LETTER TO THE EDITOR
ON THE BIOCHEMICAL DIFFERENCES BETWEEN ADH ALLOZYMES IN DROSOPHILA

McDONALD, ANDERSON and SANTOS (1980) reported apparent biochemical differences between the allozymes of alcohol dehydrogenase from *Drosophila melanogaster* and ascribed certain adaptive properties to the polymorphism on the basis of the specific nature of the observed biochemical differences. We believe that the authors' approach to the study of biochemical variation is an important one and that functional characterization of allozymes can provide information about the evolutionary significance of enzyme polymorphism. However, their data do not provide evidence for co-enzyme (NAD)-alcohol interactions in enzyme affinity between the allozymes, and their adaptive interpretations for the maintenance of the polymorphism fail to take into account certain data that they reported.

On the first point, McDONALD, ANDERSON and SANTOS did not consider the catalytic mechanism of ADH in their analyses. They incorrectly claim that the intersections of the family of lines above or below the abscissa in their Figure 1 constitute evidence for either a positive or negative effect of binding one substrate upon the enzymes' "affinity" for binding the other. They refer to these effects as "heterotrophic" [sic], or "cooperative-like," interactions between the co-enzyme and alcohol substrates. This inappropriate use of terminology is the source of some confusion. Heterotropic binding phenomena customarily refer to interactions between dissimilar ligands that bind to *topologically distinct sites* on the enzyme (see MONOD, WYMAN and CHANGEUX 1965, pp. 88–89). An example of a heterotropic interaction is the effect of modulator binding at an allosteric site on the binding/catalysis of substrate(s) at a catalytic site elsewhere on the enzyme. However, co-enzyme binds, as in all dehydrogenases, at the same catalytic site as the other substrate (*e.g.*, alcohol in ADH); therefore, there can be no heterotropic interactions, in the conventional use of this term. McDONALD, ANDERSON and SANTOS used this terminology in an unorthodox sense to refer to interactions between alcohol and co-enzyme substrates in their respective affinities to ADH at the *same* catalytic site. Use of "heterotropic interaction" between substrates in this sense implies that either the alcohol or co-enzyme can productively bind first to ADH, *i.e.*, that ADH possesses some kind of random bisubstrate kinetic mechanism. While the authors' Lineweaver-Burk plots (Figure 2) are certainly consistent with such a mechanism (CLELAND 1970; FROMM 1975), there are two reasons why these data are not evidence for interactions in this latter sense.

First, the authors' double-reciprocal plots are *also* consistent with a steady state, obligatorily sequential bisubstrate mechanism, where the substrates *must* bind in a specific order to achieve catalysis. This mechanism is thought to be

generally typical of dehydrogenases (DIXON and WEBB 1979; CORNISH-BOWDEN 1979; but see HANES *et al.* 1972). In this case, interactions of the sort envisioned by MCDONALD, ANDERSON and SANTOS are, of course, impossible. In fact, intersections of Lineweaver-Burke plots above, below, or on the abscissa for either random or steady-state sequential mechanisms reflect nothing more than the relative values of the dissociation constant of an enzyme-substrate complex and the corresponding Michaelis constant (K_m). These relative values are strictly a fortuitous consequence of the values of the *different* rate constants that comprise the dissociation and Michaelis constants (CLELAND 1970, p. 8; FROMM 1975, p. 73). The relative positions of the intersection points, therefore, cannot be diagnostic of interactions in either sense. While the intersection points do reflect allozyme functional differences in some respect, their significance in terms of relative contributions to catalysis cannot be evaluated on the basis of this information alone.

Second, for either random or sequential bisubstrate kinetic mechanisms, the various "K_m" differences reported by MCDONALD, ANDERSON and SANTOS do not necessarily reflect any simple relationship to substrate "affinity" or binding, contrary to the claims of the authors. For example, the K_m for the first substrate to bind (for a dehydrogenase exhibiting a steady-state sequential mechanism) is the ratio of the apparent first-order rate constant for the decomposition of the enzyme-NAD-substrate complex (the "turnover number," or k_{cat}) to the microscopic association *rate* constant of this substrate and enzyme (FROMM, p. 284). Recently, GREANEY and SOMERO (1980) reported that variation in NADH K_m values among lactate dehydrogenase homologues do not reflect differences in substrate affinity, as would be manifested in different values of this association rate constant, but instead reflect differences among the enzymes' turnover numbers. Therefore, the authors cannot equate relative K_m measures to substrate affinity in the absence of data on substrate binding. Such information may be obtained in a variety of ways. For example, co-enzyme equilibrium binding constants may be measured by taking advantage of their fluorescent properties (*e.g.*, STINSON and HOLBROOK, 1973).

