CHEMICAL SELECTION OF MUTANTS THAT AFFECT
ALCOHOL DEHYDROGENASE IN DROSOPHILA
II. USE OF 1-PENTYNE-3-OL

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ABSTRACT

We describe a procedure for the selection of alcohol dehydrogenase negative mutants in Drosophila. The method consists of exposing eggs and larvae to low concentrations of 1-pentyne-3-ol dissolved in the culture medium. Only those flies with greatly reduced levels of alcohol dehydrogenase activity survive. In addition, genotypically negative flies die if their mothers are alcohol dehydrogenase positive. Using this procedure and formaldehyde to generate mutants, we were able to detect seven alcohol dehydrogenase negative mutants out of 350,000 individuals subjected to selection. At least five of the mutants contain small deletions that include the alcohol dehydrogenase locus.

We have been developing the Drosophila alcohol dehydrogenase (ADH) gene-enzyme system for use as a model for the study of gene control in higher organisms. From a biochemical viewpoint, this system has the advantages that ADH is present in large amounts, is readily purified, and has a relatively simple structure (SOFER and URSPRUNG 1968; JACOBSON and PFUDEERER 1970.)

However, it is in the potential for genetic analysis that this system excels, primarily because two chemical selection procedures have been developed. The first makes use of the observation that flies lacking detectable ADH activity (Adh-negatives) die when exposed to low concentrations of ethanol (GRELL, JACOBSON and MURPHY 1968). The experimental details of this selection system that allow one to select rare revertants, recombinants and suppressors affecting ADH activity are described in the next paper in this series (VIGUE and SOFER 1974a).

The second chemical selection scheme is complementary to the first, in that it allows for the isolation of rare Adh-negative mutants among large numbers of wild-type flies. In this procedure the vapor from a 5% solution of 1-pentene-3-ol (pentenol) is used to specifically poison Adh-positive flies. Adh-negatives are unaffected (SOFER and HATKOFF 1972). We have used pentenol to isolate eleven Adh-negative mutants after mutagenesis with ethyl methanesulfonate.

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However, the pentenol procedure has several drawbacks. (a) For optimal selection flies must be aged for four or more days after eclosion. Presumably this time is required for the development of maximal ADH activity. (b) Unless the sexes are separated before this aging period, any female mutants present may have mated, necessitating relatively complicated techniques for the recovery of the mutant chromosome. (c) Much space is required to maintain the adult flies while they are awaiting selection. (d) Leaky mutants are not readily detectable by the pentenol technique.

We report here a new procedure for the detection of Adh-negative mutants. It is based on the same principle as the previous scheme but treats eggs and larvae instead of adults, and uses 1-pentyne-3-0l (pentynol) instead of pentenol. With this procedure hundreds of thousands of eggs can be treated at one time in a very limited arena. Flies which survive may be separated before they have a chance to mate. Moreover, leaky mutants sometimes survive the test.

MATERIALS AND METHODS

Mutants

Most of these second chromosome mutants are described more completely in LINDSLEY and GRELL (1967).

*wo; Adh*—This stock carries the X-linked gene white and a naturally occurring electrophoretic variant of ADH.

*Bethylie*—a wild-type stock containing the naturally occurring Adh allele.

*Adh*/*pr cn*—a strain carrying an electrophoretic variant of ADH. It was derived from *Adh*/* by GRELL, JACOBSON and MURPHY (1968).

*Df(2L)64j*—a chromosome containing a deletion which includes Adh and genes on both sides of the Adh locus.

*CyO = In(2LR)O,p*/*Cy pr cn*—a balancer chromosome which contains an active *Adh* allele.

*CyO n1* and *CyO n2*—two Adh-negative mutants (generated with ethyl methanesulfonate) on the *CyO* chromosome (GERACE and SOFER 1972).

*Adhn1-n2*—five Adh-negative strains generated by GRELL, JACOBSON and MURPHY (1968). *Adhn1-n2* is a temperature-sensitive mutant (VIGUE and SOFER 1974b).

