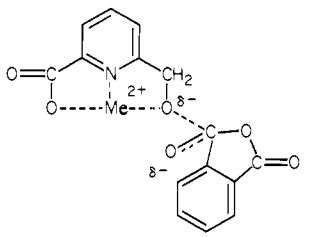
have a $k_0'$ much larger than that of $Ni^{2+}$ or $Zn^{2+}$.

These findings are quite important in regard to possible mechanisms for carboxypeptidase A catalyzed hydrolysis of esters. Breakdown of an anhydride intermediate appears to be rate limiting in enzyme-catalyzed hydrolysis of substituted cinnamoyl-L-β-phenyllactate esters.[5-7] The $k_{cat}$ vs. pH profiles suggest both $OH^-$- and $H_2O$-catalyzed reactions that are very likely promoted by the zinc ion. The observation of such reactions in hydrolysis of II, with which metal ion binding is strong, shows that a water reaction can be competitive with metal ion promoted $OH^-$ catalysis.[26]

The rate constants for hydrolysis of II at saturating concentrations of metal ions are approximately 4 orders of magnitude greater than in the intramolecular carboxyl nucleophilic reaction of 2-(6-carboxypyridyl)methyl hydrogen phthalate, with which the leaving group has a $pK_a$ comparable to that of β-phenyllactic acid.[14] The steric fit of the carboxyl and the carbonyl group of a phthalate monoester is, of course, excellent. Metal ions bind strongly to the ester and catalyze the nucleophilic reaction through a transition-state effect in which the leaving group is stabilized (VI). The rate enhancements in the metal ion catalyzed reactions

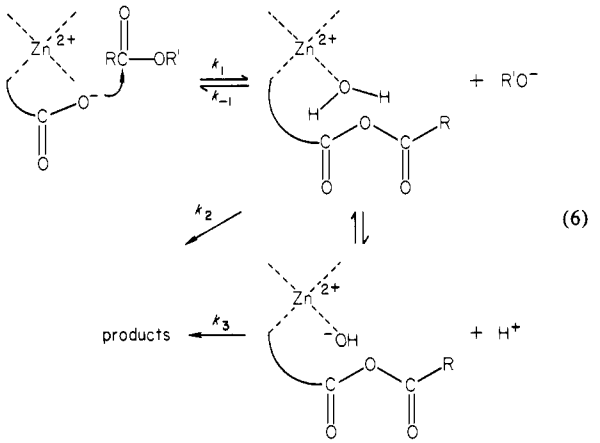


VI

are however comparable in the carboxyl nucleophile reaction and in water-catalyzed hydrolysis of the anhydride II($\sim 10^2$).

(26) The possibility of a metal ion promoted water reaction in carboxypeptidase A catalyzed reactions is in accord with the proposed structure of the metal ion, anhydride intermediate complex: Kuo, L. C.; Makinen, M. W. *J. Biol. Chem.* **1982**, *257*, 24.

Therefore, in view of the reactivity of mixed cinnamic acid anhydrides, for anhydride hydrolysis to be rate determining in carboxypeptidase A catalyzed hydrolysis of cinnamate esters, either Glu-270 attack (anhydride formation) is facilitated to an extent not duplicated in the chemical reactions of the phthalate monoester (VI) and/or the enzymatic reaction is reversible as in the simplified scheme of eq 6 with $k_{-1} > k_2$. Phenyllactic acid is bound strongly



(6)

in the active site ($K_i$ = 5.8 × $10^{-5}$ M at pH 7.5)[8] and should be in excellent position to reverse the reaction, although reversibility has not as yet been detected in reactions catalyzed by carboxypeptidase A.[7,27]

**Acknowledgment.** This work was supported by research grants from the National Institutes of Health.

**Registry No.** I, 83693-14-5; II, 83693-15-6; III, 83693-16-7; IV, 25388-78-7; $Ni^{2+}$, 14701-22-5; $Co^{2+}$, 22541-53-3; $Zn^{2+}$, 23713-49-7; $Cu^{2+}$, 15158-11-9; cinnamoyl chloride, 102-92-1; 2,6-pyridinedicarboxylic acid, 499-83-2.

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# Equilibration of 1-Octanol with Alcohol Dehydrogenase. Evidence for Horse Liver Alcohol Dehydrogenase Responsibility for Exchange of the 1-*pro-S* Hydrogen Atom‡

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*Contribution from the Worcester Foundation for Experimental Biology, Inc., Shrewsbury, Massachusetts 01545.* *Received July 12, 1982*

**Abstract:** Equilibration of hydrogen atoms of 1-octanol with water, mediated by the system horse liver alcohol dehydrogenase–NAD/NADH–diaphorase, involves a rapid exchange of 1-*pro-R* hydrogen atoms and a slow exchange of 1-*pro-S* hydrogen atoms. Yeast alcohol dehydrogenase has an apparent absolute stereospecificity for the 1-*pro-R* hydrogen atom of 1-octanol; replacement of horse liver alcohol dehydrogenase by yeast alcohol dehydrogenase in the above system results in exchange of only the 1-*pro-R* hydrogen atom of 1-octanol. In the absence of horse liver or yeast alcohol dehydrogenase, no exchange of C-1 hydrogen atoms of 1-octanol occurs. Thus, horse liver alcohol dehydrogenase is directly responsible for promoting exchange of the 1-*pro-S* hydrogen atom of 1-octanol with water hydrogen atoms.

