Enzyme-Catalyzed Organic Synthesis: Regeneration of Deuterated Nicotinamide Cofactors for Use in Large-Scale Enzymatic Synthesis of Deuterated Substances[1]

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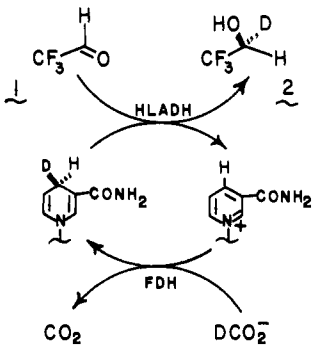
Abstract: We have developed procedures for in situ regeneration of deuterated nicotinamide cofactors based on ethanol-*1,1,-d*$_2$/alcohol dehydrogenase/aldehyde dehydrogenase, deuterioformate (DCO_2^-)/formate dehydrogenase, and glycolaldehyde-*1,2,2-d*$_3$/aldehyde dehydrogenase. These procedures can be used in the enzyme-catalyzed synthesis of deuterated organic products; representative procedures are illustrated by syntheses of (*R*)-trifluoroethanol-*1-d*$_1$ and L-glutamic-α-*d*$_1$ acid on 0.1-mol scales. For most synthetic applications, the first of these procedures (CH_3CD_2OH/ADH/AldDH) seems to provide the most practical method for in situ regeneration of deuterated nicotinamide cofactors. The high turnover numbers (TN $\simeq$ 1000) observed for the nicotinamide cofactors in these syntheses make possible the preparation of deuterated chiral substances with high isotopic purity, regardless of the stereochemistry of the hydride transfers to and from the nicotinamide cofactors.

Results and Discussion

We describe in this report several practical procedures for preparing deuterated substances on 0.1-mol scales with in situ regeneration of deuterated nicotinamide cofactors. The procedures are based on cofactor recycling systems which we and others have described previously for nondeuterated system.[2-4] Organic compounds isotopically labeled at chiral centers are particularly useful for studies of biochemical reaction mechamisms.[5,6] Nicotinamide cofactors with ^2H or ^3H labels in the C-4 position of the nicotinamide moiety have been prepared previously in small quantities ($\leq$0.1 mmol) and used to study the stereochemistry of enzyme-catalyzed hydride-transfer reactions.[7-11] Larger-scale enzymatic preparations of ^2H- or ^3H-labeled substances by procedures which involve labeled nicotinamide cofactors as the ^2H or ^3H source have not been described. Reactions which are stoichiometric in the nicotinamide cofactor are clearly economically impractical for this purpose; practical procedures must incorporate in situ cofactor regeneration. Both enzyme-catalyzed reductions of the nicotinamide cofactors and enzyme-catalyzed transfers of hydrogen (^2H, ^3H) from the cofactors to substrate are stereospecific, but these reactions may have *different* stereospecificities. Thus, any procedures used for cofactor regeneration should give high turnover numbers (TN) for the cofactors, to ensure that possible proton transfers from starting unlabeled cofactor in the first cycles of the reaction make an insignificant contribution to the isotopic purity obtained after many cycles. The enzyme formate dehydrogenase[12] (FDH, E.C. 1.2.1.2), for examples, catalyzes the transfer of the hydrogen bonded to carbon of formate to the *re* face of NAD and generates H_R; glucose 6-phosphate dehydrogenase (G-6-PDH) catalyzes the transfer of C_1-H of G-6-P to the *si* face of NAD and generates H_S; horse liver alcohol dehydrogenase (HLADH, E.C. 1.1.1.1) catalyzes the transfer of the H_R hydrogen from NADH to the *re* face of its aldehyde substrate.[13] Thus, preparation of (*R*)-trifluoroethanol-*1-d*$_1$ (**2**) from trifluoroacetaldehyde by a reaction sequence involving HLADH, G-6-PDH, and G-6-P-1-d (**10**) requires a high TN for NAD if it is to yield product with high isotopic purity, since the *first* cycle of reduction for each NAD generates unlabeled trifluoroethanol.

