Drosophila Alcohol Dehydrogenase Polymorphism and Carbon-13 Fluxes: Opportunities for Epistasis and Natural Selection

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ABSTRACT

The influence of genetic variations in Drosophila alcohol dehydrogenase (ADH) on steady-state metabolic fluxes was studied by means of 13C NMR spectroscopy. Four pathways were found to be operative during 8 hr of ethanol degradation in third instar larvae of Drosophila. Seven strains differed by 18–25% in the ratio between two major pathway fluxes, i.e., into glutamate-glutamine-proline vs. lactate-α,α-trehalose. In general, Adh genotypes with higher ADH activity exhibit a twofold difference in relative carbon flux from malate into lactate and alanine vs. α,α-trehalose compared to low ADH activity genotypes. Trehalose was degraded by the pentose-phosphate shunt. The pentose-phosphate shunt and malic enzyme could supply NADPH necessary for lipid synthesis from ethanol. Lactate and/or proline synthesis may maintain the NADH/NAD⁺ balance during ethanol degradation. After 24 hr the flux into trehalose is increased, while the flux into lipids declines in Adh² larvae. In Adh¹ larvae the flux into lipids remains high. This co-ordinated nature of metabolism and the genotype-dependent differences in metabolic fluxes may form the basis for various epistatic interactions and ultimately for variations in organismal fitness.

FROM 1960 to 1980, investigations of Drosophila evolutionary genetics were often aimed at the establishment of the amount of genetic variation in proteins in populations. Much genetic variation is expressed in enzyme activity, based on enzyme quantity and/or catalytic efficiency, and protein thermostability (Lewontin and Hubby 1966; Zera et al. 1985). More recently, variation in the DNA sequences of alleles have also been examined (e.g., Kreitman 1983). The role of genetic variation in gene–enzyme systems in determining metabolic fluxes is of growing interest (Middleton and Kacser 1983). Fluxes form the ultimate link between the biochemistry of the cells and the physiology of the organism. Relating the genotype via its biochemical physiological phenotype to ecological function and ultimately to fitness represents one of the major challenges in evolutionary genetics (Zera et al. 1985; Watt 1985; Powers et al. 1991).

Cavener and Clegg (1981a) found that the flux through the pentose-phosphate shunt was significantly affected by genetic variations in 6-phosphogluconate dehydrogenase (EC 1.1.1.43) in Drosophila melanogaster larvae. Selection favoring Drosophila alcohol dehydrogenase (Adh) genotypes with high alcohol dehydrogenase (ADH, EC 1.1.1.1) activity was found in population studies (Van Den Den 1982; Zera et al. 1985; Heinstra 1993). Larval ADH activity variation was shown to be partly related to differential alcohol-elimination rates and 14C-fluxes and to larval-to-adult survival (Heinstra et al. 1987; Heinstra and Geer 1991; Freriksen et al. 1994a). However, ADH variation in adults was not associated positively with fluxes (Middleton and Kacser 1983). The relation of one pathway flux to fitness is difficult to ascertain, because of the co-ordinated nature of metabolism as an integrated process.

Therefore, 13C nuclear magnetic resonance (NMR) spectroscopy has been used to study the complex intermediary metabolic fates of the carbons of ethanol in Drosophila larvae (Heinstra et al. 1990). 13C NMR is a non-invasive method with a low sensitivity, only compounds that reach levels above 200 μM will be detected. The natural abundance of 13C is only 1.1% which facilitates the measurement of de novo synthesized products from administered 13C-enriched substrates (Gohen 1989). When [2-13C]ethanol was provided as dietary carbon source to Drosophila larvae, the overall intermediary metabolism of its breakdown was derived from the 13C enrichment patterns in the different (end)products (depicted in Figure 1). Carbon atoms from ethanol flow at several branch points in the tricarboxylic acid (TCA) cycle into different directions. At the first branch point citrate flows into its cytosolic pool as precursor for de novo synthesis of fatty acids and triacylglycerols. ADH apparently controls this pathway in larvae, but probably not in adults (Heinstra and Geer 1991; Freriksen et al. 1991, 1994b). Glutamine and proline are formed from 2-oxoglutarate at a second branch point. Flow of malate to the cytoplasm forms the third branch point. From here, two directions can be followed, (i) into pyruvate as precursor for alanine and lactate synthesis, or (ii) into

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a pathway called gluconeogenesis, resulting in de novo synthesis of α,α-trehalose (Figure 1). This 13C NMR method thus allows relative fluxes in different larval Adh genotypes to be assessed. Flux variation within and between the sibling species D. melanogaster and Drosophila simulans were examined in a previous study (FRERIKSEN et al. 1991) and is subject in the current one.

