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

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Manuscript received May 20, 1993
Accepted for publication April 28, 1994

ABSTRACT

The influence of genetic variations in Drosophila alcohol dehydrogenase (ADH) on steady-state metabolic fluxes was studied by means of ^{13}C 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-alanine-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 Adh^F larvae. In Adh^S 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 DELDEN 1982; ZERA *et al.* 1985; HEINSTRA 1993). Larval ADH activity variation was shown to be partly related to differential alcohol-elimination rates and ^{14}C-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, ^{13}C 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). ^{13}C 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 ^{13}C is only 1.1% which facilitates the measurement of *de novo* synthesized products from administered ^{13}C-enriched substrates (COHEN 1989). When [2-^{13}C]ethanol was provided as dietary carbon source to Drosophila larvae, the overall intermediary metabolism of its breakdown was derived from the ^{13}C 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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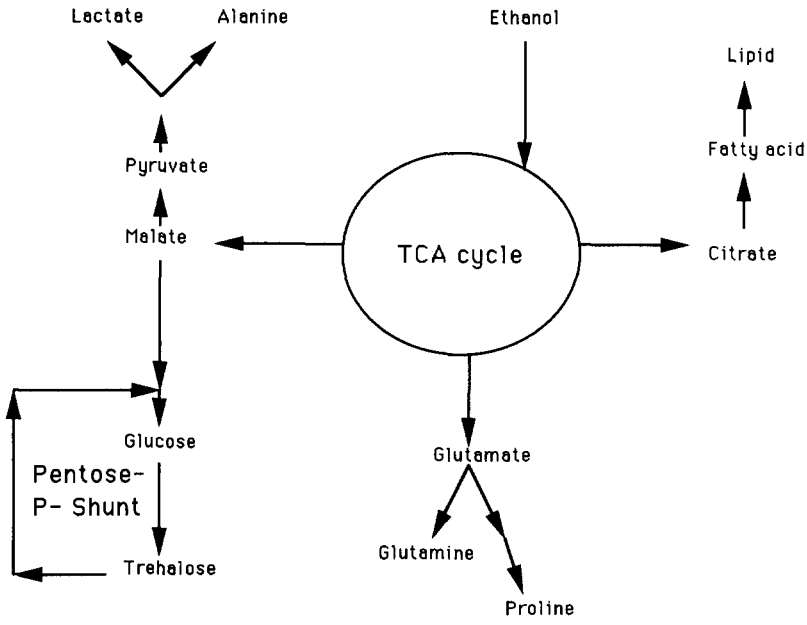

FIGURE 1.—Simplified scheme of the intermediary metabolism of ethanol degradation in Drosophila larvae (HEINSTRA *et al.* 1990).

a pathway called gluconeogenesis, resulting in *de novo* synthesis of α,α-trehalose (Figure 1). This ^{13}C 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 Adh^F or the Adh^S gene and two *D. simulans* strains were used (FRERIKSEN *et al.* 1991). The strains Fg1, Fg2 (both homozygous for Adh^F), Sg1 and Sg2 (both homozygous for Adh^S) have similar genetic backgrounds (for a full account see OUDMAN *et al.* 1991). All strains were cultured at 25° 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-^{13}C]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 ^{13}C 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/C4) 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 ^{13}C NMR spectrum that is typical for larvae that have been fed [2-^{13}C]ethanol for 8 hr. The specific carbon enrichments are thought to occur in the following way (see also Figure 3). [2-^{13}C]Ethanol enters the TCA cycle via [2-^{13}C]acetyl-CoA as [4-^{13}C]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 ^{13}C-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 ^{13}C-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).

