Reprinted from the Journal of the American Chemical Society, 1985, *107*, 6999.

Enzyme-Catalyzed Organic Synthesis: A Comparison of Strategies for in Situ Regeneration of NAD from NADH

Linda G. Lee and George M. Whitesides*

Contribution from the Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138, and Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received February 25, 1985*

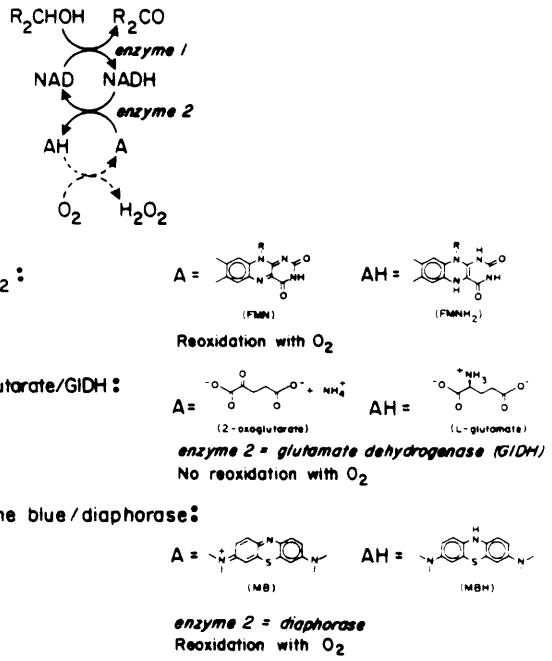
Abstract: This paper compares three different types of enzymatic systems for in situ regeneration of NAD from NADH for use in practical-scale enzyme-catalyzed organic synthesis. The first, and the most generally useful, uses an organic oxidant in stoichiometric quantities (2-oxoglutarate, with catalysis by glutamate dehydrogenase); the second involves dioxygen as the terminal oxidant, with an intermediate electron carrier dye (methylene blue, with a diaphorase as catalyst); the third is based on a stoichiometric inorganic oxidant (ferricyanide, with diaphorase as catalyst). The relative merits of these and other NAD regeneration systems are discussed with particular reference to intrinsic kinetic and thermodynamic limitations to practical application. The paper includes representative examples of oxidations using each regeneration system. For 2-oxoglutarate/glutamate dehydrogenase and methylene blue/diaporase/O_2, the conversion of *cis*-cyclohexanedimethanol to (+)-(1*R*,6*S*)-*cis*-8-oxabicyclo[4.3.0]nonan-7-one catalyzed by horse liver alcohol dehydrogenase was carried out on 70- and 30-mmol scales, respectively. The less useful ferricyanide/diaphorase system was tested on a 5-mmol scale in the oxidation of glucose to gluconate catalyzed by glucose dehydrogenase. For many dehydrogenase-catalyzed oxidations, the most important limitations to synthetic application seem to lie not in NAD regeneration but in the unrelated problem of noncompetitive inhibition by product. The paper describes empirical relationships between the equilibrium constants for the oxidation or reduction reactions being considered and the values of Michaelis and product inhibition constants. These relationships are useful in identifying reactions which are plausible candidates for practical-scale enzymatic catalysis.

Introduction

The NAD(P)(H)-requiring oxidoreductases are potentially useful catalysts in chiral synthesis.[1,2] Practical use of this class of enzymes has been inhibited by several factors: the cost of the enzymes, the requirement for efficient cofactor regeneration procedures, and the frequent requirement for operation using dilute solutions of reactants or products. In addition, oxidoreductase-catalyzed reactions have often proved less efficient on a preparative scale than might have been expected from analytical-scale reactions. The reasons for this inefficiency have not been clearly defined, and one function of this paper is to suggest the importance of product inhibition in determining efficiency.

The cost of an enzyme used in synthesis is a function both of its *initial* cost (that is, the cost to purchase or prepare the enzyme) and the turnover number (TN = mol of product/mol of enzyme) achieved in reaction. For large-scale preparations, reactor size and productivity also become important. Improved methods of enzyme stablization, especially immobilization in suitable polymer matrices[3] or on solid supports,[4] have dramatically increased the lifetimes (i.e., turnover numbers) obtainable for the oxidoreductases under the conditions used for organic synthesis. Regeneration of NAD(P)H from NAD(P) is now relatively straightforward.[5,6] The reverse regeneration—that of NAD(P) from NAD(P)H—remains a more difficult problem for three reasons: first, most enzymatic oxidations are thermodynamically unfavorable; second, oxidations are often strongly inhibited by products; third, many of the organic oxidants of potential use in enzyme-catalyzed oxidations are unstable at the higher pH values required for maximal activity of the enzymatic catalysts (pH ≃9).

Scheme I. Methods for in Situ Regeneration of NAD from NADH



The regeneration of NAD(P) from NAD(P)H is important in both analytical assays (nmol of substrate) and in preparative enzymatic syntheses (mol of substrate).[7,8] Analytical-scale regeneration methods are highly developed and include enzyme-catalyzed regenerations,[8,9] oxidations by dioxygen with electron-transfer reagents,[10] stoichiometric chemical oxidations,[8] and

* Harvard University.

(1) For review articles, see: Findeis, M. A.; Whitesides, G. M. *Annu. Rep. Med. Chem.* **1984**, *19*, 263–272. Whitesides, G. M.; Wong, C.-H. *Aldrichimica Acta* **1983**, *16*, 27–34. Jones, J. B. In "Asymmetric Synthesis"; Morrison, J. D., Ed.; Academic Press: New York, 1985.

(2) Hirschbein, B. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1982**, *104*, 4458–4450. Wandrey, C.; Buckmann, A. F.; Kula, M. R. *Biotech. Bioeng.* **1981**, *23*, 2789–2802.

(3) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–6336.

(4) Klibanov, A. M. *Science* **1983**, *219*, 722–727. Chibata, I. In "Immobilized Enzymes—Research and Development"; Halsted Press: New York, 1978.

(5) Shaked, Z.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 7104–7105. Levy, H. R.; Loewus, F. A.; Vennesland, B. *Ibid.* **1957**, *79*, 2949–2953.

(6) Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc. 103*, 4890–4899.

(7) For review articles, see: Lowe, C. R.; *Phil. Trans. R. Soc. Lond. Ser. B* **1980**, *300*, 335–353. Wang, S. S.; King, C.-K. In "Advances in Biochemical Engineering"; Springer-Verlag: Berlin, 1979; Vol. 12, pp 119–146.

(8) Pinder, S.; Clark, J. B.; Greenbaum, A. L. In "Methods of Enzymology"; McCormick, D. B., Wright, L. D., Eds.; Academic Press: New York, 1971; Vol. 18B, pp 20–30.

(9) For the use of malate dehydrogenase, see: Campbell, J.; Chang, T. M. S. *Biochem. Biophys. Res. Commun.* **1976**, *69*, 562–569. For use of NADH oxidase, see: Schmidt, H. L.; Grenner, G. *Eur. J. Biochem.* **1976**, *67*, 295–302.

(10) Greenbaum, A. L.; Clark, J. B.; McLean, P. *Biochem. J.* **1965**, *95*, 161–166. Smith, P. J. C. *Nature (London)* **1961**, *190*, 84–85.

0002-7863/85/1507-6999$01.50/0

electrochemical oxidations.[11] To date, regeneration by dioxygen with flavin mononucleotide (FMN)[12] as the electron-transfer reagent has been the system most often used for preparative enzymatic synthesis. Oxidation with 2-oxoglutarate catalyzed by glutamate dehydrogenase (GlDH) has been demonstrated to be useful,[13] but has not been extensively developed.

The goals of this work were to identify the NAD regeneration system most suited to large-scale work, and to identify the most important factors—whether connected with cofactor regeneration or not—that limit application of oxidoreductases as catalysts in synthesis. Our approach to the first problem was to consider the regeneration methods used in analytical-scale work, to test likely candidates in preparative-scale reactions, and to modify these methods to meet the requirements for synthetic practicality. The criteria by which the usefulness of the regeneration methods were measured were cost, rate of regeneration, turnover number achieved for NAD, and simplicity of execution (in operation of the system, in monitoring the progress of the reaction, and in the workup). Analytical methods which we judged to be unlikely candidates for immediate preparative use are mentioned in the following discussion, but were not tested experimentally; these included several chemical methods and most electrochemical oxidations.

The current preparative regeneration methods can be judged and compared by these criteria. The method most widely used and thoroughly developed is that of Jones and Taylor,[12] in which NADH is converted to NAD by direct reaction with oxidized flavin mononucleotide (FMN), and the resulting reduced flavin ($FMNH_2$) is reoxidized to FMN by dioxygen (Scheme I). This scheme has been used successfully in preparations of ketones on a 2-g scale.[14] The advantages of the procedure based on FMN/O_2 are that it requires no enzymes for regeneration and that it is simple to carry out. Its serious disadvantage (aside from the incompatibility of certain enzymes with O_2) is that the rate constant for reaction of FMN with NADH is low ($k_2 = 0.2\ M^{-1}\ s^{-1}$ at pH 8).[15] This low rate constant requires that concentrations of FMN and NAD be high to achieve useful rates. A typical published reaction contains substrate (2 g, 14 mmol), NAD (0.7 g, 1 mmol), and FMN (9 g, 20 mmol).[14] The very high ratio of FMN to substrate complicates product isolation and uses this species almost stoichiometrically (TN = 1.4 for FMN). The high concentration of NAD results in inefficient use of this expensive cofactor (TN = 24). Following the progress of the reaction is both inconvenient and inaccurate: a portion of the reaction solution is extracted with an organic solvent and the contents of the extract analyzed by GLC. In certain cases, inaccuracies inherent in this method lead to lowered yields and lowered enantiomeric excess (% ee).[16] The result of these difficulties is an upper limit on the scale of the reaction; no more than ~20 mmol of any substrate has been oxidized using this method.[17]

The advantages of the 2-oxoglutarate/GlDH regeneration method over FMN/O_2 are several. The maximal rate of oxidation of NADH is achieved at low concentrations of the cofactor; turnover numbers for NAD of 500–1000 are routine.[13] The progress of the reaction is easily and simply monitored by enzymatic analysis of 2-oxoglutarate.[18] The cost per mole of 2-oxoglutarate is less by a factor of 15 than that for FMN (2-oxoglutarate: $12/mol; FMN: $200/mol).[19] The method is applicable on scales >0.1 mol.[13] The disadvantages of this method relative to FMN/O_2 are two: it generates an organic product (glutamate) and thus complicates workup; it requires a second enzyme (GlDH) and thus increases costs.

