Physiological Significance of the Alcohol Dehydrogenase Polymorphism in Larvae of Drosophila

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ABSTRACT

This study deals with biochemical and metabolic-physiological aspects of the relationship between variation in *in vivo* alcohol dehydrogenase activity and fitness in larvae homozygous for the alleles Adh^{71k}, Adh^{F}, Adh^{S}, of *Drosophila melanogaster*, and for the common *Adh* allele of *Drosophila simulans*. The *Adh* genotypes differ in the maximum oxidation rates of propan-2-ol into acetone *in vivo*. There are smaller differences between the *Adh* genotypes in rates of ethanol elimination. Rates of accumulation of ethanol *in vivo* are negatively associated with larval-to-adult survival of the *Adh* genotypes. The rank order of the maximum rates of the ADHs in elimination of propan-2-ol, as well as ethanol, is ADH-71k > ADH-F > ADH-S- > *simulans*-ADH. The ratio of this maximum rate to ADH quantity reveals the rank order of ADH-S > ADH-F > ADH-71k > *simulans*-ADH, suggesting a compensation for allozymic efficiency by the ADH quantity in *D. melanogaster*.—Our findings show that natural selection may act on the *Adh* polymorphism in larvae via differences in rates of alcohol metabolism.

GENETIC variation of proteins in natural populations has been extensively documented. However, its significance for adaptive changes is less elaborated. An important issue in population genetics is whether polymorphic loci (structural and/or regulatory) are submitted to random genetic drift and/or are controlled by natural selection (*e.g.*, NEI 1983; KIMURA 1983; KOEHN, ZERA and HALL 1983). In order to study effects of natural selection on a polymorphic locus, one comes face to face with several questions. Knowledge is required of the function(s) of the gene-product(s) in the physiology. When known, the relation between genetic variation of the locus involved and the variation in physiology should be studied under different environmental conditions (CLARKE 1975; SCHARLOO *et al.* 1977; CAVENER and CLEGG 1981a; ZERA, KOEHN and HALL 1985). It has been argued even when genetic variants (*e.g.*, allozymes) differ in their biochemistry that their ultimate effects in the physiology of the organisms are rather small (KACSER and BURNS 1981).

The alcohol dehydrogenase (*Adh*, II—50.1) polymorphism of *Drosophila melanogaster* provides one of the few cases in which variation in gene-products can be studied on metabolic action and differences in fitness (VAN DELDEN 1982). Genetic variation at the *Adh* locus involves three major alleles in natural and laboratory populations, *i.e.*, Adh^{F}, Adh^{S} and Adh^{71k}. Previous studies have shown differences between their alcohol dehydrogenase allozymes (ADH, EC 1.1.1.1.) with respect to *in vivo* quantity, total ADH activity *in vitro*, thermostability and kinetic-catalytic properties, respectively (THÖRIG, SCHOONE and SCHARLOO 1975; LEWIS and GIBSON 1978; MCDONALD, ANDERSON and SANTOS 1980; WINBERG, THATCHER and MCKINLEY-MCKEE 1982a,b; HOVIK, WINBERG and MCKINLEY-MCKEE 1984; EISSES *et al.* 1985a; HEINSTRA *et al.* 1986b). A major part of the difference in ADH quantity between $Adh^{F/F}$ and $Adh^{S/S}$ flies can be explained by regulation of transcriptional activity (ANDERSON and MCDONALD 1983). Allele frequencies of the *Adh* can be affected by some dietary alcohols in laboratory populations, suggesting the importance of natural selection (VAN DELDEN 1982).

The relation between genotypic and metabolic variation has barely been investigated for enzyme-polymorphisms (ZERA, KOEHN and HALL 1985). The study of *Adh* polymorphism in adults generated apparently contradicting results. MIDDLETON and KACSER (1983) have found no significant differences in flux through the whole catabolic pathways from ethanol into lipids and carbon dioxide between different *Adh* genotypes. HEINSTRA *et al.* (1986b) have found differences in the one-step conversion of propan-2-ol into acetone between Adh^{71k} and Adh^{F} homozygotes. However, it is known that differences in *in vitro* activity between ADH allozymes are greater with propan-2-ol as substrate than with ethanol due to intrinsic properties of the allozymes studied (WINBERG, HOVIK and MCKINLEY-MCKEE 1985). In our previous report (HEINSTRA *et al.* 1986b), we have suggested that selective action by alcohols may be stronger in the larval stages than in adults. Therefore, we have decided to study the biochemistry and physiology of different *Adh* genotypes in third-instar larvae of *D. melanogaster* and its sibling species *Drosophila simulans*. Previous work has

shown that it is possible to quantify alcohols and related substances *in vivo* by means of gas liquid chromatography (HEINSTRA *et al.* 1986a,b). Here, we report on the physiological differences between various larval genotypes when fed different alcohols and the quantification of the relevant metabolites *in vivo*.

MATERIALS AND METHODS

Strains and rearing of flies: Two strains homozygous for the Adh^{71k} and Adh^{F} allele were already present (EISSES *et al.* 1985a; HEINSTRA *et al.* 1986b). Two strains homozygous for the Adh^{F} and Adh^{S} allele were provided by D. R. CAVENER. These strains were derived from Providence, Rhode Island (CAVENER 1979; CAVENER and CLEGG 1978, 1981b). We used two ADH negative strains both with wild-type aldehyde oxidase activity, $bAdh^{n4}$ (EISSES 1986) and a $bAdh^{n2}$ *pr;cn* strain provided by M. ASHBURNER. A wild-type strain of *D. simulans* caught in Malaga (Spain) in the autumn of 1983 was provided by C. MOLLEMA. Another strain of *D. simulans* homozygous for the scarlet (*st*) mutant was provided by J. S. F. Barker. Both strains of *D. simulans* appeared to be monomorphic at the *Adh* gene.

Strains were reared on the MITTLER-BENNETT (1962) diet which contained half the normal dead-yeast content and propionic acid (8 ml per liter medium). Third-instar larvae raised on this medium contained no endogenous alcohols and related substances. The strains were reared at 25° and 60% relative humidity (RH), whereas metabolic studies were conducted at room temperature (20–22°) on mid third-instar larvae.

