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The Stereospecificities of Seven Dehydrogenases from *Acholeplasma laidlawii*

THE SIMPLEST HISTORICAL MODEL THAT EXPLAINS DEHYDROGENASE STEREOSPECIFICITY*

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Stereospecificities are reported for seven dehydrogenases from *Acholeplasma laidlawii*, an organism from an evolutionarily distinct branch of life which has not previously been studied from a stereochemical point of view. Three of the activities examined (alcohol dehydrogenase, lactate dehydrogenase, and alanine dehydrogenase) catalyze the transfer of the pro-*R* (A) hydrogen from NADH. Four other activities (3-hydroxy-3-methylglutaryl-CoA reductase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and NADH oxidase) catalyze the transfer of the pro-*S* (B) hydrogen from NAD(P)H. The stereospecificity of hydroxymethylglutaryl-CoA reductase is notable because it is the opposite of that of hydroxymethylglutaryl-CoA reductases from yeast and rat. These data are used to derive the simplest historical model capable of explaining available experimental facts.

Although stereospecificity in dehydrogenases has been studied for nearly 40 years, only recently have logically formal functional and historical models been offered to interpret this behavior (1–4). Distinguishing between these models involves a question central to biochemistry: what is the relative importance of natural selection, conservation, and neutral drift in the evolution of behavior in proteins (5–11)?

Stereospecificity in dehydrogenases is not random. For example, regardless of their sources, enzymes accepting the same substrates generally have the same stereospecificities (12–14). This generalization ("Bentley's first rule") (14) has proven to be remarkably broad. For example, malate dehydrogenases from archaebacteria, eubacteria, and eukaryotes all transfer the pro-R hydrogen of NADH (15, 16).

Traditionally, stereospecificity in dehydrogenases has been viewed as a nonselected trait (17). The "nonrandomness" in the experimental data has been explained with two assumptions: (*a*) enzymes from all organisms catalyzing the same reaction are (nearly always) homologous and (*b*) stereospecificity is (nearly always) conserved during divergent evolution.

These assumptions remain widely accepted today (17–21), even though they have never been formally incorporated into a logically coherent historical model. For this to be done, the significance of the parenthetical "nearly always" must be estimated. If nearly always means "except in one or two isolated cases," a historical model incorporating these assumptions remains a serviceable paradigm capable of guiding experimental work. However, if these assumptions have many exceptions, the historical model must incorporate many *ad hoc* assumptions to account for them. If the *ad hoc* assumptions are mechanistically based and general, they are testable and potentially valuable. If, however, they are introduced only to explain single results and apply arbitrarily only to single enzymes, they destroy the explanatory and experimental value of a model. An example of how the need for arbitrary *ad hoc* assumptions led to the rejection of a *functional* model in enzyme stereochemistry has been published recently (22).

To ascertain the generality of assumptions *a* and *b*, stereochemical data must be obtained for enzymes from organisms that are widely divergent in evolution (23). The mycoplasma *Acholeplasma laidlawii* is one such organism (24). *Acholeplasma* is evolutionarily quite distant from better-studied organisms (although it is still classified as a eubacteria). In some mycoplasma, the genetic code has diverged (25), suggesting that certain members of the order are quite distant evolutionarily from better studied microorganisms. Based on ribosomal sequence data, Woese (27) has placed the order as a separate branch with an ancient divergence from the clostridial lineage (26, 27). No enzymes from this order have been examined previously stereochemically. Thus, the stereospecificities of enzymes from *Acholeplasma* might contain some surprises that will help develop and better understand historical models for stereospecificity in dehydrogenases.

We report here the stereospecificities of seven dehydrogenases from *A. laidlawii*. These data are then combined with data from the literature to modify assumptions *a* and *b* (above) to yield the simplest historical formalism that is consistent with available facts.

EXPERIMENTAL PROCEDURES[1]

RESULTS

The stereospecificities of seven dehydrogenases from *A. laidlawii* are shown in Table I. The alcohol dehydrogenase,

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[1] Portions of this paper (including detailed "Experimental Procedures" for isolating the dehydrogenases examined here and determining their stereospecificities and Refs. 51–66) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press. Archival material describes the detailed kinetic behavior of the HMG-CoA reductase. This material can be obtained directly from the author.

TABLE I

Results of stereospecificity determinations of dehydrogenases isolated from Acholeplasma

Enzyme	EC No.	pro-*S*	pro-*R*
		%	
Alcohol dehydrogenase	1.1.1.1	2[a]	94[a]
Lactate dehydrogenase	1.1.1.27	19[b]	99[b]
Glucose-6-phosphate dehydrogenase	1.1.1.49	95[c]	3[d]
HMG-CoA reductase	1.1.1.88	100[b]	4[b]
Glyceraldehyde-3-phosphate dehydrogenase	1.2.1.12	97[e]	1[f]
Alanine dehydrogenase	1.4.1.1	3[b]	97[b]
NADH oxidase	1.6.99.3	89[a]	1[a]

[a] Percent activity in volatile fraction after reaction of 4*S* or 4*R* [^{3}H]NADH with enzyme and substrate.

[b] Percent activity that elutes away from origin by chromatography after reaction of 4*S* or 4*R* [^{3}H]NADH with enzyme and substrate.

[c] Percent activity that elutes after reaction of [4-^{3}H]NADPH (formed by enzyme and substrate and labeled $NADP^+$) with glutamate dehydrogenase and 2-oxoglutarate.

[d] Percent activity that elutes after reaction of [4-^{3}H]NADPH (formed by enzyme and labeled substrate and $NADP^+$) with glutamate dehydrogenase and 2-oxoglutarate.

[e] Percent activity in volatile fraction after reaction of [4-^{3}H]NADH (formed by GAPDH and substrate and labeled NAD^+) with NADH oxidase.

[f] Same as for footnote *e* except that [4-^{3}H]NADH is reacted with HLADH.

lactate dehydrogenase, and alanine dehydrogenase activities catalyze the transfer of the pro-*R* (A) hydrogen from NADH. The hydroxymethylglutaryl (HMG)-CoA[2] reductase, glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, and NADH oxidase activities all catalyze the transfer of the pro-*S* (B) hydrogen from NAD(P)H.