The equilibration of C-1 hydrogen atoms of primary alcohols with water, mediated by horse liver alcohol dehydrogenase–NAD/NADH–diaphorase, has been presumed to involve the stereospecific exchange of the 1-*pro-R* hydrogen atom of the alcohol.[1,2] Recently we found that this reaction is *not* stereospecific: the 1-*pro-S* hydrogen atom of 1-octanol is also exchanged,

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‡ Dedicated to Prof. E. Lederer on his 75th birthday.

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albeit at a much slower rate than the 1-*pro-R* hydrogen.[3-7] We now present evidence that horse liver alcohol dehydrogenase is directly responsible for promoting exchange of the 1-*pro-S* hydrogen atom of 1-octanol with solvent hydrogen atoms. We further show that in contrast to equilibration in the system containing horse liver alcohol dehydrogenase; the system yeast alcohol dehydrogenase–NAD/NADH–diaphorase catalyzes the *stereospecific* exchange of *only* the 1-*pro-R* hydrogen atom of 1-octanol with water hydrogen atoms.

## Experimental Section

**Chemicals and Instrumentation.** Horse liver alcohol dehydrogenase (EC 1.1.1.1, lots 128C-8050, 1.9 U/mg protein and 40F-8015, 2 U/mg protein), yeast alcohol dehydrogenase (lots 29C-8135, 338 U/mg solid and 81F-82051, 365 U/mg solid), chicken ovalbumin (grade V), NAD (grade III), NADH (grade III), flavin mononucleotide (FMN) (commercial grade, 95–97%), DL-$\alpha$-lipoamide, and deuterium oxide (99.8 atom % $^2H$) were purchased from Sigma Chemical Co. (St. Louis, MO). Porcine heart diaphorase (EC 1.6.4.3, grade I, control nos. 1319322/Sept 1980, 1509322/Jan 1981, 1360124/Oct 1981, 1271324/Aug 1982, and 1421324/Nov 1982) was obtained from Boehringer Mannheim GmbH (Mannheim, West Germany). $^3H_2O$ (1 Ci/mL) was obtained from International Chemical and Nuclear Corp. (ICN) (Irvine, CA) and lithium aluminum deuteride (99 atom % $^2H$) from Kor Isotopes (Cambridge, MA). (−)-Camphanoyl chloride was a product of Fluka A. G. (Zürich, Switzerland), and Eu(dcm)$_3$ [tris(*d,d*-dicampholylmethanato)-europium(III)] a product of Alfa/Ventron (Danvers, MA). Silica gel 60 HF 254 + 366, purchased from E. Merck A. G. (Darmstadt, West Germany), was used for thin-layer chromatography.

Radioactive samples were counted with a Mark II liquid scintillation system (Nuclear-Chicago, Des Plaines, IL) in 15 mL of Liquifluor (New England Nuclear, Boston, MA). Analytic GC was performed on a Model 3700 gas chromatograph (Varian Associates, Inc., Walnut Creek, CA) fitted with a Varian stainless steel 3% OV-17 Chromosorb W-HP column (80–100 mesh, 2-mm-width × 2-m length) and a flame ionization detector. Mass spectra were recorded with a Model 12-90G mass spectrometer equipped with a DA/CS I.2 data analysis/control system (Nuclide Corp., State College, PA). Proton magnetic resonance spectra were recorded on a Model EM 390 90-MHz spectrometer equipped with a CAT scanner (Varian Associates, Inc.). Deuterium magnetic resonance spectra were recorded at 38 MHz on a Model WM-250H spectrometer (Brucker Instruments, Inc., Billerica, MA). Melting points were determined on a Monoscop IV hot stage apparatus (H. Bock, Frankfurt am Mein, West Germany), and are corrected. The pD deuterium oxide based reaction mixtures was determined by measuring the pH with a Model E-5M glass electrode (Fisher Scientific Co., Pittsburgh, PA) and applying the formula pD = pH + 0.4.[8,9]

(1*RS*)-[1-$^3$H,-1-$^1$H;1-$^{14}$C]Octanol was synthesized from [1-$^{14}$C]octanal and lithium borotritide as previously described.[4] (1*RS*)-[1-$^2$H,1-$^1$H]Octanol and [1,1-$^2H_2$]octanol were prepared by reduction of octanal and methyl octanoate, respectively, with lithium aluminum deuteride in diethyl ether.

**Synthesis of (1*RS*)-[1-$^2$H]Octyl (−)-Camphanate.** (1*RS*)-[1-$^2$H]Octanol (130 mg, 1 mmol) was added to a solution of (−)-camphanoyl chloride (260 mg, 1.2 mmol) in pyridine (2 mL) and stirred for 4 h. After the mixture was diluted with cold water (15 mL), the product was extracted into ether (3 × 25 mL). The ether extract was successively washed with 5% HCl (3 × 10 mL), 5% $NaHCO_3$ (3 × 10 mL), and water (3 × 10 mL). The ether solution was dried ($Na_2SO_4$), concentrated in vacuo to a gummy residue, and purified by thin-layer chromatography [20 × 40 cm plate; hexane–ethyl acetate (3:1)] to yield (1*RS*)-[1-$^2$H]octyl (−)-camphanate (240 mg, 0.77 mmol): $^1$H NMR ($CCl_4$) $\delta$ 0.92 (s, $CH_3$), 1.02 (s, $CH_3$), 1.06 (s, $CH_3$), 1.3 (br s, $CH_2$), 4.20 (C-1 H, t, $J$ = 6 Hz). Upon addition of 1 molar equiv of Eu(dcm)$_3$, the triplet at $\delta$ 4.20 was resolved into a pair of triplets, $\delta$ 5.56 ($H_R$) and $\delta$ 5.86 ($H_S$) (Figure 1A).

