10570-67-9; **2i**, 2040-83-7; **2j**, 5438-36-8; **2k**, 70331-81-6; **2l**, 99282-18-5; **2m**, 99298-06-3; **2o** (2-iodo), 24381-12-2; **2o** (4-iodo), 61748-87-6; 2n, 2033-42-3; **3a**, 609-23-4; **3b**, 4186-52-1; **3c**, 2432-18-0; **3d**, 75908-75-7; **3e**, 15459-50-4; **3g**, 18071-50-6; **3l**, 99282-19-6; **3m**, 99282-20-9; **3o**, 17756-86-4; 4-amino-2-hydroxybenzoic acid, 65-49-6; 4-azido-2-hydroxybenzoic acid, 66761-27-1.

FMN Reductase Catalyzed Regeneration of NAD(P) for Use in Enzymatic Synthesis

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The synthetic utility of horse liver alcohol dehydrogenase (HLADH) in the oxidation of alcohols to many potentially useful chiral synthons has been demonstrated.[1-3] These reactions and many other synthetically useful enzymatic oxidations require the cofactor nicotinamide adenine dinucleotide, NAD (or nicotinamide adenine dinucleotide phosphate, NADP), which is reduced to NADH (or NADPH) during the course of the reaction. While several practical procedures are available for the regeneration of reduced nicotinamide cofactors NAD(P)H, the problem of regeneration of *oxidized* nicotinamide cofactors NAD(P) is still not completely solved.[4] The high cost of NAD(P) requires the regeneration of NAD(P) from NAD(P)H to make the process economically feasible. The procedure for NAD(P) regeneration generally considered best is that using flavin mononucleotide (FMN) with dioxygen as the ultimate oxidizing agent.[5,6] This procedure is convenient, the reaction is energetically favorable and, FMN is innocuous to enzymes. However, this *noncatalytic* regeneration system is not efficient enough for routine use in large-scale synthesis because large quantities of FMN and NAD(P) are required to achieve useful rates, and separation of products from the cofactors is inconvenient. Many NAD(P)-dependent reactions are carried out at a substrate concentration of 0.1–0.5 M in order to be practical and to avoid inhibition by substrate and product. At these concentrations the NAD(P) concentration must be set at 0.1–0.5 mM to achieve a high turnover number (1000 or more) and to minimize the cost of the cofactor. Since the rate for the noncatalytic oxidation of NADH with FMN is governed by the bimolecular rate constant 0.2 M^{-1} s^{-1},[6] the number of moles of product produced per day by the synthesizing enzyme can be calculated and the results are shown in Table I. These data demonstrate the the productivity using this nonenzymatic regeneration system is quite low, especially at the desired low concentration of cofactor.

We describe in this paper an improvement of this NAD regeneration system in which the enzyme FMN reductase (EC 1.6.8.1) from *Photobacterium fischerei* (ATCC 7744)[7] is added to the reactor (Scheme I). This enzyme catalyzes the oxidation of NAD(P)H by FMN, thereby increasing

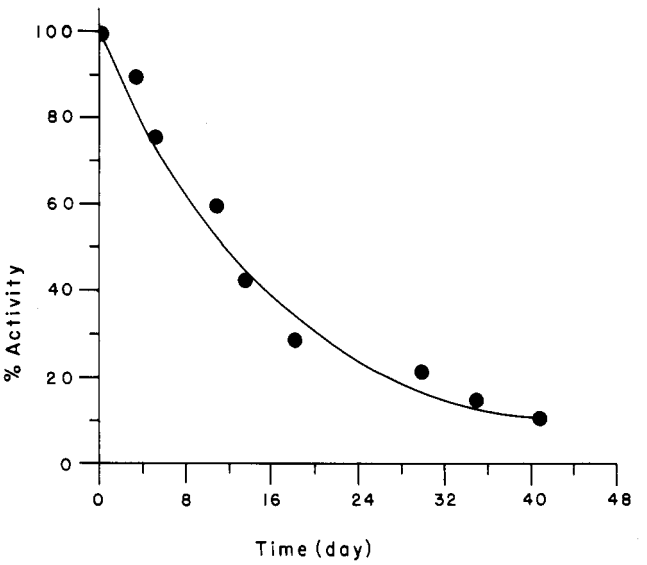
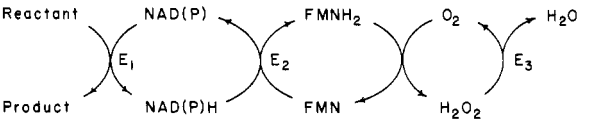
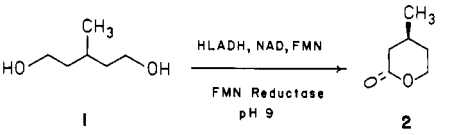


Figure 1. Stability of soluble FMN reductase in solution under air at pH 9.

