the dissociation equilibrium of the G_s subunits. This conclusion agrees with the tendency of R–H to stabilize reconstituted G_s and to increase its apparent affinity for nucleotide (Asano et al., 1984).

While the mechanism we propose for the hormonal stimulation of G_s activation seems to be consistent with our kinetic and quasi-equilibrium data, it is still clearly speculative. Direct physical measurements of the receptor–G_s interaction and the dissociation of the subunits of G_s must be made in the vesicles, and the reconstitution of receptor with the resolved subunits of G_s will simplify this analysis.

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Variation of Transition-State Structure as a Function of the Nucleotide in Reactions Catalyzed by Dehydrogenases. 1. Liver Alcohol Dehydrogenase with Benzyl Alcohol and Yeast Aldehyde Dehydrogenase with Benzaldehyde†

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ABSTRACT: Primary intrinsic deuterium and ^{13}C isotope effects have been determined for liver (LADH) and yeast (YADH) alcohol dehydrogenases with benzyl alcohol as substrate and for yeast aldehyde dehydrogenase (ALDH) with benzaldehyde as substrate. These values have also been determined for LADH as a function of changing nucleotide substrate. As the redox potential of the nucleotide changes from –0.320 V with NAD to –0.258 V with acetylpyridine–NAD, the product of primary and secondary deuterium isotope effects rises from 4 toward 6.5, while the primary ^{13}C isotope effect drops from 1.025 to 1.012, suggesting a trend from a late transition state with NAD to one that is more symmetrical. The values of ^{D}k (again the product of primary and secondary isotope effects) and ^{13}k for YADH with NAD are 7 and 1.023, suggesting for this very slow reaction a more stretched, and thus symmetrical, transition state. With ALDH and NAD, the primary ^{13}C isotope effect on the hydride transfer step lies in the range 1.3–1.6%, and the α-secondary deuterium isotope effect on the same step is at least 1.22, but ^{13}C isotope effects on formation of the thiohemiacetal intermediate and on the addition of water to the thio ester intermediate are less than 1%. On the basis of the relatively large ^{13}C isotope effects, we conclude that carbon motion is involved in the hydride transfer steps of dehydrogenase reactions.

Very little is known about the structure of the transition states for hydride transfer in enzymatic reactions catalyzed by dehydrogenases, since until recently it was not even possible to determine the intrinsic isotope effects on the hydride transfer step except in rare cases where this step was totally rate limiting (as for formate dehydrogenase; Blanchard & Cleland, 1980). Hermes et al. (1982), however, have developed methods that allow the determination of the intrinsic isotope effects within narrow limits by measurement of ^{13}C isotope effects on C–H cleavage with an unlabeled substrate and one that is deuterated in the primary position. Further, if the ^{13}C isotope effect can be measured with a nucleotide deuterated at the 4-position (this causes an α-secondary deuterium isotope effect), one can obtain an exact solution for all of the intrinsic isotope effects and commitments in the system (Hermes et al., 1982). These techniques allow one to vary the nucleotide substrate and determine the effects on the intrinsic primary

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deuterium and ^{13}C isotope effects, and it is the intent of this study to attempt to deduce from these values the way in which the transition state varies with the nature of the nucleotide and to see whether the nature of the variation is the same for all enzymes or depends more heavily on the specific interactions of the nucleotide with a given enzyme rather than solely on the redox potential of the nucleotide.

In order best to identify the transition-state structure, one would ideally determine all possible isotope effects on the atoms involved in the hydride transfer reaction, including in particular the primary ^{13}C isotope effect at C-4 of the nucleotide. Techniques are being developed to determine these values, but in the meantime we report preliminary interpretations based on the primary ^{13}C and deuterium isotope effects with benzyl alcohol and liver alcohol dehydrogenase (LADH)[1] and with benzaldehyde and yeast aldehyde dehydrogenase (ALDH), since the well-behaved pyrolysis of benzoic acid to CO_2 and benzene (O'Leary, 1966) makes possible the determination of accurate ^{13}C isotope effects in these systems by isotope ratio mass spectrometry.

Materials and Methods

Chemicals. Benzyl alcohol-*1,1-d*$_2$ was synthesized by the procedure of Klinman (1976) from benzoyl chloride and $LiAlD_4$ (Merck, 99 atom % D). The isotopic purity was confirmed by the absence of methylene protons at C-1 by 270-MHz proton NMR.

NAD deuterated at the 4-position of the nicotinamide ring (NAD-*4-d*) was synthesized by reduction of NAD with ethanol-d_6 (Merck, 99 atom % D) and YADH to the A-side NADD as described by Viola et al. (1979), followed by oxidation by the method of Cook et al. (1980), by using the B-side enzyme, glutamate dehydrogenase. The enzyme was removed by passage of the solution through an Amicon PM 10 ultrafilter, and the filtrate was lyophilized and desalted by gel filtration on a 1.6 × 44 cm column of Bio-Gel P-2. Further purification was obtained by ion-exchange chromatography on a 1.7 × 23 cm column of AG-MP-1-Cl^- (Bio-Rad) with 0.3 M LiCl, pH 8.0, as the eluant, followed by another desalting step on Bio-Gel P-2. Final purification was by high-pressure liquid chromatography (Waters semiprep C-18 column, flow rate 3 mL/min, elution with 7.5 mM potassium phosphate, pH 6.0, 0.5 mg/injection). The coenzyme was 99% deuterated on the basis of 270-MHz proton NMR.

