

# Communications

## Enzyme-Catalyzed Organic Synthesis: Electrochemical Regeneration of NAD(P)H from NAD(P) Using Methyl Viologen and Flavoenzymes

**Summary:** A procedure for enzyme-catalyzed organic synthesis is described in which regeneration of NAD(P)H from NAD(P) is accomplished by the electrochemical reduction of oxidized to reduced methyl viologen ( $MV^{2+} \rightarrow MV^{1+}$ ) followed by flavoenzyme-catalyzed reduction of the nicotinamide cofactor by this  $MV^{1+}$  (Scheme I).

**Sir:** Direct electrochemical methods for reduction of NAD(P) to NAD(P)H have not proved sufficiently selective for reduction at the 4-position to give high turnover numbers (TN) for these cofactors.<sup>1-3</sup> A combined electrochemical/enzymatic method based on cathodic reduction of lipoamide and lipoamide dehydrogenase (LipDH, EC 1.6.4.3) catalyzed reduction of NAD gave TN  $\approx$  900,<sup>4</sup> and hydrogenase-catalyzed reduction of  $MV^{2+}$  by  $H_2$  combined with lipoamide dehydrogenase or ferredoxin reductase (FDR, EC 1.6.99.4) catalyzed reduction of NAD(P) gave similar values.<sup>5</sup> Although the use of electrochemically generated  $MV^{1+}$  to produce NADPH from NADP via reaction with FDR has been reported previously,<sup>6,7</sup> application of this redox cycle to regeneration of NAD(P)H for practical organic synthesis has not been described; here we report such a procedure.

Reactions were performed under argon in a 1-L three-necked flask containing a magnetic stirring bar and a working electrode of 10 m of coiled tungsten wire<sup>8</sup> (0.020-in. diameter,  $\sim$ 160-cm<sup>2</sup> surface area). The counter electrode was a 2  $\times$  5-in. section of 80-mesh platinum gauze and was separated from the working solution in a glass tube sealed to a fine-porosity sintered glass frit inserted through the center neck of the flask. The reference electrode (SCE) was isolated from the working solution in the same manner. The total charge passed during electrolyses was determined by graphical integration of the recorded current-time curve. Current efficiency was calculated by subtraction of the integrated background current<sup>9</sup> from the total in-

tegrated current and comparison of this value with the value calculated for the amount of product present, determined by enzymatic assay.

In a typical procedure, a 600-mL solution containing imidazole (0.2 g, 3 mmol, pH 8.0), sodium pyruvate (10 g, 91 mmol), NAD (0.090 mmol, 0.15 mM), 1,1'-dimethyl-4,4'-bipyridinium dichloride ( $MV^{2+}$ , 0.31 g, 1.2 mmol),  $\beta$ -mercaptoethanol (0.94 g, 1.2 mmol), and  $K_2SO_4$  (5.23 g, 30 mmol) was added to a flask containing LipDH (from pig heart, 1400 U, immobilized in 50 mL of PAN gel<sup>10</sup>) and D-lactate dehydrogenase (D-LDH, EC 1.1.1.28, 30 U, 1 mL of gel).<sup>10</sup> The counterelectrode and reference electrode compartments were filled with 50 mM  $K_2SO_4$  solution, and the three compartments were deoxygenated with ultrahigh purity argon for 30 min. The potential of the tungsten working electrode was adjusted to  $-0.72$  V<sup>11</sup> (vs. SCE) and the reaction mixture was stirred at ambient temperature, while the solutions in both the counterelectrode and working electrode compartments were continuously purged with argon. After 9 days, the reaction was 94% complete and D-lactate was isolated as its calcium salt (as described previously<sup>12</sup> but with  $CaCO_3$  instead of  $ZnCO_3$ ). A white crystalline material (11.4 g) composed of 94%  $Ca(D-lactate)_2 \cdot 5H_2O$  (36.9 mmol) and 3%  $Ca(L-lactate)_2 \cdot 5H_2O$  was obtained, corresponding to an 81% yield and 94% ee.<sup>12</sup> Turnover numbers (and residual activities) for cofactor and enzymes were as follows: NAD, 940 (51%); LipDH,  $5.4 \times 10^5$  (65%); D-LDH,  $3.5 \times 10^7$  (84%). The current efficiency was  $104 \pm 10\%$ .