Scheme I. FMN Reductase Catalyzed Regeneration of NAD(P): E_1, Horse Liver Alcohol Dehydrogenase; E_2, FMN Reductase; E_3, Catalase



Scheme II. HLADH-Catalyzed Oxidation Coupled with FMN Reductase Catalyzed Regeneration of NAD



the rate of the reaction and making the process more efficient. The productivity depends on the amount of enzyme used. In a typical reaction condition of 0.1 mM NAD, 10 mM FMN, and 10 mg/L of FMN reductase, a productivity of 4×10^2 mmol/day/L can be accomplished. Without the enzyme the productivity would be only 17 mmol/day/L under otherwise identical conditions. This comparison and the comparison of productivities under other conditions is shown in Table I.

Isolation of the enzyme FMN reductase used in this study was straightforward. Eight hundred grams of wet cells would produce about 260 units of the enzyme. The enzyme is also available commercially from Boehringer but is quite expensive.

The relative activity of the enzyme was determined at pHs 7, 8, and 9 with NADH and FMN as substrates. The enzyme had the greatest activity at pH 7, with 87% of this activity at pH 8 and 72% of this activity at pH 9. This indicates that the enzyme is sufficiently active to be useful synthetically at all of these pH regions. The high activity at alkaline region is particularly useful when the FMN reductase catalyzed reaction is coupled with the HLADH-catalyzed oxidations which have an optimal activity at pH 9.[5,6] The enzyme also shows comparable activity using NADPH as a substrate.[8] Stability studies

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Table I. Comparison of Enzymatic and Nonenzymatic Oxidation of NADH with FMN

concn, mM		rate (V)		productivity, mmol/L/day		
		nonenzymatic,[a]	enzymatic,[b]		enzymatic	
FMN	NADH	mM min^{-1}	μmol/min/μmol enz	nonenzymatic	10 mg enz	100 mg enz
1[c]	0.1[d]	1.2×10^{-3}	1.2×10^{3}	1.7	4.0×10^{2}	4.0×10^{3}
10[c]	0.1[d]	1.2×10^{-2}	1.2×10^{3}	1.7×10	4.0×10^{2}	4.0×10^{3}
20[c]	0.1[d]	2.4×10^{-2}	1.2×10^{3}	3.4×10	4.0×10^{2}	4.0×10^{3}
10[c]	0.5[c]	6.0×10^{-2}	2.1×10^{3}	8.6×10	7.2×10^{2}	7.2×10^{3}
20[c]	0.5[c]	1.2×10^{-1}	2.1×10^{3}	1.7×10^{2}	7.2×10^{2}	7.2×10^{3}

[a] The second-order rate constant for the nonenzymatic reaction at pH 8.0 is 12 M^{-1} min^{-1}. [b] The specific activity of the FMN reductase from *Photobacterium fisheri* measured at V_{max} is 50 units/mg (K_m(NADH) = 0.08 mM; K_m(FMN) = 0.07 mM) and the molecular weight of the enzyme is 43 000 (see ref 7 and 8). 1 unit = 1 μmol product formed per min. [c] At this concentration, the enzyme is saturated with the substrates and operated at its V_{max}. [d] At this concentration, the enzyme is operated at its $0.56V_{max}$.

indicate that the enzyme is quite stable, the soluble FMN reductase having a half-life of about 11 days in solution (Figure 1). No loss of activity was observed when the immobilized enzyme was stored in a refrigerator for 2 months.

