Reprinted from The Journal of Organic Chemistry, 1982, 47, 2816

Enzyme-Catalyzed Organic Synthesis: NAD(P)H Cofactor Regeneration Using Ethanol/Alcohol Dehydrogenase/Aldehyde Dehydrogenase and Methanol/Alcohol Dehydrogenase/Aldehyde Dehydrogenase/Formate Dehydrogenase[1]

Summary: An enzyme-catalyzed system potentially applicable to large-scale synthesis is described.

Sir: We have recently described a number of methods for regeneration of NAD(P)H from $NAD(P)^+$ for use in enzyme-catalyzed organic syntheses requiring nicotinamide cofactors.[2-8] In this paper we compare two additional useful schemes, and apply these schemes to syntheses producing 0.1–0.5-mol quantities of products (Figure 1). The first method is based on catalysis by two enzymes—alcohol dehydrogenase (ADH, EC 1.1.1.1) and aldehyde dehydrogenase (AldDH, EC 1.2.1.5)—and converts ethanol to acetate. This scheme has been demonstrated previously in millimole-scale syntheses.[9,10] The second uses ADH, AldDH, and formate dehydrogenase (FDH, EC 1.2.1.2) and converts methanol to CO_2. The first generates 2 equiv of reduced nicotinamide cofactor/equiv of ethanol, and accepts either NAD^+ or $NADP^+$; the second generates 3 equiv of reduced nicotinamide cofactors/equiv of methanol but accepts only NAD^+. The relevant kinetic parameters for the enzymes in these schemes are summarized in Table I. The only feature of these parameters which requires specific comment concerns ADH: the enzyme from *Saccharomyces cerevisiae* has high specific activity with ethanol but is specific for NAD^+; that from *Leuconostoc mesenteroides* reduces both NAD^+ and $NADP^+$ but has lower specific activity.

In a representative procedure for NADH regeneration using ethanol as ultimate reducing agent (A, Figure 1), a 500-mL solution containing potassium pyruvate (15.1 g, 120 mmol), NAD^+ (50 μmol), ethanol (3.3 g, 70 mmol), and β-mercaptoethanol (39 mg, 0.5 mmol) was mixed with PAN-immobilized yeast ADH (90 units, 0.5 mL of gel),[11] AldDH (110 units, 10 mL of gel), and L-lactic dehydrogenase (L-LDH, 200 units, 0.5 mL of gel). The reaction mixture was stirred at 25 °C under argon, and the pH of the solution was controlled automatically at 8.0–8.2 by adding 2 N KOH through a peristaltic pump. More ethanol (3.3 g, 60 mmol) was added after 1 day. The reaction was complete in 2 days. The solution, after removal of the enzyme-containing gel, was concentrated to 20 mL and acidified with concentrated H_2SO_4 to pH 2.8, followed by addition of ethanol (200 mL). The precipitates were separated by filtration and discarded, and the filtrate was concentrated at room temperature to an oily residue. The residue was diluted with water (150 mL) and neu-

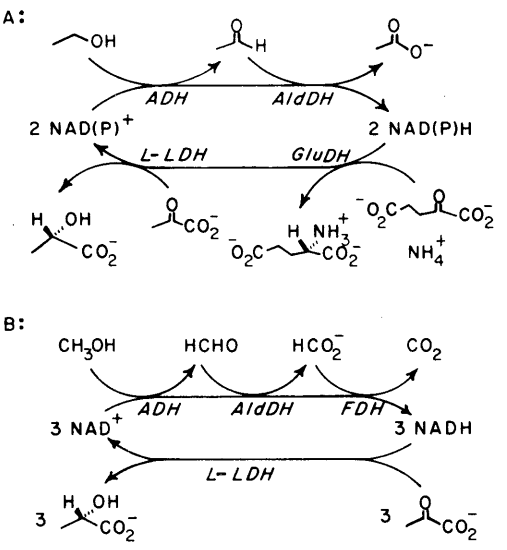

Figure 1. A: Regeneration of NAD(P)H using ethanol and alcohol dehydrogenase/aldehyde dehydrogenases. B: Regeneration of NADH using methanol and alcohol dehydrogenase/aldehyde dehydrogenase/formate dehydrogenase. Abbreviations: ADH, alcohol dehydrogenase from yeast (for NAD) or from *L. mesenteroides* (for NAD or NADP); AldDH, aldehyde dehydrogenase from yeast (for NAD or NADP); L-LDH, L-lactic dehydrogenase; GluDH, glutamic dehydrogenase; FDH, formate dehydrogenase.