**Diaphorase Activity.** Porcine heart diaphorase was assayed as lipoamide dehydrogenase.[10] Reactions were initiated in cuvettes containing

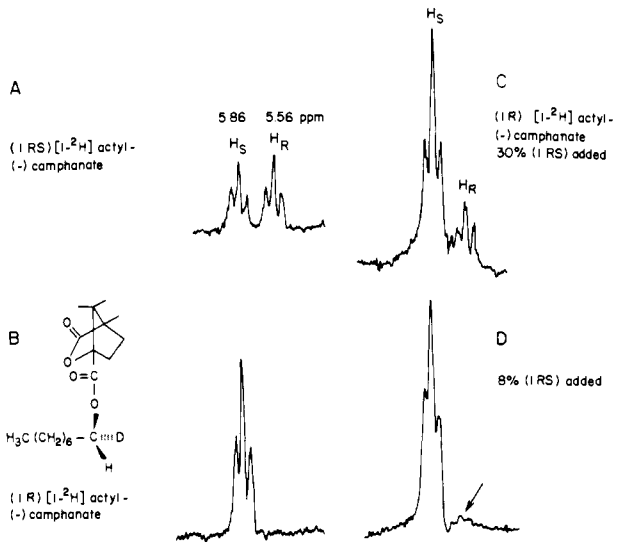


**Figure 1.** Proton NMR spectra of the 4–6 ppm region of solutions of C-1 monodeuterated octyl (−)-camphanates in $CCl_4$ (400 $\mu$L) containing 1 molar equiv of Eu(dcm)$_3$. Sweep width, 5 ppm: (A) synthetic 1*RS* ester (8 mg); (B) enzymically derived (see text) 1*R* ester (6.2 mg); (C) mixture of B (8 mg) containing A (30%); (D) mixture of B (10 mg) containing A (8%).

2.8 mL of 71 mM potassium phosphate buffer (pH 6.5[11]), 0.1 mL of 8.3 mM NADH in 50 mM potassium phosphate (pH 7.2), and 0.1 mL of 48.7 mM DL-$\alpha$-lipoamide in 95% ethanol by addition of 0.8–4.6 $\mu$g of diaphorase (10–60 $\mu$L of commercial suspension appropriately diluted with 0.1 M potassium phosphate, pH 6.5). Initial velocities of NADH oxidation were plotted and extrapolated to give a specific activity of 138 U/mg protein for the undiluted commercial suspensions (1 U = 1 $\mu$mol NADH oxidized per minute) at 20 °C.

**Exchange Procedures Utilizing (1*RS*)-[1-$^3$H;1-$^{14}$C]Octanol. A. Horse Liver Alcohol Dehydrogenase–NAD/NADH–Diaphorase.** To a 30-mL serum bottle containing 10 mL of Günther–Simon[12,13] buffer (77 m*M* potassium phosphate + 3.4 mg $Na_2EDTA{\cdot}2H_2O$, pH 8.0)[12,13] were added ovalbumin (7 mg), NAD (0.62 $\mu$mol), NADH (0.62 $\mu$mol), horse liver alcohol dehydrogenase (10 mg, 19–20 U), and porcine heart diaphorase (0.13 mL, 1.3 mg, 180 U). The bottle was flushed with $N_2$ (20 min) and sealed, and the exchange reaction was initiated by injection of (1*RS*)-[1-$^3$H;1-$^{14}$C]octanol (20 $\mu$L, 0.13 mmol). The reaction mixture was vigorously shaken in the dark at 35 °C. Equilibrations were terminated after 24 h by the addition of NaCl (3 g). Reaction mixtures were extracted with ether (2 × 10 mL), following which the aqueous phase was acidified (0.5 mL of 10% $H_2SO$) and reextracted with ether (2 × 10 mL). The ether extract was dried ($MgSO_4$) and distilled through a 15-cm Vigreux column to a small volume. An aliquot (ca. 5 $\mu$L) of residual octanol was added to a solution of 3,5-dinitrobenzoyl chloride (250 mg) in dry benzene (3 mL). Pyridine (2 drops) was added and the solution refluxed (90 min). The product was recovered with ether (30 mL), and the ether extract washed with 1 N HCl (2 × 10 mL), $H_2O$ (10 mL), saturated aqueous $NaHCO_3$ (2 × 10 mL), $H_2O$ (10 mL), and saturated aqueous NaCl (10 mL). The ether solution was dried ($Na_2SO_4$), and concentrated in a rotary evaporator, and the residue was fractionated by thin-layer chromatography [20 × 20 cm plate; benzene–ethyl acetate (150:1)]. Octyl 3,5-dinitrobenzoate was recovered (ether), crystallized as fine white needles from 95% ethanol (mp 61 °C; lit.[14] mp 61 °C), and counted.