MATERIALS AND METHODS

Five D. melanogaster strains carrying either the AdhF or the AdhS gene and two D. simulans strains were used (FRERIKSEN et al. 1991). The strains Fgl, Fg2 (both homozygous for AdhF), Sgl and Sg2 (both homozygous for AdhS) have similar genetic backgrounds (for a full account see OUDMAN et al. 1991). All strains were cultured at 25°C and 60% relative humidity on a modified Mittler-Bennet diet (54 g sucrose, 16 g dead yeast, 19 g agar in 1 liter tap water and 8 ml propionic acid).

Larvae were exposed to 5% (v/v) [2-13C]ethanol for 8 or 24 hr as described by HEINSTRA et al. (1990) and FRERIKSEN et al. (1991). For the analysis of the metabolites both the neutralized perchloric acid (PCA) and chloroform extracts were used (HEINSTRA et al. 1990).

The proton decoupled 13C NMR spectra were recorded at 50.32 MHz on a Bruker WP 200 WB spectrometer equipped with an Aspect 2000 computer. Acquisition parameters: sweep width 10,000 Hz, pulse width 9.5 μsec corresponding to a 90° flip angle, data size 16,000, relaxation delay 2.0 sec, number of scans 20,480. A relaxation delay of 2.0 sec was found to produce no saturation effects in the regions of interest (D. SEYKENS, unpublished results). The total time to scan one larval extract was 16 hr, and overall this limited the number of replicates that were measured.

The chemical shifts in part per million (ppm) are referred to the chemical shift of ethanol-C2, which is 17.8 under our conditions relative to tetramethylsilane at 0 ppm. Standard solutions and literature data were employed for the identification of different carbon atoms (JOHNSON and JANKOWSKI 1972). The intensities of the peaks were determined by cutting out and weighing the peaks, to verify the data obtained by integration of the peak area. The glutamate, glutamine and proline C3/C4 ratios were corrected for small differences in nuclear Overhauser enhancement as follows: (C3/CA) corrected = 0.90 (C3/C4) observed.

Analysis of variance and the Tukey or Scheffé test (for groups with unequal sizes) were performed using SPSS (Statistical Data Analysis; SPSS Inc., 444 North Michigan Avenue, Chicago, Illinois 60611).

RESULTS

Theoretical background and TCA cycle activities: Background spectra of all strains only showed a small and comparable amount of the storage carbohydrate α,α-trehalose (see also HEINSTRA et al. 1990). Figure 2a shows a 13C NMR spectrum that is typical for larvae that have been fed [2-13C]ethanol for 8 hr. The specific carbon enrichments are thought to occur in the following way (see also Figure 3). [2-13C]Ethanol enters the TCA cycle via [2-13C]acetyl-CoA as [4-13C]citrate through the action of citrate synthase (EC 4.1.3.7). The first turn of the TCA cycle will enrich glutamate C4 via 2-oxoglutarate C4, and two derivatives, glutamine and proline, will be C4-enriched as well. Again in the TCA cycle, scrambling of label will occur in C2 and C3 of the symmetrical intermediates, succinate and fumarate. Multiple turns of the TCA cycle will result in a pool of glutamate that is C1-, C2-, C3- and C4-enriched, even within the same molecule. Two adjacent 13C-labeled carbons give rise to C-C scalar coupling thus resulting in the appearance of doublets, triplets, et cetera in the spectrum. Such multilabeled molecules of glutamate, glutamine and proline were identified (Figure 2b). These interactions between adjacent 13C-labeled nuclei may provide useful information on (relative) fluxes of metabolic pathways [for recent overviews, see COHEN (1989) and JEFFREY et al. (1991)].

Most of the carbons enter the TCA cycle via acetyl-CoA. However, carbon may also enter the TCA cycle via other pathways, called anaplerosis (KORNBERG 1966).
When anaplerosis and carbon disposal are not active, glutamate will be equally labeled at C2, C3 and C4; if active, unequal labeling occurs. Therefore, the ratio \( y \) between GluC3 and GluC4 represents the activity of the anaplerotic routes \( a \) and it is expressed as a fraction of the citrate synthase flux \( c \); thus GluC3/C4 = 1/(2\( y \) + 1) with \( y = a/c \) (Malloy et al. 1987). The ratio between the total glutamate multiplet gives the fractional enrichment \( F_e \) of the acetyl-CoA entering the TCA cycle as [2-\( ^{13} \)C]acetyl-CoA; thus GluC4\(_{doublet}\)/C4\(_{triplet}\) = \( F_e / (2\( y \) + 1) \) (Malloy et al. 1988). These values have been determined for each strain (Table 1). On average,
the $F_c$ value is $73 \pm 7\%$ and $\gamma = 54 \pm 7\%$ with nonsignificant differences between the strains. In general for the strains, about 70% of the total acetyl-CoA entering the TCA cycle comes from [2,13C]ethanol, whereas anaplerotic routes are active too.