Metabolic studies: About 40 larvae were transferred directly from the glass-well of the rearing bottles into small Petri dishes (40 mm diameter, 10 mm high). Two milliliters of the appropriate solution were added and the dishes were glass-covered. Larvae crawled freely in the solutions and could easily lift their posterior spiracles through the surface of the solution for respiration. Only active and feeding larvae were further used. When needed, larvae withdrawn from the solution are carefully dried on paper filters. In most cases, three replicates of three larvae were homogenized in 100 μl ice-cold pure water at 4° and 1-μl aliquots analyzed by means of gas liquid chromatography [for details, see HEINSTRA *et al.* (1983, 1986b)]. Retention times at a column temperature of 90°: acetaldehyde after 30 sec, propanal after 60 sec, acetone after 80 sec, ethanol after 110 sec, propan-2-ol after 160 sec and propanol-1 after 300 sec. Calculations from concentrations in extracts into concentrations per individual have already been outlined elsewhere (HEINSTRA *et al.* 1986b). Replicate injections of the same extract over periods of hours gave deviations lower than 5% of average concentration. Preliminary studies showed no differences in metabolic activity of ADH between larval sexes. Studies with acetone, propan-2-ol, propanol-1, and ethanol (5% v/v) were performed on larvae of the same generation within one week for each substance. However, with 10% (v/v) ethanol, larvae of Adh^{71k} Adh^{F} (Europe), and $bAdh^{n4}$ were studied in one experiment; larvae of Adh^{F} (USA), Adh^{S}, $bAdh^{n2}$ and *D. simulans* in another. Therefore, this ethanol experiment was considered separately (*e.g.*, in Table 8).

Electrophoresis: Disc electrophoresis was performed according to HEINSTRA *et al.* (1983). Fresh larval extracts, three larvae homogenized in 0.1 ml 50 mM phosphate buffer (pH 6.7) plus EDTA and sucrose (THÖRIG, HEINSTRA and SCHARLOO 1981) were applied with 25 μl loading/gel. After electrophoresis, the gels were stained for ADH activity with 100 mM propan-2-ol, 1 mM NAD, 1.5 mM 3-(4-5-dimethyl-2 thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 1 μM phenazine methosulfate (PMS) in a 50 mM phosphate buffer (pH 7.4) for 90 min at 22° in the dark. After some rinsing with water, the gels were scanned at 550 nm in a Gilford Response spectrophotometer supplied with a scanning holder and data analyzed by a computer program loaded by the manufacturer.

Immunological characterization: Antibodies against purified ADH-71k and ADH-F protein were obtained according to the procedures described by HEINSTRA *et al.* (1986b). Immunodiffusion tests were performed according to OUCHTERLONY (1953). The gels were incubated overnight at 4° and thereafter stained for ADH activity. Radial immunodiffusion tests were according to MANCINI, CARBONARA and HEREMANS (1965); see HEINSTRA *et al.* (1986b) for further details.

Viability studies: Ethanol was thoroughly mixed with the MITTLER-BENNETT medium at 50° leading to 10% (v/v) ethanol concentrations in the final medium. Thereafter, medium was poured into bottles (25 ml medium/bottle) and rapidly cooled. Within 1 hr, 25 larvae of the different *Adh* genotypes were stocked per bottle with 3–6 independent replicas in each experiment. The bottles were then kept at 25° and 60% RH. Emerging of adults was counted twice a day.

RESULTS

Immunological studies: Antibody preparations raised against purified ADH-71k and against ADH-F protein showed single and continuous precipitin lines without spurs with crude homogenates containing ADH proteins (Figure 1, A and B). This strongly suggests immunological identity between the ADH variants tested. The absence of a precipitin line with the homogenate of $bAdh^{n4}$, an ADH-negative mutant without ADH protein (PELLICA and SOFER 1982) (Figure 1B, well 3) and the presence of precipitin lines with purified ADH-71k, ADH-F, ADH-S, and *simulans*-ADH (Figure 2) indicate the mono-specificity of the antibodies toward ADH.

Radial immunodiffusion revealed that the quantity of ADH protein [$E_{t=0}$] was 30% higher in Adh^{71k} homozygotes than in $Adh^{F/F}$ in both larvae and adults (Table 1). The ADH quantity in $Adh^{F/F}$ larvae and adults was about twice that found in Adh^{S} homozygotes. In larvae of *D. simulans*, ADH quantities approached those of $Adh^{F/F}$ of *D. melanogaster*. However, adults of *D. simulans* contained only about 60% of the ADH quantity found in $Adh^{S/S}$ of *D. melanogaster* (Table 1). Our findings are in close agreement with data reported for other strains (LEWIS and GIBSON 1978; MCDONALD, ANDERSON and SANTOS 1980; JUAN and GONZÀLEZ-DUARTE 1980; ANDERSON and MCDONALD 1983; DICKINSON, ROWAN and BRENNAN 1984; HEINSTRA *et al.* 1986b).

Alcohols are known to modulate ADH quantity and thereby total ADH activity in third-instar larvae during a 2-day culture (GEER, MCKECHNIE and LANGEVIN 1983; MCKECHNIE and GEER 1984). Because this