**B. Yeast Alcohol Dehydrogenase–NAD/NADH–Diaphorase.** Equilibration reaction mixtures were prepared as described above, except that yeast alcohol dehydrogenase (11.75 mg, 3970 U) was used in place of horse liver alcohol dehydrogenase. Reaction vessels were vigorously shaken in the dark at 25 °C for 168 h. Aliquots (1 mL) were withdrawn at various intervals and injected into suspensions of NaCl (0.3 g) and 1-octanol (2.5 $\mu$L) in ether (5 mL). Each suspension, corresponding to a single time point, was shaken at room temperature (30 min), and the

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Table I. Incubation of [1,1-$^1H_2$; 1-$^{14}C$]Octanol with $^3H_2O$–HLAD–NAD/NADH–Diaphorase

| | octanol | | octanoic acid | | octanal | |
|---|---|---|---|---|---|---|
| expt | % $^{14}C$ recovered[a] | $^3H$:$^{14}C$ ratio[b] | $^3H$:$^{14}C$ ratio[c] | % $^3H$ lost | $^3H$:$^{14}C$ ratio[d] | % (1S)-octanol obtained |
| 1 | 76 | 5.94 | 0.06 | 99 | 0.56 | 9.0 |
| 2 | 82 | 5.94 | 0.07 | 99 | 0.61 | 10.0 |

[a] Based on recovery of incubated [1-$^{14}C$]octanol. [b] Counted as the 3,5-dinitrobenzoate ester in 15 mL of Liquifluor. [c] Counted as *p*-toluidide in 15 mL of Liquifluor. [d] Counted as the 4-phenylsemicarbazone in 15 mL of Liquifluor.

aqueous and organic phases were separated by centrifugation. The ether layer was dried ($MgSO_4$) and distilled through a 10-cm Vigreux column, and residual octanol was converted to the 3,5-dinitrobenzoate ester and counted.

After 168 h, an aliquot (1 mL) of reaction mixture was assayed for yeast alcohol dehydrogenase[15] and diaphorase activities.

**Synthesis of [1-$^3$H;1-$^{14}$C]Octanol with $^3H_2O$–Horse Liver Alcohol Dehydrogenase–NAD/NADH–Diaphorase.** To a 10-mL round-bottom flask containing Günther–Simon buffer[12,13] (1 mL), ovalbumin (0.7 mg), NAD (0.07 μmol), NADH (0.07 μmol), horse liver alcohol dehydrogenase (1 mg, 1.9 U) and diaphorase (12.5 μL, 125 μg, 17 U) were added [1-$^{14}$C]octanol (40 μL, 0.26 mmol, 0.32 μCi) and $^3H_2O$ (25 μL, 1.4 mmol, 25 mCi). The air in the flask was replaced with nitrogen, and the mixture was sealed and vigorously shaken in the dark at 35 C. Two such exchange reactions were carried out. After 1 h, each reaction mixture was chilled in an ice bath, saturated with NaCl, and extracted with ether (2 × 3 mL). Unlabeled 1-octanol (50 μL) was then added to the aqueous phases, which were further extracted with ether (2 × 2.5 mL). The combined ether layers from each reaction were washed with saturated aqueous NaCl (1 mL) and dried ($MgSO_4$). The ether solutions were transferred to 10-mL volumetric flasks, which were filled to mark with ether. Portions of [1-$^3$H;1-$^{14}$C]octanol were withdrawn, freed of ether, converted to the 3,5-dinitrobenzoate ester, and counted. Aliquots of [1-$^3$H;1-$^{14}$C]octanol were also oxidized with Jones' reagent, and the resulting acids were converted to *p*-toluidides and counted. Ether was then distilled from the remaining [1-$^3$H;1-$^{14}$C]octanol samples, and the enantiomeric composition of each recovered octanol was estimated by oxidation to octanal using horse liver alcohol dehydrogenase–NAD (Table I).

**Analysis of [1-$^3$H;1-$^{14}$C]Octanols Obtained by Enzymic Exchange with Tritiated Water. A. Enzymic Oxidation of [1-$^3$H;1-$^{14}$C]Octanol.** To a 30-mL serum bottle containing 10 mL of buffer (50 mM potassium phosphate + 30 mM 4-phenylsemicarbazide·HCl, pH 6.8) were added horse liver alcohol dehydrogenase (5 mg, 9.5 U) and NAD (0.035 mmol). The bottle was flushed with nitrogen (20 min), sealed, and injected with 5 μL (0.032 mmol) of [1-$^3$H;1-$^{14}$C]octanol. The bottle was vigorously shaken in the dark at 35 °C (90 min), and the reaction was terminated by chilling the mixture in an ice bath and saturating it with NaCl. The reaction mixture was extracted with $CH_2Cl_2$ (2 × 10 mL). The combined $CH_2Cl_2$ extracts were diluted with ether and washed with 1 N HCl (2 × 10 mL), $H_2O$ (10 mL), and saturated aqueous NaCl (10 mL), then dried ($Na_2SO_4$), and concentrated in vacuo. The residue was fractionated by thin-layer chromatography [20 × 20 cm plate, hexane–ethyl acetate (17:3), developed five times]. The band corresponding to octanal 4-phenylsemicarbazone was eluted [ether–$CHCl_3$ (2:1)]. Unlabeled octanal 4-phenylsemicarbazone was added to the eluate, and the product was crystallized from 95% ethanol as fine white needles [mp 128–130 °C (lit.[16] mp 128–129 °C)] and counted.