The stereospecificity of HMG-CoA reductase is notable because it is the *opposite* of the stereospecificities of HMG-CoA reductases from yeast and rat (28, 29). However, lactate dehydrogenase, alanine dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, and glucose-6-phosphate dehydrogenase from *Acholeplasma* all have the *same* stereospecificities as the corresponding dehydrogenases from yeast, rat, and related organisms. Thus, HMG-CoA reductase is the only enzyme in *Acholeplasma* that violates Bentley's first rule; the others obey it (13).[3]

DISCUSSION

Like any model in science, a historical model is a logical formalism consisting of assumptions that are deductive precursors of experimental facts. The experimental data reported here are logically incompatible either with assumption *a* (enzymes from all organisms catalyzing the same reaction are homologous) or with assumption *b* (stereospecificity is conserved during divergent evolution) (*vide supra*). Therefore, for a historical model to be consistent with fact, at least one of these assumptions must be modified. Because no rigorously formulated historical model has been published to date, it is worthwhile here to briefly outline one such model given these facts.

Table II lists the assumptions that must be incorporated into the formalism of the simplest historical model consistent with available facts. It is immediately clear from the table that the model is not at all simple. Assumptions *a* and *b* are essential to explain the identical stereospecificities in the the first set classes of dehydrogenases listed in Table II. Yet they must be modified by no fewer than 16 *ad hoc* hypotheses to account for the stereochemical facts listed elsewhere in Table II. From these *ad hoc* hypotheses arise further paradoxes, which themselves must be resolved *ad hoc*.

To show that the simplest historical model consistent with experimental fact is quite complicated, consider the fact that the stereospecificity of HMG-CoA reductase from *Acholeplasma* is different from that of HMG-CoA reductases from yeast and rat. Either there existed two ancestral HMG-CoA reductases (an exception to assumption *a*) or stereospecificity has diverged during the divergent evolution of a single pedigree of HMG-CoA reductases (an exception to assumption *b*). Clearly, a historical model must incorporate at least one *ad hoc* assumption to explain the stereochemical diversity in HMG-CoA reductases.

Weakening assumption *a* or *b* by *ad hoc* modification need not profoundly damage a historical model. *Acholeplasma* may simply be so evolutionarily distant from "mainstream" organisms that *all* of their enzymes have independent pedigrees or that sufficient time has passed since the divergence of *Acholeplasma* that the stereospecificities of *all* of its enzymes from have drifted. However, the other data reported here rule out this as an easy solution. The stereospecificities of four other dehydrogenases from *Acholeplasma* are the *same* as in yeast, rat, and related organisms. Normally, the historical model would explain *these* stereospecificities by assuming that these other dehydrogenases *shared* a common ancestor with analogous dehydrogenases from other organisms and that stereospecificity *cannot* drift in the evolutionary time that has passed since the divergence of *Acholeplasma*.

The historical model is therefore faced with a dilemma that arises from the fact that in the *same* organisms, the stereospecificities of *some* dehydrogenases are conserved whereas others are not. To resolve this dilemma via a special assumption, the historical model must either assume that the HMG-CoA reductase is the only one of these dehydrogenases that arose independently in *Acholeplasma* or that the HMG-CoA reductase is the only one whose stereospecificity can drift.

An assumption of multiple ancestry is generally more manageable than an assumption that stereospecificity in some dehydrogenases can drift more easily than in others. Stereospecificity apparently can be reversed in all dehydrogenases simply by rotating the nicotinamide ring 180 ° around the glycosidic bond to present the opposite face to the substrate (30–32). This rotation is permitted by alteration in amino acids that interact with the nicotinamide ring (32), and there is no obvious reason why the ease with which these mutations can be introduced into a dehydrogenase depends on the nature of its substrate. Thus, Table II incorporates the *ad hoc* assumption that the HMG-CoA reductases from *Acholeplasma* and yeast/rat have separate pedigrees.

Although such an assumption might appear arbitrary, it would perhaps not be serious if it could be grounded in mechanism or physiology in a way that makes testable predictions in other systems. For example, the HMG-CoA reductase in rat catalyzes the first step in the biosynthesis of cholesterol; the enzyme from *Acholeplasma* is believed to

[2] The abbreviation used is: HMG-CoA 3-hydroxy-3-methylglutaryl coenzyme A.

[3] There seems to be no consensus regarding the classification of NADH oxidases, making the comparison of the NADH oxidase from *Acholeplasma* to other NADH oxidases problematic. The alcohol dehydrogenase was found to prefer short chain aldehydes (acetaldehyde, propionaldehyde) as substrates, suggesting (but certainly not proving) that its physiological role is the interconversion of ethanol and acetaldehyde. As both pro-*R*- and pro-*S*-specific alcohol dehydrogenases with this physiological role are known, the stereospecificity of the alcohol dehydrogenase from *Acholeplasma* certainly must be the same as that of at least one previously studied alcohol dehydrogenases of this type.

TABLE II

Facts and explanations in the simplest historical model accounting for stereospecificity in dehydrogenases

The Table does not include all possible mechanisms to account for individual stereospecificities; among the less likely is lateral transfer of genetic information, which assumes that the pedigree of enzymes is not parallel to the pedigree of organisms from which they are isolated.

Fact	Explanation
(*a*) Enzymes displaying extreme conservation in stereospecificity[a]	
Malate dehydrogenase[b]	All mechanisms for creating stereochemical diversity (multiple ancestors, domain shuffling, divergence of stereo- or substrate specificity, deletion-replacement events) must be inaccessible for these enzymes
Glucose-6-phosphate dehydrogenase	
Lactate dehydrogenase	
Alanine dehydrogenase	
Glutamate dehydrogenase	
Isocitrate dehydrogenase	
3-Hydroxybutyrate dehydrogenase[b]	
(*b*) Enzymes catalyzing similar reactions with opposite stereospecificity	
HMG-CoA reductase	Two ancestors[c]
Enoyl-CoA reductase	Two ancestors[d]
Ethanol dehydrogenase	Domain shuffling[e]
(*c*) Nonhomologous enzymes catalyzing similar reactions with identical stereospecificity[f]	
Dihydrofolate reductase[g]	Coincidence
D- and L-lactate dehydrogenase[h]	Coincidence
D- and L-aldose dehydrogenases	Coincidence
(*d*) Allowed divergence in substrate specificity[i]	
Ethanol, glucose, polyols[j]	Structural similarity of substrates
Ethanol, polyols, 3-hydroxysteroids[k]	Structural similarity of substrates
Within alcohols with similar redox potentials[l]	Structural similarity of substrates
Lactate to malate[m]	Structural similarity of substrates
(*e*) Forbidden divergence in substrate specificity	
Malate to 3-hydroxybutyrate	Structural dissimilarity of substrates[n]
Between alcohols with dissimilar redox potentials[l]	Mechanistic imperative (?)
(*f*) Allowed divergence in substrate specificity that has not happened[o]	
Pro-*R*-specific ethanol dehydrogenase to become an aldose dehydrogenase	
Pro-*S*-specific ethanol dehydrogenase to become an aldose reductase	

[a] These are just some of the enzymes that conform to Bentley's first rule; the historical model must assume that all of the mechanisms that create stereochemical diversity in enzymes listed elsewhere in this table are not operative in these enzymes. This assumption might be justified by an assumption that dehydrogenases whose stereochemical preferences are highly conserved are more "essential" to the survival of the host organism than dehydrogenases which display stereochemical diversity.