Regeneration of NAD using 2-oxoglutarate appeared to us to be superior to regeneration using FMN/O_2. A clear choice between the two could not, however, be made until the two methods were compared directly in the same preparative, enzyme-catalyzed oxidation. This comparison, using horse liver alcohol dehydrogenase (HLADH) catalyzed oxidation of *cis*-1,2-cyclohexanedimethanol to the chiral [4.3.0] lactone is described here.

There are, in principle, several potential advantages to using electron-transfer reagents to regenerate NAD. Dioxygen or an anode would be the ultimate oxidants (the electron-transfer reagent and NAD would be used catalytically). In this work we have examined only systems using dioxygen as the oxidant. The assembly and workup of reactions which consume O_2 can, in principle, be inexpensive and uncomplicated. The most important potential disadvantages of dioxygen-based systems are the sensitivity of many enzymes toward dioxygen and its reduction products, the slow rates of many electron-transfer reactions involving dioxygen, and the lack of simple methods to monitor the progress of certain of these reactions. We have examined several systems based on electron-transfer dyes. In our opinion, the best of these procedures uses O_2 as the ultimate oxidizing agent, methylene blue as the intermediate electron-transfer agent, and diaphorase to catalyze the reaction between methylene blue and NADH (Scheme I).[20] The initial problem which must be addressed in designing a useful system based on a catalytic dye is that of achieving high rates of reaction at low concentrations of NADH and of oxidized dye. The regeneration system must be able to oxidize *dilute* NADH rapidly using *dilute* dye for two reasons. The first reason is economic: the nicotinamide cofactors should be used in small quantitites to minimize their contribution to the cost of the system. The second reason is ease of purification; product isolation is most convenient if the concentrations of NAD(H) and dye are kept at low values. The requirement for dilute solutions puts a minimum value on the rate constant for oxidation of NADH in order that this step *not* be rate determining. For example, if an NAD turnover number of 1000 is to be achieved, the concentration of NAD in a solution containing 0.1 M substrate will be 0.1 mM. To achieve a useful rate of overall reaction (e.g., a rate which will oxidize a 0.1 M solution of substrate in 10 h), a dye concentration of 1 mM (1% that of the product) requires an effective bimolecular rate constant for the reaction between oxidized dye and NADH (whether enzyme-catalyzed or not) of $30\ M^{-1}\ s^{-1}$. For comparison, the rate constant for the reaction of FMN with NADH in the procedure of Jones and Taylor is $k_2 = 0.2\ M^{-1}\ s^{-1}$ at pH 8. This low value underlies the high concentrations of both FMN and NAD required in this scheme.

Regeneration sequences related to MB/diaphorase and FMN/O_2 have been used previously (using phenazine methosulfate (PMS),[10] ferricyanide and diaphorase,[21] dichloroindophenol with PMS,[9] and methylene blue)[9,20] but only on an analytical scale. This paper summarizes kinetic data needed to optimize methods

(11) Moiroux, J.; Elving, P. J. *Anal. Chem.* **1979**, *51*, 346–350. Aizawa, M.; Coughlin, R. W.; Charles, M. *Biochim. Biophys. Acta* **1975**, *385*, 362–370. Moiroux, J.; Elving, P. J. *J. Am. Chem. Soc.* **1980**, *102*, 6533–6538.

(12) Jones, J. B.; Taylor, K. E. *Can. J. Chem.* **1976**, *54*, 2969–2973.

(13) Wong, C.-H.; Pollak, A.; McCurry, S. D.; Sue, J. M.; Knowles, J. R.; Whitesides, G. M. *Methods Enzymol.* **1982**, *89*, 108–121. Wong, C.-H.; McCurry, S. D.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 7938–7939.

(14) Jakovac, I. J.; Goodbrand, H. B.; Lok, K. P.; Jones, J. B. *J. Am. Chem. Soc.* **1982**, *104*, 4659–4665.

(15) Jones, J. B.; Taylor, K. E. *Can. J. Chem.* **1976**, *54*, 2974–2980.

(16) An example in which inaccurate analysis of the extent of reaction, *R*, led to lowered enantioselectivity is the HLADH-catalyzed oxidation of racemic *cis*-3,5-dimethyltetrahydropyran-2-ol to *cis*-(3*S*,5*R*)-3,5-dimethyltetrahydropyran-2-one. In order to achieve maximal % ee of both the unreacted acetal and the product, exactly 50% of the racemic starting material must be oxidized ($R = 0.35$). The low enantiomeric excess (35% ee) of the unreacted starting material was attributed to an inaccurate GLC analysis (which indicated $R = 0.5$) resulting from "incomplete extraction of the starting diol". The product of the oxidation, the 3*S*,5*R* lactone, was enantiomerically pure (100% ee), indicating the enzyme-catalyzed oxidation was completely stereoselective. See: Ng, G. S. Y.; Yuan, L.-C.; Jakovac, I. J., Jones, J. B. *Tetrahedron* **1984**, *40*, 1235–1243.

(17) Dodds, D. R.; Jones, J. B., *Chem. Commun.* **1982**, 1080–1081.

(18) Bergmeyer, H. U. In "Methods of Enzymatic Analysis", 2nd ed.; Verlag Chemie Weinheim, Academic Press: New York, 1974. For specific assays, see: p 1577 (2-oxoglutarate), p 1604 (oxalacetate), p 2048 (NAD), p 2053 (NADH), p 650 (GlDH), p 428 (HLADH), p 458 (G-6-PDH). The assay for G-6-PDH was used for GlcDH, substituting glucose and NAD for glucose-6-phosphate and NADP, respectively.

(19) Prices are from a Sigma Chemical Co. catalog, 1984.

(20) Viche, H. G.; Fuks, R.; Reinstein, M. *Angew. Chem.* **1964**, *76*, 571.

(21) Wallace, T. C.; Coughlin, R. W. *Biotech. Bioeng.* **1978**, *20*, 1407–1419. May, S. W.; Landgraff, L. M. *Biochem. Biophys. Res. Commun.* **1976**, *68*, 786–798.

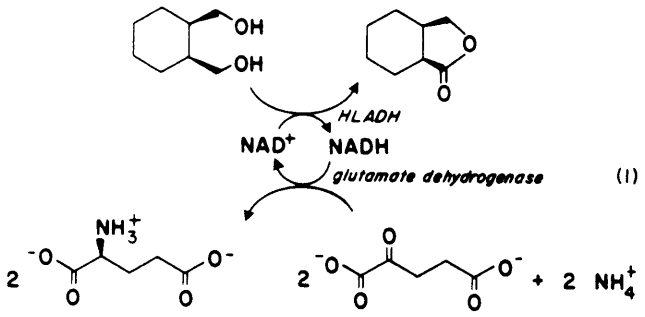
based on electron-transfer dyes and demonstrates MB/diaphorase regeneration with the HLADH-catalyzed oxidation of *cis*-1,2-cyclohexanedimethanol.

Our decision to demonstrate NAD regeneration with the oxidation of *cis*-1,2-cyclohexanedimethanol to the bicyclic lactone was based on the effective irreversibility (V_f/V_r = 150) and favorable kinetic parameters (K_m/K_i = 0.3)[22] of the reaction (V_f and V_r are the maximal rates of the forward and reverse reactions per mg of enzyme; K_m is the Michaelis constant for the substrate; K_i is the inhibitor constant of the final product, (+)-(1*R*,6*S*)-*cis*-8-oxabicyclononan-7-one).[23] Arguments detailed later in this paper establish that oxidations using most dehydrogenases for which $K_m/K_i > 1$ give low yields of product regardless of the method of NAD regeneration, because most of these reactions are characterized by noncompetitive product inhibition (or kinetically similar mixed inhibition). These types of inhibition result in a decrease in the rate of the forward reaction as product is formed. This inhibition *cannot* be overcome through the use of a more powerful oxidant or by high concentrations of substrate; although the overall reaction will be thermodynamically favorable, the low reaction rates will result in unacceptably long reaction times. The only effective methods for minimizing the effects of noncompetitive or mixed product inhibition is to remove the product from the reaction system as it is formed (for example, by extraction into a second phase).[24]

The HLADH-catalyzed oxidation of *cis*-cyclohexanedimethanol to the bicyclic lactone is also attractive as a demonstration system because the method provides a chiral product which is not easily obtained by nonenzymatic methods. There are now other, simpler methods to obtain the chiral lactone (using, e.g., pig liver esterase).[25] We wished, however, to compare this type of procedure with a number of other enzymatic methods for generating chiral centers, and the large body of useful information developed by Jones on chiral oxidations with HLADH provides an important comparison.

Results and Discussion

NAD Regeneration with 2-Oxoglutarate/GlDH. In our experience, this method is the most useful now available for regeneration of NAD from NADH. It requires ammonia, 2-oxoglutarate, and glutamate dehydrogenase (GlDH). The Experimental Section details a procedure for the oxidation of *cis*-cyclohexanedimethanol (**1**) to (+)-(1*R*,6*S*)-*cis*-8-oxabicyclononan-7-one (**2**) on a 70-mmol (10-g) scale using 2-oxoglutarate



(1)

(22 g) and NAD (0.1 g) in a mixture of water (0.8 L) and hexane (1 L). The reaction was carried out using soluble HLADH and immobilized GlDH in a 1:1 v:v mixture of water (pH 8) and hexane. The progress of the reaction was followed by periodic, enzymatic assay of unreacted 2-oxoglutarate. The reaction was complete in 3 days and the lactone **2** was isolated in 85% yield.

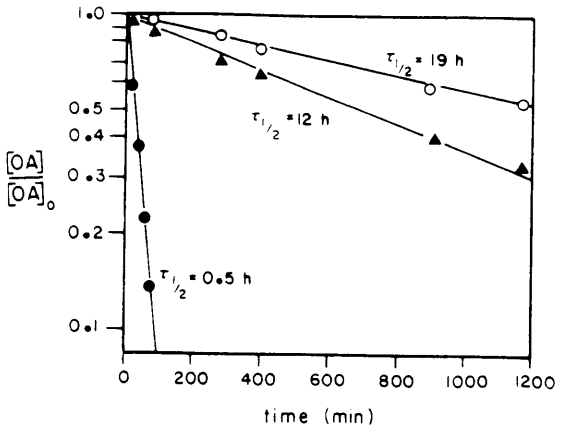


Figure 1. First-order plot of the decomposition of oxalacetate (OA) in different buffer solutions. Values of k_1 were: 4.2×10^{-4} s^{-1} for 0.1 M Hepes with 0.1 M glycine, pH 7.6 (●); 1.6×10^{-5} s^{-1} for 0.1 M Hepes with 5 mg/mL of soluble HLADH, pH 7.6 (▲); 9.9×10^{-6} s^{-1} for 0.1 M Hepes, pH 7.6 (○).

