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Primary and Secondary Deuterium Isotope Effects on Equilibrium Constants for Enzyme-Catalyzed Reactions†

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ABSTRACT: Primary deuterium equilibrium isotope effects for the reaction of five secondary alcohols with nicotinamide adenine dinucleotide (DPN) to give reduced deuterionicotinamide adenine dinucleotide (DPND) (cyclohexanol-*1-d*, 1.18; 2-propanol-*2-d*, 1.175; *threo*-DL-isocitrate-*2-d*, 1.168; L-malate-*2-d*, 1.173; L-lactate-*2-d*, 1.19) are all ~1.18, while for a primary alcohol, ethanol, the value is 1.07, for an amino acid, L-glutamate-*2-d*, it is 1.14, and for a hemiacetal, glucose-*1-d*, it is 1.28. In each case deuterium becomes enriched in the alcohol, amino acid, or hemiacetal with respect to DPNH (TPNH). β-Secondary equilibrium isotope effects for reduction of ketones by DPNH (cyclohexanone-*2,2,6,6-d*$_4$, 0.81; acetone-d_6, 0.78; pyruvate-d_3, 0.83; α-ketoglutarate-*3,3-d*$_2$ reduced to glutamate, 0.898; oxaloacetate-*3,3-d*$_2$, 0.877; oxaloacetate-*3R-d*, 0.945) give an average value of 0.946/D, with deuterium becoming enriched in the alcohol or amino acid with respect to the ketone. For reduction of acetaldehyde-*1-d* by DPNH, the observed value of 0.953 includes the equilibrium effect on the hydration equilibrium in addition to that on the reduction, and the calculated values for reduction of the free aldehyde and the hydrate are 0.78 and 1.07. For reduction of benzaldehyde-*1-d*, which is not hydrated, the observed value was 0.79. The secondary equilibrium isotope effect for conversion of DPN-*4-d* to DPNH is 0.89, with deuterium becoming enriched in DPNH, and, for conversion of fumarate-*2,3-d*$_2$ to malate, the value is 0.69, with deuterium becoming enriched in L-malate. The equilibrium isotope effect for reaction of cyclohexanol-*1-d* with DPN is temperature independent over the range 15–35 °C.

Changes in the magnitude of the equilibrium constant when deuterium is substituted for hydrogen in the reactants are predicted by the calculations of Hartshorn & Shiner (1972). The magnitude of the equilibrium isotope effect reflects the different "stiffness" of the bonds in substrate and product, with deuterium becoming enriched in the stiffest bond and hydrogen in the looser one. Hartshorn & Shiner (1972) suggested that the bond stiffness depends only on the atoms bonded directly to the carbon substituted with deuterium, with more remote atoms having only small effects. There are relatively few experimental data available to test the predictions of Hartshorn & Shiner (1972), and most of these are not of sufficient precision to be useful. The aim of this report is to present simple but accurate means for obtaining deuterium isotope effects on equilibrium constants for enzyme-catalyzed reactions and to report a number of primary and secondary equilibrium isotope effects which have been determined in this laboratory.

Materials and Methods

Chemicals. DL-Isocitrate lactone, D_2O (99.8 atom %), and all enzymes were from Sigma Chemical Co. Ethanol-d_6 (99 atom %), 2-propanol-*2-d* (98 atom %), fumarate-*2,3-d*$_2$ (98 atom %), glucose-*1-d* (98 atom %), glutamate-*2,3,3,4,4-d*$_5$ (98

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atom %), cyclohexanone-*2,2,6,6-d*$_4$ (98 atom %), acetone-d_6 (98 atom %), and acetaldehyde-*1-d* (98 atom %) were from Merck. Dimethyl sulfoxide, δ-gluconolactone, and cyclohexanone were from Aldrich. L-Glutamate-*2-d* was prepared by J. E. Rife in this laboratory by exchange in D_2O in the presence of glutamate–oxaloacetate transaminase. DL-Isocitrate-*2-d* lactone was the gift of Dr. M. H. O'Leary. A-side reduced deuterionicotinamide adenine dinucleotide (DPND) and L-malate-*2-d* were synthesized by the method of Viola et al. (1979). 2-Propanol-*2-d* and cyclohexanol-*1-d* were synthesized by the reduction of acetone and cyclohexanone with $NaBD_4$ and recovered by distillation. For preparation of benzaldehyde-*1-d*, 397 mg of solid $NaBD_4$ was added over 15 min to 2 g of benzil in 50 mL of methanol. After neutralization of the preparation to pH 5, 1.2 equiv of $NaIO_4$ was added, and after 1 h the product was extracted with 2 volumes of ether and recovered by evaporation of the ether. DPN from Boehringer-Mannheim contained a large amount of ethanol, which was removed by adsorbing a 10 mM solution on a 30 × 1.2 cm column of DEAE[1]-cellulose equilibrated with 0.5 M ammonium bicarbonate, pH 10. After being washed with 5 column volumes of water, DPN containing no ethanol was eluted with 25 mM ammonium bicarbonate, pH 10, titrated to pH 7 with 1 N HCl, and used without further treatment. Some of the DPN may have been converted at pH 10 into compounds which are known to inhibit some of the enzymes used in these studies, but the presence of such inhibitors in DPN will only increase the time required to attain equilibrium and will not affect the equilibrium values, since DPN concentrations were determined enzymatically.