[b] Enzymes that presumably play different metabolic roles in different organisms.

[c] A postulate of two independent pedigrees for these enzymes may be based on an assumption of different physiological roles for the two enzymes; this assumption raises questions about other pairs of dehydrogenases that also play different physiological roles but nevertheless have the same stereospecificities.

[d] Some fatty acid synthetase complexes may have arisen by gene fusion (47–50), and it is conceivable that some components of the complexes of different organisms might be homologous, whereas others are not.

[e] Sequence data virtually require that the model assume that the dinucleotide binding domains at least of pro-*R*-specific alcohol dehydrogenase from yeast and the pro-*S*-specific alcohol dehydrogenase from *Drosophila* are homologous (40). However, alcohol dehydrogenase is the only enzyme from *Drosophila* known to have divergent stereospecificity (23). Thus, the model must propose either that the drift in stereospecificity is faster for enzymes acting on ethanol than for other enzymes or that ethanol dehydrogenases are more easily replaced by cross-evolution of other dehydrogenases than are other dehydrogenases.

[f] The probability of two randomly selected nonhomologous dehydrogenases having the same stereospecificity is 50%. The model predicts that as more examples are discovered, the number of pairs of *non*homologous proteins acting on the *same* substrate and having the *same* stereoselectivity will approach 50% of the total number of such pairs examined.

[g] Nonhomology suggested by crystal structure.

[h] It is not likely that stereospecificity with respect to substrate can drift in a dehydrogenase, whereas stereospecificity with respect to cofactor is retained. Therefore, the historical model assumes that enzymes acting on enantiomeric substrates have independent pedigrees.

[i] Divergence of substrate specificity is indisputable in enzymes that obey Bentley's first rule, contradicting the assumption in footnote *g*. Thus, the historical model must include a set of assumptions to govern allowed and disallowed patterns of substrate specificity to account for the fact that in these enzymes, dehydrogenases with opposite stereospecificities have not evolved to replace deleted dehydrogenases.

[j] The polyol here is sorbitol. In general, polyol dehydrogenases that form aldoses transfer the pro-*R* hydrogen.

[k] The polyol here is ribitol. Polyol dehydrogenases that form ketoses sometimes transfer the pro-*S* hydrogen of NADH; polyol dehydrogenases that form ketose phosphates universally transfer the pro-*S* hydrogen.

[l] The correlation between the stereospecificity of a dehydrogenase and the redox potential of its natural substrate is discussed elsewhere (5). The assumption that this correlation is based on a restricted pattern of divergence in substrate specificity requires many additional assumptions not discussed here.

[m] This transformation has recently been accomplished by site-directed mutagenesis by Holbrook and his coworkers (51).

[n] The model must assume that the structure of malate is more dissimilar to the structure of 3-hydroxybutyrate than it is to the structure of lactate; see Fig. 2.

[o] The historical model predicts that examples of this type should eventually be found in future studies.

catalyze the first step in the biosynthesis of carotenoids (33). Furthermore, one enzyme uses NADH, the other NADPH. We might propose that the two pathways in the two organisms are not homologous and develop this proposal into a working hypothesis worth examining experimentally; enzymes catalyzing identical reactions in different pathways are exempt from Bentley's first rule.

Unfortunately, this hypothesis does not apply universally. Hydroxybutyrate dehydrogenases in eubacteria play different roles than hydroxyacyl-CoA dehydrogenases in eukaryotes, yet they have the same stereospecificities. Malate dehydrogenases in anaerobic archaebacteria have different metabolic roles than malate dehydrogenase in aerobic organisms, yet their stereospecificities are the *same*. Furthermore, enoyl-CoA reductases play the *same* role in fatty acid synthesis, yet their stereospecificities are *different* (5, 34–39). Finally, enzymes catalyzing analogous reactions, but using NADH instead of NADPH (generally indicating different catabolic and anabolic roles), have the *same* stereospecificity (13). Thus, any formalism of this type must itself be modified by special assumptions concerning the enzymes to which it is intended to apply.

Even without a mechanistic or physiological rationalization, such *ad hoc* assumptions would perhaps not be serious if they were needed only in this single case. However, HMG-CoA reductases are not the only dehydrogenases displaying stereochemical diversity. For example, the stereospecificity of ethanol dehydrogenase from *Drosophila melanogaster* is *opposite* to that from mammals and yeast, yet five other dehydrogenases from *Drosophila* have the *same* stereospecificities as dehydrogenases from these other organisms (23). Likewise, the stereospecificity of enoyl-CoA reductases from a variety of organisms is widely *divergent* both with respect to cofactor and to substrate (Fig. 1 and Refs. 34–39), yet 3-hydroxyacyl thioester dehydrogenases acting in the same pathway (and in the same multienzyme complex) in the same organisms all have the *same* stereospecificity, both with respect to cofactor and to substrate (5, 10).

Thus, assumptions *a* and *b* must again be modified to explain these additional cases. A historical model must posit two separate pedigrees for ethanol dehydrogenases, HMG-CoA reductases, and enoyl-CoA reductases (set *b*, Table II), but only a single pedigree for malate dehydrogenases, lactate dehydrogenases, and 3-hydroxyacyl thioester dehydrogenases (set *a*, Table II); the last pair of assumptions are the most remarkable as enoyl-CoA reductases and 3-hydroxyacyl thioester dehydrogenases normally function in the same complex. In the historical model presented here, assumptions of this type are incorporated arbitrarily. The reader is challenged to devise mechanistic explanations that might make these *ad hoc* assumptions less arbitrary.

Even here the model is not complete, as assumption *b* of historical models must be further modified to accommodate the fact that homology does not appear to be an absolute determinant of stereospecificity in dehydrogenases. For example, the dinucleotide binding domains of glyceraldehyde-3-phosphate dehydrogenases and lactate dehydrogenases appear *homologous* (based on comparisons of their crystal structures, Ref. 31), yet these enzymes have *opposite* stereospecificities. Furthermore, sequence homology is detectable in ethanol dehydrogenase from *Drosophila* and yeast, enzymes with opposite stereospecificities (40). Even closely homologous enzymes may not have the same stereospecificities, as stereospecificity can be reduced by point mutation without a corresponding loss in catalytic activity (32), and small changes in substrate structure can apparently reverse the stereospecificity of dehydrogenases with respect to cofactor (41, 42).

