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REVIEW

Regeneration of Nicotinamide Cofactors for use in Organic Synthesis

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ABSTRACT

The high cost of nicotinamide cofactors requires that they be regenerated *in situ* when used in preparative enzymatic synthesis. Numerous strategies have been tested for *in situ* regeneration of reduced and oxidized cofactors. Regeneration of reduced cofactors is relatively straightforward; regeneration of oxidized cofactors is more difficult. This review summarizes methods for preparation of the cofactors, factors influencing their stability and lifetime in solution, methods for their *in situ* regeneration, and process considerations relevant to their use in synthesis.

Index Entries: Nicotinamide cofactors, regeneration of; nicotinamide cofactors, and organic synthesis; organic synthesis; regeneration; nicofinamide cofactors, preparation of; nicotinamide cofactors, stability; enzymes, dehydrogenases.

INTRODUCTION

With the demonstrated, successful application of noncofactor-requiring enzymes as catalysts for preparative synthesis (*1–10*), attention is turning to the use in synthesis of the more complex cofactor-requiring enzymes. Among these enzymes, the oxidoreductases requiring nicotinamide cofactors are of central interest. Although these enzymes have long been indispensable in their analytical applications (*11*), they have seen only limited use in preparative organic synthesis. One problem has

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been the initial expense and physical instability of the nicotinamide cofactor-requiring enzymes themselves. Many oxidoreductases are multimeric when active (*12,13*). Their activity in solution or on immobilization supports requires maintenance both of their correct secondary and tertiary structures and of their interactions between subunits. Like many enzymes, oxidoreductases may be inactivated by oxidizing or reducing agents, compounds particularly likely to be present in biochemical redox reactions. Improved methods of enzyme preparation, manipulation, and immobilization have addressed these difficulties in part. Further improvements of economy and stability will eventually emerge through protein engineering and recombinant DNA technology (*14–20*). A second problem is that oxidoreductases exhibit kinetics that are more complex than those of simple hydrolases and isomerases. Allosteric effects and the influence of noncompetitive product inhibition are particularly important (*21*). One fundamental obstacle to the large-scale use of NAD(P)(H)-dependent oxidoreductases has, however, been the expense of their cofactors. Nicotinamide cofactors cost too much to be used as stoichiometric reagents for other than small-scale syntheses (Table 1). If nicotinamide cofactor-dependent enzymes are to be employed as catalysts for preparative chemistry, effective methods for *in situ* cofactor regeneration must be available.

In fermentations, cells provide the enzymes necessary for substrate conversion and cofactor regeneration. The nicotinamide cofactors are synthesized and regenerated as a part of cellular metabolism. In processes using isolated enzymes *ex vivo*, nicotinamide cofactors must be regenerated explicitly. In addition to the system of enzymes used for synthesis, a second reaction system—the *regenerative system*—must be used. The regenerative system may involve oxidizing or reducing reagents, enzymes, photochemical activators, electrodes, or some combination of these elements.

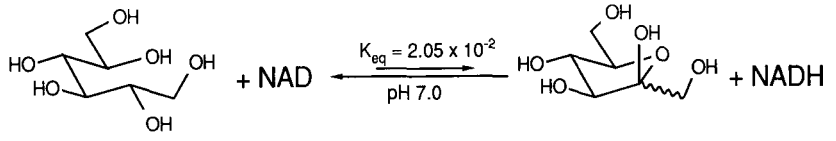
Cofactor regeneration can accomplish three objectives in addition to reducing the contribution of cofactor to the cost of synthesis. First, it can be used to influence the position of equilibrium. At pH 7.0, the equilib-

TABLE 1
Cost of Nicotinamide Cofactors

Cofactor	$/mol
NAD	710[a]
NADH	3,050[a]
NADP	25,780[b]
NADPH	216,100[b]

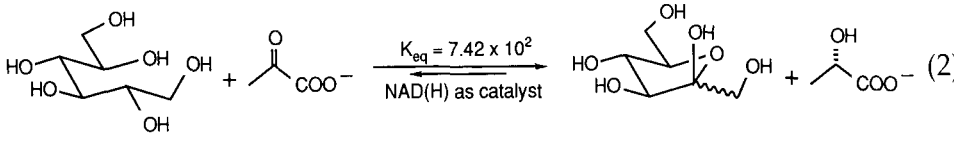
[a]Kyowa Hakko Kogyo Co., Ltd.
[b]United States Biochemical Corp.
[c]Sigma Chemical Co.

rium for the oxidation of sorbitol to fructose by NAD [Eq. (1)] strongly favors the formation of sorbitol and NAD (*22*).



(1)

By contrast, regeneration of NAD by the lactate dehydrogenase-catalyzed reduction of pyruvate leads to an equilibrium constant for the conversion of sorbitol and pyruvate to fructose and lactate, [Eq. (2)]

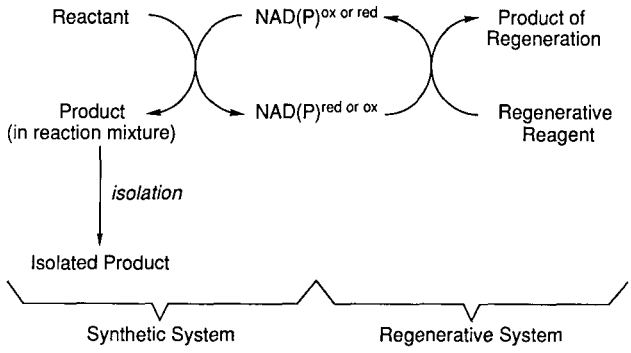


(2)

which strongly favors the formation of fructose and lactate (*23*). Cofactor regeneration thus couples substrate conversion to a redox reaction capable of driving the equilibrium toward product formation. Second, cofactor regeneration can obviate the problem of product inhibition from the cofactor produced by the synthetic reaction. Cofactor regeneration prevents the accumulation of cofactor byproduct both by reducing to a catalytic quantity the total amount of cofactor required and by consuming the cofactor produced by the synthetic reaction as it forms. Third, by eliminating the need for stoichiometric quantities of NAD(P)(H), cofactor regeneration can simplify the reaction work-up.

Any method for regenerating nicotinamide cofactors must be practical and should enable an enzymatic process to be inexpensive and convenient. Enzymes, reagents, and equipment required should be readily available, inexpensive, and easily manipulated. The reaction used for regeneration should proceed in high yield, and total turnover numbers for the cofactor (TTN = total turnover number = mol product formed/mol cofactor present) should be high. Cofactor regeneration should facilitate the thermodynamically and kinetically favorable formation of product. It should allow convenient monitoring of the extent of reaction. Any reagents or byproducts of the regenerative system should not interfere with product isolation and should be compatible with the components of the synthetic system.

No one method of regenerating cofactor is perfect in its advantages and lack of disadvantages. A combination of many factors determine the regenerative process best suited to an application. In selecting a method, the entire system must be considered. Cofactor regeneration functions not in isolation but coupled to a synthetic process [Eq. (3)].



(3)

In practice, synthetic chemists will probably require a number of methods for cofactor regeneration—each with particular characteristics (low cost, high redox potential, optimal isolation of products)—for use with a range of synthetic applications requiring cofactors.

Regeneration schemes leading to enzymatically inactive forms of cofactor are useless. The lifetime of active cofactor depends on its stability under operating conditions and the selectivity of its regeneration. We will see that the criterion of long lifetime for the cofactor effectively eliminates most chemical and electrochemical methods of regeneration from serious consideration. Although we mention these methods for completeness, our review will focus on enzymatic methods of regeneration.

Our review first examines the nicotinamide cofactors themselves: their preparation, their stabilities in solution, and their redox properties. It summarizes various general strategies for nicotinamide cofactor regeneration and then details several of the most useful or interesting methods for recycling cofactors. Finally, it discusses reactor configuration as it relates to the implementation of cofactor regeneration for synthesis. Our evaluation of methods for cofactor regeneration is based on cost, rate of reaction, and ease of operation.

Throughout the review, we will often refer to the oxidized cofactors, NAD and NADP, generically as NAD(P) and to the reduced cofactors as NAD(P)H. Two definitions concerning turnover numbers for the nicotin amide cofactors are also important. The "turnover number" (TN) for a cofactor [Eq. (4)] is a rate—typically, the average number of complete cycles of oxidation and reduction experienced by a molecule of cofactor per unit time—and has units of s^{-1}. This definition is equivalent (discounting multiple pathways leading to products) to the number of moles of product formed per mole of cofactor per unit time. The TTN is the total number of moles of product formed per mole of cofactor during the course of a complete reaction [Eq. (5)].

$$\text{TN} = \frac{\text{cofactor redox cycles}}{\text{time}} = \frac{\text{mol product}}{(\text{mol cofactor})(\text{time})} \tag{4}$$

$$\text{TTN} = \frac{\text{mol product formed}}{\text{mol cofactor present in reaction}} \tag{5}$$

This number reflects several factors: the loss of cofactor caused by degradation or incorrect regiochemistry of regeneration, the length of time the reaction is allowed to proceed, and the turnover number. It is useful because it provides an estimate of the contribution, C, of the cost of cofactor to the cost of the product [Eq. (6)]. Reports of TTN are not consistent.

$$C = \frac{\text{cost of cofactor}}{\text{TTN}} \tag{6}$$

Some authors consider the moles of cofactor in calculating C to be the number added to the reactor. Some consider the number to be only the number destroyed during the reaction, presuming that cofactor still active at the conclusion of the reaction might be recovered and reused in subsequent reactions. We will follow the former, more conservative practice.

In calculating the contribution of enzyme to the cost of a synthetic process, one must consider the initial expense of the enzyme, the cost of enzyme activity lost during enzyme immobilization, and the effects of single or repeated use of the enzyme. For a one-time synthesis, the cost of the enzyme is correctly taken to be its full initial cost (taking into account activity lost during immobilization). For a repeated process, though, the cost of the enzyme may be calculated as the cost of *only the activity lost during the reaction* (taking into account activity lost during immobilization).

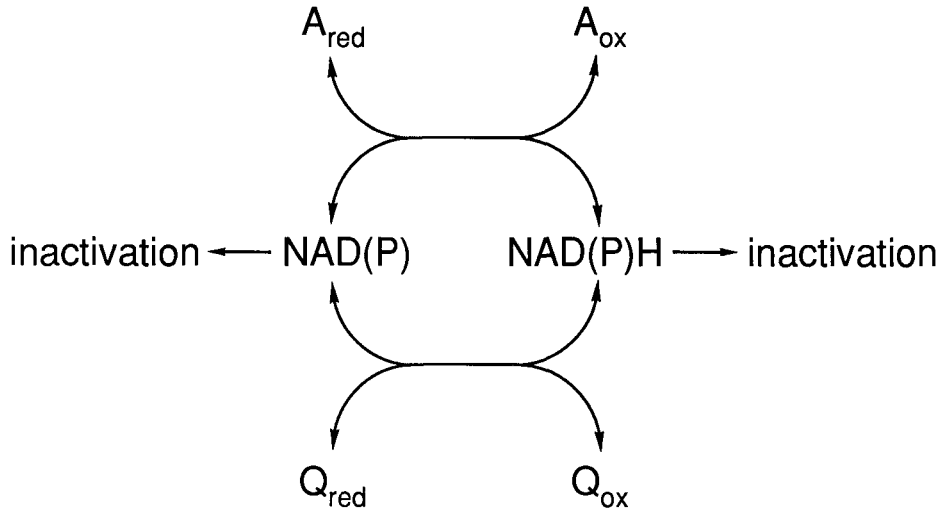
Finally, the rate of a reaction deserves consideration when estimating the cost of the reaction. A process that is inexpensive *per unit rate of product formation* may be more desirable than a process that is inexpensive *per mole of product formed*. The regeneration cost (RC) may be defined as the cost of the components (enzymes, reagents, cofactor) required to regenerate one mole of cofactor per day [Eq. (7)]. The RC is particularly dependent on the cost and amount used of any catalytic species (enzymes) in the regenerative reaction.

$$\text{RC} = \frac{[\text{cost of component(s)}](\text{d})}{\text{mol of cofactor regenerated}} \tag{7}$$

NICOTINAMIDE COFACTORS

Preparation of Cofactors

The effective cost of cofactor used in a process is proportional to its initial cost and inversely proportional to its total turnover number [Eq. (6)]. The maximum possible value of the TTN for a batch reactor is set by the maximum allowed substrate concentration (determined by substrate solubility, product inhibition, and enzyme stability) divided by the minimum cofactor concentration required to effect acceptable reaction rates. In practice, the TTN is further limited by the competing rates of produc-



Scheme 1. Processes involved in cofactor utilization, regeneration, and inactivation.

tive (cofactor utilization and regeneration) vs nonproductive (cofactor degradation) reactions (Scheme 1). Thus, both the initial cost of the cofactor and the efficiency with which it is utilized and recycled during the synthetic process determine its contribution to the cost of the product.

The NAD is presently isolated from yeast (*24–31*). Reported fermentation yields (*24,25*) range from 1.2 g/kg of dry cells for *Saccharomyces cerevisiae* to 4.2 g/kg of dry cells for *Saccharomyces carlsbergensis*. Fermentation of *Brevibacterium ammoniagenes* yields 2.3 g/L of NAD (*32*). Because NAD suitable for use in assays normally must be free from contaminating ethanol or acetaldehyde, its preparation requires the use of bakers yeast rather than the less expensive brewers yeast. The cost of the yeast [$1/kg (*33*)] is a significant part of the cost of the NAD. Although minor contamination by alcohols would not be a problem for NAD(P)(H) used with cofactor regeneration in organic synthesis, economical microbial starting materials for the production of such NAD(P)(H) have not been developed.

The NADP has been isolated from microbes and liver (*34*), but is normally prepared (*35–37*) by the phosphorylation of NAD using NAD kinase (EC 2.7.1.23) and ATP. The NAD(P)H can be prepared from NAD(P) by chemical (*38*), enzymatic (*39*), or microbial (*40*) reduction. Thus, an improved method for preparing NAD would provide improved routes to all of the nicotinamide cofactors. Combined chemical and enzymatic syntheses of NAD (*41–45*) from AMP or ATP have been developed, but these procedures are inferior to isolation.