The preparation of D-lactate was repeated with FDR instead of LipDH. A 600-mL solution containing  $Na_2SO_4$  (8.5 g, 60 mmol), imidazole (0.2 g, 3 mmol, pH 8.0), sodium pyruvate (13.2 g, 120 mmol), NAD (0.2 mM),  $MV^{2+}$  (0.23 g, 0.91 mmol),  $\beta$ -mercaptoethanol (94 mg, 1.2 mmol), PAN-immobilized FDR (300 U, 90 mL of gel),<sup>13</sup> and D-LDH (30 U, 1 mL of gel)<sup>10</sup> was electrolyzed as previously described. The reaction was 90% complete in 14 days, and calcium D-lactate was isolated as a white solid (14.2 g) in 77% yield based on pyruvate and 94% ee. Turnover numbers (and residual activities) for cofactor and enzymes were as follows: NAD, 900 (20%); FDR,  $7.3 \times 10^6$  (60%); D-LDH,  $2.2 \times 10^7$  (64%). The current efficiency was  $103 \pm 10\%$ .

Regeneration of NADP was demonstrated by the synthesis of L-glutamate from  $\alpha$ -ketoglutarate. The recovered gel from the FDR/D-LDH reactor was added to a solution

(1) Cunningham, A. J.; Underwood, A. L. *Biochemistry* 1967, 6, 266-271. Janik, B.; Elving, P. J. *Chem. Rev.* 1968, 68, 295-319.

(2) Aizawa, M.; Coughlin, R. W.; Charles, M. *Biotech. Bioeng.* 1976, 18, 209-215. Aizawa, M.; Suzuki, S.; Kubo, M. *Biochim. Biophys. Acta* 1976, 444, 886-892.

(3) Schmamel, C. O.; Santhanam, K. S. V.; Elving, P. J. *J. Electrochem. Soc.* 1974, 121, 345-353. Bresnahan, W. T.; Moiroux, J.; Samec, Z.; Elving, P. J. *J. Electroanal. Chem.* 1980, 116, 125-155.

(4) Shaked, Z.; Barber, J. J.; Whitesides, G. M. *J. Org. Chem.*, in press.

(5) Wong, C. H.; Daniels, L.; Orme-Johnson, W. H.; Whitesides, G. M. *J. Am. Chem. Soc.*, in press.

(6) Day, R. J.; Kinsey, S. J.; Seo, E. T.; Weliky, N.; Silverman, H. P. *Trans. N.Y. Acad. Sci.* 1972, 34, 588-594. Weliky, N.; Day, R. J.; Dale, E.; Gale, N.; Seo, E. T.; Silverman, H. P. *Ibid.* 1972, 34, 647-663.

(7) Ito, M.; Kuwana, T. *J. Electroanal. Chem.* 1971, 32, 415-425.

(8) The tungsten electrode was not pretreated before use, and no fouling of the electrode was observed during experiments.

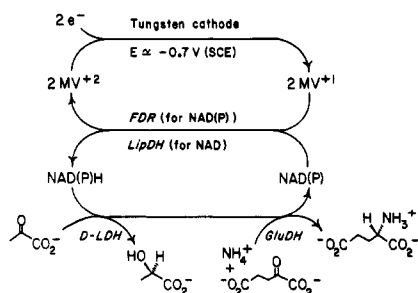
(9) Background current was measured by using a solution identical with that of a reaction mixture except that addition of enzymes was omitted. Pyruvate or NAD(P) was not reduced at the working electrode, nor was lactate or NAD(P)H oxidized at the counterelectrode, under the reported experimental conditions.

(10) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* 1980, 102, 6324-6336. The activity of LipDH was based on NADH and lipoamide as substrates and that of LDH on pyruvate and NADH.