DPN-4-d. To a 250-mL solution of 1 mM A-side DPND [synthesized by the procedure of Viola et al. (1979)], 5000 units of glutamate dehydrogenase and 250 mg of $(NH_4)_2SO_4$ were added. Solid α-ketoglutarate (monosodium salt) was added 5 mg at a time until no further decrease in A_{340} was observed. Dilute base (0.1 N KOH) was added after each addition of α-ketoglutarate to maintain the pH of the solution at 8. The enzymes were then removed by passing the solution through an Amicon PM 10 ultrafilter, and the filtrate was concentrated by rotary flash evaporation to 5 mL. The yellow solution was then applied to a 190 × 2 cm column of Sephadex G-10 equilibrated and eluted with 20 mM KCl. The peak A_{260} fractions were pooled and applied to a 30 × 1.2 cm column of Dowex-1-formate and thoroughly washed with water. The column was then eluted with 0.5 M KCl, and the peak A_{260} fractions were pooled and concentrated to a thick syrup by rotary evaporation. Further purification was obtained by using high-pressure liquid chromatography employing reverse-phase chromatography on a 4 × 250 mm column of hydrophobic Porasil-B resin eluted with 10 mM NH_4HCO_3, pH 7. Fractions containing DPN-*4-d* were pooled, concentrated to ~1 mM, and used without further treatment.

Pyruvate-3,3,3-d$_3$. The use of glutamate–pyruvate transaminase allows exchange of deuterium from solvent D_2O into the methyl group of pyruvate. Thus, 50 mM pyruvate and 2 mM alanine were incubated with 1000 units of glutamate–pyruvate transaminase in a final volume of 20 mL of D_2O, pD 7.6. Exchange of deuterium into the methyl group of pyruvate was monitored by NMR. When isotopic equilibrium was attained, the reaction mixture was passed through an Amicon PM 20 ultrafilter, and this solution of pyruvate-d_3 (>95 atom %) was used without further purification or removal of the alanine.

Malate-3,3-d$_2$. Oxaloacetate-*3,3-d*$_2$ was prepared by exchange of deuterium from D_2O at pD 1 (exchange was monitored by NMR). The pD was then increased to pD 8.0 by the addition of 1 M K_3PO_4 in D_2O, and this solution was slowly dripped into an unbuffered solution of 4 mM DPNH and 5000 units of malate dehydrogenase in D_2O, pD 8.0, until no further disappearance in A_{340} was observed. The malate solution was then acidified to pH 1.5 with perchloric acid, and nucleotide was adsorbed on 2 g of acid-washed, heat-activated charcoal. The solution was then filtered, adjusted to pH 7, and filtered again to remove more precipitated salts and protein. The filtrate was applied to a 30 × 1.2 cm column of Dowex-1-formate and eluted with a 0–4 N linear gradient of ammonium formate, pH 3.2. Fractions containing malate (determined by using malic enzyme as described below) were pooled and lyophilized for 48 h to remove ammonium formate. Neither a formate proton signal nor protons in the β position of malate were detected in the 60-MHz NMR spectrum.

Malate-3R-d. When fumarate was incubated with fumarase in D_2O, deuterium was stereospecifically incorporated into the 3*R* position of L-malate. Malate was then separated from fumarate by using a Dowex-1 column with an ammonium formate gradient as described above for the preparation of malate-*3,3-d*$_2$.

Equilibrium Isotope Effects. Equilibrium constants were determined by adding enzyme to reaction mixtures containing initially all substrates and all products other than the one which will contain deuterium when a labeled substrate is used. The final concentrations when equilibrium had been reached were determined from the change in concentration of the colored molecule (DPNH, TPNH, fumarate). Whenever possible, concentrations were chosen so that the concentrations of reactants other than the colored one changed as little as possible during the reaction. For measurement of the equilibrium deuterium isotope effect, all reactants which never contain deuterium were added to matched cuvettes at the same concentration and from the same stock solution, while aliquots of separate stock solutions of deuterated and unlabeled substrate were added. The accuracy of the measured isotope effect is only as good as that with which the relative concentrations in the separate stock solutions of deuterated or unlabeled substrates can be determined, and thus, although in these studies all reactant concentrations were determined by enzymatic assays whenever possible, we will discuss only the enzymatic assays used for the deuterated and unlabeled substrates.