Reactivity of Cofactors Under Specific and General Acid and Base Conditions

The reduced and oxidized forms of nicotinamide cofactors are both subject to decomposition in aqueous solution. The NAD(P)H is stable in base but labile in acid; NAD(P) is stable in acid but labile in base (*46,47*) (Fig. 1). Although incubation at 23°C in 0.02*N* HCl for 1 min destroys NAD(P)H (*46*), NAD(P) is relatively stable in 0.02*N* HCl. The NAD(P) is destroyed by heating to 100°C at pH 11 for 15 min (*48*) or in 0.1*N* NaOH for 5 min (*49*), but NAD(P)H withstands heating at 100°C in 0.1*N* NaOH for 30–60 min (*50,51*). Prolonged storage in base, however, does tend to promote the oxidation of NAD(P)H to NAD(P) (*46*).

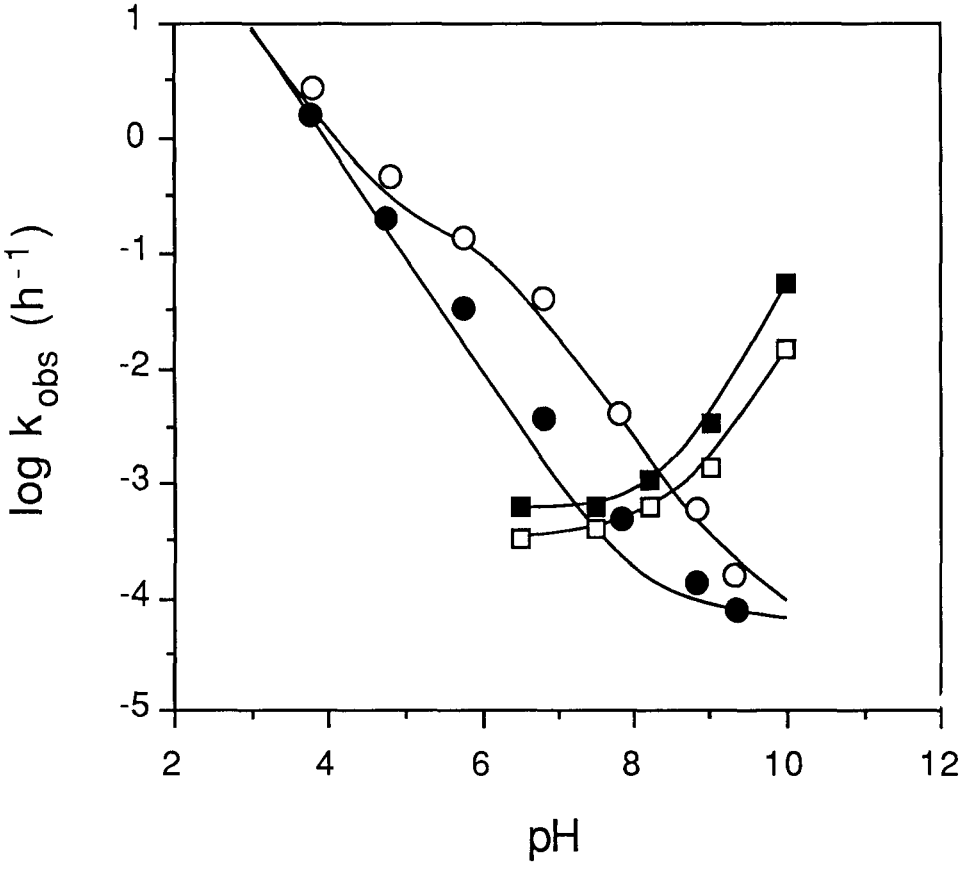
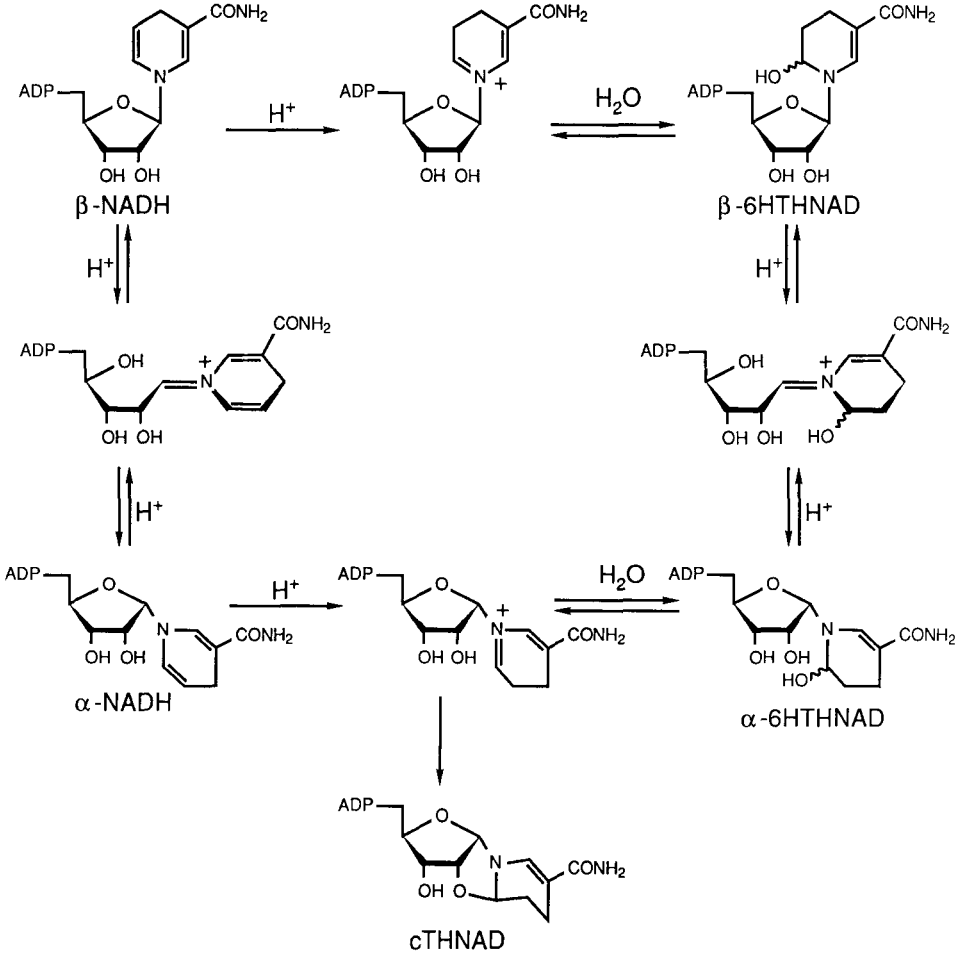


Fig. 1. Rate constants for the decomposition of NAD (■), NADP (□), NADH (●), and NADPH (○). Conditions: 0.1 m*M* cofactor, 25°C, 50 m*M* buffer (acetate, pH 3.8–5.8; triethanolamine, pH 6.5–8.8; bicarbonate, pH > 9). Data taken from ref. (*47*). Line connecting (●) calculated by Eq. (8), using the rate constants of Wong and Whitesides (*47*); line connecting (○) calculated by Eq. (10).

Acid-Catalyzed Decomposition of NAD(P)H

In acid, the decomposition of NAD(P)H involves hydration, epimerization, and cyclization (52). The order of events depends upon the pH and buffer concentration (Scheme 2) (53). As expected, the rate of decomposition of NAD(P)H is subject to general acid catalysis (Fig. 1) (47,53). Between pH 3 and 10, the rate of decomposition of NADH obeys Eq. (8), where k_w, k_H, and k_{HA} are the rate coefficients for uncatalyzed, specific acid catalyzed, and general acid catalyzed reactions, respectively.

$$\frac{-d \ln [\text{NADH}]}{dt} = k^{\text{obs}}_{\text{NADH}} = k_w + k_H [\text{H}^+] + k_{HA}[HA] \qquad (8)$$



Scheme 2. Acid-catalyzed decomposition of β-NADH [from ref. (52)]. At low pH, β-NADH first undergoes acid-catalyzed anomerization to α-NADH and then cyclizes to α-$O^{2'}$-6B-1,4,5,6-tetrahydronicotinamide adenine dinucleotide (cTHNAD). At higher pH, initial hydration of β-NADH yields β-6-hydroxy-1,4,5,6-tetrahydronicotinamide adenine dinucleotide (β-6HTHNAD), which then anomerizes and dehydrates to give cTHNAD.

Wong and Whitesides measured the values of $k_w = 7 \times 10^{-5}$/h and $k_H = 9.4 \times 10^3/M$/h (*47*). Johnson and Tuazon report $k_w = 6.6 \times 10^{-4}$/h and $k_H = 7.3 \times 10^3/M$/h (*53*). Table 2 lists values for k_{HA} for a number of buffers as well as the calculated half-life for the disappearance of NAD(P)H in a solution containing the acidic buffer, *HA* (pH = 7, [HA] + [A^-] = 0.1*M*, 25°C).

The NADPH is subject to the same mechanisms of decomposition as NADH. In addition, however, decomposition of NADPH reflects an *intramolecular* acid catalysis by the 2′-phosphate group (*47*). The rate of decomposition of NADPH obeys Eqs. (9) and (10), where $k_I = 0.15$/h is a constant reflecting intramolecular catalysis and $K_a = 10^{-6.2}$ is the dissociation constant of the 2′-phosphate.

$$\frac{-d\ \ln[\text{NADPH}]}{dt} = k^{\text{obs}}_{\text{NADPH}} \tag{9}$$

$$k^{\text{obs}}_{\text{NADPH}} - k^{\text{obs}}_{\text{NADH}} = \frac{k_I[\text{H}^+]}{K_a + [\text{H}^+]} \quad \frac{k_I}{1 + 10^{\text{pH} - pK_a}} \tag{10}$$

TABLE 2
Rate Constants for the General Acid-Catalyzed Decomposition of NAD(P)H[a]

			$t_{1/2}$,[b] h	
HA	pK_a	$k_{HA}/M/h$	NADH	NADPH
Pyruvic acid	2.5	260	460	31
Formic acid	3.8	(19)[c]	340	31
Lactic acid	3.9	(1.9)[c]	320	31
Acetic acid	4.8	2.5 (4.4)[c]	270	30
Citric acid	5.4	1.2 (2.1)[c]	180	28
Glucose-6-phosphate	6.1	0.97	59 (60)[d]	21 (21)[d]
6-Phosphogluconate	6.2	0.96	59	22
Maleic acid	6.2	0.22 (0.25)[c]	172	28
Pyrophosphate	6.5	0.33 (0.54)[c]	78	24
Imidazole	7.0	0.087	130 (180)[d]	27 (29)[d]
Methylimidazole	7.1	0.068	140 (220)[d]	27 (28)[d]
Sodium phosphate	7.2	0.41 (0.69)[c]	27 (29)[d]	13 (13)[d]
Guanadinium phosphate			(27)[d]	(15)[d]
HEPES	7.5	0.048	150 (170)[d]	28 (29)[d]
Triethanolamine	7.8	0.018	280 (330)[d]	30 (32)[d]
Tris	8.1	0.012	330 (340)[d]	31 (34)[d]
Bicarbonate	10.3	0.0081	520 (80)[d,e]	32 (28)[d]

[a]Eqs. (2)–(4). Rate constants for the disappearance of the NAD(P)H chromophore (0.1 m*M*) at 340 nm. T = 25°C. Taken from ref. (*47*).

[b]Estimated half-life for the disappearance of NAD(P)H in a solution containing 0.1 *M* [HA] + [A−], pH 7.0, T = 25°C.

[c]Values of rate constants in parentheses are from ref. (*53*).

[d]Measured values of $t_{1/2}$.

[e]The difference between observed and calculated values of $t_{1/2}$ is probably related to catalysis by H_2CO_3.

Thus, the rate of decomposition of NADPH is not linear with respect to $[H^+]$ at moderate pH and is higher than that of NADH (Fig. 1). At pH 5 or 9, NADPH decomposes 3-to-4 times faster than NADH. At pH 7, NADPH can decompose 10-to-20 times faster than NADH.

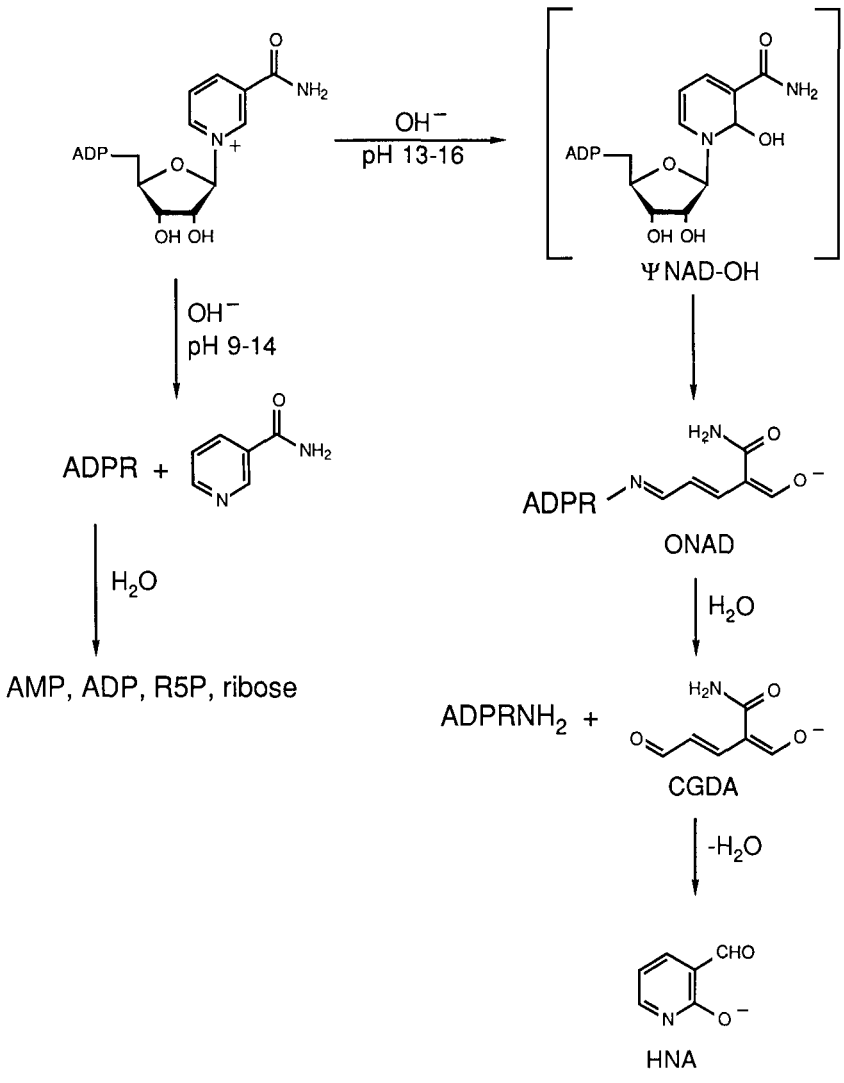
We note that the most effective general acid catalysts for the decomposition of NAD(P)H are those buffers whose pK_a are approximately equal to the pH of the solution. This relationship results from a compromise between the strength of the acid (stronger acids are better catalysts) and the degree of its ionization (only the undissociated acid can be a general acid catalyst). Exceptions to this trend include inorganic phosphate, which is a better catalyst than expected from its pK_a value, and imidazole, HEPES, triethanolamine, and Tris buffers, which are less effective than expected. In addition, imidazole and pyridine buffers exhibit nonlinear effects in which the rate of cofactor decomposition drops off at high buffer concentration (*53*). Organic cosolvents do not significantly inhibit phosphate-catalyzed decomposition of NAD(P)H.