MCDONALD, ANDERSON and SANTOS also claim that the ADH allozymes are significantly different in their kinetic properties. We find this assertion troublesome, because the two Michaelis constant measures that appear to estimate the same quantity (*i.e.*, K'_m and K_m) are often in poor agreement with each other (Table 1). In some cases, the differences might be accounted for by their use of unweighted least-square analyses of double reciprocal plots to estimate K_m values. Linearized data of this sort may lead to very large variances in $1/v$ at low reaction velocities (v), but to very small errors in $1/v$ at high reaction velocities. Because of this, estimating kinetic parameters from Lineweaver-Burk plots by this means is inappropriate and leads to unreliable parameter estimates. CORNISH-BOWDEN (1979) and ATKINS and NIMMO (1980) review the various criteria necessary for accurately estimating kinetic parameters and their standard errors. In addition, there exists a large interstrain variance in their Michaelis constants. Since the K_m is an intrinsic property of an enzyme, we conclude either

that this variance is an artefact of the acknowledged difficulties in enzyme purification, data collection or data analysis, or that the ADH's isolated from each strain within an electrophoretic class are actually different ADH proteins with different kinetic properties. At present, there is no way to evaluate these alternatives. It is important to identify the cause of the interstrain variance in kinetic parameters and to evaluate the apparent kinetic differences by some acceptable statistical procedure (ATKINS and NIMMO 1980).

If we neglect the foregoing points and suppose that the reported kinetic differences between the ADH-S and ADH-F allozymes approximate their properties *in vivo*, our last point concerns the adaptive significance the authors ascribe to the reported kinetic differences. The specific activities of ADH-F and ADH-S at saturating substrate concentrations, corrected for differences in enzyme protein concentration, are identical (Table 2). The authors find higher activities in ADH-F individuals, because the concentration of ADH-F allozyme is about 2.5-fold greater than that of ADH-S. The authors reasonably interpret this as the basis for selective advantage of ADH-F individuals in environments with increased alcohol concentrations. However, we disagree with the authors' contention that "... *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." This interpretation arises from their observation that the ADH-S allozyme has a generally lower K_m for alcohols than does ADH-F. Their discussion implies that NAD concentrations either are very constant or are close to saturating levels *in vivo*, so that variations in alcohol concentration largely govern ADH reaction velocities *in vivo*. There is some support for this assumption for vertebrates, where pyridine nucleotide concentrations are close to saturation (*e.g.*, TISCHLER 1977; GREANEY and SOMERO 1980). If their assumption is valid for Drosophila ADH, then the rate equations effectively reduce to:

$$v_f = \frac{V_f \cdot A}{K_m^f + A} \quad , \quad v_s = \frac{V_s \cdot A}{K_m^s + A} \quad ,$$

where A = alcohol concentration; v_f and v_s = reaction velocity for the ADH-F and ADH-S allozymes, respectively; V_f and V_s = maximal velocity at saturating alcohol concentrations for ADH-F and ADH-S; and K_m^f and K_m^s refer to alcohol Michaelis constants of ADH-F and ADH-S. The authors' interpretation specifies that there is some alcohol concentration, A_i, where $v_f = v_s$, so that $v_s > v_f$ when $A < A_i$, and $v_f > v_s$ when $A > A_i$. As A becomes low, v approaches $(V/K_m)A$. For the authors interpretation to be correct, $(V_s/K_m^s) > (V_f/K_m^f)$. However, if $V_f = 2.5\ V_s$ and $K_m^f = 1.6\ K_m^s$ for ethanol, as they reported, then this inequality does not hold. In other words, given the relative concentrations *and* reported kinetic parameter values of ADH-F and ADH-S, there is no ethanol concentration where the ADH-S allozyme will have a higher reaction velocity than ADH-F. Hence, the data of MCDONALD, ANDERSON and SANTOS suggest that ADH-S individuals can never enjoy a selective advantage on the basis of high

ethanol oxidation rates. On the other hand, the authors' interpretations do seem to apply to propanol and butanol, where $K^f_m \geq 2.5\ K^s_m$, which suggests that the kinetic properties of ADH-S allozyme may reflect adaptations to alcohols other than ethanol in a fermenting environment. However, if NAD concentrations remain relatively constant at subsaturating levels (*i.e.*, $\leq K_{NAD}$), then the allozymes' relative catalytic rates at different alcohol concentrations depend upon the relative values of the *apparent* alcohol K_m values at these lower co-enzyme levels. Consequently, evaluation of the allozymes' relative catalytic effectiveness awaits measurements of *in vivo* NAD concentrations at varying environmental alcohol concentrations, as well as of the *apparent* alcohol K_m values at these co-enzyme levels.

In summary, adaptive inferences based on *in vitro* kinetic behavior of an enzyme require an explicit statement regarding the assumed kinetic mechanism. A clear justification can then be made about how variation in certain kinetic parameters, such as K_m, would contribute to adaptive catalytic variation among allozymes. In the absence of such information, relating kinetic parameters to "substrate affinity" or "catalytic efficiency" is misleading and can lead to inaccurate assessments of catalytic function.

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