To demonstrate this regeneration for use in enzymatic synthesis, the HLADH-catalyzed oxidation of 3-methylpentane-1,5-diol (1) was carried out. This reaction has been demonstrated previously by using the nonenzymatic regeneration system.[3] The reaction (Scheme II) was carried out on a scale of 75 mmol in a 1.5 L buffer solution containing 0.1 mM NAD and 1 mM FMN at pH 9. The enzymes HLADH and FMN reductase were immobilized on polyacrylamide gels and added to the reactor. The enzyme catalase was added to destroy the H_2O_2 produced upon oxidation of $FMNH_2$. Upon completion of the reaction the product was isolated by extraction and the immobilized FMN reductase was recovered in 82% yield.

This enzymatic regeneration system should be a significant improvement over the nonenzymatic system used previously. The system is convenient, efficient, and practical. It can be used in the regeneration of both NAD and NADP and it can be used over a range of pH values commonly used in organic synthesis. It saves greatly in the amount of both NAD(P) and FMN required and it can allow the reaction to go at a greater rate if sufficient amounts of enzyme are used.

Experimental Section

Isolation of FMN Reductase. *Photobacterium fischeri* cells were grown in a fermenter at 24 °C in an artificial seawater medium. Cells harvested were frozen and thawed to facilitate cell lysis. An 817-g sample of this cellular material was slurried in 285 mL of 3% NaCl, causing cell lysis to occur. The slurry was poured into 8 L of cold distilled water with stirring. The mixture was centrifuged to remove cellular debris. An assay of the crude mixture indicated 7.6×10^7 units corresponding to 2235 mg of luciferase enzyme.[7]

DEAE cellulose (0.22 g per g of initial weight of cells) was added to the viscous supernatant and the pH maintained at 7 by addition of 0.5 N acetic acid. After 15 min the material was filtered through Microcloth. The luciferase and reductase activity was eluted from the DEAE cellulose with phosphate buffer. Seventy-six percent of the total luciferase activity was recovered in the eluant. The protein was precipitated by the addition of ammonium sulfate (75% saturation). The suspension was centrifuged at 8000 rpm (JA 10, 11300*g*) for 45 min. The precipitate was suspended in 0.35 M phosphate buffer, pH 7, and dialyzed overnight against 410 mL of the same buffer. The enzyme was stored frozen awaiting final purification.

Final purification was carried out by column chromatography on a DEAE Sephadex (A-50) column (3.8 × 65 cm) using 0.35 M potassium phosphate buffer, pH 7. In a representative procedure, 170 mL of the previous extract was loaded onto the column. FMN reductase and luciferase were separated, the reductase eluting first. The fractions (elution volume 630–980 mL) containing reductase activity were pooled, precipitated with 80% ammonium sulfate, and centrifuged at 14000 rpm (JA 14, 30000*g*) for 45 min. The precipitate was resuspended in 10 mL of 0.1 M phosphate buffer, pH 7, and stored in a freezer. The suspension demonstrated a total reductase activity of 110 units with substrate concentration of 0.11 mM NADH and 0.21 mM FMN at pH 9. This corresponds to a total of 265 units of enzyme from the initial 817 g of cells. The suspension was diluted with 150 mL water and ultrafiltered at 4 °C using an Amicon Model 8200 apparatus with a YM 10 semipermeable membrane filter (excludes M_r = > 10 000) using 40 psi of argon. The ultrafiltration took about 3 h and the resulting solution was then used in the immobilization.

Immobilization of Enzymes. Horse liver alcohol dehydrogenase and catalase were immobilized in polyacrylamide gels as described previously.[8] FMN reductase was immobilized by a similar procedure as described below. PAN 1000 (3 g) was placed in a 50-mL beaker and 12 mL of 0.2 M Hepes buffer, pH 7.5, containing 1.5 mM NAD, 0.5 mM FMN, and 15 mM $MgCl_2$ was added. The polymer was dissolved within 1 min by magnetic stirring and mechanical grinding with a spatula or glass rod. A solution of 0.5 M DTT (150 μL) and 2.55 mL of 0.5 M TET were added with stirring. After 10 s 1 mL of a solution containing 10 units of FMN reductase was added. In about 45 s the solution formed a resilient gel. The gel was allowed to stand for 1 h at room temperature and transferred to a mortar. The gel was ground with a pestle, and the particles were suspended in 150 mL of 50 mM Hepes buffer, pH 7.5, containing 50 mM ammonium sulfate. The suspension was agitated with a magnetic stirrer for 15 min and transferred to centrifuge tubes, and the gel was separated by centrifugation at 2000 rpm (5.2*g*). The washing procedure was repeated twice with Hepes buffer containing no ammonium sulfate and the gel was suspended in the same buffer. The gel and washes were assayed for enzyme activity by using 0.11 mM NADH and 0.21 mM FMN in 0.05 M glycine–NaOH buffer, pH 9.0, by measuring the change in absorbance at 340 nm. FMN reductase activity of 3.2 units (32%) was found in the gel and 1.8 units (18%) were recovered in the combined wash solutions.