The TN for NAD was 500. There seem to be no intrinsic problems in scaling the reaction to larger quantities. In this procedure the overall rate-limiting step was the HLADH-catalyzed oxidation, and the most expensive component of the system was HLADH.

The use of hexane as an immiscible second phase was important in that it removed the product as it formed and thus minimized product inhibition. The volume of the aqueous phase calculated to be optimal for these enzymes and quantities of reagents was 0.6 L.[26] In practice, we used a slightly larger volume (with less favorable rates of reaction) in order to solubilize 2-oxoglutarate and the cis diol.[27] The cis diol substrate was not significantly soluble in hexane and the lactone product had a partition coefficient (ρ) of 1.0 between hexane and water containing glycine buffer. The 2-oxoglutarate/GlDH system has certain intrinsic disadvantage—especially the requirement for stoichiometric quantities of 2-oxoglutarate and the fact that glutamate is produced. It is, however, clearly more practical than the procedures based on electron-transfer dyes which follow.

NAD Regeneration Using Lactate Dehydrogenase, Yeast Alcohol Dehydrogenase, and Malate Dehydrogenase. The success of the 2-oxoglutarate/GlDH regeneration system led us to consider related systems based on other readily available dehydrogenases. The requirements for a successful enzymatic method are two: an inexpensive dehydrogenase and a substrate which is a stable, inexpensive oxidizing agent. L-Lactate dehydrogenase and yeast alcohol dehydrogenase are both inexpensive, but pyruvate and acetaldehyde are both unstable in solution, particularly at high values of pH. In addition, pyruvic acid and its condensation product are significantly soluble in organic solvents and would be expected to complicate workup. Malate dehydrogenase is very inexpensive; however, oxalacetate decarboxylates in solution, particularly when amines or soluble HLADH are present (Figure 1). The half-life of oxalacetate (19 h) is unacceptably short, given its high initial cost ($160/mol).[19] It may be possible to develop useful regeneration methods based on these systems by circumventing the indicated (and other) problems, but we have not worked on them as part of this research.

Oxidation of NADH by Electron-Transfer Dyes: Uncatalyzed Reactions. Rate constants for the direct (nonenzyme catalyzed) reaction of NADH with the oxidized form of several dyes were measured by following the disappearance of NADH at 340 nm

(22) Lee, L. G.; Whitesides, G. M., to be submitted for publication.

(23) In this manuscript, K_i refers only to the concentration of noncofactor product which produces a velocity of $V_{max}/2$ at saturating substrate concentrations (>10 K_m). K_i is not equivalent to the parameters K_{ii}, K_{is}, K_I (substrate inhibition), etc.

(24) Martinek, K.; Semenov, A. N. *J. Appl. Biochem.* **1981**, *3*, 93–126. Martinek, K.; Levashov, A. V.; Khmelnitsky, Y. L.; Klyachko, N. L.; Berezin, I. V. *Science* **1982**, *218*, 889–891. Martinek, K.; Semenov, A. N.; Berezin, I. V. *Biochim. Biophys. Acta* **1981**, *658*, 76–89.

(25) Sabbioni, G.; Shea, M. L.; Jones, J. B. *Chem. Commun.* **1984**, 236–238.

(26) An equation (ref 22) which gives the optimal value of $[S_0]/K_i$ to minimize the time to complete a reaction to extent of reaction R for given values of K_m and K_i was used:

$$\frac{[S_0]}{K_i} = \left[R^{-2}\frac{K_m}{K_i} - 2 \ln (1 - R) \right]^{1/2}$$

Values of K_m (26 mM), K_i (80 mM), R (0.95), and $[S_0]$ (70 mmol) were used to calculate the optimal volume (0.6 L).

(27) A parallel reaction without hexane was not performed.

Table I. Uncatalyzed and Diaphorase-Catalyzed Oxidation of NADH to NAD by Oxidized Dyes

dye	E^0 [a]	pH	uncatalyzed k_2, M^{-1} s^{-1}	uncatalyzed v,[b] M s^{-1} × 10^7	catalyzed sp act. (U/mg)[c]	catalyzed v^b M s^{-1} × 10^6	rate of reoxidation of reduced dye, k_1 (s^{-1}) × 10^3	stability of dye system
methylene blue (MB)		9.0	5.3 ± 10.5	14			moderate, 2.4 ± 0.1	stable
	0.01	7.6	3.2 ± 0.2	8.3	10	20	moderate, 2.4 ± 0.1	
methyl viologen (MV)	−0.44	7.6	0	0	0.02	0.03	fast	stable
flavin mononucleotide (FMN)	−0.22	7.6	0.3	0.8	0.06	0.1	fast	unstable[d]
dichloroindophenol (DCIP)	+0.22	7.6	1.0	3	10	20	no reaction	stable
phenazine methosulfate (PMS)	+0.06	7.6	70	200	*e*		moderate	unstable
potassium ferricyanide ($K_3Fe(CN)_6$)	+0.36	7.6	2	5	10	20	no reaction	stable

[a] Reference 34. [b] The calculated rate of production of NAD from NADH for these concentrations: oxidized dye, 1 mM; NADH, 2.6 × 10^{-4} M; diaphorase (if present) 100 mg/L. [c] Concentrations assumed were: dye, 0.2 mM; NADH, 0.24 mM. Uncertainties are ±20%. [d] In "Handbook of Biochemistry"; Sober, H. A.; Ed.; CRC Press: Cleveland, 1970; pp K-41–K42. [e] Not measured.

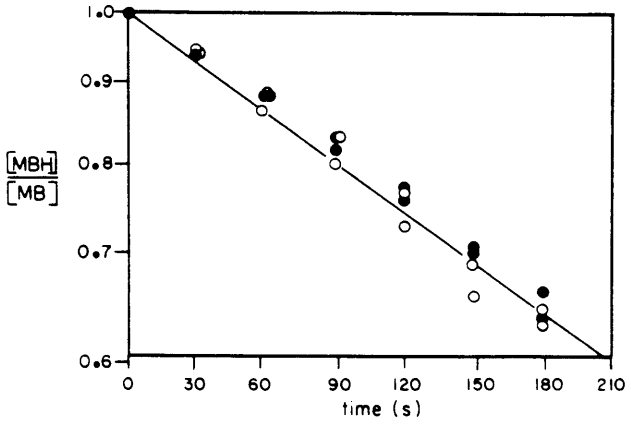


Figure 2. Pseudo-first-order plot of the oxidation of reduced methylene blue (MBH) by oxygen at pH 7.6 (●) and pH 9.0 (○). For both, k_1 = (2.4 ± 0.1) × 10^{-3} s^{-1}.

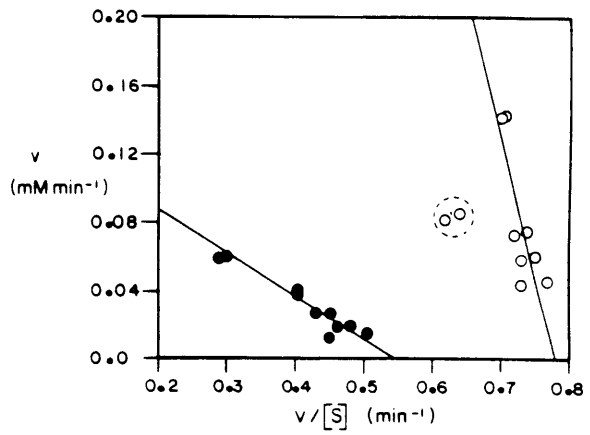


Figure 3. Eadie–Hofstee plots used in the determinationof values of K_m of methylene blue with NADH and diaphorase at pH 7.6 (●) and pH 9.0 (○). The value of K_m at pH 7.6 was 0.24 ± 0.02 mM; at pH 9.0, 1.7 ± 0.4 mM. The circled points were omitted from the analysis.

spectrophotometrically. Results are summarized in Table I. Oxidations by methylene blue were followed carefully at two values of pH, and at several concentrations of methylene blue and NADH between 0.06 and 0.25 mM. Oxidations by other dyes were followed only at a dye concentration of 0.2 mM and an NADH concentration of 0.25 mM. These data indicate that FMN, the oxidizing dye employed in the presently used procedure for direct reoxidation of NADH to NAD, is significantly slower than several of the other dyes examined (methyl viologen, dichloroindophenol, phenazine methosulfate).[29] These rates are not, of course, the only parameters of interest in choosing a reoxidation system. In particular, the rate of reaction of the reduced dye with O_2 must also be rapid to achieve high overall rates, and the dye must be stable under the reaction conditions. Dichloroindophenol oxidizes NADH reasonably rapidly, but the reduced form of DCIP is not autooxidizable.[30] PMS reacts rapidly with NADH without diaphorase and is autooxidizable; it has been used to regenerate NAD in several analytical systems. PMS is, however, unstable under reaction conditions; it undergoes reactions with oxygen which are both photocatalyzed (yielding pyocyanine) and dark (yielding 2-keto-*N*-methylphenazine and phenazine).[31] DCIP and PMS were discarded for practical synthetic applications on the basis of these characteristics.

Reduced methylene blue reacts rapidly with dioxygen in a stirred solution in contact with air. Rates of this autooxidation were measured at pH 7.6. Figure 2 shows the resulting pseudo-first-order kinetic plots. Rate constants are given in Table I. These data in combination suggest that substitution of methylene blue for FMN in the Jones and Taylor procedure would increase rates and decrease the quantity of dye required. The advantage of this substitution would, however, be marginal, and balanced by the slower rate of reoxidation of reduced methylene blue as compared to the reoxidation of reduced FMN.

We conclude that none of the systems summarized in Table I provide a genuinely practical procedure for the regeneration of NAD, because all are too slow. In searching for some procedure which would significantly increase the rate of reoxidation of NADH at low concentrations of both NADH and dye, we explored enzymatic catalysis of this step.

