Nicotinamide Coenzyme Regeneration by Dihydropyridine and Pyridinium Compounds[1a,b]

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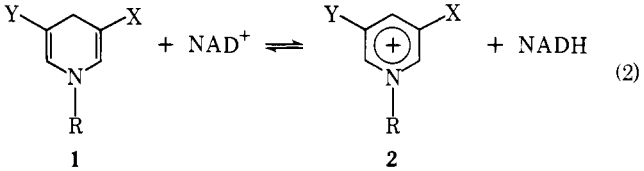
Abstract: The high cost of nicotinamide coenzymes is a major concern to chemists wishing to exploit the chiral catalyst properties of alcohol dehydrogenases in asymmetric synthesis. Of the many attempts which have been made to overcome this problem, the most promising approaches are those which employ catalytic amounts only of coenzyme in conjunction with an auxiliary in situ system capable of continuously regenerating it in its active form. H-transfer reactions between 1,4-dihydropyridines and NAD^+, and pyridinium salts and NADH, have now been shown to provide a new, practical, chemical method of this type for nicotinamide coenzyme regeneration during enzyme-catalyzed carbonyl-reduction and hydroxyl-oxidation reactions. The procedure was evaluated with the synthetically useful enzyme horse liver alcohol dehydrogenase using cyclohexanone and cyclohexanol as representative ketone and alcohol substrates. High yields of products are attainable in both reduction and oxidation directions. Coenzyme recycling of up to 14- and 21-fold, respectively, has been achieved in reductive- and oxidative-mode reactions. Furthermore, use of 4,4-dideuteriodihydropyridines permits the preparation of 1-deuterio alcohols via the recycling process. The effects of variations in reaction conditions, reactant concentrations, and pH are surveyed. This coenzyme regeneration method does not interfere with the enzymic reaction nor do the dihydropyridine and pyridinium recycling agents effect oxidoreduction of the substrates in the absence of enzyme.

Organic chemists are becoming increasingly aware of the potential of enzymes as chiral catalysts and a significant number of examples of their applications in preparative-scale reactions have now been documented for a broad range of substrates of organic chemical interest.[3] The asymmetric synthesis opportunities provided by the alcohol dehydrogenases are particularly attractive; enzymes of this group catalyze selective and stereospecific oxido reductions of the type represented in eq 1. At the present time the high costs of the nic-

$$>C{=}O + NAD(P)H + H^+ \underset{}{\overset{K\sim 10^{9-11}M^{-1}}{\rightleftharpoons}} >CH(OH) + NAD(P)^+ \quad (1)$$

otinamide coenzymes [currently \$>2000 and \$>17 000/mol for NAD^+ [4] and NADH, respectively, and an order of magnitude higher for NAD(P)/H], which are formally required in at least stoichiometric quantities, act as a deterrent to the widespread adoption of alcohol dehydrogenases in organic synthesis. This barrier has been recognized for many years and some progress toward overcoming the problem has been made by using catalytic quantities only of the appropriate coenzyme in conjunction with a system capable of continuously regenerating it in its active form.[3] However, none of the recycling methods reported are generally satisfactory. In this paper we describe a new, viable, and enzyme-compatible chemical method for nicotinamide coenzyme regeneration during preparative-scale alcohol dehydrogenase catalyzed carbonyl-reduction and hydroxyl-oxidation reactions.

Selection of Recycling Agents. The present work was prompted by the reports[5] of C-4 H-exchange between various 1,4-dihydropyridines and pyridinium salts, including NADH and NAD^+. The type of reaction involved is indicated in eq 2.



(2)

1 **2**

We hypothesized that by appropriate variation of substituents X, Y, and R of **1** and **2**, 1,4-dihydropyridine and pyridinium derivatives of higher and lower redox potentials than NAD^+ or NADH could be prepared such that the equilibrium of eq 2 could be displaced in either direction as desired. This approach contrasts those of previous chemical recycling methods[3] in that it permits nicotinamide coenzyme regeneration in either a reductive or an oxidative mode *via the same basic system.*

The groups X, Y, and R which would endow a dihydropyridine ring with greater reducing potential than NADH, or provide a pyridinium salt with greater oxidizing potential than NAD^+, were selected using the cyanide affinity approach of Wallenfels and co-workers[6] to estimate the standard redox potentials of a range of structures **1** and **2**.[7] The structures considered, together with their estimated standard redox potentials, are recorded in Table I. It was felt that the efficiency of coenzyme regeneration by these reagents might parallel the difference in magnitude between their redox potentials and that of the NAD/H couple.[6,15,16] Accordingly, the dihydropyridines and pyridinium salts are arranged in Table I in order of their decreasing and increasing $\Delta E_0'$ values, respectively.

Results

Reductive-Mode Recycling. With the exception of the Hantzsch ester **1b**,[17] the dihydropyridines **1a–e** were obtained by sodium dithionite reduction of the corresponding pyridinium salts. The abilities of **1a–e** to effect continuous in situ regeneration of catalytic quantities of NADH from NAD^+ were evaluated with horse liver alcohol dehydrogenase (HLADH, the most thoroughly documented of the synthetically useful oxidoreductases[3]) and using cyclohexanone as a representative carbonyl substrate of the enzyme. The results obtained are summarized in Table II. The yields of cyclohexanol and the recycling efficiencies attainable were markedly affected by variations in the concentrations of the reaction mixture. Table III illustrates this point. The pH dependence of the reaction under the conditions of Table II was also examined using **1c** as the recycling agent. With the concentration of $NAD^+ > 1$ mM, the pH optimum was 7; the efficiency of reduction was gradually reduced as the pH was raised to 9. In contrast, with 0.2 mM solutions of NAD^+, the recycling efficiency remained virtually constant over the pH range 7–9. The effects of lowering the pH below 7 were more dramatic and no enzyme-catalyzed reduction of cyclohexanone was observed at pH <6.