**B. Jones' Oxidation of [1-$^3$H;1-$^{14}$C]Octanol.** Jones' reagent[17,18] (0.4 mL) was added dropwise to a chilled, stirring solution of [1-$^3$H;1-$^{14}$C]-octanol (30 μL, 0.19 mmol) in acetone (1.0 mL). After 5 min, the reaction was terminated with 2-propanol, and the products were extracted with pentane (4 × 5 mL). The pentane solution was washed with saturated aqueous NaCl (2 × 1 mL), and acids were extracted with 1 N NaOH (4 × 1 mL). The alkaline solution was chilled in an ice bath, acidified (concentrated HCl), and extracted with pentane (3 × 3 mL). The pentane solution was dried ($Na_2SO_4$) and distilled. The residue was taken up in a solution of *N*,*N*′-dicyclohexylcarbodiimide (250 mg, 1.21 mmol) and *p*-toluidine (70 mg, 0.65 mmol) in dry $CH_2Cl_2$ (10 mL) and shaken overnight at room temperature. The reaction mixture was washed with 1 N HCl (2 × 10 mL), $H_2O$ (10 mL), and saturated aqueous NaCl (10 mL) and dried ($Na_2SO_4$). The filtrate was rotary-evaporated to an oily residue, and pentane–ether (1:1) was added dropwise until the mixture solidified. The solid was stirred with ether (3 × 15 mL) and the combined extract filtered and concentrated under reduced pressure. The residue was fractionated by thin-layer chromatography [20 × 20 cm plate; benzene–ethyl acetate (9:1)]. Octanoyl *p*-toluidide was eluted with ether, crystallized from *n*-hexane as tiny white needles [mp 69 °C (lit.[19] mp 70 °C)], and counted.

Table II. Incubation of [1,1-$^1H_2$]Octanol with $^2H_2O$–YADH–NAD/NADH–Diaphorase

| | relative abundance of *m/e* ($M^+ - H_2O$) | | | | |
|---|---|---|---|---|---|
| 1-octanol | 111 | 112 | 113 | 114 | 115 |
| [1,1-$^1H_2$] | 1.3 | 10 | 1.76 | | |
| (1*RS*)-[1-$^2$H] | 0.5 | 1.86 | 10 | 1.36 | |
| [1,1-$^2H_2$] | | 0.62 | 0.89 | 10 | 1.79 |
| (1*R*)-[1-$^2$H] | 0.4 | 1.76 | 10 | 1.8 | |

Table III. Requirement of Alcohol Dehydrogenase and Diaphorase for Exchange of C-1 Tritium

| | | $^3H$:$^{14}C$ ratio of (1*RS*)-[1-$^3$H; 1-$^{14}$C]octanol equilibrated for | |
|---|---|---|---|
| expt | reaction mixture[d] | 0 h | 24 h |
| 1 | complete[a] | 6.38 | 1.81 |
| 2 | (–) HLAD | 6.38 | 6.42 |
| 3 | (–) diaphorase, (+) FMN[b] | 6.38 | 6.25 |
| 4 | (–) diaphorase, (+) 10X FMN[c] | 6.38 | 6.28 |

[a] See Experimental Section. Flavin content of added diaphorase, 260 nmol (based on a molecular weight for porcine heart diaphorase of 100 000 and 2 mol of flavin adenine dinucleotide per mole of protein[20]). [b] 260 nmol. [c] 2.6 μmol. [d] (–) indicates omitted from the reaction mixture, (+) indicates added to the reaction mixture.

**Synthesis of (1*R*)-[1-$^2$H]Octanol Using $^2H_2O$–Yeast Alcohol Dehydrogenase–NAD/NADH–Diaphorase.** To a solution of $KH_2PO_4$ (0.376 g), $K_2HPO_4$ (2.468 g), and $Na_2EDTA·2H_2O$ (78 mg) in $^2H_2O$ (240 mL) (pD = 7.9) were added ovalbumin (154 mg), NAD (10 mg), NADH (10 mg), yeast alcohol dehydrogenase (260 mg, 94 900 U), and porcine heart diaphorase (2.8 mL, 28 mg, 3864 U). Aliquots of reaction mixture were transferred into serum bottles, flushed with $N_2$ (20 min), and sealed. Exchange reactions were initiated by injecting [1,1-$^1H_2$]octanol into each bottle (2 μL octanol/mL reaction mixture). Reaction vessels were vigorously shaken in the dark at 25 °C. After 24 h, the bottles were heated in a steam bath, their contents pooled, and octanol recovered (ether).

Gas chromatographic examination of the ether extract of the reaction mixtures showed a single peak which coeluted with authentic 1-octanol. The mass spectra of 1-octanol, synthetic [1,1-$^2H_2$]octanol, synthetic (1*RS*)-[1-$^2$H]octanol, and enzymically obtained (1*R*)-[1-$^2$H]octanol were recorded (Table II). The results indicate that the biosynthetically prepared [1-$^2$H]octanol contained one atom of deuterium at C-1 (~100% [1-$^2$H]).

**Determination of Enantiomeric Purity of Enzymically Prepared (1*R*)-[1-$^2$H]Octanol.** Enzymically prepared (1*R*)-[1-$^2$H]octanol was freed of ether by distillation, and an aliquot (300 μL) was taken up in a solution of (–)-camphanoyl acid chloride (635 mg, 3 mmol) in pyridine (5 mL). The reaction mixture was stored at room temperature overnight and then diluted with ice-cold water (20 mL) and extracted with ether (3 × 50 mL). The ether extract was washed with 5% HCl (3 × 20 mL), $H_2O$ (2 × 20 mL), 5% $NaHCO_3$ (2 × 25 mL), and $H_2O$ (3 × 25 mL), dried ($Na_2SO_4$), and concentrated in vacuo. The residue was fractionated by thin-layer chromatography [20 × 40 cm plates; hexane–ethyl acetate (3:1)], and octyl (–)-camphanate (435 mg, 1.40 mmol) was obtained as a viscous oil. Gas chromatographic analysis of the recovered camphanate ester showed a single peak with retention time of 175 s (column, 100 °C;

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injection port, 210 °C; detector, 210 °C; He, 30 mL/min).