Full analysis of spin-spin coupling data of glutamate and/or glutamine can also be used to test whether steady-state metabolic conditions apply in our system (MALLOY et al. 1990). This analysis requires measurements of relative areas of the singlet, doublet, triplet, and/or quartet components of the glutamate (or in our case, glutamine) C2, C3 and C4 resonances. The non-steady-state counter analysis requires measurements of the total 13C enrichment in GluC4 vs. GluC3. Using equations and computer software provided by C. R. MALLOY, we found that the larvae were in metabolic steady-state, verifying earlier independent conclusions (HEINSTRA et al. 1987; HEINSTRA and GEER 1991; FRERIKSEN et al. 1991).

**Glutamate branch point to glutamine and proline synthesis:** Part of the TCA cycle intermediate, 2-oxoglutarate, is diverted into glutamate. Subsequently, glutamine may be produced through the action of glutamine synthase (EC 6.3.1.2) and proline may be formed by a two-step reaction, the last step being catalyzed by a NADH-dependent proline dehydrogenase (BEENAKKERS et al. 1985). Total C4 resonances of Glu, Gln and Pro were analyzed for differences in flux between the strains at this branch point. The general ratio was GluC4:GlnC4:ProC4 = 0.3:0:4:0.3 with non-significant differences for the strains.

**Malate branch point to trehalose or alanine/lactate synthesis:** Malate, as TCA cycle intermediate, is apparently transported out of the mitochondrion to become part of the cytosolic pool. If gluconeogenesis occurs, C2,C3-enriched malate from the first turn in the TCA cycle will eventually label the C1, C2, C5 and C6 nuclei of glucose, and in Drosophila of α,α-trehalose. Multiple turns through the TCA cycle will enrich the C3 and C4 nuclei of α,α-trehalose, but this will be dependent on how much anaplerosis occurs. Another diversion from malate into the synthesis of pyruvate takes place through action of a NADP+-dependent malic enzyme (EC 1.1.1.40). Pyruvate serves as substrate for both alanine and lactate synthesis, the latter step via NADH-dependent lactate dehydrogenase (LDH, EC 1.1.1.37).

In all the strains, the malate branch point was found to be active in both directions. Since the absolute concentration of each product was not determined, only relative fluxes could be deduced.

We first determined the ratio of Glu + Gln + Pro/Lac + Ala + Tre as a relative measure of diversion of metabolites into two different metabolic pathways (Table 2). Differences of only 18–25% across the seven strains were deduced for this ratio (ANOVA: $d.f. = 25$, $P = 0.10$, nonsignificant). The diversion of label into the glutamate direction was always higher (27–43%) than into cytosolic malate. In any event, there were no significant differences between the different Adh genotypes studied. Secondly, the ratio of Lac + Ala/Tre for the strains revealed a twofold difference between S vs. F and simulans. That is, strains with twofold higher ADH activity (e.g., F, Fg1 and SimM) had relatively higher fluxes to lactate/alanine than those with lower ADH activity (i.e., Sg1 and Sg2; $d.f. = 25$, $P = 0.02$, significant) (for ADH activities see FRERIKSEN et al. 1991).

The carbon enrichments of TCA cycle intermediates is evident in 13C-resonances in glucose or α,α-trehalose (DEN HOLLANDER and SHULMAN 1983). The incorporation of label from [2-13C]acetate into the C1, C2, C5 and C6 atoms of α,α-trehalose is a normal phenomenon from the first turn of the TCA cycle. The C3 and C4 intensities reflect labeling as a result of additional turning of the TCA cycle, in which 2-oxoglutarate C2 becomes C1 in malate (e.g., DEN HOLLANDER and SHULMAN 1983). A comparison of C3 + C4/C2 + C5 ratios should, therefore, reveal strain differences in TCA cycle activity. However, no significant differences were found between the strains after 8 hr of ethanol degradation (Table 3).

