Adaptive response due to changes in gene regulation: A study with *Drosophila*

(alcohol adaptation/evolution/selection/alcohol dehydrogenase)

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**ABSTRACT** In spite of the critical role of the process of adaptation in evolution, there are few detailed studies of the genotypic and molecular basis of the process. *Drosophila melanogaster* flies selected for increased tolerance to ethanol exhibited higher levels of alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase; EC 1.1.1.1) activity than unslected controls. A series of tests (electrophoresis, product inhibition, temperature stability, pH optima, substrate specificity, and Michaelis constants) gave no evidence of structural differences in the enzyme of the selected and the control flies. However, quantitative immunological assays showed that the selected flies contained significantly higher amounts of alcohol dehydrogenase. Adaptation of the selected flies to higher alcohol tolerance has most likely taken place by changes in the structural gene locus coding for the enzyme, but by regulatory changes affecting the amount of gene product.

Evolution is largely the result of natural selection promoting adaptation. Few direct and detailed analyses of adaptation exist because of the difficulty in identifying biological systems in which the complex interactions among genotype, phenotype, and environment involved in adaptation can all be studied. Adaptations in higher organisms often involve polygenic control; the adaptively significant phenotypes may be identifiable, but their genetic and molecular controls are difficult to investigate. There are also simple genetic systems where specific loci and their immediate gene products are readily identifiable, but their adaptive function remains obscure. The electrophoretic studies have established correlations between allozyme variants and specific environmental variables are examples of this category (1, 2). The few adaptations successfully analyzed from the genotypic, through the phenotypic, to the environmental level are, in general, traits under simple genetic control. In some cases the molecular basis of the adaptation is known, e.g., sickle-cell trait in humans (3, 4) or DDT resistance in house flies (5). In other instances the molecular picture remains incomplete although, as in the industrial melanism of *Biston betularia* (6), a causal connection between specific genotypes and environmentally significant phenotypes may be unambiguously established.

One model situation for study of adaptation is to observe and manipulate the process in a biological system evolving under controlled laboratory conditions. This has been accomplished in bacteria and yeasts (e.g., refs. 7–11). Various sources of evidence have suggested to us that a similar approach could be successful in *Drosophila* with respect to forced adaptation to an alcohol environment. The enzyme alcohol dehydrogenase (ADH; alcohol:NAD⁺ oxidoreductase; EC 1.1.1.1) has been implicated in the ability of *D. melanogaster* to exploit alcohol environments (e.g., refs. 12–14). Moreover, the primary structure of ADH is determined by a single gene locus, but there is evidence suggesting that ADH activity is under complex regulatory control (e.g., refs. 15–17). We report here the results of a study of the genetic and biochemical basis of a selected response for increased alcohol tolerance in *Drosophila*.

**MATERIALS AND METHODS**

*Drosophila melanogaster* flies collected in Colmar, France, were used to establish a strain subsequently divided into two populations. One population, Selected (S), was subject to intense selection for ethanol tolerance for 28 generations; the other was the Control (C) (18). After the 28th generation of selection, flies from the S and C strains were transferred to Davis, where the experiments reported here were conducted.

Tolerance to alcohol was tested placing 5-day-old adult flies at room temperature in vials containing 2.5 × 5.0 cm filter-paper strips saturated with 1 ml of a water solution consisting of 3% sucrose and 8, 10, 12, 14, 16, or 18% ethanol; vials were sealed with Parafilm. For each ethanol concentration and strain, two replicate vials were prepared for each sex, containing 10 flies per vial. The number of surviving flies was monitored at regular intervals for 100 hr.

ADH was purified as described (19), except that DEAE-Sephadex (A50) replaced DEAE-cellulose and pooled column fractions were concentrated by ultrafiltration rather than by ammonium sulfate precipitation. Protein concentration was determined (20). The purification was successful to better than 95% homogeneity, as estimated by an acrylamide gel scan of electrophoresed purified product.

Crude extracts were prepared from 10 mg of flies homogenized with hand with a glass tissue grinder in 0.6 ml of Tris-HCl buffer, pH 8.5, and centrifuged at 28,000 × g for 20 min.