(11) The potentiostat employed was either a Princeton Applied Research Model 371 potentiostat/galvanostat or a potentiostat of similar design built in our laboratories. The reduction potential of  $-0.72$  V (SCE) was chosen to be slightly more negative than the reported  $E^\circ$  of 1,1'-dimethyl-4,4'-bipyridinium dichloride ( $-0.68$  V vs. SCE; see Summers, L. A. "The Bipyridinium Herbicides"; Academic Press: New York, 1980; p 106). A cyclic voltammogram of a tungsten electrode in 0.1 M  $Na_2SO_4$  (pH 7.0) indicated  $H_2$  production began at  $-1.5$  V (SCE), eliminating the possibility that  $H_2$  was the reducing agent under reaction conditions.

(12) Wong, C. H.; Whitesides, G. M. *J. Am. Chem. Soc.* 1981, 103, 4890-4899.

(13) FDR was isolated from spinach leaves: Zanetti, G.; Curti, B. *Methods Enzymol.* 1980, 69, 250-255. The activity was based on NADPH and  $K_3Fe(CN)_6$  as substrates.

Scheme I. Electrochemical Regeneration of NAD(P)H<sup>a</sup>.

<sup>a</sup> Abbreviations: MV, methyl viologen; FDR, ferredoxin-NADP reductase; LipDH, lipoamide dehydrogenase; D-LDH, D-lactate dehydrogenase; GluDH, glutamic dehydrogenase.

(total volume 600 mL) containing sodium  $\alpha$ -ketoglutarate (20 g, 120 mmol, neutralized with  $\sim$ 13 mL of 10 N  $\text{NH}_4\text{OH}$ ), NADP (0.12 mmol, 0.2 mM),  $\text{MV}^{2+}$  (0.31 g, 1.2 mmol),  $\beta$ -mercaptoethanol (0.94 g, 1.2 mmol),  $\text{Na}_2\text{SO}_4$  (4.2 g, 30 mmol), and glutamic dehydrogenase (GluDH, EC 1.4.1.3, 40 U, 1 mL of gel). The pH was controlled at 8.0 by adding deoxygenated 1 N  $\text{H}_2\text{SO}_4$  with a peristaltic pump. The reaction was complete in 7 days. The decanted solution was concentrated to  $\sim$ 100 mL and the pH adjusted to 6.5, followed by addition of ethanol (60 mL). Crystalline monosodium L-glutamate (17.8 g) was obtained after cooling. This material contained 96% of monosodium L-glutamate (101 mmol),<sup>14</sup> corresponding to a 84% isolated yield. The turnover numbers (and residual activities) for cofactor and enzymes were as follows: NADP, 1000 (68%); GluDH,  $1.1 \times 10^7$  (92%); FDR,  $7.5 \times 10^5$  (80%). The current efficiency was  $105 \pm 10\%$ .

The relative activities of flavoenzymes for NADH regeneration under the conditions employed in these reactions were LipDH (yeast)/LipDH (pig heart)/FDR = 1:4:7 [ca. 3  $\mu\text{mol}$  of NAD reduced  $\text{min}^{-1}$  (mg of FDR)<sup>-1</sup>]. FDR-catalyzed NADPH regeneration is 5 times as fast as the reaction using FDR-catalyzed NADH regeneration, while LipDH-catalyzed NADPH regeneration is 10% as fast as that for NADH regeneration. Under the reaction conditions studied, FDR is more stable ( $\tau_{1/2} = 16$  days) than LipDH ( $\tau_{1/2} = 7$  days).

The electrochemical method for NAD(P)H regeneration summarized in Scheme I is more convenient than that based on hydrogenase,<sup>5</sup> since hydrogenase is not commercially available and requires a nonroutine fermentation for its preparation. In both schemes, LipDH has relatively low stability and catalytic activity ( $\sim$ 1 U/mg, 2 mM  $\text{MV}^{2+}$ , pH 7.8,  $-0.72$  V vs. SCE); FDR is more expensive but more stable and active ( $\sim$ 3 U/mg under the same conditions). The overall reaction rate in systems using FDR or LipDH is limited by the reduction of NAD(P) by  $\text{MV}^{1+}$  under FDR or LipDH catalysis.<sup>15</sup> Increasing the concentration of  $\text{MV}^{1+}$  increases this rate but may lead to increased side reactions.

**Acknowledgment.** This research was supported by grants from the NIH (GM 26543) and NSF (80-12722 CHE). R.D. held a Chevron Fellowship.