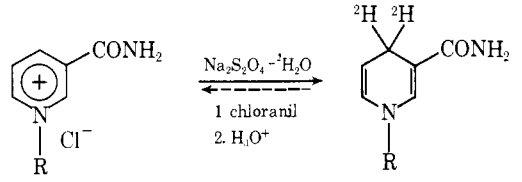
The above results led us to investigate the possibility of extending H-transfer reactions of the eq 2 type to the preparation of 1-deuteriocarbinols by using 4,4-dideuterio-1,4-dihydropyridines to effect $NAD^+ \rightarrow NAD\text{-}^2H$ recycling. The dideuterio compounds **1a**-$^2H_2$ and **1c**-$^2H_2$ were prepared as shown

Table I. Dihydropyridine and Pyridinium Recycling Agents Considered and Their Estimated Standard Redox Potentials

No.	X	Y	R	E_0', mV[a]	$\Delta E_0'$, mV[b]
			Reductive Mode		
1a	$CONH_2$	H	CH_2Ph	−361	−46
1b	$CO_2C_2H_5$	CO_2-C_2H_5	H	(−370)[d]	(−55)[d]
1c	$CONH_2$	H	n-C_3H_7	−387	−72
1d	$CON(CH_3)_2$	H	n-C_3H_7	−434	−119
1e	COOH	H	n-C_3H_7	−442	−127
			Oxidative Mode		
α-**2f**	$CONH_2$	H	α-TAG[c]	(−292)[e]	(23)
β-**2f**	$CONH_2$	H	β-TAG	−267	48
β-**2g**	$COCH_3$	H	β-TAG	−222	93
2h	NO_2	H	DCB[c]	(−195)[f]	(120)[f]
2i	NO_2	H	PNB[c]	(−195)[f]	(120)[f]
2j	NO_2	H	CH_2COPh	[g]	[g]
2k	$COCH_3$	$COCH_3$	DCB	(−120)[h]	(195)[h]
2l	$COCH_3$	$COCH_3$	TAG	(−100)[h]	(215)[h]
2m	NO_2	NO_2	CH_3	(>−100)[h]	(>215)[h]

[a] Unless indicated otherwise, the standard redox potentials E_0', defined at 20 °C and pH 7, were calculated (or estimated if in parentheses) by the cyanide affinity method of Wallenfels and Gellrich[6] using literature[6,9] data. Full details of the method followed are provided in ref 10. [b] With respect to E_0' for the NADH–NAD^+ couple as −315 mV when measured against the normal hydrogen electrode.[11] [c] For abbreviations, see ref 4. [d] No information is available on the cyanide affinity of the oxidized form of **1b**; the E_0' value is estimated from a comparison of its reactivity in model reactions[12] with that of **1a**. [e] By analogy with the E_0' differences between α- and β-NAD^+.[13] [f] These values were estimated using bisulfite[14] and hydroxide ion (see present work) affinities as additional guides. [g] No quantitative information available on the anion affinity of this compound. [h] Estimated assuming that additivity of substituent effects[6b] remains valid for 1,3,5-trisubstituted pyridinium salts.

Scheme I



2a, R = $CH_2C_6H_5$ **1a**-$^2H_2$
c, R = n-Pr **c**-$^2H_2$

in Scheme I. The recycling experiments were carried out under the conditions given in Table II using the N-benzyl compound **1a**-$^2H_2$ containing 1.92 ^2H/molecule. The reactions effected are summarized in Scheme II. A 72% yield of [1-^2H]cyclohexanol (isolated as its α-naphthylurethane) was obtained which contained 0.65 ^2H/molecule.

Table II. Survey of the NADH-Regenerating Efficiency of the Dihydropyridines **1a–e** in the HLADH-Catalyzed Reduction of Cyclohexanone[d]

Recycling agent	[NAD^+], mM	Cyclohexanol, % yield[a]	$NAD^+ \rightarrow$ NADH recycles
1a	0.25	26	6.5
1b	0.2	39[b]	12.1
1c	0.2	35	10.9
1d	0.25	28[c]	6.9
1e	0.2	25	7.8

[a] By GLC analysis. [b] Solution contained 14% dioxane by volume; no recycling occurred in the absence of organic cosolvent. [c] Heterogeneous reaction mixture with a nominal [**1d**] of 40 mM. [d] All reactions were carried out for 24 h at 20 °C using stirred solutions of aqueous 0.05 M Tris–HCl buffer, pH 7.0, 6.2 mM in cyclohexanone, 13 mM in dihydropyridine, and 4×10^{-7} M in HLADH.

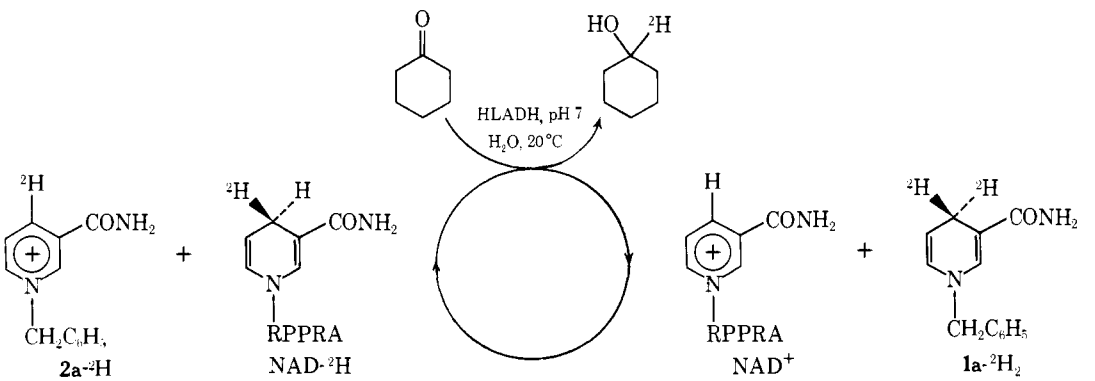
Table III. Effects on Recycling Efficiency of Variations in Coenzyme and Enzyme Concentrations[c]

Recycling agent (mM)	HLADH, $\times 10^7$ M	[NAD^+], mM	Cyclohexanol, % yield[a]	$NAD^+ \rightarrow$ NADH recycles
1a (13)	4.0	0.1	16	9.9
	4.0	0.25	26	6.5
	4.0	0.5	29	3.6
	4.0	1.0	49	3.0
	4.0	0.9	35	2.4
	2.0 + 2.0[b]	0.9	65	4.4
	4.0 + 4.0[b]	0.1	23	14.3
	4.0 + 4.0[b]	0.25	32	7.9
	4.0 + 4.0[b]	0.5	47	5.8
	4.0 + 4.0[b]	1.0	65	4.0
1c (13)	4.0	0.2	35	10.9
	4.0	0.5	59	7.3
	4.0	1.0	95	5.9
	4.0	2.0	100	3.1
1c (6)	4.0	0.5	30	3.9
1c (13)	4.0	1.9	90	2.9
	2.0	1.9	71	2.3
	0.9	1.9	14	0.5
	0.4	1.9	7	0.2

[a] By GLC analysis. [b] Enzyme added in two equal portions, one-half at the beginning of the reaction and the second half after 18 h. [c] The reaction monitored was the HLADH-catalyzed reduction of cyclohexanone and, unless otherwise noted, the reaction conditions and concentrations were as cited for Table II.

