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Stereospecificity in Enzymology: Its Place in Evolution

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Glossary

Adaptive trait: A behavior in a protein that arises in response to natural selection searching for behaviors that contribute optimally to the survival of a host organism

can (in principle) be structurally coupled to another (adaptive) trait. If this coupling is tight, the non-adaptive trait cannot drift without altering the adaptive trait at the same time. Such non-adaptive traits will not drift, but rather will be conserved over significant evolutionary time.

It is impossible to interpret enzymatic behavior without knowing whether it is adaptive, non-adaptive/conserved, or drifting. As natural selection is the only mechanism for obtaining functional behavior in enzymes, only adaptive traits can reflect biological function and underlying chemical principles (9, 13–15). Non-adaptive/conserved traits reflect ancient historical accidents. While not interpretable in terms of chemical principles, they may be useful in reconstructing the biochemical details of ancient organisms and interrelating the pedigrees of modern organisms (15). Drifting traits reflect recent historical accidents. They are the random blots of painting on Picasso's canvas, the trivia of biochemistry, and reflect neither history, pedigree, nor chemistry.

Surprisingly little concern has been given to these issues in enzyme stereochemistry, and little tolerance is displayed by the biochemical community when these issues are raised. In enzyme stereochemistry, discussions focus almost exclusively on the technology needed to determine the stereochemical course of enzymatic reactions, and stereochemical results are generally interpreted only at the level of chemical mechanism. For example, if a methyl group is enzymatically transferred with inversion of configuration, this is viewed only as evidence for a one step mechanism. In contrast, transfer with retention is viewed as evidence for a reaction proceeding via an enzyme-methyl intermediate; in the two-step mechanism, inversion at each step produces overall net retention (16).

While such views are sound, they cannot "explain" stereospecificity in any fundamental sense and do not permit conclusions to be drawn regarding the chemical or biological significance of cryptic stereospecificity. Nor can they answer the underlying question: "So what?" Even if stereochemical diversity (for example, inversion versus retention in the example above) is correlated with mechanistic diversity (for example, one- or two-step mechanism), we still cannot say whether the choice of a particular mechanism is adaptive or not, or whether the mechanistic choices displayed by natural enzymes reveal something fundamental about biochemical behavior, reflect ancient historical accident, or are simply random.

Unfortunately, similar statements can be made about most bio-organic data. However, evolutionary interpretations that can be found in the literature remain, with only a few notable exceptions, casual. Most referees accept only the most cautious statements of the evolutionary significance of the data collected. Only rarely is evolutionary understanding the goal of the research.

Thus, as a result of intensive experimental effort we now know that a particular alkaline phosphatase from *E. coli* operates via a two-step mechanism

(17), a particular chorismate mutase catalyzes a reaction via a chair transition state (18), and that the elimination of water from fumarate catalyzed by a particular fumarase is not concerted (19). However, we cannot say whether such behaviors are adaptive, conserved, or drifting; thus, we cannot say whether they have any chemical or biological significance.

This review seeks to fill this gap for cryptic stereospecificity. Other types of enzymatic behavior are discussed elsewhere (9, 13–15). The discussion of cryptic stereochemical distinctions made by enzymes will be directed towards developing competing "functional" and "historical" models as explanations for the stereochemical results observed for each case (9).

II. INTERPRETATIONS

Like any biological trait, enzymatic stereospecificity is a product of two competing evolutionary processes, natural selection and neutral drift. To be selected, the trait must influence the ability of the host organism to survive and reproduce.

In principle, adaptive and non-adaptive stereospecificity might be distinguished by examining the stereospecificities of enzymes that are homologous (related by a common ancestor) and analogous (performing similar functions in different organisms). In homologous enzymes, non-adaptive stereospecificity is expected to drift during divergent evolution (11). In contrast, stereospecificity that performs a selected function should be conserved during divergent evolution.

In non-homologous enzymes, non-adaptive stereospecificity is expected to be similar only to the extent anticipated by random statistics. Conversely, if a set of non-homologous enzymes catalyzing the same reaction have all convergently evolved to have the same stereospecificity, this is a strong argument that the stereochemical distinction is functionally adaptive.

In practice, several factors complicate this analysis. First, enzymes catalyzing analogous reactions from different organisms are far more likely to be homologous than non-homologous. Thus, the number of non-homologous enzymes catalyzing analogous chemical processes is often insufficient to permit a statistically significant comparison for a variable (such as stereospecificity) that can adopt only two values.

Second, methods for determining homology may fail to detect distant homology that might be relevant to an understanding of the stereochemical behavior of a class of enzymes. Homology between two proteins is generally identified by sequence comparisons or immunological cross-reactivities; other approaches are demonstrably inadequate. However, the similarity between two highly divergent sequences may be insufficient to provide a statistically

convincing case for homology. This creates problems, especially since tertiary structure is more highly conserved than either sequence or immunological cross-reactivity in proteins (20). To the extent that cryptic stereospecificity depends on tertiary structure, similar stereospecificities in distantly homologous proteins would properly be explained as a result of homology (not adaptation). Because sequence similarities are inadequate to prove homology, however, the identical stereospecificities would be misinterpreted as convergent evolution, leading to the opposite conclusion that they are adaptive.

Third, non-functional traits can be constrained from drifting simply by being coupled structurally to selected traits *(vide supra)* (9). Thus, a cryptic stereochemical distinction might be highly conserved in divergent evolution, even though it itself is not adaptive, simply because it is tightly coupled to another adaptive trait. Thus, not all conserved traits need be directly functional.

Fourth, when enzymes catalyzing analogous reactions in two different organisms have opposite stereospecificities, it is possible that this divergence does *not* mean that the stereospecificity is neutral but rather may reflect functional adaptation in the two proteins for two different environments.

These reservations make single items of stereochemical data difficult enough to interpret. However, another obstacle to the development of an understanding of the evolutionary significance of cryptic stereospecificity is the attitude of biological chemists themselves. To some, cryptic stereochemical distinctions appear to be too "subtle" to influence the survival of a host organism. To others, the fact that stereochemical diversity correlates with mechanistic diversity, and the presumption that mechanistic diversity must be important are sufficient to conclude that such stereochemical diversity must be adaptive.

For example, cryptic stereospecificity is often the same in homologous enzymes. Its subtlety prompts the belief that it is not adaptive. Thus, the dogma in some quarters is that cryptic stereospecificity is more highly conserved than almost any other enzymatic behavior because it is tightly coupled to tertiary structure in a protein (21). Stereospecificity presumably cannot be reversed without altering tertiary structure and, presumably, destroying catalytic activity or some other selected behavior.

There is remarkably little basis in fact for this opinion. First, as a special example of substrate specificity, stereospecificity is expected to diverge at a rate similar to that for the divergence of substrate specificity in general (9). This rate is rapid, although some of the rate can undoubtedly be ascribed to positive selection for new functions.

Further, there are several examples where a modest change in substrate structure or enzyme structure changes the stereospecificity of an enzymatic reaction. For example, the cryptic stereospecificity of citrate synthase from

Clostridium acidi-urici is reportedly reversed upon exposure of the enzyme to oxidizing conditions (Figure 2) (22). Data are inadequate to rule out other explanations for the observation; in particular, it is possible that this organism has *two* isozymes of the enzyme with opposite stereospecificity, and that the more abundant enzyme is more sensitive to destruction by oxygen. The clear implication, however, is that enzymatic stereospecificity can be reversed with only small changes in the structure of the protein.

Stereospecificity in an enzymatic reaction can certainly be altered by small changes in substrate structure. The stereochemical orientation of substrate and cofactor in dihydrofolate reductase is reversed upon binding of methotrexate, a close structural analog of dihydrofolate (Figure 3) (23). Likewise, acetoacetate decarboxylase from *Clostridium acetobutylicum* decarboxylates 2-methylacetoacetate with 98% retention of configuration (Figure 4, top) (24). In contrast, decarboxylation of acetoacetate proceeds essentially with racemization (25). The stereospecificity of chymotrypsin with respect to the chirality of the substrate is largely reversed by the substitution of an oxygen for a nitrogen (Figure 4, bottom) (26). Orcinol hydroxylase transfers the 4′-*pro-R* hydrogen of NADH with "natural" substrates. With substrate analogs, the 4′-*pro-S* hydrogen is reportedly removed in several cases (27).

If small changes in the structure of substrates can reverse stereospecificity in an enzyme, it seems possible that a small number of amino acid replacements in the protein will also reverse stereochemistry. However, there are few pertinent data. Although several examples are now known where distantly homologous proteins have opposite stereospecificity, there are no cases where proteins with greater than 50% sequence identity have opposite stereospecificity.

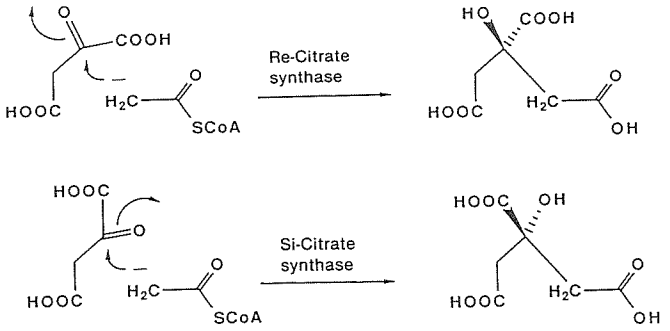
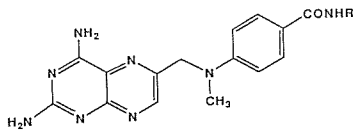
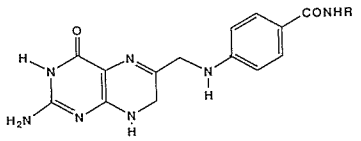


Figure 2. Alteration of stereospecificity in citrate synthase.



Methotrexate
(substrate analog)



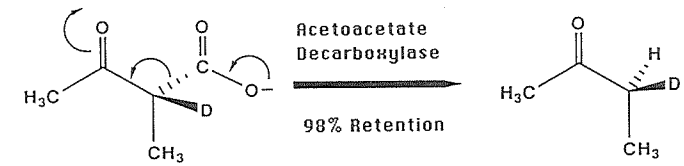
Dihydrofolate
(substrate)

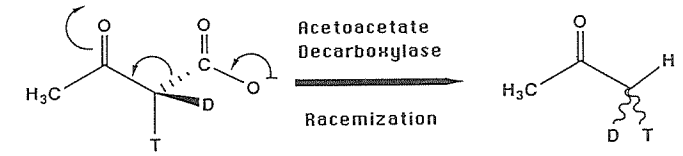
Figure 3. Alteration of stereospecificity in dihydrofolate reductase.

A. Historical and Functional Models

It is a theme of this review that conclusions about adaptation and molecular evolution based on intuition or casual analysis are largely suspect. Further, it is clear that in practice, models are never either proven or disproven. In the face of contradicting experimental data, models generally are modified in an *ad hoc* fashion to accommodate the new experimental result. While multiple *ad hoc* modification of a model at some point makes it unacceptable, taste, more than logic, determines when that point arrives.

Thus, to assess the likelihood that cryptic stereospecificity is adaptive or non-adaptive, an intellectual method is needed for systematically collecting and analyzing biochemical data. The approach that we have developed involves constructing contrasting historical and functional models (9). Experiments that are relevant to the models are then done and the models modified to accommodate the new results as they appear. The predictive and experimental value of each of the modified models becomes increasingly limited with further modification. After successive rounds of experiment and modification, the contrasting models are compared and evaluated relative to each other.





L-amide preferred
by chymotrypsin

D-ester preferred
by chymotrypsin

Figure 4. Alteration of stereospecificity by minor alteration in substrate structure.

The cryptic stereochemical outcome of an enzymatic reaction cannot in itself be a selectable trait. Thus, functional models begin with the assumption that the stereochemical distinction reflects an evolutionarily selectable mechanistic distinction in the enzymatic reaction. Identifying this distinction and explaining the basis for its selection then become the foci of the model. In many cases, functional models predict the convergence of stereospecificity in analogous, non-homologous enzymes. In cases where different stereospecificities are found in analogous enzymes from different organisms, a functional model must provide and defend arguments that explain how this behavioral difference reflects adaptation to different environments.

Historical models deny a selectable role for the stereochemical distinction. Rather, historical models explain the distribution of stereospecificities in terms of the pedigree of the enzymes involved. Historical models generally

make no predictions about the relative behaviors of non-homologous enzymes. They generally predict, however, that closely homologous enzymes display the same behavior. For more distantly related enzymes displaying similar stereospecificity, historical models must provide and defend arguments that explain why this non-functional behavior has been conserved during divergent evolution. These "conservation principles" generally must refer to functional traits that are coupled to stereospecificity, or they argue that reversal of stereospecificity requires complicated alteration of the active site.

Each model supports its own type of experimental test. The chemical basis of functional models can be examined experimentally in non-enzymatic systems. Historical models rely on assumptions regarding pedigree in modern enzymes. As the enzymes from extinct organisms generally cannot be retrieved (but see reference 28 for an exception), such assumptions are difficult to test in the laboratory. However, these assumptions can often be examined by sequencing enzymes.

In contrast with kinetic behavior in enzymes (13), where functional models are most appropriate, and the structures of cofactors, where historical models are strongest (15), neither functional nor historical models for cryptic stereospecificity enjoy a dominant position. Indeed, they both can be quite controversial. Thus, cryptic stereospecificity in enzymology is an excellent topic to illustrate the development and testing of functional and historical models in enzymology, and is proving to be valuable for defining the boundary between selected and non-selected behaviors in biological macromolecules.

B. Classes of Enzymes

Enzymes can be divided into classes based on the reaction type that they formally catalyze. In some classes, cryptic stereospecificity is different in different enzymes in the class (8). Some oxidoreductases transfer the 4′-*pro-R* hydrogen from NADH, while others transfer the 4′-*pro-S* hydrogen. Some decarboxylases, replacing a carboxylate group on their substrate with a hydrogen, retain stereochemistry at the reacting center; others invert stereochemistry at that center. Some phosphotransferases produce inversion of stereochemistry at the transferred phosphorus, while others produce retention.

In other classes, cryptic stereochemical distinctions are the same for each enzyme in the class. As far as is known, all microscopic steps in which methyl groups are transferred proceed with inversion (16). All transaminases so far studied abstract the *pro-S* hydrogen of pyridoxamine (8). All enzymatic condensations of acetyl CoA with electrophilic substrates proceed with inversion at the carbanionic center (8).

The process of model building is different for these two classes, and we discuss them separately below.

III. CLASSES SHOWING STEREOCHEMICAL HOMOGENEITY

In many cases, classes of enzymes catalyzing similar reactions have the same stereospecificities. As discussed nearly 15 years ago by Rose and Hanson (8, 29), this generalization is consistent with two alternative models.

A historical model explains stereochemical uniformity within a class of enzymes by assuming that all of the enzymes are descendants of a common ancestor with an arbitrary stereoselectivity, with stereoselectivity highly conserved in the divergent evolution of these proteins. Functional models explain the uniformity by arguing that a particular stereospecificity is needed for optimal catalysis of the reaction in question. Therefore, either by convergent evolution or by functional conservation during divergent evolution, enzymes catalyzing the mechanistically similar reactions should have the same stereospecificity.

It is extremely difficult to distinguish experimentally between these two models. Naively, the historical and the functional models make the same predictions: both predict that the "next" enzyme of the class to be studied will have the same stereospecificity as those already examined. For enzymes outside this class, historical models cannot make predictions unless pedigrees and conservation principles are clearly defined. Further, functional models are predictive only to the extent that the functional model can be applied to different reaction types.

Of course, certain experimental results might contradict either model. For example, if a functional model is constructed to be "universal" (i.e., applying to all enzymes in the class), one might examine many different enzymes in the hope of encountering one that does not conform in stereospecificity. This is conceptually simple. Most scientists (and most funding agencies), however, would find quite uninteresting a research proposal to examine 100 pyridoxal-dependent enzymes in the hope of finding one with an "aberrant" stereospecificity.

Further, a non-conforming enzyme weakens historical models as well. The historical model that accommodates a non-conforming stereospecificity must be modified *ad hoc* to include postulates that there existed more than one ancestral enzyme for the reaction, or must weaken its conservation principles to allow stereospecificity to drift during divergent evolution. Either *ad hoc* modification weakens the predictive power and testability of the model.

A. Amino Acid Decarboxylases

Models explaining the stereospecificity of enzymes catalyzing the decarboxylation of amino acids illustrate these points. These decarboxylases are known in two mechanistic classes (Figure 5). In one class, the amino acid forms a

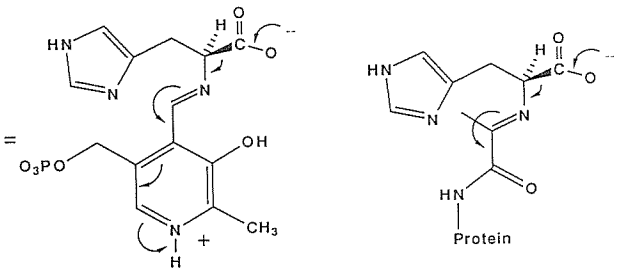


Figure 5. Cryptic stereospecificity of decarboxylases dependent on pyridoxal cofactors.

Schiff's base with a pyridoxal phosphate cofactor in the active site. In the other, the amino acid forms a Schiff's base with a pyruvyl residue covalently embedded in the enzyme's polypeptide chain. In both cases, electrons move from the carboxylate to an electron withdrawing group, and the carboxylate is replaced by a proton that ultimately comes from the solvent.

By 1979, several decarboxylases from both mechanistic classes had been examined. All were reported to catalyze decarboxylation with retention of configuration (Figure 6) (29); the proton replacing the carboxyl group was added to the same side of the molecule as the carbon dioxide departed. Enzymes from the first mechanistic class included tyrosine decarboxylase (30), glutamate decarboxylase (31), and the analogous pyridoxal-dependent serine hydroxymethylase (32). Enzymes from the second mechanistic class included the histidine decarboxylases from *Lactobacillus, Clostridium,* and mammals (33, 34).

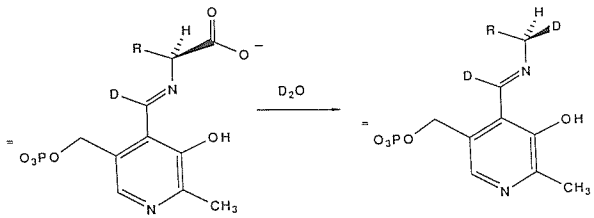


Figure 6. Decarboxylation of an L-amino acid with retention.

This uniformity was widely noted (8, 29), and discussed in terms of the stereoelectronic hypotheses of Dunathan (35). Dunathan argued that breaking of a carbon–carbon bond can occur only when the substrate is in a conformation where the bond overlaps with the pi orbitals of the Schiff's base. This argument is a functional one. It assumes that organisms containing decarboxylases that do not obey this stereoelectronic principle are less fit to survive (14).

Stereoelectronic considerations are not, however, directly relevant to the problem of explaining the choice between retention or inversion. The removal of carbon dioxide and the addition of a proton occur in separate reaction steps. The stereoelectronic argument addresses only the geometric requirements for these individual steps. In contrast, "retention" or "inversion" are overall stereochemical outcomes that depend on the relative geometries of the two steps. Reaction paths that produce either retention or inversion can be constructed so as to be equally satisfactory from a stereoelectronic point of view. Thus, whether Dunathan's hypothesis is affirmed or denied has no impact on any conclusion as to whether decarboxylation is "retentive" or "invertive" for functional or historical reasons.

In enzymes that catalyze the decarboxylation of *beta-ketoacids,* both "retentive" and "invertive" modes are known. Rose argued that this fact makes functional explanations of the stereochemical homogeneity observed in *amino acid* decarboxylases less satisfactory (29). The argument assumes that both classes of decarboxylases (those acting on beta ketoacids and those acting on amino acids) are subject to the same functional constraints, and that stereochemical heterogeneity among beta-ketoacid decarboxylases indicates that there are no functional constraints governing stereochemistry in this class of decarboxylases.

Arguments for either premise are not compelling. It is not clear why enzymes decarboxylating beta-ketoacids should be subject to the same functional constraints as enzymes decarboxylating amino acids, as the reactions proceed via rather different mechanisms. Nor is it certain that the mechanistic heterogeneity observed in beta-ketoacid decarboxylases is not itself functional.

Most authors choose not to distinguish among these possibilities, even though this choice precludes a biological interpretation of the data. For example, in their discussion of the apparent uniform stereospecificities in amino acid decarboxylases, Allen and Klinman simply listed possible interpretations without adopting one preferentially (36).

> "The possibility exists that the observed conservation is mechanism based. Alternatively, both classes of amino acid decarboxylases may have risen from a common progenitor. The final possibility is that the choice of retention versus inversion occurred once, in a random manner, for each class of enzymes."

Given stereochemical uniformity in a class of enzymes, none of the alternatives is obviously preferred. However, depending on which is correct, the significance of the data is considerably different. If the uniformity is "mechanism based," this means that the survival of the host organism is influenced by this rather subtle stereochemical distinction. If both classes of enzymes arose from a common ancestor, then drift in stereospecificity must be extremely difficult, as it has been conserved during the same divergent evolution that has seen pyridoxal replaced by pyruvyl as a cofactor. Finally, if the choice is truly random, stereoselectivity cannot be informative about either function or history, suggesting that the trait is not particularly interesting to study.

Without explicit functional or historical models, the only experimental option is to examine more enzymes. This was done in many laboratories. For example, Orr and Gould (37) examined decarboxylases for ornithine, lysine, and arginine from *E. coli,* and for lysine from *Bacillus cadaveris.* All enzymes produced retention. Allen and Klinman (36) examined the stereochemical fate of substrates in S-adenosylmethionine decarboxylase, an enzyme dependent on an active-site pyruvyl residue. The enzyme catalyzed decarboxylation with retention.

Unfortunately, enzymes were not selected for study with the goal of resolving contrasting functional and historical explanations for the uniform stereochemical behavior of these decarboxylases. First, the enzymes examined all came from eubacteria (from the genera *Escherichia, Bacillus, Streptococcus, Lactobacillus,* and *Clostridium*). Though many examples of divergent behavior in proteins can be found within eubacteria, eubacteria are only one of the three kingdoms of life (archaebacteria, eubacteria, and eukaryotes) available for bio-organic study (38). If the goal is to find examples of non-uniform stereospecificity within a class of enzymes, one is advised to examine organisms from different kingdoms and, if possible, from all three kingdoms.

Further, all of the decarboxylases studied act on the L-enantiomer of the amino acid. Several simple functional models would predict that the preferred stereochemical mode (retention or inversion) should be the same for both D- and L-amino acid. Yet enzymes acting on opposite enantiomers of a substrate are more likely to be non-homologous, or if homologous, likely to have diverged more, and therefore are more likely to have altered stereoselectivities than enzymes acting on the same enantiomers.