Test of pH Dependence of Activity. The activity was tested at pH 7 in 50 mM sodium phosphate buffer, at pH 8 in 0.1 M Tris buffer, and at pH 9 in 50 mM glycine–NaOH buffer. To 1 mL of each of the buffer solutions in a cuvette containing 0.107 mM NADH and 0.212 mM FMN was added 0.15 unit of FMN reductase. After addition of the enzyme the activity was monitored by measuring the decrease in absorbance at 340 nm (disappearance of NADH) vs. time by using a Beckman DU-6 UV/vis spectrophotometer. Initial rates were calculated and compared.

Test of Relative Activity with NADH and NADPH. To 1 mL of 0.05 M glycine–NaOH buffer, pH 9, containing 0.107 mM NADH and 0.212 mM FMN was added 20 μL of an FMN reductase solution (0.15 unit). To 1 mL of the same buffer containing 0.212 mM FMN and 0.107 mM NADPH was added 0.15 unit of FMN reductase (20 μL). The activity was followed by measuring the decrease in absorbance at 340 nm as a function of time. The initial rates were the same within experimental error.

Stability Study of FMN Reductase. A solution of 3 units of FMN reductase in 200 μL 0.05 M glycine–NaOH buffer, pH 9, was incubated at room temperature. A 10-μL aliquot was taken periodically and added to a cuvette containing 1 mL of the pH 9 buffer containing 0.107 mM NADH and 0.212 mM FMN. The

change in absorbance at 340 nm was measured as before and initial rates were calculated.

Preparation of 3-Methylpentane-1,5-diol (1). 3-Methylglutaric anhydride (16 g, 125 mmol) from Aldrich and $LiAlH_4$ (7.2 g, 190 mmol) were refluxed in dry THF for 4 h. The solution was cooled, quenched with 70 mL of saturated aqueous NH_4Cl, and decanted from the residual sluge. The sluge was washed with methylene chloride. The organic phases were combined, dried with $MgSO_4$, and evaporated. The residual was extracted into acetone and the acetone evaporated. Distillation of the resulting oil under reduced pressure yielded 1 (10.1 g, 68%): bp 83–85 °C (0.1 torr) [lit.[3] bp 139–146 °C (17 torr)]; NMR ($CDCl_3$) δ 3.6–4.0 (6 H, s overlapping t, J = 6.6 Hz), 1.3–1.9 (5 H, m), 0.9 (3 H, d, J = 5.9 Hz).

HLADH-Catalyzed Oxidation of 3-Methylpentane-1,5-diol (1). Soluble Enzyme System. The diol 1 (1.5 g, 12.7 mmol), NAD (11.2 mg, 0.02 mmol), and FMN (0.5 g, 1 mmol) were dissolved in 0.05 M glycine–NaOH buffer (200 mL, pH 9). The pH of the resulting solution was adjusted to 9 with 4 M NaOH. HLADH (25 mg) and FMN reductase (10 units) were added and the solution was kept at room temperature. The pH was readjusted to 9 periodically with 4 M aqueous NaOH. The progress of the reaction was monitored by removing a 2-μL sample from the reactor and adding to 1 mL of glycine buffer, pH 9, containing 1.4 M NAD in a cuvette. The enzyme HLADH (1 unit) was added to the cuvette and the subsequent increase in absorbance at 340 nm was measured. From this the concentration of substrate remaining in the reactor was calculated. Upon completion of the reaction in 1 day the pH was raised to 12 with 4 M aqueous NaOH, and the solution was continuously extracted with chloroform for 2 days. The aqueous solution was acidified to pH 3 with 12 M HCl and continuously extracted with chloroform for 1 day. Evaporation of the chloroform yielded 2 in 68% yield (see the following for characterization).