The camphanate ester, as well as other deuteriooctyl camphanates, was investigated by deuterium and proton NMR (Figure 1) (see Results and Discussion).

## Results and Discussion

The equilibration of C-1 hydrogen atoms of primary alcohols with water using horse liver alcohol dehydrogenase–NAD/NADH–diaphorase involves the redox interconversion of alcohol and aldehyde.[6] Exchange with water hydrogen atoms requires the presence of both alcohol dehydrogenase and diaphorase in the reaction mixture (Table III) flavin mononucleotide at the same or tenfold higher concentration as diaphorase could not replace the flavoprotein in the equilibration reaction.

Horse liver alcohol dehydrogenase–NAD/NADH–diaphorase has been used in conjunction with $^3H_2O$ for the preparation of (1*R*)-[1-$^3$H] primary alcohols.[1,2] However, our earlier results obtained for equilibration of (1*S*)-[1-$^3$H;1-$^{14}$C]octanol and (1*RS*)-[1-$^3$H;1-$^{14}$C]octanol with $H_2O$ showed that 1*S* hydrogen (tritium) atoms were also exchanged.[3-7] In view of these observations, it was necessary to assess the chiral composition of tritiated octanol obtained by equilibration of protiated alcohol with $^3H_2O$. To this end, [1,1-$^1H_2$;1-$^{14}$C]octanol was incubated with $^3H_2O$–horse liver alcohol dehydrogenase–NAD/NADH–diaphorase; the results are summarized in Table I. Jones oxidation of the recovered [1-$^3$H;1-$^{14}$C]octanol to octanoic acid proceeded with the loss of 99% of tritium, demonstrating the location of the incorporated tritium atom at C-1. Samples of the [1-$^3$H;1-$^{14}$C]octanol were oxidized to octanal by using horse liver alcohol dehydrogenase–NAD, a reaction claimed to proceed with the stereospecific abstraction of the 1-*pro-R* hydrogen atom.[21] The recovered octanol retained ca. 10% of tritium, indicating that a minimum 10%[3-7] of [1-$^3$H]octanol produced in the equilibration reaction was (1*S*)-octanol (see below).

The stereospecificity of most enzymes toward their substrates is very high, as evidenced by their reactivity with only one enantiomer or enantiotopic group.[22,23] It is clear, however, that 1-octanol incubated with horse liver alcohol dehydrogenase–NAD/NADH–diaphorase exchanges not only its 1-*pro-R* hydrogen atom but also its 1-*pro-S* hydrogen atom.[3-7] Equilibrations were carried out anaerobically in the dark, thereby excluding the possibility of nonstereospecific autooxidation of octanol to octanal.

Under the equilibration conditions employed, 100% of the 1*R* tritium atoms and 15–20% of the 1*S* tritium atoms of (1*RS*)-[1-$^3$H]octanol are exchanged with water protium atoms by 12 h with horse liver alcohol dehydrogenase–NAD/NADH–diaphorase.[5,6] Comparison of the initial velocities of tritium loss from (1*R*)- and (1*S*)-[1-$^3$H]octanol[5,6] indicates that the rate of 1-*pro-S* hydrogen exchange is ca. 2–4% that of 1-*pro-R* hydrogen exchange. Thus, exchange of C-1 hydrogen of 1-octanol by the system horse liver alcohol dehydrogenase–NAD/NADH–diaphorase is not absolutely stereospecific. The loss of 1-*pro-S* hydrogen atoms from 1-octanol is relatively infrequent and thus not readily detectable in the *unidirectional oxidation* of octanol to octanal by horse liver alcohol dehydrogenase–NAD. However, the cumulative *effect* of the equilibration involving *reversible* oxidation–reduction allows for the accurate quantitation of the loss of 1*S* (isotopic) hydrogen atoms.

Yeast alcohol dehydrogenase and horse liver alcohol dehydrogenase are homologous but distantly related proteins.[24,25] Both primary sequence data and extensive chemical modification studies support similar acive-site configurations in these enzymes.[26] We therefore undertook a comparison of the stereospecificity of equilibration of the C-1 hydrogens of octanol using yeast alcohol dehydrogenase–NAD/NADH–diaphorase with the preceding results.

Table IV. Deuterium NMR Spectra of [1-$^2$H]Octyl (–)-Camphanates in the Presence of Eu(dcm)$_3$[a]

| parent octanol | chemical shift, ppm *R* | chemical shift, ppm *S* |
|---|---|---|
| 1*RS* | 5.13 | 5.41 |
| 1*R* | 5.13 | |

[a] Spectra were obtained on solutions of the esters (4 mg) in 0.4 mL of $CCl_4$ containing 1 molar equiv of Eu(dcm)$_3$ with $C_6{}^2H_6$ (5 μL) as internal reference. In the absence of Eu(dcm)$_3$, the 1*RS* ester gave a single peak at 4.11 ppm.

[1,1-$^1H_2$]Octanol was equilibrated for 24 h with yeast alcohol dehydrogenase–NAD/NADH–diaphorase in deuterium oxide (99.8 atom % $^2$H). The mass spectrum of recovered octanol showed it contained one atom of deuterium (~100% $^2$H) (Table I). To determine the enantiomeric purity of the [1-$^2$H]octanol, the alcohol was converted to the (–)-camphanate ester and its deuterium and proton magnetic resonance spectra analyzed.