In this light, studies of D-amino acid decarboxylases became interesting. Soda and his coworkers examined an enzyme that decarboxylates a D-amino acid, a diaminopimelate decarboxylase from *Bacillus sphaericus.* The enzyme produces decarboxylation with *inversion* of configuration (Figure 7) (39).

We must pause for a moment to consider how this result constrains the logical form of historical or functional models that might be used to explain

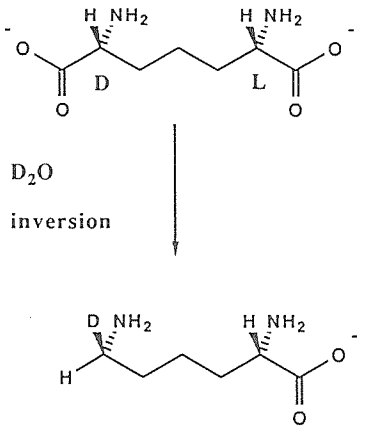


Figure 7. Decarboxylation of a D-amino acid with inversion.

it. These constraints in turn have an impact on the predictions that the models can make, and the experiments that one might do to test the models. We begin with a discussion of functional models.

The products of the enzymatic decarboxylation are the same whether the reaction proceeds with inversion or retention. Thus, the cryptic stereochemical outcome of the reaction cannot in itself be a selectable trait. Further, intermediates in the two reactions, the Schiff's base of a D-amino acid and the Schiff's base of an L-amino acid, are formally enantiomeric, if we ignore the chirality of the enzyme. (Although perhaps offensive to a stereochemical purist, we use the term "locally enantiomeric" to describe two enantiomeric species in a chiral environment where, for convenience in discussion, we overlook the surrounding chirality and the possibility of conformational diastereomerism). Thus, a functional explanation must identify a behavioral difference between the two locally enantiomeric transition states, and then correlate this with an explanation as to why retention is better for one, and inversion is better for the other. As enantiomeric species are energetically identical in an achiral environment, such a model must identify another chiral center in the environment and explain how this chiral center creates a functional difference.

First, and most obviously, the enzymic active site is "globally" chiral; this chirality might certainly be the basis of distinguishing between the two enan-

tiomeric transition states. But the fact that a chiral active site can make this distinction is not (by itself) a satisfactory functional explanation for the opposite stereospecificities. The global chirality of an active site is a variable over evolutionary time. It could have been different, and it could evolve.

Of course, the amino acid components of the proteins themselves are "intrinsically" chiral, meaning that their chirality is not an evolutionary variable. (Indeed, the use of L- instead of D-amino acids might be a prominent example of a non-adaptive trait that is nevertheless highly conserved). It is possible that inversion is functionally optimal for the decarboxylation of D-amino acids because L-amino acids are the building blocks of the polypeptide catalyst. Conversely, retention might be functionally optimal for L-amino acids because of the intrinsic chirality the L-amino acids used in the catalyst. This explanation would be the first to invoke the chirality of the amino acids building blocks of a protein to explain functionally a stereochemical course of enzymatic reactions.

Further, diaminopimelate itself has a second chiral center, and interaction with this center may (at least in principle) be the basis for a distinction between the two transition states. Possibly relevant to this notion is the fact that LL- and DD-diaminopimelates are neither substrates nor inhibitors of the enzyme, implying that the second center has some interaction with the enzyme (40).

Finally, a functional explanation might be constructed that incorporates some historical assumptions. One mode of decarboxylation (let us say, retention) might in fact be optimal for both D- and L-amino acids. However, for historical reasons, one set of amino acid decarboxylases (let us say the D-amino acid decarboxylases) may not have had the opportunity yet to evolve to produce the catalytically optimal retention. Perhaps D-amino acid decarboxylases arose only recently from L-amino acid decarboxylases and have not had time to accumulate the mutations required to become a superior retentive enzyme.

Thus, the stereochemical data from diaminopimelate decarboxylase logically constrain functional models. They must either:

a. Assume that the second chiral center in diaminopimelate is responsible for the stereochemical distinction; this center is more remote than any center so far suggested to be important in determining stereospecificity in an enzymatic reaction;
b. Assume that the chirality of the amino acids in an enzyme (the fact that, in the modern world, proteins must be made from L-amino acids) provides a functional basis for different stereospecificities in decarboxylases acting on D- and L-centers (this explanation is unprecedented);

c. Assume that a stereochemical imperative exists (for either retention or inversion), but that one of the sets of enzymes (either the D- or the L-amino acid decarboxylases) has not evolved to attain it (perhaps because not enough evolutionary time has passed); or
d. Be abandoned.

None of these options is especially attractive. Several examples are known where pairs of enzymes catalyze identical reactions via enantiomeric transition states. For example, citrate synthases are known that have opposite stereospecificities, a *Re*-citrate synthase from *Clostridium acidi-urici* (41, 42), and *Si*-citrate synthases from other organisms (43). The transition states in these two enzymes are locally enantiomeric (ignoring possible conformational differences in the transition states). Likewise, ethanol dehydrogenases from yeast and *Drosophila* have been recently shown to catalyze reactions via transition states that, except for the chirality of the NADH cofactor, appear to be locally enantiomeric (44). Thus, assumption (b) is known not to apply universally, making it less likely that it applies here.

Thus, at least in some cases, it is not obvious that the chirality intrinsic in proteins by virtue of the fact that their constituent amino acids are chiral selects for one enantiomeric transition state over another. One must note, however, that the citrate synthases mentioned above have different mechanisms, and different properties. The *Re*-specific enzyme requires Mn^{2+} and has a V_{max} of 5.5 I.U./mg, in contrast with the *Si*-enzyme, with a V_{max} of 150 and no metal requirement. Likewise, ethanol dehydrogenase from yeast requires a metal and is much faster than ethanol dehydrogenase from *Drosophila,* which requires no metal ion for catalysis. One cannot absolutely rule out the possibility that these behavioral differences are the result of a sub-optimal selection of a transition state with the incorrect chirality.

Assumption (a) might be evaluated if crystallographic data were available for the enzyme-substrate complex. Such data are almost certainly not immediately forthcoming. However, one might find support for such a hypothesis in the stereospecificities of other decarboxylases that act on substrates with two chiral centers (for example, threonine or isoleucine decarboxylases).

Assumption (c) appears to us to be the most plausible. However, a functional model that is modified to incorporate the possibility that some enzymes have not yet evolved to meet the functional "imperative" is not predictive unless an independent measure of optimality is available.

Such independent measures of optimality are conceivable. For example, one might argue that optimal enzymes have high k_{cat}/K_M values (13), and that D-amino acid decarboxylases are sub-optimal, both stereochemically and kinetically. Indeed, diaminopimelate decarboxylases appear to be kinetically rather slow. Kinetic parameters are shown in Table 1 for a number of amino

Table 1
Kinetic Parameters of Amino Acid Decarboxylases from Microorganisms

Substrate	Source	k_{cat} (sec^{-1})	K_M (mM)	$\left(\frac{k_{cat}}{K_M}\right)$ mM^{-1} sec^{-1}	Ref.
L-AMINO ACID DECARBOXYLASES USING PYRIDOXAL					
Lysine	*B. cadaveris*	86	.37	250	a
Arginine	*E. coli*	400	.65	600	b
Glutamate	*E. coli*	115	.5	230	c
D-AMINO ACID DECARBOXYLASES USING PYRIDOXAL					
Diaminopimelate	*E. coli*	7.5	1.7	4	d
Diaminopimelate	*B. sphaericus*	28	1.7	17	38
L-AMINO ACID DECARBOXYLASES USING PYRUVYL RESIDUES					
S-Aden.Met	*E. coli*	40	0.1	400	e
Histidine	*Lactobacillus*	69	0.9	75	f

[a]Soda, K.; Moriguchi, M. *Method. Enzymol.* **1971**, *17B*, 677. [b]Boeker, E. A.; Snell, E. E. *Meth. Enzymol.* **1971**, *17B*, 657. [c]Hager, L. P. *Method. Enzymol.* **1971**, *17A*, 857. [d]White, P. J. *Method. Enzymol.* **1971**, *17B*, 142. [e]Wickner, R. B.; Tabor, C. W.; Tabor, H. *Method. Enzymol.* **1971**, *17B*, 647. [f]Rosenthaler, J.; Guirard, B. M.; Chang, G. E.; Snell, E. E.; *Proc. Nat. Acad. Sci.* **1965**, *54*, 152.

acid decarboxylases; the D-amino acid decarboxylases producing inversion have considerably lower k_{cat}/K_M values than L-amino acid decarboxylases producing retention, for enzymes involving both pyridoxal and pyruvyl groups. This is consistent with the notion that amino acid decarboxylases that produce inversion have not yet evolved enough to have the catalytically favored stereospecificity.

Again, the caveat must be stated that specific activities are preferable to turnover numbers or k_{cat} as a measure of optimality in an enzyme, as the former number reflects the size of the enzyme and, presumably, its biosynthetic "cost" to the organism (13). Further, one must remain aware of the fact that differences in kinetic parameters measured *in vitro* need not reflect differences *in vivo* (13).

Thus, a single result can greatly constrain the form of a model, in this case a functional one. New experiments are suggested by these constraints. However, the same result also constrains historical models with the same productive result. In historical models, a functional role for stereospecificity is denied. The historical model must consider two possibilities: The first is that D- and L-decarboxylases are homologous, the second is that they are not.

If the enzymes are not homologous, the historical model postulates that stereospecificity arose randomly in several unrelated ancestral decarboxylases. The model builder may argue that there were three ancestral enzymes, one using pyridoxal acting on L-amino acids, the second using pyridoxal acting on D-amino acids, and the third using pyruvyl acting on L-amino acids. The first and third randomly evolved to decarboxylate with retention, the second with inversion. Again, there must be a conservation principle; once the stereochemical mode is chosen in the ancestral enzyme, it must be conserved during subsequent divergent evolution.

Alternatively, the historical model might postulate that the pyridoxal enzymes producing retention are homologous with the pyruvyl enzymes. Here, stereospecificity is presumed to diverge less rapidly than mechanism. This presumption seems implausible if the stereospecificity is assumed to serve no functional role, as replacing a pyruvyl residue by a pyridoxal residue (or *vice versa*) would seem to require more than enough rearrangement in the active site to permit stereospecificity to drift. Thus, the similar stereospecificities of the two mechanistic classes of decarboxylases probably must again be viewed as the result of accident.

Historical models that assume that "retentive" and "invertive" enzymes are homologous also deny function. The strongest conservation hypotheses then is that stereoselectivity is conserved within a class and that the kinetically most accessible path for evolving a D-decarboxylase from an L-decarboxylase will convert a "retentive" enzyme" into an "invertive" enzyme. A less restrictive conservation hypothesis argues that stereoselectivity is randomized in a divergence that leads to enzymes accepting enantiomeric substrates.

Evaluations of both historical and "historically modified" functional models (for example, models that presume that evolution towards functionally optimal stereospecificity is currently in progress) must ultimately be based on comparisons of sequence data. For example, aspartate aminotransferases from mitochondria and cytoplasm from several organisms are clearly homologous. Other enzymes dependent on pyridoxal, including transaminases, D-serine dehydratase, and the B subunit of tryptophan synthetase, may also share this pedigree (45). The similarities in sequence that support such models are few, so homology must be distant and remains somewhat speculative. Nevertheless, homology among all retentive decarboxylases supports historical arguments explaining the common stereospecificity in terms of a shared pedigree.

However, even the amino acid sequence of diaminopimelate decarboxylase from *E. coli* (46) shows limited similarities with those of other pyridoxal enzymes (47). This suggests (again weakly) that these enzymes with *opposite* stereospecificities are also homologous, a suggestion that supports the "historically modified" functional model (where retention is assumed to be opti-

mal, but that D-amino acid decarboxylases have not yet evolved to this optimum), and weakens the conservation principle (stereospecificity in pyridoxal-dependent enzymes is highly conserved) needed by historical models.

Conversely, more recent studies have shown that the diaminopimelate decarboxylase from wheat germ also produces inversion (48). This result probably argues *against* the historically modified functional model, as it implies that D- and L-amino acid decarboxylases diverged before the divergence of bacteria and plant enzymes. This implies that the D-amino acid decarboxylases should have had enough time to be optimized. The alternative interpretation, that the diaminopimelate decarboxylase evolved independently in plants and bacteria, seems less likely, although the notion could be tested with sequence data.

More data relevant to this discussion were collected in 1979 by Gerdes and Leistner (49). These authors investigated the stereochemical course of lysine decarboxylation in *Bacillus cadaveris* and in *Sedum* plants. They demonstrated that decarboxylation of L-lysine by *B. cadaveris* proceeded with retention, but that the decarboxylation of L-lysine by *Sedum* proceeded with inversion.

Regrettably, the lysine decarboxylase was not purified, and it is not known whether this enzyme contains pyridoxal phosphate, a pyruvyl residue, or neither. Nor are kinetic parameters available. However, if a *bona fide* L-lysine decarboxylase from *Sedum* proceeds with inversion, historical and functional models must be further constrained.

We consider the functional model first and assume that the new enzyme uses pyridoxal as a cofactor. A functional model that explains inversion in L-lysine decarboxylase must identify some functionally relevant difference between the environments of this enzyme and the analogous enzyme from animals and bacteria. This must then form the basis of an argument that inversion is the optimal mode of catalysis in some environments and retention in others. The enzymes might operate in opposite directions physiologically (not likely in this case, but possible in others). Different stereochemical modes might be preferred at different ambient temperatures or *p*H optima.

Alternatively, a historically modified functional model might be constructed. The enzyme from *Sedum* may be presumed to have evolved from optimized retentive decarboxylases, but itself is not optimized. The prediction would be that the lysine decarboxylase from *Sedum* has a poor k_{cat}/K_M value in contrast to the analogous enzyme from other sources. Alternatively, historical models must propose yet another ancestral decarboxylase, perhaps one unique to plants. The amino acid sequence of the decarboxylase from *Sedum* is then needed as a test of the theory. There should be no homology. Further, L-amino acid decarboxylases from plants should all produce inversion.

A historical model so modified becomes unpredictive except in uninteresting cases, those where high (>50%) sequence similarity is already known. Not only can stereospecificity not make any statement about biological function and chemistry in this case, but it also cannot make statements about very ancient history. It may, however, serve as a tool for understanding evolution within biological kingdoms.

B. Conclusions

Stereospecificity in amino acid decarboxylases appears not to be a selected trait. However, sequence data currently available are insufficient to estimate whether the stereochemical diversity in this class of enzymes arises from drift or multiple ancestry.

While this discussion may seem hopelessly pedantic, it is important. The exercise of constructing formally precise models and the experimental pursuit of their logical implications illustrate the methods that must be applied to elucidate the biological significance of any enzymatic behavior. At the very least, the process forces the experimenter to examine his prejudices concerning the validity of historical and functional pictures of enzymatic behavior. At best, the process suggests new experiments.

Those studying cryptic stereospecificity in enzymes should chose systems with these issues in mind. In pyridoxal enzymes, further studies of enzymes from eubacteria seem to be of little value. However, fungi might contain homologous amino acid decarboxylases that have different stereospecificities. Further, it is interesting to determine whether enzymes catalyzing reactions other than decarboxylations, but dependent on pyridoxal cofactors, act differently on D-amino acids and on L-amino acids. For example, all amino acid transaminases so far studied abstract the *pro-S* hydrogen from pyridoxamine. It is important to determine whether this is true as well in transaminases acting on D-amino acids.

IV. CLASSES SHOWING STEREOCHEMICAL HETEROGENEITY

If members of a class of enzymes catalyzing similar reactions have different stereospecificities, functional models that ascribe a selectable function to stereospecificity must provide a chemical basis for the heterogeneity. Contrasting historical models must either argue that the trait is drifting, or that members of the class arose from two (or more) non-homologous ancestors. Four important classes of enzymes display stereochemical heterogeneity: phosphoryl transferases, beta-ketoacid decarboxylases, dehydrogenases dependent on nicotinamide cofactors, and enzymes catalyzing additions to olefins.

A. Phosphoryl Transfers

Methods for determining the absolute chirality of phosphates substituted with isotopes of oxygen (50) have been used to examine the stereospecificities of many phosphoryl transferases (51–57). Transfer is found in all cases to occur either with retention or with inversion of configuration at the phosphate center, and these results are generally interpreted in terms of two mechanistic alternatives. Inversion of configuration is interpreted as evidence for direct transfer of phosphorus from donor to acceptor in a single-step sequence involving no intermediate. Retention of configuration is interpreted as evidence for a two-step reaction with a phosphoryl–enzyme intermediate. While overall inversion is also consistent with more complicated mechanisms involving an odd number of displacement reactions at phosphorus, and retention is consistent as well with mechanisms postulating an even number of displacements, little evidence supports these more complicated mechanisms. Thus, discussion of the stereospecificity of phosphoryl transferases can move directly to a discussion of why intermediates are functionally better for some phosphoryl transfer reactions than for others.

Table 2 comprises the stereospecificities of a variety of phosphotransferases. It reveals an interesting distribution. Enzymes formally transferring phosphorus within a single molecule produce retention of configuration. Enzymes transferring phosphate from ATP to an alcohol or amine produce inversion. Enzymes transferring phosphorus from an alcohol to water, ATPases, and enzymes transferring phosphorus from phosphate to phosphate show stereochemical heterogeneity.

Table 2
Stereospecificity of Phosphotransferases

TRANSFERING PHOSPHORUS WITHIN A SINGLE MOLECULE	
Phosphoglucomutase	retention
Phosphoglycerate mutase (rabbit)	retention
Phosphoglycerate mutase (wheat)	retention
TRANSFERRING PHOSPHORUS FROM DONOR TO ACCEPTOR MOLECULES OF EQUAL SIZE	
Nucleoside diphosphate kinase	retention
Nucleoside phosphotransferase	retention
TRANSFERING PHOSPHORUS FROM DONOR TO ACCEPTORS OF DIFFERENT SIZE	
Acetate kinase	inversion
Acetyl CoA synthetase	inversion
Adenosine kinase	inversion

Table 2
(Continued)

TRANSFERING PHOSPHORUS FROM DONOR TO ACCEPTORS OF DIFFERENT SIZE	
Adenylate kinase	inversion
Adenylosuccinate synthetase	inversion
Aminoacyl tRNA synthetases	inversion
Creatine kinase	inversion
DNA-dependent RNA polymerase (initiation)	inversion
(elongation)	inversion
Galactose-1-phosphate uridylyltransferase	retention
Glycerol kinase	inversion
Hexokinase	inversion
Phosphofructokinase	inversion
Phosphoglycerate kinase	inversion
Polynucleotide kinase	inversion
Polynucleotide phosphorylase (exchange)	retention
(elongation)	inversion
Pyruvate kinase	inversion
tRNA nucleotidyl transferase	inversion
UDP-glucose pyrophosphorylase	inversion
TRANSFERING PHOSPHORUS TO WATER	
Acid phosphatase (bovine)	retention
Alkaline phosphatase (coli)	retention
Cyclic AMP phosphodiesterase	inversion
Exonuclease (bovine spleen)	retention
Non-specific phosphohydrolase	inversion
5′-Nucleotidase	inversion
Nucleotide pyrophosphatase	retention
Phosphodiesterase (snake venom)	retention
Phosphodiesterase (bovine intestine)	retention
Phosphohydrolase (Enterobacter)	inversion
Phospholipase D (cabbage)	retention
Ribonuclease (pancreatic)	inversion, inversion
Ribonuclease T_1	inversion
Ribonuclease T_2	inversion
Staph nuclease	inversion
ATPases	
Mitochondrial ATPase	inversion
Myosin ATPase	inversion
Ribosome-dependent GTPase	inversion
Sarcoplasma reticulum ATPase	retention
Elongation factor G GTPase	inversion
Elongation factor T GTPase	inversion

Only in the first case is there a simple explanation. On geometric grounds, transfer of a phosphate group in a 1,2-diol can occur only via either an enzyme–phosphoryl (or cofactor–phosphoryl) intermediate, or via a cyclic intermediate that must undergo pseudorotation (Figure 8). In both cases, the stereochemical outcome is retentive (58).

In the other cases, more discussion is necessary. Frey has proposed a fascinating functional explanation for the last subclass of phosphoryl transferases, those that transfer phosphoryl groups between nucleotides. The transfer of phosphate from a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP) proceeds via an enzyme–phosphoryl intermediate, but transfer from NTP to a nucleoside monophosphate (NMP) is direct. Frey noted that in the first case the phosphoryl donor and acceptor are approximately the same size (Figure 9) (59). Therefore, a single binding site can accommodate each, the enzyme holding the phosphoryl group while the donor (minus the phosphate) leaves the active site and the acceptor enters. In contrast, with donor and acceptor molecules of different size, the same binding site would not accom-

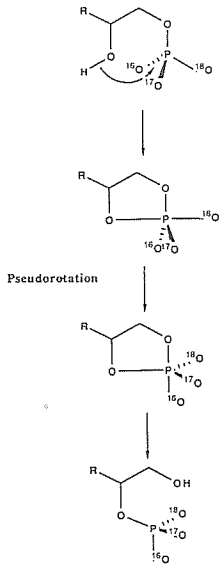


Figure 8. Phosphate transfer involving pseudorotation.

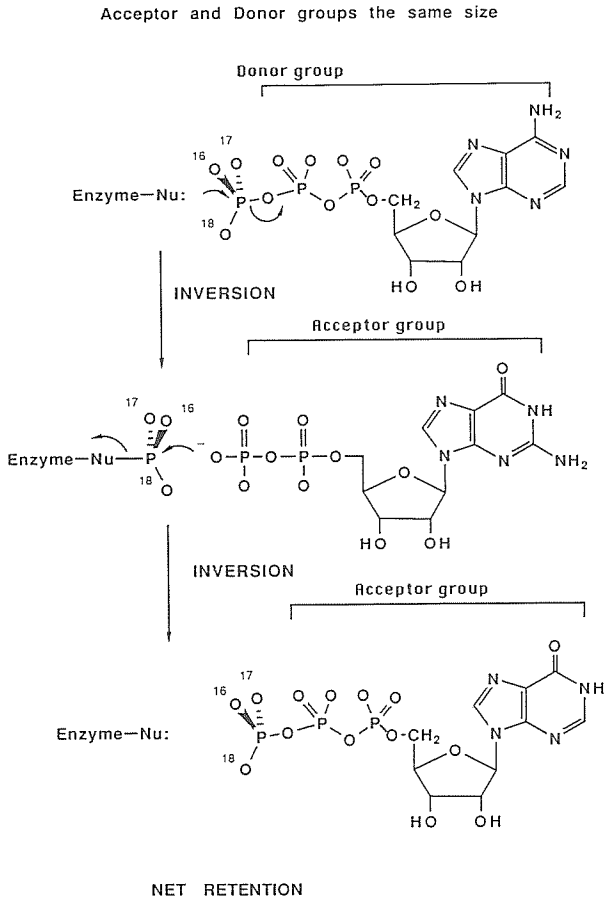


Figure 9. Retention of configuration in enzymes transferring phosphate between molecules with similar structure.

modate both (Figure 10). Two binding sites must be built into the enzyme, and the transfer is direct from phosphoryl donor in one site to acceptor in the second.