Oxidation of NADH by Methylene Blue/O_2 Catalyzed by Diaphorase. Diaphorase catalyzes the oxidation of NADH by oxidized methylene blue. Diaphorase activity is found in lipoamide dehydrogenases obtained from many sources (e.g., porcine heart, yeast, and *Clostridium kluyveri*). The *Clostridium* enzyme used in these studies was commercially available and relatively inexpensive. Kinetic parameters for the diaphorase-catalyzed reaction of NADH by MB were determined using standard procedures. Figure 3 shows Eadie–Hofstee plots used to determined K_m for methylene blue. Values obtained were: pH 7.6, K_m(MB) = 0.24 ± 0.02 mM; pH 9.0, K_m(MB) = 1.7 ± 0.4 mM. It was more difficult to obtain an accurate value of K_m for NADH, since it was low. Examination of rates of oxidation of NADH at pH 7.6 over the concentration range 2 × 10^{-5} to 1.8 × 10^{-3} M with a methylene blue concentration of 0.6 mM (approximately twice K_m(MB)) shows no variation in rate. We thus infer that K_m(NADH) is less than 10^{-5} M. An NAD regeneration system was demonstrated with the enantioselective oxidation of *cis*-1,2-cyclohexanedimethanol catalyzed by immobilized horse liver alcohol dehydrogenase (HLADH) at pH 7.6. The best results were obtained in a two-phase system; hexane was again used to remove the lactone selectively from the aqueous phase.[32] Oxygen was slowly bubbled through the mixture. The reactor color

(28) Pederson, K. J. *J. Am. Chem. Soc.* **1938**, *60*, 595–601.
(29) The reaction is accelerated by light. See: Chambers, R. P.; Ford, J. R.; Allender, J. H.; Baricos, W. H.; Cohen, W. In "Enzyme Engineering"; Pye, E. K., Wingard, L. B., Eds.; Wiley: New York, 1974; Vol. 2, pp 195–202.
(30) Gurr, E.; Anand, N.; Unni, M. K.; Ayyangar, N. R. In "The Chemistry of Synthetic Dyes"; Venkataraman, K., Ed.; Academic Press: New York, 1974; Vol. 7, pp 305–308.
(31) McIlwain, H. *J. Chem. Soc.* **1937**, 1704–1711.
(32) HLADH has also been used in homogeneous solutions of water and organic solvents. See: Jones, J. B.; Schwartz, H. M. *Can. J. Chem.* **1982**, *60*, 1030–1033.

changed from an initial deep blue to colorless; then, after 24 h, back to deep blue. The diol (4 g, 28 mmol) was converted to lactone (3.2 g, 83%, 100% ee) in 4 days. The TNs for NAD and for methylene blue were 350 and 580, respectively.

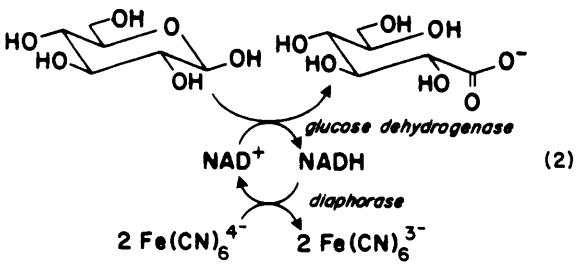
This procedure provides a practical method for regenerating NAD from NADH in situ. It is marginally superior in convenience and practicality to the procedure based on FMN/O_2.[12] Much smaller quantitites of dye are used, and the reaction volume is smaller, but the system is rendered more complex and expensive by the requirement for an additional enzyme (diaphorase). For FMN/O_2, the slowest step is reaction of FMN with NADH; for $MB/diaphorase/O_2$, the slowest step is reaction of O_2 with MBH. Although the latter reaction is slightly faster than the former, FMN is much more soluble in water than MBH and the effective rates are higher.

For comparison with the 2-oxoglutarate/GlDH method, preparation of 10 g of lactone by oxidation of *cis*-cyclohexanedimethanol using MB/O_2/diaphorase would require NAD (0.25 g) and methylene blue (0.08 g) in a mixture of water (0.25 L) and hexane (1.3 L) and would require a reaction time of 4 days. A synthesis of **2** on the same scale using FMN/O_2 would be extrapolated from literature data to require NAD (3.5 g) and FMN (45 g) in 5 L of water,[33] and a reaction time of 3 days. Thus the methods based on 2-oxoglutarate/GlDH and MB/O_2/diaphorase require smaller volumes. The use of smaller volumes simplified isolation of the product and, in the case of the 2-oxoglutarate/GlDH method, resulted in a workable isolation of residual HLADH by ultrafiltration. The method based on 2-oxoglutarate/GlDH is intrinsically cleaner than the FMN/O_2 system. The product in the first case is isolated as pale yellow oil; with FMN/O_2 regeneration it is isolated as "black oil" presumably due to decomposition of FMN. The 2-oxoglutarate/GlDH method allows the reaction to be followed simply and accurately by assaying residual 2-oxoglutarate enzymatically; this case of analysis is a significant advantage over the FMN/O_2 method when achieving a high % ee, for the product depends on accurate analysis of *R*.[16]

The method based on MB/O_2/diaphorase does not provide significantly faster rates than the FMN/O_2 system. Smaller amounts of MB than FMN are required to achieve similar rates; however, MB is slightly soluble in organic solvents and workup of reactions using MB/O_2/diaphorase requires an additional step (filtration through charcoal).

NAD Regeneration with Ferricyanide Catalyzed by Diaphorase. The slow rate of oxidation of reduced methylene blue by O_2 in the dye-based systems led us to examine $Fe(CN)_6^{3-}$ as the terminal oxidant. This species is inexpensive and very water soluble, and reacts rapidly with NADH (Table I). The reduced form, ferrocyanide ($Fe(CN)_6^{4-}$), does not react with dioxygen, and 2 equiv of the one-electron transfer reagent is used for each two-electron oxidation. The reaction of $Fe(CN)_6^{3-}$ with NADH is also catalyzed by diaphorase.[21]

This NAD regeneration system was not tested using the oxidation of the cis diol **1**, but rather with the conversion of glucose to gluconate (eq 2) because the oxidation is not complicated by



(2)

product inhibition by gluconate and the extent of reaction *R* is easily monitored by measuring the volume of added base required to maintain the pH at 7.0. In addition, glucose dehydrogenase

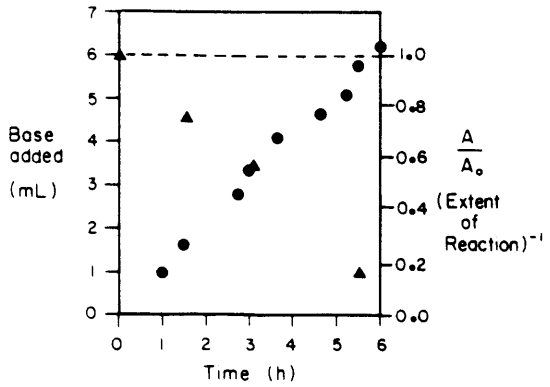


Figure 4. Two methods used to follow the progress of glucose oxidation. Plot of KOH (2.5 N) added to maintain neutrality vs. time (●) and plot of the ratio of absorbance to initial absorbance at 420 nm vs. time (▲). The reaction contained glucose, NAD, $K_3Fe(CN)_6$, GlcDH, and diaphorase.

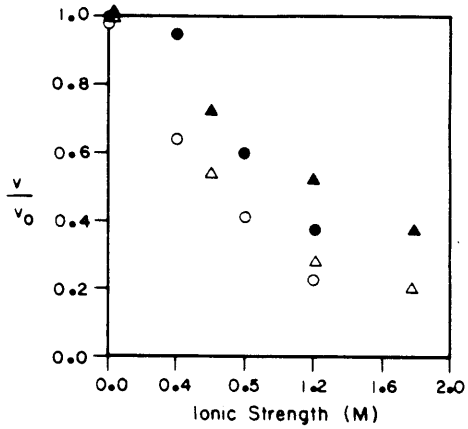


Figure 5. Effect of ionic strength on the activities of HLADH in the oxidation of *cis*-cyclohexanedimethanol and GlDH in the reductive amination of 2-oxoglutarate. The symbols ● and ▲ represent activity of GlDH with added NaCl and Na_2SO_4, respectively. The symbols ○ and △ respresent activity of HLADH with added NaCl and Na_2SO_4, respectively.

(GlcDH) is easily immobilized (compared to HLADH). We hoped that this characteristic might help to retard denaturation of the enzyme by $Fe(CN)_6^{3-}$. This oxidation was conducted only on small scale (5 mmol of glucose), using NAD (0.03 g) and immobilized diaphorase and GlcDH in 0.07 L of water. The reaction was conducted on a small scale because of the large reaction volumes required in order to keep the ionic strength of the solutions at reasonably low values (see below). The progress of the reaction was followed by two methods: monitoring the quantity of base added to maintain the pH at 7 and measuring the decrease in absorbance at 420 nm, ω_{max} of $Fe(CN)_6^{3-}$. Figure 4 shows plots of the results of both methods. The reaction was complete in 6 h; the TN for NAD was 125.

The usefulness of stoichiometric ferricyanide NADH regeneration for large-scale synthesis is limited by the high ionic strength of a solution of the reagent. Many enzymes have decreased activities at increased ionic strength. Figure 5 plots the activity of HLADH and GlDH at various values of ionic strengths using two electrolytes: NaCl and Na_2SO_4. The ionic strength of a solution of potassium ferricyanide is eight times its molarity; of potassium ferrocyanide, 10 times its molarity. The rate of oxidation of *cis*-cyclohexanedimethanol at an initial substrate concentration of 70 mM ($5K_m$) would be only 20% of the optimal rate. In addition, ferricyanide is a potent oxidizing agent ($E° = +0.36$ V)[34] and high concentrations may oxidize thiol moieties and deactivate enzymes.

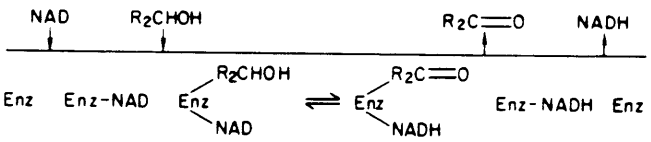
(33) These numbers are based on literature procedures; it is not clear why such dilute solutions are used or if they are actually needed.

(34) Loach, P. A. In "Handbook of Biochemistry"; Sober, H. A., Ed.; CRC Press: Cleveland, 1970; pp J-33–J-40.