The C5/C4 ratio of α,α-trehalose is indicative of the equilibrium in the triose-phosphate isomerase reaction during gluconeogenesis (DEN HOLLANDER and SHULMAN 1983). There were no significant differences between the strains for this ratio, and the ratios were all close to

### Table 1

**Fractional enrichment and activity of anaplerotic routes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>$F_c$</th>
<th>$\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>0.81 ± 0.27</td>
<td>0.50 ± 0.13</td>
</tr>
<tr>
<td>Fg1</td>
<td>0.95 ± 0.11</td>
<td>0.67 ± 0.18</td>
</tr>
<tr>
<td>Fg2</td>
<td>0.72 ± 0.15</td>
<td>0.54 ± 0.13</td>
</tr>
<tr>
<td>Sg1</td>
<td>0.67 ± 0.07</td>
<td>0.58 ± 0.24</td>
</tr>
<tr>
<td>Sg2</td>
<td>0.71 ± 0.01</td>
<td>0.45 ± 0.11</td>
</tr>
<tr>
<td>SimM</td>
<td>0.67 ± 0.10</td>
<td>0.60 ± 0.16</td>
</tr>
<tr>
<td>Simst</td>
<td>0.67 ± 0.13</td>
<td>0.45 ± 0.09</td>
</tr>
</tbody>
</table>

Each value represents mean ± se of at least three independent replicates based on glutamine multiplets. No significant differences in $F_c$ and $\gamma$ were present between the strains.

### Table 2

**Relative ratios of Gln, Glu, Pro, Lac and Ala and Tre**

<table>
<thead>
<tr>
<th>Strain</th>
<th>G + G + F/L + A + T</th>
<th>L + A/T</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>1.30 ± 0.20</td>
<td>0.54 ± 0.23</td>
</tr>
<tr>
<td>Fg1</td>
<td>1.21 ± 0.04</td>
<td>0.69 ± 0.37</td>
</tr>
<tr>
<td>Fg2</td>
<td>1.76 ± 0.11</td>
<td>0.48 ± 0.14</td>
</tr>
<tr>
<td>Sg1</td>
<td>1.22 ± 0.09</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>Sg2</td>
<td>1.37 ± 0.04</td>
<td>0.25 ± 0.13</td>
</tr>
<tr>
<td>SimM</td>
<td>1.67 ± 0.24</td>
<td>0.53 ± 0.14</td>
</tr>
<tr>
<td>Simst</td>
<td>1.79 ± 0.22</td>
<td>0.44 ± 0.10</td>
</tr>
</tbody>
</table>

Each ratio represents the mean ± se of at least three independent replicates. No significant differences are found between any of the strains for the ratio Gln + Glu + Pro/Lac + Ala + Tre.
TABLE 3
Ratios between specific carbon atoms of α,α-trehalose

<table>
<thead>
<tr>
<th>Strain</th>
<th>C2/C5</th>
<th>C3/C4</th>
<th>C3 + C4/C2 + C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>F(0)</td>
<td>0.94 ± 0.20</td>
<td>1.16 ± 0.12</td>
<td>1.02 ± 0.11</td>
</tr>
<tr>
<td>F</td>
<td>0.90 ± 0.13</td>
<td>1.22 ± 0.16</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td>Fg1</td>
<td>0.77 ± 0.08</td>
<td>0.95 ± 0.27</td>
<td>0.74 ± 0.10</td>
</tr>
<tr>
<td>Fg2</td>
<td>0.72 ± 0.02</td>
<td>1.11 ± 0.15</td>
<td>0.66 ± 0.05</td>
</tr>
<tr>
<td>Sg1</td>
<td>0.76 ± 0.08</td>
<td>0.97 ± 0.06</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>Sg2</td>
<td>0.67 ± 0.02</td>
<td>1.20 ± 0.26</td>
<td>0.72 ± 0.06</td>
</tr>
<tr>
<td>SimM</td>
<td>0.77 ± 0.11</td>
<td>1.00 ± 0.05</td>
<td>0.82 ± 0.06</td>
</tr>
<tr>
<td>Simst</td>
<td>0.72 ± 0.11</td>
<td>1.07 ± 0.18</td>
<td>0.72 ± 0.04</td>
</tr>
</tbody>
</table>

Each value represents the mean ± se of at least three independent replicates. C2/C5 and C3/C4 values are not significantly different from the background data [F(0)]. All C3 + C4/C2 + C5 ratios at t = 8 hours are significantly different from the background data.