A survey of enzyme activity was conducted by published methods (21) for ADH, glycerol-3-phosphate dehydrogenase (gGPDH, EC 1.1.1.8), isocitrate dehydrogenase (IDH, EC 1.1.1.41), and phosphoglucone isomerase (PGI, EC 5.3.1.9). Aldehyde oxidase (AO) was assayed using acetaldehyde as substrate and coupling its oxidation with dichloroindophenol (22). All assays were done in a Gilford model 250 spectrophotometer.

Starch gel electrophoresis followed described procedures (23). Polycrylamide gels were prepared as described (24), using the same staining procedures as for the starch gels (23).

Product inhibition of ADH activity in the ethanol to acetaldehyde direction was studied (25). ADH was assayed in the presence of 0, 1, 2, 5, 7, 10, and 15 mM acetaldehyde.

The thermal stability of ADH was studied in two ways. (i)

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Abbreviations: ADH, alcohol dehydrogenase; AO, aldehyde oxidase; gGPDH, glycerol-3-phosphate dehydrogenase; IDH, isocitrate dehydrogenase; PGI, phosphoglucone isomerase.

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The time was kept constant but the temperature varied: 20 µl of purified ADH was incubated in a water bath for 5 min at 22, 25, 40, 45, 47.5, and 50°. (ii) The temperature was held constant but the time varied: 20 µl of purified ADH was incubated in a 45° water bath for 0, 10, 20, 30, 40, 50, 60, and 70 min. After incubation, the samples were placed on ice until assayed for ADH activity.

The effect of pH on the reaction rate of ADH was ascertained as described (26), with either 10 mM glycopyllicine buffer (pH 7.0-9.5) or 10 mM boric acid 0.1 M KCl (with pH adjusted to 6.5-9.3 with 0.1 M NaOH). Both tests were at room temperature with ethanol as substrate and 10 µl of purified ADH for each assay.

Michaels constants were estimated by methods similar to those described (25). Purified ADH as well as crude extracts were tested; extract from the S strain was diluted until its activity was within 5% of the activity of the C strain. Tests were at 22° and pH 8.6. Assays were made over a range of five NAD (0.05-0.4 mM) and five ethanol (5-40 mM) concentrations; each assay was done twice and the average was taken as the activity reading for the particular concentration of NAD and ethanol. The whole experiment was performed twice for each strain with purified ADH and four times on separate crude extracts.

Substrate specificity of crude extracts was tested using solutions of five alcohols (isopropyl, ethyl, allyl, and n-buty1) in 55.5 mM Tris-HCl buffer, pH 8.6. For these tests we define one unit of activity as that which causes an increase of 0.001 absorbance unit/min at 340 nm.

For immunological tests, New Zealand White rabbits were injected subcutaneously with 0.5 mg of purified D. melanogaster ADH (Fast allele) and 0.5 ml of Freund's adjuvant. Two injections were made at 1-week intervals; 3 weeks later a booster of 0.5 mg of purified enzyme was injected. The rabbits were bled 4 weeks after the booster; immunological activity of the antiserum and the antigenic identity of ADH from the C and S strains were determined as described (27, 28). Antiserum was used to determine the amounts of ADH protein by antigen-antibody immunoelectrophoresis (29). The procedure was modified from that in ref 30. Crude extract (1 µl) was inserted in wells 1.5 mm in diameter. The gel dimensions were 4.5 X 7.5 X 0.1 cm. Gel plates were obtained from Antibodies Inc., Davis, CA. Electrophoresis was for 10 hr at 10 mA; the gels were then stained for ADH activity.

RESULTS

Progenies of a large sample of D. melanogaster were divided into two populations; one (S) was selected for each of 28 generations for ethanol tolerance, while the other population served as control (C).
strain, and no differences were detectable between the strains.

Product Inhibition. Acetaldehyde inhibits the ability of ADH to convert ethanol into acetaldehyde. This inhibition exhibits kinetics consistent with the notion that aldehyde is a competitive hyperbolic inhibitor (25). The inhibitory effect of acetaldehyde on the reaction catalyzed by purified ADH is shown in Fig. 3: the inhibitory kinetics were effectively identical for the S and the C strains.

Temperature Stability. The rate of thermal denaturation of proteins is a function of both the temperature to which the protein is exposed and the duration of exposure. Protein denaturation was tested in purified ADH from the S and C strains. Fig. 4 shows the percent activity remaining after 5 min of exposure to various temperatures. The ADH activity remained unaffected up to 40°, but rapidly decreased above this temperature. The response of the S and C strains was identical.