Demonstration of Product Inhibition. We stated earlier that the HLADH-catalyzed oxidation of *cis*-cyclohexanedimethanol to the chiral lactone is a useful reaction with which to demonstrate NAD regeneration not only because the reaction is irreversible but because the product is a poor inhibitor. If thermodynamic irreversibility were the only criterion for successful reaction, addition of acetaldehyde—the product of HLADH-catalyzed oxidation of ethanol—to a reaction mixture containing cis diol and HLADH should not affect the rate of production of the lactone. In fact, acetaldehyde inhibits oxidation of the cis diol to the same extent that it inhibits oxidation of ethanol; the values for K_i for acetaldehyde were the same for both oxidations (K_i = 1 mM at pH 9).

Influence of Product Inhibition on Syntheses Catalyzed by Oxidoreductases. Even with a good cofactor regeneration system, many (perhaps most) oxidations of simple alcohols to ketones (and related transformations using dehydrogenases) will remain problematic. The fundamental difficulty in this area often lies not in cofactor regeneration but in inhibition of the participating oxidoreductases by the products of the reactions. Although product inhibition can sometimes be circumvented by removing the product as it forms (either by physical methods such as extraction or by further chemical transformation in cases of noncompetitive or mixed inhibition, or by using high concentrations of substrates in cases of competitive inhibition), such methods are not always convenient.

Many oxidoreductases which catalyze the oxidation of alcohols (yeast alcohol dehydrogenase, horse liver alcohol dehydrogenase, glycerol dehydrogenase, lactate dehydrogenase)[35] are believed to follow ordered bibi mechanisms.[36] The system shows mixed



inhibition by product ketone. This inhibition reflects a binding of ketone to the Enz–NADH complexes which has the effect of lowering the apparent concentration of enzyme. This inhibition *cannot*, as with competitive inhibition, be overcome simply by increasing the concentration of alcohol.

The use of these oxidoreductases for large-scale synthesis will be practical, regardless of the availability of an effective NAD regeneration system, only if the kinetics of the fundamental reaction (eq 3) are favorable under conditions which might be used

$$NAD + R_2CHOH \rightleftharpoons R_2C{=}O + NADH + H^+ \quad (3)$$

$$K'_{eq} = 10^7 K_{eq} = [R_2CHOH][NAD]/[R_2C{=}O][NADH] \quad (4)$$

in a plausible synthetic procedure (that is, relatively concentrated solutions of reactants and products). The most important parameters in determining the practicality of an enzyme-catalyzed oxidation are the Michaelis constant for the alcohol ($K_m^{R_2CHOH}$) and the inhibition constant for the ketone ($K_i^{R_2CO}$). The concentration of NAD can be maintained greater than K_m^{NAD} [37] by choosing appropriate starting concentrations of this cofactor and maintaining efficient cofactor recycling. The constant $K_i^{R_2CO}$ indicates the quantity of ketone which lowers the maximum velocity of the oxidation by half at kinetically saturating concentrations of NAD and R_2CHOH. Its magnitude determines the maximum concentration of product which can be achieved in the reaction before the rate of reaction drops to an unacceptably low value due to product inhibition. The constant $K_m^{R_2CHOH}$ is the quantity of alcohol which produces half the maximal velocity at saturating concentrations of NAD in the absence of any products. Its magnitude determines the minimum concentration of alcohol required to achieve a forward rate which makes acceptable use

(35) Dalziel, K. In "The Enzymes", 3rd ed; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol. XI, pp 1–60.
(36) Segel, I. H. In "Enzyme Kinetics"; Wiley: New York, 1975; pp 561–590.
(37) Values of K_m for NAD for the enzymes in Table II range from 10^{-7} to 10^{-4} M.

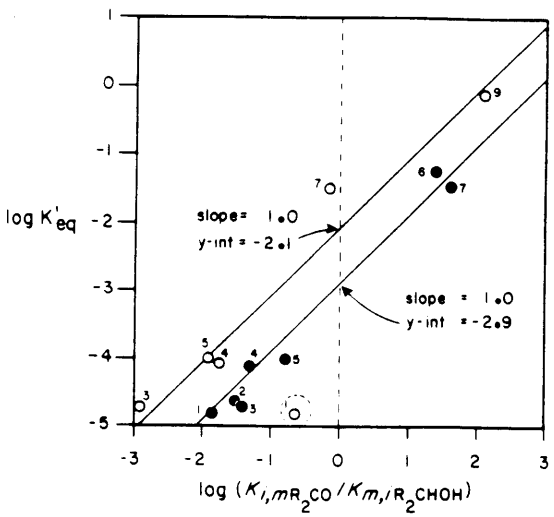


Figure 6. Plot of log K'_{eq} vs. log ($K_{i,m}^{R_2CO}/K_{m,i}^{RCHOH}$) for NAD(P)-(H)-dependent dehydrogenases. The symbol ● represents oxidations with $NAD(P)^+$; the symbol ○ represents reductions with NAD(P)H. K'_{eq} is defined in eq 4; the same number is used for both oxidative and reductive reactions of each enzyme-catalyzed transformation. For oxidations, $K_{i,m}^{R_2CO}/K_{m,i}^{RCHOH}$ refers to $K_i^{R_2CO}/K_m^{RCHOH}$; for reductions, $K_{i,m}^{R_2CO}/K_{m,i}^{RChOH}$ refers to $K_m^{R_2CO}/K_i^{RCHOH}$. Data are from Table II and are fitted to the equation $K'_{eq} = c(K_{i,m}/K_{m,i})$. Points are labeled with numbers corresponding to reactions in Table II. The value of c for oxidations is $(1.3 \pm 0.7) \times 10^{-3}$. The value of c for reductions is $(7.9 \pm 1) \times 10^{-3}$. The circled point, which represents malate dehydrogenase catalyzed reduction of oxalacetate, was omitted from the analysis.

of the catalytic potential of the enzyme. If the ratio $K_i^{R_2CO}/K_m^{R_2CHOH} < 1$, the reaction can never proceed efficiently—that is, with both high catalytic rates and high conversions of reactants to products—because concentrations of reactant high enough to saturate the enzyme active site will produce concentrations of product high enough to be strongly inhibitory at low conversions. If $K_i^{R_2CO}/K_m^{R_2CHOH} > 1$, the reaction can, in principle, proceed to acceptable conversions. Even if $K_i^{R_2CO}/K_m^{R_2CHOH} > 1$, however, the reaction may still not be practical if $K_i^{R_2CO}$ is low, because only dilute solutions of product can be generated without serious inhibition of the enzyme.

Since knowledge of both the ratio $K_i^{R_2CO}/K_m^{R_2CHOH}$ and the individual constants K'_{eq}, $K_i^{R_2CO}$, and $K_m^{R_2CHOH}$ are useful in evaluating an enzyme-catalyzed oxidation (or reduction), we wished to know whether it might be possible to estimate these parameters without conducting explicit experimental evaluations of them. In considering this problem, we have discovered an interesting, potentially useful, and presently largely empirical correlation between $K_i^{R_2CO}/K_m^{R_2CHOH}$ and K'_{eq} (eq 3) for a number of oxidoreductase-catalyzed reactions which interconvert ketone and alcohol moieties (Figure 6, Table II). Figure 6 indicates that within the group of reactions examined, K'_{eq} is linearly related to the ratio of the Michaelis constant for the reactant and the inhibitor constant of the product (eq 5). The ratio of the kinetic

$$K_{eq} = c(K_{i,m}^{R_2CO}/K_{m,i}^{R_2CHOH}) \quad (5)$$

parameters for either oxidations or reductions is expressed as $K_{i,m}^{R_2CO}/K_{m,i}^{R_2CHOH}$; for oxidations it is defined as $K_i^{R_2CO}/K_m^{R_2CHOH}$, and for reductions it is defined as $K_m^{R_2CO}/K_i^{R_2CHOH}$. Equations 6 and 7 provide the empirical values of c for oxidations and reductions.

$$K'_{eq} = [(1.3 \pm 0.7) \times 10^{-3}](K_i^{R_2CO}/K_m^{R_2CHOH}) \quad \text{(oxidations)} \quad (6)$$

$$K'_{eq} = [(7.9 \pm 6) \times 10^{-3}](K_m^{R_2CO}/K_i^{R_2CHOH}) \quad \text{(reductions)} \quad (7)$$

The empirical observation that eq 3 correlates data for a number of apparently only loosely related enzymatic rections does not, so far as we presently understand, derive directly from elementary thermodynamic and kinetic constants.

Table II Kinetics and Thermodynamic Data for Dehydrogenases

reaction	enzyme	reaction		$K_m^{R_2CHOH}$ (M)	$K_i^{R_2CO}$ (M)	$K_m^{R_2CO}$ (M)	$K_i^{R_2CHOH}$ (M)	K'_{eq}[a]
		R_2CHOH	R_2CO					
1[b]	malate dehydrogenase	L-malate	oxalacetate	3.9×10^{-4}	5.5×10^{-6}	8.0×10^{-5}	3.6×10^{-4}	1.5×10^{-5}
2[c]	glycerol dehydrogenase	glycerol	dihydroxyacetone	9×10^{-3}	3×10^{-4}	1.1×10^{-4}		2.4×10^{-5}
3[d]	lactate dehydrogenase	lactate	pyruvate	6.7×10^{-3}	2.8×10^{-4}	1.6×10^{-4}	1.3×10^{-1}	2.8×10^{-5}
4[e]	yeast alcohol dehydrogenase	ethanol	acetaldehyde	1.3×10^{-2}	6.7×10^{-4}	7.8×10^{-4}	4.3×10^{-2}	8×10^{-5}
5[f]	HLADH	ethanol	acetaldehyde	5.5×10^{-4}	8.7×10^{-5}	2.4×10^{-4}	1.9×10^{-2}	9.2×10^{-5}
6[g]	HLADH	cyclohexanol	cyclohexanone	1.6×10^{-0}	4×10^{-2}	7.6×10^{-3}		5.5×10^{-2}
7[h]	malic enzyme	L-malate	pyruvate + CO_2	2.9×10^{-4}	5.2×10^{-2} (pyr) 2.6×10^{-3} (CO_2)	1.1×10^{-2} (pyr) 4.5×10^{-4} (CO_2)	1.6×10^{-0}	3.1×10^{-2}
8[i]	3-hydroxy-butyrate	3-hydroxy-butyrate	acetoacetae	4.1×10^{-4}		2.8×10^{-4}		1.4×10^{-2}
9[j]	isocitrate	isocitrate	2-oxoglutarate + CO_2	1.2×10^{-6}		1.6×10^{-5} (2-ox) 1.9×10^{-3} (CO)2)	1.3×10^{-6}	1.3