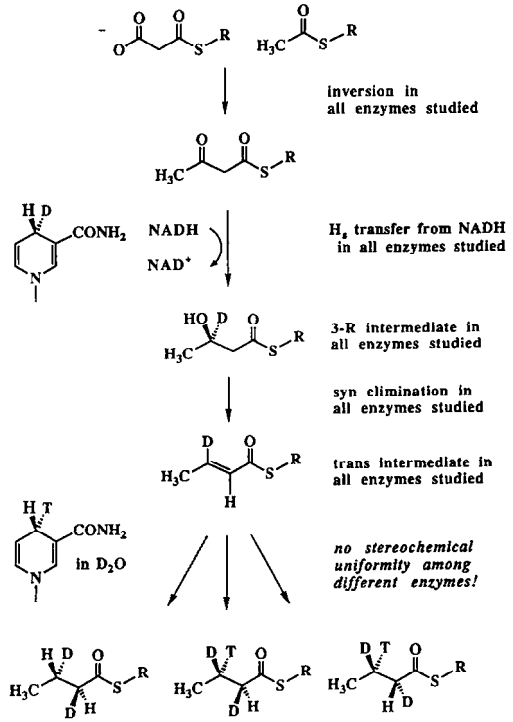

FIG. 1. **Stereochemical details of fatty acid biosynthesis.** The stereospecificities of the sequential steps in the biosynthesis of fatty acids present a special challenge to the historical model builder. In different organisms, fatty acid biosynthesis is catalyzed by either a multienzyme complex or by a multifunctional enzyme. The stereospecificities of the first three steps appear to be absolutely identical in all organisms studied. However, in the reduction of the α,β-unsaturated thioester (run here in D_2O to show the stereochemistry of the addition of a proton at carbon 2), three of the four possible stereochemical outcomes have been documented. Particularly relevant to the problem discussed in the text are the following facts: (*a*) acetoacetyl thioesters (where known) are always reduced by the pro-*S* hydrogen of NADH (consistent with the correlation between redox potential and stereospecificity discussed in Ref. 5) and (*b*) enoyl thioesters are sometimes reduced with the pro-*S* hydrogen and sometimes with the pro-*R* hydrogen of NADH (where the correlation does not apply). No simple historical model can explain these results. An assumption that the fatty acid synthetases are homologous explains fact *a*, but then fact *b* can only be explained by assuming that stereospecificity can diverge in a series of homologous enzymes, which undermines the assumption that stereospecificity *cannot* diverge needed to explain fact *a* by the assumption that the fatty acid synthetases are homologous. An assumption that fatty acid synthetases are *not* homologous might account for fact *b*, but then fact *a* (and the general stereochemical similarities found throughout the pathway) must all be assumed to be accidental. Thus, a historical model must assume a more complex ancestry for fatty acid synthetases, one that assumes that only *some* subunits are homologous. Alternatively, the historical model must postulate that the stereospecificity of dehydrogenases catalyzing some reactions can diverge more readily than those catalyzing others.

Furthermore, *non*homology does not appear to be a good indicator of stereochemical *diversity* (set *c*, Table II). D- and L-lactate dehydrogenases are presumably not homologous (30), yet have the same stereospecificities with respect to cofactor (13). Two nonhomologous dihydrofolate reductases appear to bind cofactor in the same way to yield the same stereospecificities (43).

Finally, the frequency of occurrence of stereochemical diversity is not predictable based on evolutionary distance. In enoyl-CoA reductases and ethanol dehydrogenases, stereochemical diversity is observed within a kingdom. In HMG-CoA reductases, stereochemical diversity is observed between

kingdoms. Yet in lactate and 3-hydroxybutyrate dehydrogenases, stereochemical diversity is *not* seen between kingdoms. And in malate dehydrogenases, stereochemical diversity is *not* seen among enzymes drawn from all branches of all three kingdoms. Indeed, stereospecificity in malate dehydrogenase is apparently more highly conserved than ribosomal protein sequence, membrane composition, and the genetic code (44).

Even here, the historical model is not complete. In those cases where stereospecificity is highly conserved, and in the absence of a directly selected functional role for the conserved stereospecificity, a historical model must assume that stereospecificity is tightly coupled to another selectable function in the enzyme and that stereospecificity cannot drift without disrupting this function.

However, this assumption removes only one possible mechanism for the divergence of stereospecificity. A deletion-replacement mechanism also exists for producing stereochemical diversity in a class of enzymes, whereby the gene for an enzyme transferring the pro-*R* hydrogen (*e.g.* a malate dehydrogenase) is deleted and replaced by the evolution of the substrate specificity of an enzyme transferring the pro-S hydrogen (*e.g.* a 3-hydroxybutyrate dehydrogenase) with conservation of cofactor stereospecificity (to create a malate dehydrogenase with pro-*S* stereospecificity).

Such deletion-replacement processes are facile; they are known on the laboratory time scale (45, 46). Thus, assumptions that they do not occur in dehydrogenases seem weak. *A priori,* it is not inconceivable that divergence of substrate specificity in dehydrogenases can interconvert only those enzymes acting on substrates with structures, perhaps explaining the correlation between stereospecificity in alcohol dehydrogenases and the redox potential of the natural substrate (5). Unfortunately, this does not seem to be the case. For example, dehydrogenases acting on sorbitol and ethanol (from yeast, both transferring the pro-*R* hydrogen) are clearly homologous (40). Likewise, dehydrogenases acting on glucose, ribitol, and ethanol (from *Drosophila,* all transferring the pro-*S* hydrogen) are homologous (40). Casual inspection of Fig. 2 reveals no simple structural rules that explain the allowed and disallowed patterns of substrate divergence. Therefore, the simplest historical model must incorporate *ad hoc* assumptions explaining the patterns observed. Here again, the reader is challenged to suggest mechanistic bases for these assumptions.

Even with these considerations, paradoxes remain in the historical model. Aldose dehydrogenases from two kingdoms (acting on both D- and L-sugars) all transfer the pro-*S* hydrogen. Most simply, a historical model must assume that all modern D-aldose dehydrogenases are descendants of a single ancestral D-aldose dehydrogenase, all modern L-aldose dehydrogenases are descendants of a single ancestral L-aldose dehydrogenase, both ancient enzymes happened by chance to have the same stereospecificity, and aldose reductase cannot be deleted and replaced by cross-evolution of a pro-*R*-specific dehydrogenase (for example, a pro-*R*-specific ethanol dehydrogenase). Unfortunately, an ethanol dehydrogenase *has* evolved to oxidize an aldose (set *d,* Table II) (40). Thus, even these arbitrary assumptions cannot explain why deletion-replacement events have not disrupted the pattern of conservation in aldose dehydrogenases. The historical model must posit that such events simply have not occurred in these cases. Parallel paradoxes make explanations of the stereospecificity of aldose reductases difficult as well.

