the cation is outside the channel. For the two cases, the nine water molecules inside the channel are well ordered and will give almost the same contribution, since W9/T is almost constant (see Figure 9b). Therefore, the entropy of these molecules will be small and almost the same. The ΔS contribution from the cation will be different for the two cases, because the standard deviation of the ion coordinates (STD in Table II) shows that when the cation is outside the channel, the cation is more mobile. On the other hand, the water molecules outside the channel are less mobile due to the strong solvation with the cation than those inside the channel. Thus, the entropy contributions from the water molecules outside the channel and that from the cation have opposite signs. However, since water molecules in the first and second solvation shells are strongly bound to the cation (particularly true for our simulations, owing to the simplified model), the entropy decrease of water due to the solvation of the cation is expected to be more important. In fact, when K^+ and Na^+ are solvated, the experimental solvation energies[24] of K^+ and Na^+ are reduced by 22.2 and 32.6 kJ/mol, respectively, due to the entropy effects. In our model, the upper bound of the reduction of the solvation energies due to the entropy effect can be estimated to be less than 50 kJ/mol by considering an extreme case that all the water molecules solvating the cation were frozen. Since water–water interactions along the X and Y directions were not properly considered due to an imposed hard wall, most water molecules solvating the cation oriented toward the cation, especially when the cation was outside of the channel.

Although the total energy in the asymptotic region for Na^+ has not been calculated, the internal energy activation barrier for Na^+ may be estimated to be less than 70 kJ/mol from the energy of the MC simulation calculation near $Z = 17$ Å (Figure 9c and ref 11). The free energy activation barrier will be much smaller due to the entropy effects than the internal energy activation barrier (by more than 20 or 30 kJ/mol from the aforementioned argument). Compared with experimental free energy activation barriers of 20–30 kJ/mol,[2,25,26] our result is not far from this range, but it overestimates the barrier owing to the simplified model by the following reason: (i) the bulk water characteristics outside the channel were not properly considered and (ii) these results were obtained from a rigid GA model without including phospholipids.

In order to better model the experimental system, we must first include more water by extending the boundaries to properly treat ion solvation energetics, since the energy difference between the maximum and the minimum is very different from experiment compared to the case of the activation barrier. In other words, the solvation near the minimum was exaggerated because the second solvation shell around the cation would not have proper water–water interactions owing to the limited number of water molecules used in our MC simulations. For the explanation of the first minima, i.e., experimental binding sites, the theoretical model needs to include the libration of the carbonyl oxygens 11, 13, and 15 and the hydroxyl oxygen motion of the tail. These structural changes will possibly allow the minima near $Z = \pm11$ Å. Also, the free energy difference between the maximum and the minimum might well be reduced by the following argument. If the theoretical model were to include the phospholipid interactions with GA, the energies near the estuary region will be less negative because the solvation energy of the cations will be reduced due to the phospholipid effect. If the model includes the dynamical motion of GA such as libration, bending, stretching, contraction, and vibration of GA, energy transfer among GA, cation and water, etc., then the energies inside the channel will be more negative. Therefore, in a real system, the maximum will be lowered and the minimum will be raised, resulting in a lowering of the energy difference, and this would be consistent toward the experimental value.

Work is now in progress attempting to introduce the intramolecular motions, increase the number of water molecules, and approximate the phospholipids effect. Since membranes very mobile, the latter is expected to be very important, but, also most difficult to quantitatively analyze either with laboratory or computer experiments. We note, finally, that this work has been performed by using a parallel supercomputer, 1CAP1, described elsewhere,[27] which was essential for this type of very demanding computer experiments.

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A Stereochemical Imperative in Dehydrogenases: New Data and Criteria for Evaluating Function-Based Theories in Bioorganic Chemistry

Steven A. Benner,*[1] Krishnan P. Nambiar, and Geoffrey K. Chambers

Contribution from the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and The Museum of Comparative Zoology, Harvard University, Cambridge, Massachusetts 02138. Received August 29, 1984

Abstract: We present data that show that a pair of ethanol dehydrogenases from yeast and *Drosophila* have opposite stereoselectivities. These data support the notion that the stereoselectivity in dehydrogenases reflects a "stereochemical imperative" (Nambiar et al. *J. Am. Chem. Soc.* **1983**, *105*, 5886–5890), and further weaken "historical" arguments explaining dehydrogenase stereoselectivity as a trait conserved during the divergent evolution of modern dehydrogenases from a limited number of ancestral dehydrogenases. Finally, we note that the recent challenges (Oppenheimer, N. J. *J. Am. Chem. Soc.* **1984**, *106*, 3032–3033) to our theory explaining the stereoselectivity of alcohol dehydrogenases reflect a misstatement of our thesis and overlook most available data. Furthermore, the challenge overlooks the general requirement that the physiological role of an enzyme must be "well-defined" if data from that enyzme are to be used to test a functional theory in bioorganic chemistry.

