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Biochemical Differences Between Alcohol Dehydrogenases of *Drosophila melanogaster*

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Abstract

This paper describes substrate specificities, developmental changes in activity, pH profiles, and heat stabilities of isozymes produced by four *Adh* genotypes in *D. melanogaster*. No differences are found in the substrate specificities of isozymes from the different genotypes but studies of the other three properties reveal significant differences between the isozymes. Thus relatively low activities are found among extracts of Adh^{F} Adh^{n2} larvae and among extracts of Adh^{F} Adh^{F} adults aged 44 days. Also Adh^{F} Adh^{S} and Adh^{S} Adh^{S} extracts have relatively high activities at pH 6·5, and Adh^{F} Adh^{n2} extracts have relatively low activities at pH values above 10·0. Finally, extracts of Adh^{F} Adh^{F} and Adh^{F} Adh^{S} are more stable at 40°C than are those of Adh^{S} Adh^{S} and Adh^{F} Adh^{n2}.

Introduction

Recently, several authors have found biochemical differences between alcohol dehydrogenases (EC 1.1.1.1) produced by different *Adh* genotypes in *Drosophila melanogaster*. Most reports (see Ward 1975 for a summary) have shown that flies homozygous for the Adh^{F} allele (hereinafter denoted *F*) produce about twice the *in vitro* alcohol dehydrogenase activity expressed by flies homozygous for the Adh^{S} allele (denoted *S*), whilst heterozygotes, *FS*, express intermediate activity levels.

It has also been shown that relative activities of extracts of these genotypes can be altered by varying several aspects of the culture conditions or assay procedures. These aspects include the substrate and pH for the assay (Vigue and Johnson 1973; Day *et al.* 1974), age at assay (Hewitt *et al.* 1974), temperature before or during assay (Gibson 1970; Vigue and Johnson 1973; Day *et al.* 1974) and ethanol concentration in the culture medium (Gibson 1970).

Different authors, however, have found different substrate specificities and heat stabilities for isozymes with similar electrophoretic mobilities. Although these discrepancies might simply have reflected different experimental procedures or inadequate analyses, they might also have indicated the existence of more *Adh* alleles, electrophoretically indistinguishable from those described above. It was important to resolve this ambiguity as there have been recent precedents for alleles with similar electrophoretic phenotypes but different heat stability phenotypes. Several such alleles have been found at *Xdh* loci in 11 species of the *Drosophila virilis* group (Bernstein *et al.* 1973), *Odh* loci in 10 species of the same group (Singh *et al.* 1975), and the *Odh* locus in *Drosophila psuedoobscura* (Singh *et al.* 1974).

Accordingly the present author has developed appropriate methods of analysis and investigated the substrate specificities, developmental changes in activities, pH profiles, and heat stabilities of isozymes produced by *Adh* genotypes. In addition to the

F and *S* alleles the null allele Adh^{n2} (denoted *n2*), induced with ethyl methane sulphonate by Grell *et al.* (1968), has been studied. The extra allele was studied in order that particular *Adh* alleles could be studied in wider varieties of *Adh* genotypes.

Materials and Methods

Derivation of Strains

The population studied, LS, was obtained by mixing a laboratory stock bearing *n2*, a laboratory stock bearing *F* and a stock, captured in Adelaide, South Australia, bearing *S*. Ten generations after the mixing, three strains, each homozygous for a different *Adh* allele, were extracted from LS. *Adh* genotypes were ascertained by starch gel electrophoresis, the methods for which have been described elsewhere (Oakeshott 1976). Each strain contained the progenies of seven pairs of homozygous parents and each of these parents was obtained independently from LS. *Adh* genotypes required for biochemical tests were taken from these strains and the appropriate crosses of these strains.

LS was chosen for study because it lacked inversions containing the *Adh* locus (Oakeshott 1976). This fact and the breeding system described above enabled *Adh* genotypes to be tested against similar genetic backgrounds.

Assays of Alcohol Dehydrogenase Activity

Samples for assays of alcohol dehydrogenase activity each contained 15 flies previously maintained on media lacking yeast. Each sample was homogenized in 150 μl of physiological saline and centrifuged at 1500 *g* for 20 min. The supernatant was then collected and kept at 4°C until assayed (within 8 h of preparation). The assay mixture contained 0·2 ml of 0·003 M NAD^+, 0·2 ml of 0·05 M propan-2-ol, 0·25 ml of 0·1 M glycine–sodium hydroxide buffer, pH 9·5, and 10 μl of sample. The conversion of NAD^+ to NADH was monitored at 340 nm for 3 min in a Shimadzu QV50 spectrophotometer. Alcohol dehydrogenase activity was expressed as the change in absorbance at 340 nm per milligram live weight in 3 min. All assays were conducted at 25°C.