All enzymes (except lactate dehydrogenase from Boehringer) and coenzymes (except NAD and deamino-NAD from P-L Biochemicals) were from Sigma. The activity of LADH was determined at pH 9 with ethanol and NAD, while the activities of YADH and ALDH were determined at pH 8 with acetaldehyde and either NADH or NAD, respectively.

Initial Velocity Studies. These were carried out with a Cary 118 spectrophotometer by monitoring the change in absorbance at 340 nm resulting from production of NADH. Deuterium isotope effects on initial velocities were obtained at saturating nucleotide concentration by comparing deuterated and nondeuterated substrates.

Conditions for determining isotope effects with LADH and deuterated benzyl alcohol were 7.5 mM semicarbazide, 10 mM glutathione, 1.4 mM NAD ($K_m \simeq 26$ μM) and 6 milliunits of LADH (with 10 mg/mL bovine serum albumin) in 0.2 M potassium phosphate, pH 8.0 at 25 °C. With the alternate nucleotides, 3.33 mM nucleotide was used, except that thio-NAD was 10 mM. To oxidize ethanol present in the enzyme, the reaction mixture was incubated for 10 min prior to addition of 15–90 μM benzyl alcohol (K_m = 15 μM). For YADH, mixtures were similar except that NAD was 8 mM [K_m = 5 mM (Klinman, 1976)] and 30 units of YADH was used. Benzyl alcohol (10–100 mM; K_m = 230 mM) was added after a 5-min preincubation. Isotope effects for ALDH with deuterated benzaldehyde were measured with 0.2 mM NAD (K_m = 25 μM), 10 mM glutathione, 5–225 μM benzaldehyde (K_m = 10 μM), and 5 milliunits of ALDH in 0.2 M potassium phosphate, pH 8.0 at 25 °C. The concentrations of the alternate nucleotides used were 50 μM deamino-NAD, 40 μM pyridinealdehyde–NAD, and 2 mM acetylpyridine–NAD. The α-secondary deuterium isotope effect for LADH was determined with 7.5 mM semicarbazide, 10 mM glutathione, 400 μM NAD or NAD-*4-d*, and 8 milliunits of LADH in 0.2 M potassium phosphate, pH 8.0 at 25 °C. Benzyl alcohol (10–150 μM) was added after a 5-min preincubation. For ALDH, reaction mixtures contained 5 mM glutathione, 380 μM NAD or NAD-*4-d*, 7–150 μM benzaldehyde, and 16 milliunits of ALDH in 0.2 M potassium phosphate pH 8.0 at 25 °C.

The alcohol dehydrogenase used for initial velocity studies was dialyzed overnight at 4 °C against 0.2 M potassium phosphate, pH 8.0, containing 1 mM dithioerythritol, 0.1 M KCl, 1 unit/mL ALDH, and 5 mM NAD to remove most of the contaminating ethanol, followed by dialysis against 0.1 M potassium phosphate, pH 6.5, containing 0.2 mM dithioerythritol.

Calibration of Substrate Solutions. Benzyl alcohol concentrations were determined enzymatically with 5 mM glutathione, 1.8 mM NAD, 2 units of LADH, 2 units of ALDH in 0.2 M KPO_4, pH 8.0. Benzyl alcohol (10–100 μM) was added as soon as the residual ethanol was oxidized. Benzaldehyde solutions were calibrated with 5 mM glutathione, 0.72 mM NAD, 20–300 μM benzaldehyde, and 2 units of ALDH. Assays for NAD contained 10 mM glucose-6-P, 25–150 μM NAD, and 35 units of glucose-6-P dehydrogenase from *L. mesenteroides*. Reactions went to completion, and concentrations from several determinations were identical within 1%. Assays for pyruvate contained 0.25 mM NADH, 0.05–0.1 mM pyruvate (reaction mixture diluted 1:2 with 1 N HCl) and 5 units of lactate dehydrogenase in 1 mL of 0.2 M potassium phosphate, pH 8.0 at 25 °C.

^{13}C Kinetic Isotope Effects. ^{13}C isotope effects were determined by using the natural abundance of ^{13}C in the substrate as label. The alcohol dehydrogenase catalyzed oxidation of benzyl alcohol to benzaldehyde at pH 8.0 was investigated by coupling the reaction to the ALDH-catalyzed oxidation to benzoic acid. Reaction mixtures contained 2 mM glutathione, 3.6 mM NAD, 3.5 mM benzyl alcohol or benzyl alcohol-*1,1-d*$_2$, 5 units of LADH or 90 000 units of YADH, and 50 units of ALDH in 500 mL of 0.2 M potassium phosphate, pH 8.0 at 25 °C. Reactions were quenched with HCl at 20% conversion (based on absorbance at 340 nm). Complete conversions were carried out in a similar 1-L system containing 1.8 mM NAD, 0.3 mM unlabeled or deuterated benzyl alcohol, 40 units of LADH, and 150 units of ALDH.

For the ALDH-catalyzed reaction the mixture contained 2.5 mM glutathione, 0.9 mM NAD, 50 units of ALDH, and 3.8 (20% conversion) or 0.8 mM (complete conversion) benzaldehyde or benzaldehyde-*1-d* in 500 mL of 0.2 M potassium phosphate, pH 8.0 at 25 °C.