Enzymatic assays were always carried out with several different volumes of the stock solution whose concentration was being determined to ensure that the reaction was going to completion. If the apparent concentration of the stock solution was higher when a smaller volume was used in the assay, either the conditions were changed to drive the reaction to completion or the correct value was determined by extrapolation to zero level of added material. When one determination of the equilibrium isotope effect was completed, additional determinations were carried out with different concentrations of all reactants.

Isocitrate Dehydrogenase. DL-Isocitrate-*2-(h,d)* was prepared by hydrolysis of the corresponding lactone for 30 min in 1 N NaOH at 100 °C. The resulting solution was neu-

[1] Abbreviations used: DPN-*4-d*, 4-deuterionicotinamide adenine dinucleotide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Taps, 3-[tris(hydroxymethyl)methyl]aminopropanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DEAE, diethylaminoethyl; TPN, nicotinamide adenine dinucleotide phosphate; TPNH, reduced TPN; DPNH, reduced DPN; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

tralized and assayed in the following system: 100 mM potassium phosphate, pH 8, 1 mM TPN, 2 mM $MgSO_4$, 100 μM dithiothreitol, and 1.33 units/mL isocitrate dehydrogenase. The reaction goes to completion since CO_2 (the true substrate) is trapped as HCO_3^-, and under these conditions the equilibrium is far toward TPNH formation. Concentrations from several determinations were identical within 0.5%.

A typical equilibrium isotope effect was determined under the following conditions: 100 mM potassium phosphate, pH 8, 200 μM TPN, 32 μM isocitrate-*2*-(*h*,*d*), 6 mM α-ketoglutarate, 100 mM HCO_3^-, 100 μM dithiothreitol, 2 mM $MgSO_4$, and 0.3 unit/mL isocitrate dehydrogenase.

Fumarase. Fumarate-*2*,*3*-(h_2,d_2) concentration was determined by coupling fumarase to malic enzyme so that the final TPNH concentration was equal to the initial fumarate concentration. Assays contained 100 mM Tris-HCl, pH 8, 2 mM $MgSO_4$, 1 mg/mL bovine serum albumin, 100 μM dithiothreitol, 1 mM TPN, 0.67 unit/mL chicken liver malic enzyme, and 1.67 units/mL fumarase. This reaction goes to completion for reasons similar to those given above for isocitrate dehydrogenase. Concentrations from several determinations were identical within 0.8%.

A typical assay for measuring the equilibrium isotope effect contained 100 mM Tris-HCl, pH 8, 0.4 mM fumarate-*2*,*3*-(h_2,d_2), and 1.67 units/mL fumarase. There is a difference in the ϵ_{240} when deuterium is substituted for hydrogen in the 2 and 3 positions of fumarate, and the ratio of ϵ_{240} for fumarate (2255) and dideuteriofumarate (2210) is 1.02. The change in fumarate concentration (from A_{240}) was used to calculate malate and fumarate concentrations, and thus the equilibrium constants.

Glucose-6-phosphate Dehydrogenase. The enhanced glucose dehydrogenase activity of glucose-6-phosphate dehydrogenase in the presence of 40% dimethyl sulfoxide and phosphate (Viola et al., 1979) was used to measure the primary equilibrium isotope effect for reaction of glucose-*1*-(*h*,*d*) with DPN to give DPNH(D). Two different concentration assays were used to determine the concentration of glucose. The first contained 100 mM Tris-HCl, pH 8, 40% dimethyl sulfoxide, 50 mM phosphate, 3 mM DPN, and 15 units/mL glucose-6-phosphate dehydrogenase from *Lactobacillus mesenteroides*. The second system coupled yeast hexokinase to glucose-6-phosphate dehydrogenase in the absence of dimethyl sulfoxide, with TPN as the nucleotide. These assays contained 100 mM Tris-HCl, pH 8, 5 mM $MgSO_4$, 3 mM ATP, 1 mM TPN, 67 units/mL glucose-6-phosphate dehydrogenase, and 6.7 units/mL yeast hexokinase. All assays went to completion as a result of the spontaneous hydrolysis of gluconolactone or 6-phosphogluconolactone at pH 8. Concentrations from several determinations in each assay system were identical within 0.3%.