Frey's model has several implications. First, it implies that there is a "cost" associated with evolving an enzyme with an extra binding site. This cost could be either "thermodynamic" (two identical organisms, one with an "inexpensive" NTP-NDP transferase having a single binding site, the other with an "expensive" enzyme with two binding sites, have different survival abilities) or "kinetic" (proteins with one binding site evolve more rapidly). Further, it implies that there is a smaller "cost" for enzymes using two binding sites for donor and acceptor with different sizes than for enzymes using one site (where, for example, a conformational change occurs to accommodate two substrates with different steric requirements). Finally, it suggests that once an enzyme has two binding sites, direct transfer is preferable over a phosphoryl-enzyme intermediate.

These implications have broader impact. If the expense of synthesizing large proteins is sufficiently great to influence the survival of a host organism in phosphoryl transferases, minimizing the size of proteins should be a goal of natural selection with other proteins as well. It is conceivable that such selective pressures might create trade-offs between size and other behaviors. These

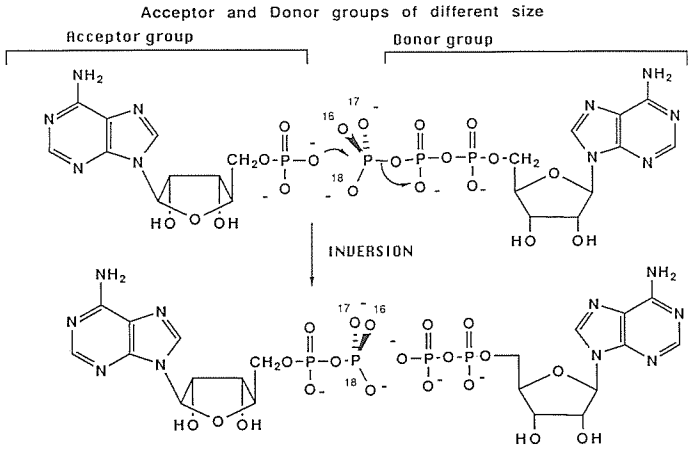


Figure 10. Inversion of configuration in enzymes transferring phosphate between molecules with different structure.

considerations are discussed at length elsewhere (9, 13-15). Finally, it implies that the large size of some enzymes confers selective advantage.

Isolated examples from other classes of enzymes suggest that Frey's hypothesis is not general. For example, two classes of enzymes catalyze the transfer of hydrogen from NAD^+ and $NADP^+$. One class catalyzes direct transfer in an active site with *two* binding sites, the other proceeds via an "enzyme-hydride" intermediate where the reducing equivalent is bound to flavin (60). The existence of these two classes suggests that the cost of constructing additional binding sites may not in fact have a significant impact on survival.

Functional theories can be constructed for the other classes of phosphoryltransferases. Frey's model would predict that transfer from ATP to an alcohol or amine should proceed with inversion. The donor and acceptor are sufficiently different as to require two binding sites and, once two binding sites are needed, direct transfer is presumed to be optimal. Inversion is observed in these enzymes.

Given this theoretical context, the phosphotransferases that appear to have functionally anomalous stereospecificities are those that transfer phosphoryl groups to water. A functional model based on principles of chemical reactivity can be constructed that suggests that these enzymes should proceed via enzyme-phosphoryl intermediates (and hence with retention) when the enzyme has evolved to have low substrate specificity. The argument is based on the fact that for catalysis to occur an enzyme must bind to the transition state of a reaction more tightly than it binds to the ground state (61).

There are several ways of stating this argument. Drawing on the language of Jencks (62) (which, although theoretically problematical (63), might be the most familiar to the reader), it is difficult for an enzyme to bind and activate small nucleophiles such as water. Thus, in phosphoryl transfers *to* water, catalysis must be achieved by interactions between the enzyme and the phosphoryl donor.

If the phosphatase has evolved to be non-specific, the enzyme does not have many strong interactions with the phosphoryl donor; thus, the enzyme has few opportunities to "activate" the phosphate donor to achieve catalysis. In these cases, nucleophilic catalysis by a residue in the active site is the only mechanism remaining for catalyzing the transfer of the phosphoryl group. Hence, transfers to water are likely to involve an enzyme-phosphoryl intermediate, especially if the enzyme has low substrate specificity. For an enzyme with high substrate specificity, direct transfer to water from an activated phosphate donor is preferred for the reasons outlined above. In this case, the "binding energy utilized" for catalysis is obtained by interactions between the enzyme and the phosphoryl donor.

This notion is consistent with the fact that many nonspecific phosphatases

produce retention (Table 2). For example, glucose-6-phosphatase, an enzyme with broad substrate specificity, produces retention, consistent with an enzyme-phosphoryl intermediate and the notion that neither the nucleophilic substrate nor the phosphate donor can be activated in an enzyme where water is the nucleophile and the enzyme must accept phosphate donors with a range of structures. In contrast, phosphatases that have narrow substrate specificity (for instance, staphylococcal nuclease and cyclic phosphodiesterases, the second acting on a substrate that is already somewhat reactive), produce inversion. This model is predictive, although the scale that describes the "narrowness" of substrate specificity is only semi-quantitative.

The divergent stereospecificities of ATPases remain a significant problem. These enzymes all catalyze (apparently) chemically identical reactions. While inversion occurs most often when phosphate is transferred from nucleoside triphosphates to water (as expected by the functional model), the ATPase from sarcoplasmic reticulum operates with retention (54). Though one might dismiss this stereochemical difference as the result of random origin or neutral drift, functional models can be constructed.

Most ATPases participate in specific physiological processes (64–69). Normally, the hydrolysis of ATP is coupled to muscle contraction, Ca^{2+} transport, or other metabolic processes. In other cases, "ATPases" are, in fact, ATP synthetases under physiological conditions. Only rarely is the destruction of ATP likely to be the physiological role for an ATPase.

Enzyme-phosphoryl intermediates have different values depending on how the energy of ATP hydrolysis is used. Clearly, enzyme phosphoryl intermediates are kinetically more stable than intermediates where this energy is stored in a high-energy conformation of the enzyme or in a tightly bound enzyme–ADP complex. The increased kinetic stability is advantageous if the high-energy intermediate must undergo conformational contortions, or must survive for periods of time (for example, to permit an ion to diffuse to or from it). It is presumably disadvantageous in other cases, as the need for a second step (the hydrolysis of the covalent enzyme-phosphorus bond) slows down the turnover rate.

These considerations offer a rationalization for the fact that enzyme–phosphoryl intermediates are used uniformly for ion transport, where considerable conformational change must take place in the activated intermediate. It also suggests an explanation for the lack of such a covalent intermediate in myosin ATPases, where the conformational change is presumably itself the work that is physiologically intended, occurs immediately upon the hydrolysis of ATP, and where slowing turnover numbers would be distinctly disadvantageous in muscle tissue, because power per unit volume is extremely important. Finally, ATPases that act physiologically as phosphoryl donors to water should produce inversion unless they are relatively nonspecific for substrate, following the rationale outlined above.

These hypotheses are generated *post hoc,* given the information that ATPases involved in ion transport do indeed catalyze reactions via an enzyme–phosphoryl intermediate, though other ATPases do not. Their value comes, again, in their predictive utility.

Sequence data are rapidly becoming available that make interesting comments on historical views of the stereospecificity of ATPases. Sequences suggest that major classes of phosphoryl transferases with the same stereospecificities are homologous (69, 70). However, there also appear to be sequence similarities in the active site of ATPases that produce retention and those that produce inversion (69), especially around the aspartate that is phosphorylated in the first class (Figure 11). Remarkably, the aspartate that is phosphorylated in the first class is found as a threonine in the second class. Further, the conserved sequence is found between the two nucleotide binding domains predicted in the second class based on structural homologies with adenylate kinase and near Tyr 311, believed to interact with certain inhibitors of ATPase. Thus, the short sequence similarities appear to be significant and suggest that some invertive and retentive ATPases are homologous.

If this suggestion is correct, it again indicates that reaction mechanisms (and therefore stereospecificity) are remarkably adaptable in the face of different functional demands. Aside from suggesting that stereospecificity is a poor indicator of homology in this case, such an indication raises doubts about historical explanations for stereospecificity in general in the presence of alternative functional models.

A final stereochemical point should be noted. Cleland and his coworkers have pioneered the use of chiral complexes between ATP and chromium as stereochemical probes of the active sites of phosphatases (71). Two stereochemical classes of enzymes have been identified; certain members of both classes produce inversion. This may indicate independent origin of two inver-

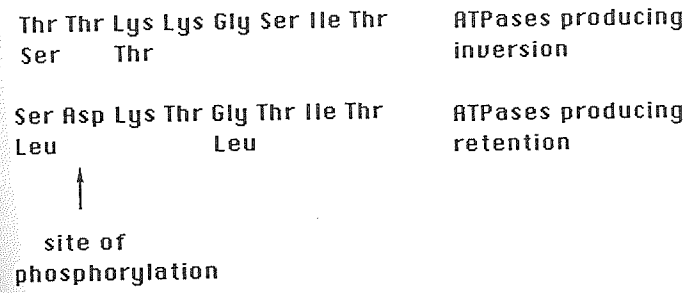


Figure 11. ATPases producing retention and inversion, respectively.

tive classes of enzymes. Alternatively, it may indicate that specificity for the chirality of the conformation in which ATP binds may drift.

B. Beta-Ketoacid Decarboxylases

Decarboxylases acting on beta-ketoacids constitute another class of enzymes that displays stereochemically heterogeneity. In 1973, Rose and Hanson noted that of five decarboxylases studied, three decarboxylated substrate with retention of configuration and two with inversion (29). Rose interpreted this as evidence that stereospecificity is selectively "neutral" in these enzymes, arguing that it makes no difference to the survival of the host organism which stereochemical mode was followed. Thus, the stereospecificity of each individual enzyme evolved randomly. A sixth enzyme, acetolactate decarboxylase, has subsequently been examined; it produces inversion (72).

As with pyridoxal-dependent decarboxylases, stereospecificity in decarboxylases acting on beta-ketoacids does not reflect a stereochemical choice in a single transition state but rather the relative stereochemical choices in two separate transition states. The first involves the removal of a carbon dioxide to give an intermediate enol or enol equivalent. The second leads to the protonation of the intermediate to yield the product ketone.

Two mechanisms for catalyzing beta-decarboxylations are well documented by model studies (Figure 12). In the first, metal ions chelated to an alpha ketoacid unit act as an "electron sink," facilitating decarboxylation (73). The decarboxylation of oxaloacetate catalyzed by divalent manganese apparently proceeds via such a mechanism. Alternatively, decarboxylation can be catalyzed by amines. Here, the amine reacts with the keto group to form a Schiff's base. Presumably, the protonated Schiff's base acts as an "electron sink" (24).

Both forms of catalysis are apparently exploited by enzymes, depending on the structure of the substrate (Table 3). For the six enzymes listed in the table, enzymes acting on substrates that possesses an alpha-ketoacid moiety

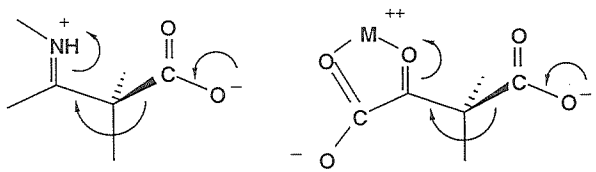


Figure 12. Schiff's base and metal-dependent decarboxylation of beta-ketoacids.

Table 3
Substrates, Stereospecificities, and Requirements for Metal Ions in Beta-ketoacid Decarboxylases

Enzyme	alpha-Keto acid as substrate?	Stereo-selectivity	Metal ion?
Isocitrate dehydrogenase (NAD^+)	yes	retention	yes
Isocitrate dehydrogenase ($NADP^+$)	yes	retention	yes
Malic enzyme	yes	retention	yes
Phosphogluconate dehydrogenase	no	inversion	no
UDP-glucuronate decarboxylase	no	inversion	no
Acetolactate decarboxylase	no	inversion	no

capable of coordinating a metal ion require a metal ion catalytic activity. Those that act on substrates lacking this moiety do not require a metal ion.

This observation permits a correlation to be drawn connecting substrate structure, mechanism, and stereospecificity (25, 74). Whenever a metal ion is required for catalysis, the decarboxylation proceeds with retention of configuration; whenever a metal ion is not required, decarboxylation proceeds with inversion of configuration. The two stereochemical modes observed in beta decarboxylases appear to reflect the existence of two mechanisms for enzyme-catalyzed decarboxylations. As a working hypothesis, this correlation predicts the stereochemical preference of any beta decarboxylase whose substrate is known.

The model makes predictions. For example, the biosynthesis of 5-aminolevulinate involves two stereochemically significant steps, the condensation of glycine with succinyl-CoA to yield 2-amino-3-ketoadipate and the decarboxylation of this intermediate to yield 5-aminolevulinate (Figure 13) (75). The functional model predicts that the condensation step proceeds with retention, and the decarboxylation with inversion. Consistent with this, the reaction of succinyl CoA and glycine to form aminolevulinic acid was found to proceed via overall inversion (75), requiring that one of the steps is invertive and the other retentive. Further work is necessary to determine whether the model has correctly predicted which step is which.

The functional model was recently scrutinized by a study of the stereochemical course of decarboxylations catalyzed by acetoacetate decarboxylase (25). This enzyme, predicted to produce inversion, surprisingly was found to produce retention and inversion with roughly equal frequencies. This appeared to reflect stereo-nonspecific protonation of an intermediate enamine, not racemization of starting material or equilibration of label in product or Schiff's base between product and enzyme (Figure 14). This interpretation was confirmed by a series of control experiments.

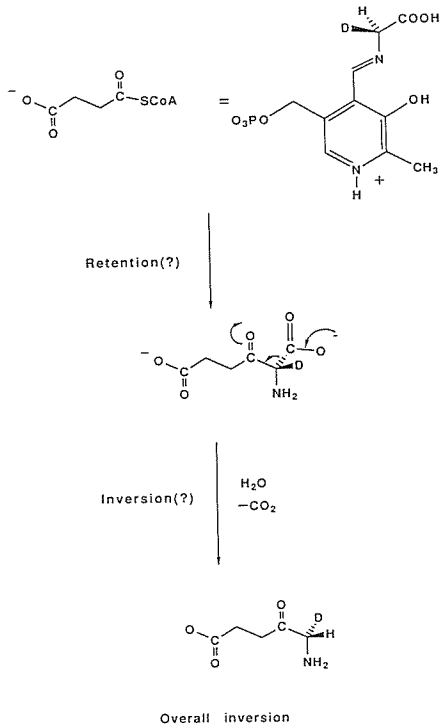


Figure 13. Cryptic stereospecificity in aminolevulinate synthase.

The functional model predicted that acetoacetate decarboxylase would produce inversion; retention would have been considered a contradictory result. The observed result neither confirmed or contradicted the model cleanly. To explain this result functionally, an *ad hoc* hypothesis was introduced. Noting that the rate or protonation of the enamine of acetone in solution at the physiological *p*H (*p*H 6) was on the same order of magnitude as the steady state turnover rate of the enzyme, the argument was made that protonation from the solvent would be kinetically competent to carry the flux through the active site. Thus, there appears to be little need for enzymatic catalysis of the protonation of the enamine. Protonation directly from solvent

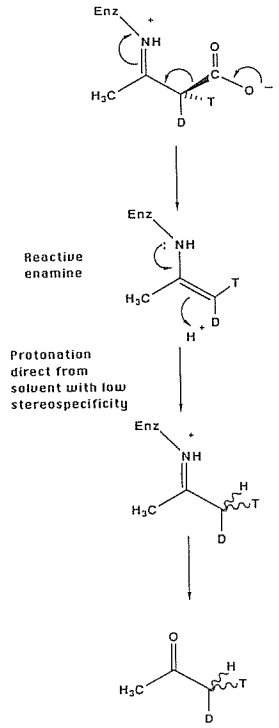


Figure 14. Reactive intermediate proposed to explain racemization in the decarboxylation of acetoacetate.

plausibly would proceed with lower stereoselectivity than protonation by a general acid provided from the active site. Thus, when the intermediate is extremely reactive, one might expect low cryptic stereospecificity.

While the introduction of *ad hoc* arguments might be excused for a theory at an early stage of development, the *ad hoc* modification could be tested, as a similar argument should apply to other unsubstituted enamines that are intermediates in enzymatic reactions. For example, a similar enamine (here, of pyruvate) is an intermediate in the conversion of aspartate to alanine catalyzed by aspartate beta-decarboxylase (Figure 15). As the enamine of pyru-

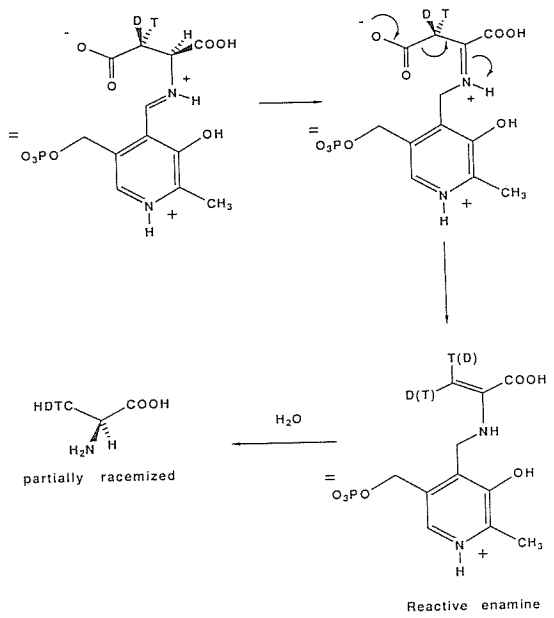


Figure 15. Reactive intermediate proposed to explain partial racemization in the decarboxylation of aspartate.

vate with pyridoxamine should have a chemical reactivity similar to that of the intermediate in the reaction catalyzed by acetoacetate decarboxylase, the enamine might also be protonated directly from solvent with incomplete stereospecificity.

Indeed, alanine formed as a product of aspartate beta-decarboxylase was found to be substantially racemized (76). This is consistent with the modified hypothesis. Unfortunately, control experiments have not yet been done to distinguish between racemization as a result of partial stereospecificity in the

decarboxylation step (consistent with the *ad hoc* explanation) and racemization resulting from subsequent enzyme-catalyzed exchange of the protons on the product.

Recently, the stereospecificity of oxaloacetate decarboxylase (OAD) from *Pseudomonas putida* was examined as a further test of the model. The OAD requires a divalent metal cation; the metal presumably chelates the alpha-ketoacid moiety of the substrate providing an electron sink for decarboxylation (Figure 12) (25). The natural substrate is presumably oxaloacetate. Thus, on mechanistic considerations, OAD from *Pseudomonas* is expected to catalyze the decarboxylation of oxaloacetate with retention, as do other metal-dependent OAD's.

In fact, OAD from *Pseudomonas* produces inversion (77). This stereochemical result violates the correlation in Table 3. Indeed, the result is inconsistent with any simple functional explanation for stereospecificity in decarboxylases based on a property intrinsic in the substrate, as OAD from *Klebsiella aerogenes* (biotin dependent) (78, 79), pyruvate carboxylase (biotin dependent) (80), and malic enzyme all produce retention (81). In the last case, the enzyme producing retention appears to operate via the same mechanism as the OAD from *Pseudomonas*. The inescapable conclusion is that mechanistic diversity in beta-decarboxylases does not always correlate with stereochemical diversity. However, the result is also inconsistent with "historical" explanations that presume common ancestry for beta-decarboxylases, with stereospecificity highly conserved during divergent evolution.

Thus, beta-ketoacid decarboxylases display a full range of stereochemical diversity: retention, inversion, and racemization. Three explanations must be considered for these results: (a) there exist several independent pedigrees of decarboxylases descendent from several ancestral decarboxylases, where stereospecificity is non-functional but highly conserved; (b) stereospecificity is a non-functional trait capable of facile neutral "drift" as homologous enzymes diverge; or (c) stereospecificity is a functional trait, where a mechanistic imperative is different in different decarboxylases because the enzymes perform subtly different roles in subtly different environments.

Data are insufficient to distinguish between these three alternatives, although possibilities (a) and (b) seem to be the most likely. It is important to collect sequence data to distinguish between these two possibilities, as the outcome here will influence our view of functional and historical models for stereospecificity in general. In particular, if stereospecificity in decarboxylases can easily drift, this would suggest that other stereochemical behaviors of enzymes can drift as well.

C. Dehydrogenases Dependent on Nicotinamide Cofactors

Of the dehydrogenases dependent on nicotinamide cofactors that have been studied, about half transfer the *pro-R* hydrogen of NAD(P)H, while half transfer the *pro-S* hydrogen (Table 4, Figure 16) (21). This stereochemical choice does not obviously correspond to a mechanistic choice. Rather, stereospecificity appears to be determined only by the relative orientation of the substrate and the cofactor in the active site, an orientation that in turn is determined by the relative positions of active site residues. These positions seem to be arbitrary. Therefore, historical explanations for stereospecificity in dehydrogenases have been predominant in the literature.