The $^2$H NMR spectra of the esters are summarized in Table IV. (1*RS*)-[1-$^2$H]Octyl (–)-camphanate gave a signal at δ 4.11, which following the addition of 1 molar equiv of Eu(dcm)$_3$, was resolved into two peaks at δ 5.13 and 5.41. The two sharp signals were distinctly separated but were not resolved to the base line. (1*R*)-[1-$^2$H]Octyl (–)-camphanate (see below), in the presence of 1 molar equiv of Eu(dcm)$_3$, gave a single peak at δ 5.13. It follows that the signal at δ 5.41 arises from (1*S*)-[1-$^2$H]octyl (–)-camphanate.

The $^1$H NMR spectrum of (1*RS*)-[1-$^2$H]octyl (–)-camphanate showed a triplet at δ 4.20, which, in the presence of 1 molar equiv of Eu(dcm)$_3$, was resolved into two triplets at δ 5.86 and 5.56 (Figure 1A). The downfield and upfield triplets are derived from the 1*S* and 1*R* protium atoms, respectively, of (1*RS*)-[1-$^2$H]octanol.[27] In the presence of 1 molar equiv of Eu(dcm)$_3$, the $^1$H NMR spectrum of the (–)-camphanate ester of [1-$^2$H]octanol obtained by equilibration of 1-octanol with deuterium oxide showed a single triplet at δ 5.86 (Figure 1B).

The spectrum of the enzymically derived [1-$^2$H]octyl (–)-camphanate admixed with (1*RS*)-[1-$^2$H]octyl (–)-camphanate (30% w/w) was then recorded in the presence of Eu(dcm)$_3$ (Figure 1C) The spectrum showed, in addition to a large downfield triplet for the 1*S* hydrogen, a smaller upfield triplet for the 1*R* hydrogen, thus confirming the 1*R* chirality of enzymicaly derived [1-$^2$H]-octanol.

For determination of the enantiomeric purity of enzymically derived (1*R*)-[1-$^2$H]octanol, the sensitivity of the 1H NMR method was assessed by titrating the sample under examination with a solution of (1*RS*)-[1-$^2$H]octyl (–)-camphanate –Eu(dcm)$_3$ (1:1) in $CCl_4$. The addition of 8% (w/w) of (1*RS*)-[1-$^2$H]octyl (–)-camphanate ester–Eu(dcm)$_3$ to the ester of the enzymically generated species gave rise to an upfield triplet for the 1*R* protron (Figure 1D). It follows that 4% of (1*S*)-[1-$^2$H]octyl (–)-camphanate in the enzymically derived ester would have been detected by the NMR procedure. In practice, the signal corresponding to the presence of ca. 3% of 1*S* ester could be discerned. Thus, the octanol recovered from equilibration of protiated 1-octanol with dueterium oxide in the system yeast alcohol dehydrogenase–NAD/NADH–diaphorase contained at least 96–97% of (1*R*)-[1-$^2$H]octanol. This establishes, *within the limits of sensitivity of* the *NMR procedure*, the absolute stereospecificity of yeast alcohol dehydrogenase toward the 1-*pro-R* hydrogen atom of octanol *under our equilibration conditions*. The fact that exchange of C-1 hydrogen of octanol with deuterium oxidde of the medium proceeded stereospecifically in the presence of diaphorase militates against a role for diaphorase in the exchange of the 1-*pro-S* hydrogen of octanol with water in the system horse liver alcohol dehydrogenase–NAD/NADH–diaphorase.

We also incubated (1*RS*)-[1-$^3$H;1-$^{14}$C]octanol with yeast alcohol dehydrogenase–NAD/NADH–diaphorase in $H_2O$. After 6 h, the tritium content of equilibrated octanol had fallen to ca.

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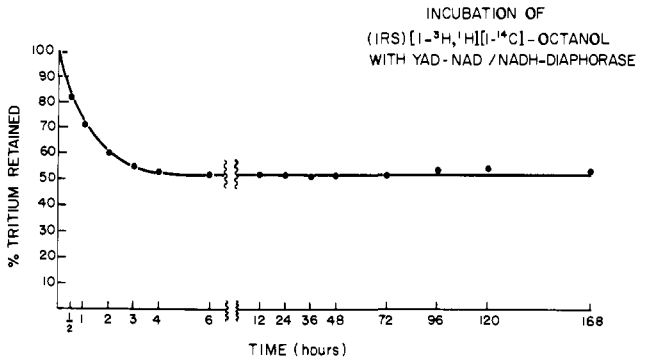


**Figure 2.**

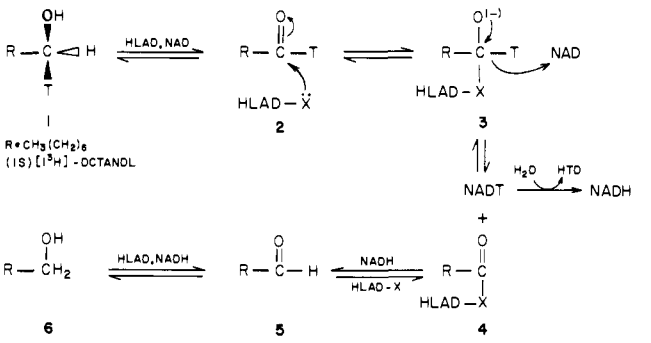


**Figure 3.**

50% that of the initial value, and remained unchanged for 1 week (Figure 2). After 7 days, both the yeast alcohol dehydrogenase and diaphorase were still active, retaining 85% and 51%, respectively, of their initial activities. It is clear that whereas yeast alcohol dehydrogenase is completely stereospecific with respect to promoting exchange of the 1-*pro-R* hydrogen atom of 1-octanol with solvent hydrogen atoms, horse liver alcohol dehydrogenase is not.