1.0 (Table 3). The dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate interconversion should be at equilibrium in Drosophila, similar to the situation found in rat liver (COHEN et al. 1979).

When the pentose-phosphate shunt is active, the C5 of α,α-trehalose will increase at the expense of the C2 enrichment (COHEN et al. 1979). This occurred after 4 hr of ethanol digestion by the larvae (HEINSTRA et al. 1990), and 8 hr of feeding (Figure 2). Overall, the strains had ratios of approximately 0.75 (Table 3; F ratio of 0.9 is nonsignificantly different from other ratios), suggesting that approximately 25% of the trehalose was used to spark the pentose-phosphate shunt.

Metabolic features after 24 hr of ethanol degradation:
Third instar larvae of only the F, Fg1 and Sg1 strain were subjected to [2-13C]ethanol for 24 hr, and the PCA extracts were analyzed for their 13C enrichment patterns. Only the F and Fg1 strain exhibited spin-spin coupling at the C1, C2, C5 and C6 atoms of α,α-trehalose, with each doublet peak being about one-third of the singlet peak at 58 ppm might also be assigned to phosphatidylcholine, a common phospholipid for Drosophila, but this was not checked with a standard solution. Therefore, the identities of the two peaks remain unknown.

Chloroform extracts from the same pool of larvae showed a continuous fatty acid synthesis in the F and Sg1 strain (Table 5; Fg1 strain not determined). However, a more continuous fatty acid synthesis in time was apparent for the Sg1 strain, but this seems not the case for the F strain. Three other features were also revealed from the chloroform extracts after 24 hr of ethanol degradation. First, the glycerol-backbone peaks at 62 and 69 ppm increased by a factor of 1.5 over the 8-hr peak (FRERIKSEN et al. 1991). Free fatty acids (FA) apparently were diverted to their triacylglycerol forms, which supports 13C-tracer studies (GEER et al. 1991). The NMR findings further substantiate the role of sn-glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) in supplying sn-glycerol-3-phosphate as the backbone for lipids (GEER et al. 1983). Spin-spin couplings at both glycerol-backbone peaks were sometimes observed in the F strain only, possibly due to metabolic turnover (BEENAKKERS et al. 1985). Second, the average FA chain lengths of the two strains were similar after 24 hr (Table 5), suggesting that the average FA chain length cannot be adequately determined after 8 hr (FRERIKSEN et al. 1991).

Third, we observed novel peaks at 18 and 58 ppm in several samples. We first suspected the formation of free fatty acid ethyl esters, analogous to the situation in adipose, pancreatic, liver and heart tissue in mammals (e.g., LAPOSATA and LANGE 1986). Comparing our data with a standard of lauric acid ethyl ester, however, revealed that a peak at 14 ppm should be evident upon ester-bonding rather than at 18 ppm (MILLER et al. 1992). The peak at 58 ppm might also be assigned to phosphatidylcholine, a common phospholipid for Drosophila, but this was not checked with a standard solution. Therefore, the identities of the two peaks remain unknown.

DISCUSSION
Understanding the complex relations between the genotype, its phenotype(s), and fitness requires consid-
eration of the interactions between the metabolic pathways involved. $^{13}$C NMR spectroscopy provides a tool to achieve this. The relation between the Adh genotype and its biochemical and metabolic phenotypes is more complex than previously envisaged. The activities of the D. simulans ADHs were already shown not only to be determined by the Adh locus, but to be also dependent on the time and pathway under study, even under the same environmental conditions. Nevertheless, the specific differences in ADH activity are also based on their regulatory factors located outside the Adh locus and its biochemical and metabolic phenotypes is more complex than previously envisaged. The activities of the D. simulans ADHs were already shown not only to be determined by the Adh locus, since trans-acting regulatory factors located outside the Adh locus were found to be responsible for the difference in ADH mRNA/ADH protein levels (LAURIE et al. 1990; THOMSON et al. 1991). The interspecific differences in ADH activity are also based on their $k_{on}$ turnover number which are caused by two or three amino acid replacements (GEER et al. 1990). For the ADH-Fast/Slow polymorphism in D. melanogaster ADH activity differences are due to turnover number differences (GEER et al. 1990) and cis-acting regulatory factors (LAURIE et al. 1991; CHOUHARY and LAURIE 1991).