We develop the historical model in Table II not because we necessarily believe that it is attractive in comparison with alternative models (1–5), but because none of the many advocates of historical models have ever rigorously described what such models must entail to be consistent with facts. The *ad hoc* assumptions in Table II offer many opportunities for a biochemist to construct mechanistic rationales to make them less arbitrary. Nevertheless, one should appreciate how complex a historical model must be to explain available data and how this complexity weakens its explanatory power and its value as a paradigm for directing experimental work. The reader is then invited to contrast the simplest historical model described here with alternative functional models discussed elsewhere (2, 3, 5), which address the same facts in terms of the assumption that in some cases, stereospecificity in dehydrogenases is a selected trait, and draw his own conclusions as to which are more plausible.

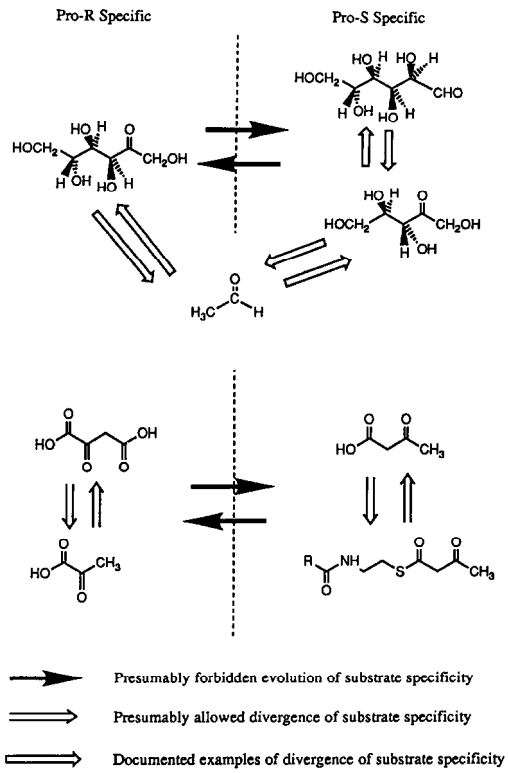

FIG. 2. **Divergence of substrate specificity offers a mechanism for obtaining stereochemical heterogeneity in a class of enzymes acting on a single substrate, even if stereospecificity with respect to cofactor is presumed to be absolutely conserved during divergent evolution, as deletion-replacement events offer the opportunity for pro-*R*-specific enzymes to be "recruited" to perform a catalytic role performed by a deleted pro-*S*-specific dehydrogenase.** The extremely highly conserved stereospecificities in some enzymes suggests that this has never happened in the time separating archaebacteria, eubacteria, and eukaryotes. In contradiction to this is the evident fact that divergence in substrate specificity is facile in enzymes generally (8–11) and is known in several dehydrogenases (indicated by the *completely enclosed white arrow*). No simple structural rule explains the patterns of known, presumably allowed, and presumably forbidden divergence in substrate specificity. Indeed, compounds on the *left* and *right side* of the *dotted line* (separating stereospecificities) often have quite similar structures (*e.g.* fructose and ribulose, oxaloacetate, and 3-hydroxybutyrate), differences often smaller than structural differences observed in the substrates of dehydrogenases known (by sequence analysis) to be homologous (*e.g.* ethanol and sorbitol, ethanol and glucose). Thus, the patterns of allowed and forbidden divergence in substrate specificity must be explained individually and *post hoc.*

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Additional references are found on p. 11699.

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Supplemental Material To: The Stereospecificities of Seven Dehydrogenases from Acholeplasma laidlawii: The Simplest Historical Model that Explains Dehydrogenase Stereospecificity. By Arthur Glasfeld, Fary F. Leanz, and Steven A. Benner. Laboratory for Organic Chemistry, Swiss Federal Institute of technology, CH-8092 Zurich, Switzerland.

Supplemental Material

While determining the stereospecificities of dehydrogenases is routine, several features prominent in the biochemistry of *Acholeplasma* are relevant to the experimental work reported here.

First, *Acholeplasma* contain substantial NADH oxidase activity, which can interfere with the determination of the stereospecificity of other dehydrogenases. Thus, although enzymes need not generally be purified to permit their stereospecificity to be studied, dehydrogenases from *Acholeplasma* must be purified at least to the extent that the NADH oxidase activity is removed. We have found (as have previous workers) that the NADH oxidase in *Acholeplasma* is associated with the membrane fraction of crude lysates if the cells have been gently disrupted. The procedure described below, where *Acholeplasma* were disrupted by freeze-thaw, successfully separated NADH oxidase (in the membrane fractions) from the cytosolic dehydrogenases discussed here, and is a general method for studying the stereospecificity of soluble enzymes from *Acholeplasma*.

NADH oxidase from *Acholeplasma* has already been purified to homogeneity, and is the dominant activity found in membrane fractions prepared by osmotic shock. Thus, its stereospecificity can be easily and reliably measured, even though the label appeared in the protons of water which, by their exchangable nature, cannot be directly converted to a solid derivative that can be recrystallized to constant specific activity.

As the hydroxymethylglutaryl-CoA reductase proved to have an unusual stereospecificity, a rather detailed study of the properties of the enzyme was justified. The enzyme was purified many thousand fold to homogeneity, and its various kinetic, physical, stereochemical, and catalytic properties investigated. Results of these investigations are presented in archival material. Further, the stereospecificity of HMG-CoA reductase was checked independently several times by two of us (GFL and AG), using two different preparations of organisms grown on two different continents. HMG-CoA reductase is now one of the best characterized enzymes from *Acholeplasma*.

The alcohol dehydrogenases raised another concern. Many organisms contain several isozymes of alcohol dehydrogenase, often with different ranges of substrate specificity. As such isozymes could in principle have different stereospecificities, it was important to be certain that a single activity was being examined. Therefore, the alcohol dehydrogenase activity examined was purified to yield a single band by activity staining on a native gel, using the procedure described below. To characterize the activity further, the substrate specificity of the enzyme was examined. Evidence concerning the mechanism of the dehydrogenase was also of interest, as at least two mechanistic types of alcohol dehydrogenases are known: those that require zinc for catalytic activity, and those that are independent of zinc for catalytic activity. Therefore, the effect of phenanthroline as an inhibitor of the catalytic activity was examined. The results are reported below.