Oxidative-Mode Recycling. The pyridinium salts **2f–j** were obtained (as their bromide salts) by alkylation of the corresponding substituted pyridine; **2m** was prepared as its fluorosulfonate salt. Compounds **2k** and **2l** could not be made in this way. Although the electrophilic nature of **2h–j,m** made them

Scheme II



attractive candidates for effecting NADH–NAD^+ oxidations, it was anticipated that this same property would reflect itself negatively in their proclivity to undergo pseudobase formation under the basic assay conditions to be applied. Accordingly, this aspect was examined in some detail. Hydroxide addition to **2h** and **2i** was reversible at pH <9 but the compounds reacted irreversibly at pH >10. The more electrophilic ion **2j** was irreversibly inactivated at pH >8.9 and **2m** rapidly formed *N*-methyl-3,5-dinitro-2-hydroxy-1,2-dihydropyridine even at pH 7. The hydroxide affinities of **2h** and **2i** were determined spectrophotometrically; their pK_{ROH}[18] values were 8.2 and 8.0, respectively.

The abilities of the pyridinium salts **2f–j,m** to regenerate NAD^+ during HLADH-promoted oxidations were surveyed at pH 9 with cyclohexanol as the representative substrate in a manner similar to that employed for the reductive systems. No recycling was observed with **2h–j,m**. The results for **α-2f**, **β-2f**, and **β-2g** are recorded in Table IV. For the **β-2f** experiments, the consequences of doubling the initial enzyme concentration, or of portionwise addition of NAD^+ or the recycling agent, were minor. However, portionwise addition of the enzyme did result in somewhat (<10%) increased yields of cyclohexanone. The pH dependence of recycling by **β-2g** was evaluated under the conditions given in Table IV for the pH range 7–10.7. A pH optimum of 9 was observed within the 0.1–1.0 mM NAD^+ concentration studied.

Table IV. Survey of the NAD^+-Regenerating Efficiency of the Pyridinium Salts **α-2f**, **β-2f**, and **β-2g** in the HLADH-Catalyzed Oxidation of Cyclohexanol[c]

Recycling agent	$[NAD^+]$, mM	Cyclohexanone, % yield[a]	NADH → NAD^+ recycles[b]
α-2f	0.25	11	1.6
	0.9	20	0.3
β-2f	0.2	22	5.6
	0.9	48	2.2
β-2g	0.1	37	21.2
	0.25	52	11.5
	0.5	68	7.2
	1.0	80	3.8

[a] By GLC analysis. [b] The first "cycle" of coenzyme has not been included since the NAD^+ added initially can function directly in the oxidation. [c] Reactions were carried out for 24 h at 20 °C using stirred solutions of aqueous 0.05 M Tris–HCl buffer, pH 9.0, 6.0 mM in cyclohexanol, 12 mM in recycling agent, and 4×10^{-7} M in HLADH.

Discussion

Reductive-Mode Recycling. In contrast to the behavior of **1a,c,d**, and to the situation reported for *N*-2,6-dichlorobenzyl-1,4-dihydronicotinic acid (**1**, X = COOH; Y = H; R = DCB),[19] isolation of *N*-propyl-1,4-dihydronicotinic acid (**1e**) following the dithionite reduction step was not possible owing to its sensitivity to acid-catalyzed hydration.[20] Accordingly the efficiency of **1e** as a recycling agent was determined on solutions prepared in situ prior to the enzymic reaction and using a twofold excess of the precursor pyridinium salt in order to exclude the possibility that any recycling observed might be due to unreacted dithionite itself.[21]

In order to ensure that the up to 12-fold recycling efficiencies of Table II reflected the behavior of the systems under truly preparative-scale conditions, reaction solutions containing up to 1 g of cyclohexanone were employed. In each experiment, the coenzyme was supplied as its oxidized NAD^+ form, and thus no substrate reduction was possible in the absence of a recycling agent. Control experiments were carried out to ensure that (a) no reduction of cyclohexanone to cyclohexanol occurred when any one of NAD^+, the enzyme, or the dihydropyridine was not added to the reaction mixture; (b) none of the dihydropyridines **1a–e** used, nor their corresponding pyridinium salts, had any inhibitory effects on the enzyme; and (c) product inhibition by high levels of cyclohexanol was not occurring. Of the five dihydropyridines tested initially, **1a** and **1c** were chosen for more detailed evaluation since they were more soluble in aqueous solution than **1b** and **1d** and were also more stable than **1d** and **1e**. The additional data obtained (Table III) showed that increasing the NAD^+ concentration resulted in increased yields of the cyclohexanol product but with correspondingly reduced efficiencies of coenzyme regeneration. However, for those experiments where reduction proceeded almost to completion (95–100% yields of cyclohexanol), the low recycling factors should not be taken at their face value since they obviously do not necessarily represent the maximum levels of coenzyme regeneration achievable under those conditions.