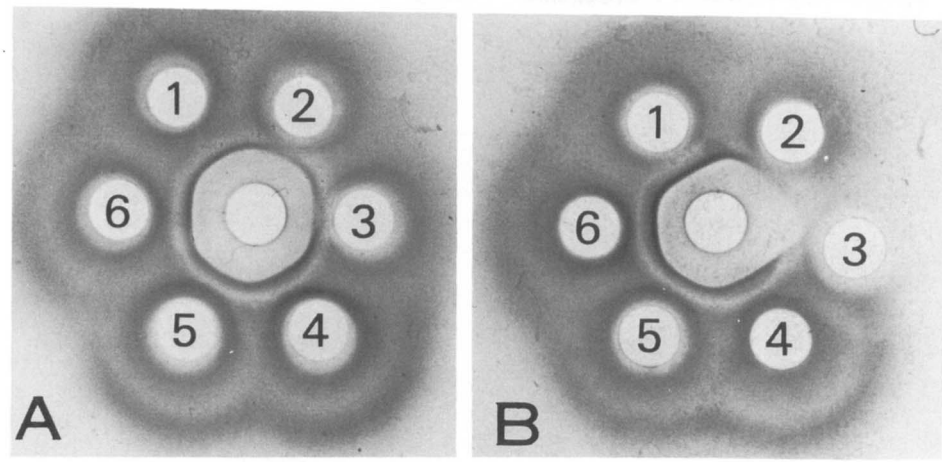

FIGURE 1. Immunodiffusion of ADH variants from Drosophila. Antibody preparations against ADH-F and ADH-71k were placed in the center wells of A and B, respectively. The outer wells contained aliquots of crude adult extracts of (1) $Adh^{S/S}$, (2), *simst*, (A,3) sim (M), (B,3) $bAdh^{n4}$, (4), $Adh^{71k/71k}$, (5), $Adh^{F/F}$, and (6), $Adh^{F/F}$ (USA).

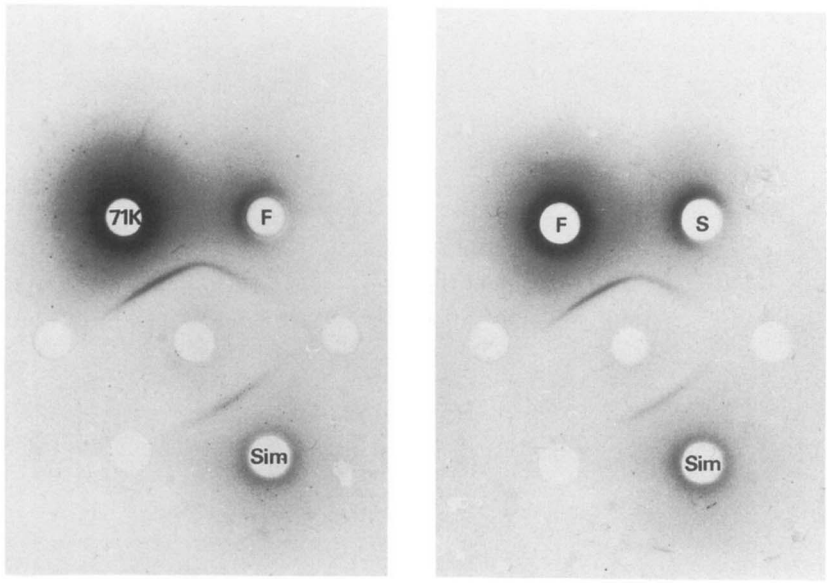

FIGURE 2. Immunodiffusion of ADH variants from Drosophila. An antibody preparation against ADH-71k was placed in the center wells. The outer wells contained aliquots of purified protein of ADH-71k, ADH-F, ADH-S, and *simulans*-ADH (*st*).

TABLE 1

Relative quantities of ADH in *D. melanogaster* and *D. simulans*

Stage	*Adh* genotypes							
	71k/71k	*F/F*	*F/F* (USA)	*S/S*	*simst*	*sim* (M)	*n2*	*n4*
Larvae	100	73	69	44	64	58	4	0
Adults	100	69	68	33	22	21	ND	0
Ratio	1.00	1.06	1.01	1.33	2.91	2.76		
Ratio in larvae								
Control/18 hr 5% ethanol	0.99	0.99	1.00	0.97	0.95	0.98		
Control/2 hr 10% ethanol	1.00	ND	ND	ND	ND	0.99		
Control/2 hr 4% propan-2-ol	1.00	ND	ND	ND	ND	1.02		

The relative quantities of the ADH protein variants were determined by radial immunodiffusion. Larvae were of mid third-instar, and adults were males of 6 days (± 12 hr). Values represent averages of at least six (for control conditions), and three (for ratio control/substance exposure in time) independent analyses (standard errors <5% of averages). ND, not determined.

could interfere with our experiments on alcohol metabolism, we have studied such potential modulations in the *Adh* genotypes during the short-term exposure to alcohols. Radial immunodiffusion runs performed on control larvae and larvae exposed to ethanol or propan-2-ol did not reveal effects on ADH quantity (Table 1).

Metabolic studies

Feeding rates of the larvae: After oxidation of secondary alcohols into ketones no further oxidation is possible. Only a step in the reverse direction can then occur (HEINSTRA *et al.* 1986a,b). If ketones are administered, a similar situation can be anticipated. If

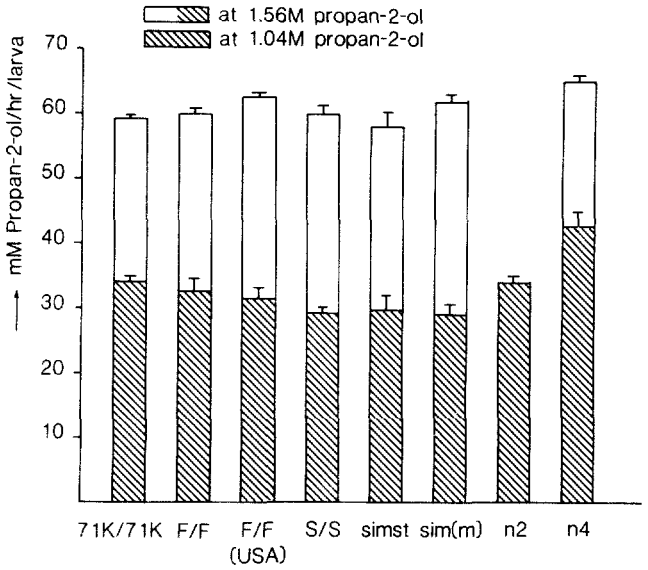

FIGURE 3.—Sum of acetone and propan-2-ol after larvae were exposed to 1.04 and 1.56 M propan-2-ol for 60 min. Evaluation of this total propan-2-ol intake per hour has been given in Table 2. Bars indicate standard deviations ($n \geq 4$).