Drosophila larvae utilize at least four pathways to avoid the toxic effects of ethanol: (i) the pathway from ethanol to lipids, (ii) from ethanol to glutamate, that branches to glutamine and proline, (iii) from ethanol to pyruvate that branches to lactate and alanine and (iv) from ethanol to α,α-trehalose, which also shows high turnover (see overall in Figure 1). The fluxes through these complex pathways, which involve at least eight branch points, strongly complicate the straightforward application of the so-called metabolic control theory (see e.g., KACSER and PORTEOUS, 1987; HEINSTRA, 1993). Our NMR approach provides circumstantial evidence that the choice of point to monitor a metabolic pathway will determine the value of the flux control coefficient for the enzyme under study. For example, the flux control coefficient for larval ADH was shown in our previous studies to be close to 1.0 for the flux from ethanol into lipids (HEINSTRA and GEER 1991; FRERIKSEN et al. 1991). This merited the idea that natural selection can act on the larval Adh polymorphism. Based on the current results, we anticipate a very low flux control coefficient for ADH in the combined pathways into Glu + Gln + Pro/Lac + Ala + Tre and Lac + Ala/Tre after 8 hr of ethanol degradation. If we had monitored only the $^{13}$C-enrichments in α,α-trehalose, again a low coefficient would be evident after 8 hr, but possibly not after 24 hr of ethanol feeding (Table 5). Thorough examination of several different F and S strains has to substantiate this hypothesis. There were also shifts in the ratio between the fluxes through different pathways in time (Table 2 vs. 5). This suggests that estimation of flux control coefficients is dependent on the time and pathway under study, even under the same environmental conditions. These metabolic complications have not been adequately considered (MIDDLETON and KACSER 1983; KACSER and PORTEOUS 1987). Nevertheless, the specific differences in fluxes through intermediary metabolism between these Adh genotypes might form the basis for differences in fitness, thus leading to an opportunity for natural selection to occur.

The function of these metabolic pathways in the larval physiology is not completely clear. The synthesis of proline might be related to the role of soluble proline dehydrogenase in regeneration at basal levels of NADH.
into NAD$^+$ (Beenackers et al. 1985). A similar argument for maintaining the NADH/NAD$^+$ balance during ethanol degradation can be made for the action of LDH in synthesis of lactate. It would be worthwhile to test whether the Drosophila ADH:LDH pair involves metabolic channeling (Ovad 1991). Remarkably, strains with generally higher ADH activity show relatively more flux into lactate and alanine, suggesting a surplus mechanism in transferring reducing equivalents. Moreover, malic enzyme functions in the same pathway to produce pyruvate. Enough data are available to support its important role in supplying NADPH for lipid synthetic purposes in Drosophila larvae (Geer et al. 1978; Clark and Keith 1988). Synthesis and further metabolic turnover of α,α-trehalose is another intriguing aspect during degradation of ethanol. Trehalose is known as blood sugar in insects and even may act as an antifreeze (Mullins 1985). Metabolic turnover of trehalose through the pentose-phosphate shunt is important in Drosophila larvae also to generate NADPH by the glucose-6-phosphate and 6-phosphogluconate dehydrogenase reactions (Geer et al. 1981). This NADPH in turn is used in lipid synthesis as well (Clark and Keith 1988). After 24 hr of ethanol degradation, $^{13}$C-enrichments in trehalose were higher in the F than in S strains. It remains to be seen whether this is a normal phenomenon for other F vs. S strains. If so, one may anticipate that the allele frequency of the Adh$^b$ may be higher in colder climates (Van Delden 1982), when trehalose acts as an antifreeze in Drosophila.

Metabolic pathways were found to interact in a complex way during the degradation of ethanol in Drosophila larvae. This also explains the occurrence of various epistatic interactions and covarying latitudinal clines (Oakeshott et al. 1982) that have been observed in population-genetical research (Van Delden 1982; Cavener and Clegg 1981b; Voudiibio et al. 1989). More specifically, correlated variation in Adh-Gpdh-G6pdh alleles would fit a concept involving sn-glycerol-3-phosphate dehydrogenase in the supply of glycerol backbone for triacylglycerol synthesis from ethanol. This in turn would require high amounts of NADPH, supplied by malic enzyme and the pentose-phosphate shunt via action of glucose-6-phosphate dehydrogenase in Drosophila larvae.

Our current approach has shown that $^{13}$C NMR spectroscopy allows an integral look into complex organismal processes. The NMR method may be applicable in several other evolutionary genetic studies with similar questions, e.g., genetic variation in glycolysis, pentose-phosphate shunt, and tricarboxylic acid cycle. Such an approach would be highly complementary to the other techniques available for evolutionary geneticists.

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