The pH of the quenched solutions was raised with KOH to 7, and the enzymes were removed by filtration in an Amicon

[1] Abbreviations: LADH, horse liver alcohol dehydrogenase; YADH, yeast alcohol dehydrogenase; ALDH, yeast aldehyde dehydrogenase; glucose-6-P, glucose 6-phosphate.

cell with a PM 10 membrane. Each filtrate was extracted 3 times with ether, titrated to pH 2, and extracted 3 times with ether to remove benzoic acid. The ether extract was dried over $MgSO_4$ and rotary evaporated to dryness. The crude benzoic acid was dried under vacuum for 2 h to remove all of the ether and finally sublimed in vacuo (<5 μm) at 40–50 °C. The sublimed benzoic acid was transferred with a spatula to a Vycor test tube that was cleaned in chromic acid and preheated in vacuo almost to the softening point.[2] The pyrolysis tubes were then sealed under vacuum, and the benzoic acid was pyrolyzed for 1 h at 550 °C. After the pyrolysis the tubes were opened under vacuum, and the CO_2 was isolated as described by O'Leary (1966, 1980).

To measure ^{13}C kinetic isotope effects with nucleotides other than NAD, we reduced expense by recycling the coenzyme. This also decreases problems from reversibility of the alcohol dehydrogenase reaction and product inhibition by the reduced coenzyme. Lactate dehydrogenase is a suitable A-side dehydrogenase to recycle the coenzymes. Reaction mixtures contained coenzyme (1 mM thio-NAD or pyridinealdehyde–NAD, 0.3 mM acetylpyridine–NAD or deamino-NAD, or 0.1 mM NAD or NAD-*4-d*), 7.4 mM pyruvate, 2 mM glutathione, 15 mM benzyl alcohol and 12 units of each of LADH and LDH in 100 mL of 0.2 M potassium phosphate, pH 8.0 at 25 °C. To make sure that the oxidation of benzyl alcohol was rate limiting, the absorbance at the λ_{max} of the reduced nucleotide was monitored to be sure that it did not increase. Quantitative assays for pyruvate and the generated benzaldehyde were used during the reactions and for the final determination of the percentage conversion to benzaldehyde. Since pyruvic and lactic acids would sublime with benzoic acid, it was necessary to isolate the benzaldehyde and oxidize it with NAD without recycling. The enzymes were thus denatured with HCl at 20% conversion to benzaldehyde, and after the pH was readjusted to 8, the solution was distilled in vacuo and the benzaldehyde trapped with liquid nitrogen. The benzaldehyde was then completely converted to benzoic acid, which was isolated as usual.

Sorbitol dehydrogenase (sheep liver) was a suitable A-side dehydrogenase to recycle the coenzyme in the aldehyde dehydrogenase reaction, since the substrate and the product are the sugars fructose and sorbitol, which are not extracted by ether. The reaction mixture contained 2 mM glutathione, 3.5 mM benzaldehyde, 10 mM fructose, coenzyme (0.25 mM acetylpyridine–NAD or 0.1 mM of the others), and 30 units each of ALDH and sorbitol dehydrogenase in 400 mL of 0.2 M potassium phosphate, pH 8.0. The absorbance at the maximum wavelength of the reduced coenzyme did not increase during the reaction. Quantitative determination of the remaining benzaldehyde was used to follow the reaction and for the final determination of the percentage of the conversion. At 20% conversion the reaction was stopped with HCl, and the isolation of the benzoic acid was accomplished as usual.

^{13}C Equilibrium Isotope Effect for the LADH-Catalyzed Reaction. A reaction mixture with 3 mM glutathione, 2.5 mM NAD, 10 mM benzyl alcohol, and 150 units of LADH in 500 mL of 0.2 M potassium phosphate and 0.1 M pyrophosphate, pH 9.0, was incubated at 25 °C for 15 h (chemical equilibrium achieved in 30 min as detected by absorbance at 340 nm). The enzyme was denatured with HCl, and the isolation of the benzaldehyde and the oxidation to benzoic acid were done as described above.

Instrumentation. The $^{13}C/^{12}C$ ratios in the CO_2 samples were measured with a Nuclide Associates RMS 6-60 isotope ratio mass spectrometer with a dual inlet system. All the samples of one series of reactions were analyzed on the same day to minimize variations in instrument response.

Data Processing. To determine deuterium isotope effects, reciprocal initial velocities were plotted vs. reciprocal substrate concentrations. The data were fitted to eq 1 (or where ap-

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \quad (1)$$

propriate to similar equations where $E_{V/K}$ or E_V were zero or identical) by the least-squares method using the Fortran programs of Cleland (1979). In eq 1 F_i is the fraction of deuterium label in the substrate, and E_V and $E_{V/K}$ are the isotope effects minus one on the respective parameters. ^{13}C kinetic isotope effects were calculated with

$$^{13}(V/K) = \log (1 - f)/\log (1 - f(R_f/R_0)] \quad (2)$$

where R_f is the $^{13}C/^{12}C$ ratio in product CO_2 at fractional reaction f and R_0 is the ratio in product after complete reaction. We use the nomenclature of Northrop (1977), where isotope effects are indicated by a leading superscript. Thus, $^{13}(V/K)$ is the ^{13}C isotope effect on V/K. The ^{13}C equilibrium isotope effect was calculated with

$$^{13}K_{eq} = \frac{1 + x_1(1 - y_1/y_2)}{y_1/y_2} \quad (3)$$

where x_1 = [benzaldehyde]/[benzyl alcohol] at equilibrium (here, 0.075), y_1 = $^{13}C/^{12}C$ ratio in the benzaldehyde at equilibrium, and y_2 is the mass ratio in benzyl alcohol before equilibration.

