BIOCHEMICAL DIFFERENCES BETWEEN PRODUCTS OF THE *Adh* LOCUS IN DROSOPHILA

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ABSTRACT

An analysis of the molecular properties of the major alcohol dehydrogenase (E.C.1.1.1.1) allozyme variants found segregating in natural populations of *D. melanogaster* is presented. Our results indicate: (1) ADH-S enzyme has generally lower Michaelis-Menten constants than those of ADH-F; (2) ADH-S and ADH-F enzymes display opposite interactions for both co-factor and substrate; and (3) higher levels of ADH are associated with the *Adh*-fast genotype. The possible adaptive significance of these findings is discussed.

THE enzyme alcohol dehydrogenase (ADH:NAD$^+$ oxidoreductase:EC 1.1.1.1.) has been found to play a key role in the ability of Drosophila to exploit alcoholic environments. Species that have high levels of alcohol dehydrogenase activity are able to tolerate greater concentrations of environmental alcohol than are species having relative low ADH activity levels (McDONALD and AVISE 1976). These interspecific findings seem to be consistent with intraspecific studies as well. In *Drosophila melanogaster*, for example, the *Adh*-fast allele is generally associated with higher ADH activity than the electrophoretically distinguishable *Adh*-slow allele (*e.g.*, McDONALD and AYALA 1978; DAY, HILLER and CLARKE 1974; VIGUE and JOHNSON 1973; HEWITT *et al.* 1974; GIBSON 1970). Population cage experiments in which ADH polymorphic populations are exposed to environmental alcohol generally undergo a significant increase in the frequency of the *Adh*-fast allele within relatively few generations (CAVENER and CLEGG 1978; VAN DELDEN, KAMPING and VAN DIJK 1975). In addition, a number of laboratories have reported that *Adh*-fast genotypes tend to survive better in alcohol stress environments than do *Adh*-slow genotypes (*e.g.*, KAMPING and VAN DELDEN 1978; AINSLEY and KITTO 1975; BRISCOE, ROBERTSON and MALPICA 1975). Collectively, these findings suggest that the structural differences between ADH-F and ADH-S protein (recently shown to involve at least one amino acid substitution; FLETCHER *et al.* 1978) in some way affects catalytic efficiency. The exact nature of this difference, however, has remained unclear (DAY, HILLER and CLARKE 1974). For example, reported estimates of K_{ethanol} measured on partially purified ADH-F extract of the major ADH-5 isozyme are generally high (~20

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mM) (DAY, HILLER and CLARKE 1974), while those measured on pure enzyme vary from 2 mM to 8 mM (VIGUE and JOHNSON 1973; ELLIOTT and KNOPP 1975).

In those few cases where comparisons were made between the ADH-F and ADH-S enzyme under the same conditions, no significant differences were reported (DAY, HILLER and CLARKE 1974; VIGUE and JOHNSON 1973). However, since the number of replicates and strains examined in these studies was small, it has been difficult to come to any general conclusion about the functional significance of the ADH polymorphism that exists in natural populations (O'BRIEN and MACINTYRE 1969).

In order to help clarify this situation, our laboratory has recently engaged in an analysis of the molecular properties of the major enzyme variants of ADH that are found segregating in natural populations of *D. melanogaster*. We report here the results of detailed kinetic and quantitative immunological studies designed to characterize the relative catalytic efficiency of Drosophila ADH.

MATERIALS AND METHODS

Strains: Five Drosophila strains made completely homozygous for their entire second and third chromosome were used in our study. The chromosomes were extracted from wild males collected at McDonald Ranch, Napa County, CA, according to previously published techniques (MCDONALD and AYALA 1978). Strains S-1 and S-2 carry electrophoretically identical *Adh*-slow alleles; strains F-1, F-2 and F-3 carry the electrophoretic *Adh*-fast allele.

Biochmeical techniques: ADH was purified to better than 90% homogeneity (MCDONALD *et al.* 1977). Protein concentrations were determined according to BRADFORD (1976). Antibody to purified ADH was prepared according to MCDONALD *et al.* (1977). The antisera's cross-reactivity with both ADH-F and ADH-S was determined by the technique of OUCHTERLONY (1953). Antiserum was used to estimate the relative amount of ADH protein by the technique of radial immunodiffusion (MANCINI, CARBONARA and HEREMANS 1965). Diameters were plotted against log concentrations (FAHEY and MCKELVEY 1965), and serial dilutions of each sample verified that a linear relationship exists (Figure 2). Amounts were estimated on 4 samples per strain. Each sample was prepared by homogenizing 30 $\pm$0.5 mg male Drosophila 5 to 10 days post-eclosion in 1 ml of 100 mM Tris-HCl, pH 8.6. Samples were spun at 10,000 rpm for 30 min and the supernatant recovered for application to immunodiffusion plates (1% agarose, 1% antibody). The average weight of flies taken from each strain was estimated by dividing the total weight of the flies taken for each sample by their number.