For the reaction conditions applied in Tables II and III, the enzyme activity showed a first-order decrease with a half-life of ~7 h. The adverse effect of progressive loss of HLAD activity is illustrated by the data in Table II for **1c** recycling of 1.9 mM NAD^+ at various HLADH concentrations. The effect is most noticeable at high coenzyme concentrations and, when the NAD^+ concentration is lowered to <0.25 mM, the reaction system becomes relatively insensitive to the concentration of HLADH. This observation is in accord with the report[22] that thermal denaturation of HLADH is accelerated by high levels of NAD^+. The deleterious effects of long reaction times on HLADH activity can be offset by portionwise addition of the enzyme. The increased yields of cyclohexanol when a second portion of HLADH is added after 18 h (Table II, **1a** recycling) support this conclusion. Portionwise addition of NAD^+ or of the recycling agent is not of significant benefit despite the fact that the effects of reducing the concentration of the dihydropyridine are unfavorable. For example, lowering the **1c** concentration by ca. twofold (from 13 to 6 mM) adversely affects both the yield of cyclohexanol and the recycling efficiency by a similar factor. The practical upper limit of solubility of **1a** and **1c** in aqueous solutions is 13 mM and thus the potential benefits of operating with higher dihydropyridine concentrations could not be explored. Increasing the solubility of the dihydropyridines by the addition of organic cosolvents, as with **1b** (Table II), was not generally beneficial. For example, no improvement in yields was observed for reactions carried out with **1a** in the presence of 6% dioxane or acetonitrile and equivalent levels of dimethyl sulfoxide and tetrahydrofuran inhibited the extent of reduction by two–threefold.

The absence of recycling by **1c** at pH <6 is attributed to the rapid acid-catalyzed hydration of dihydropyridines at low pH's.[20] The pH optimum of 7 observed with NAD^+ >1.0 mM reflects the pH dependence of HLADH-mediated reductions in the presence of saturating NADH levels.[23]

Attempted preparations of the 4,4-dideuterio derivatives of **1a** and **1c** via cyanide-mediated C-4-H exchange[24,25] of **2a** and **2c**, respectively, were unsuccessful. However, the C-4-dideuterio-*N*-benzyl derivative **1a**-$^2H_2$, containing 1.92 ^2H/molecule, was obtained in 43% yield when **1a** was subjected to 3.5 (Scheme I) oxidoreduction cycles.[26] The method was less successful for **1c**-$^2H_2$ with 3.5 oxidoreduction cycles producing a 45% yield of material containing only 1.65 ^2H/molecule. Subjection of the latter material to further redox cycles did not augment the deuterium content to any significant degree. These results are in accord with previous reports on analogous *N*-alkyl derivatives.[27]

The feasibility of applying the reductive recycling approach to the preparation of deuterio alcohols was evaluated using **1a**-$^2H_2$ as the coenzyme regenerating agent, again with cyclohexanone as a representative substrate (Scheme II). Al-

though the reduction process was much slower than for the corresponding protio reaction due to kinetic isotope effects, a good (72%) yield of 1-deuteriocyclohexanol was obtained. Taking into account the deuterium contents of the recycling agent **1a**-$^2H_2$ (1.92 ^2H/molecule) and the product alcohol (0.65 ^2H/molecule), the isotope effect of 4.8[10] for H transfer from **1a** to NAD^+, and that HLADH utilizes C-4-H_R of NADH stereospecifically in the reduction of its carbonyl substrates,[3] it is calculated that the nonenzymic **1a**-$^2H_2 \rightarrow NAD^+$ transfer is 78% specific for the *re* face of NAD^+. This degree of enantiotopic specificity is similar to that observed with NAD^+ dithionite, cyanide, and lactate reactions[28] but contrasts those of NADH-$NAD\cdot$,[5d] -acetyl NAD^+,[5c] and -riboflavin[29] reductions where no stereospecificity of H transfer is observed. The current result is extremely promising in this regard since, by choosing an appropriate, perhaps chiral, [4,4-$^2H_2$]dihydropyridine, it should be possible to achieve completely stereospecific ^2H transfer to the *re* face of NAD^+ thereby providing a chemical alternative to the coupled-enzyme and coupled-substrate recycling methods currently used in enzyme-catalyzed preparations of chiral [1-^2H] alcohols.[3]

Oxidative-Mode Recycling. The preparation of the pyridinium salt recycling agents listed in Table I was approached via alkylation of the appropriate pyridine precursors. However, attempts to prepare **2k** and **2l** by alkylation of 3,5-diacetylpyridine with DCB bromide and 1-Br-TAG, and **2m** by treatment of 3,5-dinitropyridine with methyl iodide, were unsuccessful. This was not altogether unexpected since the failure of relatively mild alkylating agents to react with electron-deficient pyridines has been documented.[30] The *N*-methyl-3,5-dinitropyridinium salt **2m** was subsequently obtained using the more powerful alkylating agent methyl fluorosulfonate.

Delineation of the susceptibility of the strongly electrophilic pyridinium derivatives **2h–j,m** to undergo pseudobase formation[31] was an important practical consideration since alcohol dehydrogenase catalyzed oxidations of alcohols are usually carried out at pH 9 in order to offset to some degree the unfavorable equilibrium of eq 1.[3] The uv maxima of ~390 nm for solutions of **2h** and **2i** under pH <9 conditions are in accord with those expected[31] for 2-hydroxy-3-nitropyridines although the possibility that other isomers[32,33] or unstable ions[34] are formed cannot be ruled out. The nature of the irreversible reactions of **2h–j** at pH >9 was not investigated. The situation is straightforward in the case of **2m,** the most electron-deficient pyridinium salt of the series, with the 2-hydroxy adduct forming rapidly at neutral pH. The pK_{ROH}[18] values of 8.2 and 8.0 for **2h** and **2i**, respectively, indicate that they would exist predominantly as the pseudobases under pH 9 reaction conditions.

The oxidative-mode recycling experiments of Table IV were monitored on solutions containing up to 1 g of cyclohexanol. Control experiments similar to those outlined for reductive recycling established that none of the recycling agents were themselves capable of oxidizing cyclohexanol, that they were not inhibitors, and that concentrations of up to 6 mM of the cyclohexanone product did not affect the oxidation. In view of the facilities with which **2h–j,m** undergo pseudobase formation, their ineffectiveness as recycling agents was not unexpected. However, the less electrophilic salts **2f,g** were capable of regenerating NAD^+ from NADH, with β-**2g** being clearly superior to the other two.