The other enzymes act in standard biochemical pathways known in *Acholeplasma* and, except for alanine dehydrogenase (*vide infra*), these activities are used for classification of the organism.(51,52) In all cases except alanine dehydrogenase, the enzymatic activity described in the text is already known and at least partially characterized in *Acholeplasma*, and several of the more obvious competing activities are reported to be absent.

The alanine dehydrogenase activity reported here was discovered accidentally when it interfered with assays for lactate dehydrogenase involving the reduction of pyruvate in buffers containing ammonium salts. As this activity appears to have never been reported in *Acholeplasma*, special care was taken to characterize it, included making solid derivatives of labelled alanine produced from pyruvate and ammonia, which were isolated by two chromatographic systems and recrystallized to constant specific activity.

All stereospecificities were re-examined many times, using both diastereomers of labeled NADH when appropriate. Experiments using or generating labeled NADH nearly always detect small amounts of apparent stereochemical "infidelity"; ratios of transfer of label from pro-R and pro-S labeled NADH are commonly 95:5 (or, conversely, 5:95). Much of this apparent infidelity appears to arise from a non-enzymatic transhydrogenation reaction between NADH and NAD^+, a reaction that has been studied by Kellogg and his coworkers.(53) There is no evidence that it reflects microscopic stereochemical infidelity at a single active site. Indeed, when stereochemical studies have been done under conditions that exclude such reactions, stereochemical fidelity is found to better than one part in 100 million.(54)

Thus, although the results reported here concern the predominant stereospecificity of hydride transfer, it is reasonable to assume that it represents the only stereochemical mode of hydride transfer, within limits similar to these.

Acholeplasma

Acholeplasma are the genus of the *Mycoplasma* family that do not require sterol for growth; this family includes the smallest self-propagating organisms known (80-400 nm in diameter). *Acholeplasma* requires a rich growth medium, and one successful chemically defined medium contained over 65 nutrients,(55) showing that many biosynthetic pathways are absent. Their size and metabolic requirements suggest that *Mycoplasmas* can help define the minimal set of biochemical functions necessary for independent growth.

The expression of the isoprenoid pathway in *Acholeplasma* is significant: the genus neither synthesizes nor requires sterol for growth, but it synthesizes several different carotenoids in large amounts.(56) These may serve to reinforce the membrane bilayer, to protect against damage from light and singlet oxygen, and to participate in electron transport, among other postulated functions.(57) The isoprenoid pathway is perhaps the richest and most varied in all of biochemistry, leading to a fascinating array of structural, metabolic, and regulatory products (58): isopentenyl adenine, quinones, carotenoids, sterols, undecaprenyl pyrophosphate, bile acids, squalene, dolichols, steroid hormones, essential oils and resins, the isoprenoid side chains of other molecules, pentacyclic hopanoids, and many more. The specific branches of the pathway which are expressed vary among organisms, but isoprenoid compounds exist universally, and evidence suggests that this pathway is quite old.(58)

The bacteria of the *Mycoplasma* family lack a cell wall,(59) and so would be not expected to synthesize bactoprenol. Menaquinones have been identified,(60) but the pathways of respiration and electron transport have not been fully elucidated.(61,62)

HYDROXYMETHYLGLUTARYL COA REDUCTASE

In cases where it has been studied, HMG-CoA reductase uses a two-step mechanism without the release of intermediates.(63) Further, HMG-CoA contains a chiral center at position 3 of the glutarate unit, and the dehydrogenase is expected to distinguish between these enantiomers.

The HMG-CoA reductase from *Acholeplasma* was studied in great depth in this work. Some of these studies are reported in archival material. Summarizing the data reported there, the HMG-CoA reductase from Acholeplasma was found to accept substrate with the same chirality at the 3 position as the analogous enzymes from other sources, accepted mevaldehyde when added separately as a substrate, and displayed ordered reaction kinetics.

NADH OXIDASE

NADH oxidase from *Acholeplasma laidlawii* was first purified to homogeneity and characterized by Reinhards et al.(64) The following procedure is based on the first steps of this purification.

Acholeplasma cells were gently lysed by osmotic shock. Frozen cell paste (0.9 g) was resuspended in a 0.2 M NaCl solution (2 ml) in 20% v/v glycerol. An aliquot of the suspension (0.3 ml) was rapidly injected into 30 ml of distilled water through a 20 gauge needle. After incubation at 37°C for 20 min, the solution was centrifuged at 45,000 x g for 30 min at 5°. The bright yellow pellet was washed twice alternately with 0.25 M NaCl (1 ml) and water (1 ml). The pellet was resuspended in buffer (2.5 mM Tris, pH 8.0, 10 mM NaCl, 0.5 mM 2-mercaptoethanol and 3% Triton X-100), allowed to stand at room temperature for two hours, and centrifuged in a Fisher microcentrifuge. The supernatant contained the NADH oxidase activity.

NADH oxidase from Acholeplasma was found to remove one hydrogen from NADH which appears as an exchangable proton of water. To determine stereospecificity of hydride removal, pro-R and pro-S labelled (4-^{3}H)NADH were each separately oxidized in the presence of 20 µl of the NADH oxidase preparation in buffer (50 mM sodium phosphate, pH 7.0, 1 ml). After the reaction was complete, the water was separated from cofactor by bulb to bulb lyophilization using the following procedure. An aliquot of the reaction mixture (0.5 ml) was placed in a 25 ml flask and frozen in liquid nitrogen. The flask was then connected to a tube that had connections to a second, empty flask and to a vacuum pump. While the flask containing the solution was immersed in liquid nitrogen, the entire system was evacuated by vacuum and then sealed. The receiving flask was then cooled with liquid nitrogen. After the distillation was complete, the lyophilizate in the initial flask was redissolved in distilled water (0.5 ml), the contents of the residue were identified by thin layer chromatography, and the tritium present in both the distillate and the residue was counted by scintillation counting.

ALCOHOL DEHYDROGENASE

Cells were lysed by a gentle freeze thaw method to enable the separation of alcohol dehydrogenase activity from NADH oxidase activity, which was present in the membrane fraction under these conditions.

Frozen cell paste (1 g) was resuspended in freeze-thaw buffer (10 ml, 0.01 M Tris, pH 7.6, 0.25 M NaCl, and 0.001 M, 2-mercaptoethanol). The suspension was frozen in a dry ice/acetone bath and subsequently warmed in a 37°C water bath. The cycle was performed three times with constant agitation. Cellular debris was removed by centrifugation (40000 x g, 5°C). The supernatant was retained and fractioned by the addition of solid ammonium sulfate. For alcohol dehydrogenase, the pellet collected between 1 and 1.5 M $(NH_4)_2SO_4$ (based on original sample volume) contained greater than 90% of the activity. The pellet was from precipitation at these concentrations was resuspended in 1 ml of buffer (50 mM sodium phosphate, pH 7.0) and dialyzed against 1 l of the buffer at 4°C overnight. Native gel electrophoresis followed by activity staining (ethanol, nitrotetrazolium blue) showed that this preparation contained a single band with ethanol dehydrogenase activity.