ADH activity was measured in crude extract according to the techniques of MCDONALD and AVISE (1976).

Michaelis constants were estimated by measuring initial velocity on 2-, 3- and 4-carbon primary alcohols over a range of 5 alcohol (1 to 20 mM) and 5 NAD (0.1 to 0.5 mM) concentrations. In no instance did substrate concentrations exceed $10 \times K_m$, nor drop below $0.1 \times K_m$. All assays were measured on a Beckman Acta III spectrophotometer at 22° in 100 mM Tris-HCl, pH 8.6; each assay was done twice and the average taken as the velocity value for that particular concentration of NAD and alcohol.

Two methods were used to calculate the Michaelis constants. Graphical estimates of primary and secondary plots were obtained by the method of FLORINI and VESTLING (1957). The reciprocals of the appropriate velocity and substrate concentrations were fit to 5 constant substrate and 5 constant co-factor lines by least squares to Lineweaver-Burke plots, using the linear regression program of the Texas Instruments model Ti-58 mini-computer. The coordinates of the intercept of every regression line with every other were calculated. For 5 lines, there are 10 such intercepts. The median value on the X axis of these 10 intercepts was taken as the best

estimate of the reciprocal of the K_m^{app}, *i.e.*, the best estimate of K_m within the range of substrate concentrations used in the experiments.

Two secondary plots were derived from the inverses of the velocities calculated from the primary plots in order to obtain K'_{alc} and K'_{NAD}, the true Michaelis-Menten constant for the substrate at infinite co-factor concentration and co-factor at infinite substrate concentration, respectively.

In addition to these graphical estimates of K_m, we have calculated the kinetic constants computationally, utilizing the initial rate analysis program of SIANO, ZYSKIND and FROMM (1975). Such computational methods are generally considered preferable to the more classical graphical approach, for they permit all of the experimental data to be analyzed simultaneously (CORNISH-BOWDEN 1976).

RESULTS

The results of our study are presented in Tables one and two. Three observations are especially relevant to the question of the possible adaptive significance of the *Adh* polymorphism that exists in natural populations.

(1) *ADH-S enzyme has generally lower Michaelis-Menten constants than those of ADH-F*: The results of our kinetic studies are presented in Table one. Six values are given for each strain and alcohol tested. K_m^{app} and K'_m are values derived graphically from the Lineweaver-Burk primary and secondary plots, respectively. K_m^{app} is a best estimate of the affinity of enzyme for substrate and co-factor over the range of substrates used in our experiments. K_m^{app} is not the true Michaelis-Menten constant, for it may fluctuate depending upon the concentration and/or time of incubation of co-factor and alcohol substrate (see below). The utility of K_m^{app} is in its comparison with K'_m, the true Michaelis-Menten constant of the reaction.

TABLE 1

Kinetic constants (mM) *for ADH purified from five Drosophila strains on 2, 3 and 4-carbon alcohols (see text for details)*

ADH	Alcohol	K_{NAD}^{app}	K'_{NAD}	K_{NAD}	K_{alc}^{app}	K'_{alc}	K_{alc}
F–1	Ethyl	0.08±0.08	0.14	0.04 ±0.002	2.61±1.00	5.67	5.20±0.06
F–2	Ethyl	0.08±0.01	0.08	0.08 ±0.001	3.92±0.50	4.29	5.62±0.03
F–3	Ethyl	0.10±0.03	0.15	0.23 ±0.007	2.72±0.97	3.32	6.69±0.06
S–1	Ethyl	0.08±0.04	0.04	0.04 ±0.001	7.31±0.67	4.46	4.02±0.03
S–2	Ethyl	0.22±0.10	0.07	0.09 ±0.001	5.58±0.51	3.07	3.44±0.01
F–1	Propyl	0.08±0.02	0.15	0.18 ±0.001	2.68±1.10	5.27	4.16±0.04
F–2	Propyl	0.10±0.03	0.10	0.10 ±0.001	1.92±0.35	2.65	2.62±0.06
F–3	Propyl	0.10±0.03	0.14	0.18 ±0.001	1.74±0.30	2.48	3.66±0.05
S–1	Propyl	0.20±0.06	0.08	0.11 ±0.002	2.63±1.00	0.89	1.03±0.02
S–2	Propyl	0.09±0.03	0.06	0.08 ±0.001	1.71±0.42	1.00	1.46±0.01
F–1	Butyl	0.09±0.02	0.21	0.21 ±0.001	1.72±0.73	4.58	4.71±0.03
F–2	Butyl	0.10±0.02	0.41	0.18 ±0.001	1.73±0.69	2.55	2.98±0.01
F–3	Butyl	0.14±0.02	0.22	0.29 ±0.005	0.69±0.04	1.48	2.18±0.04
S–1	Butyl	0.21±0.02	0.15	0.04 ±0.001	1.02±0.29	0.64	0.70±0.01
S–2	Butyl	0.16±0.02	0.12	0.15 ±0.001	1.49±0.28	0.82	1.01±0.02