Base-Catalyzed Decomposition of NAD(P)

The major pathways leading to decomposition of the oxidized nicotinamide cofactors involve initial hydrolysis of the nicotinamide—ribose bond or nucleophilic addition to the nicotinamide ring (Scheme 3) (*48, 54–56*). These reactions are base catalyzed. Although relatively stable in acid, NAD(P) does seem to hydrolyze slowly at the pyrophosphate linkage at pH < 2 (*46*). Between pH 2 and 7, the rate of hydrolysis of NAD(P) is constant (Fig. 1) (*46,57*). Above pH 8, the rate of hydrolysis increases with increased pH. The rate of base-catalyzed hydrolysis of NAD(P) is increased by the presence of phosphate, citrate, maleate, sulfate, oxalate, carbonate, or chloride anions (*46,57*). At pH 7.6, 0.1*M* phosphate or citrate increases hydrolysis 4- or 5-fold, respectively. Even 1 m*M* phosphate or citrate doubles the rate. Tris buffer, however, exhibits a protective effect against this acceleration by phosphate or citrate (*57*).

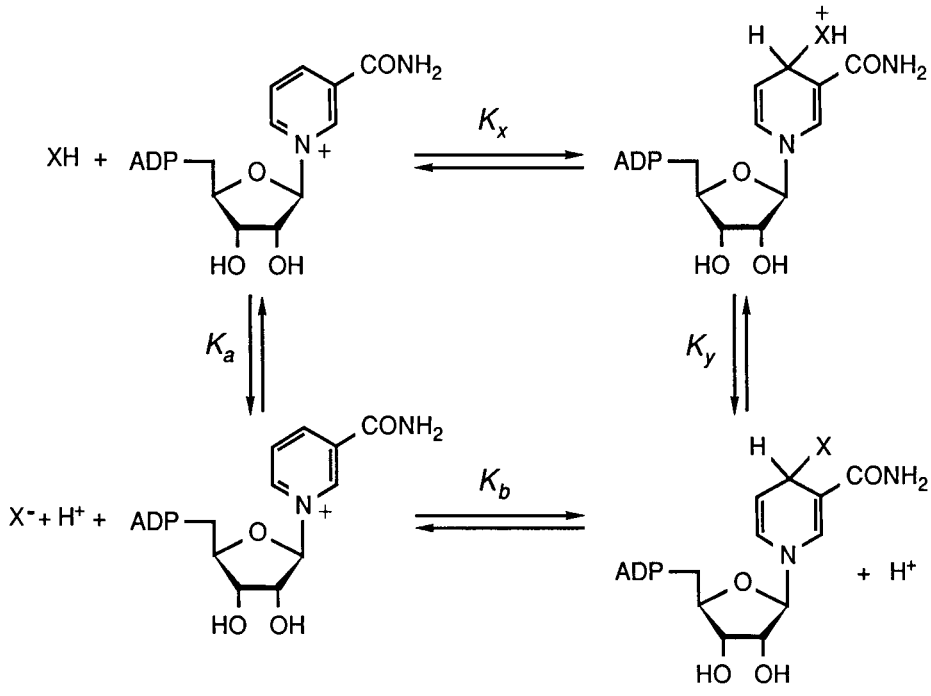
Nucleophilic Addition to NAD(P)

In addition to alkaline hydrolysis, the oxidized nicotinamide cofactors are susceptible to nucleophilic attack (Scheme 4). Whereas attack by hydroxide at C-2 of the pyridinium ring (Scheme 3) leads to opening of the ring, nucleophilic attack at C-4 leads to 1,4-dihydropyridine structures, which are stable against alkaline hydrolysis. Sulfite (*58*), phosphate (*59*), cyanide (*60*), thioglycolate (*61*), and pyruvate (*62,63*) have all been shown by NMR or deuterium exchange to add at C-4 of the pyridinium ring. Other nucleophiles are presumed to add at C-4 because of the spectral similarities between their addition products and known 1,4-dihydropyridine compounds. Thiols (*64,65*), enols (*63,66,67*), imidazole (*68*), hydrazine, hydroxylamine, and borate (*61*) all add nucleophilically to NAD. Table 3 lists rate and equilibrium constants for the addition of nucleophiles to NAD.



Scheme 3. Base catalyzed decomposition of NAD. In dilute base, the nicotinamide—ribose bond cleaves more rapidly than the pyrophosphate bond, and hydrolysis yields nicotinamide and ADP—ribose (ADPR) (*54*). The ADPR hydrolyzes further to release AMP. The ADP has also been reported as a product of NAD hydrolysis (*48,55*). In concentrated base, hydroxide adds reversibly to C-2 of the nicotinamide ring and forms a "transient pseudobase," ψNAD–OH. This intermediate converts irreversibly to a ring-opened form of NAD, ONAD, which then hydrolyzes and yields ADP-ribosylamine ($ADPRNH_2$) and 2-carboxamideglutacondialdehyde (CGDA) (*56*). The CGDA further decomposes to form 2-hydroxynicotinaldehyde (HNA).

Scheme 5 depicts the mechanism for the addition of pyruvate to NAD. Enol-pyruvate adds to C-4 of the nicotinamide ring and forms a compound that absorbs at 340 nm, but does not fluoresce. In a subse-



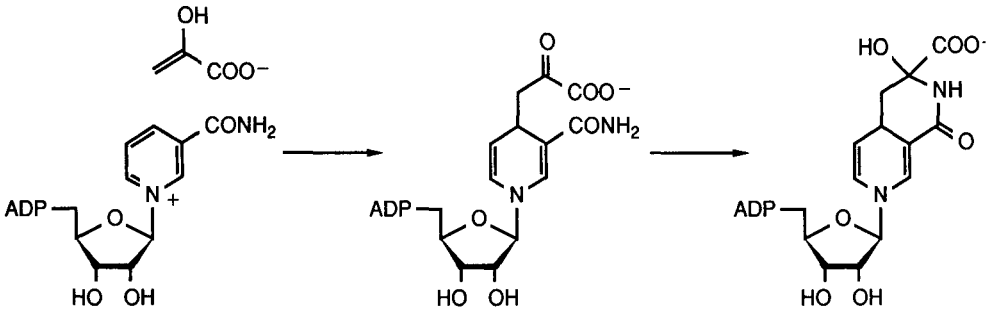
Scheme 4. Nucleophilic addition to NAD.

quent, slower reaction, the amide nitrogen attacks the carbonyl carbon to form a ring-closed compound absorbing at 340 nm and fluorescing at 450 nm (*63*). According to Eq. (11), NAD in the presence of 0.1*M* pyruvate, pH 7.0, should have a half-life of 6.9 h (*69*). The rate of nonenzymatic

TABLE 3
Rate and Equilibrium Constants for Nucleophilic Attack on NAD[a]

Nucleophile	pK_a^b	log k_1	log K_b^c	log (K_xK_y)
CN^-	9.4	0.74	2.34	–7.00
OH^-	15.75	1.45	–0.75	–16.50
SO_3^{2-}	7.0	3.3	1.61	–5.39
Thioglycolate (thiolate)	10.3	>4	0.55	–9.75
Mercaptoethanol (thiolate)	9.50	>4	0.26	–9.24
$MeONH_2$	4.60	1.38		–11.2
NH_2OH	5.97	2.2		–10.76
NH_2NH_2	8.1	3.3		–11.8

[a]T = 25°C, μ = 0.6*M*, except for NH_2OH, for which μ = 1.2*M*. Rate and equilibrium constants refer to Scheme IV. From ref. (*61*).
[b]K_a is for dissociation of conjugate acid.
[c]Based on actual concentration of nucleophile listed in first column. Units of k_1 are /*M*/s–.
[d]Units of K_b are /*M*.



Scheme 5. Nucleophilic addition of Pyruvate to NAD.

adduct formation is proportional to and of the same magnitude as the rate of pyruvate enolization (*70*). Lactate dehydrogenase and Mg^{2+} ion both catalyze enolization and accelerate the addition of pyruvate to NAD (*69,71*).

$$\frac{-d[\text{NAD}]}{dt} = (2.7 \times 10^{-4}/M/\text{s} + 93/M^2/\text{s}\ [\text{OH}^-])[\text{NAD}][\text{Pyr}] \quad (11)$$

pH for Optimal Stability of a Steady State Mixture of NAD(P) and NAD(P)H

The different sensitivities of NAD(P) and NAD(P)H to spontaneous decomposition in aqueous solution make predicting the pH that minimizes cofactor destruction in an enzymatic reactor difficult. Destruction of oxidized cofactor is minimized by acidic conditions, and destruction of reduced cofactor is minimized by alkaline conditions. In a coupled-enzyme reactor, nicotinamide cofactor cycles between its oxidized and reduced forms. Selective destruction of either form would deplete the total concentration of nicotinamide cofactor. An estimate of the rate of destruction of total cofactor requires a knowledge of the rate constant for the destruction of each form of the cofactor and of the steady-state concentration of each of the two forms. The steady-state concentrations of the two forms depend on three parameters: the total concentration of nicotinamide cofactor (oxidized and reduced), the relative Michaelis constants (K_m) for NAD(P) and NAD(P)H of the enzymes catalyzing oxidation and reduction, respectively, and the relative activities present of the two enzymes. If these parameters are known, [NAD(P)] and [NAD(P)H] and thus [NAD(P)]/([NAD(P)] + [NAD(P)H]) at steady state may be calculated. Typically, calculated values of [NAD(P)]/([NAD(P)] + [NAD(P)H]) at steady state range from 0.20 to 0.95 (*72*). Once the relative steady-state concentrations of NAD(P) and NAD(P)H are known, the rate of decomposition of the mixture may be calculated.

The rate of destruction of a mixture of NAD(P) and NAD(P)H is expressed by Eq. (12). If we assume the steady-state reactor concentration of NAD(P) to be 90% of the total cofactor concentration ([NAD(P)]: [NAD(P)H] = 9:1), Eq. (12) simplifies to Eq. (13). Equations (12) and (13) combine to give Eq. (14).

$$\frac{-d([\text{NAD(P)}] + [\text{NAD(P)H}])}{dt} = k_{obs}([\text{NAD(P)}] + [\text{NAD(P)H}]) \quad (12)$$
$$= k_{NAD(P)}[\text{NAD(P)}] + k_{NAD(P)H}[\text{NAD(P)H}]$$

$$\frac{-d([\text{NAD(P)}]+[\text{NAD(P)H}])}{dt} = [9k_{NAD(P)} + k_{NAD(P)H}][\text{NAD(P)}] \quad (13)$$
$$= \frac{9k_{NAD(P)} + k_{NAD(P)H}}{10}([\text{NAD(P)}] + [\text{NAD(P)H}])$$

$$\frac{-d \ln([\text{NAD(P)}] + [\text{NAD(P)H}])}{dt} = k_{obs} = \frac{9k_{NAD(P)} + k_{NAD(P)H}}{10} \quad (14)$$

$$\text{For } \frac{[\text{NAD(P)}]}{[\text{NAD(P)H}]} = 1 \qquad k_{obs} = \frac{k_{NAD(P)} + k_{NAD(P)H}}{2} \quad (15)$$

Similar treatment of Eq. (12) leads to an expression for k_{obs} for a 1:1 steady-state ratio of NAD(P) and NAD(P)H [Eq. (15)].

Figure 2 depicts the calculated pH stability of 9:1 and 1:1 steady-state mixtures of NAD(P) and NAD(P)H. In calculating values of k_{obs}, values for $k_{NAD(P)}$ are taken from Fig. 1. The value of $k_{NAD(P)}$ is taken to be constant between pH 5 and 6.5 (*46,47,57*). Values of $k_{NAD(P)H}$ are calculated according to Eqs. (8)–(*10*), using parameters from Table 2. The effects of enzymes, such as enzyme-catalyzed hydration or possible protection against hydrolysis caused by binding of cofactor by enzymes, are not taken into account.

In unbuffered solution, the pH for minimum destruction of a 9:1 steady-state mixture of NAD and NADH is calculated to be 7.0. In HEPES, this minimum shifts to pH 7.5 Not surprisingly, a 1:1 steady-state mixture of cofactor is generally less stable than the 9:1 mixture, especially at acidic pH. In unbuffered and 0.1*M* HEPES solutions, destruction of cofactor is calculated to be minimal at pH 7.7 and 8.5, respectively. The stability of NADP(H) is less dependent on the buffer than is that of NAD(H). In both unbuffered and HEPES-containing solutions, the pH for minimal destruction of either cofactor mixture is calculated to be approximately 8.5. In all of these systems, the cofactor is stable enough at pH 7.5 to allow syntheses to run for at least 2–3 d. Phosphate buffers would be expected to decrease cofactor lifetimes since both oxidized and reduced forms decompose more rapidly in the presence of phosphate (*46,47,57*).