Table 4a
Stereospecificity of Dehydrogenases Arranged by E.C. Number

E.C.	Name	Stereochemistry
1.1.1.1	Alcohol dehydrogenase (yeast)	*pro-R*
1.1.1.3	Homoserine dehydrogenase	*pro-R*
1.1.1.6	Glycerol 2-dehydrogenase	*pro-R*
1.1.1.8	Glycerol-3-phosphate dehydrogenase	*pro-S*
1.1.1.26	Glyoxylate reductase	*pro-R*
1.1.1.27	L-Lactate dehydrogenase	*pro-R*
1.1.1.28	D-Lactate dehydrogenase	*pro-R*
1.1.1.29	Glycerate dehydrogenase	*pro-R*
1.1.1.30	3-Hydroxybutyrate dehydrogenase	*pro-S*
1.1.1.35	3-Hydroxyacyl CoA dehydrogenase	*pro-S*
1.1.1.37	Malate dehydrogenase	*pro-R*
1.1.1.38	Malic enzyme	*pro-R*
1.1.1.40	Malic enzyme (NADP)	*pro-R*
1.1.1.50	3-Hydroxysteroid dehydrogenase (P. test.)	*pro-S*
1.1.1.51	beta-Hydroxysteroid dehydrogenase	*pro-S*
1.1.1.60	Tartronate semialdehyde reductase	*pro-R*
1.1.1.62	Estradiol 17-beta-dehydrogenase	*pro-S*
1.1.1.64	Testosterone beta-dehydrogenase	*pro-S*
1.1.1.72	Glycerol dehydrogenase (NADP)	*pro-R*
1.1.1.79	Glyoxylate reductase (NADP)	*pro-R*
1.1.1.81	Hydroxypyruvate reductase	*pro-R*
1.1.1.82	Malate dehydrogenase (NADP)	*pro-R*
1.1.1.100	3-Oxoacyl ACP reductase	*pro-S*
1.1.1.108	Carnitine dehydrogenase	*pro-S*

Table 4b
Stereospecificity of Dehydrogenases Arranged by the pK_{eq} for their Physiological Reaction

E.C.	Name	pK_{eq}	Stereochemistry
1.1.1.26	Glyoxylate reductase	17.5	*pro-R*
1.1.1.79	Glyoxylate reductase (NADP)	17.5	*pro-R*
1.1.1.60	Tartronate semialdehyde reductase	13.3	*pro-R*
1.1.1.29	Glycerate dehydrogenase	13.3	*pro-R*
1.1.1.72	Glycerol dehydrogenase (NADP)	12.8	*pro-R*
1.1.1.81	Hydroxypyruvate reductase	12.4	*pro-R*
1.1.1.82	Malate dehydrogenase (NADP)	12.1	*pro-R*
1.1.1.37	Malate dehydrogenase	12.1	*pro-R*
1.1.1.38	Malic enzyme	12.1	*pro-R*
1.1.1.40	Malic enzyme (NADP)	12.1	*pro-R*
1.1.1.27	L-Lactate dehydrogenase	11.6	*pro-R*
1.1.1.28	D-Lactate dehydrogenase	11.6	*pro-R*
1.1.1.1	Alcohol dehydrogenase (yeast)	11.4	*pro-R*
1.1.1.6	Glycerol 2-dehydrogenase	11.3	*pro-R*
1.1.1.8	Glycerol-3-phosphate dehydrogenase	11.1	*pro-S*
1.1.1.3	Homoserine dehydrogenase	10.9	*pro-R*
1.1.1.108	Carnitine dehydrogenase	10.9	*pro-S*
1.1.1.35	3-Hydroxyacyl CoA dehydrogenase	10.5	*pro-S*
1.1.1.30	3-Hydroxybutyrate dehydrogenase	8.9	*pro-S*
1.1.1.50	3-Hydroxysteroid dehydrogenase	8.0	*pro-S*
1.1.1.62	Estradiol 17-beta-dehydrogenase	7.7	*pro-S*
1.1.1.64	Testosterone beta-dehydrogenase	7.6	*pro-S*
1.1.1.100	3-Oxoacyl ACP reductase	7.6	*pro-S*
1.1.1.51	beta-Hydroxysteroid dehydrogenase	7.6	*pro-S*

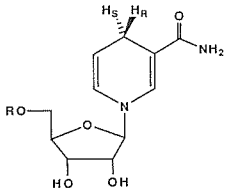


Figure 16. Cryptic stereospecificity at cofactor in dehydrogenases.

1. Correlations

With stereospecificities known for nearly 200 dehydrogenases, empirical correlations with satisfactory statistics are possible. These serve as the starting point for constructing testable models, both historical and functional. Here as above, exceptions to such correlations, and how they are treated, determine the development of the models. Failure to discard a correlation in the face of a significant exception runs the risk of destroying the rigor and predictability of a model. Yet, discarding a poorly understood correlation because of a single exception runs the risk that a valuable model will be overlooked; this risk is especially high in the early stage of model-building, where the significance of the exception may not be understood or where the reported data themselves may be incorrect.

Conservatism is the rule in literature interpreting these correlations. Interesting and experimentally testable correlations have been discarded in the face of a single (and often poorly understood) counterexample. For example, Davies and coworkers suggested that dehydrogenases involved in consecutive steps in a biochemical pathway should have the same stereospecificity at the nicotinamide cofactor (82). Though originally an empirical generalization, the model has clear "historical" basis. If enzymes catalyzing consecutive steps in a metabolic pathway are homologous, and if stereospecificity is highly conserved, this rule follows deductively. These assumptions have received some independent experimental support from work by Ornston and his coworkers (83). Further, Davies' model makes the general statement that reaction type, mechanism, and substrate specificity all diverge faster than stereoselectivity. This may be true or false, but it is interesting, and deserves exploration.

However, exceptions exist to the empirical rule. Nitrate reductase (forming nitrite) and nitrite reductase (forming ammonia) from *Canadida utilis* have opposite stereospecificities at NADH (84). Further, enzymes presumed to act consecutively in the metabolism of cinnamyl alcohol in plants have opposite stereospecificities (85). In light of these two exceptions, the correlation has been dismissed (21), and it appears as if no further investigations of this generalization have been undertaken. This is unfortunate. It is certainly conceivable that *some* pairs of enzymes catalyzing consecutive steps in a metabolic pathway are not homologous. Further, there are independent ways of assessing homology in these cases, sequence comparisons being the most direct. Thus, if sequence data suggest that these pairs of consecutive enzymes with opposite stereospecificities are not homologous, no serious damage is done to the assumptions underlying the historical model. Indeed, it would be interesting to know how often enzymes catalyzing consecutive steps in metabolism *are* homologous. Conversely, if the consecutive enzymes with opposite stereoselectivities prove to be homologous, the result strongly contradicts a conservation principle that assumes that stereospecificity is highly conserved, and casts doubt on historical models that depend on it. Thus, the model stimulates experimental work that is useful regardless of the results.

What constitutes an unacceptable level of exception to a rule is often a matter of taste. For example, a recent review criticized fourteen generalizations concerning the stereospecificities of enzymes dependent on nicotinamide cofactors (21). Generalizations that had "too many" exceptions were discarded. However, a rule correlating stereospecificity in dehydrogenases with the mode of cofactor binding (where *pro-S* and *pro-R* stereospecificities correlate with a syn or anti orientation of the nicotinamide ring around the glycosidic bond, Figure 17) with 6 confirming instances and 1 exception (14%) was regarded as "the only mechanistic explanation" for stereospecificity that was "receiving growing acceptance." A rule with some 50 confirming instances and 6 exceptions (12%) was dismissed as having "untenability" that is "overwhelmingly evident."

Here, as above, we believe that a more productive approach evaluates generalizations by their ability to suggest testable functional or historical models. Exceptions are treated as logical constraints on the form of the historical or functional model. Further, exceptions must be critically evaluated with respect to the model they are intended to disprove. If an exception is presumed to challenge a functional model, its evolutionarily relevant function must be known. If an exception is presumed to challenge a historical model, information about its pedigree is relevant.

An important set of empirical generalizations for dehydrogenases was introduced in slightly different forms by Vennesland, Colwick, and Bentley. Often referred to as "Bentley's rules," these generalizations are (86, 87):

1. The stereospecificity of a particular enzyme does not depend on the source of the enzyme.
2. The stereospecificity of a particular reaction is the same in those cases where both NAD^+ and $NADP^+$ can be used as coenzymes.
3. If a single enzyme uses a range of substrates, the stereospecificity with respect to cofactor will be the same with all substrates.

These generalizations have only a few exceptions, and some remarkable confirmations. Lactate dehydrogenases all transfer the *pro-R* hydrogen (21), regardless of whether they are isolated from mammals (bovine, rabbit, and pig), amphibians (frog), fish (halibut), birds (turkey), dogfish, plants (potato), arthropods (horseshoe crab), lower eukaryotes (sea worm, abalone), or bacteria *(E. coli, Lactobacillus)* (21). Malate dehydrogenases from many sources also transfer the *pro-R* hydrogen, including those from mammals (pig), plants (potato, wheat germ), birds (chicken), arthropods *(Drosophila),*

fungi *(Neurospora)*, bacteria *(B. subtilis)*, and archaebacteria *(Sulfolobus acidocaldarius, Thermplasma acidophilum, Halobacter halobium)* (88, 89). The 3-hydroxybutyryl-CoA and 3-hydroxybutyryl-acyl carrier protein dehydrogenases transfer the *pro-S* hydrogen, including those from mammal (bovine, rat, pig), bacteria *(Rhodopseudomonas, Escherichia, Brevibacter)*, from birds (pigeon), and from yeast (21, 90). Together, these organisms include several representatives from every kingdom of life on the planet.

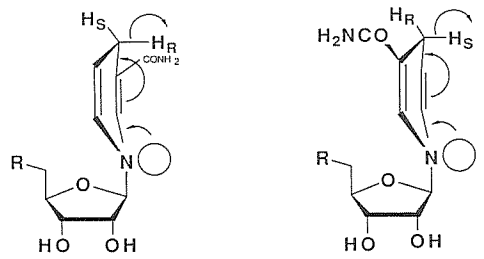
2. *Historical Models*

A historical model stating that stereoselectivity (a) is not functional, (b) is "random," and (c) can drift randomly is inconsistent with the generalizations above. For example, if stereospecificity can drift, the stereospecificities of lactate and malate dehydrogenases would not be as uniform as they are. Thus, a constrained historical model must assume that when cofactor stereospecificity originated in an enzyme, it was random. However, the model must assume that once established, stereospecificity in the dehydrogenases was highly conserved during subsequent divergent evolution. Further, the model must assume that all modern dehydrogenases acting on a particular substrate are homologous.

The range of organisms over which Bentley's first rule applies suggests that stereoselectivity was conserved in the time since the divergence of plants, animals, insects, eubacteria, and archaebacteria. This is the most ancient divergence that can be identified in modern biology. Thus, a historical model seeking to explain this rule must presume that stereoselectivity with respect to cofactor is very rigorously conserved indeed. Not surprisingly, several arguments can be found in the literature that it is *impossible* for a *pro-R* enzyme to evolve to become a *pro-S* enzyme (91, 92).

Such extreme conservation of stereospecificity is remarkable, as it appears that stereospecificity in a dehydrogenase can be reversed by simply reversing the geometry in which the nicotinamide ring is bound in the active site (Figure 17). Rotating the nicotinamide ring 180° around the glycosidic bond (for example, from a syn conformation to an anti conformation) would present the opposite face of the cofactor to the substrate and therefore produce an enzyme with opposite stereospecificity. Such a rotation leaves all other features of the enzyme–cofactor complex unchanged and appears to take place when dihydrofolate is replaced with methotrexate in complexes with NAD^+ and dihydrofolate reductase *(vide supra)*.

Extreme conservation of the mode of binding (syn versus anti) of the nicotinamide cofactor might be explained by an assumption that reversing the mode of cofactor binding requires simultaneous replacement of a large number of amino acids in the active site. Enzymes with only some of these replacements are presumed to be inactive catalytically. Organisms containing these "intermediate" enzymes would die. In this view, mode of cofactor binding (and hence, stereospecificity) is presumed to be tightly coupled to catalytic activity, tertiary structure, or some other functional trait. Therefore, stereospecificity is conserved, even though it itself serves no selectable function.



Anti conformation, H_R transferred Syn conformation, H_S transferred

Figure 17. Correlation of stereospecificity with conformation of bound cofactor in dehydrogenases.

Although this model cannot be ruled out *a priori*, it is problematical. Rossman has argued from structural data that the dinucleotide binding domains of various dehydrogenases are similar; this similarity presumably reflects a common pedigree (93, 94). However, by this argument, the dinucleotide binding domains of glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase (dehydrogenases with opposite stereospecificity with respect to nicotinamide cofactor) are homologous. If Rossmann's argument is correct, stereospecificity with respect to cofactor is not absolutely conserved. Indeed, it diverges faster than general tertiary structure in a domain and faster than the divergence of primary sequence, at least of key residues in the dinucleotide binding domain of these proteins.

Similarly, the stereospecificities of ethanol dehydrogenases from yeast and *Drosophila* are opposite (95). Yet the dinucleotide binding domains of the two enzymes appear to be homologous based on sequence analysis (96).

A modified historical model to explain these facts relies on the notion of "domain shuffling" in the evolution of proteins (97). In this model, only the dinucleotide binding domains of glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase are homologous. This homology indicates only a very ancient common ancestry. The modern enzymes in each class arose fol-

lowing the appending of two non-homologous catalytic domains to each of the proteins, a process that is presumed to create random stereospecificities in two new proteins with different substrate specificities. In subsequent divergent evolution, both stereospecificity and substrate specificity was conserved.

Such a model is consistent with the fact that the relative positions of the catalytic and dinucleotide binding domains in the polypeptide chains of the ethanol dehydrogenases from yeast and *Drosophila* are reversed. Indeed, there is a possible (but imperfect) correlation between stereospecificity and the relative positions of the two domains.

Simply assuming, however, that stereospecificity with respect to cofactor (and hence, presumably, the mode of cofactor binding, Figure 18) is highly conserved is insufficient to explain Bentley's first rule. We must also presume that enzymes catalyzing analogous reactions in different organisms generally share a common ancestor. This requires the additional assumption that drift in *substrate specificity* is also constrained. Strictly, the general substrate specificity of one primordial enzyme cannot have evolved to encompass substrates within the general substrate specificity of the other. This conservation principle is necessary because divergence in substrate specificity can create stereochemical diversity just as easily as divergence in the mode of cofactor binding.

For example, if a gene for a dehydrogenase is lost by deletion, the lost catalytic function can be replaced by the evolution of substrate specificity of a second dehydrogenase to assume the role of the deleted enzyme. This process is facile in molecular evolution; indeed, it occurs on the laboratory time scale. For example, a new chorismate mutase has evolved from a prephenate binding site in less than 40 years in a strain of *Bacillus subtilis* with the native chorismate mutase deleted (98). In *E. coli* lacking beta-galactosidase, a new beta-galactosidase emerges after another protein (with unknown function) undergoes two point mutations (99).

This process provides a mechanism for creating stereochemical diversity in a class of dehydrogenases, even given the assumption that mode of cofactor binding is rigorously conserved. For example, if the gene for malate dehydrogenase (transferring the *pro-R* hydrogen) is lost, and the activity is replaced by the evolution of a 3-hydroxybutyrate dehydrogenase (*pro-S* specific) with conservation of cofactor stereospecificity, a *pro-S* specific malate dehydrogenase is the result. Because this process is so facile, it is to be expected, certainly over long periods of evolutionary time. The fact that malate dehydrogenase does not display the expected stereochemical diversity indicates, in the historical view, that deletion-replacement events have not occurred in the time separating archaebacteria, eubacteria, and eukaryotes.

The implications of this second conservation principle are quite interesting

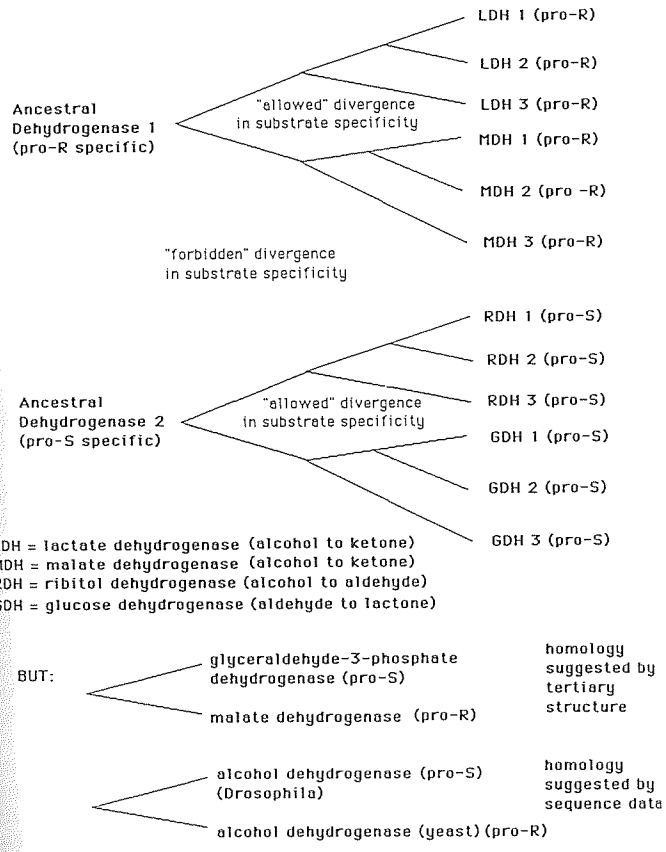


Figure 18. Evolutionary trees used for historical explanation of stereospecificity in dehydrogenases.

and quite general, both in terms of how proteins evolve and how structure and function are related in proteins. If all malate dehydrogenases (*pro-R* specific) are related and had pedigrees independent of 3-hydroxybutyrate dehydrogenases (all *pro-S* specific), the separation of the major subclasses of dehydrogenases must have been quite ancient (Figure 18). Further, if enzymes do

not alter their general substrate specificities in the evolutionary time separating mammals, plants, and archaebacteria, with no malate dehydrogenases becoming 3-hydroxybutyrate dehydrogenases, and *vice versa,* the constraint on substrate specificity is quite remarkable.

This view is significant because it contradicts other data that suggest that substrate specificity can diverge rather easily (9). For example, substrate specificities are quite different in the E (ethanol) and S (steroid) isozymes of horse liver alcohol dehydrogenase; yet amino acids sequences of these two enzymes differ at only 6 positions. Further, rapid divergence of substrate specificity is known in nature. The sequences of dehydrogenases acting on ribitol (from *Klebsiella*) glucose *(B. megaterium),* and ethanol (from *Drosophila*) show that these enzymes are homologous. Further, *pro-R* specific dehydrogenases acting on sorbitol (sheep) and ethanol (yeast) are also homologous (100).

These caveats do not require abandoning the "consensus" historical model. For example, in organisms having both lactate and 3-hydroxybutyrate dehydrogenases, it might be easier ("kinetically") to evolve a new lactate dehydrogenase from an old lactate dehydrogenase than from a 3-hydroxybutyrate dehydrogenase (9). This assumption may then be used to explain an absence of examples of homologous modern lactate and 3-hydroxybutyrate dehydrogenases. Likewise, divergence of substrate specificity might be influenced by positive selection pressures, while divergence in cryptic stereospecificity might occur only via neutral drift. The former might be expected to be faster than the latter, although little evidence supports this expectation.

Further, lactate and malate have considerable structural similarity, and the substrate conservation principle could be modified to allow substrate specificity to evolve within a general structural class. However, malate and 3-hydroxybutyrate also have similar structures (Figure 19). Whether lactate (enzymatically formed by transfer of the *pro-R* hydrogen) is viewed as having a structure more similar to 3-hydroxybutyrate (enzymatically formed by transfer of the *pro-S* hydrogen) or to malate (enzymatically formed by transfer of the *pro-R* hydrogen) is difficult to judge objectively. Further, glyoxylate reductase and L-lactate dehydrogenase might be considered to act on the same class of substrates; thus, their identical cofactor stereospecificity *(pro-R)* could be interpreted as evidence that the two are related. However, the stereospecificities of the two enzymes with respect to small molecule substrate are opposite (101).

Thus, the minimal historical model consistent with the data presented so far must presume: (a) a functionally constrained stereospecificity having random origin and no selectable value, yet rigorously conserved since the divergence of archaebacteria, eubacteria, and eukaryotes; (b) a common ancestor with a defined stereospecificity for enzymes acting on the same substrate from

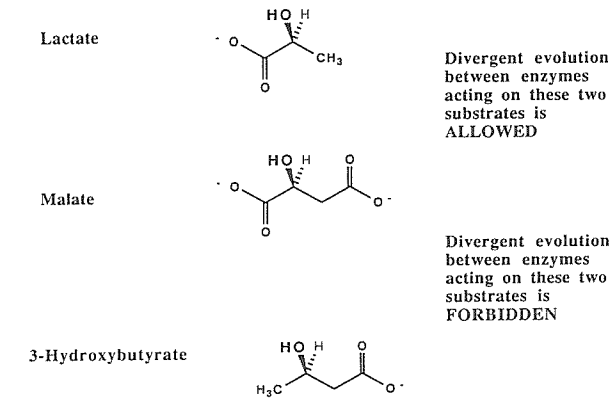


Figure 19. Historical models for stereospecificity in dehydrogenases must assume certain constraints in the divergence of substrate specificity.

plants, animals, and eubacteria (generally), and archaebacteria in the case of malate dehydrogenase; and (c) a substrate specificity that is highly conserved, with enzymes acting on one general class of substrates unable to evolve from those acting on another. This model, although rarely stated in its complete form, makes up the modern consensus regarding dehydrogenase stereoselectivity.

This consensus historical model has recently been modified by Schneider-Bernloehr and her colleagues, who argued that dehydrogenases related to *Drosophila* ADH (DADH) will transfer the *pro-S* hydrogen and those related to yeast ADH (YADH) will transfer the *pro-R* hydrogen (102). Further, they correlate enzymes in the first class as having low molecular weights (ca. 28,000 Daltons) and require no metal ion for catalysis, while those in the second class as having higher molecular weights (ca. 36,000 Daltons) and requiring Zn^{2+} for catalysis. This correlation was similar to that suggested by Jornvall and his colleagues (96). It has, however, a corresponding functional interpretation *(vide infra).*

The model loses some of its value in view of the homology between DADH and YADH themselves. Given this fact, the model makes no absolute predictions. Rather, it again reduces to the statement that dehydrogenases that are more closely related to DADH are more likely to transfer the *pro-S* hydrogen, while those more closely related to YADH are more likely to transfer the

pro-R hydrogen. This statement is never disputed, either by functional or by historical advocates.

Further, although molecular weight and metal ion requirements might be interpreted as evidence for homology in the absence of sequence data, such evidence is weak. Indeed, the predictive weakness of a historical model based on such evidence is illustrated by a variety of dehydrogenases from mammals. These include (a) a "mevaldate reductase" from rat liver, an enzyme with low molecular weight (27–30,000), apparently requiring no metal, but nevertheless transferring the *pro-R* hydrogen (103); (b) an "aldehyde reductase" from pig kidney, an enzyme with high molecular weight (42,000), transferring the *pro-R* hydrogen, but nevertheless containing no metal ion (104, 105); (c) "aldehyde reductases" from human and rat brain, enzymes with high molecular weight (40,000), transferring the *pro-R* hydrogen, but nevertheless containing no metal ion (106); and (d) several enzymes known under a variety of names, including "carbonyl reductase," "ketoprostaglandin reductase," and "xenobiotic ketone reductase," with molecular weights reported to range from 30,000 to 40,000, mostly transferring the *pro-S* hydrogen (107).

3. *Functional Models*

The consensus model can be challenged only by an alternative functional model that makes contrasting experimental predictions. Such a functional model was proposed in 1982 by one of us (108). Another model was proposed more recently by Srivastava and Bernhard (109).

The first functional model begins with a stereoelectronic analysis of NADH. In the reduced cofactor, a lone pair of electrons on nitrogen is adjacent to an antibonding orbital associated with the ribose carbon–oxygen bond (Figure 17). Two conformations, syn and anti, permit overlap between these two orbitals. The overlap is expected to distort the nitrogen from planarity; in the original model the dihydronicotinamide ring adopts a boat conformation as a result (108, 110).