Since the $^{13}(V/K)$ isotope effect with deuterated benzyl alcohol was larger than that with undeuterated material, one can conclude that the deuterium and ^{13}C-sensitive steps of the LADH-catalyzed reaction are the same (Hermes et al., 1982). Therefore, computer programs that simultaneously solve equations 4–6 or 4–8 were used to calculate intrinsic isotope

$$^{D}(V/K) = \frac{^{D}k + c_f + c_r{}^{D}K_{eq}}{1 + c_f + c_r} \quad (4)$$

$$^{13}(V/K)_H = \frac{^{13}k + c_f + c_r{}^{13}K_{eq}}{1 + c_f + c_r} \quad (5)$$

$$^{13}(V/K)_D = \frac{^{13}k + c_f/{}^{D}k + c_r{}^{13}K_{eq}{}^{D}K_{eq}/{}^{D}k}{1 + c_f/{}^{D}k + c_r{}^{D}K_{eq}/{}^{D}k} \quad (6)$$

$$^{\alpha\text{-D}}(V/K) = \frac{^{\alpha\text{-D}}k + c_f + c_r{}^{\alpha\text{-D}}K_{eq}}{1 + c_f + c_r} \quad (7)$$

$$^{13}(V/K)_{\alpha\text{-D}} = \frac{^{13}k + c_f/{}^{\alpha\text{-D}}k + c_r{}^{13}K_{eq}{}^{\alpha\text{-D}}K_{eq}/{}^{\alpha\text{-D}}k}{1 + c_f/{}^{\alpha\text{-D}}k + c_r{}^{\alpha\text{-D}}K_{eq}/{}^{\alpha\text{-D}}k} \quad (8)$$

effects and commitments [see Hermes et al. (1982)]. In these equations ^{D}k, ^{13}k, and $^{\alpha\text{-D}}k$ are the intrinsic isotope effects on the hydride transfer step caused by (1) deuteration of benzyl alcohol (this is the product of a primary deuterium isotope effect in the position transferred to NAD and an α-secondary deuterium isotope effect in the other position), (2) ^{13}C sub-

[2] This is a critical point in the procedure. When the sublimed benzoic acid was dissolved in a solvent for transfer to the Vycor tube, the subsequent pyrolysis did not go smoothly but gave a black tar and a low yield of CO_2, regardless of how thoroughly the solvent was removed after the transfer. When the benzoic acid was scraped off the sublimation apparatus and transferred with a spatula, however, the pyrolysis went cleanly. It is also important that the sublimation be carried out at a temperature below 50 °C; higher temperatures at higher pressures gave benzoic acid that did not cleanly pyrolyze.

Table I: Mass Ratios Resulting from Pyrolysis of Benzoic Acid

treatment of benzoic acid	isotope ratio ($\times 10^5$)[a]
none	1190.85
	1191.2
	1191.4
sublimation at 50 °C	1191.4
entire purification procedure	1189.2
reaction mixture and then the purification procedure	1189.2

[a] $^{13}C/^{12}C$ ratios were adjusted for ^{17}O contribution to m/e 45 by subtracting 74 from decade settings for m/e 45/44 which were corrected to tank standard of 1260 (O'Leary, 1980).

stitution in benzyl alcohol (a primary ^{13}C isotope effect), or (3) deuterium substitution at C-4 of NAD (an α-secondary deuterium isotope effect). The same leading superscripts on V/K indicate the observed isotope effects, while the following subscripts H, D, or α-D on $^{13}(V/K)$ indicate observed ^{13}C isotope effects with otherwise unlabeled reactants, with the deuterated benzyl alcohol, or with deuterated NAD. $^{D}K_{eq}$, $^{13}K_{eq}$, and $^{\alpha\text{-}D}K_{eq}$ are the corresponding equilibrium isotope effects, and c_f and c_r are the forward and reverse commitments. [A commitment for a given reactant is the ratio of the rate constant for the unlabeled molecule for the isotope-sensitive step to the net rate constant for release of the reactant into solution from the complex that undergoes the isotope sensitive step. The term net rate constant is used here as defined by Cleland (1975)].

Results

^{13}C Kinetic and Equilibrium Isotope Effects. To determine primary ^{13}C isotope effects for the alcohol dehydrogenase catalyzed reactions, the oxidation of benzyl alcohol to benzaldehyde at pH 8 was coupled to the ALDH-catalyzed oxidation to benzoic acid, which decarboxylates quantitatively by pyrolysis (O'Leary, 1966). The $^{13}C/^{12}C$ ratio of this CO_2 can be accurately determined with an isotope ratio mass spectrometer, and ^{13}C isotope effects on V/K can be calculated from the mass ratios from low percentage and complete reactions. Control experiments with benzoic acid showed that this is a reproducible technique (Table I). This method was also used to determine ^{13}C isotope effects for ALDH and the ^{13}C equilibrium isotope effect for the conversion of benzyl alcohol to benzaldehyde. Tables II–IV show data for LADH, YADH, and ALDH.

Deuterium Isotope Effects. For all dehydrogenases, the concentrations of deuterated or unlabeled benzyl alcohol or benzaldehyde were varied at saturating nucleotide levels. The isotope effects for LADH are shown in Table V. YADH showed an isotope effect of 5.2 ± 0.3 on V/K and none on V. The isotope effects for ALDH are shown in Table IV.

The α-secondary deuterium isotope effects were determined by varying benzyl alcohol or benzaldehyde concentrations at equal saturating levels of NAD or NAD-*4-d*. $^{\alpha\text{-}D}(V/K)$ values were 1.052 ± 0.014 for LADH and 1.047 ± 0.02 for ALDH.