TABLE 2

Comparison of regression lines of total acetone and propan-2-ol intake over all the *Adh* genotypes

	Acetone intake
Number of lines = 8	F_8^2-slope = 0.114, nonsignificant
Points of time = 3	F_{15}^7-height = 0.429, nonsignificant
	Propan-2-ol intake
Number of lines = 7	F_7^6-slope = 0.075, nonsignificant
Points of time = 3	F_{13}^6-height = 0.479, nonsignificant

Independent variable of time versus dependent variable of the sum of *in vivo* acetone and *in vivo* propan-2-ol concentration after feeding acetone (Figure 4) or propan-2-ol (Figure 3) were statistically evaluated according to box 14.8 and 14.10 of SOKAL and ROHLF (1981).

larvae of the different *Adh* genotypes have similar feeding rates, as was found earlier for adults (HEINSTRA *et al.* 1986b), we would expect that the sum of endogenous secondary alcohol and its ketone would be similar. When larvae were exposed to two different propan-2-ol concentrations, nonsignificant differences between the *Adh* genotypes in the sum of propan-2-ol and acetone have been found (Figure 3 and Table 2). When larvae were exposed to acetone during a two hour period, the sum of *in vivo* acetone and propan-2-ol also showed nonsignificant differences between larval *Adh* genotypes (Figure 4 and Table 2). These two experiments strongly suggest that intake of substances through active feeding of the different larval genotypes was very similar.

Metabolism and elimination of substances: When larvae were exposed to acetone, propan-2-ol was found after 1 hr in both ADH-negative strains (Figure 4). Propan-2-ol eventually also appeared in the other strains in a pattern which was specific for each *Adh*

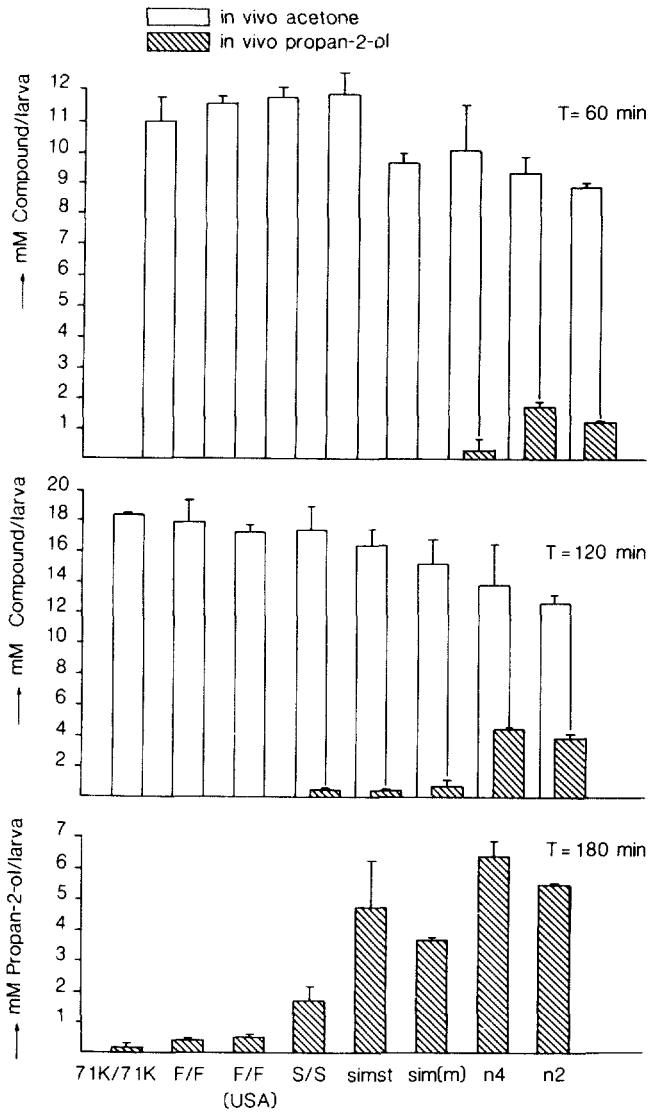

FIGURE 4.—Acetone and propan-2-ol (in mM/larva) in larvae after exposure for the indicated time (T) to 68 mM (=0.5% v/v) acetone. The sum of acetone and propan-2-ol which should reflect the total acetone intake has been evaluated in Table 2. Bars indicate standard deviations ($n \geq 4$).

genotype (Figure 4). These findings may be explained as follows. Apparently, ADH is not involved in the reduction of acetone as is shown in the ADH-negative genotypes. The enzyme responsible may be an aldo-keto reductase (ATRIAN and GONZÀLEZ-DUARTE 1985). However, the oxidation of the propan-2-ol produced can be performed by ADH in the ADH-positive strains (see also below). Because after some period acetone inhibits ADH activity [HEINSTRA *et al.* (1986a) and references therein], propan-2-ol will also accumulate in the latter genotypes. At any rate, exposure to an alcohol (see below) or to its oxidized product gives different metabolic consequences involving different enzymes. Such different metabolic consequences should be considered when, for instance, individuals are exposed to ethanol or to acet-