As in the case of the reductive recycling systems, the product yields and recycling efficiencies were affected by variations in the concentrations of the components of the reaction. The trends evident in Table IV regarding the influence of NAD^+ concentration on yield of product and degree of recycling parallel those of Table III. Both oxidatively and reductively, the efficiency of coenzyme regeneration is inversely related to the concentration of NAD^+. This phenomenon has also been observed in other systems.[21,35] For the oxidative recycling experiments performed, the effects of portionwise addition of the reagents were not as marked as those observed under reductive-mode conditions. The yield of cyclohexanone was negligible at pH 7 and reached a maximum at pH 9. This result parallels the pH dependence of HLADH-catalyzed oxidations of alcohols.[3]

The dependence (Tables II–IV and related discussions) of yields and recycling efficiencies on factors such as (a) the structure and substitution of the recycling agents, (b) the concentration of recycling agent, (c) the NAD^+ concentration, and (d) the pH independence of the reductive-mode reaction at low (<0.2 mM) levels of NAD^+ point to H transfer between dihydropyridine or pyridinium recycling agents and NAD^+/H being wholly or partially rate-limiting under many of the conditions applied. This conclusion is also supported by the large deuterium isotope effect observed in the Scheme II reaction. Furthermore, with cyclohexanone, benzaldehyde, and cinnamaldehyde as substrates, virtually identical yields of the corresponding alcohols were obtained[10] under the conditions given in Table II with **1a–c** as recycling agents despite the fact that HLADH-catalyzed reduction of these substrates occurs at markedly different relative rates (1:11:70, respectively) in the presence of saturating NADH.[3] However, the enzymic catalysis stage[36] is not faster than the nonenzymic steps under all the reaction conditions applied. For example, it appears to be at least partly rate determining at high (>1 mM) NAD^+ (Table III) and at high pH's. Superimposed on the rate effects of variations in enzyme concentration are the consequences of the enzyme's instability. This factor becomes particularly important when reaction periods in excess of 1 day are employed. Attempts to increase the rates of the nonenzymic H-transfer processes by carrying out the Table II recycling reactions at 38 °C were offset by the decreased stability of HLADH at the elevated temperature.

The prospect[6,15,16] that $\Delta E_0'$ values (Table I) might serve as useful guides to the recycling efficiencies of chemical redox reagents receives qualified support from the current data. Further data on this aspect, and on the rates of H transfer between the recycling agents discussed above, will be reported shortly.

The results obtained confirm that the use of dihydropyridine and pyridinium compounds for effecting in situ regeneration of NADH (or NAD^2H) and NAD^+, respectively, is preparatively viable. The up to 21-fold recycling efficiencies attained for the systems evaluated are already superior to those of many of the alternative recycling methods which have been employed in preparative-scale enzyme-catalyzed oxidoreductions of alcohols and ketones[3] and further improvements are anticipated as the approach is refined.

Experimental Section[37]

Preparation of Recycling Agents. The following compounds were prepared according to the literature procedures: *N*-benzyl-1,4-dihydronicotinamide (**1a**),[38] 75% yield, mp 121–123 °C (lit.[39] mp 118–120 °C); 3,5-dicarboethoxy-2,6-dimethyl-1,4-dihydropyridine (Hantzsch ester, **1b**), 37% yield, mp 186–187 °C (lit.[17] mp 183–184 °C); *N*-propyl-1,4-dihydronicotinamide (**1c**), 70% yield, mp 91–92 °C (from THF) (lit.[5d] mp 91–93 °C); *N*-(2,3,4,6-tetraacetyl-D-glycopyranosidyl)-3-carbamoylpyridinium bromide (**2f**), β-anomer, recrystallized to constant rotation from MeOH–EtOAc (1:1), 13% yield, mp 208–212 °C dec, $[\alpha]^{23}D$ −18.3° (*c* 2.7, H_2O) [lit.[40] mp 192–195 °C, $[\alpha]D$ −18.3° (*c* 2.5, H_2O)]; α-anomer, recrystallized to constant rotation from $CHCl_3$–MeOH–EtOAc (10:1:10), 10% yield, mp 205–210 °C dec, $[\alpha]^{23}D$ 33.3° (*c* 2.4, H_2O) [lit.[40] $[\alpha]D$ 20.9° (*c* 2.5, H_2O)].

***N*-Propyl-3-(*N,N*-dimethylcarbamoyl)-1,4-dihydropyridine (1d).** *N,N*-Dimethylnicotinamide[41] (11.5 g, 7.5 mmol) and *n*-propyl iodide

(27 ml, 28 mmol) were heated for 12 h under reflux. The solid obtained (80% yield) was recrystallized (five times) from EtOH to give *N*-propyl-3-(*N*,*N*-dimethylcarbamoyl)pyridinium iodide (**2d**): mp 112–113 °C; ir (KBr) 1642 cm^{-1}; NMR (^2H_2O) δ 1.00 (t, J = 7 Hz, 3 H), 2.12 (m, J = 7 Hz, 2 H), 3.12, 3.21 (2 s, 6 H), 4.70 (t, J = 7 Hz, 2 H), 8.30 (d of d, J = 6, 8 Hz, 1 H), 8.76 (d, J = 8 Hz), 9.10 (d, J = 6 Hz, 1 H), and 9.20 ppm (br s, 1 H). (This spectrum was compared with that of a sample of the previously reported *N*-ethyl analogue[42] before proceeding to the next step.)

A solution of Na_2CO_3 (14.1 g, 133 mmol) and sodium dithionite (11.6 g, 66.6 mmol) in H_2O (100 ml) was added dropwise with stirring at 0 °C under N_2 during 20 min to the *N*-propylpyridinium iodide **2d** (10 g, 33.3 mmol) in H_2O (25 ml). The yellow reaction solution was then allowed to warm to 20 °C and was stirred for a further 1 h. Extraction with $CHCl_3$ followed by evaporation of the dried ($MgSO_4$) solution gave the title compound **1d** as an unstable amber oil (2.4 g, 37% yield, >90% pure by GLC) which was used immediately after preparation: ir ($CHCl_3$) 1678, 1625, and 1586 cm^{-1}; uv (MeOH) 347 nm; NMR (C^2HCl_3) δ 1.00 (t, J = 7 Hz, 3 H), 1.57 (m, J = 7 Hz, 2 H), 3.05 (strong s + m, 10 H), 4.60 (m, 1 H), 5.77 (d of d, J = 1.5, 8 Hz, 1 H), and 6.24 ppm (s, 1 H).

