Use of Pyridinium and Flavin Derivatives for Recycling of Catalytic Amounts of NAD$^+$ during Preparative-scale Horse Liver Alchohol Dehydrogenase-catalysed Oxidations of Alcohols

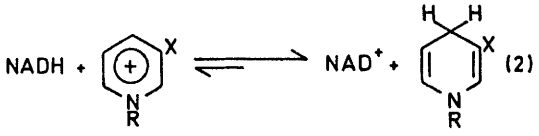
By J. BRYAN JONES* and KEITH E. TAYLOR
(*Department of Chemistry, University of Toronto, Toronto, Ontario, Canada* M5S 1A1)

Summary Use of pyridinium salts or flavin derivatives as hydrogen acceptors has provided the first practical procedure for *in situ* regeneration of catalytic amounts of coenzyme during preparative-scale enzymic oxidations of alcohols with up to 35-fold turnovers of NAD$^+$ being achieved for the horse liver alcohol dehydrogenase-catalysed oxidation of the representative substrate cyclohexanol.

THE ability of HLAD† and other oxidoreductases to catalyse selective and/or stereospecific hydroxy- and carbonyl-group oxidoreductions has not been used to a great extent,[1-6] partly because of the high cost of the coenzymes which are formally required in stoicheiometric, or greater amounts, to obtain good yields of alcohols or aldehydes/ketones [see equation (1)]. For the carbonyl reduction reaction, this problem has been partly overcome

$$\text{CHOH} + \text{NAD}^+ \overset{\text{oxidoreductase}}{\rightleftharpoons} \text{C=O} + \text{NADH} + \text{H}^+ \quad (1)$$

by recycling catalytic amounts of coenzyme *via* coupled enzyme,[1,3] coupled substrate,[4,5] and dithionite reduction[6] methods, but no similar procedure existed for the enzyme-catalysed alcohol oxidation reaction. Efficient regeneration



(1a) R = α-TAG, X = CONH$_2$
(1b) R = β-TAG, X = CONH$_2$
(1c) R = β-TAG, X = Ac

of catalytic quantities of coenzyme is even more important for such oxidative processes since the equilibrium of equation (1) favours alcohol formation at all practical (5—10) pH values.[4,7]

We have found that by using pyridinium and flavin derivatives as hydrogen acceptors, up to 35-fold turnovers of catalytic NAD$^+$ can be achieved during preparative-scale HLAD-catalysed oxidations. It appeared from the literature that addition of pyridinium salts,[8] and quinonoid or flavin derivatives,[9,10] of less negative redox potential (E_0') than NAD$^+$ to an HLAD-catalysed oxidation should regenerate the desired form of the coenzyme [see equation (2)]. The compounds examined (with their estimated[10] $\Delta E_0'$ [NAD$^+$—acceptor] values) were (**1a**) (−48 mV), (**1b**) (−48 mV), (**1c**) (−93 mV), 2,6-dichlorophenolindophenol (−532 mV), riboflavin (−140 mV), and FMN (−140 mV). Their efficiencies as hydrogen acceptors in the NADH→NAD$^+$ recycling process were evaluated on a preparative-scale using cyclohexanol (see Table). Although the pyridinium salts with the lowest ΔE_0 values (**1a,b**) were not effective, up to 25-fold recycling of NAD$^+$ was obtained with (**1c**).‡ Although the $\Delta E_0'$ of 2,6-dichlorophenolindophenol was the largest for the compounds used, its recycling capability was relatively low. The relative

TABLE

Survey of efficiency of recycling of catalytic NAD$^+$ by various hydrogen acceptors in preparative-scale HLAD-catalysed oxidations of cyclohexanol

Hydrogen acceptor	[$C_6H_{11}OH$]: [NAD$^+$]	Yield (%)[a]	NAD$^+$ recycles[b]
(**1a**)	6·7	20	0·3
	26	11	2·0
(**1b**)	6·8	48	2·0
	35	22	6·7
(**1c**)	6·2	80	3·8
	62	42	24·6
2,6-Dichlorophenol-indophenol	6·3	62	2·7
	23	37	7·5
Riboflavin[c]	6·2	74	3·2
	23	42	8·4
FMN	6·6	90	4·9
	62	44	27·2
	70[d]	53	34·7

Reactions were carried out at 22° for 16—24 h using pH 9 tris–HCl buffer solutions with cyclohexanol (6 mM), H-acceptor (12 mM), and HLAD (4×10^{-7}M). Reaction volumes of up to 160 ml (100 mg of cyclohexanol) were used. Control experiments were run to ensure that H-acceptors did not possess any coenzymic activity and that they themselves did not oxidise the substrate.

[a] From quantitative g.l.c. analysis. [b] Corrected for reaction due to NAD$^+$ added initially. [c] [Riboflavin] < 2 mM. [d] [FMN] = 30 mM.

ineffectiveness of riboflavin as an NAD$^+$ recycling agent may be due to its low solubility in the reaction solution since the more readily dissolved FMN of identical $\Delta E_0'$ gave the highest turnovers of NAD$^+$. Within the range pH 7—10, the best results were obtained at pH 9 using as large a [H-acceptor]:[cyclohexanol] ratio as possible. As in the previous reductive experiments,[6] coenzyme turnovers were highest when [NAD$^+$] was lowest. The recycling procedure helped to displace the equilibrium [equation (1)] in the unfavoured oxidation direction since the yields of cyclohexanone from experiments carried out under recycling

† HLAD, horse liver alcohol dehydrogenase; NAD$^+$, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); TAG, 1-(2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosidyl); FMN, riboflavin-5′-phosphate.

‡ Several more electron-deficient pyridinium salts were used but were ineffective as recycling agents probably due to pseudo-base formation[11] in the aqueous, pH 9, reaction solutions.

conditions were considerably higher than when an amount of NAD^+ equivalent to the total recycled was added all at once.

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