

males are due to a direct action of elevated PRL levels on the testis. Our results probably cannot be discounted as an artifact of the *in vitro* system, since both PRL and GH can affect functioning of cultured cells and incubated tissues¹⁰⁻¹². However, specific PRL binding sites have been demonstrated in the interstitium of the rat testis¹³ and PRL is capable of inhibiting steroidogenesis in long-term cultures of both testicular¹⁴ (and G.F. Erickson, personal communication) and ovarian cells¹⁰.

Another implication of the present findings may be worthy of note. Stimulation of T synthesis by dispersed mouse Leydig cells is widely used as a bioassay of LH^{15,16}. Our results suggest that the results of this assay are unlikely to be affected by PRL or GH present in serum samples.

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DISPUTANDUM

The stereoselectivity of alcohol dehydrogenases: A stereochemical imperative?¹

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Summary. The stereoselectivity of NAD⁺-dependent alcohol dehydrogenases (transferring either the pro-R or pro-S hydrogen of NADH) correlates with the thermodynamic stability of their substrates, and appears to reflect evolutionary pressure to adjust in the active site the conformation of NADH so as to match the cofactor's reducing power to the oxidizability of the substrate. A requirement that the free energies of protein-bound intermediates be matched suggests a new approach for understanding catalysis and evolution in enzymes.

Stereoselective transfer of hydrogen to and from nicotinamide cofactors is the best studied example of the power of enzymes to make stereochemical choices. Westheimer, Vennesland and coworkers² first demonstrated this selectivity in the early 1950's, showing that dehydrogenases acting on NADH occur in 2 stereochemically distinct classes. Members of 1 class catalyze the transfer of the pro-R (A) hydrogen; member of the 2nd class catalyze the transfer of the pro-S hydrogen. More recently, work on well over a 100 additional dehydrogenases has shown that roughly half of all dehydrogenases belong to the 1st class and half to the 2nd^{3,4}. The large body of data for dehydrogenases has prompted many authors⁴⁻⁷ to attempt to discern a pattern in the stereopreferences of these enzymes. Hoping to understand why a particular dehydrogenase belongs to a particular class, several investigators have formulated a variety of 'rules' regarding the stereochemical preferences of dehydrogenases⁴⁻⁷. However, most of the 'rules' have exceptions, and some appear decidedly ad hoc; none have a clear mechanistic basis. Thus, none appear to have been accepted as part of a general explanation for the stereopreferences of dehydrogenases. Still others have concluded that the data contain no pattern whatsoever, that no mechanistic explanation exists, and that the stereopreferences are 'random'⁸. Thus, despite a quarter century of speculation, the mechanistic basis for the different stereochemical behavior of dehydrogenases remains a mystery.

Stereochemical ambivalence in dehydrogenases is unusual, since stereochemical uniformity seems to be the rule for most classes of enzymes. These uniformities have been ana-

lyzed recently by Hanson and Rose⁸, who argued that uniform stereochemical preferences displayed by the members of many classes of enzymes catalyzing similar reactions might reflect the existence of a 'mechanistic imperative' in these enzymes. For chemical reasons inherent in the nature of the reaction being catalyzed, a single mode of catalysis producing a certain stereochemical outcome is presumed to be more efficient than alternative modes that produce other stereochemical outcomes. Because an organism possessing highly efficient enzymes is more likely to survive than a competing organism possessing less efficient enzymes, Hanson and Rose⁸ argued that evolutionary processes may have forced the selection of only those enzymes that are optimal catalysts, and thereby have selected only those enzymes making the optimal stereochemical choice.

The stereochemical similarities within a class of enzymes can be quite striking. For example, all enzymes using pyridoxal cofactors shuttle a proton by attacking C-4' of the pyridoxal group from the *si* face. All amino acid decarboxylases proceed with retention of configuration. Of course, these uniformities can possibly be understood in 'historical' terms; if all of the members of these classes of enzymes have evolved from a common ancestral protein that catalyzed a primeval prototypical reaction, the stereochemical preferences of the ancestor may have been conserved. Nonetheless, Hanson and Rose⁸ suggested a number of chemical explanations for why one stereochemical outcome might be catalytically superior to an alternative outcome. In the case of dehydrogenases, where non-uniformity is the rule, the arguments of Hanson and Rose force one to the

conclusion either that no stereochemical imperative exists favoring one stereochemical course over the other, or that an imperative does exist, but the processes of evolution have not been able to exploit it⁹. The 1st conclusion is remarkable from the view of bio-organic chemistry, and suggest a need for further investigation to determine which stereochemical distinctions are important catalytically and which are not. The 2nd conclusion is remarkable from an evolutionary point of view, since it contradicts the emerging belief that the processes of evolution have produced enzymes that approach catalytic 'perfection'¹⁰.