Enzyme-Catalyzed Degradation of Nicotinamide Cofactors

Enzymes such as phosphodiesterases and phosphatases are occasionally present as impurities in enzyme preparations and would be expected to degrade NAD(P)(H) (*74,75*). Other enzymes, usually expected not to be present (NAD glycohydrolase, NAD kinase, NAD pyrophosphatase), show specific activity in degradation and transformation of the nicotinamide cofactors (*76*). Enzyme-catalyzed transformations of the ni-

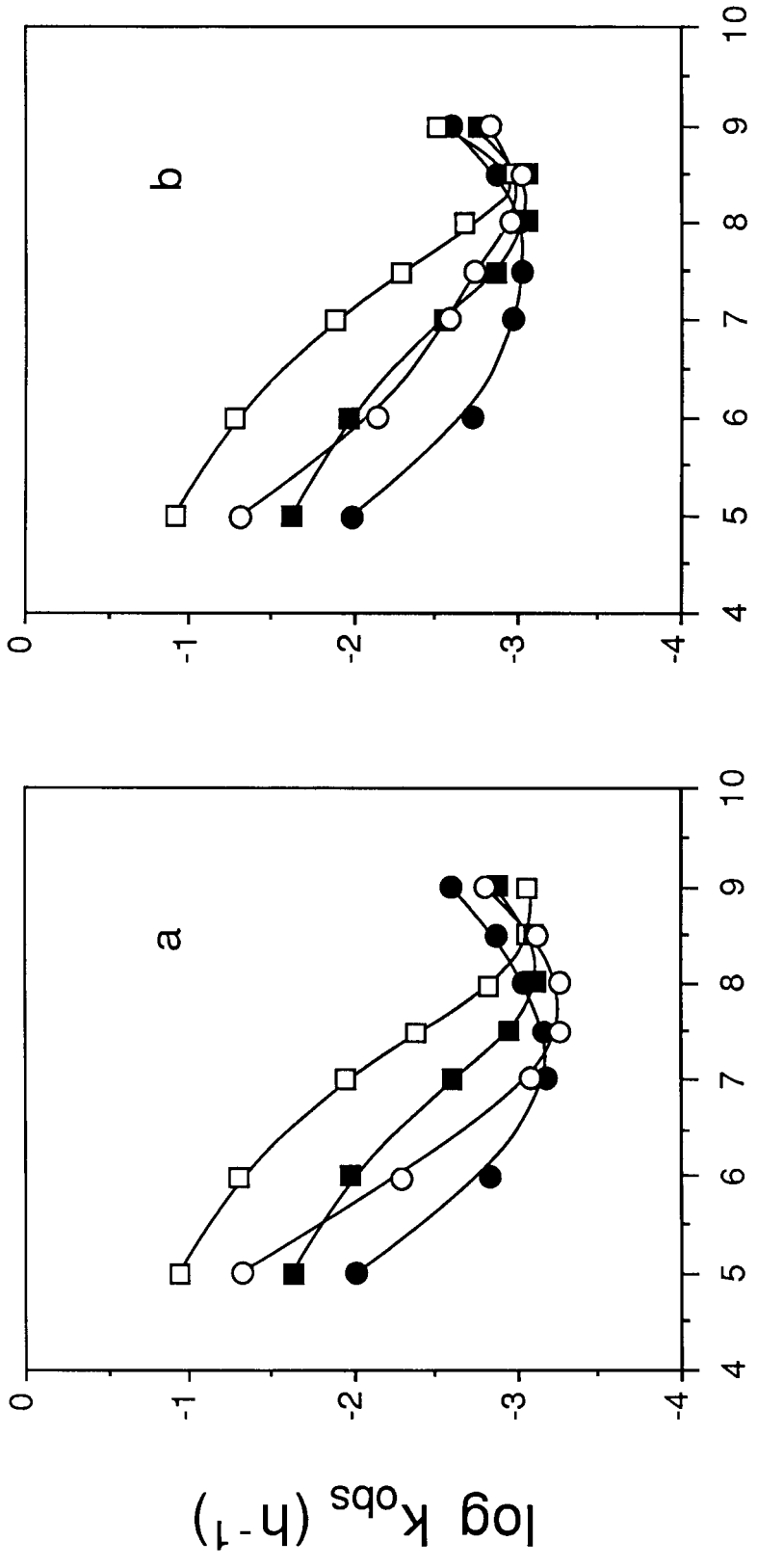


Fig. 2. Effect of pH and buffer on the rate of decomposition of a steady-state mixture of NAD and NADH (● and ○) or NADP and NADPH (■ and □). [NAD(P)]/[NAD(P)H] = 9 (● and ■) or 1 (○ and □). Solution is unbuffered (a) or 0.1 *M* HEPES (b), 25°C. Values of k_{obs} are calculated according to Eqs. (14) and (15).

cotinamide cofactors also seem to be side reactions of several common enzyme-catalyzed processes. For example, glyceraldehyde-3-phosphate dehydrogenase has been shown to catalyze the hydration of NADH to 6HTHNAD (*77*) (Scheme 2). The 6HTHNAD can be isolated; in acid, it readily converts to cTHNAD.

Several dehydrogenases catalyze nucleophilic additions to NAD: Horse liver alcohol dehydrogenase catalyzes the addition of hydroxylamine or cyanide (*78*). Lactate dehydrogenase catalyzes the addition of cyanide (*79,80*), sulfite (*81*), or pyruvate (*63,70,71,82,83*). Malate dehydrogenase catalyzes the addition of sulfite (*81*), and several dehydrogenases facilitate the addition of thiols (*84*).

Reduction Potentials of Nicotinamide Cofactors and Other Biochemical Redox Systems

The generally accepted reduction potential for the reduction of NAD to NADH at pH 7.0 is $E_0' = -0.320$ V (*85*); that of NADP is $E_0' = -0.324$ V (*86*). (The notation E_0' implies all concentrations are $1M$, except for that of H^+, which is $10^{-7}M$). The significance of the small difference between the two values is questionable (*87*). Figure 3a and Eq. (16) summarize the effect of pH on the reduction potential of NAD at 30°C (*88*). The empirical slope, $\Delta E_0'/\Delta pH = -0.0303$ V, agrees well with the theoretical value of -0.0301 V ($-2.302585\ RT/2F$).

$$E_0' = -0.1054 - 0.0303\ pH \qquad (16)$$

Figure 3b shows the effect of temperature on the reduction potential of NAD (*89*). At pH 7.0 and between 20 and 40°C, $\Delta E_0'/\Delta T = -1.31$ mV/°C. Table 4 compares the values of E_0' for a number of biochemicals and redox dyes. Many of these data were determined by equilibration with nicotinamide cofactors.

GENERAL REGENERATION STRATEGIES

We classify strategies for nicotinamide cofactor regeneration into four general categories: enzymatic, electrochemical, chemical and photochemical, and biological. A requirement of any strategy is that it be highly selective for production of the enzymatically active cofactor. This criterion is especially demanding for NAD(P)H regeneration and requires that reduction of the pyridinium ring be highly regioselective.

To be useful, a regeneration scheme must be capable of recycling cofactor 10^2 to $> 10^5$ times (depending on the cofactor species utilized and the application; cofactor prices are listed in Table 1). If 50% of the original cofactor activity is to remain after 100 turnovers, each cycle of regeneration must be 99.3% selective for the formation of active cofactor. At present, only enzymatic catalysis provides such high selectivity for

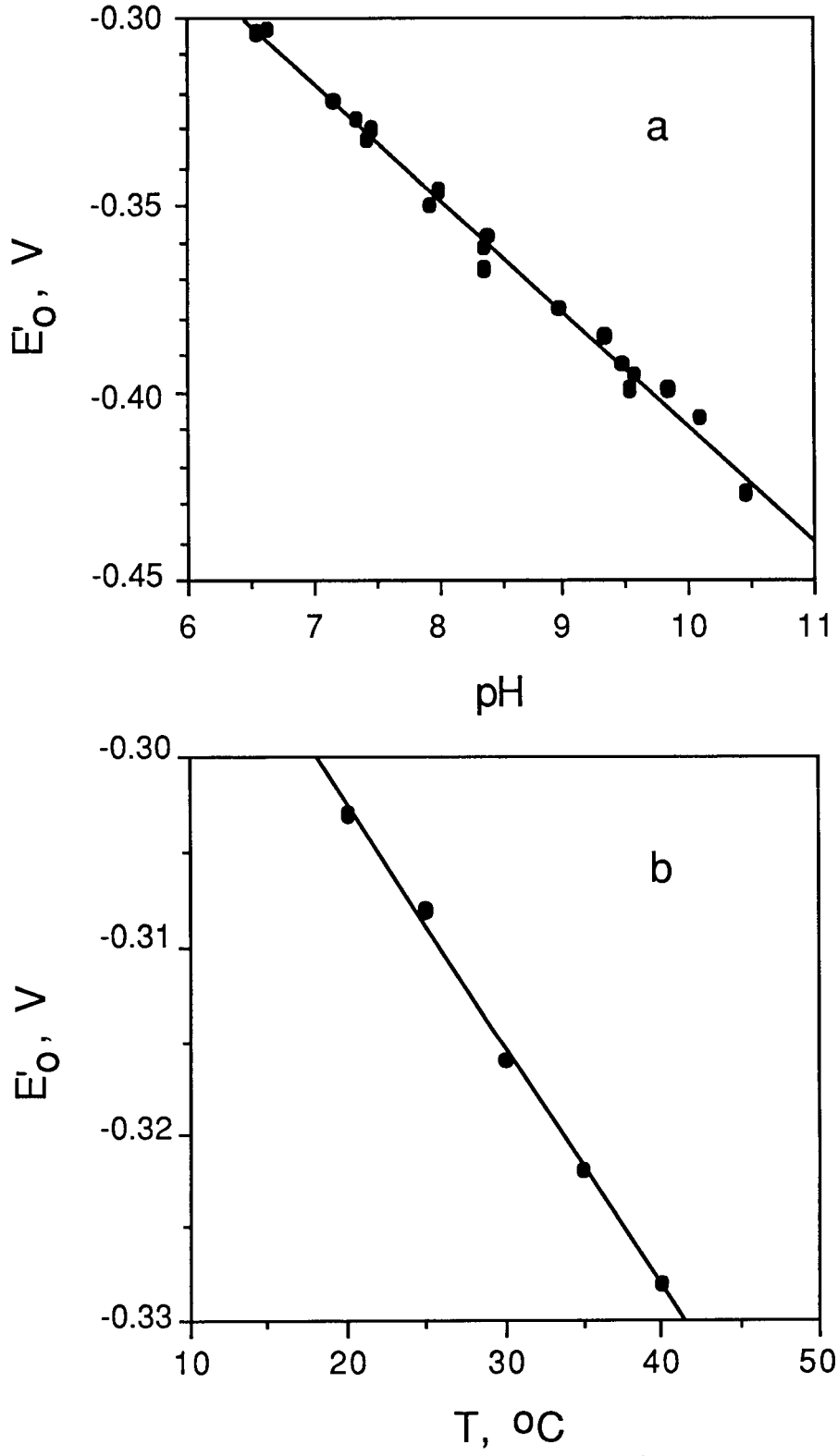


Fig. 3. (a) Dependence of the reduction potential of NAD on pH. Data taken from ref. (*88*). (b) Dependence of the reduction potential of NAD, pH 7.0, on temperature. Data taken from ref. (*89*).

TABLE 4
Biochemical Redox Potentials

Reduction	E_0',[a] V	Ref.
O_2/H_2O_2	0.816	(*90*)
$[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$	0.36	(*91*)
O_2/H_2O_2	0.295	(*90*)
Dichlorophenolindophenol$_{(ox)}$/DCPIP$_{(red)}$, 30°C	0.217	(*92*)
Phenazine methosulfate$_{(ox)}$/PMS$_{(red)}$, 30°C	0.080	(*93*)
Dehydroascorbate/ascorbate	0.058	(*91*)
Phenazine ethosulfate$_{(ox)}$/PES$_{(red)}$, 30°C	0.055	(*93*)
Fumarate/succinate	0.031	(*90*)
Methylene blue $_{(ox)}$/MB$_{(red)}$, 30°C	0.011	(*92*)
Glyoxylate/glycolate	–0.090	(*90*)
Oxaloacetate, NH_4^+/aspartate	–0.097	(*92*)
Pyruvate, NH_4^+/alanine	–0.119	(*92*)
2-Oxoglutarate, NH_4^+/glutamate	–0.121	(*92*)
Hydroxypyruvate/glycerate	–0.158	(*90,94*)
Oxaloacetate/malate	–0.166	(*23*)
Pyruvate/lactate	–0.185	(*23,91,92*)
Dihydroxyacetonephosphate/glycerol-1-phosphate	–0.192	(*85,91,92*)
Acetaldehyde/ethanol	–0.199	(*91*)
FMN/$FMNH_2$, 30°C	–0.211	(*95*)
FAD/$FADH_2$, 30°C	–0.219	(*95*)
3-Hydroxy-2-butanone/2,3-butanediol	–0.244	(*90,96*)
Fructose/sorbitol	–0.270	(*22,91*)
Acetone/2-propanol	–0.286	(*91,92*)
1,3-Diphosphoglycerate/3-phospho-glyceraldehyde,P_i	–0.286	(*90*)
Lipoamide$_{(ox)}$/lipoamide$_{(red)}$	–0.288	(*91*)
Lipoate$_{(ox)}$/lipoate$_{(red)}$	–0.29	(*97*)
NAD/NADH	–0.320	(*85*)
NADP/NADPH	–0.324	(*86*)
Pyruvate, CO_2/malate	–0.330	(*90*)
Glutathione$_{(ox)}$/glutathione$_{(red)}$	–0.340	(*92*)
Cystine/cysteine	–0.340	(*92*)
Acetoacetate/3-hydroxybutyrate	–0.346	(*92*)
2-Oxoglutarate, CO_2/isocitrate	–0.375	(*92*)
CO_2/formate, 30°C	–0.420	(*98,99*)
$2\ H^+/H_2$, 30°C	–0.421	(*92*)
6-Phosphogluconate/glucose-6-phosphate	–0.43	(*98*)
Gluconate/glucose	–0.47	(*90,100*)
Oxalate/glyoxylate	–0.50	(*92*)
$2\ HSO_3^-/S_2O_4^{2-}$	–0.527	(*92*)
3-Phosphoglycerate/3-phosphoglyceraldehyde	–0.55	(*101*)
Methyl viologen$_{(ox)}$/MV$_{(red)}$, 30°C	–0.440	(*92*)
Acetate/acetaldehyde	–0.598	(*90*)
Succinate, CO_2/2-oxoglutarate	–0.673	(*90*)
Acetate, CO_2/pyruvate	–0.699	(*90*)

[a]Standard conditions: Unit activity of all components except H^+, which is $10^{-7}M$; 25°C, unless noted otherwise.

the reduction of NAD(P) to NAD(P)H. Because selectivity is not as difficult to accomplish in regenerating NAD(P) from NAD(P)H, enzymatic catalysis is not required. Still, in this review, we will emphasize enzymatic methods for regeneration of NAD(P) for three reasons: selectivity, compatibility with other components of enzymatic reactors, and ease of monitoring (using enzymatic assays). A comparison of advantages and disadvantages of enzymatic and other strategies appears in Table 5.