If the affinity of an enzyme for substrate is dependent upon the concentration of co-factor, and/or *vice versa*, the system is said to display heterothropic interaction (FROMM 1975). In such situations, the primary Lineweaver-Burke plots will not intersect on the X-axis (Figure 1) and thus K_m^{app} values will not be equivalent to their corresponding K'_m values. A K_m^{app} value higher than its corresponding value of K_m indicates *positive interaction*, *i.e.*, a greater ease of catalysis as the concentration of substrate or co-factors rises. In such situations, the primary plots will show a decreasing K_m with increasing substrate and increasing co-factor concentration. Conversely, a K_m^{app} value lower than its corresponding value of K' is indicative of *negative interaction*, *i.e.*, there is a greater ease of catalysis as the

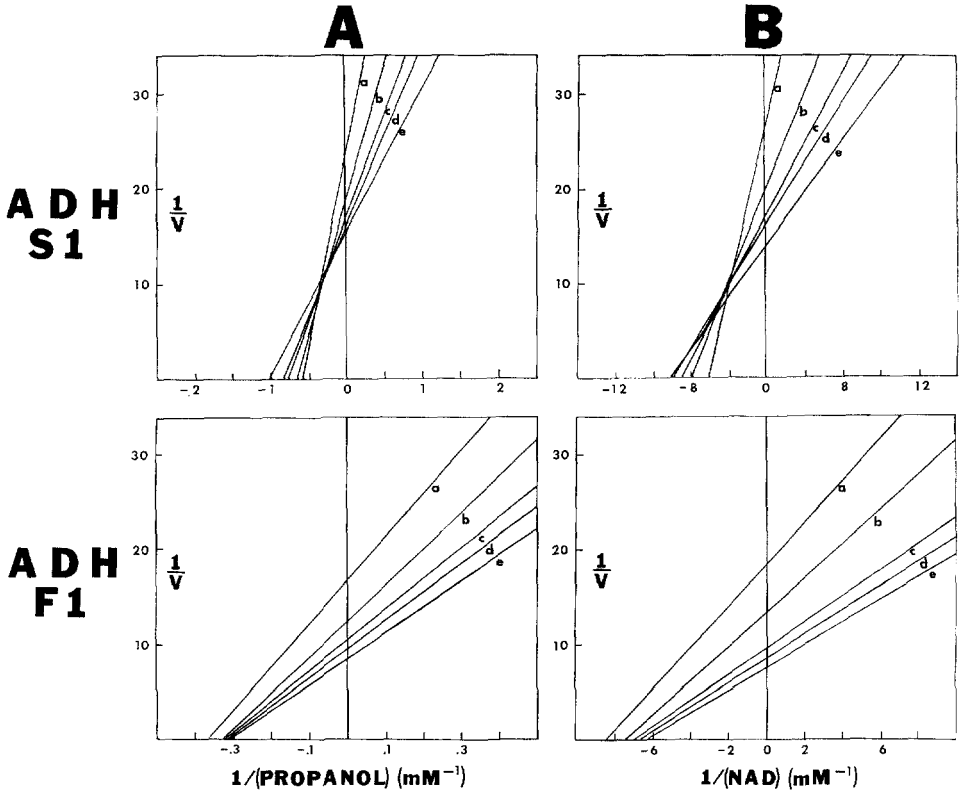

FIGURE 1.—Lineweaver-Burke plots for ADH-F and ADH-S enzyme purified from two homozygous strains of *Drosophila melanogaster*. Intersection of lines above the *X* axis is indicative of positive interaction between co-factor and substrate (ADH-S), while intersection of the lines below the *X* axis is indicative of negative interaction (ADH-F). Each line represents a separate experiment in which either (A) concentration of propanol varied and that of NAD was kept constant, or (B) concentration of NAD varied and that of propanol was kept constant. The slope of the lines decrease as the concentrations of (A) NAD, [NAD] for both F-1 and S-1: a = 1.0 mM, b = 2.0 mM, c = 3.0 mM, d = 4.0 mM, e = 5.0 mM). (B) propanol, [propanol] for S-1: a = 10.0 mM, b = 7.5 mM, c = 5.0 mM, d = 2.5 mM, e = 1.2 mM; for F-1: a = 20.0 mM, b = 15.0 mM, c = 10.0 mM, d = 5.0 mM, e = 2.5 mM, used in each experiment increases. (See text for details.)

concentration of substrate or co-factor is lowered. In this situaton, the prmary plots will show an increase K_m with decreasing substrate and decreasing co-factor concentration.

The values labeled "K_m" in Table 1 are the best estimates of the Michaelis-Menten constants of the reaction as computed by the "initial rate program" utilizing all of the data simultaneously (SIANO, ZYSKIND and FROMM 1975). The values K'_m and K_m are thus both estimates of the same constant arrived at by different numerical methods.