The data. We report here a hitherto unnoticed regularity which suggests that the stereochemical outcome in dehydrogenases acting on alcohols is neither random nor evolutionarily neutral, but reflects the evolution of these enzymes to produce an active site that is catalytically optimal. A plot (fig. 2) of stereochemical outcome (pro-R or pro-S) versus equilibrium constant:

$$(K_{eq} = [\text{ketone}] [\text{NADH}] [\text{H}^+] / [\text{alcohol}] [\text{NAD}^+])$$

for the overall reaction in dehydrogenases acting on simple alcohols shows this correlation most clearly. If the K_{eq} for a particular enzyme-catalyzed reaction is less than 10^{-12} M^{-1} , the pro-R hydrogen is generally transferred; if the K_{eq} is greater than 10^{-12} M^{-1} , the pro-S hydrogen is transferred. We have interpreted these data as suggesting that more reactive carbonyls are reduced by the pro-R hydrogen, while less reactive carbonyls are reduced by the pro-S hydrogen of NADH.

The data summarized in figure 2 are experimental facts that stand independent of any hypothesis advanced to explain them. However, in view of such a striking correlation, we may attempt to provide an explanatory hypothesis, especially one that can be tested by experiment.

The postulates. We suggest that the stereochemical preferences of dehydrogenases reflect their evolution under selective pressure to become optimal catalysts. Specifically, we propose that dehydrogenases have evolved active sites that adjust the conformation of the nicotinamide cofactor so as to match its reduction potential to the ease with which the co-substrate is reduced. Thus, the stereochemical preferences observed in dehydrogenases can be understood on the basis of the following:

1. Enzymatic transfer of the pro-R hydrogen of NADH occurs when the nicotinamide ring is in the anti conformation, while transfer of the pro-S hydrogen occurs when the nicotinamide ring is in the syn conformation (fig. 3).
2. Anti-NADH is a weaker reducing agent than syn-NADH.
3. Enzymes are optimally efficient when the intermediate states along the reaction coordinate are of equal free energies.
4. Dehydrogenases have evolved to be optimally efficient catalysts.

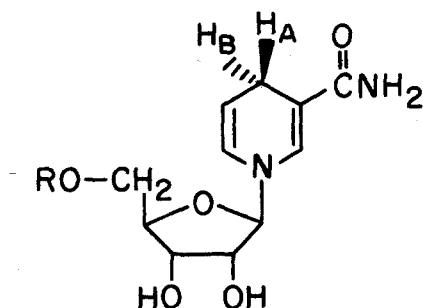


Figure 1. The pro-R (A) and pro-S (B) hydrogens of reduced nicotinamide adenine dinucleotide are diastereotopic.

Support for each of these hypotheses is enumerated below.

Evidence for the postulates. 1. Crystal structures of four dehydrogenases¹³ have led several commentators^{4,14} to suggest that dehydrogenases that are pro-R specific bind the NAD^+ cofactor in an anti conformation, while those that are pro-S specific bind the cofactor in the syn conformation. In view of the principles of stereoelectronic control¹⁵, one might expect this rule to be general for all dehydrogenases. Transfer of the pro-R hydrogen from an anti conformation permits a desired 'cis' arrangement between the leaving hydride and the pair of electrons on nitrogen¹⁶, distorted from planarity to optimize electron donation into the antibonding orbital of the C—O bond in the ribose ring¹⁷. Such a transfer would be from the sterically less hindered face of the dihydropyridine ring. Further, experimental work with NAD^+ analogs having methyl groups at the 2 or 6 positions¹⁸, (which are thereby constrained to occupy the anti and syn conformations respectively) suggest that the arguments based on crystallography are valid in solution as well.

2. The 2nd hypothesis is difficult to confirm directly, but since the equilibrium constants between the syn and anti forms of both oxidized and reduced cofactors can be estimated from NMR-data on nicotinamide mononucleotides¹⁹, this relationship can be obtained qualitatively from the thermodynamic diagram shown in figure 4. If for the reduced cofactor the anti conformation is lower in energy than the syn, while for the oxidized cofactor the anti conformation is higher in energy than the syn, then anti NADH will be a weaker reducing agent than syn NADH²⁰. Unfortunately, the experimental data are not sufficient to