Disadvantages of enzymatic methods include the expense and limited stability of enzymes. Also, enzyme immobilization may be a complication. Less obviously important in considering enzymatic methods of regeneration is the ease with which the desired product can be isolated, the specific activity of the enzyme, and the detailed kinetics of the coupled-enzyme reactor. Most enzymatic methods convert a stoichiometric reagent to byproduct, from which the desired product must be isolated. An enzyme with low specific activity will be required in substantial quantity if high rates are to be achieved. Apart from the cost of the enzyme, its use in immobilized form may require inconveniently large quantities of immobilization support. Enzyme kinetics are important in several respects. Enzymes having high values of K_m for reactant or cofactor require high concentrations of these materials. A high K_m for reactant may make it impractical to achieve complete conversion and efficient utilization of the reactant. A high K_m for the nicotinamide cofactor requires a corresponding increase in the initial cost of cofactor added to the reaction, although undestroyed cofactor can sometimes be recovered at the end of a reaction. Product inhibition (especially non- or uncompetitive product inhibition) can also result in slow rates, incomplete conversions, and inefficient reactions.

In the two following sections we describe systems that have been developed to regenerate reduced and oxidized cofactors, respectively. To help in comparing these systems, characteristics of the most important methods are compared in Tables 6 and 7.

REGENERATION OF REDUCED NICOTINAMIDE COFACTORS: NAD(P)→NAD(P)H

Many systems for regenerating reduced nicotinamide cofactors have been tested. The best methods presently available are based on the use of formate, glucose, glucose-6-phosphate, or alcohols as reducing agents. Enzymatic systems give the largest number of successful cycles of regeneration, and our discussion will focus on them. Equations (17)–(26) summarize the reactions used to reduce NAD(P), and Table 6 compares features of the most important of these methods. Table 8 lists properties of the enzymes used, and Table 4 lists the reduction potentials of the reducing reagents.

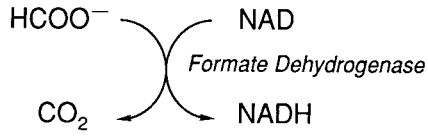
TABLE 5
Advantages and Disadvantages of Strategies for Nicotinamide Cofactor Regeneration

Strategy	Advantages	Disadvantages
Enzymatic	High selectivity [especially for NAD(P) → NAD(P)H]	Enzyme cost and instability
	Compatibility with enzyme-catalyzed synthesis	Immobilization in some cases
	High rates for some systems	Complexity of product isolation in some cases
	Easy monitoring of reaction progress	Low rates for some systems
		Low reactor volume productivities for some systems
Electrochemical	Low cost of electricity	Incompatibility with many biochemical systems
	No stoichiometric regenerating reagent	Poor selectivity (especially for reductive regeneration)
	Readily controlled redox potential	Complex apparatus and procedures
	Easy product isolation	Rapid fouling of electrodes
	Easy monitoring of reaction progress	Requirement in many systems for mediating redox dyes or enzymes

Chemical	Generally inexpensive and commercially available reagents No requirement for added enzymes High redox potentials	Limited compatibility with biochemical systems Complexity of product isolation in some cases Low product yields Low TTN Slow rates for some systems (especially for oxidative regeneration)
Photochemical	No stoichiometric regenerating reagent in some systems No requirement for added enzymes	Complex apparatus Limited compatibility with biochemical systems Limited stabilities Requirement for photosensitizers and redox dyes
Biological	Inexpensive, self-assembling enzyme activity Inexpensive regenerating reagents (O_2, glucose) High selectivity	Relatively primitive state of development Low reactor volume productivity Complicated product isolation Limited or unknown stability Reduced enantiomeric purity of product in some systems Difficulty of controlling relative activities of enzymes Possible incompatibility with some chemical or biochemical components

TABLE 6
Comparison of Methods for Regenerating Reduced Nicotinamide Cofactors

Method	Advantages	Disadvantages
Formate/formate dehydrogenase	High reducing potential Inexpensive reductant Easy product isolation Compatibility with biochemical systems	Initial expense of enzyme Low specific activity of enzyme Inability to reduce NADP directly
Glucose-6-P/glucose-6-P dehydrogenase	High reducing potential Inexpensive enzyme High specific activity of enzyme Stability of enzyme Ability to reduce NAD or NADP Compatibility with biochemical systems	Need to prepare glucose-6-P Possible complication of product isolation from 6-P-gluconate General acid-catalyzed hydration of NAD(P)H by phosphates
Glucose/glucose dehydrogenase	High reducing potential Inexpensive reductant High specific activity of enzyme High stability of enzyme Ability to reduce NAD or NADP	Initial expense of enzyme Possible complication of product isolation by gluconate
Ethanol/alcohol dehydrogenase (yeast)	Inexpensive enzyme Inexpensive reductant High specific activity of enzyme Volatility of ethanol and acetaldehyde	Low reducing potential Product inhibition by acetaldehyde Incompatibility of ethanol and acetaldehyde with some biochemical systems Inability to reduce NADP directly
H_2/hydrogenase	High reducing potential Inexpensive reductant Easy product isolation Compatibility with biochemical systems	Isolation of hydrogenase High sensitivity to O_2 Requirement for auxiliary enzyme and redox dye



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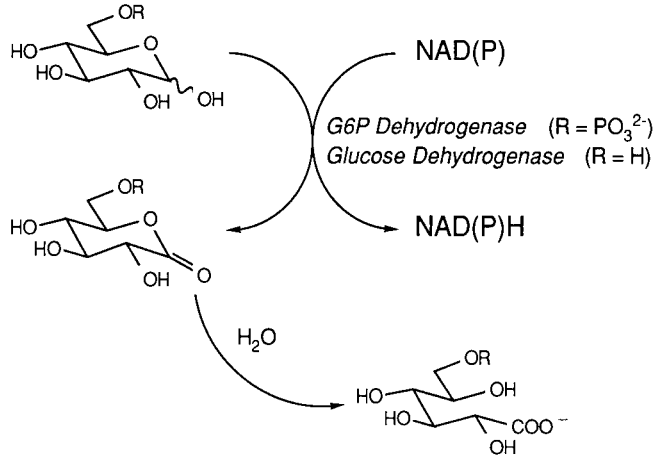

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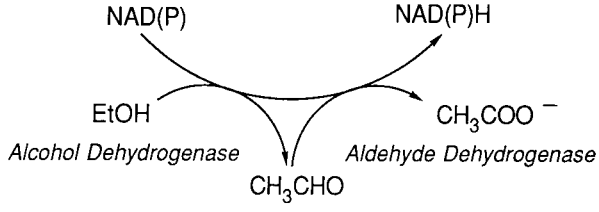

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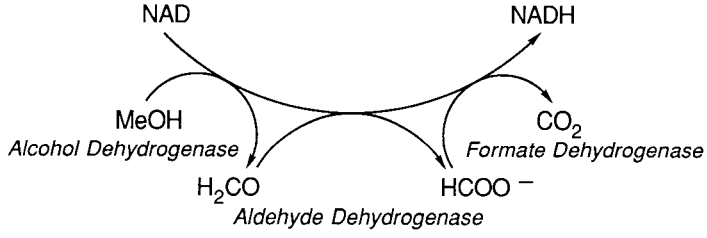

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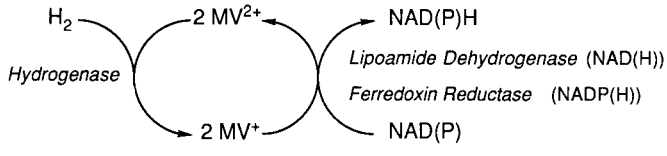

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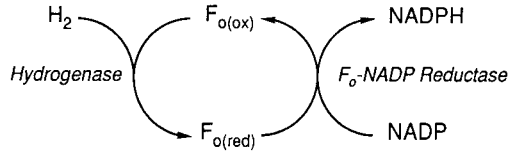

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TABLE 7
Comparison of Methods for Regenerating Oxidized Nicotinamide Cofactors

Method	Advantages	Disadvantages
α-Ketoglutarate/glutamate dehydrogenase	High oxidizing potential Inexpensive enzyme Inexpensive oxidant Ability to oxidize NADH or NADPH Compatibility with biochemical systems	Moderate specific activity of enzyme Possible complication of product isolation by glutamate Reactivity of α-ketoglutarate
Pyruvate/L-lactate dehydrogenase	Inexpensive enzyme Inexpensive oxidant High specific activity of enzyme Compatibility with biochemical systems	Moderate oxidizing potential Inability to oxidize NADPH Possible complication of product isolation by lactate Reactivity of pyruvate
Acetaldehyde/alcohol dehydrogenase (yeast)	Very inexpensive enzyme Very inexpensive oxidant High specific activity of enzyme Volatility of acetaldehyde and ethanol	Moderate oxidizing potential Incompatibility of ethanol and acetaldehyde with some biochemical systems

		Inability to oxidize NADPH directly
O_2/NAD(P)H oxidase or diaphorase	High oxidizing potential Inexpensive oxidant Easy product isolation in some systems	High cost of or need to isolate enzymes Low specific activity of enzymes Incompatibility of O_2 with many biochemical systems Slow rates of unmediated electron-transfer reactions involving O_2 in some systems Requirement for auxiliary redox dyes in some systems
O_2/FMN	Simplicity	Slow rate of reaction between NAD(P)H and FMN Expense of FMN Possible complication of product isolation by FMN Incompatibility of O_2 with many biochemical systems

TABLE 8
Properties of Enzymes Used to Regenerate NAD(P)H

Enzyme	Substrate[a] (K_m, m*M*)	U/mg[b]	K_m^{NAD}, m*M*	K_m^{NADP}, m*M*	$/1000 U[b]	Stability
Formate dehydrogenase, *C. boidinii*	HCO_2^- (13)	3	0.09	—	620	Sensitive to [0]
Glucose-6-P dehydrogenase, *L. mesenteroides*	G6P (0.053)	700, 400	0.106	0.0057	12, 21	Stable
	G6S (50)	2, 10	0.106	0.0057	4200, 840	
Glucose-6-P dehydrogenase, *S. cerevisiae*	G6P (0.051)	400	—	0.0075	26	Stable
	G6S (30)	60	—	0.002	170	
Glucose dehydrogenase, *B. cereus*	glucose (7)	250	0.1	0.1	280	Very stable
Alcohol dehydrogenase, *S. cerevisiae*	EtOH (13)	400	0.074	—	0.04	Sensitive to [0]
	MeOH (80)	10		—	1.6	
Alcohol dehydrogenase, *L. mesenteroides*	EtOH (50)	70, 90	0.5	0.085	11	Sensitive to [0]
	MeOH (70)	1.5, 2			500	

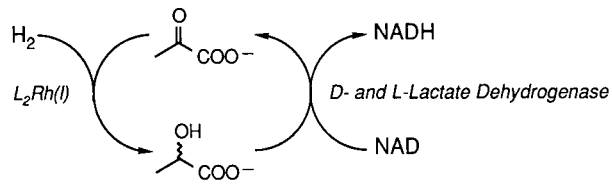
Alcohol dehydrogenase, *S. cerevisiae*	CH_3CHO (0.009)	80, 8	0.03	0.03	200, 2000	Sensitive to [0]
	H_2CO (0.7)	36, 4			440, 4000	
Hydrogenase, *M. thermoautotrophicum*	MV (0.45) F_o (0.034) H_2 (0.02)	6	—	—		Very sensitive to [0]
Lipoamide dehydrogenase, yeast	Lip·H_2 (0.7) MV (0.45)	20	0.14	—	9.5	Sensitive to [0]
Lipoamide dehydrogenase, pig heart	Lip·H_2 (0.3) MV	60 45	0.2	—	26 35	Sensitive to [0]
Ferredoxin reductase	Ferredoxin	1.5	3.8	0.007	8570[c]	Sensitive to ionic strength

Abbreviations: G6P, glucose-6-phosphate; G6S, glucose-6-sulfate; MV, methylviologen; F_o, cofactor F_o (a flavin); Lip·H_2, dihydrolipoamide.

When two values are shown, first is for NAD as cofactor; second is for NADP as cofactor. Prices are those for the enzyme as a research biochemical and should be considered as upper limits for any large-scale applications.

[a]Not commercially available; requires isolation (*106, 107*).

[b]May be isolated from spinach leaves (*108*).



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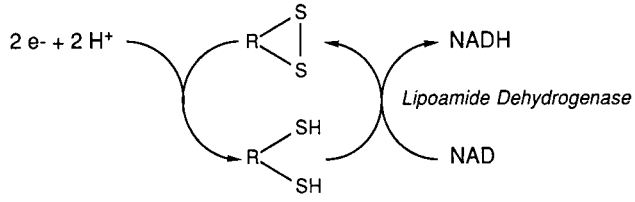

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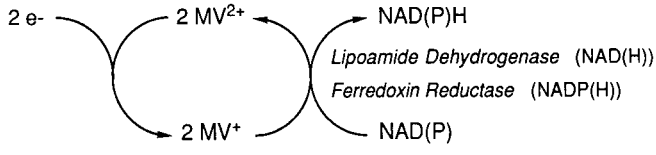

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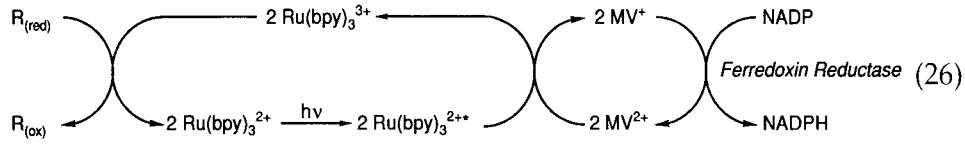

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Formate/Formate Dehydrogenase

The use of formate and formate dehydrogenase (FDH) [Eq. (17)] is presently the best developed procedure for regenerating NADH (*102–104*). This method has the advantage that formate is inexpensive and is a strong reducing agent. The byproduct, CO_2, is easily removed from the reaction and does not complicate the work-up. Formate and CO_2 are stable and are innocuous to enzymes [and to NAD(H) at near neutral pH]. Reaction progress is easily monitored by assaying for formate. The FDH is commercially available, readily immobilized, and stable if protected from autoxidation. The major disadvantages of this system are that FDH is presently expensive if purchased [although an excellent fermentation route to it is available (*105*)] and has low specific activity. The FDH does not reduce NADP, and so regenerating NADPH with FDH requires a transhydrogenase.