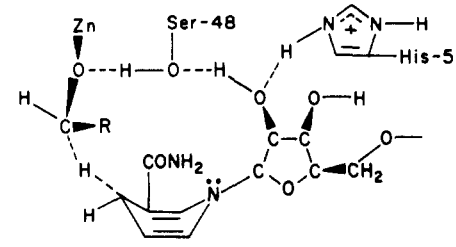
Intrinsic Isotope Effects with Alcohol Dehydrogenases. When $^{13}(V/K)_H$, $^{13}(V/K)_D$, $^{13}(V/K)_{\alpha\text{-}D}$, $^{D}(V/K)$, and $^{\alpha\text{-}D}(V/K)$ values for benzyl alcohol and NAD with LADH were used in eq 4–8 along with the $^{13}K_{eq}$ value of 0.9847 ± 0.0005 from the present work and $^{D}K_{eq}$ and $^{\alpha\text{-}D}K_{eq}$ values of 1.35 ± 0.02[3] and 0.887 ± 0.015 from Cook et al. (1980), we obtained the following values from the computer program which solves these equations simultaneously (Hermes et al., 1982): ^{D}k = 3.85 ± 0.39 (this is the product of the primary and α-secondary deuterium isotope effects in benzyl alcohol, since it was dideuterated), ^{13}k = 1.026 ± 0.002, $^{\alpha\text{-}D}k$ = 1.10 ± 0.03, c_f = 0.48 ± 0.33, and c_r = 0.13 ± 0.21. The intrinsic isotope effects are well determined, but the commitments are not, and c_r is not significantly different from zero. Previous studies by Cook & Cleland (1981a–c) have suggested that reverse commitments are negligible for alcohol dehydrogenases, so it is probable that this is true here as well. If c_r is assumed to be zero, the simultaneous solution of eq 4–7 gives 4.05 for ^{D}k, 1.0254 for ^{13}k, 1.088 for $^{\alpha\text{-}D}k$, and 0.7 for c_f; we will use these values for comparison with those derived below for nucleotides other than NAD.

For YADH we have only $^{13}(V/K)_H$, $^{13}(V/K)_D$, and $^{D}(V/K)$ values, and solution of eq 4–6 establishes limits on ^{D}k, ^{13}k, and the commitments. If c_r is zero, which seems very likely in view of the low V_{max} for oxidation of benzyl alcohol with this enzyme, ^{D}k = 7.1, ^{13}k = 1.0234, and c_f = 0.45. (The other limits when c_f is zero are ^{D}k = 6.2, ^{13}k = 1.0241, and c_r = 0.25.)

Table V shows intrinsic isotope effects calculated from $^{13}(V/K)_H$, $^{13}(V/K)_D$, and $^{D}(V/K)$ values for LADH with five nucleotide substrates, assuming that c_r is zero in each case. The K_m values for the nucleotides and for benzyl alcohol and the relative V_{max} values are also shown.

Discussion

Alcohol dehydrogenases have been shown by Cook & Cleland (1981a–c) and Morris et al. (1980) to have two-step chemical mechanisms in which proton transfer from a zinc-bound alcohol to an acid–base catalytic group (which we believe to be His-51 in LADH) precedes hydride transfer. This proton transfer is proposed to proceed via a proton shuttle involving Ser-48 and the 2′-hydroxyl of the ribose attached to the nicotinamide ring of NAD (Eklund et al., 1982). In a step prior to hydride transfer, the large ^{15}N isotope effect of 1.06 shows that N-1 of the nicotinamide ring of the nucleotide becomes pyramidal (presumably due to geometric distortion caused by the enzyme), so that the aromaticity of the ring is destroyed and a carbonium ion forms at C-4 (Cook et al., 1981). Hydride transfer thus occurs between a metal-bound alkoxide and a carbonium ion or, in the reverse reaction, between a metal-bound carbonyl group and the electron-rich dihydronicotinamide ring. We can diagram the expected transition state as the following:



Three hydrogens are in flight in this transition state: (1) the primary hydrogen which is transferred as a hydride ion (that is, as a proton and an accompanying electron pair), (2) the α-secondary hydrogen on the alcohol, which undergoes a 54° bending motion during the reaction from its tetrahedral position in the alkoxide to its trigonal position in the aldehyde, and (3) the α-secondary hydrogen at C-4 of the nucleotide, which also undergoes a 54° bending motion from in plane in NAD to out of plane in NADH. The motions of these three hydrogens appear to be coupled in the transition state, since the α-secondary deuterium isotope effects are normal (in

[3] This value is the product of 1.069 for the primary deuterium and 1.266 for the secondary deuterium.

Table II: ^{13}C Isotope Effects at pH 8.0, 25 °C[a]

enzyme	substrate	% reaction	isotope ratios (×10⁵)[b] low conversion	isotope ratios (×10⁵)[b] 100% conversion	$^{13}(V/K)$
LADH	benzyl alcohol	20	1173.5	1188.6	1.0149
			1172.5	1188.7	
	benzyl alcohol-d_2	20	1164.95	1187.0	1.0217
			1164.8	1187.9	
	benzyl alcohol ↔ benzaldehyde		1204.5		
			1204.75	1187.6	0.9847[c] ± 0.0005
			1205.3		
YADH	benzyl alcohol	20	1171.5	1188.6	1.0164
				1188.7	
	benzyl alcohol	20	1170.6	1187.6	1.0159
	benzyl alcohol-d_2	15	1163.9	1187.0	1.022
			1163.6	1187.9	
ALDH	benzaldehyde	14	1167.4	1183.0	1.0144
			1168.0	1183.6	
	benzaldehyde-*d*	21	1172.4	1194.0	1.0202
			1173.0	1193.6	

[a] Isotope effects were determined with NAD as coenzyme and without its recycling. [b] Ratios were adjusted for ^{17}O contribution to m/e 45 by subtracting 74 from decade settings for m/e 45/44 which were corrected to tank standard of 1260 (O'Leary, 1980). [c] The reaction was allowed to come to chemical and isotopic equilibrium, so the value determined is $^{13}K_{eq}$.