ADH-F enzyme has significantly higher K_{alc} values than those of ADH-S. The K_{NAD} values are also, on the average, higher for the ADH-F enzyme, but the differences are not consistent. Nonsignificantly higher $K_{ethanol}$ and K_{NAD} values for ADH-F have been reported previously (DAY, HILLER and CLARKE 1974, VIGUE and JOHNSON 1973). It should be noted that our K_{alc} values are substantially lower than some of those previously reported. At least part of the discrepancy may be due to the fact that our assays were carried out on 90 to 95% pure enzyme rather than less homogeneous preparations. It has previously been demonstrated that the degree of purity of Drosophila ADH used in kinetic analyses can significantly influence estimates of K_m (MCDONALD *et al.* 1977).

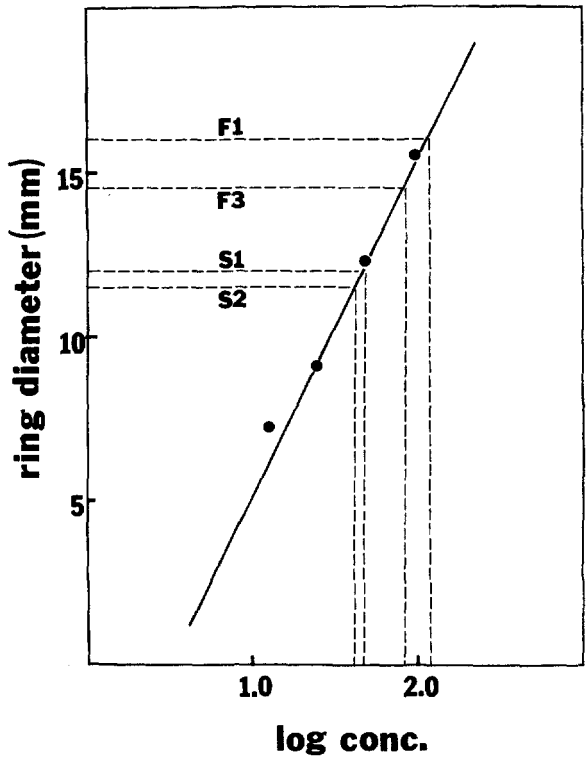

FIGURE 2.—Standard curve for radial immunodiffusion experiment. Ring diameter of samples from strains F-1, F-3; S-1, S-2 is plotted against log of relative amount of ADH present in strain F-1 (●). (See text for details.)

(2) *ADH-S and ADH-F enzymes display opposite interactions for both co-factor and substrate:* Our results indicate that ADH-S enzyme generally displays positive interaction for both substrate and co-factor, *i.e.*, as the concentration of NAD increases, values of K_{alc} go down. Likewise, as the concentration of alcohol increases, K_{NAD} values decrease. ADH-F enzyme, in contrast, displays negative interaction. As the concentration of NAD and alcohol are increased, K_{alc} and K_{NAD} values increase correspondingly (see Figure 1). Both positive and negative interactions have been observed in yeast ADH (WILLS 1976). Positive interaction was observed in horse liver ADH (NYGAARD and THEORELL 1955). Differences in the direction of interactions for ADH-F and ADH-S have not been consistently observed previously in Drosophila. However, in an ethanol affinity study of partially purified preparations of the ADH-5 isozyme of Drosophila, DAY, HILLER and CLARKE (1974) observed, on the average, positive interactions for the two fast and the two slow strains they examined. Their finding of positive interaction for ADH-F enzyme is in disagreement with the results presented here.