Scheme I. Synthesis of (*R*)-Trifluoroethanol-*1-d*$_1$ Using DCO_2^-/FDH for Cofactor Regeneration



One of the characteristics which determines the usefulness of a particular cofactor regeneration scheme for preparing deuterated substances is the availability of the deuterated reagents required as the ultimate deuterium donors. Equations 1-4 summarize simple procedures for the preparation of the deuterated reeagents we have used (or considered) for the in situ (re)generation of deuterated nicotinamide cofactors. All of these reactions have been described previously, and all are easily carried out on substantial scale in laboratory. If, however, only small quantities of these deuterated substances are required, several are commercially available (although expensive): deuterioformate (~$800/mol); perdeuterated ethanol (~$800/mol) glucose-*1-d*$_1$ (~$30 000/mol). Table I summarizes kinetic parameters for enzymes when these reagents for cofactor regeneration are used.

We have used $DCO_2^-Na^+$ (**3**) (prepared by the procedure of eq 1) with FDH to prepare **2** on 0.1-mol scale (Scheme I. The TN for NAD during this synthesis was 1000, and both the deuterium content and the enantiomeric excess (ee) of isolated **2** were >97% (a minimum number set by our analytical procedure: NMR spectroscopy of the MPTA ester (Figure 1)). This DCO_2^-/FDH cofactor regeneration system has the advantage that CO_2 is the only other product of the reaction, and that product isolation is particularly straightforward as a result. The disadavantages of

(1) Supported by National Institutes of Health Grants GM 30367 and GM 26543. Inquiries should be addressed to G.M.W. at Harvard University.
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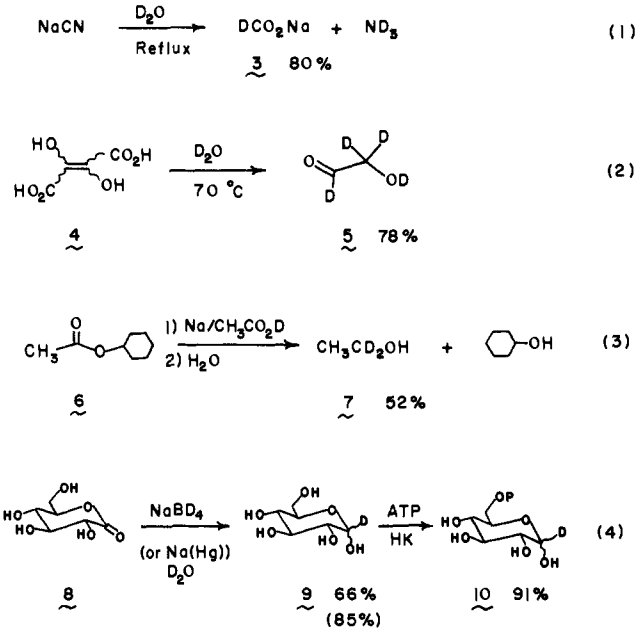


this system are that FDH from *C. boidinii* accepts only NAD and that the specific activity of this enzyme for DCO_2^- (1–2 U mg^{-1}) is relatively low (significantly lower than that for HCO_2^-, probably due to the kinetic isotopic effect in the hydride-transfer reaction).

Glycolaldehyde is a good substrate for aldehyde dehydrogenase (AldDH) and glycolaldehyde-*1,2,2,*-d_3 (**5**) is easily prepared by thermal decarboxylation of dihydroxymaleic acid or dihydroxyfumaric acid (**4**) in D_2O. Regeneration of deuterated NADH(D) with use of **5** and AldDH is illustrated by the synthesis of L-glutamic-α-d_1 acid (**11**; Scheme II). AldDH from baker's yeast accepts both NAD and NADP;[4] the system is therefore applicable in synthetic reactions which require either NAD or NADP (or both). The potential disadvantage of this system (relative to DCO_2^-/FDH) is that the byproduct glycolic acid may complicate the workup, although in practice we had no difficulties in isolation of **11** on 0.1-mol scale.

Use of ethanol-*1,1*-d_2 (**7**) as a source of deuterium is also illustrated by the synthesis of **11** (Scheme II). This reaction requires two enzymes (ADH and AldDH) for cofactor regeneration, and 2 equiv of deuterated NAD(P)H are generated from 1 equiv of **7**. The byproduct acetic acid does not complicate the workup. The enzyme ADH from baker's yeast has high specific activity for ethanol but is specific for NAD; that from *L. mesenteroides* accepts both NAD and NADP, but has lower specific activity. We believe this system is in most circumstances the best and most convenient for the regeneration of deuterated nicotinamide cofactors. For certain specific reactions this system is, however, not applicable; for example, it cannot be used for the synthesis of **2** because trifluoroacetaldehyde is a substrate for ADH and AldDH.