For substrate specificity studies propan-2-ol was replaced in the assay mixture by 0·2 ml of a 0·05 M solution of another alcohol. pH profiles were studied by varying the concentration of hydrogen ions in the buffer. Heat stability was investigated following the methods of Gibson (1970). Each extract was assayed for alcohol dehydrogenase activity immediately before and after a 10-min incubation in a 40°C water bath.

Results

Substrate Specificities

Table 1 shows alcohol dehydrogenase activities, with each of 12 substrates, of four *Adh* genotypes. Extracts of third-instar larvae (aged 6 days since hatching) were used. Extracts of *Sn2* and *n2n2* flies were not tested as they expressed little activity (Oakeshott 1976) and experimental errors probably would have concealed differences in their substrate specificities.

If relative activities of isozymes of different genotypes were similar with different substrates then the effects of genotype and substrate on activity scores would have been related multiplicatively, and their effects on logarithmically transformed scores would have been related additively. Thus the transformed scores were appropriate to a factorial analysis of variance, shown in Table 2, in which effects of changing substrates on relative activities were partitioned into the mean square for the interaction between genotype and substrate. In fact, as several types of alcohol were tested, terms were obtained for interactions between genotypes and substrates within types, and also between genotypes and types.

Conveniently, the transformation also restored homogeneity to the variances within different subclasses.

No significant interaction terms were found, indicating that isozymes of different genotypes had similar relative activities on all the alcohols and types of alcohols tested. Other criteria could have been used to classify alcohols, but the small interaction terms in Table 2 suggested that other chemically meaningful classifications would not have yielded significant interaction terms either.

Table 1. Alcohol dehydrogenase activities with different substrates

Each value is the mean activity (expressed as the change in absorbance at 340 nm per milligram live weight in 3 min) of at least three third-instar larval extracts. Some representative 95% confidence limits are shown in parentheses

Substrate	Genotype			
	FF	*FS*	*SS*	*Fn2*
Saturated alcohols				
(A) Straight chain, primary				
Methanol	2·5 (0·8, 7·1)	1·8	0·8	1·0
Ethanol	23 (20, 27)	15	10	9
Propan-1-ol	46 (33, 63)	33	17	28
Butan-1-ol	49 (40, 61)	39	20	23
Pentan-1-ol	75 (72, 79)	44	31	30
Hexan-1-ol	47 (44, 50)	33	21	19
(B) Branched chain, primary				
2 Methyl propan-1-ol	25 (23, 26)	16	9	9
3 Methyl butan-1-ol	28 (20, 39)	23	14	14
(C) Straight chain, secondary				
Propan-2-ol	100 (78, 129)	71	44	46
Butan-2-ol	127 (79, 206)	80	43	64
(D) Branched chain, tertiary				
2 Methyl butan-2-ol	27 (21, 34)	20	11	14
Unsaturated alcohol				
(E) Straight chain, secondary				
1 Pentene-3-ol	112 (74, 170)	87	46	53

Table 2. Analysis of effects of different substrates on logarithmically transformed alcohol dehydrogenase activity scores

Source of variation	d.f.	Mean square	*F* ratio	Probability
Genotypes (G)	3	1·2790	270·40	<0·001
Alcohols of type A (A)	5	2·6579	561·92	<0·001
A×G	15	0·0080	1·69	n.s.[A]
Alcohols of type B (B)	1	0·1211	25·61	<0·001
B×G	3	0·0054	1·14	n.s.
Alcohols of type C (C)	1	0·0477	10·08	<0·01
C×G	3	0·0034	0·71	n.s.
Types of alcohol (T)	4	2·5138	531·45	<0·001
T×G	12	0·0030	0·64	n.s.
Error	100	0·0047		

[A] n.s. = not significant.