The initial expense (or inconvenience of isolation) of FDH is likely to be the greatest contribution to product cost in a one-time synthesis using formate/FDH to regenerate NADH. The FDH accounted for approximately 85% of the product cost in a one-time synthesis of D-lactate (*102*). For repeated processes, though, in which product cost is a greater concern, formate/FDH is one of the least expensive methods for regenerating

NADH. The low cost and ease of product isolation make this system one of the best for large-scale synthesis. The low specific activity of FDH is a drawback, however, necessitating relatively large quantities of protein, large gel volumes, and long reaction times. Formate/FDH has been used successfully to regenerate NADH in a membrane-reactor synthesis of L-leucine (*103*) and in a reduction of dehydrocholic acid by 3α-hydroxysteroid dehydrogenase (*109*). Deuterioformate and FDH have been used to synthesize (*R*)-trifluoroethanol-1-d_1 (*110*) and (*R*)-1,2-butanediol-2-d_1 (*111*).

Glucose-6-phosphate/Glucose-6-phosphate Dehydrogenase

Reductions of NAD(P) with derivatives of glucose provide satisfactory methods for laboratory-scale syntheses. Glucose-6-phosphate (G6P), coupled with glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides,* has been used to reduce NAD(P) in syntheses of chiral α-hydroxy acids and alcohols (*47,112*). The G6P/G6PDH system [Eq. (18), $R = PO_3^{2-}$] has nine advantages. It uses a single enzyme, which is commercially available, inexpensive ($1–2/100 U), and easily manipulated. The G6PDH from *L. mesenteroides* has no active thiol groups and so is not sensitive to autoxidation or alkylation (*113*). The G6PDH from *L. mesenteroides* accepts *either* NAD or NADP at concentrations and at rates favorable for synthesis (yeast G6PDH accepts only NADP). The G6PDH has a high specific activity. The G6P is stable in solution under reaction conditions, and G6P and 6-phosphogluconate (6PG) are innocuous to most enzymes. Thermodynamically, NAD(P) reduction is strongly favored. The product of the enzymatic oxidation of G6P, 6-phosphogluconolactone, hydrolyzes spontaneously and forms 6PG, rendering the reaction essentially irreversible (Table 4). Reaction progress is easily monitored by assaying for G6P.

The G6P/G6PDH system has three disadvantages. The major disadvantage is that G6P must be prepared. The G6P is commercially available, but expensive ($1300/mol). Methods to prepare G6P are well developed (*47,114*): The best uses hexokinase (HK) and an ATP regeneration system based on acetyl phosphate (AcP) (*115*) and acetate kinase (AK). This method readily affords G6P from materials costing $50–115/mol of product formed, depending on whether the immobilized enzymes are reused or not. Another disadvantage is that the presence of 6PG in solution may complicate the isolation of products. Finally, G6P, 6PG, and the inorganic phosphate present as an impurity in G6P all catalyze the hydration of NAD(P)H (Table 2). In syntheses using 0.06–0.17*M* G6P and lasting 4 d, however, phosphate-catalyzed degradation of NAD(P)H proved to be of no practical significance (*47*). Nicotinamide cofactors retained 50–85% of their original activity. At the initial steady-state rates, 15–50% of the cofactor present was in the reduced form.

To avoid the problems of G6P preparation and acid-catalyzed cofactor degradation, glucose-6-sulfate (G6S) has been examined as a reducing

agent for NAD(P) (*116*). The G6S is a substrate for both yeast and *L. mesenteroides* G6PDH. It is not an acid catalyst of NAD(P)H hydration and is easily prepared by the reaction of glucose with pyridine–sulfur trioxide (*117*). Although the cost of the materials used to prepare G6S ($70/mol product formed) is about the same as that of G6P, G6S is much simpler to make. Unfortunately, the specific activity of *L. mesenteroides* G6PDH with G6S is so low as to make the regenerative enzyme prohibitively expensive. Yeast G6PDH, however, utilizes G6S at an acceptable rate, and a system of G6S and G6PDH (yeast) might be a practical system for regeneration of NADPH from NADP.

The G6P/G6PDH system is one of the best methods available for regenerating NADPH. It is less attractive for regenerating NADH, although it may be preferable over formate/FDH for applications in which the stability of enzymes is important. The major expense in systems using G6P/G6PDH to regenerate NAD(P)H is the G6P itself. This expense may be partially recovered in instances in which the byproduct, 6PG, has value (*42,117*).

Glucose/Glucose Dehydrogenase

Wong et al. (*118*) have described the use of glucose and glucose dehydrogenase (GDH) from *Bacillus cereus* to regenerate NADH [Eq. (18), $R = H$]. Glucose/GDH had previously been used in syntheses of ethanol-1-d_1 (*119*) and L-carnitine (*120*). The outstanding feature of GDH from *B. cereus* is its stability. When immobilized on PAN gel (*121*), the enzyme withstands heating at 55°C for 7 d in 0.5*M* NaCl, pH 7.5, with no loss of activity. In a 6-d synthesis of D-lactate, NADH was cycled 36000 times with no loss in GDH activity. The GDH from *B. cereus* is stable in O_2 and accepts either NAD or NADP with high specific activity. Like G6P, glucose is stable and is a strong reducing agent. It does not, however, catalyze the hydration of NAD(P)H. It is very inexpensive and requires no preparation. Glucose not only is innocuous to enzymes, but actually enhances the stability of many enzymes. The initial cost of GDH is a disadvantage of the system, and, like 6PG, gluconate may complicate the reaction work-up. For repeated processes, though, in which gluconate does not complicate isolation of products, the low cost of glucose and the stability and high specific activity of GDH make this method one of the least expensive methods for regenerating NAD(P)H. For laboratory-scale synthesis, this method is also excellent.

Methods Based on the Oxidation of Alcohols

Ethanol and alcohol dehydrogenase (ADH) have been used extensively for NADH regeneration (*122,123*). Their combination is particularly suited to analytical procedures (*124–126*). Ethanol is an effective reductant in coupled-substrate reductions of active aldehydes, such as

lactaldehyde or benzaldehyde (*127,128*). Ethanol/ADH has even been used to reduce cyclic ketones (*127,129*). The ready availability and low cost of both reagent and enzyme and the volatility of both ethanol and acetaldehyde make the system attractive. For general synthetic purposes, however, ethanol and ADH alone prove to be unsatisfactory.

Because ethanol is only weakly reducing, only the most active aldehydes are reduced in good yields. With other substrates, equilibria must be driven by great excesses of ethanol or by removing acetaldehyde. Low concentrations of acetaldehyde inhibit yeast ADH (K_i = 0.67 m*M*) and horse liver ADH (K_i = 0.087 m*M*) (*130*). This inhibition is partially noncompetitive and thus particularly difficult to overcome (*20*). Both ethanol and acetaldehyde deactivate enzymes, and acetaldehyde condenses with NADH to give 1,2-dihydro-2-ethylidenenicotinamide adenine dinucleotide (*131*). If EtOH/ADH is to regenerate NAD(P)H for preparative synthesis, acetaldehyde must be removed during the reaction.

One method for removing acetaldehyde is to sweep the volatile aldehyde from the reaction vessel by a stream of nitrogen and subsequently to trap it as its semicarbazone (*126,132*). A second strategy uses yeast aldehyde dehydrogenase (AldDH) to oxidize acetaldehyde to acetate (*120*), concomitantly generating a second equivalent of reduced cofactor [Eq. (19)]. The EtOH/ADH/AldDH system has been used in 0.1-mol syntheses of L-lactate, L-glutamate, and, using ethanol-1,1-d_2, L-glutamate-α-d_1 (*110,133*). With ADH from yeast, the system reduces NAD; with ADH from *L. mesenteroides*, it reduces NADP. The advantages of this system are its high potential for NAD(P) reduction, its applicability to either NAD or NADP, and the low cost of its reducing agent, ethanol. Both enzymes are commercially available, with moderate-to-high specific activities. Acetate does not harm enzymes or cofactor and rarely complicates product isolation. Reaction progress is monitored by assaying for ethanol. Disadvantages include the requirement for two enzymes and the sensitivity of both enzymes to autooxidation. The specific activity of AldDH with NADP is low. Unfortunately, because acetaldehyde formation should be rate limiting, the more expensive and more sensitive enzyme, AldDH, must be used in excess. A third strategy to remove acetaldehyde might be to trap acetaldehyde *in situ* as its cyanide or bisulfite addition product (*120*) or as a hydrazone or semicarbazone derivative. These methods undoubtedly would face problems of enzyme inactivation, nucleophilic degradation of NAD (*61*), reaction with reactants, such as aldehydes and ketones, or slow rates of reaction with acetaldehyde.

A regeneration scheme similar to EtOH/ADH/AldDH oxidizes methanol to CO_2 with catalysis by ADH, AldDH, and FDH (*133*). Each equivalent of MeOH generates three equivalents of NADH [Eq. (20)]. This system has the advantages of high reducing potential and low cost of MeOH. Carbon dioxide is a particularly convenient final product. Disadvantages of the system include its requirement of three enzymes and its restriction to NAD(H). All three enzymes are sensitive to oxidation.

Specific activities with MeOH, H_2CO, and HCO_2 are low. Formaldehyde may deactivate enzymes more rapidly than acetaldehyde (*134*).

The EtOH/ADH/AldDH and MeOH/ADH/AldDH/FDH systems are less practical for laboratory-scale cofactor regeneration than those based on FDH, GDH, or G6PDH alone. They involve more enzymes and are more complex to assemble and manipulate. They may be useful for large-scale synthesis, however. The EtOH/ADH/AldDH system, especially, provides an inexpensive method for cofactor regeneration. Microorganisms designed to have high levels of the necessary enzymes in the correct proportions—or crude extracts from these cells—might efficiently afford the enzyme activity required for these two- and three-enzyme regeneration schemes.

Methods Based on the Oxidation of Dihydrogen

In principle, dihydrogen is an attractive chemical reductant. It is inexpensive, strongly reducing, and innocuous to enzymes and nicotinamide cofactors. Its consumption leaves no byproducts and is easily monitored to provide a quick and simple measure of the extent of reaction.

Several anaerobic bacteria produce hydrogenase (Hase) enzymes that catalyze the direct reduction of NAD, methyl viologen (MV), or other redox dyes by H_2 [Eq. (27)].

$$H_2 + \text{NAD(or 2 } MV^{2+}) \xrightarrow{\text{Hase}} \text{NADH} + H^+\text{(or 2 } MV^+ + 2\ H^+) \quad (27)$$

Whole cells containing Hase activity or partially purified Hase enzymes have been immobilized and shown to reduce NAD (*135–138*). A major problem with the use of purified Hase is the enzyme's extreme susceptibility to inactivation by dioxygen or other oxidizing species. Hase immobilized in whole-cell form shows increased stability and gives productivity numbers [Eq. (28), refs (*139–141*)] for biochemical reductions one-to-two orders of magnitude greater than those for cofermentations with yeasts.

$$\text{PN} = \text{productivity number} = \frac{\text{(mmol product)}}{\text{(kg dry weight catalyst)(h)}} \quad (28)$$

Hase from *Methanobacterium thermoautotrophicum* has been partially purified, immobilized, and shown to be stable in preparative regenerations of NADH and NADPH (*106*). For NADH regeneration [Eq. (21)], Hase catalyzes the reduction of MV^{2+} by H_2. Lipoamide dehydrogenase (LipDH) or ferredoxin reductase (FR) then catalyzes the reduction of NAD by MV^+. For NADPH regeneration [Eq. (22)], Hase catalyzes the reduction of a soluble flavin analog, F_0, by H_2. The F_0-NADP reductase (F_0NR) then catalyzes the reduction of NADP by reduced F_0. Compared to other Hase enzymes, Hase from *M. thermoautotrophicum* has the ad-

vantages that large quantities can be obtained with high specific activity from a nonpathogenic organism, that it is stable, and that it is not irreversibly inactivated by O_2. A disadvantage of using Hase to activate H_2 is that the enzyme is not commercially available. It requires a fermentation. One fermentation of *M. thermoautotrophicum*, however, yields a mixture of Hase, F_0, and F_0NR (all the components needed for NADPH regeneration). Because F_0NR is NADP(H)-specific, MV and either LipDH or FR must be added to regenerate NADH. The LipDH is unstable under the reaction conditions, and FR either is expensive ($816/100 U) or requires isolation from spinach leaves. Thus, this approach is more useful for NADPH regeneration than for NADH regeneration. Presently, isolated Hase systems are sufficiently complex and expensive as to be impractical. Reduction of cofactors by H_2 is attractive, however, for systems in which reactant costs and ease of product isolation are important. Systems based on permeabilized whole cells warrant further development.

In an effort to reduce NAD by H_2 without using Hase, a water-soluble rhodium complex catalyzed the reduction of pyruvate to racemic lactate by H_2 (*142*). D- and L-lactate dehydrogenase then catalyzed the reduction of NAD by lactate [Eq. (23)]. The limited stability of the rhodium catalyst under the reaction conditions and the requirement for high initial concentrations of NAD and lactate make this system impractical. The strategy of combining a nonenzymatic catalyst for activation of H_2 with an enzymatic catalyst for reduction of NAD(P) remains an attractive one and deserves further exploration.

Electrochemical Methods

Direct cathodic reduction of NAD(P) generates the correct regioisomer of NAD(P)H in only 0–75% yield (*143*). A major problem in this process is the one-electron reduction of NAD, followed by radical coupling at C-4 of the nicotinamide ring to form an enzymatically inactive 4,4′ dimer (*143–146*). Presumably, nonselective reduction to give 1,2- and 1,6-dihydronicotinamide species also occurs. Immobilization of NAD suppresses intermolecular radical coupling and so facilitates reduction to enzymatically active NADH (*147*). A coated electrode (*148*) has been used to reduce NAD to NADH with good selectivity (≥95%).