*Adhn6-n14*—nine Adh-negative strains generated in *Adh*/**cn*/*vg* flies after ethyl methanesulfonate mutagenesis. These were selected using the pentenol procedure (GERACE and SOFER 1972).

*Be*—Black cell, a dominant larval marker. This chromosome carries an active Adh allele.

Collecting and Counting Eggs

Flies were allowed to lay overnight in bottles containing the following medium: 3% agar, 3.2% killed brewers yeast, 5.4% sucrose, 0.025% mold inhibitor (Carolina Biological), and either 2 oz. of concentrated grape juice or one package of grape flavored Kool-aid (6.5 g) per liter of water.

In most experiments the number of eggs was counted directly. In the mutant selection experiment the egg number was estimated by counting an aliquot of diluted eggs.

Formaldehyde Mutagenesis

Larvae were treated with formaldehyde according to the procedure of AUERBACH and MOSER (1953). Formaldehyde (0.1%) was mixed with freshly prepared medium (1.25% agar, 13% cornmeal, 15% molasses and 10% killed brewers yeast) when it had cooled to about 55°. The medium was used within a few hours of preparation.
Eggs and first instar larvae were placed on this medium and raised at 25°. Upon eclosion, treated males were collected and mated with appropriate untreated virgin females (formaldehyde has no mutagenic effect on females [AUERBACH and KILBEY 1971]). The progeny from this cross were subjected to selection on pentynol as described below.

**Histochemical Tests for ADH Activity**

The histochemical test is a variation of that used by GRELL, JACOBSON and MURPHY (1968) and by URSPRUNG, SOFER and BURROUGHS (1970). The staining mixture consists of 2 ml of 0.05 M sodium phosphate buffer, pH 7.5, 0.5 ml nitroblue tetrazolium (5 mg/ml), 0.1 ml 2-butanol, and 0.05 ml phenazine methosulfate (2 mg/ml). Single etherized flies were squashed in a spot plate (Disposo-Trays, Linbro Chemical Co.) in two drops of the reaction mix. If the tested flies contain wild-type levels of ADH, the reaction mix turns purple in about 5 minutes. The reaction mix remains yellow if an Adh-negative fly is tested.

**Selection of Adh-Negative Mutants**

An aqueous solution of 0.15% pentynol (v/v) was added in place of H2O to an equal volume of Formula 4-24 Blue Instant Drosophila Medium (Carolina Biologicals) in 3 x 9 cm vials. The medium was prepared immediately before use.

Eggs or larvae were placed in the vials which were then covered with Parafilm. After three days, the Parafilm was replaced with foam stoppers and a few grains of yeast were added to the medium. The number of eggs or larvae placed in a vial varied with the experiment, but ranged up to several thousand in those vials in which only a few survivors were expected. To ensure optimum survival and fertility, survivors were removed from vials as soon after eclosion as possible.

**RESULTS**

In the first experiment we tested a number of alcohols for their ability to distinguish between Adh-negative and -positive larvae. In analogy to previous results with 1-pentene-3-01 (SOFER and HATKOFF 1972), we reasoned that secondary alcohols of the general formula

\[
\begin{align*}
R & - C = C - C - C - R' \\
& \downarrow \quad \downarrow \quad \downarrow \\
H & \quad H & \quad OH & \quad H
\end{align*}
\]