One appropriate (but often neglected) goal of bioorganic chemistry is to distinguish between those details of enzymic catalysis that are the products of natural selection and those that are not.

This distinction is vital to the interpretation of all data collected by bioorganic chemists. As natural selection is the only mechanism admitted by modern biology for obtaining functional behavior in living systems, only those details of enzymic catalysis that are largely the result of selective forces may be interpreted in terms of function. It is these details that may contain clues to general and fundamental theories concerning biological catalysis. In contrast, details that are randomly preserved historical accidents are interesting only as a catalog of natural phenomenology. They reflect history, not chemistry. Generalizations drawn from these details will apply only to systems sharing the same pedigree and need not reveal fundamental principles.

Stereochemistry is one detail of enzymic reactions that has received especially careful attention from bioorganic chemists.[2,3] Stereochemical details of enzymic reactions are interpreted variously as reflecting enzymic mechanism (with mechanism being either an "optimal" one chosen in response to selective forces or a conserved ancestral mechanism), as reflecting an optimal arrangement of functional groups in the active site (a functional interpretation that presumes selection), or as reflecting pedigree. An excellent review of these different interpretations is provided by Hanson and Rose.[2a]

One particularly prominent stereochemical problem in enzymology relates to dehydrogenases dependent on nicotinamide cofactors.[4] Dehydrogenases are known to distinguish between the diastereotopic hydrogens at the 4-position of the nicotinamide ring of NADH. Approximately half of the dehydrogenases examined transfer the *pro-R* (A) hydrogen, while half transfer the *pro-S* (B) hydrogen.[4] Because of the heterogeneity in stereochemical outcome, this stereoselectivity is often cited as an example of "randomness" in enzymology.[2,3]

However, the stereoselectivities of dehydrogenases are not truly "random"; even casual inspection of a list of dehydrogenases and their accompanying stereoselectivities[4] reveals certain patterns. Several of these patterns have been formulated as rules by Bentley.[4c] Particularly important is "Bentley's first rule",[4c] which states that all dehydrogenases acting on the same substrates have the same stereospecificity, regardless of what organism they come from.[4c] One possible (and commonly invoked) explanation of this generalization is a "historical" one; a presumption is made that all enzymes from all organisms acting on a particular substrate have descended from a single ancestral enzyme that had an arbitrary stereospecificity. Further, both the stereospecificity of this ancestral protein and its general substrate specificity are presumed to have been highly conserved during this divergent evolution.[5]

However, "historical" explanations are not the only ones consistent with Bentley's first rule. Indeed, any functional explanation for dehydrogenase stereoselectivity that focuses on the substrate and its properties also predicts that all enzymes handling the same substrate will have the same stereoselectivity.

For example, we recently proposed a theory that attributes patterns observed in dehydrogenase stereospecificity to a functional constraint on the stereoselectivity of dehydrogenases.[6] This theory was prompted by a correlation between the stereoselectivity of various alcohol dehydrogenases and the redox potential of the enzyme's natural substrate.[6] The theory is based on hypotheses

that evolutionary selection pressures have produced enzymes that both (a) conform to stereoelectronic principles[7] and (b) adjust the free energies of intermediate states to achieve catalytic optimality.[8] These hypotheses have made predictions, both stereochemical and thermodynamic, that subsequently have been verified experimentally.[6c,d]

Nevertheless, the theory has been controversial.[9] In particular, Oppenheimer[9a] recently challenged the theory, asserting that (a) three dehydrogenases that appear to violate our correlation between the stereoselectivity of dehydrogenases and the reduction potential of their substrate are critical exceptions that dissolve the correlation; (b) certain "adaptive" enzymes of microbial origin should not have been included in the same correlation; and (c) in any case, the "essential features" of our structural hypotheses were not new. Oppenheimer concluded that although "discerning ordered patterns from chaos is a noble goal for scientists, dehydrogenases remain as intractable as ever".[9]