An attractive alternative to direct electrochemical reduction of NAD(P) is indirect electrochemical reduction, using electron transport agents as mediators (*149,150*). In principle, catalytic quantities of an inexpensive, water-soluble redox dye could be reduced by an electrode in a chemically well defined manner and a potential less cathodic than the overpotential for the reduction of NAD(P) [−1.1 V (*149*)]. The reduced mediator could then reduce NAD(P) in a spontaneous (*150*) or enzyme-catalyzed (*149*) step. Methylviologen has been used as such an electron transfer agent. Its reduction of NAD(P) requires an enzyme catalyst, but many organisms contain MV-dependent NAD(P) reductases (*141,149*).

In particular, *electromicrobial* reductions of NAD(P) have been investigated (*140,141,151–154*). In these systems, whole cells or crude cell extracts are used to catalyze the reduction of NAD(P) by electrochemically produced MV [Eq. (29)].

$$NAD(P) + 2\ MV^{+} + H^{+} \rightarrow NAD(P)H + 2\ MV^{2+} \qquad (29)$$

Unfortunately, these methods have not been demonstrated in preparatively useful regenerations of NAD(P)H. One system using tris(bipyridine)ruthenium as mediator and no enzyme as catalyst has demonstrated TTNs for NAD(H) of 2–3 (*150*). (It should be noted that some enzymes avoid the requirement for NAD(P)(H) as cofactor altogether. Enoate reductase and 2-oxo-acid reductase catalyze the direct reduction of substrates by MV^{+} (*140,141,151–153,155,156*). Reduced MV can be generated by either electrochemical reduction or hydrogenation.)

In order to couple enzymatic selectivities with the use of an electrode as the ultimate source of electrons, two combined electrochemical/enzymatic methods have been tested for NAD(P)H regeneration in 0.1 mol-scale syntheses (*157,158*). One method [Eq. (24)] involves electrochemical reduction of a mixture of oxidized dithiothreitol (DTT) and lipoamide ($LipNH_2$). Reduced $LipNH_2$ then reduces NAD in a reaction catalyzed by LipDH. In the other method [Eq. (25)], LipDH or FR mediates the reduction of NAD(P) by MV^{+}, which arises from cathodic reduction of MV^{2+}. These systems differ from strictly enzymatic systems only in that the stoichiometric reductant (regenerating reagent) has been replaced by a cathode and a catalytic electron transfer agent. On the basis of cost, the two hybrid electrochemical methods are comparable to purely enzymatic systems. Although the electron transfer agents are used in catalytic (0.5–7%) quantities, their expense is significant. The main advantage of the hybrid electrochemical methods is the absence of stoichiometric quantities of byproduct from which the product must be separated. Disadvantages are the reduced stabilities of LipDH and NAD(P)(H), the need to isolate FR, and the complexity of the equipment required.

Chemical and Photochemical Methods

Dithionite has been used as a reductant for *in situ* conversion of NAD to NADH with turnovers for NAD(H) of up to 105 (*38,49,51, 120,159*). Although dithionite is inexpensive and its use requires no regenerative enzyme, it has disadvantages. Total turnover numbers for NAD(H) are not high. Dithionite is unstable in solution (*160*) and can reductively inactivate dehydrogenase enzymes. Furthermore, direct reduction of the substrate by dithionite can be a problem.

A photochemical scheme for NAD(P) reduction uses tris(bipyridine)ruthenium(II) as a photosensitizer (*161,162*). In one system [Eq. (26)], photochemically excited $Ru(bpy)_3^{2+}$ reduces MV^{2+} to MV^{+}; MV^{+} then reduces NADP in a reaction catalyzed by FR. The ruthenium photo-

sensitizer can be regenerated by a variety of reducing agents: EDTA, thiols, or triethanolamine. A similar system uses *meso*-tetramethylpyridinium-porphyrinzinc(II) as photosensitizer for the regeneration of NAD (*163*). These systems have been employed in chiral reductions of ketones and α- and β-keto acids (*161,163*), in preparations of optically active amino acids (*163*), in CO_2 fixation to form malic and isocitric acids (*164*), and in H_2 evolution from alcohols, lactic acid, and alanine (*165*). Light energy is harnessed to drive otherwise endergonic processes. So far, however, TTNs for NAD(P)(H) and conversions have been low (10–40 and 10–40%, respectively), and these systems are complex.

Biological Methods

An alternative to the use of purified enzymes for NAD(P)H regeneration is the use of whole cells or organelles possessing the enzyme activity desired. The principle advantages of this strategy are reduced costs for enzyme activity and, in certain cases, increased enzyme stability. As mentioned previously, whole cells possessing Hase activity have been immobilized and used to reduce NAD (*135,136*). Anaerobically grown *S. cerevisiae* cells reduce extracellular NAD in the presence of ethanol. When immobilized, these cells have been found to reduce NAD at rates of up to 15 μmol/min/g gel (*166*) and to remain active through repeated uses. If immobilized with purified dehydrogenases or aerobically grown yeast cells, they accomplish cyclic regeneration of NADH. Using water as the ultimate electron donor, frozen-thawed blue-green alga (*167*) or immobilized chloroplasts (*168*) reduce NADP. When mixed with bacterial Hase and ferredoxin, chloroplasts regenerate NADPH for the production of H_2 from water (*169,170*). The stabilities of these systems are limited.

Conclusion

Various methods to generate NAD(P)H *in situ* from NAD(P) have been developed and characterized. Merely replacing NAD(P)H by NAD(P) as the starting material in preparative enzymatic reductions lowers the cost of cofactor by 75–90%. Recycling the cofactor reduces its effective cost even further. High total turnovers of cofactor require a high selectivity for the formation of enzymatically active NAD(P)H. At present, only methods in which the reduction of NAD(P) is enzyme-catalyzed provide this high selectivity. Even within this constraint, however, many recycling systems exist that have additional advantages of low cost, stability, or ease of operation.

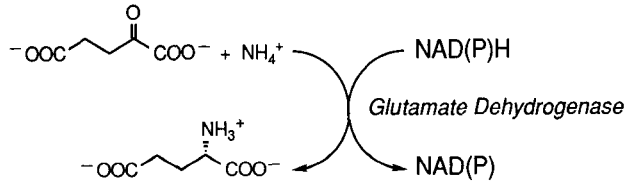
The best methods for laboratory-scale regeneration of NADH are formate/FDH, glucose/GDH, and G6P/G6PDH. Although the applications for NADPH regeneration are more limited than those for NADH regeneration, glucose/GDH and G6P(G6S)/G6PDH are the best systems for this task. The question of best methodology becomes more complex in large-scale synthesis. Formate/FDH is the best developed and charac-

terized method. It has been used in membrane reactors on up to the pilot-plant scale for the continuous production of amino acids (*103, 171,172*). It should be useful when large amounts of enzyme or long reaction times are not problematic. Glucose/GDH is a good method when gluconate byproduct is not a problem. Systems based on EtOH/ADH are a practical alternative for NAD(P)H regeneration, especially when acetaldehyde can be efficiently removed or reacted further.

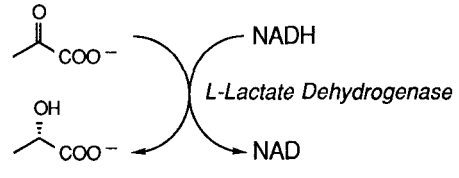
Cofactor regeneration schemes for both NADH and NADPH are successful to the extent that cofactor is no longer the dominant cost of preparative reductions. The cost of enzymes or reagents is equal to or, for systems using NAD(H), much greater than the cost of cofactor. Further advances in cofactor regeneration will come through improved methods of enzyme isolation and stabilization or through the development of chemical or electrochemical methods having selectivities for enzymatically active NAD(P)H greater than 99.3%.

REGENERATION OF OXIDIZED NICOTINAMIDE COFACTORS: NAD(P)H → NAD(P)

Although NAD(P) is more stable in solution than NAD(P)H, synthetic schemes involving *in situ* regeneration of NAD(P) are less well developed and more poorly understood than those involving regeneration of NAD(P)H from NAD(P). The NAD(P)-linked oxidations often face obstacles of unfavorable thermodynamics and product inhibition. Because regioselectivity is not the problem in the oxidation of NAD(P)H that it is in the reduction of NAD(P), chemical and electrochemical strategies are more practicable for regeneration of NAD(P) than for regeneration of NAD(P)H. Still, low rates of regeneration and inactivation of cofactor tend to limit the total turnover numbers achieved by these methods. We favor enzymatic methods of cofactor regeneration over chemical or electrochemical strategies because they are intrinsically more compatible with biochemical systems than are chemical or electrochemical systems. Equations (30–33) summarize the reactions used to oxidize NAD(P)H. Table 7 compares the methods of regenerating NAD(P), and Table 9 lists properties of the enzymes used.



(30)

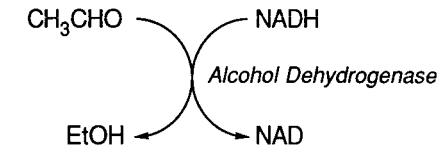


(31)

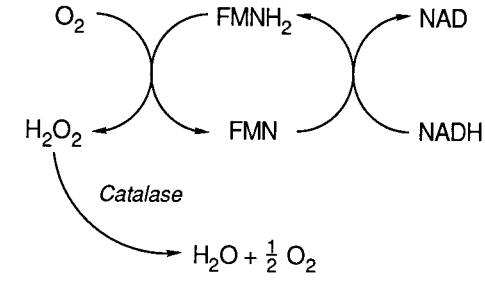
TABLE 9
Properties of Enzymes Used to Regenerate NAD(P)

Enzyme	Substrate (K_m, mM)	U/mg[a]	K_m^{NADH}, mM	K_m^{NADPH}, mM	$/1000 U[a]	Stability
Glutamate dehydrogenase, bovine liver	αKG (0.7) NH_4^+ (3.2)	40	0.024	0.025	3.1	Stable
Lactate dehydrogenase, rabbit muscle	Pyruvate (0.16)	1000	0.01	—	0.6	Stable
	Glyoxylate (31)	1000	0.01	—	0.6	
Alcohol dehydrogenase, yeast	Acetaldehyde (0.78)	300	0.01	—	0.4	Sensitive to [0]

[a]When two values are shown, first is for NAD as cofactor; second is for NADP as cofactor. Prices are those for the enzyme as a research biochemical and should be considered as upper limits for any large-scale application.



(32)



(33)

α-KETOGLUTARATE/GLUTAMATE DEHYDROGENASE

Ammonium α-ketoglutarate (αKG; 2-oxoglutarate) and glutamate dehydrogenase (GluDH) have been used to regenerate NAD(P) [Eq. (30)] in syntheses of up to 0.1 mol (*21,111,117,173–175*). This system allows the oxidation of either NAD or NADP under anaerobic conditions. The reductive amination of αKG is thermodynamically sufficient to drive most biochemical oxidations (Table 4). Both αKG and GluDH are inexpensive, and both αKG and glutamate are stable and innocuous to enzymes. The progress of the reaction may be monitored by assaying for αKG. One disadvantage of this system is the formation of stoichiometric quantities of glutamate, from which the product must be isolated. A second is that the specific activity of GluDH is only moderate. The αKG/GluDH is presently the best method for regenerating NAD(P) in most enzyme-catalyzed reactions requiring NAD(P).

Pyruvate/L-Lactate Dehydrogenase

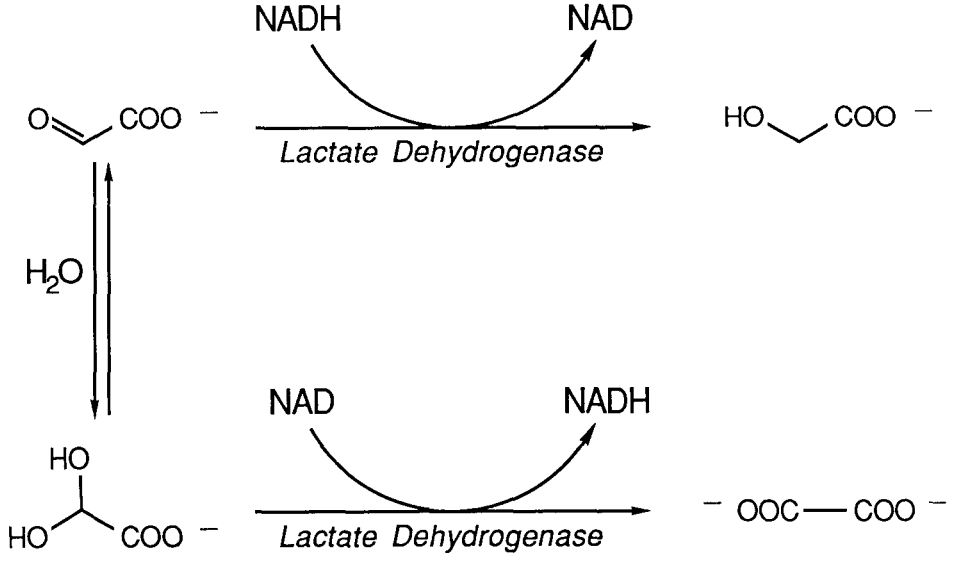
The use of pyruvate and L-lactate dehydrogenes (LDH) to regenerate NAD [Eq. (31)] has the advantage that LDH is less expensive than and has a higher specific activity than GluDH (Table 9). Pyruvic acid is less expensive than α-ketoglutaric acid. The pyruvate/LDH system, as that based on GluDH, does not require O_2, but is itself stable in air. Progress of the reaction can be monitored by assaying for pyruvate. Disadvantages of the pyruvate/LDH system are its inability to oxidize NADPH and a reduction potential less favorable than that of αKG/GluDH (Table 4). Lactate formed as a byproduct may complicate work-up.

There has been some concern about the stability of pyruvate in solution (*21*). Pyruvate may condense with itself and may add nucleophilically to NAD (*62,63*). The addition to NAD is catalyzed by LDH (*63,70,71,82,83*). We and others, however, have used pyruvate/LDH

without problem to regenerate NAD in enzymatic oxidations of 10–100 mmol of material (*133,176*). Soluble NAD(H) is stable under reaction conditions. In our preliminary work, LDH seems to be more stable than GluDH, and turnover rates are better with pyruvate/LDH than with αKG/GluDH.