(where R and R' represent alkyl groups or H) might kill Adh-positive flies without harming negatives. Consequently, eggs from an Adh-positive stock (w; Adh") and from an Adh-negative stock (Adh" b cn vg), were mixed and placed on medium containing 0.1% (by volume) of various alcohols, and the development of the flies was followed. The results are shown in Figure 1. Pentynol and 1-hexene-3-01 were most effective in killing Adh-positives and at the same time relatively innocuous to Adh-negatives. The primary alcohol, 2-pentene-1-01, did not discriminate between Adh-positive and negative flies, while all the secondary alcohols did. This correlates well with the known substrate preference of ADH for secondary alcohols (SOFER and URSPRUNG 1968). In all subsequent experiments, pentynol was used as the selective agent. This unsaturated alcohol, differing from the others in having a triple bond in place of the double bond, was found to be slightly more stable in preliminary experiments. Nevertheless, the
FIGURE 1.—Effect of various unsaturated alcohols on Adh-negative and Adh-positive flies. Groups of one hundred eggs were placed on medium prepared by adding 0.1% (v/v) of various alcohols to water. The hatched bars represent the eclosion of Adhn6 b cn ug flies; the solid bars, eclosion of w;AdhF flies, both expressed as a percentage of eclosion of control eggs placed on food without added alcohol. The symbol (o) indicates that no eggs developed into adults in these cultures.

a. no alcohol added (control)
b. 3-methyl-3-butene-2-ol
c. 1-penetene-3-ol
d. 3-penetene-2-ol
e. 4-hexene-3-ol
f. 1-hexene-3-ol
g. 2-penetene-1-ol
h. 1-butene-3-ol
i. 1-pentyne-3-ol

other unsaturated secondary alcohols tested might be useful for selection experiments under some circumstances where pentynol could not be used.

In a second series of experiments, we tested several strains of both Adh-positive and -negative flies on pentynol under a variety of conditions. Table 1 shows the effects of 0.1% pentynol on three different positive and eleven different negative strains. It is evident that all the negatives, even those derived from several different positive strains, are relatively unaffected by the presence of pentynol in the food. On the other hand, all Adh-positive strains are killed by this treatment.

In Figure 2, the results of placing different concentrations of pentynol in the food are shown. Two representative negative strains Df(2L)64j CyO as, are virtually unaffected at concentrations of up to 0.2% pentynol, while the positive strain, w; Adh b, shows significant mortality in the presence of 0.05% pentynol. Figure 2 also shows the results of subjecting Adh as flies to pentynol. Adh as is interesting in that it is a leaky mutant, exhibiting about 5% of wild-type activity at 25°C (Vigue and Sofer 1974b). As might be expected given these low levels of activity, the Adh as flies are more resistant to pentynol than wild-type flies but more sensitive than the other negative mutants which have no demonstrable ADH activity.
TABLE 1
Effects of pentynol on Adh-positive and negative strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Without pentynol</th>
<th>0.1% pentynol</th>
<th>Percent pentynol survivors relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survivors</td>
<td>Total</td>
<td>Percent survivors</td>
</tr>
<tr>
<td>AdhP</td>
<td>734</td>
<td>1155</td>
<td>64</td>
</tr>
<tr>
<td>Bethylie</td>
<td>119</td>
<td>150</td>
<td>79</td>
</tr>
<tr>
<td>AdhP b cn bw</td>
<td>39</td>
<td>50</td>
<td>78</td>
</tr>
<tr>
<td>w; Adhn1</td>
<td>201</td>
<td>350</td>
<td>57</td>
</tr>
<tr>
<td>Adhn2 b cn pr</td>
<td>483</td>
<td>700</td>
<td>69</td>
</tr>
<tr>
<td>Adhn3 b cn</td>
<td>83</td>
<td>139</td>
<td>60</td>
</tr>
<tr>
<td>Adhn4 b</td>
<td>78</td>
<td>100</td>
<td>78</td>
</tr>
<tr>
<td>Adhn5 b cn vg</td>
<td>161</td>
<td>250</td>
<td>64</td>
</tr>
<tr>
<td>Adhn7 b cn vg</td>
<td>102</td>
<td>140</td>
<td>73</td>
</tr>
<tr>
<td>Adhn8 b cn vg</td>
<td>91</td>
<td>178</td>
<td>51</td>
</tr>
<tr>
<td>Adhn9 b cn vg</td>
<td>113</td>
<td>161</td>
<td>70</td>
</tr>
<tr>
<td>Adhn10 b cn vg</td>
<td>111</td>
<td>178</td>
<td>62</td>
</tr>
<tr>
<td>Df(2L)64j/CyoA</td>
<td>143</td>
<td>584</td>
<td>25</td>
</tr>
<tr>
<td>Df(2L)64j/CyoB</td>
<td>62</td>
<td>150</td>
<td>41</td>
</tr>
</tbody>
</table>

Embryos and first instar larvae were counted and then placed on “instant” food prepared with and without added 0.1% pentynol. Adult flies emerging were counted. Survivors on pentynol-containing food usually eclosed one or two days after the controls.