Fig. 5 shows the loss of ADH activity when the enzyme was exposed to 45° for various lengths of time. The level of activity gradually decreased to zero as the length of exposure increased from 0 to 70 min. The response of the S and C strains was again effectively identical.

pH Optima. The influence of pH on the activity of purified ADH was tested for both strains using borate buffer (Fig. 6) and glycylglycine buffer (Fig. 7). The peaks of maximum activity occurred at pH 8.9 in borate buffer and at pH 9.2 in glycine buffer. The responses of the S and C strains were effectively identical. The pH optima points are similar to those of ref. 25 for strains of D. melanogaster homozygous for the F allele at the Adh locus.

Michaelis Constants. Purified ADH as well as crude extract from the S and the C strains were assayed over a range of five NAD and five ethanol concentrations, producing a 5 × 5 data matrix. The regression lines were calculated for all Lineweaver-Burk plots. For each substrate the 5 × 5 data matrix generates five regression lines. The median value on the X-axis of their 10 intercepts with each other was taken as the best estimate of the reciprocal of the Michaelis constant (K_m). Two other Michaelis constants were derived from secondary replots of 1/activity (when 1/[ethanol] = 0) against 1/[NAD], and 1/activity (when 1/[NAD] = 0) against 1/[ethanol]. The intercept on the X-axis of each of these secondary replots was taken as the reciprocal of the Michaelis constant, K'_m. Thus, the two primary and the two secondary plots produce four Michaelis constants: K_{ethanol}, K_{NAD}, K'_{ethanol}, and K'_{NAD}.

Table 1 presents the Michaelis constants. The purified ADH generally showed lower K_m values (the exception being K_{NAD} in the S strain). This result agrees with published values for purified ADH and crude extracts of D. melanogaster ADH. The values obtained here for pure ADH are close to those of ref. 31; our results for crude extracts are comparable to the values obtained in ref. 25 using partially purified ADH. The reasons for the differences between the K_m values for crude extract and purified enzyme are not known. However, it is known that enzymes other than ADH present in crude extracts can use the two substrates used in our ADH assays (ref. 32; and unpublished results). The higher K_m values obtained with crude extracts could then be due to the fact that other enzymes utilize ethanol and NAD; alternatively, they could be due to the interference of low molecular weight compounds that are removed during purification.

The significant point is that the K_m values of the S and C strains are not significantly different. True differences could, of course, be masked by the large experimental errors, but there is no consistent superiority of one over the other strain; the S strain has higher average value in five comparisons but lower in the other three. The experimental errors observed are rather large, but not untypical for studies with Drosophila ADH (25).

Substrate Specificity. The activity of ADH in crude extracts was tested on five alcohol substrates. Table 2 shows that ADH from the S strain exhibits higher activity on all five substrates than ADH from the C strain. The ratios of the activity of the S strain to that of C strain are virtually identical for all...
Table 1. Michaelis constants (mM) of purified and crude ADH from the S and C strains of D. melanogaster

<table>
<thead>
<tr>
<th>ADH preparation</th>
<th>$K_{\text{ethanol}}$</th>
<th>$K'_{\text{ethanol}}$</th>
<th>$K_{\text{NAD}}$</th>
<th>$K'_{\text{NAD}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selected strain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pure</td>
<td>3.86 ± 0.62</td>
<td>8.93</td>
<td>0.037 ± 0.010</td>
<td>0.033</td>
</tr>
<tr>
<td>1.91 ± 0.53</td>
<td>2.97</td>
<td>0.178 ± 0.152</td>
<td>0.042</td>
<td></td>
</tr>
<tr>
<td>$\bar{X} \pm$ SEM</td>
<td>2.88 ± 0.97</td>
<td>5.95 ± 2.98</td>
<td>0.108 ± 0.070</td>
<td>0.038</td>
</tr>
<tr>
<td>Crude</td>
<td>9.09 ± 3.67</td>
<td>7.27</td>
<td>0.170 ± 0.092</td>
<td>0.034</td>
</tr>
<tr>
<td>15.36 ± 3.22</td>
<td>7.27</td>
<td>0.018 ± 0.092</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>8.31 ± 6.34</td>
<td>6.08</td>
<td>0.026 ± 0.010</td>
<td>0.044</td>
<td></td>
</tr>
<tr>
<td>14.70 ± 7.43</td>
<td>16.61</td>
<td>0.087 ± 0.014</td>
<td>0.210</td>
<td></td>
</tr>
<tr>
<td>$\bar{X} \pm$ SEM</td>
<td>11.86 ± 3.88</td>
<td>9.31 ± 2.45</td>
<td>0.075 ± 0.035</td>
<td>0.078</td>
</tr>
</tbody>
</table>