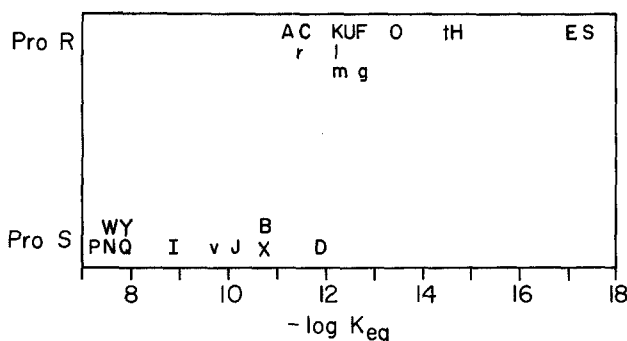


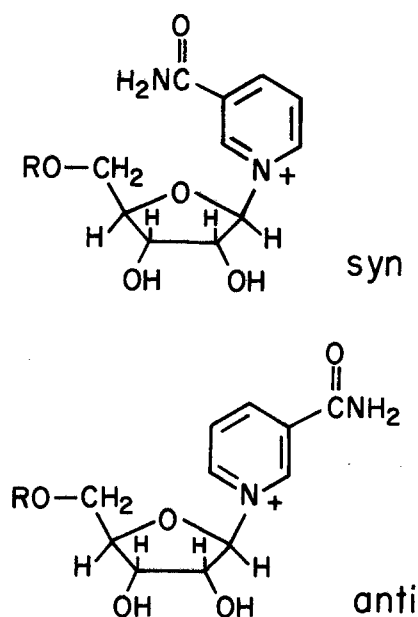
Figure 2. A plot of the stereochemical preference of dehydrogenases (pro-R or pro-S) versus the negative logarithm of the equilibrium constant for the reactions they catalyze. ($K_{eq} = [\text{H}^+] [\text{NADH}] [\text{ketone}] / [\text{NAD}^+] [\text{alcohol}]$). A key is found in the table. I have attempted to include in the plot all dehydrogenases acting on simple, unconjugated ketones where both the stereochemical preference and equilibrium constant has been reported for the reduction of the biologically significant substrate (upper case letters). Occasionally, the K_{eq} -values, although not reported for the specific enzyme in question, have been obtained from measurements where the equilibrium was established on the physiological substrate using other enzymes or methods (lower case letters). A number of enzymes with defined stereoselectivity were omitted because of the difficulty of obtaining a K_{eq} -value that reflects the actual redox step. For example, the reaction catalyzed by isocitrate dehydrogenase (pro-R specific) is highly exergonic, since the overall reaction involves the loss of CO_2 in addition to a dehydrogenation. Similarly, the equilibrium constants obtained for the oxidation of polyalcohols to sugars are invariably between the hemiacetal form of the sugar and the polyalcohol, again obscuring the equilibrium constant for the oxidation/reduction reaction. Also on occasion, an enzyme with defined stereochemistry was omitted if the physiological substrate is in question, or the substrate specificity was too broad to define reasonably the natural substrate. Alcohol dehydrogenase from horse liver is an example of this sort of enzyme.

Data for figure 2

Symbol	E.C.	Reference	Name	Stereochemistry
A	1.1.1.1	25	Alcohol dehydrogenase (Yeast)	A
B	1.1.1.3	26	Homoserine dehydrogenase	B
C	1.1.1.6	27	Glycerol dehydrogenase	A
D	1.1.1.8	28	Glycerol-3-phosphate dehydrogenase	B
E	1.1.1.26	29	Glyoxylate reductase	A
F	1.1.1.27	30	L-Lactate dehydrogenase	A
g	1.1.1.28	30	D-Lactate dehydrogenase	A
H	1.1.1.29	31	Glycerate dehydrogenase	A
I	1.1.1.30	32	3-Hydroxy butyrate dehydrogenase	B
J	1.1.1.35	33	3-Hydroxy acyl CoA dehydrogenase	B
K	1.1.1.37	34	Malate dehydrogenase	A
l	1.1.1.38	34	Malic enzyme	A
m	1.1.1.40	34	Malic enzyme (NADP)	A
N	1.1.1.51	35	β -Hydroxysteroid dehydrogenase	B
O	1.1.1.60	36	Tartronate semialdehyde reductase	A
P	1.1.1.62	37	Estradiol 17 β -dehydrogenase	B
Q	1.1.1.64	35	Testosterone β -dehydrogenase	B
r	1.1.1.72	27	Glycerol dehydrogenase (NADP)	A
S	1.1.1.79	29	Glyoxylate reductase (NADP)	A
t	1.1.1.81	31	Hydroxypyruvate reductase	A
U	1.1.1.82	34	Malate dehydrogenase (NADP)	A
v	1.1.1.91	38	Aryl alcohol dehydrogenase	B
W	1.1.1.100	39	3-Oxoacyl acyl carrier protein reductase	B
X	1.1.1.108	40	Carnitine dehydrogenase	B
Y	1.1.1.50	35	3- α -Hydroxysteroid dehydrogenase (P. test.)	B

permit extensive discussion of this point, but they are certainly suggestive. This 2nd postulate must await further experimental examination.