Due to the chemical instability of the lactone at high pH and chemical instability of DPNH at low pH, pH 6.5 was used for all determinations of equilibrium isotope effects. Equilibrium determinations were run at 15 °C with a large amount of enzyme to further decrease the rate of chemical degradations (dimethyl sulfoxide also appeared to enhance the stability of the lactone). A typical assay contained 100 mM K–Mes, pH 6.5, 50 mM phosphate, 40% dimethyl sulfoxide, 100 mM gluconolactone, 2 mM DPN, 5 mM glucose-*1*-(*h*,*d*), and 67 units/mL glucose-6-phosphate dehydrogenase from *L. mesenteroides*. DPNH appearance was used to monitor approach to equilibrium (over in the mixing time), and the DPNH concentration was used to adjust the concentrations of other reactants.

Lactate Dehydrogenase. The primary equilibrium isotope effect for L-lactate-*2*-*d* was measured by comparing the reduction of pyruvate by A-side DPND or DPNH, instead of by measuring the oxidation of L-lactate-*2*-(*h*,*d*) by DPN, since it is difficult to determine accurately the concentration of lactate enzymatically, and chemical determinations are subject to too large an error. DPNH(D) was assayed in all studies with 6.7 units/mL lactate dehydrogenase, 100 mM Tris-HCl, pH 8, and 10 mM pyruvate. Reactions went to completion, since at pH 8 the apparent equilibrium constant is 5000. Concentrations from several determinations were identical within 0.3%.

A typical assay for determining the equilibrium isotope effect contained 100 mM K–Taps, pH 9, 10 mM DPN, 220 μM pyruvate, 220 μM DPNH(D), and 5.7 units/mL lactate dehydrogenase. The secondary equilibrium isotope effect with pyruvate-d_3 was measured similarly. Pyruvate-(h_3,d_3) was assayed by using 100 mM Tris-HCl, pH 8, 300 μM DPNH, and 2.7 units/mL lactate dehydrogenase. Concentrations from several determinations were identical within 0.6%.

Glutamate Dehydrogenase. Glutamate-*2*-(*h*,*d*) concentrations were determined at pH 9 with 100 mM K–Ches, 5 mM TPN, and 3.3 units/mL glutamate dehydrogenase. Reactions did not go to completion, since the calculated concentration of stock glutamate increased with decreasing volume of glutamate added to the concentration assay. As a result, four different levels of glutamate were used to obtain apparent concentrations of the stock solution, and the true concentration was obtained by extrapolating to zero volume added to the concentration assay. Concentrations were reproducible to within 1%.

For measurement of the equilibrium isotope effect, the assay contained 100 mM Tris-HCl, pH 8, 1.09 mM TPN, 20 mM glutamate-*2*-(*h*,*d*), 1.12 mM α-ketoglutarate, 400 μM NH_4^+ (as sulfate), and 0.7 unit/mL glutamate dehydrogenase.

The secondary equilibrium isotope effect for conversion of glutamate-*3*,*3*-d_2 into α-ketoglutarate was measured by comparing glutamate-*2*-*d* and glutamate-*2*,*3*,*3*,*4*,*4*-d_5 and assuming that the effect of deuterium at the 4 position is negligible (a reasonable assumption since no hybridization change occurs at this position during the reaction). Glutamate concentrations were determined as discussed above. For determination of the equilibrium isotope effect, the assay contained 100 mM K–Taps, pH 8.5, 1 mM DPN, 1.3 mM glutamate, and 0.83 unit/mL glutamate dehydrogenase.

Yeast Alcohol Dehydrogenase. 2-Propanol-*2*-(*h*,*d*) concentrations were determined by using yeast alcohol dehydrogenase at pH 9.5 where the equilibrium constant favors acetone production. Assays contained 100 mM K–Ches, pH 9.5, 5 mM DPN, and 6.7 units/mL yeast alcohol dehydrogenase. Reactions went to completion and concentrations from several determinations were identical within 1%.

For measurement of the equilibrium isotope effect, assays were carried out in 3-mL stoppered cuvettes sealed with a small amount of stopcock grease to prevent volatilization of acetone. A typical assay contained 100 mM potassium phosphate, pH 7.6, 200 μM DPN, 100 mM acetone, 36.6 mM 2-propanol-*2*-(*h*,*d*), and 67 units/mL yeast alcohol dehydrogenase.

Since acetone is such a poor substrate for yeast alcohol dehydrogenase ($K_{acetone}$ = 500 mM at pH 8), the concentrations of acetone-(h_6,d_6) used for determining the secondary equilibrium isotope effect were determined by the formation of the semicarbazone of acetone. A fixed amount of acetone was titrated with semicarbazide at pH 8 until no change in A_{224} was obtained, and an ϵ_{224} of 11 600 was used to calculate

concentration. Concentration determinations were identical within 1.5%.

For determination of the equilibrium isotope effect, assays were carried out in stoppered cuvettes as above. A typical assay contained 100 mM Tris-HCl, pH 8, 2.11 mM DPN, 200 μM DPNH, 500 μM acetone-(h_6,d_6), and 83 units/mL yeast alcohol dehydrogenase.