Sodium *N*-Propyl-1,4-dihydropyridine-3-carboxylate (1e). Nicotinic acid (6.17 g, 50 mmol), *n*-propyl iodide (20 ml, 201 mmol), and *n*-PrOH (75 ml) were refluxed for 2 days. The cooled solution was then treated with 10 N aqueous NaOH (5 ml, 50 mmol) and Et_2O (100 ml) added. The slowly crystallizing oil was collected and recrystallized (twice) from MeOH to give the Na salt of *N*-propylpyridinium-3-carboxylic acid (**2e**, 2.7 g, 33% yield): mp 233–235 °C; ir (KBr) 3445, 1670, 1658, 1625, and 1586 cm^{-1}; NMR (^2H_2O) δ 1.08 (t, J = 7 Hz, 3 H), 2.18 (m, J = 7 Hz, 2 H), 4.77 (t, J = 7 Hz, 2 H), 8.30 (m, 1 H), 9.05 (m, 2 H), and 9.32 ppm (s, 1 H).

A solution of Na_2CO_3 (1.22 g, 12 mmol) and sodium dithionite (520 mg, 3 mmol) in H_2O (1 ml) was added with stirring at 20 °C under N_2 to the pyridinium salt **2e** (995 mg, 6 mmol). The resulting yellow solution was stirred for a further 1 h at 20 °C and was then acidified to pH 7.5 with 6 N HCl. This solution of the Na salt of the 3-carboxydihydropyridine **1e** was then used immediately in the reductive recycling experiments.

An attempt to prepare **1e** via an alternative route involving hydrolysis of *N*-propyl-3-carboxymethylpyridinium iodide with 1 equiv of NaOH did not yield the desired product.[10]

***N*-Benzyl-4,4-dideuterio-1,4-dihydronicotinamide (1a-$^2H_2$).** *N*-Benzyl-3-carbamoylpyridinium chloride (**2a**), mp 240–242 °C dec (lit.[13a] mp 234–235 °C), was obtained in quantitative yield by the published[38] procedure. Conversion of **2a** (4.6 g, 18.5 mmol) into **1a**-$^2H_2$ was accomplished by the general method of Caughey and Schellenberg[26] except that the chloranil oxidations were carried out at −50 °C. Application of 3.5 redox cycles (Scheme I) without intermediate recrystallizations yielded a product which was recrystallized from EtOH–H_2O (1:1) to give **1a**-$^2H_2$ (2 g, 43% yield, 1.92 ^2H: mp 118–120 °C; ir ($CHCl_3$) 1682, 1643, and 1600 cm^{-1}; NMR (C^2HCl_3) δ 3.10 [m, 0.075 H (=1.92 ^2H at C-4)], 4.25 (s, 2 H), 4.69 (d, J = 8 Hz, 1 H), 5.70 (d of d, J = 1.5, 8 Hz, 1 H), 7.12 (d, J = 1.5 Hz, 1 H), and 7.30 ppm (s, 5 H).

***N*-Propyl-4,4-dideuterio-1,4-dihydronicotinamide (1c-$^2H_2$).** *N*-Propyl-3-carbamoylpyridinium chloride (**2c**), mp 194–195 °C (lit.[5d] mp 193–194 °C), was prepared in 90% yield by the literature method. It was then converted into **1c**-$^2H_2$ as described above for **1a**-$^2H_2$. After recrystallization from THF, the sample of **1c**-$^2H_2$ (35% yield, 1.64 ^2H) had mp 92–93 °C; ir ($CHCl_3$) 1675, 1643, and 1591 cm^{-1}; NMR (C^2HCl_3) δ 0.97 (t, J = 6 Hz, 3 H), 1.60 (m, J = 6 Hz, 2 H), 3.22 [m and t, J = 6 Hz, 2.36 H (=1.64 D at C-4)], 4.77 (d of t, J = 1, 8 Hz, 1 H), 5.82 (d, J = 8 Hz, 1 H), and 7.14 ppm (s, 1 H).

***N*-(2,3,4,6-Tetraacetyl-β-D-glucopyranosidyl)-3-acetylpyridinium Bromide (β-2g).** A mixture of the α- and β-anomers of **2g** was obtained by alkylation of 3-acetylpyridine (3 g, 25 mmol) with 1-bromo-2,3,4,6-tetraacetylglucose (9 g, 40 mmol) by the general method of Haynes and Todd.[40] Recrystallization (three times) from CH_3COCH_3–MeOH–Et_2O (20:1:15) gave β-**2g** (1.8 g, 15% yield): mp 192–193 °C dec (lit.[9] mp 158–159 °C); $[\alpha]^{23}D$ −17.3° (*c* 2.5, H_2O); ir ($CHCl_3$) 1753 and 1723 cm^{-1}; uv (H_2O) 268 nm (ϵ 4200); NMR (^2H_2O) δ 2.0–2.2 (3 s, 12 H), 2.84 (s, 3 H), 4.47 (s, 2 H), 5.66 (m, 4 H), 6.46 (d, J = 8 Hz, 1 H), 8.46 (d of d, J = 6, 8 Hz, 1 H), 9.32 (d, J = 8 Hz, 1 H), 9.45 (d, J = 6 Hz, 1 H), and 9.72 ppm (s, 1 H). Anal. Calcd for $C_{21}H_{26}O_{10}NBr$: C, 47.38; H, 4.92; N, 2.63; Br, 15.01. Found: C, 47.49; H, 5.10; N, 2.67; Br, 14.99.

The α-anomer α-**2g** could not be obtained in pure form owing to the partial epimerization which occured during each successive recrystallization from $CHCl_3$–EtOAc–Et_2O.