Developmental Variation in Activity

Table 3 shows alcohol dehydrogenase activities of extracts of *Adh* genotypes of different ages and life cycle stages. For all genotypes late pupae showed least activity

whilst peaks of activity occurred in 3-day-old larvae and 34-day-old adults. Ursprung *et al.* (1970), using unspecified *Adh* genotypes, Hewitt *et al.* (1974), using *FF* and *SS* genotypes, and Ward (1975), using the *SS* genotype, also observed reduced activity in late pupae but they found the first peak in 4- or 5-day-old larvae. The discrepancy with the present study probably reflects the use of different activity units as the earlier workers cited activity per individual whilst the present author used activity per milligram live weight. The second peak was found by Hewitt *et al.* (1974) in flies aged 3 days since eclosion and by Ward (1975) in flies about 8 days after eclosion. Again the use of different units probably contributed to the discrepancies with the present report but Ward (1975) has also suggested that such differences might reflect differences in culture conditions or genetic differences between strains.

Table 3. Changes in alcohol dehydrogenase activities during development

Each value is the mean activity (expressed as the change in absorbance at 340 nm per milligram live weight in 3 min) of about seven replicates. Representative 95% confidence limits are shown in parentheses

Age (days since hatching)	Genotype			
	FF	*FS*	*SS*	*Fn2*
Larvae[A]				
3·00±0·25	228 (177, 292)	171	83	94
6·00±0·25	109 (85, 139)	78	41	37
Pupae[A]				
8·00±0·25	101 (75, 134)	79	27	51
9·00±0·25	100 (90, 110)	81	33	50
11·00±0·25	68 (57, 82)	51	20	33
Adult females				
12·00±0·25	185 (144, 230)	159	51	78
14·0±0·5	195 (163, 234)	132	62	89
24·0±0·5	245 (206, 291)	149	88	125
34·0±0·5	336 (308, 367)	202	93	130
44·0±0·5	96 (35, 263)	114	51	63
Adult males				
12·00±0·25	229 (211, 247)	157	63	86
14·0±0·5	278 (220, 352)	199	88	130
24·0±0·5	374 (323, 432)	312	116	168
34·0±0·5	668 (584, 764)	380	223	268
44·0±0·5	180 (114, 284)	204	81	91

[A] The sexes of larvae and pupae were not recorded.

Table 4 summarizes an analysis, analogous to that in Table 2, conducted on logarithmic transformations of data in Table 3. Neither the interaction between age of larvae and genotype, nor that between age of pupae and genotype, was significant, but that between age of adults and genotype was significant. The latter probably reflected the relatively low activity of *FF* flies aged 44 days because this term was not significant ($F^{11}_{247} = 1{\cdot}26$, $P > 0{\cdot}10$) if the latter data were omitted from the analysis.

The interaction between life cycle stage and genotype was also significant and this probably reflected the relatively low activity of *Fn2* larvae. An analysis in which these data were omitted did not show a significant interaction between stage and genotype ($F^{5}_{243} = 0{\cdot}85$, $P > 0{\cdot}50$).

Table 4. Analysis of effects of different developmental stages on logarithmically transformed alcohol dehydrogenase activity scores

Source of variation	d.f.	Mean square	*F* ratio	Probability
Genotypes (G)	3	3·2183	309·45	<0·001
Ages of larvae (L)	1	1·3560	130·38	<0·001
L×G	3	0·0040	0·38	n.s.[A]
Ages of pupae (P)	2	0·2315	22·26	<0·001
P×G	6	0·0052	0·50	n.s.
Ages of adults (A)	4	1·0895	104·76	<0·001
Sexes of adults (S)	1	1·8480	177·69	<0·001
A×G	12	0·0324	3·12	<0·001
S×G	3	0·0040	0·38	n.s.
A×S	4	0·1210	11·63	<0·001
A×S×G	12	0·0138	1·33	n.s.
Stages (St)[B]	2	4·8935	470·53	<0·001
St×G	6	0·0300	2·88	<0·01
Error	255	0·0104		

[A] n.s. = not significant. [B] Larvae, pupae and adults.