(14) Bergmeyer, H. U. "Methods of Enzymatic Analysis"; Verlag Chemie, Academic Press: New York, 1974.

(15) The reduction of NADP by  $\text{MV}^{1+}$  catalyzed by FDR has been reported to be first order in FDR (ref 7), which is consistent with our result. Since LDH was in excess in the reaction system, and the rate of production of lactate was proportional to the concentration of FDR, LipDH or  $\text{MV}^{2+}$  (but not LDH), we concluded that the rate-limiting step was the reduction of NAD(P) catalyzed by flavoenzymes.

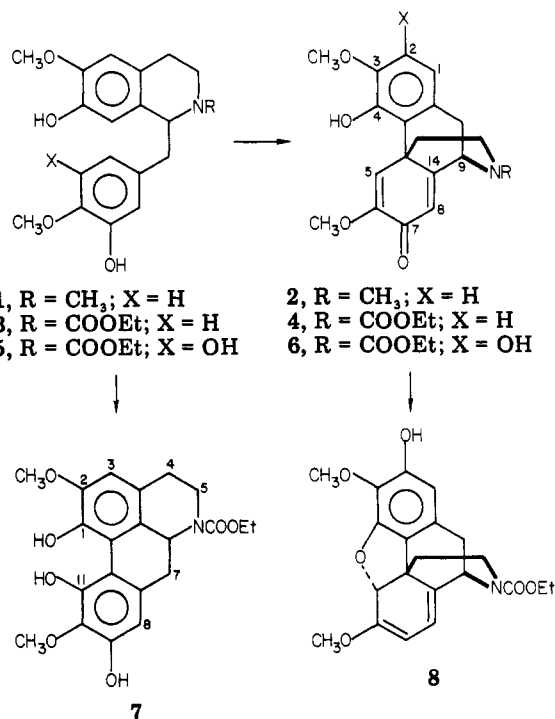
Registry No. NAD(P)H, 53-57-6; NAD(P), 53-59-8;  $\text{MV}^{2+}$ , 1910-42-5;  $\text{MV}^{1+}$ , 79028-21-0.

Robert DiCosimo, Chi-Huey Wong  
Lacy Daniels, George M. Whitesides\*  
Department of Chemistry  
Massachusetts Institute of Technology  
Cambridge, Massachusetts 02139  
Received August 14, 1981

### Biomimetic Approaches to Morphine Alkaloids. Total Synthesis of ( $\pm$ )-2-Hydroxycodone and ( $\pm$ )-Noroxycodone<sup>1</sup>

**Summary:** Oxidative coupling of a triphenolic hydroxynorreticuline substrate with  $\text{VOCl}_3$  affords the corresponding 2-hydroxynorsalutaridine derivative in good yield; the latter is readily converted to the title compounds via ( $\pm$ )-*N*-(ethoxycarbonyl)-2-hydroxynorthebaine.

**Sir:** The key step in the biosynthesis of the morphine alkaloids is the regioselective para-ortho oxidative cyclization of reticuline (1) to salutaridine (2).<sup>2</sup> The discovery in our laboratory that oxidative coupling of the reticuline derivative 3 with thallium(III) trifluoroacetate gave the salutaridine 4 as the major product resulted in the first biomimetic synthetic route to these alkaloids<sup>3</sup> and was latter extended to yield some morphine alkaloid analogues.<sup>4</sup> Szántay and co-workers very recently reported remarkable success in achieving the same regioselectivity with a variety of oxidants in the presence of certain organic acids.<sup>5</sup>



(1) Taken in part from Zoda, M. F. Ph.D. Dissertation, The Florida State University, 1981.

(2) For a succinct review, see: Herbert, R. B. In "Comprehensive Organic Chemistry"; Barton, D., Ollis, W. D., Ed.; Pergamon: Oxford, 1979; Vol 5, p 1076.

(3) (a) Schwartz, M. A.; Mami, I. S. *J. Am. Chem. Soc.* 1975, 97, 1239. (b) Schwartz, M. A. U.S. Patent 4 003 903, 1977; *Chem. Abstr.* 1977, 86, 155848g.

(4) Schwartz, M. A.; Wallace, R. A. *Tetrahedron Lett.* 1979, 3257.