3. The 3rd assumption is supported by recent experimental data which show that for nearly a dozen enzymes (including a dehydrogenase), the 'internal' equilibrium constant (that between the enzyme's bound species) is close to unity, even when the 'external' equilibrium constant (between free species) favors reactants or products by 3 orders or magnitude or more²¹. While this 3rd postulate can be justified on these empirical grounds, an argument by Alberly and Knowles suggests that such an arrangement might be expected for an efficient catalyst, especially if it operates reversibly²².

Figure 3. Syn and anti conformations of NAD⁺.

Together, these postulates permit the interpretation of the observed correlation in figure 2. Dehydrogenases select the weaker reducing agent (anti NMNH, and hence the pro-R hydrogen) to reduce more reactive carbonyls, and the stronger reducing agent (syn NMNH, and hence the pro-S hydrogen) to reduce less reactive carbonyls. This matching of reduction power to the ease with which the substrate is reduced must help match the free energies of the 2 ternary complexes (enzyme-NAD⁺-reduced substrate, and enzyme-NADH-oxidized substrate), and thereby optimize the catalytic efficiency of the enzyme.

We emphasize that it is the free energies of the bound states that actually occur along the reaction coordinate in the enzyme catalyzed process that are matched, not those of the unbound species, which the enzyme cannot alter. Yet it is the equilibrium constants between unbound species, which are far easier to measure, that are plotted in figure 2. We presume that this external equilibrium constant correlates reasonably with the equilibrium constants for the bound states. Because the chemistry of the intermediates along the

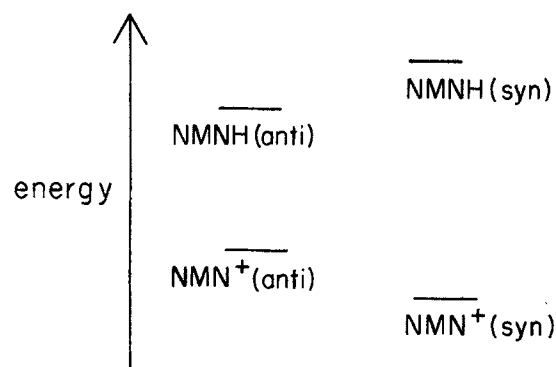


Figure 4. An energy diagram based on the preference of NMN⁺ for the syn conformation and of NMNH for the anti conformation shows that anti NMNH is a weaker reducing agent than syn NMNH.

reaction coordinate is not well understood, it is not possible to define this correlation exactly. Where one expects the 'break' in figure 2 to occur depends entirely on the nature of the correlation²³.

Finally, consideration of the thermodynamics of these systems lends some support to our suggestion that the conformation of the cofactor might be chosen to match the reducing power of the cofactor to the reducibility of the substrate. The equilibrium constants for the reactions catalyzed by these dehydrogenases span a range of approximately 8 orders of magnitude, equivalent to a $\Delta\Delta F$ of about 11 kcal/mole. Based on the data for NMN⁺ and NMNH, rotation around the glycosidic bond of the cofactor can be

estimated to change the reducing strength of NADH by 1.3 kcal/mole, which is roughly 10% of the total range involved. Thus, while dehydrogenases clearly must have available a number of strategies in addition to the one suggested here for affecting the energies of bound species, the energetic consequence of rotating about the glycosidic bond is a significant part of the overall energetic requirements. If our proposed interpretation proves to be valid, dehydrogenases will reflect a most remarkable instance of convergent evolution, in which dozens of enzymes from organisms from the 3 kingdoms of life have all evolved to exploit a device that provides a catalytic advantage of only a factor of 10.