The conflict between two interpretations of dehydrogenase stereospecificity, one based on an assumption that stereospecificity reflects a selectable function[6,10] and the other that stereospecificity reflects only "chaos", is quite general and arises regardless of the details of the functional theory.[10] However, to resolve this conflict requires careful attention not only to the details of enzymic catalysis in individual enzymes but also to how reliable the information is regarding the physiological role assigned to an enzyme. *If natural selection is the mechanism for obtaining functional behavior in living systems, we can interpret the behavior of an enzyme in terms of function only to the extent that we understand the function that the enzyme has evolved to fulfill.*

An intense discussion of stereochemistry in dehydrogenases has emerged in the last few years from a field that just a decade ago was widely believed to be expended.[6,9-11] The discussion, set in a background rich with structural and catalytic information, now makes dehydrogenases excellent systems for exploring the distinction between historical and functional explanations in bioorganic chemistry.

This article has two purposes. First, we shall point out that recent challenges[9] to our theory are based either on enzymes whose physiological roles are poorly defined or misapprehensions of our theory. Therefore, these challenges do not endanger either our functional theory or the correlation that suggested it. However, we consider these challenges to help clarify the issues involved.

Second, we present new data from experiments that are designed to help distinguish between historical and functional explanations of dehydrogenase stereospecificity. The results of these experiments support the functional interpretation in preference to the historical interpretation.

The Controversy

First, is there a correlation between stereoselectivity and redox potential in alcohol dehydrogenases? A recent challenge[9a] by Oppenheimer argues that some reasonable criteria exist for selecting alcohol dehydrogenases which, when applied, "dispel" the correlation we have proposed. This argument is incorrect.

Let us consider two strategies for constructing correlations, the first that includes all alcohol dehydrogenases, the second that includes a subset of those dehydrogenases selected to exclude those

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Function-Based Theories in Bioorganic Chemistry

J. Am. Chem. Soc., Vol. 107, No. 19, 1985 5515

enzymes with poorly defined evolutionary roles. No matter which strategy is chosen, a correlation between redox potential and stereoselectivity exists.

A correlation of the first type, that includes all dehydrogenases listed under catalog numbers EC 1.1.1 with no critical evaluation of their mechanism, physiological role, or evolutionary position, comprises 130 enzymes in 68 classes. Of these, at least 120 appear to fit our correlation. Five do not.[12] If we consider all dehydrogenases, a good relationship between stereochemistry and the redox potential of the substrate in alcohol dehydrogenases is seen.

Any argument to the contrary ignores most of the available data. For example, Oppenheimer identifies one enzyme in the Enzyme Commission's class EC 1.1.1.50 (3α-hydroxysteroid dehydrogenase), and notes that its stereochemical preference (*pro-R*) formally violates our correlation. However, he mentions only one of four enzymes listed in this class. The three not mentioned fit the correlation.[4] Likewise, stereospecificities are known for 13 steroid dehydrogenases in eight Enzyme Commission classes. Oppenheimer[9] cites three (the hydroxysteroid dehydrogenase mentioned above and two others) that formally violate our correlation; the 10 not mentioned fit the correlation.[4] Alternatively, there are 19 enzymes isolated from liver in 18 classes; Oppenheimer[9] mentions the one (the hydroxysteroid dehydrogenase mentioned above) that formally violates the correlation; the 18 not mentioned fit the correlation.[4] With use of the first strategy, a good correlation between stereochemistry and the redox potential of the substrate in alcohol dehydrogenases is an evident fact.

However, the central thesis of our previous papers[6] is that evolutionary selection pressures have produced dehydrogenases that are optimally adapted to catalyze specific reactions on specific natural substrates.[6] Therefore, only enzymes where these specifics are well defined are useful as critical tests of our functional theory, as these are the only enzymes where the information is available that is needed for the theory to make predictions.

Therefore, as stated in previous papers,[6] we have attempted to follow the second strategy in constructing our correlation, trying to include only those enzymes for which the natural substrate is "well-defined".[6c] As the substrate reported in the literature need not be the evolutionarily relevant one, some critical evaluation of the literature is appropriate before including any particular enzyme in the correlation. While such an evaluation is difficult to make, the extent to which the properties of a single enzyme can force the rejection of a functional theory in bioorganic chemistry depends, in part, on the strength of the argument assigning the physiological role.