Large-Scale HLADH-Catalyzed Oxidation of 3-Methylpentane-1,5-diol (1). Immobilized-Enzyme System. The diol 1 (8.9 g, 75 mmol), NAD (0.114 g, 0.15 mmol), and FMN (0.755 g, 1.5 mmol) were dissolved in 0.05 M glycine–NaOH buffer (1.5 L, pH 9). The immobilized enzymes HLADH (16 units), FMN reductase (9 units), and catalase (100 units) were added. The pH of the solution was readjusted to 9 with 4 M NaOH. The solution was agitated with a magnetic stirrer at room temperature. The pH was readjusted to 9 occasionally by adding 4 M NaOH. The progress of the reaction was monitored by assaying the remaining substrate using HLADH as described for the soluble enzyme reaction. After 14 days the assay procedure showed little substrate remaining. The contents of the reactor were centrifuged and the liquid was decanted from the enzyme containing gel. The gel was washed with 200 mL of distilled water and the suspension was again separated by centrifugation. The washing procedure was repeated with a second 200-mL portion of water and the washes were combined with the reactor solution. The solution was evaporated to 400 mL under reduced pressure. The pH of the solution was raised to 12 with 10 M NaOH. The solution was continuously extracted with $CHCl_3$ for 2 days. The aqueous solution was then acidified to pH 3 with 12 M HCl and continuously extracted with $CHCl_3$ for 1 day. Evaporation and distillation of the latter extract gave (–)-(3*S*)-3-methylvalerolactone (2) (5.85 g, 68% yield): bp 121–123 (0.2 torr) [lit.[3] bp 93–94 (0.02 torr)]; $[\alpha]^{27}$ –24.8° (c 5.6, $CHCl_3$) (90% optical purity); NMR ($CDCl_3$) δ 4.3 (2 H, m), 1.1–2.8 (5 H, m), 1.05 (3 H, d).

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Registry No. 1, 4457-71-0; 2, 61898-56-4; NAD, 53-84-9; NADP, 53-59-8; NADH, 58-68-4; NADPH, 53-57-6; FMN, 146-17-8; FMN-reductase, 39346-42-4; catalase, 9001-05-2; alcohol dehydrogenase, 9031-72-5; 3-methylglutaric anhydride, 4166-53-4.

New Method for Preparation of Superoxide Ion by Use of Amino Oxide

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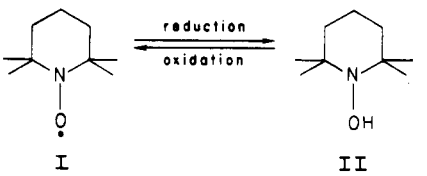
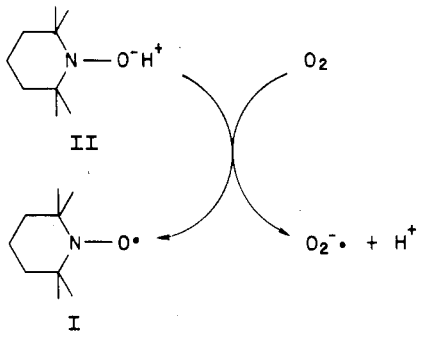
2,2,6,6-Tetramethylpiperidine-1-oxyl (I) is known as a stable radical[1] and is used as a spin trap[2] or spin label[3] reagent. As shown in eq 1, nitroxyl radical I is reduced



I II (1)

to the corresponding hydroxylamine II with ascorbic acid,[4] phenylhydrazine,[5] or hydrazine.[6] The hydroxylamine II in turn can be oxidized with Ag_2O,[7] PbO_2,[8] $NaIO_4$,[8] or $Pb(OAc)_4$[7] to the corresponding nitroxyl radical, demonstrating a reversible redox reaction between I and II.

Hydroxylamine II may be obtained as a colorless crystals which are very sensitive to air. Solutions of II in solvents containing small amounts of dissolved oxygen are oxidized especially easily. This fact suggests that II is oxidized by oxygen to give I and oxygen itself may be converted to superoxide ion by a one-electron reduction as shown in eq 2.



(2)

We now report a new method for the preparation of superoxide ion by use of an analogue of II, 4-(benzyloxy)-1-hydroxy-2,2,6,6-tetramethylpiperidine (V),[9] as a reducing agent of oxygen. Compound V was prepared from 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (III) in two steps (eq 3).

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