NAD-*4-d*), or more normal than expected (in the aldehyde) as the result of the bending motion becoming part of the reaction coordinate motion (Cook et al., 1981; Welsh et al., 1980).

The purpose of the present work is to study the way in which this transition-state structure varies as the nature of the nucleotide changes. For a nonenzymatic reaction, one would expect that as the redox potential of the nucleotide became more positive the transition state would become earlier, that is, that the primary hydrogen would be farther from C-4 of the nucleotide and closer to C-1 of the alkoxide.[4] Since the ^{13}C isotope effect at C-1 of benzyl alcohol should largely be a function of the C–H bond distance in the transition state, ^{13}k should get smaller as the redox potential of the nucleotide rises. This is the basic trend seen in Table V, with ^{13}C isotope effects varying from 2.5% with NAD to half this for acetylpyridine–NAD, except for pyridinealdehyde–NAD, which appears out of order. It must be remembered that the redox potentials on the enzyme may be different from those in solution.

Table III: ^{13}C Isotope Effects at pH 8, 25 °C, for LADH[a]

nucleotide	% reaction	isotope ratios (×10⁵)[b]	$^{13}(V/K)$
With Benzyl Alcohol			
NAD-*4-d*	15.4	1171.6, 1171.5	1.0159
NAD	13.3	1172.7, 1172.9	1.0146
deamino-NAD	19.6	1174.3, 1174.3	1.0137
thio-NAD	29.9	1181.5, 1181.3	1.0074
pyridinealdehyde–NAD	19.2	1177.5, 1177.5	1.0106
acetylpyridine–NAD	31.6	1185.3, 1185.1	1.0036
100% conversion with NAD	100	1188.9, 1188.5	
With Benzyl Alcohol-d_2			
NAD	20.0	1174.6, 1174.8	1.0217
deamino-NAD	19.6	1178.4, 1177.8	1.0182
thio-NAD	18.3	1184.8, 1185.0	1.0116
pyridinealdehyde–NAD	6.4	1179.3, 1179.7	1.0156
acetylpyridine–NAD	31.1	1189.0, 1189.0	1.0085
100% conversion with NAD	100	1197.1, 1197.5	

[a] The low conversion samples were run with recycling of the coenzyme. [b] Ratios were adjusted for ^{17}O contribution to m/e 45 by subtracting 74 from decade settings for m/e 45/44 which were corrected to tank standard of 1260 (O'Leary, 1980).

Another possible distorting influence is stretching of the reaction coordinate when the rate of hydride transfer is low; this should lead to higher isotope effects. The deuterium isotope effects in Table V show that the rate-limiting step differs with the different nucleotide substrates. With NAD, hydride transfer is reported from stopped-flow studies with benzyl alcohol to have a rate of 18 s^{-1}, while NADH release, which largely limits V, has a rate of 5.4 s^{-1}, (Kvassman & Pettersson, 1978). With deamino-NAD, hydride transfer is not rate limiting since ^{D}V is 1.0, so hydride transfer must be at least as fast as with NAD. With thio-NAD and acetylpyridine–NAD, equal deuterium isotope effects are seen on V/K and V, so hydride transfer is largely rate limiting and can be estimated to have rates of >2 and 8 s^{-1}, respectively. With pyridinealdehyde–NAD, V is not limited by hydride transfer, and in view of the high K_m for the nucleotide, it is hard to believe that release of reduced nucleotide is rate limiting, either. Possibly isomerization of the initial E–nucleotide complex to the form capable of productive binding of benzyl alcohol limits V [this is the case with alanine dehydrogenase (Grimshaw & Cleland, 1981)]. In any case, hydride transfer would have to have a rate over 5 s^{-1} for no V isotope effect to be seen. Thus, the hydride transfer rates are not that different, and the overall length of the reaction coordinate

[4] The apparent variation in transition-state structure with redox potential of the nucleotide is greater than one would expect for hydride transfer reactions with rates of only 2–20 s^{-1}. According to the theory of Marcus (1968), appreciable changes in transition-state structure occur only when $RT \ln (\Delta K_{eq})$ for the change in equilibrium constant is of the same order of magnitude as the activation energy for the reaction where $K_{eq} = 0$. This theory holds for hydride transfer reactions between NAD analogues in 4:1 2-propanol–water mixtures (Roberts et al., 1982). The 100-fold change in K_{eq} value between NAD and acetylpyridine–NAD corresponds to an activation energy of ~2.8 kcal/mol or rate constants of $\sim 5 \times 10^{10}$ s^{-1} [calculated from $(kT/h)e^{-2.8/RT}$]. The actual rate constants for hydride transfer could be much higher than the observed values of 2–20 s^{-1} if a highly unfavorable conformation change preceded the actual hydride transfer in either direction, but the reverse rate constant for this change would have to exceed that for the actual hydride transfer by at least an order of magnitude, and it seems unlikely that any conformation change could have a rate constant as high as 10^{12} s^{-1}. Another possibility is that the transition-state structure is more sensitive to K_{eq} values than predicted by Marcus theory because of nonclassical effects such as tunneling. The much smaller α-secondary deuterium isotope effects seen in dehydrogenase reactions when deuteride rather than hydride transfer occurs (Hermes et al., 1984) can only be modeled by raising the imaginary frequency to ~1000 cm^{-1}, at which point tunneling becomes a major path for the reaction (Huskey & Schowen, 1983; Saunders, 1984). No adequate theory exists for predicting how transition-state structure will vary with K_{eq} in such a situation, and it may be that the sensitivity is greater than that predicted by Marcus theory; theoretical work is clearly needed on this point.