(1) Supported by the National Institutes of Health, Grant GM 26543.
(2) Shaked, Z.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 7104–5.
(3) Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *103*, 4890–9.
(4) Wong, C.-H.; Daniels, L.; Orme-Johnson, W. H.; Whitesides, G. M. *J. Am. Chem. Soc.*, **1981**, *103*, 6227–8.
(5) Shaked, Z.; Barber, J.; Whitesides, G. M. *J. Org. Chem.*, **1981**, *46*, 4100–1.
(6) DiCosimo, R.; Wong, C. H.; Daniel, L.; Whitesides, G. M. *J. Org. Chem.*, **1981**, *46*, 4622–3.
(7) Abril, O.; Whitesides, G. M. *J. Am. Chem. Soc.* **1982**, *104*, 1552.
(8) Wong, C.-H.; Gordon, J.; Cooney, C. L.; Whitesides, G. M. *J. Org. Chem.*, **1981**, *46*, 4676–9.
(9) Chambers, R. P.; Ford, J. R.; Allender, J. H.; Baricos, W. H.; Cohen, W. *Enzyme Eng.* **1973**, *2*, 195–202.
(10) *Chem. Eng. News.* **1974**, Feb. 15, p 19.
(11) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324–36. AldDH was immobilized with PAN 1000 in the presence of acetaldehyde (3 mM) and NAD^+ (2 mM), in 50% yield. FDH was immobilized in the presence of formate (20 mM) and NAD^+ (2 mM), in 40% yield.

Table I. Kinetic Parameters for Alcohol Dehydrogenase, Aldehyde Dehydrogenase, and Formate Dehydrogenase

enzyme	substrate K_m, mM	sp act., units mg^{-1}
alcohol dehydrogenase[a] *S. cerevisiae*	EtOH (13), NAD (0.074)	400
	MeOH (80), NAD	10
alcohol dehydrogenase[b] *L. Mesenteroides*	EtOH, (50), NAD (0.5)	70
	EtOH (50), NADP (0.085)	90
	MeOH (70), NAD	1.5
	MeOH (70), NADP	2
aldehyde dehydrogenase[c] *S. cerevisiae*	CH_3CHO (0.009), NAD (0.03)	80
	CH_3CHO (0.009), NADP (0.03)	8
	HCHO (0.7), NAD	36
	HCHO (0.7), NADP	4
formate dehydrogenase[d] *Candida boidinii*	HCO_2^- (13), NAD (0.09)	3

[a] Wratten, C. C.; Cleland, W. W. *Biochemistry* **1963**, *2*, 935-41. Eys, J. V.; Levy, H. R.; Loewus, F. A.; Vennesland, B. *J. Am. Chem. Soc.* **1957**, *79*, 2949-53. [b] DeMoss, R. D. *Methods Enzymol.* **1955**, *1*, 504-8. Hatanaka, A.; Adachi, O.; Chiyonobu, T.; Ameyama, M. *Agric. Biol. Chem.* **1971**, *35*, 1304-6. [c] Clark, J. F.; Jakoby, W. B. *J. Biol. Chem.* **1970**, *245*, 6065-71. Steinman, C. R.; Jakoby, W. B. *J. Biol. Chem.* **1968**, *243*, 730-4. Bradbury, S. L.; Clark, J. F.; Steinman, C. R.; Jakoby, W. B. *Methods Enzymol.* **1975**, *41*, 354-60. [d] Schutte, H.; Flossdorf, J.; Sahm, H.; Kula, M. R. *Eur. J. Biochem.* **1976**, *62*, 151-60.

Table II. Advantages and Disadvantages of NAD(P)H Regeneration Systems

system	advantages	disadvantages
EtOH/ADH/AldDH	1. high equilibrium constant for formation of NAD(P)H	1. requires two enzymes
	2. applicable to NAD^+ and $NADP^+$	2. ADH and AldDH are sensitive to oxidation
	3. generates a relatively innocous product (Ac^-)	3. HOAc may complicate some product isolations
	4. good specific activities for NAD^+ and EtOH	4. specific activities are only moderate for $NADP^+$ (AldDH)
	5. enzymes commercially available	
	6. good TN for NAD(P)(H)	
CH_3OH/ADH/AldDH/FDH	1. inexpensive reducing agent	1. requires three enzymes
	2. ultimate product (CO_2) is volatile	2. ADH, AldDH sensitive to oxidation
	3. restricted to NAD^+ (at least while using this FDH)	3. specific activities are only moderate for MeOH, H_2CO, and HCO_2H
	4. enzymes commercially available	
	5. good TN for NAD(H)	