The substrate specificity of the alcohol dehydrogenase activity was determined by measuring the initial reaction velocities for the oxidation of NADH by carbonyl compounds the presence of the enzyme. Each reaction mixture contained enzyme solution (20 µl), NADH (0.2 µmol), and buffer (1 ml of 50 mM sodium phosphate, pH 7.0) containing 50 mM aldehyde or ketone. Initial reaction rates were measured by following the decrease in absorbance at 340 nm due to the oxidation of NADH. The relative rates of reduction of various aldehydes and ketones was (aldehyde or ketone, velocity relative to acetaldehyde): acetaldehyde, 1.00; propionaldehyde, 0.96; crotonaldehyde, 0.37; formaldehyde, 0.12; benzaldehyde, 0.07; acetone, 0.07; 2-butanone, 0.03.

To gain evidence for or against participation of a metal ion in catalysis, the sensitivity of the alcohol dehydrogenase activity to inhibition by 1,10-phenanthroline was determined. The solution of the enzyme (50 µl) was incubated in buffer (50 mM sodium phosphate, pH 7.0, 0.85 ml) containing a range of concentrations (from 10 µM to 5 mM) of phenanthroline at 0°C for 30 min. Ethanol (to a final concentration of 1 M) and 0.020 mM and NAD^+ (to a final concentration of 0.020 mM) were then added, and the initial rate of the oxidation reaction was measured spectrophotometrically (340 nm).

The alcohol dehydrogenase activity was found to be inhibited by 1,10-phenanthroline. A 50% reduction in the catalytic activity of the protein was observed at [phenanthroline]=0.1 mM. At (phenanthroline]=1 mM, the enzymatic activity was 90% inhibited.

To determine stereospecifity of the alcohol dehydrogenase, (4-S-^{3}H)NADH or (4-R-^{3}H)NADH was oxidized by acetaldehyde or propionaldehyde (each alcohol was examined seeparately, 10 µmol of each alcohol) catalyzed by a solution (20 µl) of the alcohol dehydrogenase in buffer (50 mM sodium phosphate, pH 7.0, 1 ml). After the reaction was completed (ultraviolet absorbance at 340 nm), the product alcohols were separated from cofactors by bulb to bulb distallation (*vide supra*) or by DEAE cellulose chromatography (in the bicarbonate form). The latter involved applying 0.2 ml of the reaction solution to a DEAE cellulose column (column volume 1.5 to 2 ml), and eluting first with water (5 ml, fractions pooled). Cofactors were then eluted from the column in a solution of 1 M ammonium bicarbonate (5 ml, fractions pooled). The non-volatile products were identified chromatographically, and the tritium present in the two fractions was counted by liquid scintillation counting.

To prove the structures of the volatile products (presumably ethanol and 1-propanol), solid naphthylurethane derivatives were prepared. The distillates (containing a mixture of water and the alcohol) were saturated with NaCl, the appropriate alcohols (1 ml of each) were added as carrier, and the mixtures were extracted with ether. The ether solutions were dried over molecular sieves, evaporated at room temperature under vacuum and the remaining alcohols were reacted with 1-naphthylisocyanate (0.5 g). The resulting solid products were recrystallized several times from pentane to constant specific activity. The structures were confirmed by melting point and NMR, and the radioactivity they contained was determined by liquid scintillation counting.

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

To isolate glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity, *Acholeplasma* cells were disrupted by the freeze-thaw method (*vide supra*, with the addition of 1 mM phenylmethylsulfonyl fluoride). Activity could be detected in crude extracts. A series of studies showed that enzymatic activity could be purified free of interfering activities by precipation with ammonium sulfate. Optimal recovery was found when precipitation was carried out between 2.5 and 3.0 M $(NH_4)_2SO_4$. The pellet of protein was isolated by centrifugation, and then resuspended in 1 ml of buffer (50 mM Tris, pH 8.0). The protein was then dialyzed overnight against the same buffer (1 l) to remove any remaining small molecules.

The substrate for this enzyme, glyceraldehyde-3-phosphate, was prepared by mixing the barium salt of the diethyl acetal of glyceraldehyde-3-phosphate (Sigma, 100 mg) in distilled water (5 ml) with Dowex 50 W X8 cation exchange resin (1 ml, H^+ form) for 5 min. The solution was filtered, incubated at 37°C overnight, and diluted to 20 mM aldehyde in 50 mM Tris, pH 8.0.

The stereospecificity of GAPDH was determined by reducing 1.5 nmol of (4-^{3}H)NAD^+ with an equivalent of glyceraldehyde-3-phosphate in a buffer containing arsenate (15 mM sodium phosphate/30 mM sodium arsenate, pH 8.5) in the presence of 30 µl of the enzyme solution. The mixture was incubated at 30°C for 15 min. Then unlabelled NADH (0.1 µmol) was added as carrier, and the NADH was purified by HPLC (DEAE-cellulose, bicarbonate form, gradient 0 to 1 M ammonium bicarbonate as eluant). The (4-^{3})NADH was isolated in a 1 ml eluant, and then was oxidized stereospecifically, either by removing the pro-R hydrogen with 20 µmol of acetaldehyde in the presence of horse liver alcohol dehydrogenase (0.04 units), or by removing the pro-S hydrogen through the addition of 50 µl of a solution of NADH oxidase from *Acholeplasma* (found to be pro-S specific, *vide supra*). The resulting solutions were then distilled, and the relative activities of the volatile and non-volatile fractions were determined.

LACTATE DEHYDROGENASE

Lactate dehydrogenase from *Acholeplasma* has been previously studied in some detail by O'Carra and Barry (65). Cells from *Acholeplasma* were disrupted using the freeze-thaw method (*vide supra*). The lactate dehydrogenase activity was found to be precipitated by ammonium sulfate, and was collected in the pellet formed between 2.0 and 2.5 M $(NH_4)_2SO_4$. The pellet was resuspended in buffer (0.02 M sodium phosphate pH 7.0, 0.5 M NaCl, 1 ml), and the solution was dialyzed against the same buffer (1 l) overnight.