Malate Dehydrogenase. L-Malate concentrations were determined by using malic enzyme, where the reaction goes to completion at pH 8 as a result of trapping CO_2 as HCO_3^-. Assays contained 100 mM K–Hepes, pH 8, 2 mM $MgSO_4$, 1 mg/mL bovine serum albumin, 100 μM dithiothreitol, 2 mM TPN, and 0.7 unit/mL malic enzyme. Concentrations from several determinations were identical within 0.5%.

For measurement of the equilibrium isotope effect for malate-*2*-(*h*,*d*) and DPN to give DPNH(D) and oxaloacetate, assays contained 100 mM K–Taps, pH 9, 2 mM DPN, 2.5 mM L-malate-*2*-(*h*,*d*), 100 μM oxaloacetate, and 1.3 units/mL malate dehydrogenase.

The concentrations of L-malate-*3*,*3*-d_2 and L-malate-*3R*-*d* were calibrated in the same fashion as described above for L-malate-*2*-*d*. For measurement of the secondary equilibrium isotope effect for either of these molecules, assays contained 100 mM K–Taps, pH 9, 3.1 mM DPN, 1.8 mM L-malate-(*3*,*3*-h_2,*3*,*3*-d_2,*3R*-*d*), and 133 units/mL malate dehydrogenase. A large excess of malate dehydrogenase was used in these experiments to prevent the loss of a significant amount of deuterium from the product, oxaloacetate, due to enolization. Under these conditions, equilibrium was attained in the mixing time ($\sim$3 s), and readings were taken immediately.

Liver Alcohol Dehydrogenase. The primary equilibrium isotope effect for cyclohexanol with liver alcohol dehydrogenase was determined by using either cyclohexanol-*1*-*d* or A-side DPND. DPNH(D) concentrations were determined as discussed above for lactate dehydrogenase. Cyclohexanol concentration assays contained 100 mM potassium phosphate, pH 8, 5 mM DPN, 10 mM semicarbazide, pH 8.2, and 0.6 unit/mL liver alcohol dehydrogenase. Reactions went to completion, and, for several determinations, concentrations were identical within 0.8%.

For determination of the equilibrium isotope effects with labeled ethanol-*1R*-*d* assays contained 100 mM potassium phosphate, pH 8, 1 mM cyclohexanone, 1.8 mM cyclohexanol-*1*-(*h*,*d*), 200 μM DPN, and 0.6 unit/mL liver alcohol dehydrogenase. When DPNH(D) was used as the labeled reactant, a typical assay contained 100 mM Tris-HCl, pH 8, 2 mM DPN, 100 μM DPNH(D) (A-side), 250 μM cyclohexanone, and 0.17 unit/mL liver alcohol dehydrogenase.

The primary equilibrium isotope effect for the formation of ethanol-*1R*-*d* also was determined with A-side DPND or DPNH and acetaldehyde. The concentration assay for acetaldehyde (also used for acetaldehyde-*1*-*d* and benzaldehyde-*1*-*d* in determining the secondary equilibrium isotope effects discussed below) contained 100 mM Tris-HCl, pH 8, 10 mM KCl, 100 μM dithiothreitol, 5 mM DPN, and 1.7 units/mL yeast aldehyde dehydrogenase. Since the equilibrium constant for aldehyde dehydrogenase is far toward the production of DPNH, the reaction goes to completion. Concentrations from several determinations were identical within 1%.

For measurement of the equilibrium isotope effect, assays were carried out in stoppered cuvettes as a result of the volatility of acetaldehyde. A typical assay contained 100 mM Ches, pH 9, 4.2 mM DPN, 200 μM DPNH(D) (A-side), 200 μM acetaldehyde, and 0.3 unit/mL liver alcohol dehydrogenase. The acetaldehyde and benzaldehyde secondary equilibrium isotope effects were determined with acetaldehyde-*1*-*d* or benzaldehyde-*1*-*d* and unlabeled DPNH under similar conditions.

The DPN-*4*-*d* secondary equilibrium isotope effect was determined by using liver alcohol dehydrogenase. The assay for DPN concentrations contained 100 mM Ches, pH 9.5, 50 mM cyclohexanol, and 0.3 unit/mL liver alcohol dehydrogenase. Reactions went to completion since under these conditions the equilibrium favors alcohol oxidation. Concentrations from several determinations were identical within 0.3%.

For measurement of the equilibrium isotope effect, assays contained 100 mM Tris-HCl, pH 8, 2 mM cyclohexanol, 100 μM DPN-*4*-(*h*,*d*), 200 μM cyclohexanone, and 0.17 unit/mL liver alcohol dehydrogenase.