![Graph](image)

**Figure 2.**—Effect of varying concentrations of pentynol on Adh-negative and Adh-positive flies.

Each point represents at least two hundred eggs placed on pentynol containing medium at 25°C. The number eclosing is expressed as a percentage of control eggs of the same strain that eclose when placed on food without added alcohol.

- ○ = w; AdhP
- ■ = Adhn5
- X = Adhn2
- ● = Df(2L)64j/CyoA

Adhn5 exhibits almost 5% of wild-type activity at this temperature.
Embryos and first instar larvae were counted and then placed on "instant" food containing 0.1% pentynol. The vials were incubated at 17°, 25° or 29° until eclosion of all the flies was completed.

Finally, since we intend to use this procedure to search for a temperature-sensitive mutant, we tested flies on pentynol at 17° and 29°. The results are seen in Table 2. They are similar to those observed at 25°; that is, the positive flies die in 0.1% pentynol and the negatives are virtually unaffected.

### The Maternal Effect

When *Df(2L) 64j/CyO* males are crossed to homozygous *Adh*-negative females, one-half the progeny are *Adh*-negative and should survive 0.1% pentynol. When this cross is performed and the progeny placed on pentynol-containing food, the results are exactly as expected. (This experiment also illustrates that heterozygous *Adh*-positive flies are sensitive to pentynol.) If, however, the cross is set up with the heterozygote as the mother, and the father *Adh*-negative, none of the progeny survive pentynol, even though one expects exactly the same genotypes among the progeny as in the first cross. The death of the *Adh*-negative progeny in the latter case could be due to the presence of maternal ADH known to be present in very young larvae (Wright and Shaw 1970). To examine this phenomenon further, we carried out the two crosses shown in Table 3. Here *Bc* is a dominant marker which can be detected in larvae; *Adh;*, a positive allele of *Adh*; and *CyO*, a *CyO* chromosome which contains an *Adh*-negative allele.
The maternal effect: effect of pentynol on larvae derived from Adh-positive and Adh-negative mothers

<table>
<thead>
<tr>
<th>Time of pentynol treatment</th>
<th>Adults emerging</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st instar</td>
</tr>
<tr>
<td>Adh\textsuperscript{ns} b cn pr</td>
<td>21</td>
</tr>
<tr>
<td>Cy\textsuperscript{om4}</td>
<td>0</td>
</tr>
<tr>
<td>Adh\textsuperscript{ns} b cn pr</td>
<td>0</td>
</tr>
<tr>
<td>Bc (Adh\textsuperscript{+})</td>
<td>0</td>
</tr>
</tbody>
</table>

Fifty larvae of the indicated ages were placed on “instant” food containing 0.1% pentynol. The number of adults emerging of the two possible genotypes is recorded.

Larvae of different ages were placed on pentynol food and their development followed. When the female parent is homozygous-negative, about half the progeny survive and are all Adh-negative as expected (Table 3). When the mother is a heterozygous-positive fly, however, few if any progeny survive, even when placed on the selective medium as third instar larvae. Other crosses demonstrate that this phenomenon appears to be a general one and seems independent of the positive or negative strain used (unpublished data).