In this light, Oppenheimer's three alleged "violations" of our correlation[9] are , in fact, three examples of enzymes where the natural substrate is disputed (1.1.1.149),[13] doubtful (1.1.1.50),[14] or misassigned (1.1.1.150).[15] While arguments can proceed indefinitely as to what the "natural substrate" is for these enzymes,

they cannot form the basis for a credible challenge to a functional theory in bioorganic chemistry.

For example, bovine EC 1.1.1.150, one of Oppenheimer's "violations" of our correlation,[9] is stated to be a 21-dehydrocortisone reductase. However, 21-dehydrocortisone is almost certainly not the evolutionarily relevant natural substrate for this enzyme. This compound was first synthesized in 1952 at Merck,[16] it has never been detected in natural tissues,[17] and it has no known biological function even when administered as a drug.

The second of Oppenheimer's "violations", EC 1.1.1.50 from rat liver, is stated to be a 3α-hydroxysteroid dehydrogenase. Because of the difficulties associated with defining the physiological function of enzyme from liver, we included no enzymes from liver in our correlation, even though 18 out of 19 liver enzymes appear to fit the correlation. However, Oppenheimer asserts that the natural substrate of this particular liver enzyme is "well-defined" because it "is specific for reduction of the 3-keto group of steroids". This assertion is simply not correct.

The enzyme from rat liver catalyzes a wide range of reactions on a broad range of substrates. With the facility with which it oxidizes 3-hydroxysteroids, the enzyme also converts benzene dihydrodiol to catechol,[14a] reduces quinones to hydroquinones, and catalyzes redox reactions on phenylglyoxal, a variety of nitrobenzaldehydes and acetophenones, and chloral hydrate.[14b] In fact, the enzyme is believed to be identical with both the dihydrodiol dehydrogenase[14b] and the chloral hydrate-reducing isozyme F4[14f,g] that have been isolated independently from rat liver.

Finally, the natural substrate of the third "violation" discussed by Oppenheimer, EC 1.1.1.149, from both ovary and placenta, is also disputed.[13] Catalysis by all three enzymes is substantially slower than catalysis by analogous enzymes from other sources.[13,14d,e,15]

These enzymes do not have "well-defined" natural substrates or physiological roles; indeed, for alcohol dehydrogenases, they are among the least well understood enzymes in this respect. These are the reasons why these enzymes were not included in our original correlation, and why they cannot form the basis of a convincing challenge to our theory.

In this regard, nowhere has our thesis been severely more misstated than in Oppenheimer's argument[9] that our correlation should *exclude* microbial dehydrogenases that degrade steroids because they "are adaptive enzymes", not "merely" "fortuitous degradative enzymes" necessary for the microorganism to grow on steroids "as a sole carbon source".[9] In fact, "adaptive enzymes" are the paradigms on which our theory is based: enzymes that have adapted to become catalytically optimal, and therefore to conform to a "stereochemical imperative".[18] Enzymes from rapidly evolving bacteria that permit the bacteria to use a compound as a sole carbon source may be especially good paradigms, particularly in view of the k_{cat}/K_m values of these enzymes, which are two to three orders of magnitude higher than those of the analogous enzymes that Oppenheimer cites.[14d,e]

Finally, concerns about the novelty of our structural hypotheses[9a] appear to originate in a misstatement of them. The "essential features" of our hypotheses are neither the pucker of the reduced nicotinamide ring per se nor the correlation of the stereoselectivity of hydrogen transfer with glycosidic conformation, for which we referenced previous literature.[6] Rather, the essential feature is the proposed stereoelectronic basis for these geometric details, and the corollary that enzymes in general have evolved to conform to stereoelectronic requirements.[7] There are three places in the critical analysis where these arguments appear to

(12) 77 of these 120 enzymes have redox positions removed from the break in the correlation between *pro-R* and *pro-S* specificity. Possible exceptions are EC 1.1.1.25 (two enzymes), EC 1.1.1.50 (from rat liver, not seminal vesicle, rooster comb, or bacteria), EC 1.1.1.149, and EC 1.1.1.150. Uncertain are EC 1.1.1.86, EC 1.1.1.156, EC 1.1.1.168, and EC 1.1.1.169. You, K. *Methods Enzymol.* **1982**, *87*, 101–126.