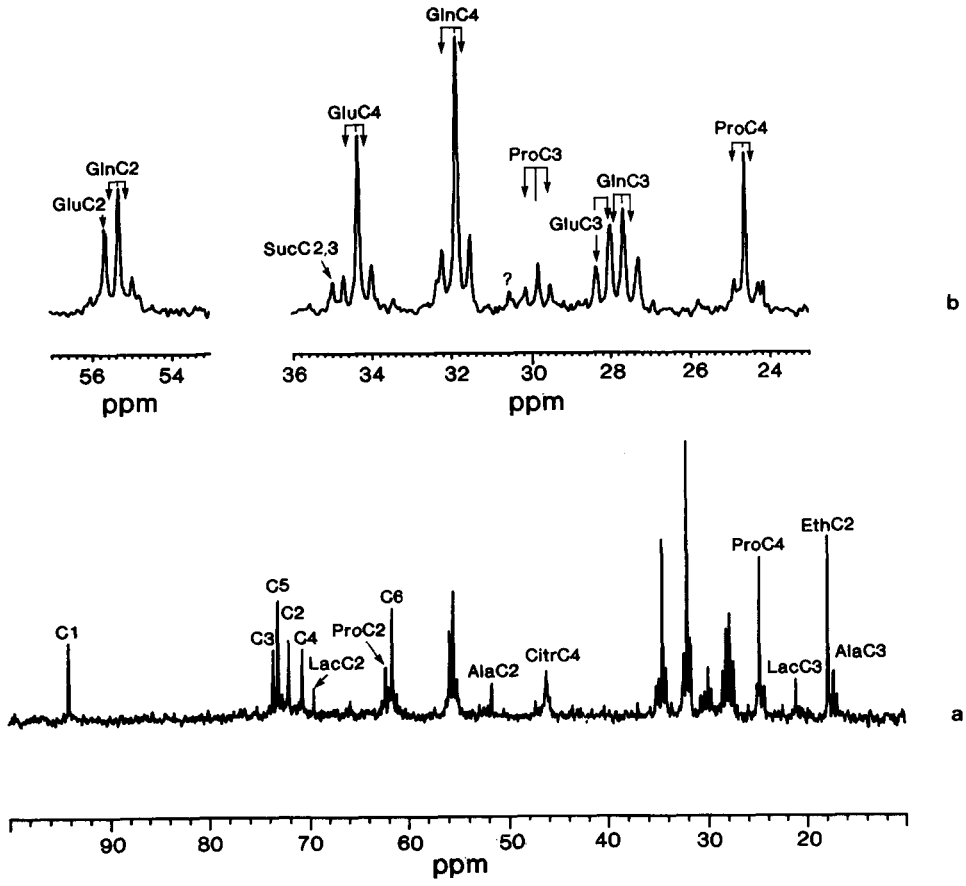

FIGURE 2.—Proton-decoupled ^{13}C NMR spectrum of Adh^{Sg1} larvae. (a) Total spectrum after 8 hr of [2-^{13}C]ethanol degradation. (b) Enlarged parts of spectrum containing multilabeled species of glutamate, glutamine and proline. Ala, alanine; Eth, ethanol; Lac, lactate; Pro, proline; Gln, glutamine; Glu, glutamate; Suc, succinate; Citr, citrate; ?, unknown compound. C1–C6 represent the carbons of the carbohydrate α,α-trehalose.

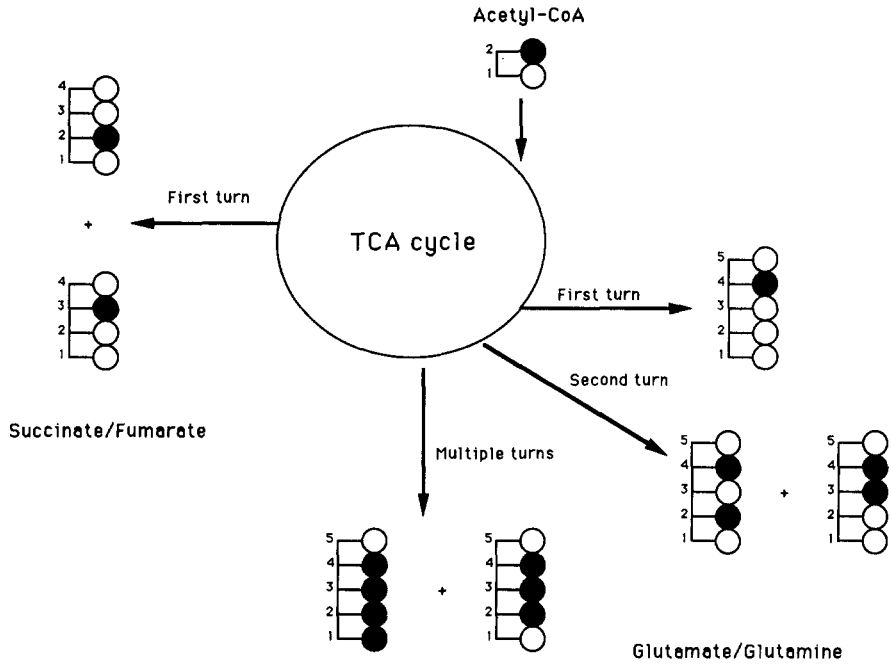

FIGURE 3.—Degradation of [2-^{13}C] ethanol in the intermediary metabolism of Drosophila larvae. The filled circles indicate those carbon atoms that are enriched with ^{13}C. The open circles represent carbon-12 atoms.