Control strain

| Pure            | 3.96 ± 0.47     | 5.59            | 0.016 ± 0.005  | 0.010           |
| 2.12 ± 0.86     | 3.42            | 0.019 ± 0.002  | 0.040           |
| $\bar{X} \pm$ SEM | 3.04 ± 0.92   | 4.51 ± 1.08     | 0.018 ± 0.002  | 0.025           |
| Crude           | 8.01 ± 1.94     | 10.46           | 0.098 ± 0.015  | 0.100           |
| 4.30 ± 1.20     | 19.49           | 0.052 ± 0.132  | 0.132           |
| 6.48 ± 3.20     | 9.12            | 0.052 ± 0.008  | 0.131           |
| 10.11 ± 3.77    | 9.61            | 0.048 ± 0.019  | 0.078           |
| $\bar{X} \pm$ SEM | 7.22 ± 1.23   | 12.17 ± 2.45    | 0.062 ± 0.012  | 0.110           |

Five alcohols tested (the mean and SEM for this ratio are 1.32 ± 0.01). This indicates that selection for ethanol tolerance has increased the activity of ADH equally with respect to all these alcohol substrates.

Immunological Determination of Amount of ADH Protein. The relative amounts of ADH in the S and C strains were determined by means of quantitative immunoelectrophoresis (29). Fig. 8 shows a typical gel exhibiting "Laurell rockets." The results of four replicate gels are shown in Table 3; ADH activity was measured on the samples used for these gels. The amount of ADH protein present in a sample (as measured by the length of the "rocks") correlates well with the level of ADH activity. (Fig. 9 gives a standard curve showing the direct relationship between rocket length and amount of ADH protein; see also ref. 33.) The males of both strains have more ADH and greater activity than the females. The critical finding is, however, that flies of the S strain contain significantly greater amounts of ADH protein than flies of the C strain. The mean differences in rocket length between the S and C strains (Table 3, "Selected − Control") are statistically significantly greater than zero; 3.63 ± 0.51 and 3.13 ± 1.26 for females and males, respectively.

DISCUSSION

Two basic questions are investigated in this study. The first question concerns the adaptive significance of levels of enzyme activity. Earlier studies have shown that ADH activity in Drosophila correlates with the ability of the flies to tolerate alcohol in the environment (14). If this correlation reflects, at least in part, a causal role of ADH in alcohol tolerance, then flies selected for alcohol tolerance should exhibit increased ADH activity as well. Our activity survey corroborates this hypothesis. Flies of a strain selected for alcohol tolerance exhibit higher ADH activity than flies of an unselected and less tolerant strain.

Our survey of enzyme activity indicates no differences in enzyme activity between the S and C strains with respect to other enzyme systems not related to alcohol tolerance. This further supports the hypothesis that higher ADH activity, at least in part, is responsible for the increased alcohol tolerance. These results do not preclude the possibility that other biochemical factors (e.g., membrane permeability and the microsomal ethanol-oxidizing system) associated with alcohol tolerance in other organisms might be involved as well (34).

The second question concerns the genetic basis of the increase in ADH activity and the associated increase in alcohol tolerance. ADH activity is a measure of the overall rate at which NAD and alcohol are converted to NADH and aldehyde. An increase in this rate, as is found in the S strain, may be due either to changes in the activity of the enzyme or to changes in the concentration of co-factors.