These data strongly implicate horse liver alcohol dehydrogenase and not diaphorase as the agent responsible for promoting exchange of the 1-*pro-S* hydrogen atom of 1-octanol. Were diaphorase rather than horse liver alcohol dehydrogenase, responsible for the observed exchange of 1-*pro-S* hydrogen, then octanol incubated with yeast alcohol dehydrogenase–NAD/NADH–diaphorase should have exchanged *more than* 50% of its C-1 hydrogen.

According to the Bränden–Eklund–Jörnvall model for the ternary complex horse liver alcohol dehydrogenase–NAD–primary alcohol,[28,29] the C-1 hydrogen atom to be transferred is 2.5 Å from C-4 of the nicotinamide ring of NAD and points toward the *re* face of that ring. Ordinarily the 1-*pro-R* hydrogen atom is so oriented as to be readily abstracted.[30] Rare misbindings in which the 1-*pro-S* hydrogen of substrate faces NAD could lead to abstraction of this hydrogen atom. The existence of degenerate noncatalytic binding sites each able to accommodate groups of various sizes was advanced by Dickinson and Dalziel[31] to explain the lack of absolute stereospecificity in the oxidation of acyclic secondary alcohols by horse liver alcohol dehydrogenase (cf. ref 32). Presumably, then, the active site of yeast alcohol dehydrogenase cannot bind 1-octanol in such a manner that the 1-*pro-S* hydrogen atom is suitably positioned for abstraction.

Alternatively, abstraction of 1-*pro-S* hydrogen atoms in lieu of 1-*pro-R* hydrogen atoms may be a consequence of torsional displacements or "wobble" of C-1 of 1-octanol within the active site of horse liver alcohol dehydrogenase. Kinetic studies have raised the possibility of conformational flexibility of alkylcyclohexanones *within* the active site of horse liver alcohol dehydrogenase.[33] If the amplitude of torsional motion of C-1 of enzyme-bound octanol is sufficient to occasionally sweep the 1-*pro-S* hydrogen atom to within 2.5 Å of C-4 of NAD, then it may be fortuitously abstracted. The residues lining the substrate-binding pocket are bulkier for yeast alcohol dehydrogenase than for horse liver alcohol dehydrogenase.[25] This is believed to restrict the substrate specificity of the yeast enzyme relative to the equine enzyme[25] (e.g., ref 31), and may conceivably restrict the wobble of C-1 of yeast alcohol dehydrogenase bound octanol so as to preclude its ever directing the 1-*pro-S* hydrogen atom toward NAD.

A different mechanism can be formulated on the basis of the known ability of horse liver alcohol dehydrogenase to oxidize octanal to octanoic acid with concomitant reduction of NAD to NADH.[34] According to this scheme (Figure 3), stereospecific abstraction of 1*R* protium from (1*S*)-[1-$^3$H]octanol (**1**) gives [1-$^3$H]octanal (**2**). Attack at the carbonyl function of [1-$^3$H]-octanal by a nucleophilic moiety ("X") of horse liver alcohol dehydrogenase (HLAD–X) yields an enzyme–substrate complex (**3**). Tritide transfer from **3** to NAD gives an octanoyl–HLAD complex **4** and [4-$^3$H]-NADH. The tritium atom of [4-$^3$H]-NADH is ultimately lost to water.[5,6] It is proposed that under the conditions of equilibration, complex **4** *may be reduced* by NADH to octanal (**5**) and thence to protiated octanol (**6**). In contrast, yeast alcohol dehydrogenase may yield a (YAD–H) complex similar to **4** which is not reduced, but is hydrolyzed to octanoic acid. Therefore, the sequence **4–5–6** is eliminated and the loss of 1-*pro-S* hydrogen (tritium) in the recovered equilibrated octanol is not observed. Pyridine nucleotide coupled oxidations of aldehydes to acids are invariably found to proceed via enzyme–thioester intermediate (e.g. ref 35–37). Aldehyde dehydrogenases also exhibit esterase activity (e.g., ref 38–40); the same sulfydryl nucleophile active in aldehyde oxidation is believed to be active in ester hydrolysis. In addition to its aldehyde dehydrogenase activity, horse liver alcohol dehydrogenase possesses esterase activity.[41,42] A reactive sulfhydryl moiety is apparently required for both esterase[41,42] and aldehyde dehydrogenase[43] activities. Should the postulated nucleophilic moiety be a cysteinyl residue, then hypothetical complexes **3** and **4** (Figure 3) would correspond to a horse liver alcohol dehydrogenase–thiohemiacetal and horse liver alcohol dehydrogenase–thioester, respectively.

**Acknowledgment.** This work was supported by National Institutes of Health Grant GM 19882. Deuterium NMR spectra were recorded at the Worcester Academic Consortium NMR facility supported in part by National Science Foundation Equipment Grant DMR-8108697. We thank Clifford McDonald for recording the proton NMR spectra.

**Registry No.** Alcohol dehydrogenase, 9031-72-5; 1-octanol, 111-87-5; (1*RS*)-[1-$^3$H;1-$^1$H;1-$^{14}$C]octanol, 80226-93-3; (1*RS*)-[1-$^2$H;1-$^1$H]octanol, 84519-52-8; [1,1-$^2$H$_2$]octanol, 78510-02-8; [1-$^2$H]octyl camphanate, 62012-40-2; (−)-camphanoyl chloride, 39637-74-6; [1-$^3$H;1-$^{14}$C]octanol, 84472-91-3; (1*R*)-[1-$^2$H]octanol, 62012-42-4.

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