3-Nitropyridine. The literature method[43] for the preparation of this compound is reported to give 10% yields. However, significantly improved yields (45%) of 3-nitropyridine are obtainable using the following procedure. A mixture of 30% H_2O_2 (44 ml), concentrated H_2SO_4 (14 ml), and 30% fuming H_2SO_4 (w/w 74 ml) (*caution:* high heat of mixing) was added slowly during 45 min to a cooled (5 °C) solution of 3-aminopyridine (3.8 g, 40 mmol) in concentrated H_2SO_4 (40 ml) with stirring. The resulting mixture was stirred for 20 min, then warmed to 20 °C during 45 min, stirred for a further 2 h at 40–45 °C, and then kept overnight at 20 °C. The solution was then poured on to ice and neutralized carefully with solid NaOH and the mixture extracted with $CHCl_3$. The dried (Na_2SO_4) $CHCl_3$ solution was evaporated and the residue was purified by TLC (on 0.5-mm silica gel G plates developed with anhydrous Et_2O–C_6H_6 (5:1)) to give 3-nitropyridine (2.25 g, 45% yield): mp 35–38 °C (lit.[43] mp 39.5 °C); ir ($CHCl_3$) 1601, 1573, 1350, and 854 cm^{-1}; NMR (C^2HCl_3) δ 7.57 (3 d, J = 1.0, 4.7, 8.3 Hz, 1 H), 8.53 (3 d, J = 1.5, 2.5, 8.3 Hz, 1 H), 8.95 (d of d, J = 1.5, 4.7 Hz, 1 H), and 9.48 ppm (d, J = 2.5 Hz, 1 H).

***N*-(2,6-Dichlorobenzyl)-3-nitropyridinium Bromide (2h).** A solution of 3-nitropyridine (1.00 g, 8 mmol) and 2,6-dichlorobenzyl bromide (1.93 g, 8 mmol) in dry CH_3CN (5 ml) was refluxed overnight and then kept for 2 weeks at 0 °C. Et_2O (15 ml) was then added and the solid produced (1.63 g, 55% yield) was filtered and recrystallized from EtOH to give **2h**: mp 168–169 °C dec; ir (KBr) 1550 and 1357 cm^{-1}; uv (H_2O) 270 nm (ϵ 4030); NMR (^2H_2O) δ 6.48 (s, 2 H), 7.70 (s, 3 H), 8.45, 8.56 (2 d, J = 7 Hz, total of 1 H), 9.31 (d, J = 6 Hz, 1 H), 9.51 (d, J = 8 Hz, 1 H), and 10.0 ppm (s, 1 H). Anal. Calcd for $C_{12}H_9N_2O_2BrCl_2$: C, 39.59; H, 2.49; N, 7.70; Cl, 21.95; Br, 19.48. Found: C, 39.59, H, 2.56; N, 7.64; Cl, 21.78; Br, 19.33.

***N*-(4-Nitrobenzyl)-3-nitropyridinium Bromide (2i).** A solution of 3-nitropyridine (525 mg, 4.2 mmol) and *p*-nitrobenzyl bromide (1.83 g, 8.5 mmol) in dry CH_3CN (7 ml) was stirred for 2 days at 20 °C and then heated under reflux for 1 h. After dilution of the cooled mixture with Et_2O (20 ml) the solid product was filtered and then recrystallized (three times) from MeOH to give **2i** (789 mg, 55% yield): mp 208–209 °C dec; ir (KBr) 1550 and 1349 cm^{-1}; uv (H_2O) 266 nm (ϵ 14 100); NMR (^2H_2O) δ 6.20 (s, 2 H), 7.72 (d, J = 8.5 Hz, 2 H), 8.30 (d, J = 8.5 Hz, 2 H), 8.44 (m, 1 H), 9.44 (m, 2 H), 10.05 ppm (s, 1 H). Anal. Calcd for $C_{12}H_{10}N_3O_4Br$: C, 42.37; H, 2.96; N, 12.35; Br, 23.49. Found: C, 42.56; H, 3.15; N, 12.28; Br, 23.29.

***N*-Benzoylmethyl-3-nitropyridinium Bromide (2j).** 3-Nitropyridine (494 mg, 4 mmol) and phenacyl bromide (1.60 g, 8 mmol) in dry CH_3CN (10 ml) were allowed to react and worked up as described above for **2i**. Recrystallization of the crude product from MeOH gave **2j** (1.06 g, 83% yield): mp 226–227 °C dec; ir (KBr) 1548 and 1358 cm^{-1}; uv (H_2O) 253 nm (ϵ 19 800); NMR (Me_2SO-$^2H_6$) δ 6.80 (s, 2 H), 7.80 (m, 3 H), 8.15 (m, 2 H), 8.54 (d, J = 7.5 Hz) plus 8.70 (d, J = 7.5 Hz, total of 1 H), 9.50 (m, 2 H), and 10.20 ppm (s, 1 H). Anal. Calcd for $C_{13}H_{11}N_2O_3Br$: C, 48.32; H, 3.43; N, 8.67; Br, 24.73. Found: C, 48.06; H, 3.59; N, 8.59; Br, 24.60.

Determination of Hydroxide Affinities of the 3-Nitropyridinium Salts 2h and 2i. The equilibrium constants were determined by the method of Bunting and Meathrel[33] in a series of 0.01 M borate and phosphate buffers spanning the pH range 7.30–9.04 at 25.0 °C. Stock solutions of the nitropyridinium salts **2h** and **2i** in H_2O were prepared and spectral measurements were made on 200-μl aliquots of these stock solutions diluted in 3.0 ml of buffer in 1.0-cm path length cuvettes. Final concentrations of **2h** and **2i** were 4.73 and 3.73×10^{-5} M, respectively. Absorbance measurements were taken at wavelengths of 406 and 404 nm for **2h** and **2i**, respectively. The pK_{ROH} values determined in this way were **2h**, 8.2, and **2i**, 8.0.

***N*-Methyl-3,5-dinitropyridinium Fluorosulfonate (2m).** 3,5-Dinitropyridine was prepared in 40% overall yield by the method of Plazek.[44] It had mp 104–105 °C (lit.[44] mp 106 °C). Freshly sublimed 3,5-dinitropyridine (103 mg, 0.6 mmol) and methyl fluorosulfonate (0.5 ml) were mixed at 0 °C and stirred for 30 min in a dry atmosphere. The resulting hygroscopic white precipitate was washed with anhydrous Et_2O to give **2m** (162 mg, 94% yield): mp 162–75 °C; uv (6 N HCl) 275 nm (sh) and end absorption; uv (H_2O) 400 (sh), 363 and 228 nm; uv (MeOH) 388 (sh), 349, and 226 nm; NMR (CF_3COOH) δ 5.04 (s, 3 H), 10.07 (t, J = 2 Hz, 1 H), and 10.23 ppm (d, J = 2 Hz, 2 H).