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 = $1/(2y + 1)$ with $y = a/c$ (MALLOY *et al.* 1987). The ratio between the total glutamate multiplet gives the fractional enrichment (F_c) of the acetyl-CoA entering the TCA cycle as [2-^{13}C]acetyl-CoA; thus $\text{GluC4}_{\text{doublet}}/\text{C4}_{\text{triplet}} = F_c/(2y + 1)$ (MALLOY *et al.* 1988). These values have been determined for each strain (Table 1). On average,

TABLE 1

Fractional enrichment and activity of anaplerotic routes

Strain	F_c[a]	y[a]
F	0.81 ± 0.27	0.50 ± 0.13
Fg1	0.93 ± 0.11	0.67 ± 0.18
Fg2	0.72 ± 0.15	0.54 ± 0.13
Sg1	0.67 ± 0.07	0.58 ± 0.24
Sg2	0.71 ± 0.01	0.45 ± 0.11
SimM	0.67 ± 0.10	0.60 ± 0.16
Simst	0.67 ± 0.13	0.45 ± 0.09

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

[a] F_c and y values were estimated using the equations given in the text *cf.* MALLOY et al. (1987, 1988).

TABLE 2

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

Strain	G + G + P/L + A + T	L + A/T
F	1.30 ± 0.20	0.54 ± 0.23
Fg1	1.21 ± 0.04	0.69 ± 0.37
Fg2	1.76 ± 0.11	0.43 ± 0.14
Sg1	1.22 ± 0.09	0.21 ± 0.07
Sg2	1.37 ± 0.04	0.25 ± 0.13
SimM	1.67 ± 0.24	0.53 ± 0.14
Simst	1.79 ± 0.22	0.44 ± 0.10

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.

the F_c value is 73 ± 7% and y = 54 ± 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-^{13}C]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 ^{13}C 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 an 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 an 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 (end) 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; ANOVA: 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 ^{13}C-resonances in glucose or α,α-trehalose (DEN HOLLANDER and SHULMAN 1983). The incorporation of label from [2-^{13}C]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 C3/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 3

Ratios between specific carbon atoms of α,α-trehalose

Strain	C2/C5	C3/C4	C3 + C4/C2 + C5
F(0)	0.94 ± 0.20	1.16 ± 0.12	1.02 ± 0.11
F	0.90 ± 0.13	1.22 ± 0.16	0.57 ± 0.07
Fg1	0.77 ± 0.08	0.95 ± 0.27	0.74 ± 0.10
Fg2	0.72 ± 0.02	1.11 ± 0.15	0.66 ± 0.03
Sg1	0.76 ± 0.08	0.97 ± 0.06	0.70 ± 0.04
Sg2	0.67 ± 0.02	1.20 ± 0.26	0.72 ± 0.06
SimM	0.77 ± 0.11	1.00 ± 0.05	0.82 ± 0.06
Simst	0.72 ± 0.11	1.07 ± 0.18	0.72 ± 0.04

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 continued to 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-^{13}C]ethanol for 24 hr, and the PCA extracts were analyzed for their ^{13}C 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 (Figure 4). Trehalose is the insect blood sugar and turns over rapidly (FRIEDMAN 1985). This may explain the C-C coupling phenomenon. Pentose-phosphate shunt activity can be determined through the C2/C5 ratio in trehalose. This activity increased for F to 22%, while it remained the same in the Fg1 and Sg1 strain (Table 4). The C3/C4 ratio in trehalose in all strains was again about 1.0. Furthermore, the C3 + C4/C2 + C5 ratio for trehalose decreased in the F strains, but remained unchanged for the Sg1. Remarkably, trehalose synthesis increased in time mainly in the F strains, but only slightly in the Sg1 strain (Table 5). On the other hand, the flux in the malate branch point to alanine and lactate was much higher in the Sg1 strain. The flux in this direction was slight in the F strains (see ratio of Lac + Ala/Tre in Table 4). The ratios for the Sg1 strain generally followed the patterns deduced after 8 hr (Table 3). The ratio Glu + Gln + Pro/Lac + Ala + Tre decreased only in the F strain (Table 1 *vs.* 4).