tralized by adding $ZnCO_3$ (8.2 g, 65 mmol) to obtain crystalline zinc L-lactate (32.6 g of solid containing 96% zinc bis(L-lactate) trihydrate (106 mmol); 88% yield and 94% ee).[3] The turnover number (TN) and residual activities were as follows: NAD^+, 2400 (92%); ADH, 6×10^7 (88%); AldDH, 5×10^6 (87%); L-LDH, 1×10^7 (96%). The recovered enzymes were used for repeated preparations of L-lactate on the same scale; five preparations in a period of 11 days generated 0.5 mol of L-lactate. The recovered enzyme activities after these five cycles of synthesis were as follows: ADH, 70%; AldDH, 71%; L-LDH, 78%.

In a typical procedure illustrating the operation of ADH/AldDH for NADPH regeneration, a 500-mL solution containing monopotassium α-ketoglutarate (22 g, 120 mmol), $NADP^+$ (50 μmol), and ethanol (3.3 g, 70 mmol) was neutralized with NH_4OH to pH 8.0–8.2. β-Mercaptoethanol and PAN-immobilized ADH from *L. mesenteroides* (90 units based on $NADP^+$, 1 mL of gel), AldDH (92 units based on $NADP^+$, 25 mL of gel) and glutamic dehydrogenase (GluDH, 180 units, 1 mL of gel) were added. The reaction was conducted at pH 8.2 and was complete in 2 days. The decanted solution was concentrated to ~100 mL, adjusted to pH 6.5, and treated with ethanol (80 mL) until the solution became turbid. A crystalline solid (20 g) containing 97% of monopotassium L-glutamate (104 mmol, 86% yield) was obtained after cooling. The TN and residual activities were as follows: $NADP^+$, 2400 (82%); GluDH, 1×10^7 (88%); ADH, 2×10^7 (86%); AldDH, 1×10^6 (86%).

The operation of the redox system using NAD^+ as cofactor and methanol as ultimate reducing agent is illustrated by a preparation of L-lactate. A 600-mL solution containing potassium pyruvate (15 g, 120 mmol), NAD^+ (10 μmol), yeast ADH (30 units, 15 mL of gel, activity based on methanol as substrate), AldDH (34 units, 6 mL of gel, activity based on formaldehyde as substrate), FDH (30 units, 12 mL of gel), and L-LDH (50 units, 0.5 mL of gel) was deoxygenated with Ar, followed by addition of methanol (1.5% v:v, 0.3 M) and mercaptoethanol (final concentration, 4 mM). The pH of the reaction mixture was controlled at 8.0–8.2. After 4 days, L-lactate was isolated as its zinc salt (16.2 g, 94% purity, 86% yield, 92% ee). The TN and residual activities were as follows: NAD^+, 1200 (90%); FDH, 3.2×10^5 (88%); ADH, 3×10^5 (82%); AldDH, 9×10^5 (86%); L-LDH, 1.8×10^7 (96%).

The most important feature of these preparations is the use of ratios of AldDH/ADH/(FDH) such that the first step (formation of aldehyde) is the slow step. This protocol insures that the aldehyde concentration in the reactor remains as low as possible and protects the enzymes from deactivation.

The advantages and disadvantages of these systems are listed in Table II. The balance of these characteristics is that these systems are potentially more economical for nicotinamide cofactor regeneration in connection with large-scale synthesis than most others but are somewhat less convenient for laboratory-scale (~0.1–10 mol) syntheses than those based on glucose 6-phosphate[3] or formate.[2] We note that it should be relatively straightforward by recombinant DNA techniques to produce microorganisms having high contents of the two or three enzymes required in these schemes in the correct ratios

for optimum performance. The use of immobilized whole organisms of this type, or of crude extracts from them, would be the most efficient method for preparation of the required enzymatic activities.

Registry No. NADH, 58-68-4; NAD^+, 53-84-9; NADPH, 53-57-6; $NADP^+$, 53-59-8; ADH, 9031-72-5; AldDH, 9028-88-0; FDH, 9028-85-7; ethanol, 64-17-5; methanol, 67-56-1.

Chi-Huey Wong, George M. Whitesides*
Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139
Received March 23, 1982