Table 3. Comparison of relative amount of ADH and of ADH activity in S and C strains

<table>
<thead>
<tr>
<th>Experiment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selected</td>
<td></td>
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</tr>
<tr>
<td>Rocket length (mm)</td>
<td>18.50</td>
<td>18.50</td>
<td>20.50</td>
<td>18.00</td>
<td>18.88 ± 0.55</td>
</tr>
<tr>
<td>ADH activity*</td>
<td>5.25</td>
<td>5.25</td>
<td>6.00</td>
<td>5.50</td>
<td>5.50 ± 0.02</td>
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<tr>
<td>Control</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rocket length</td>
<td>14.00</td>
<td>16.00</td>
<td>16.00</td>
<td>15.00</td>
<td>15.25 ± 0.48</td>
</tr>
<tr>
<td>ADH activity</td>
<td>3.00</td>
<td>4.00</td>
<td>3.50</td>
<td>4.00</td>
<td>3.63 ± 0.24</td>
</tr>
<tr>
<td>Selected − Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rocket length</td>
<td>4.50</td>
<td>2.50</td>
<td>4.50</td>
<td>3.00</td>
<td>3.63 ± 0.51</td>
</tr>
<tr>
<td>ADH activity</td>
<td>2.25</td>
<td>1.25</td>
<td>2.50</td>
<td>1.50</td>
<td>1.87 ± 0.29</td>
</tr>
<tr>
<td>Males</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Selected</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rocket length</td>
<td>21.00</td>
<td>19.50</td>
<td>22.50</td>
<td>18.00</td>
<td>20.25 ± 0.97</td>
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<tr>
<td>ADH activity</td>
<td>9.00</td>
<td>6.50</td>
<td>7.50</td>
<td>9.00</td>
<td>8.00 ± 0.61</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rocket length</td>
<td>17.00</td>
<td>17.00</td>
<td>22.50</td>
<td>12.00</td>
<td>17.12 ± 1.98</td>
</tr>
<tr>
<td>ADH activity</td>
<td>6.00</td>
<td>4.75</td>
<td>7.50</td>
<td>7.75</td>
<td>6.50 ± 0.70</td>
</tr>
<tr>
<td>Selected − Control</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Rocket length</td>
<td>4.00</td>
<td>2.50</td>
<td>0.00</td>
<td>6.00</td>
<td>3.13 ± 1.26</td>
</tr>
<tr>
<td>ADH activity</td>
<td>3.00</td>
<td>1.75</td>
<td>0.12</td>
<td>1.25</td>
<td>1.50 ± 0.62</td>
</tr>
</tbody>
</table>

* One unit of activity is that amount of enzyme required to convert 9 × 10⁻⁸ mol of NAD to NADH per min.
in the enzyme structure that modify its catalytic efficiency and/or to an increase in the amount of ADH present.

Various biochemical tests, including estimation of Michaelis constants, have failed to show any differences between the ADH molecules produced by the S and the C strains. No electrophoretically detectable differences in ADH exist between the strains. Temperature stability studies have revealed in Drosophila differences between strains with identical electrophoretic phenotype (35, 36). Significant differences in substrate specificity and in pH optima have been found between the Fast and Slow electrophoretic forms of ADH in D. melanogaster (25, 26, 37). In general, the standard biochemical tests used in our study have, in the past, proven to be sensitive to relatively small differences between ADH molecules in D. melanogaster, but have given no evidence of structural ADH differences between our S and C strains. Although no amount of analysis short of amino acid sequencing may prove the structural identity of the ADH molecules present in the S and C strains, the tests performed here strongly suggest that such differences do not exist.

If the differences in activity are not due to structural differences (whether pre- or post-translational) in the ADH, then there must be a difference in the number of ADH molecules present in the S and the C strains. The immunoelectrophoretic studies support this hypothesis. Genetically, differences in amount of a given gene product may be explained by a duplication of the structural locus and/or by differences in some sort of regulatory gene(s). Present evidence favors a regulatory explanation. Gene duplications at the ADH locus have never been detected in natural populations, and have only been identified as very rare events following mutagenesis (38); the specific ADH activity in duplicated strains is roughly proportional to the number of doses of the gene locus (38). However, genes regulating ADH activity have been identified throughout the Drosophila genome and are apparently polymorphic in natural populations (17, 39). Our own unpublished results indicate that differences in ADH activity between strains identical at the ADH locus (because they have the same second chromosome) are largely due to differences in the third chromosome.

The importance of regulatory genes in bringing about adaptive genetic changes in prokaryotes is well documented (7, 11, 40). Theoretical arguments (41, 42) and indirect evidence (e.g., refs. 40, 43, and 44) have been advanced supporting the notion that changes in genetic regulation may have played a critical role in the evolution of eukaryotes as well. Our results strongly suggest that some sort of regulatory mechanism is responsible for the adaptive response observed in our study and, as such, provide direct evidence for the potential importance of gene regulation in eukaryotic evolution.