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be misstated.[19] and the criticisms of our mechanistic hypotheses reflect these misstatements.

The other published critique of our theory[9b] is based on misapprehensions of the theory that are sufficiently apparent as to require no discussion here.[20]

Thus, the "exceptions" to the correlation that are raised by various critics of our theory[9] are, in fact, enzymes whose evolutionary role is so poorly defined as to make them inapplicable as tests of a functional theory in bioorganic chemistry.

New Data

Our theory points to a general question: Do the stereoselectivities of dehydrogenases reflect a function that has been selected by evolutionary processes, or are they vestiges of randomly preserved historical accidents? Because disagreements over the "natural substrate" of enzymes with poorly defined physiological roles can continue indefinitely without being resolved, we feel that it is best at this point to introduce new data to carry the discussion forward. We present here the results of new experiments that are designed to help distinguish between historical and functional models.

The regularities reflected in the stereoselectivities of dehydrogenases (e.g., Bentley's first rule,[4c] mentioned above) constrain historical models. To explain these regularities, a purely historical interpretation of dehydrogenase stereospecificity must presume the following: (i) there existed at least two ancestral dehydrogenases having different substrate specificities, one *pro-R* specific, the other *pro-S* specific; (ii) each ancestral enzyme must have had a defined range of substrate specificity; and (iii) the ancestral stereospecificities of these enzymes must have been conserved during the divergent evolution of each within its range of substrate specificities.[21]

Purely historical models therefore predict that (a) related dehydrogenases (whose structures have diverged from these ancestral enzymes) should have the same stereospecificites with respect to their cofactors, and (b), for members of a set of enzymes handling substrates belonging to a single structural class, the stereospecificities should all be the same.[22]

In contrast, our alternative function-based theory postulates that the divergent evolution of stereospecificity in dehydrogenases is constrained by the redox potential of the enzyme's natural substrate.[6] Our functional model predicts that (a) enzymes acting on different natural substrates having different redox potentials may have different stereospecifities even if they are related;[23] (b)

different enzymes acting on the same substrate may have different stereospecificities only if the substrate has a redox potential around the midpoint of the range of substrate redox potentials;[24] and (c) enzymes acting on substrates having redox potentials removed from this midpoint should have the same stereospecificities, regardless of pedigree.

The different predictions made by historical models and function-based models provide a basis for distinguishing between them. We present new data that are consistent with prediction (b) of our functional model and inconsistent with predictions (a) and (b) of the historical model.

We have examined the stereoselectivity of an ethanol dehydrogenase from *Drosophila melanogaster*, an enzyme that acts naturally on ethanol,[25] which appears to be under substantial evolutionary selective pressure,[26] where the natural substrate has a redox potential at the midpoint of our correlation.[27] We report

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(19) (a) Stereoelectronic structures are resonance structures, not "valence tautomers". (b) The implication that our stereoelectronic argument is invalid because the result would be a nicotinamide ring "deactivated" for hydride transfer is incorrect. If "deactivation" is intended to mean "kinetic deactivation", the implication is wrong because a resonance structure cannot produce a kinetic barrier. If "deactivation" is intended to mean thermodynamic deactivation, the implication is misplaced, as such stereoelectronic interactions will merely shift the redox potential of the nicotinamide ring. In any case, "deactivation" implies a comparison (deactivated compared to what?). As stereoelectronic effects presumably occur in solution, they need not alter the redox potential of the cofactor in the active site compared to in solution. (c) We need to make no separate assumption regarding the relative orientation of substrate and cofactor or the order of binding to derive a relationship between nucleotide conformation (syn vs. anti) and stereoselectivity (*pro-S* vs. *pro-R*). Such a relationship follows directly from the stereoelectronic argument by itself.

(20) For example, the lead criticism of our work[6c] made by You[9b] is that the lactaldehyde reductase that we studied[6c] is the same enzyme as "alcohol dehydrogenase". This assertion is made despite the fact that the enzyme was shown not to dehydrogenate alcohol.[6c] The remaining criticisms misunderstand the hypotheses we present regarding the matching of the free energies of enzyme-bound intermediates and the need to use the equilibrium constant of the microscopic redox reaction in predicting the stereospecificity of any given enzyme.