As mentioned previously, at least part of the discrepency between our results and those of DAY, HILLER and CLARKE (1974) may be attributable to the degree of purity of our respective enzyme preparations. Indeed, in an earlier study it was shown that the negative interactive properties of ADH-F, which were readily apparent in pure enzyme preparations, were often obscured when tests were carried out on crude extract (MCDONALD *et al.* 1977). It is interesting to note that while DAY, HILLER and CLARKE (1974) always observed positive interaction for ADH-S enzyme, the results of their ADH-F experiments were not wholly consistent and in one instance did, in fact, demonstrate the existence of negative interaction. We believe these facts caution against the determination of K_m constants on crude or partially pure enzyme preparations, especially if one is interested in detecting the existence of slight, but significant, heterotrophic interactions.

Molecular models of interaction are often envisioned to involve mechanisms of subunit interaction; however, it is known that the initial velocities of a mixture of isozymes will also give rise to cooperative-like kinetics (FROMM 1975). Since the products of both *Adh*-fast and *Adh*-slow gene variants are known to be subject to electrophoretically detectable modifications resulting from the addition of an NAD-carbonyl complex (SCHWARTZ, O'DONNELL and SOFER 1979), the isozymic mixture interpretation cannot presently be ignored. Detailed studies are presently underway in our laboratory to elucidate the basis of our observed interactions. The significant finding being reported here, however, is that the ADH-F and ADH-S enzymes we have examined consistently display opposing interactions. This means that the relative *in vivo* catalytic efficiencies of ADH-F and ADH-S may vary with respect to one another, depending upon intracellular levels of alcohol and/or NAD co-factor.

(3) *Significantly higher levels of ADH are associated with the* Adh-*fast genotype:* Table 2 presents the relative amounts of ADH present in whole flies for the two *Adh*-slow and three *Adh*-fast strains studied. In addition, relative ADH

TABLE 2

Relative ADH activities, relative amounts of ADH and average weight per fly of five homozygous strains of Drosophila melanogaster

Strain	Weight/fly (mg)	Relative activity*			Relative amount ADH†	Relative specific activity (activity/antigenicity)		
		ethyl	n-propyl	n-butyl		ethyl	n-propyl	n-butyl
S-1	0.75	0.42	0.41	0.46	0.42	1.00	0.98	1.10
S-2	0.68	0.43	0.45	0.43	0.37	1.16	1.22	1.16
F-1	0.70	1.09	1.09	1.06	1.14	0.96	0.96	0.93
F-2	0.60	1.00	1.00	1.00	1.00	1.00	1.00	1.00
F-3	0.76	0.90	0.89	0.88	0.78	1.15	1.14	1.14

* Mean activity (one unit defined as 1 μM NAD+ reduced per ml reaction mixture per min) normalized to that of strain F-2. Strain mean activity based on six replicates per strain; within strain error (S.D./mean activity) for all strains $\leq$ 10%.

† Mean amount normalized to that of strain F-2. Strain mean based on 4 replicates per strain; within strain error (S.D./mean amount) for all strains $\leq$ 5%.

activity values measured at effectively saturating concentrations of co-factor and alcohol (100 mM alcohol, 2 mM NAD) are presented. Under these assay conditions, the observed velocities should approximate the relative *in vivo* values of apparent V_{max}. *In vivo* maximum velocities are a function of per molecule catalytic efficiency and amount of enzyme present. For ease of comparison, both activities and amounts have been normalized to those of strain F-2 (Table 2).

In general, we find that the *Adh*-fast strains have significantly higher (2× to 3×) amounts of ADH per fly than the *Adh*-slow strains have (Table 2). These findings are consistent with most earlier reports (GIBSON 1972; LEWIS and GIBSON 1978). However, an analysis of the relative amounts of ADH present in 10 Drosophila strains, DAY, HILLER and CLARKE (1974) found that one of the five *Adh*-fast strains (Kaduna F24/F24) examined had ADH levels insignificantly different from those of their *Adh*-slow genotypes. This result may suggest that the degree of difference observed between *Adh*-fast and *Adh*-slow genotypes need not be universal. Nevertheless, based upon the considerable number of findings to the contrary, the abnormally low levels of ADH in the *Adh*-fast Kaduna strain must, at this stage at least, be considered an exception rather than the rule.