Boat conformations in cyclohexadiene systems have some precedent (111), although the extent of the distortion is disputed (112). Recent crystallographic data have provided definitive data in the case of dihydronicotinamide (113). A boat conformation does not exist in simply substituted dihydronicotinamide rings, at least not in the ground state. Stereoelectronic interactions do appear to distort the nitrogen from planarity, however, and structural, spectroscopic, and computational data are consistent with the stereoelectronic model as applied to transition state structures and energies (113, 114).

The prediction from this stereoelectronic argument is that the *pro-R* hydrogen is transferred from the anti conformer of NADH, while the *pro-S* hydrogen is from the syn conformer. Thus, the stereoelectronic argument provides a chemical rationale for a correlation *(vide supra)* that was proposed much earlier on empirical grounds (94, 115).

Recently, glutathione reductase has been suggested as an enzyme that violates this correlation (116). The enzyme transfers the *pro-S* hydrogen but has been reported to bind NADH in an "anti" conformation (116). Although the coordinates needed to evaluate this possibility have not yet been published, private communication suggests that this "violation" is an interesting one (117). The crystallographic definition of the "anti" conformation in this case corresponds to a dihedral angle (C(2)-C(1)-N-C(2′) of approximately 74°. This is different from the stereoelectronic definition (where this angle is close to 0°). Indeed, NADH bound in the active site of glutathione reductase appears to have a conformation where the orbital on nitrogen containing the lone pair is orthogonal to the antibonding orbital of the carbon–oxygen bond. This may be related to the fact that glutathione reductase catalyzes a reaction involving flavin, thereby differing from other dehydrogenases that have been crystallized.

The functional model next assumes that enzymes adjust the internal equilibrium constant, defined as the ratio of enzyme-bound substrates to enzyme-bound products at equilibrium, to catalytically optimal values (118). The anti conformation of NADH is presumed to be a weaker reducing agent than the syn conformation. Thus, within a class of analogous reactions (for instance, the reduction of carbonyls to alcohols) enzymes evolved to reduce "easy-to-reduce" carbonyls should have evolved to transfer the *pro-R* hydrogen, while those evolved to reduce "hard-to-reduce" carbonyls should have evolved to transfer the *pro-S* hydrogen (108).

The functional model explains the common stereospecificities of lactate dehydrogenases, malate dehydrogenases, and 3-hydroxybutyryl CoA dehydrogenases as the products either of convergent evolution to, or of functional constraint on drift away from, *pro-R*, *pro-R*, and *pro-S* stereospecificity, respectively. The absolute stereospecificities are predictable based on the redox potentials of lactate, malate, and 3-hydroxybutyrate. As in any functional model, this argument requires no comment about the homology of any of these enzymes.

This model unifies stereochemical data for dehydrogenases interconverting alcohols and carbonyls. A correlation (108) between stereoselectivity and redox potential of natural substrate (Table 4b) divides dehydrogenases into three groups. Those reducing thermodynamically unstable carbonyl groups transfer the *pro-R* hydrogen; those reducing stable carbonyl groups transfer the *pro-S* hydrogen. In regions in between, where the equilibrium constant for the redox reaction is approximately 10^{-11} M, where the functional theory argues that stereoselectivity has little or no selective value, some enzymes transfer the *pro-R* hydrogen, while others transfer the *pro-S* hydrogen.

4. *Controversy*

A lively controversy has surrounded the functional model. The first challenge argued that three enzymes, 3-hydroxysteroid dehydrogenase from rat liver, 20-hydroxysteroid dehydrogenase from rat ovaries, and 21-hydroxysteroid dehydrogenase from bovine adrenals, all violate the correlation in Table 4b (119). The three "counterexamples" were assumed to "dispel" the correlation, rendering the mechanistic analysis above "not pertinent."

The discussion in the early sections of this review alerts the reader to three issues that must be considered before accepting this critique. First, the functional model makes experimentally testable predictions and has proven valuable in directing experimental work. Second, the three exceptions are advanced to dismiss a correlation that includes some 120 examples (95). Last, the three exceptions are advanced to dismiss a functional model. Therefore, it is relevant to ask whether the selectable functions of the enzymes discussed are correctly identified. As the functional model concerns properties of the substrate that the enzyme has evolved to act upon, the question of physiological substrate becomes central (9).

The literature almost certainly misassigns the physiological substrates of two of the three enzymes discussed (21-hydroxysteroid dehydrogenase and 3-hydroxysteroid dehydrogenase). In the first case, the k_{cat}/K_M for the alleged substrate (dehydrocortisone) is four orders of magnitude smaller than expected based on the k_{cat}/K_M values of enzymes catalyzing similar reactions. Further, dehydrocortisone has never been detected in natural tissues (95). Likewise, the "3-hydroxysteroid dehydrogenase" from liver almost certainly has not evolved to act specifically on 3-hydroxysteroids. The enzyme converts benzene dihydrodiol to catechol, reduces quinones to hydroquinones, and catalyzes redox reactions on phenylglyoxal, a variety of nitrobenzaldehydes and acetophenones, and chloral hydrate (95), all with the same facility with which it oxidizes 3-hydroxysteroids.

The natural substrate of 20-hydroxysteroid dehydrogenase from rat ovaries is also disputed (95). Nevertheless, the third violation of the correlation is the most likely of the three to be an actual challenge of the functional model.

The functional model has been the subject of other less coherent criticism. For example, it has been argued that lactate and malate dehydrogenases should not be included in the correlation as two separate entries, as the two enzymes are homologous (120). Though this may be true, the criticism begs the central question. Malate and lactate dehydrogenases are either homologous or they are not. If they are homologous, the question remains as to whether their common stereospecificity reflects conservation for functional reasons. If they are not homologous, the question remains as to whether their common stereospecificity reflects functional or accidental convergence.

Another criticism is based on the fact that some dehydrogenases act on a range of carbonyl substrates with a range of redox potentials. For example, lactate dehydrogenase and liver alcohol dehydrogenase act on substrates both with redox potentials in the *pro-R* region of the plot, and with redox potentials in the *pro-S* region (21). The criticism is confused. While the functional model argues that stereospecificity of a dehydrogenase has evolved functionally to reflect the redox potential of a natural substrate, it does not argue that the redox potential of a substrate *per se* determines stereospecificity. Stereospecificity is determined by the placement of amino acids in the active site. This placement has evolved over millions of years; it does not change in the laboratory when the enzyme is challenged with a new substrate with a different redox potential. Only if the enzyme is forced to evolve to accept the new substrate as a natural substrate does one expect stereospecificity to reverse so as to reflect a mechanistic imperative.

Of course, enzymes (such as liver alcohol dehydrogenase) may act naturally on a range of substrates. In these cases, functional theories based on a property of a specific substrate cannot make predictions, and data from such enzymes cannot be used to evaluate a functional theory.

Other criticisms are simply based on chemical misapprehensions. For example, the equilibrium constant for the overall reaction catalyzed by isocitrate dehydrogenase is less than 10^{-11}, and arguments have been made that the enzyme should transfer the *pro-S* hydrogen, not the *pro-R* hydrogen, to be consistent with the correlation in Table 4b (120). Of course, the quoted equilibrium constant for the overall reaction catalyzed by isocitrate dehydrogenase includes a decarboxylation step; the microscopic equilibrium constant for the redox reaction itself is much different. Indeed, based on an estimated equilibrium constant for the redox reaction alone, isocitrate dehydrogenases are expected to transfer the *pro-R* hydrogen. All examined (so far) do.

More extreme error is reflected by the recent direct comparison of the equilibrium constants for the reactions catalyzed by glutamate dehydrogenase, ethanol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase. The comparison was used to question the validity of the correlation in Table 4b (120). As the units of the three equilibrium constants are M^2, M, and unitless, respectively, the significance of a direct comparison of their numerical values is less than clear, and the relevance of such a comparison to an evaluation of the correlation and the functional model is minimal.

A final criticism enjoying currency at the time of this writing is that the functional model is too restricted in its scope, and that enzymes must meet too many criteria to serve as critical tests of the model. For example, a recent paper from Kozarich's laboratory argued that "the number of tests that a dehydrogenase must pass in order for it to be included in [the functional] hypothesis suggests that the NAD-dependent dehydrogenases are a too inherently complex class of enzyme to serve as a paradigm" (121).

The criteria that must be met for an enzyme presumed to serve as a critical

test for the functional model were defined in 1983: the enzymes must have well-defined physiological functions, act on unconjugated substrates (a criterion introduced to avoid enzymes that might operate via radical intermediates), and act on substrates with redox potentials one log unit from the break in the correlation in Table 4b. The first criterion is demanded by the logic of functional models; one cannot evaluate functional models for enzymatic behavior using enzymes whose physiological function is unknown. The second criterion is needed to evaluate any model based on assumptions regarding mechanism. A correlation presumably rooted in mechanism would be most remarkable if it is obeyed by enzymes employing different mechanisms. Finally, a functional model can be critically challenged only if a property that is presumed to be strongly favored by natural selection is not observed by experiment.

Analogous criteria are essential for evaluating *any* functional model; experiments that ignore them can draw no conclusions. They *must* be accepted, regardless of their "complexity." Nevertheless, in the case of alcohol dehydrogenases, these criteria scarcely limit opportunities for experimental test. Over 100 alcohol dehydrogenases fulfill the first requirement; 70 fulfill all three. Further, in the last five years, some 24 new enzymes have been examined as tests of the model. While not all of these enzymes have provided critical tests to distinguish between functional and historical models, several have *(vide infra)*.

Based on the criticisms reviewed above, one recent commentator concluded that "the drawbacks [of the functional model] are overwhelming" (21). That criticisms such as these are considered overwhelming might be regarded as an indication of the novelty of functional and historical analysis in general. However, it is unfortunate that these criticisms have led in the literature to the conclusion that "the validity of this new postulate should await the test of time" (21). Time does not test hypotheses. Given two opposing models, a historical model complicated by constraints needed to explain available data, and an untested functional model, experiments that distinguish between the two are needed.

5. *Distinguishing Functional and Historical Cases*

Many data have now been collected for the purpose of evaluating the relative merits of functional and historical explanations for the stereospecificity of dehydrogenases. In some cases (for example, the stereospecificity of "lactaldehyde reductase"), incorrect biochemical identification prevented meaningful interpretation of the data (110, 122–124). However, other data have substantially narrowed the scope of historical models. Other experiments have tested the chemical and kinetic rationales underlying the functional model, including the stereoelectronic effects *(vide supra)*, and the assumptions made by the functional model regarding internal equilibrium constants (118).

Some of the most interesting tests have examined the functional model purely as a logical formalism. The danger of a formalistic approach to model testing is that the formalism can be mistaken for underlying reality by the not-too-careful scientist. For example *(vide supra)*, the direct comparison of equilibrium constants with different units, the confusion over the physiological substrates of dehydrogenases, and the confusion over the mode of cofactor binding by glutathione reductase all reflect problems in identifying formalism. However, once the potential for such confusion is recognized and avoided, logical formality is a most valuable property in a model. In particular, the formalism offers the opportunity for "mechanical" tests of the model, and can be directly applied to new systems as a first step in exploratory research to detect other selected traits.

For example, drift of stereospecificity is expected only in alcohol dehydrogenases where the functional model predicts that stereospecificity is a weakly selected trait. The formalism of the correlation in Table 4b suggests that such enzymes act on substrates near the "break" in the correlation. Stereochemical heterogeneity is therefore expected in enzymes catalyzing the interconversion of CH_2–CH_2–OH and CH_2–CHO groups, as the equilibrium constant for this reaction is approximately 10^{-11}M.

Clear examples of such stereochemical diversity were first discovered through efforts to discover stereochemical heterogeneity in such enzymes. Ethanol dehydrogenase from *Drosophila* and hydroxymethylglutaryl-CoA (HMG-CoA) reductase from *Acholeplasma* were found to have stereospecificities opposite to those of analogous enzymes from previously studied organisms (95, 125). To rule out the possibility that the stereospecificities of dehydrogenases in *Drosophila* and *Acholeplasma* are *generally* different from stereospecificities of dehydrogenases found in other organisms, the stereospecificities of many dehydrogenases from the two organisms were determined (Table 5). The only enzymes displaying stereochemical heterogeneity were the two mentioned above (44, 125).

6. *The Simplest Historical Model*

The new data collected from these studies require a re-evaluation of the consensus historical model for dehydrogenase stereospecificity. The following facts are especially important:

Fact 1: Certain enzymes catalyzing analogous reactions appear to be non-homologous but nevertheless have the same stereospecificities. Examples include: glucose-6-phosphate dehydrogenase and glucose dehydrogenase (non-homologous judging by sequence) (126); metal-dependent alcohol dehy-

Table 5
Stereospecificities of Alcohol Dehydrogenases from a Variety of Sources. *pro-R* Stereospecificity is Designated "A", and *pro-S* Stereospecificity is Designated "B"

Prediction of functional theory for: Substrate	Experimentally Determined Stereospecificity of Dehydrogenase from:						
	mammal	insect	plant	fungus	eubac-teria	myco-plasma	archae-bacteria
A							
Malate	A	A	A	A	A	?	A
Lactate	A	A	A	A	A	A	A
A/B							
Ethanol	A	B	B	A	A	A	A
Hydroxymethyl glutaryl CoA	A	?	?	A	?	B	?
B							
Glucose-6-P	B	?	?	B	B	?	?
3-Hydroxybutyryl derivatives	B	?	?	B	B	?	?

drogenase from *Saccharomyces* and *Zymomonas* (127, 128) (non-homologous judging by sequence and metal ion requirement); dihydrofolate reductases (non-homologous judging by sequence and tertiary structure) from two bacterial plasmids (129, 130).

Fact 2: Certain enzymes that appear homologous (at least in one domain) nevertheless have the opposite stereospecificities. Examples include: the dinucleotide binding domains of the ethanol dehydrogenases from yeast (*pro-R* specific) and *Drosophila melanogaster* (*pro-S* specific) (homologous by sequence comparisons, Figure 20) (95, 96); the dinucleotide binding domains of glyceraldehyde-3-phosphate dehydrogenases (*pro-S* specific) and lactate dehydrogenases (*pro-R* specific) (homologous based on comparisons of their crystal structures) (93, 94); mammalian aldehyde dehydrogenase (*pro-R* specific) and aspartate-beta-semialdehyde dehydrogenase from E. coli (*pro-S* specific) (possibly homologous based on limited sequence similarities) (131); enoyl CoA reductase from yeast (*pro-S* specific) and from mammal (*pro-R* specific) (possibly homologous based on limited sequence similarities) (132, 133).

Fact 3: Certain enzymes that are clearly homologous act on substrates with quite different structures. Examples include: ethanol and sorbitol dehydrogenases (96); ribitol dehydrogenase, glucose dehydrogenases, and ethanol dehydrogenase from *Drosophila* (96, 126).

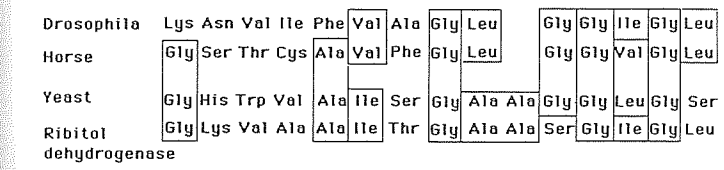


Figure 20. Sequence comparisons in alcohol dehydrogenases from four organisms.

Fact 4: Stereospecificity in alcohol dehydrogenases correlates with the redox potential of the natural substrate (Table 4b) (108).

Fact 5: In one case, enzymes with unknown pedigree acting on the same substrate have different stereospecificities: the hydroxymethylglutaryl-CoA (HMG-CoA) reductases from rat and yeast have stereospecificities opposite to that from *Acholeplasma* (134, 135).

Fact 6: Certain enzymes with unknown pedigree acting on analogous substrates with opposite chiralities have the same stereospecificities. Examples include: D- and L-lactate dehydrogenases, both transferring the *pro-R* hydrogen (21, 136); L-fucose dehydrogenase and D-glucose dehydrogenase (stereochemistry at C-1 of the sugars has the opposite absolute sense), both transferring the *pro-S* hydrogen (21, 136).

Fact 7: In general, enzymes from widely divergent sources catalyzing a redox reaction far from the break in the correlation (Table 4b) share a common stereospecificity. These are now best exemplified by the malate dehydrogenases mentioned above, including enzymes from eubacteria, archaebacteria, and eukaryotes (88, 89, 136).

A difficult task faces a historical model builder. The enzymes in group 1 appear to be examples of convergent evolution, suggesting functional adaptation. For the pair of dihydrofolate reductases, the argument for convergence is quite strong. In the other cases, however, an advocate of a historical model for dehydrogenase stereospecificity might argue that a *lack* of sequence similarity is inadequate to *rule out* distant homology, implying that stereospecificity is more highly conserved than virtually every other trait in a protein (21).

However, the enzymes in group 2 contradict this implication. Enzymes with detectable (if small) sequence similarities have opposite stereospecificities, making it difficult to state the level of sequence divergence that is required before stereospecificity can be reversed or, conversely, the level of sequence identity that safely predicts that two enzymes will have identical stereospecificity. In other words, the statement "cofactor stereospecificity is highly conserved" is difficult to define objectively. It appears safe to assume,

however, that a pair of natural dehydrogenases with sequence identities greater than 50% will have the same stereospecificities.

A historical model must account for the fact that in some classes of dehydrogenases, evolutionary processes have not created stereochemical diversity in the time separating archaebacteria, eubacteria, and eukaryotes (even though diversity is known in virtually every other biochemical behavior in this range of organisms), while in other cases, evolutionary processes have produced stereochemical diversity in much less time. Further, it must explain why divergence of substrate specificity appears facile (Fact 3), yet evolution of substrate specificity following deletion events has not created stereochemical diversity in most dehydrogenases. Finally, it must account for Fact 7, that stereospecificity within one subclass of dehydrogenases, those interconverting alcohols and ketones, correlates with the redox potential of the natural substrate. This requires a model with at least six hypotheses (125):

Hypothesis 1: To explain the absolute conservation of stereospecificity of malate dehydrogenases (MDH) (compared to HMG-CoA reductases and ethanol dehydrogenases), the drift of stereospecificity in MDH is presumed to be lower than in other dehydrogenases. Likewise, the model must presume that it is impossible to replace MDH by the evolution of the substrate specificity of a *pro-S* specific enzyme (for example, a 3-hydroxybutyrate dehydrogenase) via a deletion–replacement event *(vide supra)*. This prohibition applies also to all other dehydrogenases (with a bit less rigor) except for ethanol dehydrogenases and HMG-CoA reductases (137).

While this hypothesis is arbitrary, it might be justified by assuming that most dehydrogenases (including malate dehydrogenases and lactate dehydrogenases) are more "essential" to the survival of the host organism than ethanol dehydrogenases and HMG-CoA reductases. Thus, structural variation (as a prelude to drift in stereospecificity) and deletion are presumed to be more selectively disadvantageous in malate and lactate dehydrogenases than in ethanol dehydrogenases and HMG-CoA reductases (138).

Hypothesis 2: To explain the divergent stereospecificities of HMG-CoA reductase from *Acholeplasma* versus those from yeast and rat, these two enzymes are presumed to be not homologous. This seems more reasonable than proposing that HMG-CoA reductase is uniquely capable of drifting compared with the other enzymes.

Hypotheses 3: It is difficult to argue that stereospecificity with respect to substrate can drift while stereospecificity with respect to cofactor cannot. Therefore, D- and L-lactate dehydrogenases and D- and L-sugar oxidases each are presumed to have arisen independently, and their common stereospecificities are accidental (25% probability, assuming that stereospecificity serves no selectable function).

Hypothesis 4: The dinucleotide binding domains of the ethanol dehydrogenases from *Drosophila* and yeast are almost certainly homologous. Further, as ethanol dehydrogenase is the only enzyme from *Drosophila* that has "abnormal" stereospecificity (11), all dehydrogenases *except* ethanol dehydrogenase must be presumed to be homologous in *Drosophila* (44, 95). Either the drift of stereospecificity is more rapid for enzymes acting on ethanol than for other enzymes or ethanol dehydrogenases are more easily replaced by deletion/replacement events than are other dehydrogenases.

Hypothesis 5: To explain the correlation between redox potential and stereospecificity observed in alcohol dehydrogenases (Fact 1) drift in substrate specificity between enzymes must be allowed. To avoid a contradiction with Hypothesis 1 (where crossover of substrate specificity is forbidden except for ethanol dehydrogenases and HMG-CoA reductases), enzymes are allowed to evolve to adopt other substrate specificities only if such crossover does not create an enzyme that oxidizes an alcohol with a K_{eq} for the reaction of less than 10^{-11} M from one oxidizing an alcohol with a K_{eq} greater than 10^{-11} M, and vice versa. Thus, a malate dehydrogenase can evolve to become a lactate dehydrogenase but not a 3-hydroxybutyrate dehydrogenase. The structural similarities of oxaloacetate, pyruvate, and acetoacetate (Figure 19) make it difficult to provide a chemical rationale for this special constraint on the divergence of substrate specificity.

Hypothesis 6: The convergent evolution of the stereospecificities of dihydrofolate reductases, glucose dehydrogenases, and metal-dependent alcohol dehydrogenases (Fact 1) must now be viewed as accidental (12.5% probability, assuming that stereospecificity serves no selectable function).

We develop this historical model not because we necessarily believe that it is attractive in comparison with alternative models, but rather because none of the many advocates of historical models have ever rigorously stated what such models must entail to be consistent with fact. Nevertheless, many members of the biochemical community remain strong advocates of historical models as explanations of stereospecificity in alcohol dehydrogenases and in enzymatic stereospecificity in general.

The fact that a historical model must be so complex to be consistent with known fact does not mean that it is wrong (nor that a contrasting functional model is correct). However, the defenders of the consensus model can be faulted for not constructing a formally clear statement of their position, dismissing without sufficient justification several correlations that suggest experimentally testable historical and functional models, and not examining the logical consequences of these models in a rigorous way. Had these expedients been followed, we believe that the historical consensus would be less dogmatically defended.

7. *Further Tests for the Model*

Any set of data can be explained by a historical model that assumes an arbitrary number of ancestral enzymes interrelated by an arbitrary pedigree. However, the assumption that stereospecificity is so tightly coupled to catalytic activity that it cannot be reversed without destroying catalytic activity is critical even to the highly modified historical model outlined above. In particular, it is necessary to explain the highly conserved stereospecificities of malate dehydrogenases. This assumption can be examined experimentally.

Inspection of the crystal structures of alcohol dehydrogenase suggests that the mode of cofactor binding (anti in this enzyme) is determined by two interactions (Figure 21) (139). On one side of the nicotinamide ring, the enzyme offers residues that form hydrogen bonds to the carboxamide group of the nicotinamide ring. On the other side, the side chain of residue 203 (corresponding to Leu 182 in yeast alcohol dehydrogenase) sterically blocks the carboxamide group in the syn conformation.