(21) Strictly, the general substrate specificity of one primordial enzyme cannot have evolved to encompass substrates within the general substrate specificity of the other. This assumption is necessary to explain the empirical generalization embodied in Bentley's first rule.[4c]

(22) Several empirical rules are consistent with this prediction; e.g., most α keto acid reductases are *pro-R* specific.[4b]

(23) If the relationship proposed by Fondy and Holohan is correct, glycerol-3-phosphate dehydrogenase (*pro-R* specific) and mitochondrial malate dehydrogenase (*pro-S* specific) may be an example of such a pair: Fondy, T. P; Holohan, P. D. *J. Theor. Biol.* **1971**, 31, 229–244. Finally, a common folding topology for the dinucleotide binding domain may be an indication of relatedness of a wide range of dehydrogenases.[5]

(24) The midpoint in the redox potential of the natural substrate is defined as $K_{eq} = [NADH][H^+][ketone]/[NAD^+][alcohol] = 1 \times 10^{-11}$ M; enzymes catalyzing reactions with this equilibrium constant are presumed to be under little selective pressure favoring one stereochemical outcome over the other.

(25) "Physiological role" concerns properties of *Drosophila* ADH that are important in the survival of the host organism. The issue is somewhat confused by the ability of ADH to oxidize long chain aliphatic alcohols and secondary alcohols. Fortunately, a large body of biological evidence allows us to address the issue of selectable function directly, without relying solely on data collected in vitro. These data suggest that the primary, and perhaps only, selected role of ADH is the oxidation of ethanol, both as a source of metabolic energy and as a mechanism for detoxifying environmental ethanol. The evidence supporting this conclusion includes the following: (a) Different species of *Drosophila* have different levels of ADH, and these levels correlate with the level of ethanol in the environment where they feed (which can be as high as 15%): McDonald, J. F.; Avise, J. C. *Biochem. Genet.* **1976**, 14, 347–355. (b) Strains of flies characterized by different ADH activities position eggs depending on their different ethanol tolerances: Hougouto, N.; Lietaert, M. C.; Libion-Mannaert, M.; Feytmans, E.; Elens, A. *Genetics (The Hague)* **1982**, 58, 121–128. (c) Strains of flies genetically adapted in laboratory selection experiments to environments high in ethanol constitutively produce increased levels of ADH: McDonald, J. F.; Chambers, G. K.; David, J.; Ayala, F. J. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, 74, 4562–4566. (d) Survival of flies with ADH fed with ethanol is substantially greater than mutant flies deficient in ADH. In contrast, there is little or no difference between survival ability of wild and ADH null mutant flies fed on long chain primary alcohols: David, J.; Bocquet, C. *Comp. Biochem. Physiol., C: Cm. Pharmacol. Toxicol.* **1976**, 54C, 71–74. (e) There is no difference in survival on secondary alcohols between ADH wild and null mutants: David, J. R.; van Herrewege, J.; de Scheemaker-Louis, M.; Pla, E. *Heredity* **1981**, 47, 263–268. (f) Flies with active ADH tolerate ethanol at very high levels; mutants deficient in ADH are sensitive to ethanol, and are killed by rather low concentrations: Grell, E. H.; Jacobson, K. b.; Murphy, J. H. *Ann. N.Y. Acad. Sci.* **1968**, 151, 441–445. Vigue, C.; Sofer, W. *Biochem. Genet.* **1976**, 14, 127. (g) Tolerance to long chain primary alcohols is not substantially different in flies with and without ADH: David, J. R.; Bocquet, C.; Arens, M. F.; Fouillet, P. *Biochem. Genet.* **1976**, 14, 989–997. (h) The apparent preference of ADH for long chain alcohols is most manifest at unphysiologically high pH's: Winberg, J. O.; Thatcher, D. R.; McKinley-McKee, J. S. *Biochim. Biophys. Acta* **1982**, 704, 7–16. (i) The reported aldehyde dehydrogenase activity with endogenous acetaldehyde is almost certainly not physiological because of its high pH optimum: Heinstra, P. W. H.; Eisses, K. Th.; Schoonen, W. G. E. J.; Aben, W.; de Winter, A. J.; van der Horst, D. J.; Marrewijk, W. J. A.; Beenakkers, A. M.; Scharloo, W.; Thorig, G. E. W. *Genetica (The Hague)* **1983**, 60, 129–137. (j) Further evidence against a physiological role of ADH as an aldehyde dehydrogenase are experiments with acetaldehyde utilization by ADH null mutants: David, J. R.; Daly, K.; van Herrewegw, J.; *Biochem. Genet.* **1984**, 22, 1015–1029. (k) A major role of ADH in pheromone response (Winberg et al., vide supra) is unlikely in view of the K_M values for the substrates involved. (l) The presence of another alcohol dehydrogenase in *Drosophila* (octanol dehydrogenase) with greater activity toward long chain aliphatic alcohols diminishes the possibility that the primary physiological function of ADH is to detoxify these compounds: Madhavan, K.; Conscience-Egli, M.; Sieber, F.; Ursprung, H. *J. Insect. Physiol.* **1973**, 19, 23–241. (m) Ethanol is by far the most abundant alcohol in the natural environment of flies; long chain primary alcohols and secondary alcohols, if detectable at all, are present in concentrations well below those that are toxic: see ref 25d above.