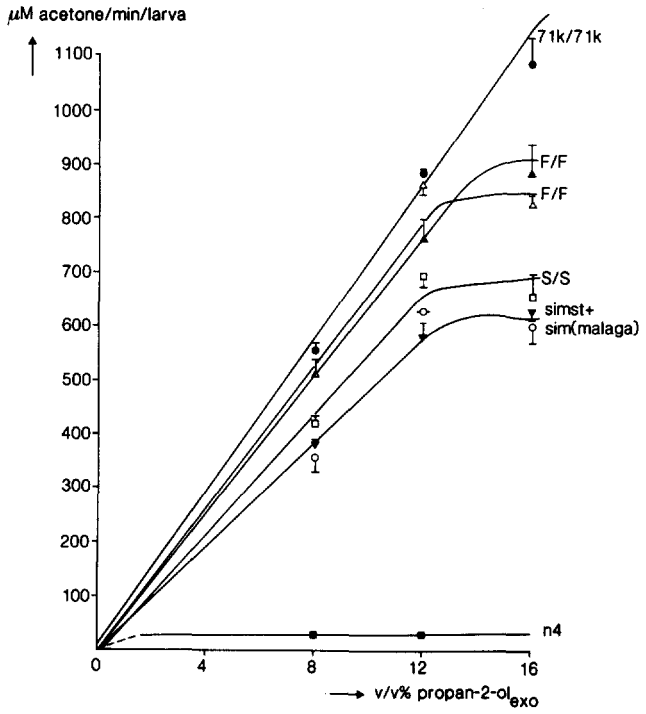

FIGURE 5.—Acetone formation (ordinate) at the different exogenous levels of propan-2-ol (abscissa) in the *Adh* genotypes. Larvae were exposed to propan-2-ol for 30 min to minimize nonmetabolic loss of compounds and to prevent an inhibitory effect of acetone on ADH action ($n \geq 4$). Bars indicate standard deviations (▲ = $Adh^{F/F}$, USA).

aldehyde (*e.g.*, DAVID, DALY and VAN HERREWEGE 1984).

Increasing external concentrations of propan-2-ol caused increased production of acetone until maximum levels which differed considerably between the different ADH-active genotypes (Figure 5). When maximum levels were attained a pathway in which ADH was not involved was responsible for about 3.5% of the acetone production (comparison of Adh^{n4} with $Adh^{71k/71k}$ and *D. simulans*). This shows that the different ADH variants exhibit a different maximum ability to oxidize propan-2-ol. A summary of their relative maximum *in vivo* oxidation rates is given in Table 8.

In contrast to secondary alcohols and ketones, primary alcohols can be utilized in metabolism (DAVID *et al.* 1981). During exposure to primary alcohols, their concentration in the larvae will reflect the difference between intake and elimination. When larvae were exposed to 5% ethanol, a large linear increase of ethanol in time was found in the two ADH-negative strains (Table 3), indicating that elimination could not cope with intake. On the other hand, genotypes with ADH activity kept internal ethanol levels low, so rates of intake and elimination are balanced. A similar picture has been obtained during exposure to 4% propanol-1 (Table 4). In both ADH-negative strains, viability of the larvae decreased as a consequence of propanol-1 toxicity. In both the ADH-negative and ADH-positive strains, we did not detect acetaldehyde and propanal during catabolism of ethanol and propanol-1, respectively. At those moderate levels of ethanol, the elimination rates could reasonably cope with intake in genotypes with ADH activity.

If external ethanol concentrations are raised, we postulated that intake would exceed elimination in the ADH-positive genotypes, too. When larvae were exposed to 10% ethanol, the ethanol elimination could not cope with intake, because all genotypes showed a linear increase in ethanol accumulation (Figure 6). This suggests that the enzyme-system(s) responsible for ethanol elimination were saturated. The potential of the different ADH variants to eliminate ethanol may now be assessed by using the differences in accumulation between the ADH-negative and ADH-positive genotypes. This gives a value for ADH-71k of 57.7 mM ethanol elimination/hr/larva and for ADH-F of 53.4 mM ethanol elimination/hr/larva; in the other experiment, ADH-F (USA) a value of 46 mM ethanol elimination/hr/larva, ADH-S of 37.1 mM ethanol elimination/hr/larva, and *simulans*-ADH a value of 36.5 mM ethanol elimination/hr/larva (see Table 8 for relative values). The differences between the *Adh* genotypes in the rate of ethanol accumulation are statistically evaluated in Table 5. Among genotypes, slopes are always significantly different. Between the Adh^{71k} and Adh^{F} homozygotes (Europe), slopes are close to a significant difference. For the experiment with the other ADH-positive genotypes, significantly different slopes occurred between Adh^{F} and Adh^{S} homozygotes on the one hand, and between $Adh^{F/F}$ and *D. simulans*, on the other (Table 5). Slopes for $Adh^{S/S}$ and *D. simulans* are nonsignificantly different.

The ethanol elimination reflects metabolic utilization and nonmetabolic loss. In the case of propan-2-ol oxidation into acetone we can monitor the fate of both substances. This cannot be done directly with the oxidation of primary alcohols. Therefore, the rate of non-metabolic loss has been assessed using the one-step conversion of propan-2-ol (Figure 7) as an estimate of the rate of nonmetabolic loss of ethanol. At a moderate concentration of propan-2-ol, the acetone produced in the $Adh^{71k/71k}$ genotype remained in the body. In the ADH-negative strain, the total amount of propan-2-ol which was the sum of predominantly propan-2-ol and traces of acetone also remained constant (Figure 7). These findings indicate that nonmetabolic loss was negligible under the moderate levels of these substances. At a higher concentration of propan-2-ol, the ADH-negative larvae showed a linear decline of propan-2-ol which represents a nonmetabolic loss of about 4 mM propan-2-ol/hr/larva. At a high ethanol concentration, elimination constituted about 7.8 mM ethanol/hr/larva which should involve both metabolic utilization (non-ADH mediated) and

TABLE 3

Ethanol levels in larvae of the different *Adh* genotypes

Genotype	Exposure time in hr			
	1	2	3	18
71k/71k	2.78 ± 0.55	1.89 ± 0.05	2.54 ± 0.31	2.23 ± 0.00
F/F	4.45 ± 0.30	4.36 ± 0.09	4.17 ± 0.83	4.01 ± 0.26
F/F (USA)	6.38 ± 0.30	5.63 ± 1.29	4.92 ± 1.03	5.19 ± 2.23
S/S	11.69 ± 0.15	9.83 ± 1.67	10.91 ± 0.67	13.73 ± 2.22
simst	ND	ND	ND	15.58 ± 4.08
sim (M)	7.73 ± 0.77	11.08 ± 0.95	17.31 ± 3.61	ND
n4	37.19 ± 2.50	67.34 ± 2.41	92.98 ± 6.30	—[a]
n2	38.40 ± 0.93	70.30 ± 0.19	98.56 ± 7.68	—

Levels ± standard deviation (in mM/larva) are based on at least four independent analyses after larvae were exposed to a 868 mM (= 5% v/v) ethanol solution. Regression analysis for *n4*, $y = 3.0 + 30.9x$, $r = 0.997$ and for *n2*, $y = 2.7 + 32.8x$, $r = 0.998$. ND, not determined.
[a] All larvae had died.