Thus, a point mutation in the active site that replaces the bulky side chain of residue 182 in yeast alcohol dehydrogenase should diminish the stereospecificity of the enzyme. (It would not, of course, destroy the stereospecificity entirely, as the hydrogen bonds favoring the anti conformation would remain, even though the steric bulk obstructing the syn conformation is gone.) The historical model predicts that any loss in stereospecificity should be accompanied by a large loss in catalytic activity. Site-specific mutagenesis techniques allow the deliberate synthesis of proteins carrying amino acid replacements (140), and this prediction was not sustained when the mutant was prepared and examined (139).

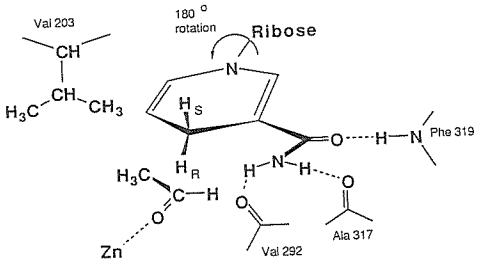


Figure 21. Structural determinants of stereospecificity in alcohol dehydrogenase.

Replacing Leu 182 in yeast alcohol dehydrogenase by Ala leads to a 10,000-fold decrease in the stereospecificity of alcohol dehydrogenase (with respect to cofactor). However, the k_{cat} of the mutant was approximately 40% and 70% (for the oxidation of ethanol and the reduction of acetaldehyde, respectively) of the wild type. Essentially no loss in catalytic activity accompanied the large loss in stereospecificity. Thus, stereospecificity in this dehydrogenase is not tightly coupled to catalytic activity, as historical models normally assume to explain the patterns of stereospecificity observed in dehydrogenases. This suggests that stereospecificity would diverge if it were not directly subject to functional constraints. The fact that stereospecificity has *not* drifted in many dehydrogenases then supports the argument that its drift is directly constrained by function. It remains to be determined whether a complete reversal of stereospecificity can be achieved in this enzyme by the introduction of point mutations.

8. *Extension of the Functional Model*

The discussion above has focused almost exclusively on dehydrogenases interconverting alcohols and the corresponding carbonyl compounds. Enzymes in this subclass of dehydrogenases should have some degree of mechanistic homogeneity and therefore should be directly comparable. However, the formalism of the functional model, especially the relationship between stereospecificity and redox potential, might be extended to other classes of dehydrogenases. For example, the *pro-R* hydrogen might be used to reduce reactive substrates (and the *pro-S* hydrogen to reduce unreactive substrates) in other subclasses of dehydrogenases acting with similar mechanisms on an analogous series of substrates.

In such exploratory research, one recognizes that the formalism is being stretched past the limits of the underlying chemical theory. This simply means that one can neither be too excited if the extension is predictive nor too disappointed if it is not. Conversely, if one is an aggressive critic of the model, one cannot be too excited if the extension is not predictive nor too disappointed if it is.

For example, redox reactions interconverting hemiacetals and their corresponding esters are analogous to redox reactions interconverting simpler alcohols and ketones. The equilibrium constant has the same units for both reactions (permitting direct formal comparison). Further, if the formalism developed for alcohol–ketone interconversions is applied to hemiacetal–lactone interconversions, the prediction is that all enzymes catalyzing the latter transformation will transfer the *pro-S* hydrogen (141).

This happens to be the case (21, 136). At present, over a dozen such enzymes have been studied stereochemically. Some come from widely divergent

organisms, and several are not obviously homologous; all transfer the *pro-S* hydrogen. This is true for L-fucose dehydrogenase and D-glucose dehydrogenase, enzymes that act on substrates of opposite chirality. Historical models that postulate that these enzymes have the same stereospecificity because they are descendants of a common ancestor must explain why stereospecificity is highly conserved with respect to cofactor but not with respect to substrate. In other cases, sequence data argue that homology within this class of dehydrogenases, if it exists at all, must be very distant.

The formalism can also be applied to enzymes catalyzing redox reactions between flavins and nicotinamide cofactors. Often, the redox potential of the enzyme-bound flavin can be measured directly. This avoids the need to correlate stereospecificity with a thermodynamic property of the substrate measured outside the active site—problematical given the acknowledged fact that enzyme-substrate binding interactions could significantly perturb this property. Values for the reduction potential of enzyme-bound flavin range from −465 mV to +145 mV (10 pK_{eq} units) (142). The formalism again predicts that flavoenzymes transfer the *pro-S* hydrogen to flavins with more negative redox potentials and enzymes transfer the *pro-R* hydrogen to flavins with less negative potentials.

Table 6 shows an apparent trend in this direction. The reader should be cautioned that the reduction potentials reported for enzyme-bound flavins are disappointingly variable from laboratory to laboratory and method to method. Solubilization of membrane-bound flavoenzymes can cause large changes in measured redox potentials. Thus, a selection of the data must be made. While the selection inevitably requires subjective decision and clearly may prejudice conclusions, the data in Table 6 were chosen based on their apparent reliability, consistency, and availability.

Another extension of the formalism explains the fact that all alcohol dehydrogenases that employ a metal ion display *pro-R* stereospecificity (128). The presence of a metal ion as an electrophilic activator is expected to destabilize all carbonyl compounds (with respect to the corresponding hydroxyl compounds) when compared to electrophilic activation by a proton (128). Thus, the break point in the correlation is expected to be shifted down in the correlation (Table 4b); in other words, it should be possible to find enzymes reducing the same substrate where the enzyme employing a metal for electrophilic activation transfers the *pro-R* hydrogen, while the enzyme employing a proton for the same purpose transfers the *pro-S* hydrogen. The recent demonstration that the iron-dependent alcohol dehydrogenase from *Zymomonas mobilis* transfers the *pro-R* hydrogen is consistent with this model (128).

In other areas, the formalism appears to be difficult to apply. For example, both *pro-R* and *pro-S* stereospecificities are known in enzymes catalyzing the oxidation of aldehydes to carboxylic acid derivatives (Table 7) (21,

Table 6
List of Stereospecificities of Flavin-Dependent Dehydrogenases Ordered by Redox Potential

Enzyme Name	Cofactor	A/B[a]	E1	E2	E3	E4 (mv)	Titrant	*p*H/T(°C)	E_{corr}[b]	Ref
Putidaredoxin Red.	NADH	B		−283			Dithionite	—/—	−283	*c*
Lipoamide DH	NADH	B		−280		−346	Dithionite	7.0/25	−280	*d*
NADPH Ox. (Superoxide)	NADPH	A	−304	−258			Dithionite	7.0/25	−280	*e*
Adrenodoxin Red.	NADPH	B		−291			NADH	7.5/—	−261	*f*
Thioredoxin Red.	NADPH	B		−243		−260	NADH	7.0/12	−260	*g*
Cytochrome b_5 Red.	NADH	A		−258			Dithionite	7.0/25	−258	*h*
Old Yellow Enzyme	NADPH	A	−245	−215			Dithionite	7.0/25	−230	*i*
Cytochrome P-450 Red.	NADPH	A	−110	−270	−290	−365	Dithionite	7.0/25	−190	*j*
FMN Reductase	NADH	A		−200			—	7.0/25	−200	*k*

[a]Reference 136; *pro-R* stereospecificity is designated "A", and *pro-S* stereospecificity is designated "B". [b]Two electron reduction potentials corrected to *p*H 7.0, 25°C (O'Donnell, M. E.; Williams, C. H. *J. Biol. Chem.* **1983**, *258*, 13795–805). [c]Marbach, W. J.; Thesis, University of Illinois, 1972. [d]Matthews, R. G.; Williams, C. H. *J. Biol. Chem.* **1976**, *251*, 3956–64. [e]Kakinuma, K.; Kaneda, M.; Chiba, T.; Ohnishi, T. *J. Biol. Chem.* **1986**, *261*, 9426–32. [f]Lambeth, J. D.; Kamin, H. *J. Biol. Chem.* **1976**, *251*, 4299–306. [g]O'Donnell, M. E.; Williams, C. H. *J. Biol. Chem.* **1983**, *258*, 13795–805. [h]Iyanagi, T. *Biochem.* **1977**, *16*, 2725–2730. [i]Stewart, R. C.; Massey, V. *J. Biol. Chem.* **1985**, *260*, 13639–47. [j]Iyanagi, T.; Makino, N.; Mason, H. S. *Biochem.* **1974**, *13*, 1701–10. [k]Fisher, J.; Walsh, C. T. *J. Am. Chem. Soc.* **1974**, *96*, 4345–6.

136). Several mechanistic types of enzymes are involved, including enzymes that presumably oxidize thiohemiacetals between the substrate and a cysteine side chain to yield thioesters covalently bound to the enzyme, enzymes that oxidize thiohemiacetals between two substrates to produce non-covalently bound thioesters, and enzymes that produce mixed anhydrides with phosphoric acid.

Enzymes forming a carboxylic acid as a product generally belong to the first mechanistic class. Five examples transfer the *pro-R* hydrogen, one transfers the *pro-S* hydrogen (21). Enzymes forming acyl phosphates transfer the *pro-S* hydrogen, however; these presumably also proceed via an acyl–enzyme intermediate (although perhaps higher in energy). Chemical intuition suggests that the thiohemiacetal–thioester equilibrium constant is smaller than the hemiacetal–ester equilibrium constant. As all of the latter transfer the *pro-S* hydrogen, the formalism makes no useful prediction regarding the stereospecificities of members of the first class. The best that the functional model at present can do is argue that the thiohemiacetal–thioester equilibrium constant is near the break in the correlation. This suggests possibility for further experiments. Historical models fare little better. Limited sequence similarities exist between the *pro-S* hydrogen and the *pro-R* hydrogen enzymes, suggesting that dehydrogenases with opposite stereospecificities are more closely homologous than enzymes with similar stereospecificities where homology is presumed to be an explanation for this similarity (131).

Amino acid dehydrogenases present another stereochemical problem. Glutamate, leucine, diaminopimelate, and phenylalanine dehydrogenases all transfer the *pro-S* hydrogen, while alanine dehydrogenase transfers the *pro-R* hydrogen (21, 136). There is no obvious difference in redox potentials that might explain this difference (Table 8). Nevertheless, limited data suggest that the stereochemical difference between alanine and other amino acid dehydrogenases is maintained in a range of organisms (21, 136). More data (especially from enzymes that are evolutionarily distant) are necessary before one can decide whether stereospecificity in amino acid dehydrogenases is adaptive or not.

Further, the stereospecificity with respect to cofactor for the reduction of alpha-beta unsaturated thioesters appears to be a non-selected trait. Examples are discussed in detail below.

Less conventional applications of the formalism are also possible. For example, the enzyme NADPH-NAD^+ transhydrogenase catalyzes the transfer of "hydride" between NAD^+ and $NADP^+$. The *pro-S* hydrogen of NADPH is removed; the *pro-R* hydrogen of NADH is removed (60). The distinction is subtle, and the functional model, combined with the notion of "descending staircase internal thermodynamics" (13), predicts that the choice of hydrogen is determined by the physiological direction of flux through this enzyme. In

Table 7
Stereospecificities and Equilibrium Data of Enzymes Catalyzing the Nicotinamide Cofactor-dependent Oxidation of Aldehydes

Enzyme	E.C.#	Spec.[a]	pK_{eq}[b]	Source
A. Aldehyde dehydrogenases producing carboxylic acids				
UDP-glucose dehydrogenase	1.1.1.22	B	—	Bovine
Histidinol dehydrogenase	1.1.1.23	A	—	*Neurospora*
Aldehyde dehydrogenase	1.2.1.3	A	—	Yeast, Bovine, Equine
Aminobutyraldehyde dehydrogenase	1.2.1.19	A	—	*Pseudomonas*
Succinate semialdehyde dehydrogenase	1.2.1.24	A	—	*Pseudomonas*
Aminopropionaldehyde dehydrogenase	1.2.1.X	A	—	Chicken
B. Aldehyde Dehydrogenases producing phosphate anhydrides				
Aspartate semialdehyde dehydrogenase	1.2.11	B	6.5[c]	*E. coli*
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	B	7.3[d]	*E. coli*, *Acholeplasma*, Yeast, Horseshoe crab, Sturgeon, Bee, Turkey, Rabbit, Pea
C. Aldehyde Dehydrogenases producing thioesters				
Hydroxymethylglutaryl-coenzyme A reductase	1.1.1.34	A,B	—	*Acholeplasma*, Yeast, Rat
Aldehyde dehydrogenase (acylating)	1.2.1.10	A	3.9[e]	*Clostridium*
Cinnamoyl-coenzyme A reductase	1.2.1.44	B	3.3[f]	*Forsythia*
Glyoxylate dehydrogenase	1.2.1.17	B	5.1[g]	*Alcaligenes*

[a] Reference 136; *pro-R* stereospecificity is designated to "A", and *pro-S* stereospecificity is designated "B". [b] K_{eq} is unitless. [c] Black, S. *Meth. Enz.* **1962**, *5*, 823. [d] Cori, C. F.; Velick, S. F.; Cori, G. T. *Biochim. Biophys. Acta* **1950**, *4*, 16. [e] Stadtman, E. R.; Burton, R. M. *Meth. Enz.* **1955**, *1*, 518. [f] Lüderitz, T.; Grisebach, H. *Eur. J. Biochem.* **1981**, *119*, 115. [g] Quayle, J. R. *Biochem. J.* **1963**, *87*, 368.

Table 8
Stereospecificities of Amino Acid Dehydrogenases

Enzyme	E.C. no.	pK_{eq}	Spec.
Alanine dehydrogenase	1.4.1.1	13.2	A
Glutamate dehydrogenase	1.4.1.2	13.3	B
Glutamate dehydrogenase ($NADP^+$)	1.4.1.4	13.3	B
Leucine dehydrogenase	1.4.1.9	13.0	B
Glycine dehydrogenase	1.4.1.10	10.6	
3,5-diaminohexanoate dehydrogenase	1.4.1.11	9.4	
2,4-diaminopentanoate	1.4.1.12	14.0	
Diaminopimelate dehydrogenase	1.4.1.16		B
Phenylalanine dehydrogenase			B

Stereochemical data is from reference 136. *Pro-R* stereospecificity is designated "A" and *pro-S* stereospecificity is designated "B". The equilibrium constant has units M^2.

the beef heart enzyme that has been studied, the direction of flux (oxidation of NADPH) and the observed stereospecificities are consistent with the functional model. It would be interesting to see if this is true for transhydrogenases from other sources, especially those that catalyze the reverse reaction (the oxidation of NADH) under physiological conditions.

Finally, in elegant studies of model compounds, Ohno and his colleagues have recently discovered that the stereospecificity of "hydride" transfer is dependent on the redox potential driving the reaction (143). This is in a model system; there is no enzyme involved, and the dihydronicotinamide ring has a structure distinctly different from that in the natural cofactor. Yet the apparent control of stereospecificity in solution by a thermodynamic property of the reacting molecules parallels directly the functional model proposed to explain the stereospecificity of dehydrogenases. The parallel between chemistry inside and outside the active site is most remarkable, especially as the analogy appears to be purely formal.

D. Addition-Elimination Reactions

Both "syn" and "anti" transition state geometries are possible for the addition of a proton and a nucleophile (HX) to an olefin and, in the microscopic reverse reaction, the elimination of the elements HX to form an olefin. Intermediary geometries form twisted olefins that presumably are not allowed (14).

Stereoelectronic considerations suggest that the "anti" transition state is slightly preferred in concerted elimination reactions, as the electrons from the bond to the departing electrophile are oriented such that they can move directly into the antibonding orbital of the bond to the departing nucleophile

(144). However, stereoelectronic principles make no explicit statement regarding the preferred stereochemical course of a stepwise addition/elimination reaction.

Many enzymes catalyze addition/elimination reactions, and both syn and anti geometries are known. "Anti" stereoselectivity is found in the addition/elimination reaction catalyzed by fumarase, aspartate ammonia lyase (145–148), arginosuccinase (149), aconitase (150), oleic acid dehydratase from *Pseudomonas* (151), adenylosuccinase (152), malease (153), enolase (154), phenylalanine ammonia lyase (155), and histidine ammonia lyase (156). Other reactions that are analogous in the broadest sense also proceed via "anti" transition states. For example, the decarboxylative elimination of 5-pyrophosphomevalonate (3,5-dihydroxy-3-methylpentanoic acid, 5 pyrophosphate) proceeds with an overall "anti" stereoselectivity (157). The decarboxylation of cis-aconitate to give itaconate also proceeds with "anti" stereochemistry (158).

Enzymes catalyzing "syn" addition/elimination reactions include cis-cis-muconate cycloisomerase (159), enoyl-CoA hydratase (160), dehydroquinate synthase (161), beta-hydroxydecanoylthioester dehydratase (162), yeast fatty acid synthetase (163), methylglutaconyl-CoA hydratase (164), and dehydroquinate dehydratase (165). Methacrylate is converted to beta-hydroxyisobutyrate, presumably via the intermediacy of methacrylyl-CoA, by a "syn" addition (166). The 3-dehydroshikimate is converted to protocatechuate via a "syn" elimination (167). Cyclization of carboxymuconic acid to give beta-carboxymuconolactone is also "syn" (168).

A simple proposal to account for the stereochemical diversity in these cases assumes that the elimination reactions catalyzed by the first class of enzymes are concerted, but that those catalyzed by the second class of enzymes are stepwise. For stepwise reactions, one has a choice of several functional hypotheses that argue that "syn" elimination is the preferred stereochemical course. The simplest is that active sites with a single base are "better" for a stepwise elimination that those with multiple functional groups (8). In a "syn" elimination, the base can act both to abstract a proton in the first step and then, in the protonated form, to assist the departure of the nucleophilic group in the second step.

Simple examination of the structure of the substrates lends support to the hypotheses. In many cases where the elimination is "syn" (and presumably stepwise), the departing proton leaves behind a relatively stable anion (e.g., alpha to a thioester or a ketone). In those cases where the elimination is "anti" (and presumably concerted), however, the proton that is leaving is adjacent to a group that is *not* chemically well suited to stabilize an adjacent carbanion (for instance, a carboxylate group). In the first case, a hypothetical "carbanion" intermediate is relatively stable; the hypothesis of a stepwise re-

action is reasonable. In the second case, the hypothetical "carbanion" is not stable; the hypothesis of a concerted reaction is therefore again reasonable.

This sort of argument has some precedent in the literature. For example, Schwab and Klassen proposed that the stereochemical course of allylic rearrangements likewise is stepwise or concerted, syn or anti, depending on the intrinsic reactivity of the substrate molecule (169). While there are perhaps some exceptions to this proposal (170–172), they are best treated in the manner discussed above. The suggestion remains important as a working hypothesis.

Recent experiments by Cleland and his coworkers, however, argue strongly against this simple interpretation. For example, isotope effect studies argue in several cases (phenylalanine ammonia lyase and fumarase) that the reaction proceeds via a stepwise mechanism (19). Though certain caveats may apply to this conclusion (173), the simplest explanation apparently cannot be correct.

A remarkably simple formal rule can be proposed to explain the stereospecificity of enzymatic elimination reactions. This rule is based on consideration of *both* the stability of the carbanion and the carbocation that would be generated in a fully disassociative mechanism. The stability of the carbanion corresponds to the pKa value of its conjugate acid. The stability of the carbocation corresponds to its pK_{R+} (174). Tables of these values for a range of compounds are now available, and values can be estimated for carbanions and carbocations that have not been studied.

Figure 22 shows a selection of enzymes arranged according to these values. Readily apparent is the fact that a syn transition state is found when *either* the carbanion or carbocation is relatively stable. Anti transition states are found in the "box" in the upper left hand side of Figure 22, where neither the cation nor the anion is stable. Indeed, for predictive purposes, the rule can be stated formally: If either the pK_a of the carbanion is less than 20, or the pK_{R+} of the carbocation is greater than -10, the syn transition state will be preferred. This formalism makes mechanistic sense in terms of the lifetime of the proposed intermediate in a stepwise mechanism (175). As a formalism, it can be tested without recourse to assumptions about the detailed mechanism of the reaction.

One might attempt to apply this rule to three enzymes that catalyze three quite similar reactions. These reactions are: (a) the cyclization of *cis-cis*-muconate to give muconolactone, a "syn" addition reaction; (b) the cyclization of *cis-cis*-carboxymuconate to give beta-carboxymuconolactone, also a "syn" addition reaction; and (c) cyclization of *cis-cis*-carboxymuconate to give gamma-carboxymuconolactone, an "anti" addition (Figure 23) (176). The stereospecificities of these enzymes were examined by Kozarich and his group to test an intriguing hypothesis of Ornston that sequential enzymes in a pathway are related (83). The last enzyme was expected to have the same stereospecificity as the first two.

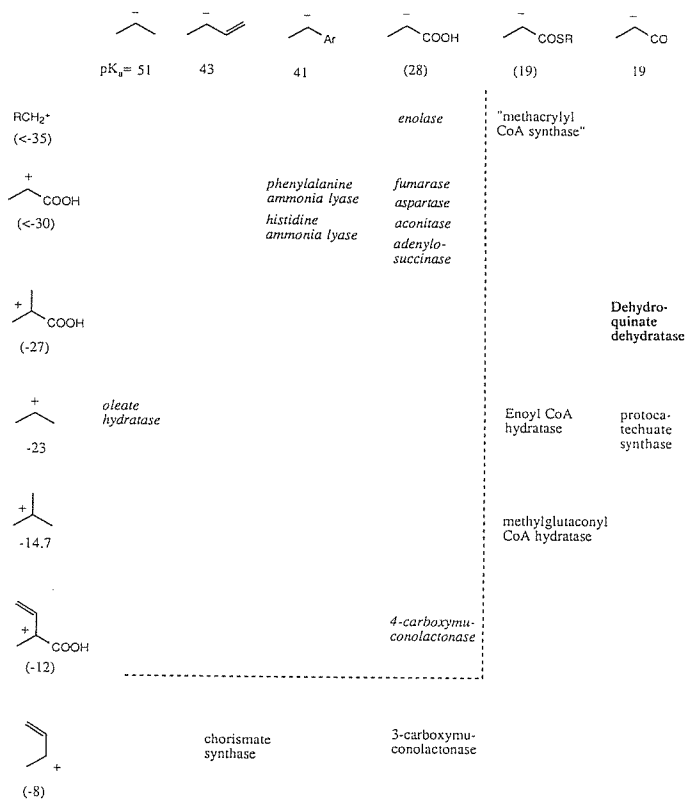


Figure 22. Stereospecificity in dehydratases correlates with substrate structure.