The lactate dehydrogenase activity was then further purified by affinity chromatography. Oxamate-agarose resin (0.35 g) was allowed to swell in a solution of NaCl (0.5 M) for 2 hours. The beads were then centrifuged and washed 3 times with NaCl solution and 3 times with distilled water prior to being resuspended in buffer (0.02 M sodium phosphate, pH 7.0, 0.5 M NaCl, 0.2 mM NADH) and loaded into a column (0.7 x 4 cm). The column was washed with five column volumes of buffer. The enzyme solution was brought to 0.2 mM NADH, and then loaded on to the column at room temperature and allowed to equilibrate for two minutes prior to elution. Non-specifically bound material was eluted with 5 column volumes of the buffer. The NADH was then omitted from the eluant, causing the release of lactate dehydrogenase from the column. The runoff was collected in 1 ml fractions. Lactate dehydrogenase activity was eluted in fractions 3-5 following the removal of NADH. The enzyme activity was unstable, do these fractions were used immediately in stereospecificity determination.

(4-S-^{3}H)NADH or (4-R-^{3}H)NADH, in 0.8 ml of buffer (50 mM sodium phosphate, pH 7.0), were (in separate experiments) oxidized by 3 µmol of sodium pyruvate in the presence of 0.2 ml of purified enzyme. The product lactate was isolated by thin layer chromatography on Whatman 3 chromatography paper using the method of Long and Kaplan (66). An aliquot of the reaction mixture (50 µl) was applied to a 12 cm piece of paper, along side of authentic NAD^+ and lactate as standards. The chromatograph was then developed with n-butanol:acetic acid:water (12:3:5) to 10 cm. The 10 cm chromatogram was then dried with heating and cut into ten 1 cm strips, each of which were placed in scintillation vials with 5 ml of scintillation fluid and counted directly. To demonstrate the presence of radioactivity in the product lactate, the phenacyl derivative was prepared and recrystallized to constant specific activity. An aliquot of the reaction mixture (0.5 ml) was diluted with carrier sodium L-lactate (1 g) in water (5 ml). Phenacyl bromide (1 g) in ethanol (10 ml) was then added, and the solution was heated at reflux for 1 hr. The solution was then cooled and evaporated under reduced pressure. The phenacyl derivative of lactate was recrystallized to constant specific activity from pentane and methylene chloride, and counted by liquid scintillation counting.

ALANINE DEHYDROGENASE

Alanine dehydrogenase activity was precipiated by ammonium sulfate, and was collected in the pellet formed between 2.0 and 2.5 M $(NH_4)_2SO_4$. The pellet was resuspended in buffer (0.02 M sodium phosphate pH 7.0, 0.5 M NaCl, 1 ml), and the solution was dialyzed against the same buffer (1 l) overnight. In the presence of NADH and ammonium sulfate (50 mM), the extract was found to yield alanine, which was positively identified both by chromatography and as a solid derivative.

(4-S-^{3}H)NADH and (4-R-^{3}H)NADH were incubated separately with sodium pyruvate (1 µmol) ammonium sulfate (50 mM) and enzyme solution (50 µl) in 1 ml buffer (50 mM sodium phosphate, pH 7.0). To separate alanine from NAD^+, an aliquot of the reaction mixture (50 µl) was spotted on a 12 cm piece of Whatman 3 MM chromatography paper. The chromatograph was developed with n-butanol:acetic acid:water (4:2:1). Alanine, lactic acid and NAD^+ were run as standards on the same chromatograph, and the spots removed and counted (*vide supra*)

To identify L-alanine unambiguously as the product of the reaction, a p-toluenesulfonamide derivative was prepared from labelled alanine. An aliquot of the reaction mixture (0.5 ml) together with carrier of L-alanine (1 g) were dissolved in 20 ml of 1 M NaOH. A solution of p-toluenesulfonyl chloride (2.0 g) in ether (25 ml) was then added, and the mixture stirred at room temperature for 3 hours. The phases were separated, and the aqueous phase was acidified with 10% HCl and extracted with three volumes of methylene chloride. The organic phase was dried over $MgSO_4$, filtered and evaporated to give the sulfonamide. The solid was recrystallized to constant specific activity. The melting point and NMR spectra corresponded with authentic material.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Glucose-6-phosphate dehydrogenase activity has been previously reported in *Acholeplasma*. Frozen cell paste (1.5 g) was resuspended in lysis buffer (5 ml), and the cells disrupted by freeze-thaw cycle (*vide sura*). The mixture was then centrifuged (20,000 x g, 15 min, 4°C), the supernatant decanted, and the pellet resuspended in the 5 ml of buffer. Glass beads (5 g) were added and cells were homogenized by grinding for 5 sec every 15 sec over a period of 3 min at 0°C in a Bio-spec bead beater. Centrifugation (40000 x g, 15 min, 4°C) was performed twice to remove cellular debris and the supernatant was treated by ammonium sulfate fractionation. The activity of glucose-6-phosphate dehydrogenase was found to precipitate between 2.5 and 3.0 M $(NH_4)SO_4$. The pellet obtained at these concentrations of salt was resuspended in 1 ml of buffer (50 mM Tris, pH 8.0), and dialyzed overnight against 1 l of buffer.

To obtain a pair of matching stereochemical results, separate incubations were run, one with labelled (4-^{3}H)$NADP^+$ and unlabelled glucose-6-phosphate, the other with unlabelled $NADP^+$ and (1-^{3}H)glucose-6-phosphate, each in the presence of an aliquot of the solution containing glucose-6-phosphate dehydrogenase activity. In the first case, (4-^{3}H)$NADP^+$ (1 nmol) was incubated with glucose-6-phosphate (100 nmol) and an aliquot of the enzyme solution (50 µl) in 125 µl of buffer (50 mM Tris, pH 8.0). In the second, $NADP^+$ (50 nmol) (1-^{3}H)glucose-6-phosphate (600 nmol, produced *in situ* from glucose, ATP, and hexokinase), and enzyme (50 µl) were incubated. (4-^{3}H)NADPH was purified by HPLC (DEAE column, 0-750 mM NaCl in 50 mM Tris, pH 8.0). To determine the prochiral position tritiated by each reaction, the labelled samples of NADPH were oxidized by sodium 2-oxoglutarate (100 nmol) in the presence of 2 units glutamate dehydrogenase (bovine). To separate glutamate from $NADP^+$, 50 µl of a reaction mixture were streaked on a piece of Whatman chromatography paper, and eluted with n-butanol:acetic acid:water (4:3:1). Authentic specimens of glutamate $NADP^+$ were chromatographed in parallel. The resulting chromatogram was treated as above for scintillation counting.

For purposes of comparison, parallel runs were made using glucose-6-phosphate dehydrogenases from *Bacillus stearothermophilus* (5 units) and bovine adrenal glands (3 units, both from Sigma). Both enzymes transfer a hydrogen to the *si* face of NAD^+.

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