[a] K'_{eq} is defined in the text (eq 4). [b] Kinetic data: Heyde, E.; Ainsworth, S. *J. Biol. Chem.* **1968**, *243*, 2413–2423. Equilibrium data: Stern, J. R.; Ochoa, S.; Lynen, F. *Ibid.* **1952**, *198*, 313–321. [c] Kinetic and equilibrium data: McGregor, W. G.; Phillips, J.; Suelter, C. H. *Ibid.* **1974**, *249*, 3132–3139; ref 22. [d] Kinetic data: Stambaugh, R.; Post, D. *J. Biol. Chem.* **1966**, *241*, 1462–1467. Equilibrium data: Hakala, M. T.; Glaid, A. J.; Schwert, G. W. *Ibid.* **1956**, *221*, 191–209. [e] Kinetic data: see footnote *f*. Equilibrium data: Backlin, K. I. *Acta Chem. Scand.* **1958**, *12*, 1279–1285. [f] Kinetic Data: Wratten, C. C.; Cleland, W. W. *Biochemistry* **1963**, *2*, 935–941. Equilibrium data: see footnote *g*. [g] Kinetic and equilibrium data: Merritt, A. D.; Tomkins, G. M. *J. Biol. Chem.* **1959**, *234*, 2778–2782; Experimental Section, this paper. [h] Kinetic data: Schimerlik, M. I.; Cleland, W. W. *Biochemistry* **1977**, *16*, 565–570. Equilibrium data: Schimerlik, M. I.; Rife, J. E.; Cleland, W. W. *Ibid.* **1975**, *24*, 5347–5354. The value of K_m/K_i was estimated by dividing the square root of the product of the values of K_m of the two substrates by the value of K_i of the product. [i] Kinetic data: Bergmeyer, H. U.; Gawehn, K.; Klotzsch, H.; Krebs, H. A.; Williamson, D. H. *Biochem. J.* **1967**, *102*, 423–431. Equilibrium data: Krebs, H. A.; Mellanby, J.; Williamson, D. H. *Ibid.* **1962**, *82*, 96–98. [j] Kinetic data: Uhr, M. L.; Thompson, V. W.; Cleland, W. W. *J. Biol. Chem.* **1974**, *249*, 2920–2927. Equilibrium data: Ochoa, S. In "Methods of Enzymology"; Colowick, S. P., Kaplan, N. C., Eds.; Academic Press: New York, 1955; Vol. 1, p 699–704. The value of the ratio K_m/K_i was calculated as in footnote *h*.

In searching for a theoretical rationalization for this empirical correlation we have considered a Haldane equation for the ordered bi–bi mechanism (eq 8: V_{maxf} refers to the maximum velocity in

$$K'_{eq} = \frac{(V_{maxf})(K_i^{NADH})(K_m^{ketone})}{(V_{maxr})(K_i^{NAD})(K_m^{alcohol})} \quad (8)$$

the direction of oxidation of alcohol; V_{maxr} refers to the maximum velocity in the direction of reduction of ketone; K'_{eq} is defined by eq 4).[36] In the course of manipulating this equation and exploring possible relations between it and eq 5, we note a second curious correlation: a plot of log K'_{eq} vs. log $(K_m^{ketone}/K_m^{alcohol})$ for the same set of oxido-reductase-catalyzed reactions included in Figure 6 also yields a reasonably straight line (Figure 7). (A plot of log K'_{eq} vs. either log K_m^{ketone} or log $K_m^{alcohol}$ is notably more scattered: correlation coefficients $r = -0.8$). The least-squares slope of Figure 7 is 1.5, rather than the value of ~1.0 observed in Figure 6. The values of K_m for the alcohol and ketone substrates were, however, generally measured at different values of pH; a closer correlation may exist for a set of values of K_m measured at a common value of pH.

These correlations between equilibrium thermodynamic data for reactants and products and kinetic parameters for the enzyme-catalyzed processes interconverting them are empirically useful, surprising, and presently theoretically unrationalized (at least by us). It is tempting to consider explanations relating differences in stability between reactants and products to their relative strength of binding to the enzyme, but we note that values of K_m and K_i for these relatively complex enzymatic processes do not necessarily correspond closely to physically interpretable dissociation constants. Further, it is unexpected that free energy relationships of the types observed here would hold through a series of (apparently quite) different enzymes. It is possible that these enzymes, as NAD(H)-dependent alcohol dehydrogenases, have substantial similarities in their active sites. Alternatively, they may share some deeper kinetic similarities reflecting the fact that all probably have a similar catalytic efficiency, in the sense discussed by Albery and Knowles.[38] These matters are of substantial mechanistic interest, but since we cannot presently discuss them intelligently, and since their theoretical rationalization is peripheral to the central points of this paper, we will not discuss them further theoretically.

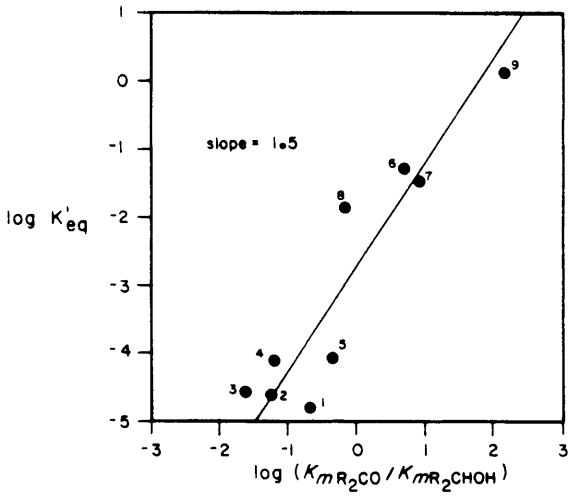


Figure 7. Plot of log K'_{eq} vs. log $(K_m^{R_2CO}/K_m^{RCHOH})$ for NAD(P)(H)-dependent dehydrogenases. K'_{eq} is defined in eq 4. Data are from Table II; points are labeled with numbers corresponding to reactions in Table II.

The value of the correlation is summarized in Figure 6 and eq 5–7 to estimate the value of K'_{eq} at which oxidations (which are generally kinetically and thermodynamically unfavorable) become kinetically favorable ($K_m/K_i \leq 0.1$) and thus suitable candidates for large-scale preparative synthesis. The answer, from eq 6, is at approximately $K'_{eq} = 1 \times 10^{-2}$. Reactions with values of $K_{eq} < 10^{-2}$ will proceed inefficiently while reactions with $K'_{eq} \geq 10^{-2}$ will be favorable enough to accomplish syntheses of "large" (≥100 mmol) quantities of substrate. By a similar analysis, reductions, which are usually kinetically and thermodynamically favorable, become kinetically unfavorable when $K'_{eq} > 8 \times 10^{-4}$ and are kinetically favorable when $K'_{eq} \leq 8 \times 10^{-4}$.

Equation 5 suggests that the value of K_m/K_i, and thus the efficiency of enzyme-catalyzed reaction, depends primarily on the equilibrium constant of the chemical transformation being considered and is not strongly influenced by the characteristics of

(38) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631–5640.

the enzyme catalyst (within the series of oxidoreductases considered here). This influence of (or correlation between) K'_{eq} (or, alternatively K_m/K_i) and reaction efficiency is illustrated by the HLADH-catalyzed oxidation of ethanol to acetaldehyde (without removal of the product)—a reaction which is inefficient at any concentration ($K'_{eq} = 9 \times 10^{-5}$; $K_m/K_i = 3$)—and the HLADH-catalyzed oxidation of cyclohexanol to cyclohexanone—a reaction which proceeds smoothly ($K'_{eq} = 5 \times 10^{-2}$; $K_m/K_i = 0.04$). An example of a difficult reduction is the preparation of *threo*-isocitrate, which has been performed in this group by two methods.[6,39] In both cases, the thermodynamics of the overall reaction were favorable; however, because of unfavorable kinetic parameters ($K'_{eq} = 1.3$ M, $K_m/K_i = 130$, $K_i = 1.3 \times 10^{-6}$ M), yields were poor and reaction times were long.

The use of NAD regeneration schemes will be useful only with preparative enzymatic oxidations which have favorable kinetic parameters ($K_m/K_i < 1$). This category of reaction includes HLADH oxidations of diols to lactones, certain oxidations generating stable ketones such as cyclohexanone, and the synthesis of ribulose 1,5-diphosphate. If the problem of product inhibition can be solved, other systems will be practical, for instance, resolution of primary or secondary racemic alcohols by enantioselective oxidation to the ketones or aldehydes, or the preparation of chiral α-hydroxy ketones or aldehydes by enantioselective oxidation of the diols.

We emphasize that the correlation in eq 3–5 described above does not in any way replace the task of finding the real value of K_i for a given K_m and K'_{eq} but merely provides an "expectation value" for the parameter. We believe eq 4 and 5 are a useful way to quantitate the enzymologist's intuition when faced with the possibility of the occurrence of product inhibition.

Experimental Section

General. Enzymes and biochemicals were purchased from Sigma. *cis*-1,2-Cyclohexanedimethanol (98%) was purchased from Aldrich and used without further purification. Methylene blue was purchased from Fisher. Hexane and 2,2,4-trimethylpentane were Fisher reagent grade. Water was doubly distilled, the second time through a Corning glass AG-1b still. Oxygen was technical grade and was used without further purification. GLC analysis was performed on a Perkin-Elmer 3920B gas chromatograph with a SE-30 column.

Kinetics. Buffered solutions were prepared with Hepes–KOH (0.1 M, pH 7.6) or glycine–KOH (0.1 M, pH 9.0). A Perkin-Elmer Model 552 spectrophotometer with thermostated cell compartment (25 °C) was used for UV measurements at 340, 610, and 665 nm. The slopes, intercepts, and errors of the Eadie–Hofstee and pseudo-first-order plots were determined with a least-squares program. Stock solutions of methylene blue and NADH were made according to their nominal molecular weights and the final concentrations determined by measuring the absorbances of diluted solutions at 665 nm (ϵ 78 000 M^{-1} cm^{-1}) and at 340 nm (ϵ 6220 M^{-1} cm^{-1}), respectively.