N-Methyl-3,5-dinitro-2-hydroxy-1,2-dihydropyridine. *N*-Methyl-3,5-dinitropyridinium fluorosulfonate (**2m**, 130 mg, 0.46 mmol) in H_2O (0.8 ml) was kept for 20 min at 0 °C and then filtered to give the title compound (59 mg, 64% yield): mp 126.0–127.5 °C; ir ($CHCl_3$) 3595, 1642, 1562, and 1315 cm^{-1}; uv (6 N HCl) 275 nm (sh) and end absorption; uv (CH_3COOH) 364 (sh), 350, and 225 nm; uv (H_2O) 400 (sh), 363, and 226 nm; NMR (CH_3COCH_3-$^2H_6$) δ 3.85 (s, 3 H), 6.55 (s, 1 H), 8.47 (d, J = 1.5 Hz, 1 H), and 8.84 ppm (d, J = 1.5 Hz, 1 H). Anal. Calcd for $C_6H_7N_3O_5$: C, 35.83; H, 3.51; N, 20.89. Found: C, 35.86; H, 3.4; N, 19.98.

Attempted Alkylations of 3,5-Diacetylpyridine. Diacetylpyridine, mp 65–67 °C (lit.[45] mp 71°), was obtained in 48% yield by oxidation of 3,5-diacetyl-1,4-dihydropyridine[46] with $NaNO_2$ in CH_3COOH.[17] No pyridinium derivatives were isolable on treatment of 3,5-diacetylpyridine with 2,6-dichlorobenzyl bromide in Me_2SO at 50 °C or with 1-bromo-2,3,4,6-tetraacetylglucose in refluxing CH_3CN.

Coenzyme Recycling Experiments. The recycling studies were carried out on solutions (10–150 ml) containing fixed concentrations of reagents. The concentration ranges used were substrate (cyclohexanone or cyclohexanol), 6.0–6.2 mM; NAD^+, 0.1–2.0 mM; recycling agent, 6–13 mM; and HLADH, $0.4–8.0 \times 10^{-7}$ M. The runs performed in the pH range 7–9 were carried out in 0.05–0.1 M Tris–HCl buffers. For the other pH ranges studied, the buffers used were phthalate–NaOH (pH 5–6) and CO_3^{2-}–HCO_3^- (pH 10–11). Both substrate and coenzyme reagents were utilized as stock solutions prepared in the appropriate buffer. The recycling agents were added in neat (solid or liquid) form to the assay mixture and HLADH as 10–100-μl aliquots of a stock solution in 0.05 M Tris–HCl, pH 7, containing 1 mg/ml of enzyme. This latter solution retained >90% of its activity when stored for 1 month at 4 °C. The reactants were combined in stoppered 10–200-ml vol, pear-shaped flasks and the mixtures agitated slowly (without foaming or cavitation) with a magnetic stirrer at 20 °C. The course of each reaction was monitored by extracting a 3-ml aliquot with $CHCl_3$ (3 × 3 ml) which was then dried (Na_2SO_4) and concentrated carefully at 0 °C (10–15 Torr). The residual solution (~0.3 ml) was analyzed by GLC. This extractive procedure led to >60% recovery of the available cyclohexanol–cyclohexanone. The marginal bias of the extraction procedure toward cyclohexanone was balanced by an equivalent compensating GLC sensitivity in favor of cyclohexanol.

In order to confirm that coenzyme recyclings performed as described above were preparatively viable, the reactions were repeated for the better recycling agents under the conditions given in Tables II and III (**1a,c**) and Table IV (β-**2g**) on solutions containing up to 1 g of cyclohexanone or cyclohexanol, respectively. When little or no residual substrate could be detected by GLC (24–48 h), the reaction mixtures were worked up via chloroform extraction followed by distillation; >60% yields of the pure alcohol or ketone product were isolated.

Preparation of [1-^{2}H]Cyclohexanol via Reductive Recycling. The reaction was performed according to the general procedure described above using cyclohexanone (103 mg, 1.05 mmol), *N*-benzyl-4,4-$^2H_2$-1,4-dihydronicotinamide (**1a**-$^2H_2$, 465 mg, 2.15 mmol), NAD^+ (trihydrate, 120 mg, 0.17 mmol), HLADH (5 mg) in 0.1 M Tris–HCl buffer, pH 7 (150 ml). The progress of the reduction was monitored by GLC on 3-ml aliquots and fresh HLADH and NAD^+ were added at 2-day intervals. After 8 days (72% reduction) the reaction mixture was extracted with Et_2O (3 × 40 ml), and the Et_2O solution dried (Na_2SO_4), diluted with *n*-heptane (4 ml), and concentrated via spinning band distillation. The resulting heptane solution (~4 ml) was heated for 4 h on a steam bath with pyridine (1 ml) and freshly distilled α-naphthylisocyanate (103 mg, 0.61 mmol). The TLC homogeneous solid (116 mg, representing 71% of available [1-^{2}H]cyclohexanol) obtained was purified by TLC on silica gel G with Et_2O–benzene (1:5) development. Recrystallization from heptane gave *N*-cyclohexyloxycarbonyl-α-naphthylamine-*1'*-$^2H_2$: mp 131–133 °C [lit.[47] (protio compound) mp 129 °C]; ir ($CHCl_3$) 3443 and 1723 cm^{-1}; NMR (C^2HCl_3) δ 1.3–2.1 (m, 10 H), 4.90 [m, 0.35 H (=0.65 ^{2}H at C-1')], 7.10 [br s, 1 H (exchangeable)], and 7.4–8.0 ppm (m, 7 H); mass spectrum *m/e* (rel abundance) 270 (32, M^+ of ^{2}H compound) and 269 (23, M^+ of protio compound).

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References and Notes

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(37) HLADH (E.C. 1.1.1.1, crystallized three times, mol wt ~84 000) was obtained from Worthington; NAD^+ was purchased from Sigma. Where not provided in the text, full spectral characterizations of each compound are given in ref 10. Due to their inherent instability, satisfactory elemental analyses could not be obtained for many of the new pyridinium–dihydropyridine compounds prepared. The deuterium contents of **1a,c**-$^2H_2$ were analyzed by NMR since the facility with which the aromatic $M^+ - 1$ cations were formed in the mass spectrometer (at all possible ionizing voltages) precluded the use of mass spectroscopy for this purpose. GLC analyses were performed using a 2% QF-1 on Chromosorb G column.
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