Another substrate of LDH that may be useful for *in situ* oxidation of NADH is glyoxylate. We have used glyoxylate/LDH to regenerate NAD in small-scale (several mmol) oxidations (*73*). The advantages of substituting glyoxylate for pyruvate are several. Glyoxylate is less expensive than pyruvate (pyruvic acid, $10/mol; glyoxylic acid as a 50% aqueous solution, $2.80/mol). The reduction of glyoxylate is thermodynamically more favorable than even the reductive amination of αKG (Table 4). Because glyoxylate cannot enolize as does pyruvate, some of the reaction pathways important to the degradation of pyruvate are not accessible to glyoxylate.

Glyoxylate/LDH does have a potential disadvantage (Scheme 6). In the LDH-catalyzed reduction of glyoxylate by NADH, glyoxylate acts as an analog of pyruvate (*177–179*). In the presence of NAD, however, LDH recognizes the hydrated form of glyoxylate as an analog of lactate and catalyzes its oxidation to oxalate. Thus, in the presence of NAD(H), LDH is capable of catalyzing the disproportionation of glyoxylate to glycolate and oxalate (*180*). Presently, practical experience is insufficient to judge the extent to which the oxidation of glyoxylate interferes with NAD regeneration. Only small scale reactions over a limited range of conditions have been examined.



Scheme 6. Lactate Dehydrogenase-Catalyzed Disproportionation of Glyoxylate.

Acetaldehyde/Alcohol Dehydrogenase

Acetaldehyde, coupled with alcohol dehydrogenase (ADH), has been used to regenerate NAD from NADH, with TTN = 1000–19000 in preparations of fructose and cyclic ketones (*181,182*). Advantages of using acetaldehyde and ADH include the low cost of acetaldehyde and ADH and the high specific activity of ADH (Table 9). Both acetaldehyde and ethanol are volatile enough not to complicate product isolation. Disadvantages of the system are the low reduction potential of acetaldehyde, the possible deactivation of enzymes by acetaldehyde or ethanol, and the instability of acetaldehyde in solution [acetaldehyde is capable of both self-condensation and reaction with NAD (*63,82*)]. Maintaining acetaldehyde at a low reactor concentration by continuous addition may obviate the problems of enzyme deactivation and acetaldehyde decomposition. Although it has not been reported, acetaldehyde/ADH should be able to regenerate NADP if ADH from *L. mesenteroides* or *Thermoanaerobium brockii* is used (*183,184*).

Other Enzymatic Methods

Although a system of oxaloacetate (OA) and malate dehydrogenase (MDH) has been used to regenerate NAD for analytical purposes (*185,186*), it is unsuitable for preparative applications. Although MDH is inexpensive, OA is expensive and decarboxylates readily in solution. The half-life of OA is 19 h or less (*21*).

In principle, O_2 as the terminal oxidant has advantages of economy and convenience. Dioxygen is inexpensive, and its ultimate reduction product is H_2O. One disadvantage of O_2 is the sensitivity of many enzymes toward dioxygen, superoxide, and peroxide. A second is that unmediated electron-transfer reactions involving dioxygen are often kinetically slow and difficult to monitor. The NADH oxidase (*187*) and old yellow enzyme (*188–190*) catalyze the direct oxidations of NADH and NADPH, respectively, by O_2. Only NADH oxidase has been investigated for use in NAD regeneration (*191–193*).

Diaphorase enzymes from many sources (yeast, pig heart, *Clostridium kluyveri*) catalyze the oxidation of NAD(P)H by electron-transfer dyes, such as methylene blue (MB), dichlorophenolindophenol (DCPIP), and potassium ferricyanide [$K_3Fe(CN)_6$] (*21,194*). Some dyes, such as MB, may be used catalytically and reoxidized by O_2. Others, such as $Fe(CN)_6^{3-}$, cannot be reoxidized *in situ* and must be used stoichiometrically as the terminal oxidant. Both O_2/MB/diaphorase and $Fe(CN)_6^{3-}$/diaphorase have been used to recycle NAD for small-scale synthesis (*21*). These systems offer little advantage over other enzymatic methods or purely chemical methods. Diaphorase is not cheap, and the reoxidation of MB by O_2 is slow. The high ionic strengths of ferricyanide solutions (two equivalents of ferricyanide must be used since it undergoes a one-

electron transfer) reduce the activities of many enzymes. Ferricyanide also may deactivate enzymes by direct oxidation of thiol moieties.

Electrochemical Methods

The direct anodic oxidation of NAD(P)H is far more successful than direct electrochemical reduction of NAD(P). The NADH oxidized at carbon or platinum electrodes yields cofactor that is 90–99.3% enzymatically active (*195–197*). Surface modifications, such as the adsorption of aromatic catechols or the covalent immobilization of LDH onto electrodes, improve the electrochemical activity of NAD(H) (*198,199*). The continuous electrochemical regeneration of NAD has been demonstrated in reactors oxidizing ethanol to acetaldehyde (*196,200,201*).

A major problem limiting the practicality of electrochemical procedures for regeneration of NAD(P) is electrode fouling by adsorbed components of the solution, including cofactor (*202–204*). Dye-mediated electron transfer between NAD(P)H and the anode might minimize the interaction of cofactor with the electrode. At present, we know of no reports of dye-mediated electrochemical regeneration of NAD(P).

Chemical and Photochemical Methods

The method most frequently reported for regeneration of NAD is that of Jones and Taylor (*205–208*), in which flavin mononucleotide (FMN) oxidizes NADH, and the reduced FMN ($FMNH_2$) is reoxidized *in situ* by reaction with O_2 [Eq. (33)]. The advantage of the O_2/FMN system is its simplicity. It requires no added enzymes, and FMN is commercially available. The major disadvantage is that the reaction of FMN with NADH is slow (*21*). The small rate constant for the reaction of FMN with NADH requires that the two species be used in such high concentrations that FMN is typically present at concentrations similar to that of the reactant, and the TTN for NAD seldom exceeds 25. Since the product is often readily separated from FMN by extraction into an organic solvent, high concentrations of FMN might be tolerable, except that FMN itself is expensive ($250/mol). Introducing FMN reductase (EC 1.6.8.1) to catalyze the reduction of FMN by NADH improves the regenerating system (*209–210*). Although the additional enzyme complicates the system, the use of NAD and FMN is more efficient.

Other electron-transfer dyes, such as MB, DCPIP, phenazine methosulfate (PMS), and potassium ferricyanide react directly to oxidize NAD(P)H. Of these, PMS reacts with NADH most rapidly, but all react more rapidly than FMN (*21*). To be useful for cofactor regeneration, however, both the reaction with NAD(P)H and the reoxidation of the reduced dye by O_2 should be rapid. The DCPIP and potassium ferricyanide are not oxidized by O_2. The PMS is reoxidized by O_2, and it is used to regenerate NAD in many analytical procedures (*125,126,211*). It has been used

to recycle NAD in small-scale oxidations of ethanol and androsterone (*212–214*). The PMS is unstable, however. It reacts with O_2 and forms pyocyanine (photocatalyzed) or 2-keto-*N*-methylphenazine and phenazine (dark) (*215*). The problem of instability can be partially overcome by replacing PMS with the more stable phenazine ethosulfate (PES) (*125*).

Photoexcitation increases the rates of NAD(P)H oxidation by several electron-transfer dyes (*192*). Catalytic quantities of MB and PMS, when irradiated by visible light, have regenerated NAD and NADP, respectively, in oxidations of ethanol and 6PG, respectively (*216*). The TTN for NAD reached 1125. Polymerized FMN (*192*) and immobilized acriflavin (*217*) have been used to regenerate NAD photochemically in reactors of specialized configuration.

Biological Methods

Escherichia coli contains in its inner membrane respiratory enzyme(s) that oxidize NADH and NADPH (*218,219*). When immobilized, whole *E. coli* cells regenerate NAD or NADP. *L. mesenteroides* exhibits a high NADH oxidase activity, and the permeabilized bacteria have been immobilized by microencapsulation and used to regenerate NAD for the oxidation of androsterone (*220*). Biological methods for regeneration of NAD(P) from NAD(P)H have not been thoroughly explored, and little is known about the critical issue of the stability of these preparations. They deserve exploration for industrial processes in which process costs are a major concern.

Conclusion

Methods for *in situ* NAD(P) regeneration are fewer and less developed than those for NAD(P)H regeneration. A frequent obstacle is not the cofactor regeneration itself, but noncompetitive or mixed product inhibition associated with the oxidation of substrate (*21,111*). Methods for identifying and minimizing probable effects of product inhibition on enzymatic conversions have been outlined (*21,111*).

The αKG/GluDH is the most satisfactory, most generally applicable, and best characterized method for laboratory-scale NAD regeneration. It is virtually the only method for NADP regeneration. Pyruvate/LDH and glyoxylate/LDH are promising as less expensive and more convenient methods for NAD regeneration in certain applications, but these procedures require further development. Acetaldehyde/ADH also deserves further development for NAD(P) regeneration, since acetaldehyde is inexpensive and ADH has high specific activity. Important questions are: Can continuous reagent addition or enzyme immobilization prevent enzyme inactivation? How can the redox potential of the system be increased? Can ADH from organisms other than yeast be used to regenerate NADP?

For industrial-scale-use, methods of regenerating NAD(P) are not fully developed, and no method is completely satisfactory. Acetaldehyde/ADH has regenerated NAD successfully in a small column reactor (*181*). Electrochemcial regeneration is more practical for oxidizing NAD(P)H than for reducing NAD(P), and methods to improve yields of active cofactor and to prevent the passivation of electrodes by components in solution warrant development. For use with enzymes that are not sensitive to O_2, NAD(P)H oxidase or diaphorase enzymes—or whole cells having such enzymatic activity—deserve further investigation.

REACTOR DESIGN

The reactor configuration most convenient for use in laboratory-scale synthesis is the batch reactor. Typically, reagents and cofactor are present in soluble form, and enzymes are immobilized on insoluble supports (*8,9,121,221,222*). Immobilization of enzymes facilitates their recovery from reactors and, generally, improves their stability under reaction conditions. If the cofactor is recycled 10^3–10^4 times, its effective cost is low enough to allow its use as an expendable reagent. Batch reactors have three disadvantages. First, the physical separation of the reaction solution from immobilized enzymes requires an additional step. Second, since products remain in the reaction vessel, inhibition of enzymes by products or poor substrate conversions caused by unfavorable thermodynamics can be problematic. Third, processing of relatively insoluble compounds, such as steroids, requires high dilutions and, thus, large reaction volumes.

More convenient for large-scale synthesis than for laboratory synthesis are column (*181*), hollow fiber (*128,200,201*), and membrane-based (*103,223*) reactors. These configurations allow continuous processing of an influent substrate solution while product is removed from the effluent stream. Continuous-flow systems are also useful when electrochemical regeneration of nicotinamide cofactors is employed. Enzyme-catalyzed synthesis, mediated or unmediated electrochemical regeneration of cofactor, and product extraction can all occur in separate cells (*224*). In column or hollow-fiber reactors, soluble nicotinamide cofactors may be present in the feed solutions. With appropriately long residence times, high TTNs for the cofactor may be achieved (*128,181*). If cofactors are to be retained within ultrafiltration membranes or on column beds, however, they must be attached to some insoluble or high-molecular-weight, soluble support. A full discussion of cofactor immobilization is not within the scope of this paper, but a brief summary of the work done in this area follows.

In an early immobilization of a nicotinamide cofactor, NAD was covalently bound to diazotized glass beads and found to be enzymatically active (*225*). The formation of N^6-(6-aminohexyl)-NAD and its sub-

sequent coupling to Sepharose 4B was the first synthesis of a defined-structure NAD polymer (*226*). A similar monomer, N^6-[(6-aminohexyl) carbamoylmethyl]-NAD (*227*), and its NADP analog (*228*) have been prepared and used extensively by Mosbach and coworkers. The NAD monomer was attached to soluble dextran and used in a membrane-bound reactor for the continuous production of alanine from pyruvate (*223*). With galactose/galactose dehydrogenase, the cofactor was recycled 90 times.

N^6-[(6-aminohexyl)carbamoylmethyl]-NAD has been co-immobilized with and covalently attached to horse liver ADH to provide complexes requiring no exogenous cofactor (*229,230*). Dextran-NAD has been co-immobilized with LDH and ADH in a collagen membrane to convert ethanol and pyruvate to acetaldehyde and lactate (*231*). Another monomer, succinyl-NAD, can be attached to formyl polyethyleneimine or polylysine and used similarly (*232,233*).

In an impressive demonstration of the efficiency of enzyme reactors, Wichmann et al. (*103*) linked NAD to polyethylene glycol of molecular weight 10^4 (PEG-10000). The PEG-10000-NAD was retained in a membrane reactor with L-leucine dehydrogenase and used to convert α-ketoisocaproate to L-leucine. Formate/FDH regenerated reduced cofactor. L-Leucine was produced continuously for 48 d, with a mean yield of 0.324 mol/L/d. The nicotinamide cofactor was recycled more than 5000 times.

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72. Calculations are based on initial reactor conditions reported in references cited by this paper. Typically, in batch reactors, the total concentration of nicotinamide cofactor is on the order of the sum of the Michaelis constants for NAD(P) and NAD(P)H of the oxidizing and reducing enzymes, respectively. Such concentrations provide an efficient compromise between maximal utilization of enzymatic activity (optimizing V/V_{max}) and maximal utilization of cofactor (optimizing *TTN*). Values for K_{NAD} tend to be on the order of 0.1 m*M*, and values for K_{NADH} tend to be on the order of 0.01 m*M*

(*13,45*). Thus, K_{NAD}/K_{NADH} is approximately 10. For coupled enzymes having $5 \leq K_{NAD}/K_{NADH} \leq 10$ and that differ in cost by no more than two orders of magnitude, the calculated total cost of enzyme per unit rate of product formation reaches a minimum when the oxidizing and reducing enzymes are present with relative activities of from 5:1 to 1:5 (*71*). When the relative enzyme activities are set so as to minimize the cost of enzyme per unit rate of product formation, these systems also give calculated values of [NAD]/([NAD] + [NADH]), which are generally in the range of 0.20–0.95.

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