Rates of Uncatalyzed Reactions between Methylene Blue and NADH. Stock solutions of methylene blue (2.2 mM) and NADH (3.1 mM) in water were prepared. To a cuvette (1.5 mL) containing Hepes buffer (0.1 M, pH 7.6, 0.94 mL) were added methylene blue (0.03 mL, 0.066 mM in the cuvette) and NADH (0.02 mL, 0.063 mM in the cuvette). The cuvette was capped and inverted several times to mix the contents. The rate of decrease in concentration of NADH was taken to be the rate of reaction between methylene blue and NADH and was calculated from the initial rate of decrease in absorbance at 340 nm. Additional rates were measured in a similar manner using approximately equimolar concentrations of methylene blue and NADH at concentrations of 0.13, 0.16, 0.19, and 0.26 mM. Two cuvettes were prepared for each concentration (a total of 10 rate measurements were taken) and an average value for the second-order rate constant, k_2, was calculated to be 3.2 ± 0.2 M^{-1} s^{-1}. The procedure was repeated at pH 9 (0.1 M glycine–KOH); the rate constant was found to be 5.3 ± 0.5 M^{-1} s^{-1} (Table I).

Determination of Values of K_m for Methylene Blue for the Diaphorase-Catalyzed Reaction between Methylene Blue and NADH. Stock solutions of methylene blue (2.0 mM in water), NADH (2.5 mM in water), and diaphorase (1.5 mL^{-1} in Hepes buffer, pH 7.6) were prepared. To a cuvette (1.5 mL) containing Hepes buffer (0.1 M, pH 7.6, 0.90 mL) were added methylene blue (0.015 mL, 0.03 mM in the cuvette) and NADH (0.06 mL, 0.15 mM in the cuvette). The cuvette was capped and inverted several times to mix the contents and the rate of decrease in absorbance at 340 nm was measured (v_1). Diaphorase (0.02 mL, 30 μg) was added and a second rate was measured (v_2). The difference in the two rates ($v_2 - v_1$) was attributed to the diaphorase-catalyzed reaction. Additional diaphorase-catalyzed rates were measured using the same concentrations of NADH and varying concentrations of methylene blue (0.04, 0.06, 0.1, and 0.2 mM). Two cuvettes were prepared for each concentration of methylene blue (a total of 10 measurements of $v_2 - v_1$ were made). The procedure was repeated at pH 9.0 (0.1 M glycine–KOH buffer). Eadie–Hofstee plots of the data are given in Figure 3.

Determination of the Value of K_m for NADH in the Diaphorase-Catalyzed Reaction of Methylene Blue and NADH. Stock solutions of methylene blue (2 mM in water), NADH (0.93 mM in water), and diaphorase (1.5 mg in 16 mL of pH 7.6 Hepes buffer) were prepared. To a cuvette (1.5 mL) containing glycine–KOH buffer (0.1 M, pH 9.0, 0.71 mL) was added methylene blue (0.25 mL, 0.02 mM in the cuvette). The cuvette was capped and inverted several times to mix the contents and the rate of decrease in absorbance at 340 nm was measured (v_1). Diaphorase (0.02 mL, 2 μg) was added and a second rate was measured (v_2). The difference in the two rates ($v_2 - v_1$) was attributed to the diaphorase-catalyzed reaction. Additional diaphorase-catalyzed rates were measured in a similar manner using the same concentration of methylene blue and varying the NADH concentration (0.03, 0.06, 0.18 mM). No difference in diaphorase-catalyzed rate with varying NADH concentration ($v_2 - v_1$) was observed at these concentrations of NADH; the value of K_m for NADH was assumed to be $\leq 1 \times 10^{-5}$ M. An exact value for the value of K_m for NADH was not measured.

Rate of Reaction between Reduced Methylene Blue and Dioxygen. A stock solution of reduced methylene blue was prepared by mixing a dark blue, argon-purged solution of methylene blue (2.0 mM, 8 mL, 16 μmol), NADH (11 mg, 13 μmol), and diaphorase (2 mg). The color of the solution changed from deep blue to light blue. The concentration of reduced methylene blue was approximately 1.6 mM (see below). A cuvette (1.5 mL) containing Hepes bufer (0.1 M, pH 7.6, 0.97 mL) was capped with a rubber septum. Inlet and outlet needles were attached and oxygen was bubbled through the solution for 20 min. An aliquot (30 μL) of the reduced methylene blue solution was transferred to the cuvette using a 100-μL gas-tight syringe. The initial absorbance at 610 nm (the extinction coefficient, ϵ_{610}, for methylene blue is 44 500 M^{-1} cm^{-1}) was measured (OD = 0.5) and the concentration of unreacted methylene blue in the stock solution was calculated to be 0.4 mM. (The UV spectrum of methylene blue has a second, smaller absorbance maximum at 610 nm; this wavelength was used so that a wider concentration range could be examined.) The difference between the initial and final concentrations of methylene blue in the stock solution was assumed to be the concentration of reduced methylene blue. The absorbance at 610 nm was read directly from the spectrophotometer every 30 s for 3 min. The procedure was repeated using glycine–KOH buffer (0.1 M, pH 9). A plot of the data is given in Figure 2.

Rates of Reactions between NADH and Other Electron-Transfer Reagents. Uncatalyzed and Catalyzed Rates. The uncatalyzed rates of reaction between NADH and various electron transfer reagents (methyl viologen, flavin mononucleotide, dichloroindophenol, phenazine methosulfate, and potassium ferricyanide) were measured at pH 7.6. Stock solutions of the electron-transfer reagent (10 mM in water), NADH (12 mM in water), and diaphorase (1 mg/mL in Hepes buffer of pH 7.6) were prepared. To cuvettes (1.5 mL) containing Hepes buffer (0.1 M, pH 7.6, 0.94 mL) were added NADH (0.02 mL, 0.24 mM in the cuvette) and electron-transfer reagent (0.02 mL, 0.02 mM in the cuvette). The solutions in the cuvettes were mixed and the rate of decrease in absorbance at 340 nm was measured (v_1). For all the electron-transfer reagents except for phenazine methosulfate, the decrease in absorbance at 340 nm was attributed to the decrease in concentration of NADH alone, and the extinction coefficient for NADH (ϵ = 6220 M^{-1} cm^{-1}) was used to calculate the rate. The oxidized form of phenazine methosulfate has a high extinction coefficient at 340 nm (ϵ = 2600 M^{-1} cm^{-1}); the sum of the two extinction coefficients was used to calculate the rate. Diaphorase (0.02 mL, 20 μg) was added and a second rate measured (v_2). The difference in the two rates ($v_2 - v_1$) was attributed to diaphorase-catalyzed reaction. The data for both the uncatalyzed and catalyzed rates are given in Table I.

Enzyme Assays. Assays were performed using literature procedures.[18] Units of enzymatic activity are μmol min^{-1}. Units of HLADH refer to activity with *cis*-cyclohexanedimethanol as the substrate (the value of V_{max} for *cis*-cyclohexanedimethanol is approximately 80% of the value of V_{max} for cyclohexanol[14]). Diaphorase was assayed as described for the kinetic determinations, above, except with methylene blue at a concentration of 0.2 mM in the cuvette. Assays of HLADH and glucose de-

(39) Wong, C.-H.; Daniels, L.; Orme-Johnson, W. H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 6227–6228.

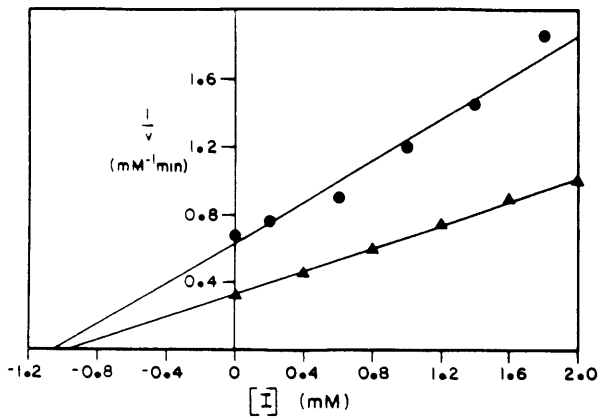


Figure 8. Dixon plot used in the determination of the value of K_i for acetaldehyde in the HLADH-catalyzed oxidations of *cis*-cyclohexanedimethanol (●) and ethanol (▲). The value of K_i was 1 mM for both reactions. The assays were performed on different days with different solutions of enzyme; the relative values of V_{max} for the two substrates shown here may not be accurate.

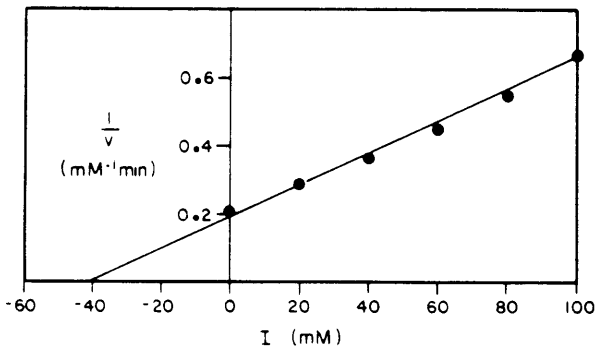


Figure 9. Dixon plot used in the determination of the value of K_i for cyclohexanone in the HLADH-catalyzed oxidation of cyclohexanol. The value of K_i was 40 mM.

hydrogenase (GlcDH) with added Na_2SO_4 or NaCl were accomplished by varying the concentration of added salt between 0 and 0.6 M for Na_2SO_4 and between 0 and 1.2 M for NaCl and assaying as usual.

Determination of K_i. The value of K_i of acetaldehyde was determined for the HLADH-catalyzed oxidations of ethanol and *cis*-1,2-cyclohexanedimethanol at pH 9.0 (glycine–KOH). Figure 8 shows Dixon plots of the data. The concentrations of ethanol and NAD were maintained at 30 mM (60 times the value of K_m for ethanol; see Table II) and 2 mM (40 times K_m), respectively, while the concentration of acetaldehyde was varied between 0 and 2 mM. The concentrations of cyclohexanedimethanol and NAD were maintained at 100 mM ($4K_m$, close to its solubility limit) and 2 mM ($40K_m$), respectively, while the concentration of acetaldehyde was varied between 0 and 1.8 mM. The value of K_i was 1 mM for both reactions.

The value of K_i of cyclohexanone for the HLADH-catalyzed oxidation of cyclohexanol was determined similarly (Figure 9).

Enzyme Immobilization. HLADH and diaphorase were immobilized on polyacrylamide gel as previously described.[3] HLADH was immobilized in 10–30% yield, and diaphorase in 30–40% yield. After the gels had been stirred in a reactor for several days, the activity of the recovered enzyme was often higher than the initial activity. This increase in activity is due to the additional grinding the gel receives under reaction conditions. This grinding reduces the size of the gel particles and decreases rate limitations due to slow diffusion of substrates into the interior of large particles.