The cyclohexanone-*2*,*2*,*6*,*6*-d_4 secondary equilibrium isotope effect was also measured by using liver alcohol dehydrogenase. Determinations of cyclohexanone concentration were carried out at pH 7.5 where the equilibrium favors ketone reduction, and the reactions go to completion. Concentration assays contained 100 mM Pipes, pH 7.5, 400 μM DPNH, and 0.17 unit/mL liver alcohol dehydrogenase. Concentrations from several determinations were identical within 0.8%.

For measurement of the equilibrium isotope effect, assays contained 100 mM Tris-HCl, pH 8, 1 mM DPN, 250 μM DPNH, 250 μM cyclohexanone-*2*,*2*,*6*,*6*-(h_4,d_4), and 0.17 unit/mL liver alcohol dehydrogenase.

Results and Discussion

As can be seen from Table I, the equilibrium isotope effect for oxidation of secondary alcohols by DPN ranges from 1.17 to 1.19, with deuterium becoming enriched in the alcohol with respect to DPNH. Thus atoms other than those directly bonded to the deuterium-substituted carbon make little, if any, difference. In addition to the isotope effects presented in Table I, the primary equilibrium isotope effect for the oxidation of L-malate-*2*-*d* by TPN was reported by Schimerlik et al. (1975) using pigeon liver malic enzyme to be 1.19 $\pm$ 0.04. This value is in excellent agreement with those reported in Table I, but the value for the oxidation of L-malate-*2*-*d* by DPN in the malate dehydrogenase reaction of 1.32 reported by these authors was clearly in error.

On the basis of the work of Hartshorn & Shiner (1972) and the estimate by Shiner that oxygen should behave like fluorine in their calculations, Schimerlik et al. (1975) suggested the rules that on a carbon attached to the isotopic H or D of interest, replacement of H with C, N, or O gives enrichment of deuterium in the resulting molecules by factors of 1.10, 1.15,[2] and 1.18, respectively. The secondary alcohols used in the present study differ from the 4 position of the nicotinamide ring of DPNH in having two carbons and an oxygen, as opposed to two carbons and a hydrogen, attached to the carbon which bears the H or D transferred in the reaction. The predicted equilibrium isotope effect is thus 1.18, in exact agreement with the experimental values.

The value of 1.14 $\pm$ 0.01 for glutamate (C-2 of glutamate differing from C-4 of TPNH in having a nitrogen in place of a hydrogen) is also in agreement with the predicted value of 1.15 [this value was earlier reported by Rife (1979) as 1.17 $\pm$ 0.04, which is not significantly different]. The value for

[2] The value of 1.15 is for a positively charged NH_3^+ group; the value for an uncharged NH_2 group is 1.12 (V. J. Shiner, Jr., personal communication).

Table I: Primary Deuterium Equilibrium Isotope Effects

dehydrogenase	substrate pair[a]	$^{D}K_{eq,ox}$[b]	$K_{eq,ox}$[c]
secondary alcohols			
liver alcohol	cyclohexanol-*1-d* DPNH	1.18 ± 0.03[d]	(1.42 ± 0.40) × 10^{-9} M
liver alcohol	cyclohexanol DPND (A-side)	1.183 ± 0.008[d]	(1.58 ± 0.39) × 10^{-9} M
lactate	L-lactate DPND (A-side)	1.19 ± 0.005	(2.19 ± 0.08) × 10^{-12} M
yeast alcohol	2-propanol-*2-d* DPNH	1.175 ± 0.010	(4.2 ± 0.2) × 10^{-9} M
malate	L-malate-*2-d* DPNH	1.173 ± 0.008	(2.1 ± 0.8) × 10^{-13} M
isocitrate	*threo*-DL-isocitrate-*2-d* TPNH	1.168 ± 0.006	1.04 ± 0.18 M
primary alcohol			
liver alcohol	ethanol DPND (A-side)	1.069 ± 0.008	(6.0 ± 0.1) × 10^{-12} M
amino acid			
glutamate	L-glutamate-*2-d* TPNH	1.14 ± 0.01	(4.4 ± 0.1) × 10^{-14} M^2
hemiacetal			
glucose-6-P	glucose-*1-d* DPNH	1.28 ± 0.02	(5.2 ± 0.5) × 10^{-7} M

[a] Reactants between which label is transferred. The labeled molecule actually used in each case is noted. [b] $^{D}K_{eq,ox}$ is the deuterium isotope effect (that is, K_{eqH}/K_{eqD}) on the equilibrium constant for oxidation by DPN or TPN. All effects were carried out at least in triplicate. [c] Equilibrium constant for oxidation of unlabeled molecule by DPN or TPN, including the concentration of H^+ when it is a reactant. For isocitrate dehydrogenase, dissolved CO_2 is considered the reactant (i.e., bicarbonate is not included), and, for glutamate dehydrogenase, NH_4^+ is considered the reactant and the amino group of glutamate is assumed to be protonated. The apparent pK used to calculate CO_2 concentrations from added bicarbonate was 6.4. [d] Since identical values are obtained for equilibrium isotope effects and for equilibrium constants with 100 mM potassium phosphate, pH 8, and cyclohexanol-*1-d* and with 100 mM Tris-HCl, pH 8, and A-side DPND, Tris apparently does not form Schiff's bases with ketones such as cyclohexanone to any extent at this concentration.