TABLE 4

Propanol-1 levels in larvae of the different *Adh* genotypes

Genotype	Exposure time in hr		
	1	2	3
71k/71k	0.78 ± 0.00	0.78 ± 0.00	1.18 ± 0.00
F/F	1.37 ± 0.15	1.37 ± 0.18	3.40 ± 1.03
F/F (USA)	2.08 ± 0.47	2.48 ± 0.73	3.72 ± 0.20
S/S	3.42 ± 0.81	3.92 ± 0.84	4.31 ± 1.17
simst	4.31 ± 0.00	5.09 ± 0.39	5.29 ± 0.58
sim (M)	4.63 ± 0.33	5.87 ± 0.48	5.88 ± 0.00
n4	17.64 ± 1.15	35.97 ± 0.69	—[a]
n2	18.69 ± 0.67	31.58 ± 3.16	—

Levels ± standard deviation (in mM/larva) are based on at least four independent analyses after larvae were exposed to a 532 mM (= 4% v/v) propanol-1 solution. Regression analysis for *n4*, $y = -0.1 + 18.0x$, r = 0.999, and *n2*, $y = 0.9 + 15.8x$, $r = 0.994$.
[a] Larvae became sluggish, leading to death.

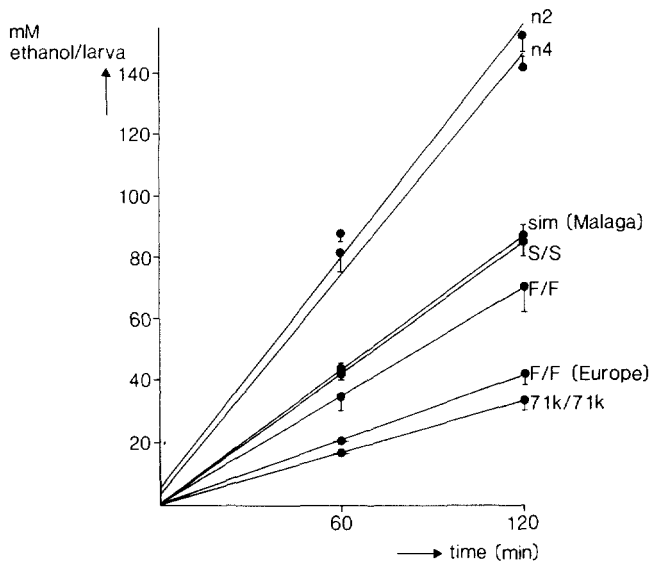

FIGURE 6.—Increasing levels of ethanol detected in time after larvae were exposed to 10% (v/v) ethanol ($n \geq 4$, and $r > 0.996$ for all lines). Bars indicate standard deviations. Experiments on *n4*, $Adh^{71k/71k}$ and $Adh^{F/F}$ (Europe) were performed separately from the other *Adh* genotypes.

TABLE 5

Comparison of regression lines of differences in slope of ethanol levels between *Adh* genotypes

Between *n4*, *71k*, and *F* (Europe)	$F_{s(2,3)} = 74.9634$	$P < 0.025$
Between *71k* and *F* (Europe)	$F_{s(1,2)} = 147.6900$	$0.05 < P < 0.1$
Between *n2*, *sim* (M), *S*, and *F* (USA)	$F_{s(3,4)} = 32.3594$	$P < 0.01$
Between *S* and *F* (USA)	$F_{s(1,2)} = 245.9557$	$P < 0.05$
Between *sim* (M) and *F* (USA)	$F_{s(1,2)} = 243.4290$	$P < 0.05$
Between *sim* (M) and *S*	$F_{s(1,2)} = 2.5864$	NS
Between *n2* and *n4*	$F_{s(1,2)} = 0.3405$	NS

Statistical evaluation was done according to box 14.8, 14.10 of SOKAL and ROHLF (1981). Data were taken from Figure 6.

non-metabolic loss. So, at least (7.8−4=) 3.8 mM ethanol elimination per hr/larva can be attributed to a non-ADH mediated pathway. In the $Adh^{F/F}$ larvae under 10% ethanol conditions, total ethanol elimination per hour was found to be on the average 50 mM (see above). So, a non-ADH mediated pathway in ethanol catabolism may account for approximately 7.5% (3.8/50 × 100%) of the total elimination. Almost an identical value was derived from radioactive tracer studies in Canton-S ($Adh^{F/F}$) larvae (GEER, LANGEVIN and MCKECHNIE 1985).

Intake of different substances: The low contribution of a non-ADH mediated pathway in alcohol metabolism allows a further comparison of the intake rates by the different *Adh* genotypes. For ADH-negative larvae submitted to 5% external ethanol this reveals 3.9% intake per hr interval (Table 3), and about 4.2% at 10% external ethanol (Figure 6). At varying external propan-2-ol concentrations for all genotypes an average of 3.8% intake per hr interval is found (Figure 3). For the *n2* genotype this is 3.8% for propan-2-ol, 3.7% for ethanol and 3.8% for the higher concentration of propan-2-ol (Figure 7). For both ADH-negative strains at 4% external propanol-1, approximately 3.4% intake per hr is produced (Table 4). A lower intake can be found for the $Adh^{71k/71k}$ genotype at low external propan-2-ol of