The preparation of glucose 6-phosphate-*1*-d_1 (**10**) is not as convenient as that of **3**, **5**, and **7**. We have, in fact, not used **10** and glucose 6-phosphate dehydrogenase (G-6-PDH) as a cofactor regeneration system for large-scale synthesis of deuterated substrates, but we point out that G-6-P/G-6-PDH has high specific activity for NAD and NADP, and that G-6-PDH is commercially available, inexpensive, and easily manipulated.

In conclusion, the cofactor regeneration systems we describe here for the preparation of deuterium-labeled compounds provide practical routes to products on scales larger than 0.1 mol. The enzymes used are commercially available and stable in immobilized form. These procedures are more efficient than those based on exchange reactions using flavoenzymes as catalysts for cofactor regeneration;[14] these latter reactions require long times to equilibrate solvent and substrate isotopically, and often require large volumes of D_2O for the reaction.

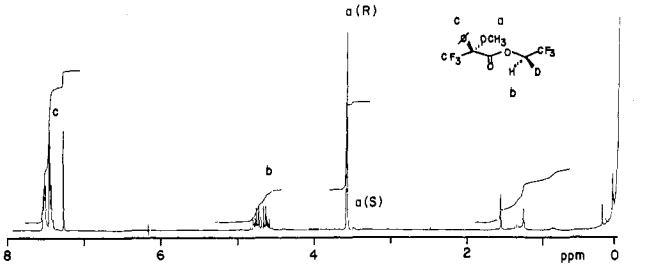
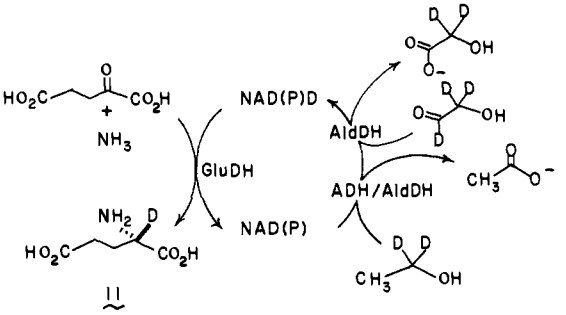


Figure 1. ^1H NMR (270 MHz) spectrum of the (*R*)-trifluoroethanol-1-d_1 MPTA ester of **2** in $CDCl_3$. The chemical shifts are expressed in ppm downfield from Me_4Si.

Scheme II. Synthesis of Glutamic-α-d_1 Acid Using Glycolaldehyde-*1,2,2*-d_3/AldDH or Ethanol-*1,1*-d_2/ADH/AldDH for Cofactor Regeneration



Experimental Section

General. Enzymes and biochemicals were from Sigma or Boehringer. Deuterium oxide (D_2O, 99.8% D) was from Merck. Deuterioacetic acid (CH_3CO_2D, 98% D) was from Sigma. Other reagents and solvents were reagent grade. Gas chromatography was performed at 150 °C on a 10-ft 10% Carbowax column. Enzyme immobilization followed the procedures described previously.[16] Enzymatic analysis was carried out following standard procedures.[17] NMR chemical shifts are expressed in ppm downfield from Me_4Si or DSS. The optical purity of CF_3CHDOH was determined by ^1H NMR spectroscopy following its conversion to a MPTA ester.[18] Racemic alcohol was prepared by reduction of CF_3CHO with $NaBD_4$ according to the procedure described previously.[2]

$DCO_2^-Na^+$ was prepared from sodium cyanide (NaCN) by a modification of the published procedure.[19] A mixture of NaCN (4.9 g, 0.1 mol), NaOH (0.2 g), and D_2O (20 g) was placed in a 100-mL flask equipped with a condenser and refluxed for 2 weeks. The outlet of the condenser was fitted with a drying tube containing anhydrous $CaCl_2$ to protect the reaction from contamination by atmosphere H_2O. After distillation to remove D_2O (14 g), the residue was dissolved in 50 mL of water and neutralized with 5 N HCl to pH 8.0. The concentration of DCO_2^- in this solution, determined by using FDH and NAD, indicated that 80 mmol of DCO_2^- had been obtained, corresponding to an 80% yield based on NaCN as starting material. This solution was used directly without further purification.