Table 5. Alcohol dehydrogenase activities at different pH values

Each value is the mean activity (expressed as the change in absorbance at 340 nm per milligram live weight in 3 min) of about four replicates. Representative 95% confidence limits are shown in parentheses

pH	Genotype			
	FF	*FS*	*SS*	*Fn2*
6·5	10·0 (5·9, 16·8)	8·5	8·0	6·5
8·0	67 (49, 92)	50	30	48
8·5	103 (65, 162)	67	39	52
9·0	92 (67, 125)	74	30	54
9·5	99 (92, 107)	71	29	48
10·0	85 (73, 99)	64	30	46
10·5	83 (71, 97)	56	29	31
11·0	66 (55, 79)	53	24	27
11·3	46 (44, 49)	34	20	19

pH Profiles

Table 5 shows alcohol dehydrogenase activities of pupal extracts over a pH range from 6·5 to 11·3. The data suggested that the pH optima of isozymes of all genotypes occurred between about 8·5 and 9·0. Vigue and Johnson (1973) and Day *et al.* (1974) also found no differences between pH optima of isozymes from *FF*, *FS* and *SS* genotypes. The positions of the optima described in these two reports differed slightly from one another and from those observed in the present study, but the earlier workers suggested that such differences simply reflected use of different buffer systems.

The data in Table 5 were transformed logarithmically and then subjected to a factorial analysis of variance. This was analogous to preceding analyses, but it contained only one interaction term because the pH range could not be meaningfully subdivided.

A highly significant interaction between pH and genotype was found ($F^{24}_{96} = 4{\cdot}11$, $P < 0{\cdot}001$). Inspection of Table 5 suggested that this was caused by the relatively high activities of *FS* and *SS* extracts at pH 6·5, and the relatively low activity of *Fn2* extracts at pH values greater than 10·0. In fact the interaction term remained significant in analyses in which data for either all genotypes at pH 6·5, or *Fn2* extracts at all pH values, were omitted ($F^{21}_{84} = 3{\cdot}37$, $P < 0{\cdot}001$ and $F^{16}_{72} = 3{\cdot}91$, $P < 0{\cdot}001$ respectively) but was non-significant in an analysis in which data from both these sources were omitted ($F^{14}_{63} = 1{\cdot}72$, $P > 0{\cdot}05$).

Table 6. Stabilities of alcohol dehydrogenases at 40°C

Stability is expressed as the percentage of activity (expressed as the change in absorbance at 340 nm per milligram live weight in 3 min) remaining after incubation at 40°C for 10 min. Each value is the mean stability of about 15 replicates. Representative 95% confidence limits are shown in parentheses

Life cycle stage	Genotype			
	FF	*FS*	*SS*	*Fn2*
Third instar larvae[A]	15·3 (13·2, 17·9)	15·8	13·8	13·5
Adult females[B]	31·4 (22·8, 43·3)	33·2	23·0	23·4
Adult males[B]	50·8 (46·6, 55·4)	53·6	51·1	48·4

[A] The sexes of larvae were not recorded.
[B] Adults were assayed 23 days after hatching.

Stabilities at 40°C

Table 6 shows stabilities of alcohol dehydrogenases in extracts of larvae and adults of different genotypes. These data were transformed logarithmically to restore homogeneity to the variances within subclasses and then subjected to factorial analysis of variance.

Interpretation of the analysis differed from those of previous analyses. Previous data simply expressed the activity of each extract after treatment, but data in Table 6 expressed the activity of each extract after treatment as a proportion of its activity before treatment. Therefore, in this analysis any differences between the effects of treatment on different genotypes were represented not in the interaction terms (as previously) but in the mean square for the main effect of genotype.

This term was highly significant ($F^{3}_{156} = 5{\cdot}76$, $P < 0{\cdot}001$). Furthermore the interactions between genotype and the sex of adults and between genotype and stage were non-significant ($F^{3}_{156} = 1{\cdot}57$, $P > 0{\cdot}10$ and $F^{3}_{156} = 0{\cdot}30$, $P > 0{\cdot}75$ respectively). Therefore it was concluded that there were significant stability differences between extracts of different genotypes and that these differences were similar in all sexes and stages tested.

The stability differences resolved the genotypes into two pairs, *FF* and *FS* in one, and *SS* and *Fn2* in the other. The stability differences between genotypes within each pair were not significant (e.g. $F^{1}_{78} = 1{\cdot}29$, $P > 0{\cdot}25$ for *FF* and *FS*), indicating

that the significant differences obtained from the analysis of all genotypes reflected the fact that alcohol dehydrogenases in *FF* and *FS* extracts were significantly more stable than those in *SS* and *Fn2* extracts.