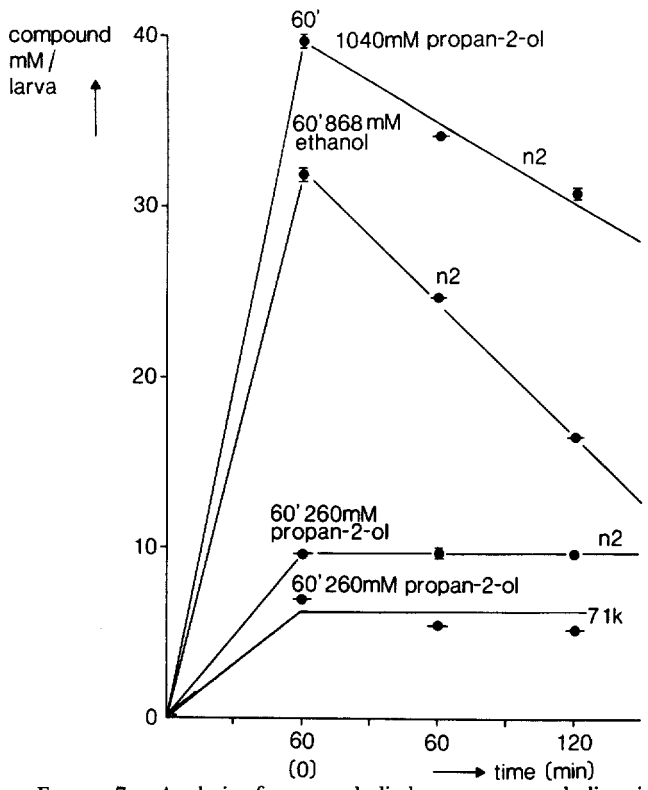

FIGURE 7.—Analysis of nonmetabolic loss versus metabolic utilization in two *Adh* genotypes. Larvae were exposed for 60 min to the indicated compounds. Thereafter, larvae were transferred to Petri dishes containing 1% (w/v) agarose medium. For an additional 60 and 120 min period, larvae were allowed to move freely on this medium in glass-covered dishes (n = 3). Bars indicate standard deviations.

approximately 2.7% per hr (Figure 7). Excluding the latter date, an average of 3.8% ± 0.2% has been deduced. This average value for larval intake is about 3.5 times higher than intake of vapors by adults (HEINSTRA *et al.* 1986b), and supports our conclusion that higher effective levels of alcohols are reached in the larval body. However, the intake of acetone is for all genotypes approximately four times higher compared with the alcohol substances. Identical higher values of acetone intake were found when adults were exposed to acetone vapors (P. HEINSTRA, unpublished results). The reason for this remains puzzling.

Biochemical studies

The fresh larval extracts showed three ADH-isozyme activity zones after electrophoresis. The ADH-5 isozyme band was stronger in ADH-S compared with ADH-71k and *simulans*-ADH (Table 6). For the ADH-3 isozyme bands, no significant differences between the ADH variants have been found. The ADH-1 isozyme band was stronger for ADH-F (Europe) compared with the ADH-71k and ADH-S. According to our hypothesis, the ADH-5 isozyme is 100% active, whereas the ADH-3 shows *in vivo* 50% and the ADH-1 100% inhibition by dead-end ternary complex formation (HEINSTRA, SCHARLOO and THÖRIG 1986). If

TABLE 6

Relative distribution of ADH-isozyme activities

Protein variant	ADH-5	ADH-3	ADH-1
ADH-71k	64.7 ± 0.1	31.1 ± 1.3	2.8 ± 0.1
ADH-F (Europe)	62.1 ± 3.8	29.5 ± 5.1	8.4 ± 1.3**
ADH-S (USA)	72.7 ± 0.9*	25.1 ± 1.5	2.2 ± 0.5
sim-ADH (M)	64.0 ± 3.9	32.4 ± 4.0	3.6 ± 0.1
sim-ADH (*st*)	61.9 ± 0.8	29.7 ± 0.3	8.1 ± 1.8

Data are based on two replicates of gel activity scannings from two independent extracts of mid third-instar larvae. The contribution of each ADH-isozyme activity is given as part of total activity of the three isozymes together for each separate ADH protein variant.

* Significantly different from ADH-71k and sim-ADH (*st*), $P < 0.01$.

** Significantly different from ADH-71k and ADH-S, $P < 0.05$.

TABLE 7

Larval-to-adult survival of *Adh* genotypes on ethanol containing media

Genotype	Percent eclosed	Total number of larvae tested
71k/71k	69.2	250
F/F	56.2	250
F/F (USA)	ND	ND
S/S	42.0	200
simst	14.7	143
sim (M)	7.3	150
n4	0.0	200

Mid third-instar larvae were placed on the MITTLER-BENNETT food medium supplemented with 10% (v/v) ethanol. The data represent the average percent of eclosed individuals from two independent experiments. Within strain error was for all strains <4%. Under control conditions, eclosion was better than 95% for all strains. ND, not determined.

applied to the ADH variants, no significant differences in percent relative active ADH molecules between the variants have been found.

Viability studies

Estimates of larval-to-adult survival on 10% (v/v) ethanol medium are given in Table 7. The general rank order of survival is $Adh^{71k/71k} > Adh^{F/F} > Adh^{S/S} > D.\ simulans$. Larvae of $bAdh^{n4}$ did not proceed to puparium formation. The viability has a negative relation with rates of ethanol accumulation (Figure 6). Both strains of *D. simulans* showed lower survival than predicted from ethanol accumulation patterns compared with $Adh^{S/S}$ larvae for example.

DISCUSSION

Intake of alcohols and related substances is an active process which depends on the feeding rates of the larvae. Once taken in, we assume a passive process of distribution of the substances in the larval body (GOLDSTEIN 1983, p. 5). The oxidation *in vivo* and elimination of the alcohols are found to be mainly mediated by ADH activity. Our question is whether

TABLE 8

Relative biochemical parameters of the ADH protein variants

Protein	Maximum oxidation rate on propan-2-ol	$[E_o]$	Ratio
ADH-71k	100	100	1.00
ADH-F	79*	73	1.08
ADH-F (USA)	82	69	1.19
ADH-S	64**	44	1.45
sim-ADH (*st*)	59***	64	0.92
sim-ADH (M)	59***	58	0.98
	Apparent maximum rate of ethanol elimination		
ADH-71k	100	100	1.00
ADH-F	93	73	1.27
ADH-F (USA)	100	100	1.00
ADH-S	81	63	1.29
sim-ADH (M)	79	84	0.94

Data on the maximum oxidation rates with propan-2-ol as substrate *in vivo* are taken from Figure 5. Data on the relative ADH quantity are taken from Table 1. Data on the apparent maximum rate of ethanol elimination are extracted from Figure 6. For further details, see text.