Table IV: Primary ^{13}C and Deuterium Isotope Effects at pH 8.0, 25 °C, for Aldehyde Dehydrogenase with Benzaldehyde as Substrate[a]

nucleotide	% reaction	isotope ratios ($\times 10^5$)[b]	$^{13}(V/K)$	K_m[c] (μM)	rel V	K_m[d] (μM)	$^{D}(V/K)$	^{D}V
NAD	20.1	1169.4 1169.0	1.0144	25	(100)	10	1.43 ± 0.06[e]	1.02 ± 0.02
NAD-*4-d*	21.1	1167.43 1168.0	1.0160					
deamino-NAD	33.1	1167.1 1166.5	1.0184	5	30	4	0.88 ± 0.06[f]	0.88 ± 0.06[f]
pyridinealdehyde–NAD	9.1	1172.25 1172.65	1.0106	3	10	140	1.24 ± 0.04[e]	1.00 ± 0.06
acetylpyridine–NAD	12.1	1167.7	1.0179	120	120	25	1.22 ± 0.22[e]	0.97 ± 0.24
complete conversion with NAD		1183.9						
same, NAD recycled		1184.15 1184.7						

[a] The low conversion samples were run with recycling of the coenzyme. [b] Ratios were adjusted for ^{17}O contribution to m/e 45 by subtracting 74 from decade settings for m/e 45/44 which were corrected to tank standard of 1260 (O'Leary, 1980). [c] K_m for the nucleotide with 1 mM benzaldehyde with NAD and 6 mM with the other nucleotides. [d] K_m for benzaldehyde with the following nucleotide levels: 200 μM NAD, 50 μM deamino-NAD, 40 μM pyridinealdehyde–NAD, and 2 mM acetylpyridine–NAD. [e] Standard error based on an assumed value of 1.00 for ^{D}V. [f] The isotope effect appeared identical on V and V/K.

Table V: Calculated Intrinsic Isotope Effects for Oxidation of Benzyl Alcohol by Various Nucleotides Catalyzed by LADH[a]

nucleotide	$E^{\circ\prime}_{pH7}$	$^{D}(V/K)$	^{D}V	calculated parameters			K_m[b] (μM)	rel V	K_m[c] (μM)
				^{D}k	^{13}k	c_f			
NAD	−0.320	2.80 ± 0.20	1.4 ± 0.3	4.05 ± 0.35	1.0254 ± 0.0013	0.69 ± 0.11	20	(100)	30
deamino-NAD	−0.320	3.45 ± 0.80	1.0 ± 0.4	4.59 ± 1.15	1.0200 ± 0.0016	0.46 ± 0.17	110	130	51
thio-NAD	−0.285	3.0 ± 0.3[d]	3.0 ± 0.3[d]	4.77 ± 0.86	1.0136 ± 0.0016	0.84 ± 0.36	>500	>40	200
pyridinealdehyde–NAD	−0.262	4.45 ± 0.34	1.0 ± 0.8	6.54 ± 0.87	1.0170 ± 0.0013	0.61 ± 0.21	240	20	670
acetylpyridine–NAD	−0.258	2.65 ± 0.27[d]	2.65 ± 0.27[d]	6.25 ± 1.92	1.0115 ± 0.0018	2.2 ± 1.1	110	200	190

[a] Calculated on the assumption that $c_r = 0$. All data are for pH 8.0, 25 °C. [b] Michaelis constant for the nucleotide with 2.5 mM benzyl alcohol. [c] Michaelis constant for benzyl alcohol with 3.33 mM nucleotide. [d] Fitted to a form of eq 1 in which $^{D}(V/K) = {}^{D}V$, since the two isotope effects did not appear significantly different.

should not differ much with the different nucleotide substrates.

The primary deuterium isotope effect should be a maximum when the hydrogen atom is symmetrically placed in the transition state and smaller for either early or late transition states (Westheimer, 1961). The trend in ^{D}k values suggests that the transition state with NAD is rather late and that it gets more symmetrical as the redox potential of the nucleotide gets more positive, and thus the overall reaction more favorable.[5] Cook & Cleland (1981b,c) have determined primary ^{D}k values of 6.3 for LADH with cyclohexanol and 5.7 for YADH with 2-propanol as the substrate. The redox potentials of these secondary alcohols are more negative than that of a primary alcohol, so that the reactions with NAD as substrate have comparable equilibrium constants to that with benzyl alcohol and acetylpyridine–NAD. It thus appears that a symmetrical transition state gives a primary ^{D}k value of 5–6, and lower values are seen for later transition states.

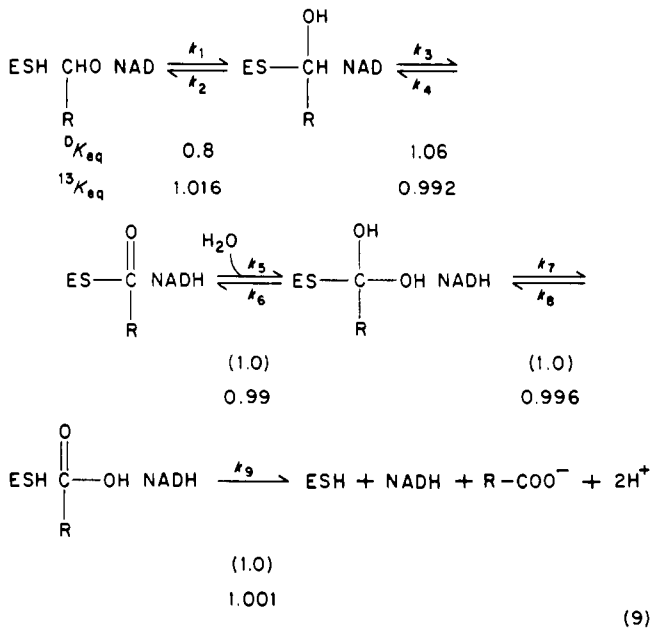
This interpretation calls for the redox potential difference on the enzyme to be considerably different than in solution, where NAD has a value of −0.320 V and benzyl alcohol one of −0.214 V. Whether the redox potential of the nucleotide is made more positive by the geometric distortion which makes N-1 pyramidal or the value of the alcohol more negative by formation of the zinc alkoxide or whether both occur to some extent, the two values must come together by about 0.05 V if they become equal for benzyl alcohol and the nucleotides with redox potentials originally about −0.26 V.