Since the Bc marker allowed us to distinguish between heterozygous-positive and -negative early third instar larvae, we could examine larvae for the presence of ADH activity. Cy\textsuperscript{om4}/Adh\textsuperscript{ns} larvae whose mothers were Adh-positive/Adh-negative heterozygotes were compared to those whose mothers were Adh-negative. (Both types of larvae are genotypically Adh-negative.) ADH activity was assayed by dissecting the larvae in ADH histochemical reaction mix. No ADH activity could be detected in either type of early third instar larvae.

Another way of testing for ADH activity is by testing adults and larvae for the ability to survive on medium containing ethanol. Those flies heterozygous for an active Adh allele survive in medium containing 4% ethanol while Adh-negatives die under these conditions (Vigee and Sofer 1974b). Accordingly, the following crosses were set up:

\[
\varnothing \varnothing \text{Df}(2L)64j/CyO[Adh^+] \times \frac{Adh^{ns} b cn pr}{Adh^{ns} b cn pr} \delta \delta
\]

and

\[
\delta \delta \text{Df}(2L)64j/CyO[Adh^+] \times \frac{Adh^{ns} b cn pr}{Adh^{ns} b cn pr} \varnothing \varnothing
\]

and the eggs placed on food with and without added ethanol.

The data in Table 4 indicate that the direction of the cross is irrelevant; Adh-negative flies die regardless of the genotype of the mother. These data may indi-
TABLE 4
Effect of ethanol on eggs and first instar larvae derived from Adh-negative and Adh-positive mothers

<table>
<thead>
<tr>
<th>% ethanol</th>
<th>Mother Adh-negative</th>
<th>Father Adh-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Adhn^b cn pr/Df(2L)64i</td>
<td>Adhn^b cn pr/CyO</td>
</tr>
<tr>
<td>4</td>
<td>173</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>70</td>
</tr>
</tbody>
</table>

Nine hundred embryos and first instar larvae were collected from the cross Adhn^b cn pr Df(2L)64i x Adhn^b cn pr CyO. Four hundred and fifty were placed on "instant" food containing 0% and 4% ethanol. Two hundred embryos and first instar larvae from the reciprocal cross were divided in the same way. The number of flies of each genotype emerging were counted.

cate that ADH activity sufficient to cope with the ethanol in the medium persists for only a very short time in progeny from Adh-positive mothers.

Use of the Technique to Detect New Mutations

In order to demonstrate the usefulness of pentynol selection we wanted to show that it could be used to detect new Adh-negative mutations. Formaldehyde was chosen as the mutagen in this study because formaldehyde, in contrast to other chemical mutagens, causes relatively few mosaic mutations in the F₁ generation (Auerbach and Kilbey 1971).

After treatment of Adh^D cn pr flies with formaldehyde as described above, adult males were collected and crossed with Df(2L)64j/CyOnB cn* pr virgin females. Eggs were collected, counted and placed in vials containing 0.15% pentynol.

About 3.5 x 10⁵ eggs were tested. The results are shown in Table 5. Twelve Adh-negative survivors were recovered, and found to be phenotypically like the female parent. They probably represent offspring of non-virgin females (last

TABLE 5
Formaldehyde mutagenesis

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of flies recovered</th>
<th>ADH phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adh^D cn pr/CyOnB cn^a pr</td>
<td>13</td>
<td>Adh-positive</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Adh-negative</td>
</tr>
<tr>
<td>Adh^D cn pr/Df(2L)64j</td>
<td>6</td>
<td>Adh-positive</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Adh-negative</td>
</tr>
<tr>
<td>Df(2L)64j/CyOnB cn^a pr</td>
<td>12</td>
<td>Adh-negative</td>
</tr>
</tbody>
</table>

Progeny from the cross:

δ δ Adh^D cn pr Df(2L)64j CyOnB cn^a pr
were placed on 0.15% pentynol food as embryos or first instar larvae. 351,150 individuals were tested. The Df(2L)64j/CyOnB progeny probably resulted from contamination of a non-virgin female parent.
line. Table 5). Nineteen survivors were found to be Adh-positive, as judged by their reaction in a spot test. Their progeny were also Adh-positive and pentynol-sensitive. We believe these nineteen represent false negatives and are the background "noise" of the screen. Eight true Adh-negative mutants were detected, of which seven were fertile. These were crossed to Df(2L)64j/CyO* flies and their progeny tested for activity. In all cases the progeny were of the expected phenotype, lacked ADH activity, and were resistant to pentynol. Five of the seven fertile mutants are lethal when homozygous or in heterozygotes with the Df(2L)64j chromosome, and are therefore maintained balanced against the CyO* chromosome. Two of the mutants are homozygous viable. From genetic and cytological analysis, it appears that all of the homozygous lethal mutants contain small deletions which include the Adh region. Further characterization of the mutants will be reported elsewhere.

DISCUSSION

The procedure described in this paper for the detection and isolation of Adh-negative mutants has several important advantages over the previous selection scheme (SOFER and HATKOFF 1972). Firstly, since eggs and early larvae are treated with the selective agent rather than adults, hundreds of thousands of individuals can be tested easily. Secondly, it is a simple matter to detect any larvae which have survived the selection procedure, and to isolate them before, or immediately after, they have eclosed, thus ensuring that female survivors are virgins. Thirdly, the pentynol selection scheme has the potential of detecting a new class of mutants: those individuals which exhibit activity as adults, and not as larvae. Fourthly, leaky mutant sometimes survive treatment with 0.1% pentynol (Figure 2).

We have demonstrated the utility of the selection procedure in a pilot experiment where 3.5 \times 10^6 eggs and larvae were subjected to selection after formaldehyde mutagenesis of their fathers. Only sixteen false negatives passed through the pentynol screen, while eight bonafide Adh mutants were isolated. It is possible that the false negatives were individuals with somewhat less than the normal amount of ADH due to mosaicism or some other mechanism. More probably, selection is not absolute and allows an occasional Adh-positive fly to survive when large numbers are tested. In any event, subsequent testing of these survivors showed that they and their progeny had ADH activities indistinguishable from wild-type levels.

Under the conditions described in this report, formaldehyde yields about 5% X-linked recessive lethals as determined by the Muller-5 procedure (unpublished data). If it is assumed that the Adh locus has average mutability, and that there are about 2000 loci on the second chromosome (BEERMANN 1972), then we should have obtained about 14 Adh-negative mutants out of the 280,000 viable eggs examined (only about 80% of the eggs examined were viable). We found 8 mutants. However, most of the mutants we detected (5/7 tested) were apparently deletions which included vital genes as well as Adh. These mutants are
not viable as $Df(2L)64j$ heterozygotes. Since our test cross is set up such that one-half of the putative mutant chromosomes are tested in heterozygotes with the $Df(2L)64j$ chromosome, we probably miss nearly one-half the potential mutants. This means that the number of mutants actually detected is nearly that expected given the assumptions made above.

One puzzling phenomenon which we noted during these studies is the "maternal effect." We have shown that genotypically $Adh$-negative flies are pentynol-sensitive through the third instar when their mothers are heterozygous $Adh$-positive. WRIGHT and SHAW (1970) have shown that maternally derived ADH persists in flies until at least the second instar. However, we could detect no ADH activity in these flies during the third instar. Furthermore, a maternal effect was not found in larvae subjected to ethanol poisoning.

These conflicts can be explained in several ways. One possibility is that a small amount of maternally derived enzyme persists in one or more tissues in third instar larvae. The small amount of activity would be insufficient to significantly influence ethanol metabolism, and would be undetected by histochemical staining but would suffice for pentynol sensitivity.

Another possible explanation is that the maternal ADH acts to produce some oxidant which in turn oxidizes the pentynol to a lethal product. This too would explain both the failure to find ADH-activity and the lack of a maternal effect with regard to ethanol poisoning.

Regardless of the correct explanation, the maternal effect is easily avoided if the progeny to be tested are derived from a cross where the mother is homozygous $Adh$-negative. We use $Df(2L)64j/CyO^w$ flies for this purpose.

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