Table II: Secondary Deuterium Equilibrium Isotope Effects

enzyme	substrate pair[a]	$^{D}K_{eq}$[b]	K_{eq}[c]
	β-Secondary Isotope Effects		
liver alcohol dehydrogenase	cyclohexanone-d_4 cyclohexanol	0.81 ± 0.02 (0.948)	(2.0 ± 0.3) × 10^{-9} M
yeast alcohol dehydrogenase	acetone-d_6 2-propanol	0.78 ± 0.02 (0.960)	(5.3 ± 0.5) × 10^{-9} M
lactate dehydrogenase	pyruvate-d_3 L-lactate	0.826 ± 0.014 (0.948)	(4.5 ± 0.1) × 10^{-12} M
glutamate[d] dehydrogenase	α-ketoglutarate L-glutamate-d_5 or -*2-d*	0.898 ± 0.016 (0.948)	(4.4 ± 0.1) × 10^{-14} M^2
malate dehydrogenase	oxaloacetate L-malate-*3,3-*d_2	0.88 ± 0.02 (0.937)	(7.1 ± 0.1) × 10^{-13} M
malate dehydrogenase	oxaloacetate L-malate-*3R-d*	0.945 ± 0.020	(7.1 ± 0.1) × 10^{-13} M
	α-Secondary Isotope Effects		
liver alcohol dehydrogenase	DPN-*4-d*[e] DPNH	0.887 ± 0.005	(2.2 ± 0.2) × 10^{-9} M
fumarase	fumarate-*2,3-*d_2 L-malate	0.690 ± 0.017	4.25 ± 0.08
liver alcohol dehydrogenase	acetaldehyde-*1-d* ethanol	0.953 ± 0.003	(2.9 ± 0.1) × 10^{-12} M
yeast alcohol dehydrogenase	benzaldehyde-*1-d* benzyl alcohol	0.79 ± 0.01	(3.1 ± 0.2) × 10^{-11} M

[a] Reactants between which label is transferred. The labeled molecule actually used in each case is noted. [b] Equilibrium isotope effect for reaction in the direction from the first reactant given to the second. The observed value which represents the equilibrium effect for all deuterium atoms in the originally labeled reactant is listed, and for β-secondary isotope effects a value per deuterium, obtained by taking the nth root of the observed value, where n is the number of deuterium atoms, is listed in parentheses. All experiments were carried out in triplicate, except that for pyruvate, which was carried out in sextuplicate, and that for acetaldehyde, which was carried out in duplicate. [c] K_{eq} for unlabeled molecules in the direction of nucleotide reduction for dehydrogenases or for conversion of fumarate to malate for fumarase. [d] Ammonia and the amino group of glutamate are protonated. [e] Cyclohexanol and cyclohexanone were the other reactants.

a primary alcohol relative to DPNH, where the carbon holding the H or D is attached to a carbon, a hydrogen, and an oxygen, is predicted by the above rules to be 1.18/1.10 = 1.07 (the rules are multiplicative, and in going from C-1 of ethanol to C-4 of DPNH, one replaces H with C, and O with H), in exact agreement with experiment. Finally, C-1 of glucose in the predominant hemiacetal form is bonded to two oxygens and a carbon, in addition to the H or D, and thus the calculated equilibrium isotope effect for transfer to C-4 of DPNH is $(1.18)^2/(1.10) = 1.27$, compared to the experimental value of 1.28 ± 0.02. The rules of Schimerlik et al. (1975) thus appear to predict very precisely the observed equilibrium isotope effects on dehydrogenase reactions and can be used with some degree of confidence for other similar reactions.

Secondary isotope effects may also be obtained on equilibrium constants. These effects indicate enrichment of deu-

terium on or adjacent to a carbon which changes its bonding during the course of the reaction. The first portion of Table II presents β-secondary equilibrium isotope effects (that is, where deuterium is substituted adjacent to the carbon undergoing a change in hybridization) for reduction of ketones to alcohols or amino acids. These effects are all very similar and give an average effect of 0.946 per deuterium (that is, deuterium becomes enriched in the alcohol or amino acid), in good agreement with the value reported by Hogg (1974) of 0.944 for a series of ketone addition reactions. Geneste et al. (1971) obtained 0.958/D for acetone-d_6 by measuring the equilibrium for hemiketal formation with methanol (which agrees with the value of 0.96/D reported in Table II) and 0.955/D for cyclohexanone-*2,2,6,6-d*$_4$, again measuring hemiketal formation with methanol (the value reported in Table II for cyclohexanone-*2,2,6,6-d*$_4$ is 0.948/D).