(26) (a) Kreitman, M. *Nature (London)* **1983**, 304, 412–417. (b) Oakeshott, J. G.; Gibson, J. B.; Anderson, P. R.; Knibb, W. R.; Anderson, D. G.; Chambers, G. K. *Evolution (Lawrence, KS)* **1982**, 36, 86–96. (c) van Delden, W. *Evol. Biol.* **1982**, 15, 187–222.

here that the enzyme is *pro-S* specific, opposite to that of an ethanol dehydrogenase from yeast,[4] which is *pro-R* specific.

Experimental Section

Alcohol dehydrogenase (ADH) was purified from a line of *Drosophila melanogaster* isogenic for the Adh^F allele,[28] isolated by chromosome extraction from a natural population from Villeurbane, France. The identity of the allele was confirmed by electrophoresis and by the specific activity and substrate specificity of the final purified product, which gave values typical for ADH-F.[29]

4'(*R*)- and 4'(*S*)-tritio-NADH were prepared as previously described[6c] and used to enzymatically reduce acetaldehyde and 2-butanone. The products, ethanol and 2-butanol, were converted to phenylurethanes, which were crystallized to constant specific activity and counted. In parallel experiments, the stereospecificity of ethanol dehydrogenase from yeast was redetermined as a control.

Results

For the *Drosophila* enzyme, the ratio of ^{3}H-specific activities of products derived from 4'(*S*)-tritio-NADH vs. 4'(*R*)-tritio-NADH was greater than 95:5; the reverse was true for products obtained with the yeast enzyme. Thus, ADH from *Drosophila* catalyzes the transfer of the *pro-S* hydrogen from NADH, a stereoselectivity opposite to that of yeast ADH.[4]

Discussion

These data confirm prediction (b) of our functional model and contradict prediction (b) of the historical model. A pair of ethanol dehydrogenases from different organisms, enzymes that catalyze a reaction where our functional theory suggests that selective pressure favoring one stereochemical outcome over the other is small,[6,24,27] have opposite stereoselectivities. The functional model has predicted a violation of Bentley's first rule[4c] that could not be predicted by historical models.

Regardless of the pedigree of this pair of enzymes, these data contradict prediction (b) of the historical model. There exists at least one set of enzymes handling substrates belonging to the same structural class where the stereospecificities ae opposite.

Furthermore, these data appear inconsistent with prediction (a) of the historical model. Jornvall et al. have noted a sequence homology between the alcohol dehydrogenases from *Drosophila* and yeast and proposed that these two enzymes are related, albeit rather distantly.[30] If their proposal is correct, these enzymes are a *related* pair of dehydrogenases that has divergently evolved to give opposite stereochemical outcomes,[31] a divergence that his-

torical models postulate does not happen.

These facts present a challenge to any purely historical model for interpreting dehydrogenase stereoselectivity. While ad hoc hypotheses may be introduced to modify purely historical models to make them consistent with these new data,[32] such hypotheses bear a price. Any mechanism for evolving two dehydrogenases with opposite stereoselectivities that act on ethanol must also be a mechanism for evolving dehydrogenases having opposite stereoselectivities that act on other substrates as well. Thus, models based on these ad hoc hypotheses, while perhaps explaining the violation of Bentley's first rule in ethanol dehydrogenases in historical terms, will find it difficult to explain the fact that Bentley's first rule is *not* violated in dehydrogenases acting on other substrates.[32]

Clearly, many additional data are needed before the relative importance of selection and pedigree in determining the stereoselectivity in dehydrogenases is fully understood. Nevertheless, these results are important as the first attempt to formulate a general approach for distinguishing between selection and history in enzymology, a distinction that is central to bioorganic chemistry.