Ethanol-*1,1*-d_2 (**7**) was prepared according to the procedure reported previously[20] with slight modifications for large-scale preparation. Deuterioacetic acid (CH_3CO_2D, 122.2 g, 2 mol) and sodium (46 g, 2 g-atom) were added simultaneously over a period of 3 h to a solution containing cyclohexyl acetate (63.2 mL, 0.5 mol), diethyl ether (600 mL), and phenolphthalein (10 mg, pH indicator). The temperature of the reation mixture was maintained between 0 and −5 °C during the period of the

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Table I. Kinetic Parameters for Enzymes Used in Regeneration of Deuterated Nicotinamide Cofactors

reagent	enzyme	cofactor (K_m, mM)	specific activity (U mg^{-1})[a]
CH_3CH_2OH (CH_3CD_2OH)	ADH/AldDH (baker's yeast)	NAD (0.74[b]/0.03[c])	80[c] (50)
	(*L. mesenteroides*/ baker's yeast)	NAD (0.5[d]/0.03[c])	70[d] (40)
		NADP (0.085[d]/0.03[c])	8[d] (5)
HCO_2Na (DCO_2Na)	FDH (*C. boidinii*)	NAD (0.09)[e]	3[e] (1.6)
glycolaldehyde (glycolaldehyde-*1,2,2-d*$_3$)	AldDH (baker's yeast)	NAD (0.029)[e]	45[c] (30)
		NADP (0.05)[c]	5[c] (3)
G-6-P-*1-d*$_1$	G-6-PDH (*L. mesenteroides*)	NAD (0.1)[f]	700[f] (400)
		NADP (0.006)[f]	400[f] (240)

[a] 1 U = 1 μmol of NAD(P)H formed per min at $\bar{V}_{max}$. The number in parentheses is the value obtained in this laboratory at pH 7.6 (0.1 M tri(2-hydroxyethyl)amine buffer) using deuterated substrate and NAD in concentrations >20K_m so that the reaction rate is near $\bar{V}_{max}$. [b] Wratten, C. C.; Cleland, W. W. *Biochemistry* **1963**, *2*, 935–41. [c] Bradbury, S. L.; Clark, J. F.; Steinman, C. R.; Jakoby, W. B. *Methods Enzymol.* **1975**, *41*, 354–60. [d] Hatanaka, A.; Adachi, O.; Chiyonobu, T.; Ameyama, M. *Agric. Biol. Chem.* **1971**, *35*, 1304–6. [e] See ref 12. [f] See ref 13.

addition. The rate of the addition was controlled so that the mixture was kept slightly acidic. After addition had beem completed, the mixture was stirred at 25 °C for at least 10 h, then the reaction was stopped by adding cold water (30 mL) slowly. The precipitate was removed by filtration and solid material washed with 200 mL of cold ether. The combined filtrates were distilled slowly through a 20-cm Vigreux column and **7** ws collected in the boiling range 78–79 °C. This material (15 mL, 52% yield based on cyclohexyl acetate) showed no α-proton (δ 3.5) in its ^{1}H NMR spectrum; a multiplet centered at 1.2 ppm arises from the methyl protons. Enzymatic analysis using ADH indicated that the sample contained 91% ethanol.

Glycolaldehyde-*1,2,2-d*$_3$ (5). Compound **4** (17 g, 113 mmol) suspended in 100 mL of D_2O was incubated at 70 °C in an oil bath. The material dissolved very rapidly at this temperature. In order to avoid contamination by adventitious moisture, the flask was fitted with a reflux condenser connected to a drying tube containing anhydrous $CaCl_2$. The progress of the reaction was determined by measuring the concentration of **5** with use of AldDH and NAD. After 6 h, 88 mmol of **5** had been produced, corresponding to 78% yield on the basis of **4** as starting material.