The fact that the third enzyme catalyzed an "anti" addition/elimination reaction contradicted these expectations. The investigators could propose only that perhaps the "anti" addition to a dicarboxylated double bond proceeded via a transition state or lower energy than a "syn" addition because

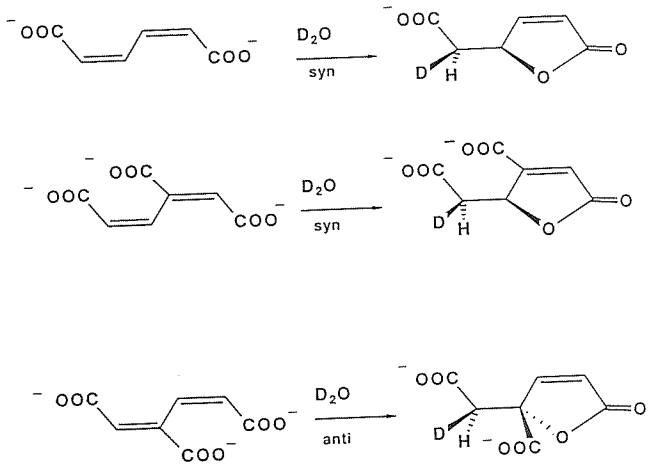


Figure 23. The different stereospecificities of dehydratases catalyzing similar reactions.

the two carboxylate anions are somewhat farther apart in the "anti" transition state than in the "syn" transition state.

This hypothesis is problematic from a theoretical point of view, as there is no evidence that suggests that electrostatic interactions of this type are a major consideration in the evolution of active sites. However, the data are consistent with the rule suggested above. The pK_a for the removal of a hydrogen from all three substrates is expected to be approximately the same; here, we use the value of 24.5 for the pK_a of the protons alpha to the carbonyl function of an ester. Thus, the reaction will be anti except in cases where there is an especially stable carbocation. This is in fact the case for the first two substrates; a secondary allyl carbonium ion has a pK_{R+} of approximately -8, and a syn transition state is expected.

In the third case, the allyl cation is adjacent to an extra electron withdrawing group, and this is expected to lower the pK_{R+} by three orders of magnitude. By the formalism proposed above, this carbocation is insufficiently stable to allow the syn transition state geometry.

This formalism is subject to many experimental tests. For example, the conversion of 2,3-dihydroxy-3-methylbutanoate to 2-keto-3-methylbutanoate

in the biosynthesis of valine involves the elimination of water. The formal carbanion (alpha to a carboxylate) has a pK_a value of 28; the formal carbonium ion (tertiary) has a pK_{R+} value of -14.7. Thus, the formalism (Figure 22) predicts that the stereospecificity of the elimination be anti.

V. FATTY ACID SYNTHESIS: MANY STEPS, MANY STEREOCHEMICAL DISTINCTIONS

Should the reader by now be convinced that the evolutionary analysis of stereochemical data in enzymology is too complicated to be worth the effort, we must repeat the statement made at the beginning of this review: one cannot interpret stereochemical data (or any other data) in enzymology without such an analysis. This is the primary incentive to examine and test the models developed above. However, a brief discussion of the stereochemical details of fatty acid biosynthesis may provide further encouragement, as it shows how assembling a critical mass of data can provide valuable insight once the task is acknowledged and work begins.

Fatty acids are synthesized in a sequence of reactions catalyzed by a fatty acid synthetase complex (Figure 24) (177). Following a Claisen condensation between a carbanion and a thioester, a 3-ketoacid group is reduced by a nicotinamide-dependent dehydrogenase. The resulting alcohol undergoes elimination to produce an olefin, and the olefin is reduced (again by an enzyme dependent on nicotinamide cofactors) to give a saturated fatty acid thioester.

Many of the cryptic stereospecificities discussed above are exemplified in one or more steps in this sequence, and an interesting pattern has emerged (178–183). With all of the synthetases, the initial Claisen condensation proceeds with *inversion* of configuration. With all of the synthetases, the *pro-S* hydrogen of NADH is used to reduce the 3-ketothioester intermediate. The elimination of water is universally syn, and the *trans* olefin is always the product.

So far, the stereospecificities of all enzymatic reactions have been found to be the same in fatty acid synthetases from all of the organisms examined. These results are consistent either with functional models that assume that all of these stereochemical distinctions are adaptive or with the historical view that all of the synthetases are homologous and stereospecificity is highly conserved.

However, in the next step in the pathway, stereochemical uniformity is no longer found. With different synthetases, the addition of hydrogen to the unsaturated thioester occurs with three of the four possible stereochemical courses (3-*Re*/2-*Re*; 3-*Re*/2-*Si*; 3-*Si*/2-*Re*; 3-*Si*/2-*Si*). Further, stereospecificity with respect to *NADH cofactor* for the reduction of the double bond of the enoyl-CoA intermediate in fatty acid synthesis is not the same in different

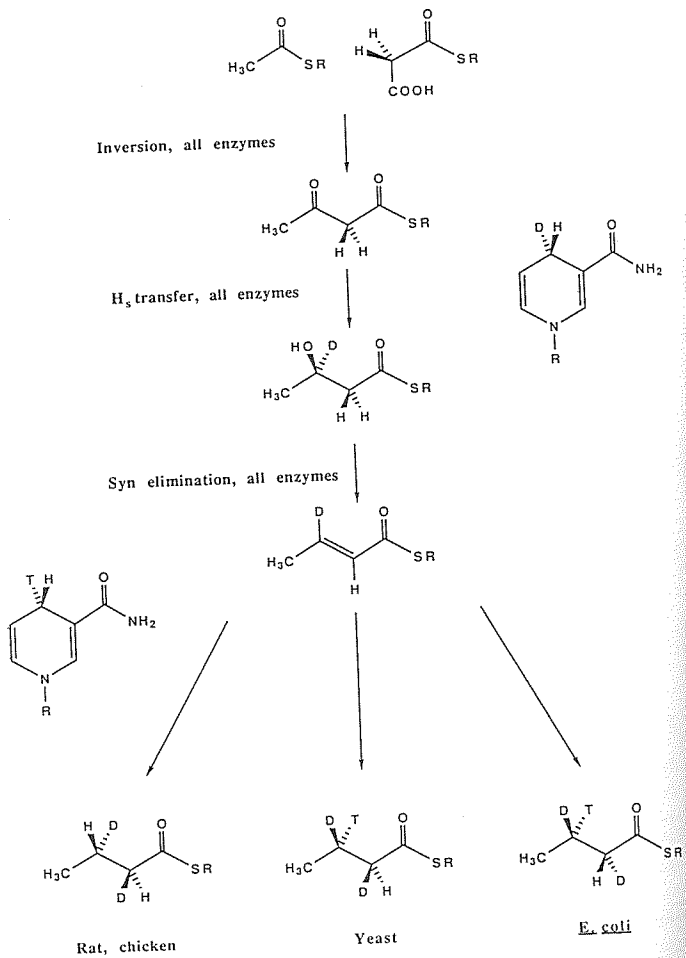


Figure 24. Stereospecificities of fatty acid biosynthesis.

organisms; some enzymes transfer the *pro-R* hydrogen of NAD(P)H and others transfer the *pro-S* hydrogen.

These stereochemical data provide one of the most controlled arguments for and against specific functional and historical models in enzymology. The uniform use of the *pro-S* hydrogen of NADH in the reduction of the 3-keto thioester intermediate is simply explained by the functional model outlined above, as the equilibrium constant for the redox reaction is in the *pro-S* region of Table 4b. The universal use of a syn elimination to form the unsaturated ester intermediate is similarly understood in functional terms. These explanations apply whether the fatty acid synthetases are homologous (in which case stereospecificity has been functionally constrained from drifting) or non-homologous (in which case, stereospecificity has converged). The divergent stereospecificities for the reduction of the carbon–carbon double bond, and the divergence in the choice of hydride of NADH to effect this reduction, are consistent with the notion that stereospecificity in this type of reaction is not a selected trait (or reflects mechanistic divergence), and the diversity reflects either drift or independent pedigree.

Accounting for these data with a historical model is more difficult. Most simply, either the fatty acid synthetase complexes are homologous or they are not. If they are not homologous, a historical model must assume that the common stereospecificities in the first steps arose by convergent evolution, expected only if the stereospecificities are directly adaptive. If they are homologous, historical models must concede that stereospecificity can drift in enzymes catalyzing addition of the elements of molecular hydrogen to a double bond. A concession that stereospecificities of this type can drift in the last steps in fatty acid synthesis makes difficult the assumption that analogous stereospecificities are highly conserved in the first steps.

The asymmetry between functional and historical models in addressing stereochemical problems in fatty acid synthetases arises from the fact that stereochemical imperatives can be different for reactions with different mechanisms, and stereochemical behaviors serving different functions can behave differently during evolution. However, historical models treat stereospecificity as consequences of an arbitrary placement of substrates and functional groups in the active site. As these arrangements serve no selected role in the historical view, the arrangements should behave the same during evolution in different enzyme classes regardless of the function of the enzyme.

Thus, it is conceivable on mechanistic grounds that stereospecificity with respect to cofactor for the reduction of 3-ketoesters is adaptive, while that for the reduction of an enoyl–CoA derivative is not. Conceivably, one stereospecificity can be highly conserved while the other drifts. However, the historical model regards stereospecificity in both cases as the result of an arbitrary arrangement of functional groups in the active site, binding the NADH cofactor

(arbitrarily) in either a syn or an anti conformation. As the orientation of binding serves no functional role (in the historical view), there seems to be no basis for explaining why the orientation drifts when the NADH is used to reduce a carbon-carbon double bond, but does not drift when the NADH is used to reduce a carbonyl group.

Of course, a historical model can explain these data if it is sufficiently complex. Gene fusions have apparently occurred during the evolution of fatty acid synthetase complexes (184–187). One might argue that the enoyl reductase subunits of the various synthetases are non-homologous, while the remaining subunits are homologous. This suggestion might be coupled with the intriguing notion that fatty acid biosynthesis originated relatively recently in evolution (15). Although this historical model is weakly contradicted by sequence data already available (132), specific hypotheses such as the ones proposed here should encourage further investigation.

VI. ENZYMES DISPLAYING STEREOCHEMICAL INFIDELITY

Enzymes need not be completely stereospecific, although the discovery of enzymes with incomplete stereochemical fidelity is undoubtedly hindered by the common prejudice that enzymes are necessarily absolutely stereospecific. Given this prejudice, evidence for incomplete fidelity is in danger of being dismissed as experimental error or artifact.

Careful studies of the intrinsic level of stereochemical error have now been made for a few enzymes. Rétey and his coworkers have studied the stereochemical infidelity of methylmalonyl CoA mutase from *Propionobacterium shermanii,* an infidelity that is quite substantial (188). In contrast, dehydrogenases display little if any stereochemical infidelity. Recent examinations of stereochemical infidelity in lactate and yeast alcohol dehydrogenases suggests that stereochemical error occurs only once in every billion turnovers (139, 189).

Where substantial stereochemical infidelity is observed, it may have both mechanistic and evolutionary implications. As discussed above, decarboxylases operating via an intermediate that is a Schiff's base of a 3-keto-acid proceed with large amounts of racemization. This was explained as a result of the high reactivity of an enamine intermediate in the reaction. Enzymes protonating such reactive intermediates directly from solvent are presumably not selectively disadvantageous compared with enzymes that maintain an active site residue to protonate the intermediate.

A particularly interesting case arises in the biosynthesis of cytidine diphosphate dideoxyhexoses in certain bacteria. In the reduction of the sugar derivative, direct hydrogen transfer from NADH to the substrate can not be demonstrated (190). Further, it appears as if the enzyme catalyzes the release of both the *pro-R* hydrogen and the *pro-S* hydrogen of NADH to solvent (191).

This remarkable lack of stereospecificity with respect to cofactor may have a mechanistic basis. Pyridoxal phosphate is a cofactor for the reaction, and a putative intermediate in the reduction is shown in Figure 25. Remarkably, this intermediate incorporates five conjugated double bonds and two positively charged nitrogens. While single electron transfer is unlikely in most dehydrogenases dependent on nicotinamide cofactors, the intermediate in Figure 25 is exceptional in its ability to accept an electron. Indeed, if any redox enzyme involves NADH radical cations as intermediates, this enzyme should. Thus, on chemical grounds, the absence of stereochemical fidelity in enzymes synthesizing CDP-dideoxyhexoses is consistent with the hypothesis of a radical intermediate in this section.

VII. CONCLUSIONS

The conclusion now is virtually inescapable that the stereospecificities of some enzymatic reactions (decarboxylation of amino acids, decarboxylation of beta-ketoacids, reduction of enoyl thioesters) behave as if they were not the targets of natural selection, while the stereospecificities of others (reduction of keto groups, enzymatic aldol condensations with acetyl CoA, polar elimination reactions, phosphoryl transfer reactions) behave as if they were the targets of natural selection. It remains a challenge to unify this collection of data into a formally simple statement regarding the distinction between selected and non-selected traits in stereochemistry.

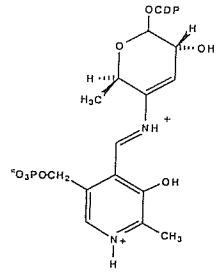


Figure 25. Intermediate in the synthesis of dideoxyhexoses.

A pattern emerges that suggests a set of hypotheses that could serve as the focus for further experimentation and controversy:

a. Discrimination between two enantiotopic groups where the distinction determines the chirality of a (non-cryptically) chiral product always reflects selective pressures.
b. Discrimination between two enantiotopic groups where the distinction does not determine the chirality of a product, but where the distinction results in the formation of one of two diastereomeric transition states generally reflects selective pressure.
c. Discrimination between two enantiomeric transition states, that are made diastereomeric only by virtue of the chirality of the catalyzing enzyme, do not reflect selective pressure (Figure 26).

These rules can be illustrated by a simple example (44). Ethanol dehydrogenases must choose between the *pro-R* and *pro-S* hydrogens of NADH, and between the *pro-R* and *pro-S* hydrogens at carbon-1 of ethanol. As discussed above, natural selection appears to accept either stereochemical outcome at the cofactor, owing to the fortuitous value of the equilibrium constant for the reaction (at the position of the break in the correlation in Table 4b). The ethanol dehydrogenase from *Drosophila melanogaster* transfers the *pro-S* hydrogen from NADH, while the enzyme from *Saccharomyces cerevisiae* transfers the *pro-R* hydrogen (Figure 27) (95).

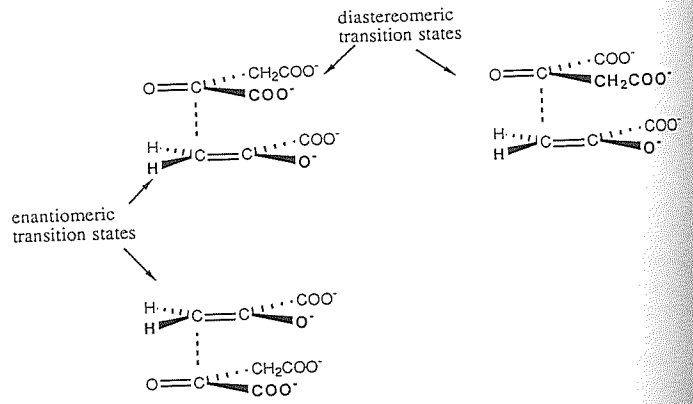


Figure 26. Locally enantiomeric transition states.

Accepting that the distinction between diastereotopic hydrogens of NADH is fortuitously neutral, one might ask whether the second choice, between the *pro-R* and *pro-S* hydrogens at carbon-1 of ethanol, is adaptive or not. Ethanol dehydrogenase from yeast transfers the *pro-R* hydrogen from ethanol to the 4-*Re* face of a nicotinamide ring. The "enantiomeric" transition state (here, considering only atoms directly attached to the reacting centers and ignoring conformation) is the transfer of the *pro-S* hydrogen from ethanol to the 4-*Si* side of nicotinamide ring. A diastereomeric transition state is the transfer of the *pro-R* hydrogen of ethanol to the 4-*Si* face of NAD^+. If the *pro-R* hydrogen of ethanol is preferred for transfer to the 4-*Re* face of NAD^+, the *pro-S* hydrogen of ethanol should be preferred for transfer to the 4-*Si* face of NAD^+. Thus, if this stereochemical distinction is adaptive, alcohol dehydrogenase from *Drosophila* should transfer the *pro-S* hydrogen from ethanol. In fact, they do (44).

This sort of test had been previously proposed by George et al. (191), who attempted to rationalize cofactor stereoselectivity by a hypothesis that enzymes "choose" between diastereomeric transition states so as to minimize steric interactions. Thus, enzymes "match" the face of the substrate to the face of the cofactor so that the bulky groups of the cofactor abut the small groups of the substrate, and vice versa. The authors attempted to test their hypothesis by predicting the stereospecificity of two steroid dehydrogenases, oxidizing respectively an α hydroxyl and a β hydroxyl group. They predicted that the cofactor stereospecificities would be opposite.

Given the functional theory for dehydrogenase stereospecificity, and the discussion above, it can be argued in retrospect that the systems available to the authors were inadequate to test their conjecture. The two steroids had different chiralities, and requirements for metabolic coupling (rule a) dictated the stereospecificity of synthesis of the two compounds. Furthermore, for compounds of this redox potential, the functional model for dehydrogenase stereospecificity suggests that the stereospecificities at cofactor should both be *pro-S* (rule b). In this view, the more subtle diastereomerism (rule c) is expected to be obscured by this functional adaptation.

The general rules formulated here are intended to be the object of experimental test. They do not represent the final word in enzymatic stereospecificity, and additional data could quite easily force their re-evaluation. They do divide selected from non-selected behavior in cryptic stereospecificity and therefore help divide enzymatic traits in general into those controlled by natural selection and those reflecting neutral drift. This division is more than academic. It also marks the line dividing behaviors that are biologically functional from those that are not, behaviors that reflect underlying chemistry from those that do not, and behaviors that are interesting to study from those that are not. To the extent that the division can be made here, it facilitates the division throughout all of bio-organic chemistry.

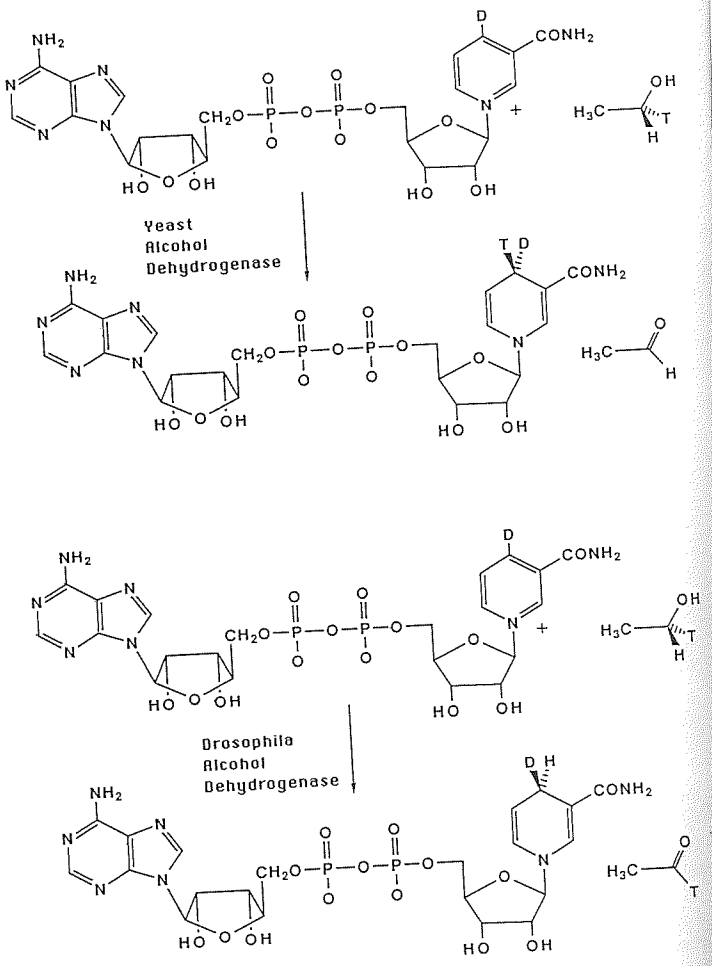


Figure 27. Opposite cryptic stereospecificity in both substrate and cofactor.

Note added in proof

With regard to functional models concerning stereospecificity in dehydrogenases, the original papers presenting the functional model focusing on a correlation between redox potential and stereospecificity (108, 110) excluded enzymes acting on carbonyl groups that were conjugated to olefinic systems. The exclusion was mechanism based: such carbonyls would be the most likely to be reduced via radical anion intermediates instead of via hydride transfer, and the correlation would not be expected to hold with enzymes acting with different mechanisms. Quite recently, Rando and his coworkers (Law, W. C., Kim, S., Rando, R. R. *J. Am. Chem. Soc.* 1989, *111*, 793) reported that the reduction of the carbonyl groups in cis and trans retinal were with the *pro-S* and *pro-R* hydrogens respectively. The first case formally violates the correlation, as the pK_{eq} is ca. 9. As these carbonyl groups are highly conjugated (to a pentaene), radical anion intermediates would be expected to be especially stable in these cases, and it will be interesting to learn the mechanisms of actions of these dehydrogenases.

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REFERENCES

1. Fisher, H. F.; Ofner, P.; Conn, E. E.; Vennesland, B.; Westheimer, F. H. *J. Biol. Chem.* **1953**, *202*, 687.
2. Martius, C.; Schorre, G. *Liebigs Ann. Chem.* **1950**, *570*, 140.
3. Wood, H. G.; Werkman, C. H.; Hemingway, A.; Nier, A. O. *J. Biol. Chem.* **1942**, *142*, 31.
4. Arigoni, D.; Eliel, E. *Topics in Stereochemistry*; John Wiley: New York, 1969; Vol. 4, p 127.
5. Evans, E. A. Jr.; Slotin, L. *J. Biol. Chem.* **1940**, *136*, 301.
6. Benner, S. *Yale Scientific* **1976**, *50*, 4.