Our results indicate that at high concentrations of alcohol, *i.e.*, $[alcohol] >> K_{alc}$, the specific activity (activity/antigenicity) of ADH-F and ADH-S are insignificantly different (Table 2). In other words, the ADH actvity differences observed between our *Adh*-fast and *Adh*-slow genotypes at saturating concentrations of substrate are effectively the result of differences *in vivo* levels of enzyme. The qualitative differences that exist between ADH-F and ADH-S enzyme are significant only at lower substrate concentrations.

It has recently been suggested that the differences in ADH observed between Drosophila strains may be largely the result of slight differences in the weight of flies (CLARKE et al. 1979). Clearly this is not the case in our study. The average weight of the flies from the five strains used in our study are nearly identical.

In addition, the slight differences that do exist are not correlated with differences in ADH levels (Table 2).

DISCUSSION

Evidence has recently been presented that the amount of ADH present in Drosophila can be influenced by "regulatory loci" mapping to positions outside the *Adh* structural locus (McDONALD and AYALA 1978; MARONI 1978; BARNES and BIRLEY 1978; THOMPSON, ASHBURNER and WOODRUFF 1977). Although it is certainly possible that the consistently higher levels of ADH associated with the *Adh*-fast genotype are due to linkage disequilibria with high activity regulatory elements, it is equally likely that at least some of the differences in amount are due to differences in the relative *in vivo* half-life of the enzymes themselves and/or to differential rates of RNA stability or processing.

The fact that *Adh*-fast genotypes have higher maximum velocities than *Adh*-slow genotypes implies that *Adh*-fast flies are capable of higher rates of alcohol degradation at high ([alcohol] $>> K_m$) concentrations of substrate. This fact is consistent with *Adh*-fast genotypes being selectively favored in alcohol-stress environments (CAVENER and CLEGG 1978; KAMPING and VAN DELDEN 1978; BRISCOE, ROBERTSON and MALPICA 1975; AINSLEY and KITTO 1975; VAN DELDEN, KAMPING and VAN DIJK 1975; GIBSON 1970).

However, in those situations where cellular concentrations of alcohol are expected to be low ([alcohol] $\leq K_m$), for example, in low or nonalcohol-stress environments, maximum velocity would not be as functionally significant a parameter as per molecule catalytic efficiency (reflected at low alcohol concentrations by the Michaelis-Menten constant). Since K_{alc} values of ADH-S are generally lower than those of ADH-F, *Adh*-slow genotypes may be expected to have a greater substrate to product turnover rate than *Adh*-fast genotypes have when cellular concentrations of alcohol approximate K_m and concentrations of NAD are not abnormally low.

We are presently in the process of estimating *in vivo* concentrations of NAD in flies subjected to a variety of alcohol-stress and nonstress situations. However, if cellular levels of NAD in Drosophila reasonably approach those values reported for other organisms in nonstress environments (IMSANDE 1961; CHAYKIN 1967) *Adh-slow* genotypes may, in some instances, be selectively favored in situations where cellular alcohol concentrations are low. This fact could in part contribute to the maintenance of the balanced polymorphism at the *Adh* locus that exists in natural populations of *Drosophila melanogaster*.

In general, the role of ADH in alcohol adaptation is emerging as a more complex phenomenon than first envisioned. Our results suggest that the probable fitness relationship that exists between *Adh*-fast and *Adh*-slow genotypes *vis-à-vis* alcohol oxidation is not rigid, but depends upon cellular concentration of both alcohol and NAD. We have also found that at high cellular concentrations of alcohol, the relative ability of flies to degrade this substrate is largely a function of the amount of ADH they possess. Since there is a growing body of evidence that *in vivo* amounts of ADH (LAURIE-AHLBERG *et al.* 1980; McDONALD and

AYALA 1978; MARONI 1978; BARNES and BIRLEY 1978; THOMPSON, ASHBURNER and WOODRUFF 1977) and NAD (RAWLS and LUCCHESI 1974; O'BRIEN and MACINTYRE 1972) are both capable of being influenced by genetic elements mapping outside the *Adh* locus, our results underscore the importance of genetic context and interaction in the adaptive process.

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