Glucose 6-Phosphate-*1-d*$_1$ (10). To a solution (50 mL, pH 7.0) containing $MgCl_2$ (6 mM) and ADP (2mM) was added phosphoenol pyruvate monopotassium salt K^+PEP^-, 2.3 g, 11 mmol)[21] and **9** (1.8 g, 10 mmol, from Stohler).[22] The solution was adjusted to pH 7.0 by adding 2 N KOH and deoxygenated by introducing a stream of argon for 10 min. Hexokinase (HK, E.C. 2.7.1.1, 200 U) and pyruvate kinase (PK, E.C. 2.7.1.40, 240 U) coimmobilized in 3 mL of PAN gel were added to the solution and the mixture was kept under argon and stirred at 25 °C with pH automatically controlled at 7.0. The reaction was complete in 6 h. After separation of the enzyme-containing gel, the solution was mixed with $BaCl_2$ (2.9 g, 14 mmol), followed by addition of ethanol (250 mL). The precipitated material (5.1 g) contained 9.1 mmol of BaG-6-P-*d*$_1$·7H_2O (92% pure) corresponding to a 91% yield on the basis of **9**.

(R)-Trifluoroethanol-*1-d*$_1$ (2). To a 1-L solution containing trifluoroacetaldehyde dihydrate (11.6 g, 0.1 mol), DCO_2Na (0.11 mol), and NAD (0.1 mmol) was added HLADH (20 mL of PAN gel containing 20 U of HLADH on the basis of trifluoroacetaldehyde as substrate) and FDH (10 mL of gel containing 40 U of enzyme on the basis of HCO_2Na as substrate). The reaction mixture was stirred under argon for 10 days with the pH maintained at 7.6. After the enzyme-containing gel was separated, the solution was mixed with 5 g of sodium bisulfite and distilled through a Vigreux column (2 × 40 cm). The fractions with boiling range 78–80 °C were collected (18 mL); these contained 72 mmol of **2** as determined by using HLADH. The combined fractions were redistilled through the same column to obtain 6.7 g of liquid (containing 60 mmol of **2**, 60% yield on the basis of trifluoroacetaldehyde). The sample showed the same GC retention time as trifluoroethanol (with a N_2 flow rate of 40 mL min^{-1}, the retention time was 196 s). No effort was made to remove the water present in the sample (~10% by weight). The MPTA derivative indicated that the ee was >97% (Figure 1).

L-Glutamic-α-*d*$_1$ Acid (11). To a solution (1 L) containing α-ketoglutarate monosodium salt (17 g, 0.1 mol), KCl (7.5 g, 50 mmol), NAD (0.1 mmol), and **5** (0.11 mol) was added ammonium hydroxide until the solution had pH 7.6. Separately immobilized GluDH (120 U in 2 mL of PAN gel) and AldDH (130 U in 2 mL of gel) were added to the solution, and the mixture was stirred under argon with pH controlled at 7.6. Enzymatic analysis indicated that the reaction was complete in 46 h, and 96 mmol of **11** was produced. After separation of the enzyme-containing gel, the solution was acidified to pH 4.0 with 5 N HCl and concentrated under reduced pressure to 20 mL. A crystalline material come out during the concentration. After being kept in a refrigerator at 5 °C overnight, the product was isolated by filtration. The crystalline solid (13.2 g) contained 86 mmol of **11** (96% purity determined by GluDH). The ^{1}H NMR spectrum of the material in D_2O, compared with that of unlabeled glutamic acid, showed that the α-H (δ 3.8) was missing, and indicated that the deuterium content at the position was >97%. The enzymes with the following percentages of their original activity remaining were recovered: GluDH, 92%; AldDH, 93%.

A very similar reaction was carried out except that **5** was replaced with **7** (2.8 g, 55 mmol, 93% pure) and yeast ADH (100 U in 0.5 mL of gel) was added to the reactor. The reaction was complete in 2 days and **11** was isolated as described in 91% yield with deuterium incorporatioon >97%. The recovered enzymatic activities were as follows: ADH, 81%; AldDH, 90%; GluDH, 92%.

Registry No. **1**, 75-90-1; **2**, 85956-73-6; **3**, 3996-15-4; **4**, 13096-38-3; **5**, 85956-74-7; **6**, 622-45-7; **7**, 1859-09-2; **8**, 90-80-2; **9**, 10390-17-7; **10**, 85956-75-8; **11**, 24778-46-9; HLADH, 9031-72-5; AldDH, 9028-86-8; FDH, 9028-85-7; G-6-PDH, 9001-40-5; NaCN, 143-33-9; α-ketoglutarate·Na, 22202-68-2.

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