Oxidation of *cis*-1,2-Cyclohexanedimethanol Using HLADH and GlDH. A 3-L, three-necked, round-bottomed flask equipped with a magnetic stirring bar was charged with 2-oxoglutaric acid (22.4 g, 153 mmol) and water (0.75 L). Ammonium hydroxide (30%, 35 mL) was added to neutralize the acid and to adjust the pH to 8.3. *cis*-1,2-Cyclohexanedimethanol (10 g, 69 mmol), soluble HLADH (250 mg, 580 U), and immobilized GlDH (230 U, 100 mL of gel) were added. An aliquot (0.5 mL) of the mixture was removed. NAD (0.20 g, 0.27 mmol) and hexane (1 L) were added. The mixture was stirred only rapidly enough to suspend the gel in the aqueous phase. The extent of the reaction was measured by removing aliquots (0.5 mL) of the aqueous

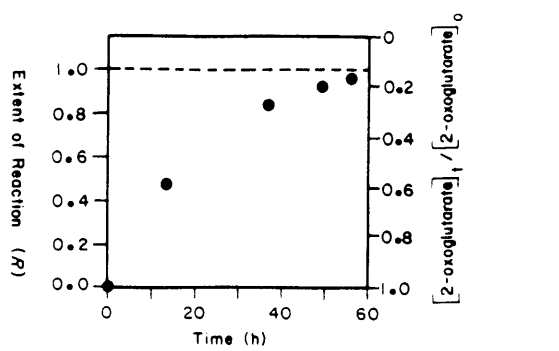


Figure 10. Plot of the progress of an oxidation catalyzed by HLADH of *cis*-1,i-cyclohexanedimethanol. Regeneration of NAD was accomplished using 2-oxoglutarate/GlDH; the reaction was followed by periodic assays of residual 2-oxoglutarate. The subscripts t and 0 on the right axis refer to concentrations of 2-oxoglutarate at time t after the start of the reaction and at time = 0.

phase, centrifuging to separate the gel, diluting a portion of the supernatant in a 1:50 ratio, and quantitatively measuring 2-oxoglutarate by enzymatic assay. Figure 10 shows a plot summarizing the extent of reaction vs. time. An accurate determination of the volume of the aqueous phase was found by enzymatic assay of 2-oxoglutarate in the aliquot removed at the start of the reaction; the known, initial quantity of 153 mmol of 2-oxoglutarate was used in the calculation and a reaction volume of 1.05 L was obtained. Accuracy in the measurement of the volume was necessary for the subsequent, quantitative determinations of residual 2-oxoglutarate.

The reaction was 94% complete after 2.5 days. Enzymatic assay of the reaction mixture showed that 0.17 mmol of NAD (60%) and 0.0 mmol of NADH remained. The turnover number (TN, equiv of product/equiv of NAD) for NAD was 500. The hexane layer was removed by forced siphon using a stainless steel cannula and concentrated to a pale yellow oil (6.3 g). The enzyme-containing gel was allowed to settle and the aqueous supernatant removed via cannula. The remaining gel was assayed and found to contain 45 U (20%) of residual GlDH activity. The aqueous solution was concentrated to 0.25 L by batchwise ultrafiltration at 40 psi using a stirred Amicon cell (350 mL) and a Diaflo membrane with a 10 000-dalton cutoff. The residual HLADH activity was found to be 140 U (24%). The aqueous solution was acidified with HCl to pH 5 and extracted with three 150-mL portions of ether. The ethereal solution was dried ($MgSO_4$) and concentrated to a yellow oil (3.0 g). The combined yield of the crude lactone was 9.3 g (96%). The oil was distilled through a vacuum-jacketed short-path distillation head to give (+)-(1*R*,6*S*)-*cis*-8-oxabicyclo[4.3.0]nonan-7-one as a colorless oil which solidified upon standing at room temperature (8.2 g, 85% yield); bp 64–74 °C (0.2 torr) (lit.[14] bp 86 °C (2 torr)); α^{23}_D +43.8° (neat), $[\alpha]^{23}_D$ +48.8° (*c* 0.5 g/100 cm^3, $CHCl_3$) (lit.[14] $[\alpha]^{25}_D$ +48.8° (*c* 0.5 g/100 cm^3)); ^{1}H NMR data were in agreement with literature values.

Oxidation of *cis*-1,2-Cyclohexanedimethanol Using HLADH and MB/Diaphorase. To a 1-L round-bottomed flask equipped with a magnetic stirring bar were added Hepes buffer (0.1 M, pH 7.6, 0.1 L), *cis*-cyclohexanedimethanol (4 g, 28 mmol), NAD (0.1 g, 0.13 mmol), methylene blue (30 mg, 0.08 mmol), immobilized HLADH (20 U, 90 mL gel), immobilized diaphorase (27 U, 40 mL of gel), and soluble catalase (10 mg). The solution turned from deep blue to colorless after 20 min. Hexane (500 mL) and 2,2,4-trimethylhexane (2.0 mL, used as an internal GLC standard) were added. Oxygen was bubbled through the aqueous solutions via a stainless steel needle. The reaction was monitored by GLC analysis of the organic phase. After 4 days, the hexane layer was decanted and the aqueous phase centrifuged to remove the gel. The gel was washed with additional buffer (100 mL) and the combined aqueous solutions were acidified to pH 3 and extracted with hexane (2 × 0.1 L). The combined organic solutions were concentrated, redissolved in ether (20 mL), filtered through decolorizing carbon (0.5 g), and concentrated to a colorless oil (3.2 g, 83%). The ^{1}H NMR spectral data agreed with literature values: $[\alpha]^{23}_D$ +48° (*c* 0.5 g/100 cm^3, $CHCl_3$); lit. $[\alpha]^{25}_D$ +48.8°. The residual enzyme activities were: HLADH, 30 U, 160%; diaphorase, 85 U, 300%. The aqueous solution was assayed for residual NAD activity (0.04 mmol, 28%). The turnover number for NAD was 350; the turnover number for methylene blue was 580.

Oxidation of Glucose Using GlcDH and $Fe(CN)_6^{3-}$/Diaphorase. A 500-mL round-bottomed flask equipped with a magnetic stirring bar was charged with glucose (0.9 g, 5 mmol), NAD (0.03 g, 0.04 mmol), immobilized GlcDH, and diaphorase (44 and 22 U, respectively, 80 mL of

gel) and Hepes buffer (0.1 M, pH 7.6, 70 mL). Potassium ferricyanide (3.3 g, 10 mmol) was added and the mixture was stirred under an argon atmosphere. The pH was maintained at 7.0 by automatic addition of 2.5 N KOH. Aliquots (0.5 mL) of the reaction were removed periodically and centrifuged, a portion of the supernatant was diluted in a 1:20 ratio, and the absorbance was measured at 420 nm. After 6 h, 6.3 mL of 2.5 N KOH (15.8 mmol, 3.2 equiv) had been added. Figure 4 shows a plot of the addition of base vs. time for the reactor and a plot of the ratio of the absorbance to initial absorbance at 420 vs. time. The mixture was centrifuged to separate the gel. The activitites of the recovered enzymes were 60 U for GlcDH (140%) and 32 U for diaphorase (150%). The turnover number for NAD was 125. The product of the oxidation, gluconate, was not isolated.

Determination of Partition Coefficients of (+)-(1*R*,2*S*)-*cis*-8-Oxabicyclo[4.3.0]nonan-7-one between Hexane and Water. A solution of the lactone (55 mg, 0.39 mmol, 78 mM) and octadecane (30 mg, 0.12 mmol, 24 mM) in hexane (5 mL) was analyzed by GLC, (10% Carbowax). The response factor, R_f, was found to be 1.91 (R_f = [mmol of lactone/mmol off octadecane]/[area of lactone/area of octadecane]). A 2-mL portion of the hexane solution was equilibrated by shaking with a 2-mL aliquot each of glycine buffer (0.25 M, pH 9.0) and of distilled water. The hexane layer was analyzed by GLC using octadecane as the internal standard. The partition coefficients (σ) with glycine buffer and distilled water were 1.0 and 1.9, respectively.

Decomposition of Oxaloacetate. A stock solution of oxaloacetate (100 mM in Hepes buffer, pH 7.6) was prepared by combining oxalacetic acid (132 mg, 1 mmol), NaOH (1.8 mL of a 1 N standard solution), and Hepes buffer (8.2 mL). Three vials (10 mL) were prepared: the first contained Hepes (0.1 M), glycine (0.1 M, pH 7.6, 4.5 mL), and oxalacetate (0.5 mL); the second contained HLADH (5 mg), Hepes (0.1 M, pH 7.6, 0.9 mL), and oxalacetate (0.1 mL); the third contained Hepes (0.1 M, pH 7.6, 4.5 mL) and oxalacetate (0.5 mL). Aliquots (50 μL) were removed and assayed for oxalacetate.[18] Figure 1 shows a plot of the first-order decomposition.

Acknowledgment. This work was supported by the National Institutes of Health, Grant GM 30367. Our colleagues Professor Jeremy Knowles and Steve Benner offered useful suggestions on several aspects of this work.

Registry No. 1, 15753-50-1; **2**, 65376-02-5; NAD, 53-84-9; NADH, 58-68-4; MB, 61-73-4; MV, 1910-42-5; FMN, 146-17-8; DCIP, 956-48-9; PMS, 299-11-6; O_2, 7782-44-7; $Fe(CN)_6^{3-}$, 13408-62-3; CH_3CHO, 75-07-0; EtOH, 64-17-5; $K_3Fe(CN)_6$, 13746-66-2; glutamate dehydrogenase, 9029-12-3; diaphorase, 9001-18-7; D-glucose, 50-99-7; D-gluconate, 526-95-4; alcohol dehydrogenase, 9031-72-5; glucose dehydrogenase, 9028-53-9; oxidoreductase, 9055-15-6; dehydrogenase, 9035-82-9; cyclohexanone, 108-94-1; cyclohexanol, 108-93-0; malate dehydrogenase, 9001-64-3; glycerol dehydrogenase, 9028-14-2; lactate dehydrogenase, 9001-60-9; malic enzyme, 9028-47-1; 3-hydroxybutyrate dehydrogenase, 9028-38-0; isocitrate dehydrogenase, 9001-58-5; 2-oxoglutarate, 328-50-7.