Discussion

Several differences in biochemical properties were found between the *Adh* genotypes tested. Developmental changes occurred in the relative activities of *FF* and *Fn2* extracts, *FS* and *SS* extracts had relatively high activities at pH 6·5, *Fn2* extracts had relatively low activities at pH values above 10·0, and *FF* and *FS* extracts were more stable to heat than those of *SS* and *Fn2*. Comparable data have not been published previously for the *Fn2* genotype but comparisons could be made with previous reports on the biochemical properties of the other three genotypes.

Changes in relative activities with the pH of the assay and during development were consistent with previous reports. Vigue and Johnson (1973) and Day *et al.* (1974) also observed decreases in relative activities of *FF* flies at pH values below 8·0. Analyses of their data were unnecessary as significant changes were obvious from their graphs. Hewitt *et al.* (1974) studied only the *FF* and *SS* genotypes but found that relative activities in these genotypes remained constant during development, except for minor changes during the first 8 days after eclosion. The significance of the changes was not established and they did not test the 44-day-old age group, at which the present author found a significant decrease in the relative activities in *FF* flies.

The substrate specificity data differed from those reported previously. Vigue and Johnson (1973) studied three primary alcohols and two secondary alcohols, propan-2-ol and butan-2-ol. They found that activity in *FF* flies relative to that in *FS* and *SS* flies was greater with butan-2-ol than with the other alcohols. However, this difference was not large and their data was not analysed or published in a form amenable to analysis.

Day *et al.* (1974) studied six alcohols, not including butan-2-ol, and found that activity in *FF* flies relative to that in *SS* flies was less with primary alcohols than with other alcohols. Although the data were not analysed rigorously their validity was confirmed indirectly by Morgan (1975) who found a correlation between the relative activities they observed with different alcohols and the relative survival scores he recorded when the same *FF* and *SS* stocks were exposed to these alcohols.

Thus substrate specificities observed by the present author possibly differed from those of Vigue and Johnson (1973) and unequivocally differed from those of Day *et al.* (1974). Unfortunately the importance of the latter difference was uncertain because the two studies used slightly different substrate specificity assay systems. The present author used third-instar larvae which were only partially deyeasted and the sexes of which were not distinguished. After centrifuging extracts, supernatants were harvested by pipette. The assay was buffered with 0·04 M glycine–sodium hydroxide, pH 9·5. Day *et al.* (1974) used thoroughly deyeasted adult males, millipore filtration of supernatants and assay mixtures containing 0·11 M phosphate, pH 8·0. The two studies also used slightly different molarities for other assay constituents.

There were also discrepancies between results of different studies on stabilities at high temperatures. Gibson (1970) found that extracts of *FF* larvae were significantly less stable, during 10 min at 40°C, than those of *FS* larvae, and both were less stable

than those of *SS* larvae. Similar relative stabilities were observed by Vigue and Johnson (1973) using extracts of adults and a variety of incubation times and temperatures. The latter differences were not analysed but were obviously significant. Day *et al.* (1974) also found that extracts of *FF* flies were clearly less stable during 10 min at 40°C than those of *SS* flies but, unlike previous authors, they were unable to distinguish between the stabilities of *FF* and *FS* extracts. In addition to this discrepancy another was provided by results of the present author who, using the method of Gibson (1970), found that stabilities of extracts of *FF* and *FS* flies were similar to one another but significantly greater than those of *SS* flies.

Unfortunately, technical differences such as those occurring between different studies of substrate specificities might have also caused the discrepancies between the heat stability results of different authors. However, the differences between relative heat stabilities observed by the present author and those in previous reports were considerably larger than the discrepancies between substrate specificity studies. In fact the magnitudes of discrepancies in relative heat stabilities suggested that they could have also reflected genetic differences between the populations studied. Such genetic differences might have occurred at the *Adh* locus, indicating the existence of at least one other *Adh* allele electrophoretically indistinguishable from one previously described. Alternatively the differences might have occurred at loci other than *Adh*. The activity modifier loci known for *Adh* (see Ward 1975 for references) might well have stability modification properties also.

While interpretation of the significance of these discrepancies between studies awaits genetic analysis of interpopulation crosses, two conclusions can be drawn from the variety of biochemical differences between isozymes observed within the present study. Firstly, the results suggest that such biochemical criteria as those studied herein might be useful, in future, to screen for genetic variability within electrophoretic morphs. Secondly, the variety of differences observed suggest that such genotypes are not selectively equivalent. However, the discrepancies with previous reports also illustrate the dangers of inferring directions of selective differences in natural populations from *in vitro* biochemical differences observed among laboratory stocks.

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