Chloroform extracts from the same pool of larvae showed a continuous fatty acid synthesis in the F and Sg1

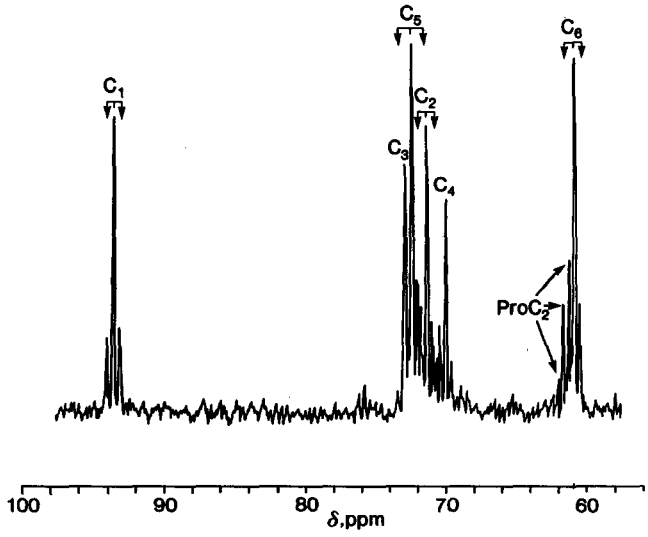

FIGURE 4.—Part of the ^{13}C NMR spectrum of Adh^F larvae after 24 hr of ethanol administration, showing the spin-spin coupling at carbon C1, C2, C5 and C6 of α,α-trehalose.

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 ^{14}C-tracer studies (GEER *et al.* 1991). These 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-

TABLE 4

Metabolite ratios after 24 hours of [2-^{13}C]ethanol degradation

Strain	Ratio				
	C2/C5	C3/C4	C3 + C4/C2 + C5	G + G + P/L + A + T	L + A/T
F	0.77 ± 0.01	1.24 ± 0.11	0.41 ± 0.02	0.55 ± 0.09	0.06 ± 0.02
Fg1	0.85 ± 0.11	1.18 ± 0.21	0.45 ± 0.05	1.00 ± 0.06	0.10 ± 0.04
Sg1	0.73 ± 0.07	0.90 ± 0.30	0.80 ± 0.24	1.40 ± 0.21	0.45 ± 0.20

Each estimate represents the mean ± SE of three independent replicates, except for Fg1, $n = 2$. The ratios of C3 + C4/C2 + C5, Gln + Glu + Pro/Lac + Ala + Tre and Lac + Ala/Tre are significantly different between the F and Sg1 strain.

TABLE 5

Some metabolic features in time

Strain	Trehalose[a]		^{13}C enrichment in lipids[b]		Average chain length[c]	
	$t = 8$	$t = 24$	$t = 8$[d]	$t = 24$	$t = 8$[d]	$t = 24$
F	13.91 ± 3.69	76.56 ± 12.55	13.93 ± 4.49	34.24 ± 9.20	15.47 ± 1.19	13.16 ± 0.82
Fg1	11.25 ± 3.82	37.30 ± 2.60	13.64 ± 2.52	ND[e]	13.68 ± 1.30	ND
Sg1	18.25 ± 9.71	22.76 ± 3.40	8.02 ± 2.24	32.54 ± 4.49	10.39 ± 1.27	12.19 ± 0.16

Each estimate represents the mean ± SE of three independent replicates, except for Fg1, $n = 2$.
[a] Total ^{13}C enrichment in carbons of trehalose.
[b] Total ^{13}C enrichment in carbons of fatty acids and lipids.
[c] Average fatty acid chain length, determined as described (FRERIKSEN *et al.* 1991).
[d] Data taken from FRERIKSEN *et al.* (1991).
[e] ND, not determined.

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* genotype, 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_{cat} 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 to *cis*-acting regulatory factors (LAURIE *et al.* 1991; CHOUDHARY 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 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^+ (BEENAKKERS *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 (OVADI 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^F 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; VOUIDIBIO *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 genetical 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.

We would like to thank D. SEYKENS for performing the NMR measurements, L. OUDMAN (University of Groningen, The Netherlands) for supplying the Groningen strains and D. SMIT for drawing Figures 2 and 3. C. R. MALLOY (Southwestern Medical Center, University of Texas, Dallas) is thanked for supplying computer software and for NMR advice, and B. W. GEER (Knox College, Galesburg, Illinois) for critically reading the manuscript. Anonymous referees are thanked for their criticisms on an earlier draft. This research was supported by grant 436-186 from the Foundation of Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

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Communicating editor: W. F. EANES