However, in view of recent challenges to our theory,[9] it is essential to note again that this issue, as with any other issue concerning functional theories in bioorganic chemistry, can only be resolved by examining data from enzymes with well-defined physiological roles. A specific physiological role must be assigned to an enzyme before it can be used to test any functional theory in bioorganic chemistry, and the quality of the evidence used to support this assignment in part determines the strength of the test. In this regard, enzymes from liver, enzymes with comparatively poor k_{cat} and K_M values, and enzymes whose alleged "natural substrates" have never been found in physiological tissue are certainly not good places to begin.

(27) K_{eq} = 8.0 × 10^{-12} M: Backlin, K. I. *Acta Chem. Scand.* **1958**, *12*, 1279.

(28) Chambers, G. K.; Fletcher, T. S.; Ayala, F. J. *Insect. Biochem.* **1984**, *14*, 359–368. *Drosophila* populations possess two major alleles, Adh^F and Adh^S, which encode electrophoretically separable allozymes, ADH-F and ADH-S.

(29) Chambers, G. K. *Biochem. Genet.* **1984**, *22*, 529–549.

(30) Jonvall, H.; Persson, M.; Jeffrey, J. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 4226–4230. Regions in *Drosophila* ADH tentatively assigned as part of the dinucleotide binding domain have 25–30% identity (P = 0.004). In the proposed divergent evolution of yeast and *Drosophila* ADH, the size of the subunit and the requirement for divalent metal ions have both diverged.[29] Thatcher has also proposed a sequence similarity between *Drosophila* ADH (*pro-S* specific) and pig heart mitochondrial malate dehydrogenase (*pro-R* specific): Thatcher, D. R.; Retzios, A. D. *Protides Biol. Fluids* **1980**, *28*, 157–60.

(31) The existence of such a pair is not entirely surprising. If stereoelectronic factors govern stereoselectivity in dehydrogenases,[6] the conformation of the cofactor around the nicotinamide glycosidic bond determined stereoselectivity.[5] This conformation is dictated by a small number of amino acids binding the carboxamide group of the nicotinamide on one side of the nicotinamide ring and sterically obstructing the carboxamide on the other. The former are often in the "catalytic domain", the latter often in the "dinucleotide binding domain". A naive analysis would suggest that stereospecificity is easily reversed simply by altering a few residues in both domains. More sophisticated analyses designed to show that reversal is impossible because of steric interactions between substrates and the carboxamide generally involve stereoelectronically questionable assumptions (e.g., nonlinear hydride transfer)[5] and make predictions that are not consistently verified.[33]

(32) One set of ad hoc assumptions for resurrecting a purely historical model in light of these data might follow from the work of Rossmann and co-workers[5] and suggestions of Gilbert, Rutter, and their co-workers.[34] For example, one might assume that the dinucleotide binding domain and the catalytic domain in dehydrogenases have evolved independently,[34] that only the binding domain is related in *Drosophila* and yeast ADH, that structural features of the catalytic domain alone determine the stereoselectivity of dehydrogenases, and that there were multiple ancestral catalytic domains that encompassed the general substrate specificity of primary alcohol dehydrogenases. Little evidence supports these assumptions. Yet even if correct, these ad hoc hypotheses greatly weaken historical models in general, as they do not explain why there were not also multiple ancestral catalytic domains (and hence multiple cofactor stereospecificities) in, for example, the lactate dehydrogenases, where all 14 D- and L-lactate dehydrogenases are *pro-S* specific,[4] as might be predicted by our function-based theory.[6]

(33) George, J. M.; Orr, J. C.; Renwick, A. G. C.; Carter, P.; Engel, L. L. *Bioorg. Chem.* **1973**, *2*, 140–144.

(34) (a) Gilbert, W. *Nature (London)* **1978**, *271*, 501. (b) Craik, C. S.; Rutter, W. J.; Fletterick, R. *Science (Washington, D.C.)* **1983**, *220*, 1125–1129.