* Significantly different from the mean of ADH-71k, $P < 0.01$.

** Significantly different from the mean of ADH-F (USA), $P < 0.02$.

*** Significantly different from the mean of ADH-S, $P < 0.05$.

the ADH variants have contributed directly to the different survival of the respective genotypes when exposed to ethanol, and what, then, are the mechanisms involved?

One would expect that differences in survival occur when ethanol accumulates in the body above some critical concentration, *i.e.*, when the enzyme-system(s) responsible for detoxification are saturated and when intake exceeds elimination. At external concentrations of 10% ethanol, we found that elimination could not keep pace with intake. Moreover, the concentration of ethanol in the ADH-negative larvae was about 80 mM/larva after 1 hr (Figure 6). This turns out to be approximately 15 times the average *in vitro* Michaelis constant for the ADH variants [this K_m^{ethanol} is about 5.5 mM calculated from data given in ZERA, KOEHN and HALL (1985), EISSES *et al.* (1985a), and EISSES (1986)]. Also on the basis of this figure, we could expect conditions in larvae of the ADH-positive genotypes which lead to saturation of ADH by ethanol. So, it seems justified to use patterns of linear accumulation of ethanol for the determination of the apparent maximum elimination rates controlled by the ADHs in the different genotypes (Figure 6; see Table 8 for relative values).

The differences found in ethanol elimination in which ADH probably acts in the first two oxidative steps (HEINSTRA *et al.* 1983, 1986a; EISSES *et al.* 1985a; GEER, LANGEVIN and MCKECHNIE 1985; MOXOM *et al.* 1985), thus can be explained by differences in direct contribution of the ADH variants. This is also supported by similar differences between the maximum oxidation rates for propan-2-ol (Table 8). Nevertheless, the relative differences between the *Adh* genotypes in oxidation of propan-2-ol are somewhat greater than in ethanol elimination. This was found earlier *in vitro* (DAY, HILLIER and CLARKE 1974; LEWIS and GIBSON 1978; WINBERG, HOVIK and MCKINLEY-MCKEE 1985). The differences in elimination rates of ethanol between genotypes are found to be smaller than between their total ADH activities measured *in vitro* (DAY, HILLIER and CLARKE 1974; EISSES *et al.* 1985a; EISSES 1986). This suggests metabolic buffering (KACSER 1957; KACSER and BURNS 1981), but not as strong as found previously after flux studies from ethanol in adults (MIDDLETON and KACSER 1983).

What are the mechanisms behind the different contribution of the *Adh* genotypes to detoxify ethanol? Two factors will be of primary importance, (i) kinetic properties of the ADH variants and (ii) the amount of active ADH protein *in vivo*. Although small differences in primary structure may give rise to changed kinetic properties (EISSES *et al.* 1985a; WINBERG, HOVIK and MCKINLEY-MCKEE 1985), the question remains whether such differences are also manifest under physiological conditions (ZERA, KOEHN and HALL 1985). Details of this latter aspect will be given elsewhere (HEINSTRA 1987). We and others have found differences in quantity of ADH between the *Adh* genotypes. We may relate these differences in protein quantity to the differences in ADH action. If we use the ratio of *in vivo* ADH activity per larva to quantity of ADH protein, a rank order of action per enzyme molecule becomes evident, on both substances, of ADH-S > ADH-F > ADH-71k > *simulans*-ADH (Table 8). This rank order is reversed when we compare the maximum activity of the ADH allozymes in *D. melanogaster*. This suggests that either the quantity of ADH compensates for apparent differences in molecular efficiency of the ADH allozymes or this effect is related to metabolic buffering. ANDERSON and MCDONALD (1983) reported differences of *Adh* mRNA between $Adh^{S/S}$ and $Adh^{F/F}$ genotypes. This could be a consequence of linkage disequilibrium between regulatory elements and the structural parts of the *Adh* locus. Whether the differences in mRNA levels are caused by differences in transcriptional activity of the *Adh* alleles involved or by differences in stability of mRNA remains to be established.

In *D. simulans*, the lower efficiency of ADH molecules is not compensated by ADH protein quantity (Table 8). Ethanol elimination also was lower than in the $Adh^{F/F}$ genotypes of *D. melanogaster* which have similar ADH quantity, whereas the survival of *D. simulans* was also substantially lower. These findings might reflect a less efficient two-step mechanism of the ADH of *D. simulans* to oxidize ethanol into acetate

as found *in vitro* (HEINSTRA *et al.* 1983; EISSES 1986).

There are indications for other functions of the ADH in metabolic pathways (EISSES *et al.* 1985b; GEER, LANGEVIN and MCKECHNIE 1985). It has been shown that the ADH-71k variant oxidizes sarcosine (EISSES *et al.* 1985b). It has been inferred from various experiments that this oxidation by ADH-71k occurs also *in vivo* (EISSES *et al.* 1986). The ADH-71k variant has a disadvantage in wild-type strains on food media without alcohol (EISSES 1986). However, a selective advantage in combination with *Notch* mutants seems to be a consequence of its broader substrate specificity (EISSES *et al.* 1986). This interaction with *Notch* mutants shows that the effect of differences between allozymes on fitness can be dependent on the rest of the genotype. Such differences in substrate specificity show that effects on fitness from different allozyme variants (*e.g.*, ADH-71k and ADH-F) cannot be predicted from protein quantities, and kinetic properties to single substrates: substrate competition could be important under moderate concentrations *in vivo*. However, under food conditions with higher alcohol concentrations it will probably be a dominant factor.

In conclusion, we have found differences in ADH-mediated ethanol elimination in different *Adh* larval genotypes. This leads to differences in accumulation rates of ethanol which can be correlated with the survival of the *Adh* genotypes in high ethanol environments. However, the relative contribution of structural, regulatory, and metabolic buffering aspects are not yet clear.

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