- Acknowledgment. This work was done in part while the author was supported by a Xerox Fellowship, and subsequently while a member of the Harvard Society of Fellows. Helpful discussions with Professors F.H. Westheimer and J.R. Knowles, Mr J.D. Rozzell, Dr J. Stackhouse, Dr H. Levine, and Dr D. Yee are warmly acknowledged. I am indebted to Professors S. Benkovic, K.R. Hanson, J.P. Klinman and I.A. Rose for their comments on an early version of this manuscript.
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- For a number of reasons, it is unlikely that the stereochemical preferences of dehydrogenases arose by the conservation of the preferences of 2 ancestral enzymes, 1 pro-R specific and 1 pro-S specific. First, sequence data suggest that certain pro-R specific enzymes and certain pro-S specific enzymes are themselves evolutionarily interrelated. Furthermore, if we are to believe that pro-R specificity and pro-S specificity have no selective advantage whatsoever, it is stunning that those specificities have been conserved for the billion years separating organisms as diverse as members of the *Lactobacillus* genus, potatoes, and mammals. Since stereochemical reversal in dehydrogenases would seem to require a change in only 2 amino acid residues, in order to reconcile the belief that the stereochemical preferences in dehydrogenases have no selective advantage with the fact that these preferences have been conserved for 10⁹ years, one is forced to assume that the randomization of stereopreferences is far slower than evolutionary data from other sources seem to tolerate²⁴.
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- Although the data are less conclusive than might be desired, the difference in reducing strength of syn and anti NMNH can be estimated to be 1.3 kcal/mole. Although not insignificant when compared to the 11 kcal/mole total spread in redox potentials of the ketones being reduced, this difference nonetheless is small, and would normally be well below the 'noise' that would accompany most chemical methods designed to detect it. However, 10⁹ years of biological evolution may have been sufficient to select in enzymes the most efficient reaction pathway, even when the efficiency differential between the path selected and the next most efficient path is small. This possibility raises the intriguing notion that by examining the paths that have evolved in enzyme active sites, we may be able to discern new chemical principles that have hitherto been overlooked because their effects are obscured beneath the 'noise' in most chemical data. I am indebted to Professor D. Arigoni for his clear statement of this point.
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PRO EXPERIMENTIS

Large scale preparation of oxidized streptolysin O using molecular filtration

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Summary. Oxidized streptolysin O, suitable for the determination of antistreptolysin O in whole blood, has been prepared from crude broth culture of *S. pyogenes* by molecular filtration using membranes with nominal mol. wt limit 10,000.

A new hemolytic method for the determination of antistreptolysin O (ASO) in whole blood has been proposed¹. The method is based on some characteristic properties of streptolysin O (SO): the oxidized form of the toxin can bind the specific antibodies but cannot interact with the red cell membrane. After addition of a reducing agent, both free and antibody-bound toxin interact with membrane receptors, but only the free toxin is able to cause red cell hemolysis. These principles form the basis of a new diagnostic method for the determination of ASO^{1,2}.

The oxidized SO was originally prepared from crude cultures of *Streptococcus pyogenes* by precipitation with ammonium sulfate, followed by centrifugation, dialysis and oxidation with hydrogen peroxide¹. The excess H₂O₂ was then removed by dialysis and the residual unoxidized SO was inactivated by addition of CuCl₂, followed by extensive dialysis. Although this method gave oxidized SO which could be used in the diagnostic test, the procedure proved to be time-consuming, and unsuitable for large-scale production.

In the present paper we report the results of experiments using molecular filtration for both concentration and purification of the initial crude broth culture and for dialysis after treatment with H₂O₂ and CuCl₂.

Crude broth cultures of *S. pyogenes* were prepared as previously described, with slight modifications. After 10 h growth, the bacteria were removed by filtration under pressure on Seitz filters type K7 and EKS, arranged in series. The titre of the toxin was determined measuring both the hemolytic units (HU) and the combining units (CU) as previously described¹.

The concentration and purification of streptolysin by molecular filtration was performed with a 'high volume plastic cassette' assembled with PTGC membranes (nominal mol. wt limit=10,000) (Millipore, USA), fed by a piston pump generating a pressure of 4 at. The total filtering surface used was 0.92 m².

The figure shows the results of a typical experiment, performed on a 24-l batch of filtered crude broth culture. During the concentration phase, an increase was observed in the protein concentration in the filtrate, expressed as the absorbance at 280 nm, due to the presence of proteins partially retained by the membrane, which were removed only at a higher concentration. During the washing phase, keeping the retentate volume constant at 3 l, the protein concentration decreased rapidly. The washing was interrupted when the absorbance at 280 nm reached about 1, corresponding to removal of about 84% of the proteins.

Analytical characteristics of streptolysin O during the various phases of the preparation procedure

Solution	Volume (ml)	CU/ml	Protein* (mg/ml)	Specific activity (UE/mg protein)	Yield (%)
Initial broth culture	24,000	10	18.3	7	—
Concentrated and purified SO	2580	92	26.7	48	100
Oxidized SO	5450	41	8.9	72	94.1
Oxidized and CuCl ₂ -treated SO	6400	33	6.7	60	88.9

*Determined with the method of Lowry et al.⁵ using bovine serum albumin as standard.