^+ is added. There are several possible explanations for this, but it seems likely that the failure to detect some AO bands with alcohol as substrate may be due to low AO activity which fails to show when alcohol rather than aldehyde—a more specific substrate—is used.

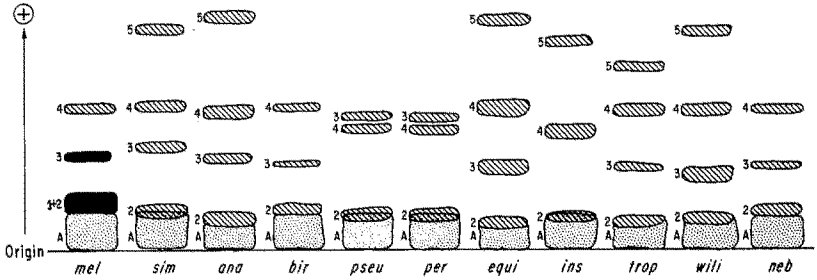

Fig. 4. Staining regions exhibited in polyacrylamide gels by 11 *Drosophila* species, using a mixture of isopropanol, octanol, and NAD^+ as substrates. All samples are from adult flies 5–10 days old; the species abbreviations are given in Materials and Methods. Bands are labeled as in Figs. 1 and 2.

DISCUSSION

A considerable number of studies have recently appeared dealing with alcohol dehydrogenase activity and its genetic control in *Drosophila*. Some studies are primarily surveys of genetic variation, while others have focused on the molecular and genetic basis of adaptation to environmental alcohol. It is therefore important to ascertain the number of loci coding for enzyme products that may be directly involved with the oxidation of alcohol, particularly the short-chain aliphatic alcohols often found in *Drosophila* environments (McKenzie and Parsons, 1972, 1974; Starmer *et al.*, 1977).

Our results (see summary in Table II) indicate that caution should be observed before characterizing adaptively significant *Adh* "loci" merely on the basis of electrophoretic assays using alcohol as substrate. The appearance of an electrophoretic band on a gel is not equivalent to the presence of a genetic locus; as pointed out above and noted by other authors (e.g., Jacobson *et al.*, 1972; Day *et al.*, 1974; Dickinson and Sullivan, 1975), two or more bands (bands 1 and 2 in the figures) may be the enzymatic product of one single allele.

Moreover, the appearance of a band in an assay using alcohol as a substrate is not sufficient evidence for the presence of an alcohol dehydrogenase. Several bands may appear in gels stained in alcohol assays, yet our results indicate that only the product of a single genetic locus, *Adh*, is an alcohol dehydrogenase (E.C. 1.1.1.1) exhibiting specificity for short-chain aliphatic alcohols. Other staining regions have been identified as enzymes with catalytic properties quite different from those of ADH. It seems, therefore, most likely that this *Adh* locus is the only one involved with the adaptation of *Drosophila* to alcohol environments.

The 13 species examined in our survey possess, with possible minor exceptions, the same basic complement of enzymes able to oxidize a variety of alcohols and aldehyde, and with similar specificities. Although amino acid substitutions have altered the electrophoretic mobilities of these enzymes from species to species in the course of evolution, the enzyme substrate specificities appear, at least under the limited conditions employed, to have remained qualitatively unaffected. However, substantial differences between species in total activity are apparent in our gels. These differences are consistent with quantitative spectrophotometric measurements of alcohol dehydrogenase and aldehyde oxidase activity (McDonald and Avise, 1976; G. K. Chambers, unpublished). These differences may be due to the existing differences in the structure of the enzymes but may also be due to changes in other modifier or regulatory loci controlling the amount of enzyme synthesized (Ward, 1975; McDonald *et al.*, 1977). The persistence of the same pattern of enzyme specificities in 13 *Drosophila* species belonging to four different groups suggests, nevertheless, that the full complement of enzymes (with the possible exception of the *Odh-3* gene product) is functionally required in these species.

Table II. Substrate Specificities and Characterization of Electrophoretic Bands on Starch Gels in *Drosophila* Species

Band	Singh's (1976) designation	Identity	Alcohol specificity	NAD requirement
1	*Adh-1*	Alcohol dehydrogenase	Relatively nonspecific, reacts with all alcohols tested but shows preference for short-chain aliphatic alcohols	Yes
2	*Adh-3*			
A	*Adh-2*	Blue artifact	Reacts directly with PMS and NBT in absence of alcohol	No
3	*Adh-6*	Aldehyde oxidase	Reacts slowly with all alcohols tested except benzyl; reacts with aldehyde	No
4	*Adh-4* (*Odh-1*)	Octanol dehydrogenase	Reacts with several aliphatic alcohols, but shows preference for long-chain alcohols, particularly octanol	Yes
5	*Adh-8* (*Odh-3*)	Octanol dehydrogenase	The same as for band 4	Yes

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