It thus appears that a carbonyl group causes a reproducible effect on the vibrational frequencies of C–H bonds on adjacent carbons, in exception to the general rule that only the nature of the atoms directly attached to the carbon involved in the C–H(D) bond has an effect. Note that similar β-secondary equilibrium isotope effects are seen for conversion of ketones to amino acids, alcohols, and hemiketals, despite the fact that the atoms that become attached to the carbonyl carbon during the reaction are different and would induce different effects on the vibrational frequencies of C–H bonds for hydrogen directly bonded to the carbonyl carbon. It is interesting to note that ionized carboxyl groups do not cause a β-secondary effect as ketones do, since the values in Table I are the same for α-hydroxycarboxylic acids as for 2-propanol and cyclohexanol.

The α-secondary equilibrium isotope effect (that is, where deuterium is substituted on the carbon undergoing a change in hybridization) for the conversion of DPN-*4-d* into DPND is 0.89 (deuterium becoming enriched in DPNH).[3] The value for fumarate-*2,3-d*$_2$ is more complicated, since not only does it involve two sp^2 carbons substituted with deuterium becoming sp^3 but also one of these carbons becomes bonded to an oxygen, rather than a hydrogen, in L-malate. If the observed equilibrium isotope effect of 0.69 is multiplied by 1.18 to allow for the oxygen, the square root of the result, 0.90, corresponds to the equilibrium isotope effect for a single sp^2–sp^3 hybridization change (that is, $=CH-$ to $-CH_2-$) and is in good agreement with the value for DPN-*4-d*. A similar value of 0.89/D has been calculated by Cleland (1980) from the data of Kresge & Chiang (1968) for trimethoxybenzene, and thus this value seems well established for the α-secondary equilibrium isotope effect for sp^2–sp^3 rehybridization.

The observed α-secondary equilibrium isotope effect of 0.953 for reduction of acetaldehyde-*1-(h,d)* by DPNH is an apparent value, since acetaldehyde is partly hydrated in solution and there is a large isotope effect on the hydration equilibrium [0.73; Lewis & Wolfenden (1977)], with deuterium becoming enriched in the hydrated aldehyde. For unlabeled acetaldehyde, [hydrate]/[aldehyde] = 1.5 (Bell, 1966), while for acetaldehyde-*1-d*, the value will be 1.5/0.73 = 2.05. Thus the proportion of free aldehyde is 1.0/2.5 = 0.40 for unlabeled acetaldehyde and 1.0/3.05 = 0.327 for deuterated acetaldehyde. The equilibrium isotope effect for conversion of free aldehyde to ethanol is then (0.953)(0.327)/0.40 = 0.779, and the value for conversion of the aldehyde hydrate to ethanol is 0.779/0.73 = 1.067. For reduction of benzaldehyde-*1-d*, which is not appreciably hydrated, to benzyl alcohol, the α-secondary isotope effect is 0.79, in close agreement with the value for acetaldehyde and in fair agreement with values of 0.82 for *p*-methoxybenzaldehyde and benzaldehyde calculated from tritium equilibrium isotope effects by Welsh et al. (1980). It thus appears that aromatic and aliphatic aldehydes show similar values.

Hogg (1974) reported that the equilibrium isotope effect for hydration of 1,3-dichloroacetone was temperature independent. We measured the primary deuterium equilibrium isotope effect for the reaction of DPN and cyclohexanol-*1-(h,d)* at 15, 25, and 36 °C and found no significant differences. It thus appears likely that over the temperature range of interest to biochemists, equilibrium isotope effects will be temperature independent. The experimental values for the isotope effects determined in these studies, as well as other published values, have been used by Cleland (1980) to construct a table of fractionation factors which reflect enrichment of deuterium in a variety of organic molecules relative to water. By the use of this table, together with the rules of Schimerlik et al. (1975) which have been verified by the present work, one can now calculate the primary and secondary equilibrium isotope effect for nearly every reaction of biological interest.

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[3] This isotope effect has been reported by Kurz & Frieden (1980) to be 0.97–0.99. However, the ratio of calculated fractionation factors for the hydrogen on C-2 of propene and propane is 0.89 (Buddenbaum & Shiner, 1977), and in view of the clear sp^2 character of C-4 in DPN, and the close agreement between the various measured values for the sp^2–sp^3 change, this discrepancy must result from experimental error.