7. Ogston, A. G. *Nature* **1948**, *162*, 963.
8. Hanson, K. R.; Rose, I. A. *Acc. Chem. Res.* **1975**, *8*, 1.
9. Benner, S. A.; Ellington, A. D. *CRC Crit. Rev. Biochem.* **1988**, *23*, 369.
10. Dobzhansky, T.; Ayala, F. J.; Stebbins, G. L.; Valentine, J. W. *Evolution;* W. H. Freeman: San Francisco, 1977; p. 19.
11. Kimura, M. *Molecular Evolution, Protein Polymorphism; the Neutral Theory;* Springer-Verlag: Berlin, 1982.
12. Lewontin, R. C. *Sci. Am.* **1979**, *239*, 156.
13. Benner, S. A. *Chem. Rev.* **1989** in press.
14. Benner, S. A. In *Molecular Structure and Energetics;* Liebman, J.; Greenberg, A., Eds., VCH: New York 1988.
15. Benner, S. A. In *Redesigning the Molecules of Life;* Benner, S. A., Ed.; Springer-Verlag: Berlin, 1988.
16. Zydowsky, T. M.; Courtney, L. F.; Frasca, V.; Kobayashi, K.; Shimizy, H.; Yuen, L.-D.; Matthews, R. G.; Benkovic, S. J.; Floss, H. G. *J. Am. Chem. Soc.* **1986**, *108*, 3152.
17. Jones, S. R.; Kindman, L. A.; Knowles, J. R. *Nature* **1978**, *275*, 564.
18. Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1985**, *107*, 5306.
19. Hermes, J. D.; Weiss, P. M.; Cleland, W. W. *Biochemistry* **1985**, *24*, 2959.
20. Lesk, A. M.; Levitt, M.; Chothia, C. *Prot. Engineering* **1986**, *1*, 77.
21. You, K.-S. *CRC Crit. Rev. Biochem.* **1985**, *17*, 313.
22. Stern, J. R.; O'Brien, R. W. *Biochim. Biophys. Acta* **1969**, *185*, 239.
23. Bolin, J. T.; Filman, D. J.; Matthews, D. A.; Hamlin, R. C.; Kraut, J. *J. Biol. Chem.* **1982**, *257*, 13650.
24. Benner, S. A.; Rozzell, J. D.; Jr.; Morton, T. H. *J. Am. Chem. Soc.* **1981**, *103*, 993.
25. Rozzell, J. D. Jr.; Benner, S. A. *J. Am. Chem. Soc.* **1984**, *106*, 4937.
26. Cohen, S. G.; Crossley, J.; Khedouri, E.; Zand, R.; Klee, L. H. *J. Am. Chem. Soc.* **1963**, *85*, 1685.
27. You, K.-S.; Arnold, L. J., Jr.; Kaplan, N. O. *Arch. Biochem. Biophys.* **1977**, *180*, 550.
28. Presnell, S. R. Ph.D. Thesis, Harvard University, 1988.
29. Rose, I. A.; Hanson, K. R. *CRC Crit. Rev. Biochem.* **1972**, *1*, 33.
30. Belleau, B.; Burba, J. *J. Am. Chem. Soc.* **1960**, *82*, 5751.
31. Yamada, H.; O'Leary, M. H. *Biochemistry* **1978**, *17*, 669.
32. Akhtar, M.; Jordan, P. M. *Tetrahedron Lett.* **1969**, 875.
33. Chang, W. C.; Snell, E. E. *Biochemistry* **1968**, *7*, 2005.
34. Battersby, A. R.; Joyeau, R.; Staunton, J. *FEBS Lett.* **1979**, *107*, 231.
35. Dunathan, H. C.; Voet, J. G. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3888.
36. Allen, R. R.; Klinman, J. P. *J. Biol. Chem.* **1981**, *256*, 3233.
37. Orr, G. R.; Gould, S. J. *Tetrahedron Lett.* **1982**, *23*, 319.
38. Woese, C. R. *Microbiol. Rev.* **1987**, *51*, 221–271.
39. Asada, Y.; Tanizawa, K.; Sawada, S.; Suzuki, T.; Misono, H.; Soda, K. *Biochemistry* **1981**, *20*, 6881.
40. Asada, Y.; Tanizawa, K.; Kawabata, Y.; Misono, H.; Soda, K. *Agric. Biol. Chem.* **1981**, *45*, 1513.

41. Gottschalk, G.; Barker, H. A. *Biochemistry* **1966**, *5*, 1125.
42. Gottschalk, G.; Barker, H. A. *Biochemistry* **1967**, *6*, 1027.
43. Faloone, G. R.; Srere, P. *Biochemistry* **1969**, *8*, 4497.
44. Allemann, R.; Hung, R.; Benner, S. A. *J. Am. Chem. Soc.*, **1988**, *110*, 5555.
45. Schiltz, E.; Schmitt, W. *FEBS Lett.* **1981**, *134*, 57.
46. Stragier, P.; Danos, O.; Patte, J.-C. *J. Mol. Biol.* **1983**, *168*, 321.
47. Christen, P.; Metzler, D. E. *Transaminases;* Wiley: New York; 1985.
48. Kelland, J. G.; Palcic, M. M.; Pickard, M. A.; Vederas, J. C. *Biochemistry* **1985**, *24*, 3263.
49. Gerdes, H. J.; Leistner, E. *Phytochem.* **1979**, *18*, 771.
50. Knowles, J. R. *Ann. Rev. Biochemistry* **1980**, *49*, 877–919.
51. Frey, P. A. *Tetrahedron* **1982**, *38*, 1541.
52. Eckstein, F. *Ann. Rev. Biochemistry* **1985**, *54*, 367–402.
53. Gerlt, J. A., In *Phosphorus-31 NMR;* Gorenstein, D. G., Ed.; Academic Press: New York, 1984.
54. Webb. M. R. *Meth. Enzymol.* **1982**, *87*, 301.
55. Buchwald, S. C.; Hansen, D. E.; Hassett, A.; Knowles, J. R. *Meth. Enzymol.* **1982**, *87*, 279–301.
56. van Pelt, J. E.; Iyengar, R.; Frey, P. A. *J. Biol. Chem.* **1986**, *261*, 15995–15999.
57. Hamblin, M. R.; Cummins, J. H.; Potter, B. V. L. *Biochem. J.* **1987**, *241*, 827–833. Cummins, J. H.; Potter, B. V. L. *Eur. J. Biochem.* **1987**, *162*, 123–128.
58. Buchwald, S. L.; Pliura, D. H.; Knowles, J. R. *J. Am. Chem. Soc.* **1982**, *104*, 845.
59. Sheu, K. R.; Richard, J. P.; Frey, P. A. *Biochemistry* **1979**, *18*, 5548. Frey, P. A. *Adv. Enzymol.* **1988**, *62*, in press.
60. Kaplan, N. O. *Methods Enzymol.* **1967**, 10.
61. Pauling, L. *Chem. Eng. News* **1946**, *10*, 1375.
62. Jencks, W. P. *Adv. Enzymol.* **1975**, *43*, 219.
63. Benner, S. A. *Chimia* **1988**, *42*, 309.
64. Askari, A., Ed.; *Properties and Functions of Na^+ and K^+ Activated Adenosinetriphosphatase Ann. N.Y. Acad. Sci.* **1974**, *242*.
65. Carafoli, E.; Scarpa, A., Eds.; *Transport ATPases Ann. N.Y. Acad. Sci.* **1982**, *402*.
66. Cross, R. L. *Ann. Rev. Biochemistry* **1981**, *50*, 681–714.
67. Downie, J. A.; Gibson, F.; Cox, G. B. *Ann. Rev. Biochemistry* **1979**, *48*, 103–31.
68. Walker, J. E.; Saraste, M.; Runswick, M. J.; Gay, N. J.; *EMBO J.* **1982**, *1*, 945.
69. Pederson, P. L.; Carafoli, E. *Trends Biochem. Sci.* **1987**, *12*, 186–188.
70. Hesse, J. E.; Wieczorek, L.; Altendorf, K.; Reicin, A. S.; Dorus, E.; Epstein, W. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 4746.
71. Cleland, W. W. *Methods Enzymol.* **1982**, *87*, 159.
72. Hill, R. K.; Sawada, S.; Arfin, S. M. *Bioorg. Chem.* **1979**, *8*, 175–189.
73. (a) Steinberger, R.; Westheimer, F. H. *J. Am. Chem. Soc.* **1949**, *71*, 4158–4159. (b) *Ibid.* **1951**, *73*, 429–435.
74. Vennesland, B. *Top. Curr. Chem.* **1974**, *48*, 39–65.
75. Mueller, W., E.T.H. Dissertation 5507, 1975.

76. Chang, C.-C.; Laghai, A.; O'Leary, M. H.; Floss, H. G.; *J. Biol Chem.* **1982**, *257*, 3564.
77. Piccirilli, J. A.; Benner, S. A. *J. Am. Chem. Soc.* **1987**, *109*, 8084.
78. Dimroth, P. *Eur. J. Biochem.* **1981**, *115*, 353–358.
79. Dimroth, P. *Eur. Biochem.* **1982**, *121*, 443.
80. Dimroth, P. *Eur. Biochem.* **1982**, *121*, 435.
81. Rose, I. A. *J. Biol. Chem.* **1970**, *245*, 6052.
82. Davis, D. D.; Teixeria, A.; Kenworthy, P. *Biol. J.* **1972**, *127*, 335.
83. Yeh, W.-K.; Fletcher, P.; Ornston, L. N. *J. Biol. Chem.* **1980**, *255*, 6342.
84. Davies, D. D.; Kenworthy, P. *Biochem. J.* **1982**, *205*, 581.
85. Mansell, R. L.; Gross, G. G.; Stockigt, J.; Franke, H.; Zenk, M. H. *Phytochem.* **1974**, *13*, 2427.
86. Colwick, S. P.; van Eys, J.; Prk, J. H. In *Comprehensive Biochemistry* 14, 1, Florkin, M.; Stotz, E. H., Eds.; Elsevier: Amsterdam, 1966.
87. Bentley, R. *Molecular Asymmetry in Biology* 2, Academic: New York, 1970.
88. R. Allemann, A. Glasfeld, L. Ge., unpublished.
89. Goerisch, H.; Hartl, T.; Grussebueter, W.; Stezowski, J. J.; *Biochem. J.* **1985**, *226*, 885.
90. S. A. Benner, unpublished.
91. Garavito, R. M.; Rossmann, M. G.; Argos, P.; Eventoff, W.; *Biochemistry* **1977**, *16*, 5065.
92. Oppenheimer, N. J.; Marschner, T. M.; Malver, O.; Kam, B.; "Proceedings of the Steenbock Symposium," Madison, Wisconsin, July 1985, 15–28, Elsevier: New York, 1986.
93. Rossmann, M. G.; Liljas, A.; Braenden, C.-I.; Banaszak, L. J.; *The Enzymes* **1975**, *11*, 61.
94. Rossmann, M. G.; Moras, D.; Olsen, K. W. *Nature* **1974**, *250*, 194.
95. Benner, S. A.; Nambiar, K. P.; Chambers, G. K. *J. Am. Chem. Soc.* **1985**, *107*, 5513.
96. Jornvall, H.; Persson, M.; Jeffrey, J. *Proc. Nat. Acad. Sci.* **1981**, *78*, 4226.
97. Gilbert, W. A. *Nature* **1978**, *271*, 501.
98. Llewellyn, D. J.; Daday, A.; Smith, G. D. *J. Biol. Chem.* **1980**, 2077.
99. Hall, B. G.; Yokoyama, S.; Calhoun, D. H. *Molec. Biol. Evol.* **1984**, *1*, 109.
100. Joernvall, H.; Bahr-Lindstrom, H. von, Jany, K. D.; Ulmer, W.; Froschle, M. *FEBS Lett.* **1984**, *165*, 190.
101. (a) Krakow, G.; Vennesland, B. *Biochem. Z.* **1963**, *338*, 31. (b) Simon, H.; Kraus, A. *Isotopes in Organic Chemistry 2* Buncel, E.; Lee, C. C.; Elsevier: Amsterdam, 1976, p 153.
102. Schneider-Bernloehr, H.; Adolph, H.-W.; Zeppezauer, M. *J. Am. Chem. Soc.* **1986**, *108*, 5573.
103. Beedle, A. S.; Rees, H. H.; Goodwin, T. W. *Biochem. J.* **1974**, *139*, 205.
104. Morpeth, F. F.; Dickinson, F. M. *Biochem. J.* **1980**, *191*, 619.
105. Bosron, W. F.; Prairie, R. L. *Arch. Biochem. Biophys.* **1973**, *154*, 166.
106. Ris, M. M.; von Wartburg, J.-P. *Eur. J. Biochem.* **1973**, *37*, 69.
107. Wermuth, B. *J. Biol. Chem.* **1981**, *256*, 1206.
108. Benner, S. A. *Experientia* **1982**, *38*, 633.
109. Srivastava, D. K.; Bernhard, S. A. *Biochemistry* **1984**, *23*, 4538.

110. Nambiar, K. P.; Stauffer, D. M.; Kolodziej, P. A.; Benner, S. A. *J. Am. Chem. Soc.* **1983**, *105*, 5886.
111. Van der Veen, R. H.; Kellog, R. M.; Vos, A. *J. Chem. Soc., Chem. Commun.* **1978**, 923.
112. Rabideau, P. W.; Mooney, J. L.; Lipkowitz, K. B. *J. Am. Chem. Soc.* **1986**, *108*, 8130.
113. Glasfeld, A.; Zbinden, P.; Dobler, M.; Benner, S. A.; Dunitz, J. D. *J. Am. Chem. Soc.* **1988**, *110*, 5152.
114. Wu, Y.-D.; Houk, K. N. *J. Am. Chem. Soc.* **1987**, *109*, 2226.
115. Levy, H. R.; Vennesland, B. *J. Biol. Chem.* **1957**, *228*, 85.
116. Pai, E. F.; Schulz, G. E. *J. Biol. Chem.* **1983**, *258*, 1752.
117. Pai, E. F., personal communication to A. G.
118. Ellington, A. D.; Benner S. A. *J. Theor. Biol.* **1987**, *127*, 491.
119. Oppenheimer, N. J. *J. Am. Chem. Soc.* **1984**, *106*, 3032.
120. These criticisms were the basis for the evaluation of a research proposal by the Biophysical Review Group of the National Institutes of Health (NIH), 1982.
121. Chari, R. V. J.; Whitman, C. P.; Kozarich, J. W.; Ngai, K.-L.; Ornston, L. N. *J. Am. Chem. Soc.* **1987**, *109*, 5514.
122. You, K.-S. *Chem. Eng. News* **1986**, *64*, 3.
123. Robinson, W. G. *Methods Enzymol.* **1966**, *9*, 332.
124. Flynn, T. G. *Biochem. Pharmacol.* **1982**, *31*, 2705.
125. Glasfeld, A.; Benner, S. A. *J. Biol. Chem.* **1988**, submitted.
126. Takizawa, T.; Huang, I.-Y.; Ikuta, T.; Yoshida, A. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4157.
127. Williamson, V. M. and Paquin, C. E. *Mol. Gen. Genet.* **1987**, *209*, 374.
128. Glasfeld, A. and Benner, S. A. *Eur. J. Biochem.* **1988**, submitted.
129. Matthews, D. A.; Smith, S. L.; Baccanari, D. P.; Burchall, J. J.; Oatley, S. M.; Kraut, J. *Biochemistry* **1986**, *25*, 4194.
130. The correlation in Table 4 does not apply to dihydrofolate reductase. However, any convergence of behavior is an argument for function; it suggests that stereospecificity is adaptive in these enzymes as well.
131. Hempel, J.; Bahr-Lindstroem, H. von, Joernvall, H. *Eur. J. Biochem.* **1984**, *141*, 21.
132. Schweizer, M.; Roberts, L. M.; Hoeltke, H.-J.; Takabayashi, K.; Hoellerer, E.; Hoffmann, B.; Mueller, G.; Koettig, H.; Schweizer, E. *Mol. Gen. Genet.* **1986**, *203*, 479.
133. In these cases, sequence homologies have been proposed in only small segments of the protein and are of insufficient statistical significant to prove (as opposed to suggest) that the proteins are homologous.
134. Dugan, R. E.; Porter, J. W. *J. Biol Chem.* **1971**, *246*, 5361.
135. Beedle, A. S.; Munday, K. A.; Wilton, D. C. *Eur. J. Biochem.* **1972**, *28*, 151.
136. You, K.-S. *Meth. Enzymol.* **1982**, *87*, 101.
137. The alternative argument, that the sequence of MDH in archaebacteria is >50% identical to MDH from eukaryotes and eubacteria, seems less likely (although not impossible) in view of the low structural similarities of other proteins from these two kingdoms. See Hardesty, B.; Kramer, G. *Structure, Function and Genetics of Ribosomes;* Springer-Verlag: Berlin, 1986.
138. This hypothesis might be regarded as demonstrably false; many organisms, including

Acholeplasma show no malate dehydrogenase activity. Further, the ethanol dehydrogenases from both *Drosophila* and yeast are evidently under enormous selective pressure. See Kreitman, M. *Nature* **1983**, *304*, 412.

139. Glasfeld, A.; Thesis, Harvard University, 1988.
140. Knowles, J. R. *Science* **1987**, *236*, 1252.
141. Brink, N. G. *Acta Chem. Scand.* **1953**, *7*, 1081.
142. Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman: San Francisco, 1979.
143. Ohno, A.; Ohara, M.; Oka, S. *J. Am. Chem. Soc.* **1986**, *108*, 6438.
144. March, J. *Advanced Organic Chemistry*, 3rd ed.; Wiley: New York, 1985; p 657.
145. Anet, F. A. L. *J. Am. Chem. Soc.* **1960**, *82*, 994.
146. Englard, S. *J. Biol. Chem.* **1958**, *233*, 1003.
147. Jones, V. T.; Lowe, G.; Potter, B. V. L. *Eur. J. Biochem.* **1980**, *108*, 433.
148. Takagi, J. S.; Tokushige, M.; Shimura, Y.; Kanehisa, M. *Biochem. Biophys. Res. Commun.* **1986**, *138*, 568.
149. Hoberman, H. D.; Havir, E. A.; Rachovansky, O.; Ratner, S. *J. Biol. Chem.* **1964**, *239*, 3818.
150. Englard, S. *J. Biol. Chem.* **1960**, *235*, 1510.
151. Schroepfer, Jr.; G. L. *J. Biol. Chem.* **1966**, *241*, 5441.
152. Miller, R. W.; Buchanan, J. M. *J. Biol. Chem.* **1962**, *237*, 491.
153. Englard, S.; Britten, J. S.; Listowsky, I. *J. Biol. Chem.* **1967**, *242*, 2255.
154. Cohn, M.; Pearson, J.; O'Connell, E. L.; Rose, I. A. *J. Am. Chem. Soc.* **1970**, *92*, 4095.
155. Givot, I. L.; Smith, T. A.; Abeles, R. H. *J. Biol. Chem.* **1969**, *244*, 6341.
156. Havir, E. A.; Hanson, K. R. *Biochemistry* **1975**, *14*, 1620.
157. Cornforth, J. W.; Cornforth, R. H.; Popjak, G.; Yengoyan, L. *J. Biol. Chem.* **1966**, *241*, 3970.
158. Martinoni, B. Q. M. E.T.H. Dissertation 5115, 1973.
159. Avigad, G.; Englard, S. *Fed. Proc.* **1969**, *28*, 345.
160. Willadsen, P.; Eggerer, H. *Eur. J. Biochem.* **1975**, *54*, 247.
161. Widlanski, T. S.; Bender, S. L.; Knowles, J. R. In Bartman, W.; Sharpless, K. B., Eds.; *Stereochemistry of Organic and Bioorganic Transformations*, 1987; Workshop Conference, Hoechst, Vol. 17, 275–282.
162. Schwab, J.; Klassen, J. B.; Habib, A. *J. Chem. Soc. Chem. Comm.* **1986**, 357.
163. Sedwick, B.; Morris, C.; French, S. J. *J. Chem. Soc., Chem. Commun.* **1978**, 193.
164. Messner, B.; Eggerer, H.; Cornforth, J. W.; Mallaby, R. *Eur. J. Biochem.* **1975**, *53*, 255.
165. Hanson, K. R.; Rose, I. A. *Proc. Nat. Acad. Sci* **1963**, *50*, 981.
166. Aberhart, J.; Tann, C.-H. *J. Chem. Soc., Perkin Trans. I* **1979**, 939.
167. Scharf, K. H.; Zenk, M. H.; Onderka, D. K.; Carroll, M.; Floss, H. G. *J. Chem. Soc. Chem. Commun.* **1971**, 765.
168. Kirby, G. W.; O'Loughlin, G. J.; Robins, D. J. *J. Chem. Soc. Chem. Commun.* **1975**, 402.
169. Schwab, J.; Klassen, J. B. *J. Am. Chem. Soc.* **1984**, *106*, 7217.
170. I am indebted to Prof. J. Schwab for calling these references to my attention.
171. Caspi, E.; Ramm, P. J. *Tetrahedron Lett.* **1969**, 181.
172. Mortimer, Niehaus *J. Biol. Chem.* **1974**, *249*, 2833.

173. For example, the kinetic isotope effects were measured on an unnatural substrate.
174. Arnett, E. M.; Hofelich, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 2889.
175. Jencks, W. R. *Acc. Chem. Res.* **1976**, *9*, 425.
176. Kozarich, J. W.; Chari, R. V. J.; Ngai, K.-L.; Ornston, N. L. In *Mechanisms of Enzymatic Reactions: Stereochemistry*, Frey, P. A. Ed., Elsevier: Amsterdam 1986; p 233.
177. Volpe, J. J.; Vagelos, P. R. *Ann. Rev. Biochem.* **1973**, *42*, 21.
178. Singh, N.; Stoops, J. K.; In *Enzyme Mechanisms*. M. I. Page, Ed.; Royal Society: London, 1987.
179. Saito, K.; Kawaguchi, A.; Seyama, Y.; Yamakawa, T.; Okuda, S. *Eur. J. Biochem.* **1981**, *116*, 581.
180. Saito, K.; Kawaguchi, A.; Seyama, Y.; Yamakawa, T.; Okuda, S. *J. Biochem.* **1981**, *90*, 1697.
181. Saito, K.; Kawaguchi, A.; Seyama, Y.; Yamakawa, T.; Okuda, S. *Tennen Yuki Kagobutsu Toronkai Koen Yoshishu, 24th* **1981**, 529.
182. Saito, K.; Kawaguchi, A.; Okuda, S.; Seyama, Y.; Yamakawa, T.; Nakamura, T.; Yamada, M. *Plant Cell. Physiol.* **1980**, *21*, 9.
183. Kikuchi, S.; Kusaka, T. *J. Biochem.* **1984**, *96*, 841.
184. Schweizer, M.; Roberts, L. M.; Hoeltke, H.-J.; Takabayashi, K.; Hoellerer, E.; Hoffman, B.; Mueller, G.; Koettig, H.; Schweizer, E. *Mol. Gen. Genet.* **1986**, *203*, 479.
185. Hardie, D. G.; McCarthy, A. D.; Braddock, M. *Biochem. Soc. Trans.* **1986**, *14*, 568.
186. McCarthy, A. D.; Goldring, J. P. D.; Hardie, D. G. *FEBS Lett.* **1983**, *162*, 300.
187. Chirala, S. S.; Kuziora, M. A.; Spector, D. M.; Wakil, S. J. *J. Biol. Chem.* **1987**, *262*, 4231.
188. Michenfelder, M.; Hull, W. E.; Rétey, J. *Eur. J. Biochem.* **1987**, *168*, 659.
189. Anderson, V. E.; LaReau, R. D. *J. Am. Chem. Soc.* **1988**, *110*, 3695–97.
190. Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3782.
191. George, J. M.; Orr, J. C.; Renwick, A. G. C.; Carter, P.; Engel, L. L. *Bioorg. Chem.* **1973**, *2*, 140.