It is interesting to compare the ^{D}k values for LADH with that for YADH. With benzyl alcohol, a very slow substrate for YADH (5000 times slower than with LADH), ^{D}k was 7.[5] We ascribe this high value to an extended reaction corrdinate; that is, the enzyme does not bring the substrates as close together as it does for better substrates such as 2-propanol. Thus, although the redox potential difference would predict a late transition state, the isotope effect is large because NAD is still so far away in the transition state that the hydrogen atom is loosely bonded. The C–H bond at C-1 of benzyl alcohol is probably stretched to a similar extent as with LADH, however, since ^{13}k shows a very similar value with both enzymes.[6] Confirmation of this model will have to await measurement of ^{13}C isotope effects at C-4 of NAD with the two enzymes.

Mechanism of Aldehyde Dehydrogenase. It is thought that the aldehyde dehydrogenase mechanism involves largely ordered addition of NAD and aldehyde, followed by thiohemiacetal formation between the bound aldehyde and an SH group on the enzyme (Weiner, 1979; Wiseman & Abeles, 1979; Wiseman et al., 1980). Hydride transfer then generates a thio ester (Buckley & Dunn, 1982), which must be subsequently hydrolyzed, presumably via a tetrahedral adduct with water. This mechanism and the deuterium and ^{13}C equilibrium isotope effects associated with each step can be diagrammed as follows:[7]

[5] The ^{D}k values determined in the present work are the product of the primary and secondary deuterium isotope effects, since the benzyl alcohol was dideuterated, but the majority of the value is the primary isotope effect. The equilibrium secondary isotope effect is 1.266, and the kinetic value could be as high as 1.5 because of the coupled motion effect (Cook et al., 1981).

[6] Klinman (1976) has concluded from structure reactivity relationships in a series of substituted benzyl alcohols with YADH that in the transition state the alcohol carbon of benzyl alcohol has little charge and thus no aldehyde character. Likewise, with secondary alcohols in the LADH reaction, Cook et al. (1981) found from β-secondary deuterium isotope effects that there was no hyperconjugation in the transition state. These data can be explained by a transition state in which bond order is conserved at the alcohol carbon, with the C–O bond order being increased by the amount needed to balance the decrease in C–H bond order.



(9)

The $^{D}K_{eq}$ value for thiohemiacetal formation is that of Lewis & Wolfenden (1977), while that for the hydride transfer step is calculated from the overall $^{D}K_{eq}$ value, which is based on the fractionation factors for benzaldehyde and NADH (Cleland, 1980). The $^{13}K_{eq}$ values are based on fractionation factors from Hartshorn & Shiner (1972), O'Leary & Yapp (1978), and measured values from this laboratory (we estimate the following values relative to CO_2 in aqueous solution: aldehyde, 0.997; thiohemiacetal, 0.981; thio ester, 0.99; tetrahedral adduct, 1.00; free acid, 1.004; carboxylate, 1.003). We suspect that k_7/k_6 and k_9/k_8 are both large in mechanism 9, so that we need only consider k_1–k_5 in evaluating isotope effects.[8]

With NAD we have measured $^{13}(V/K)$ values with unlabeled substrates and primary and α-secondary deuterated substrates (Tables II and IV), as well as $^{\alpha\text{-}D}(V/K)$ and $^{D}(V/K)$ values, so if only one step in mechanism 9 were isotope sensitive, we could solve for the intrinsic isotope effects as was done with LADH. While the α-secondary deuterium isotope effect will be only on k_3 and k_4, one expects deuterium isotope effects on both of the first two steps in the mechanism when benzaldehyde-*1-d* is used (α-secondary effects on k_1 and k_2 and a primary effect on k_3 and k_4). Primary ^{13}C isotope effects are expected on all of k_1–k_8,[8] so we can only solve for limits on the possible isotope effects. We have done this and find that rather narrow limits are obtained.

Equations 4–8 were modified to allow for the various equilibrium isotope effects noted in mechanism 9, and we further assumed that benzaldehyde was not a sticky substrate (that is, that $k_{off} \gg k_1$ in mechanism 9). The approach taken was to assume values for $^{D}k_1$, $^{13}k_1$, and $^{13}k_5$ and then solve for $^{D}k_3$, $^{13}k_3$, and the partition ratios k_3/k_2 and k_4/k_5. The possible values of $^{13}k_1$ and $^{13}k_5$ were limited and allowed the following conclusions:

(1) $^{13}k_3$ lies in the range 1.013–1.016, with 1.014 corresponding to $^{D}k_1 = 0.9$ (a symmetrical transition state for thiohemiacetal formation). This value suggests a fairly early transition state for hydride transfer, which is what one would expect for the oxidation of an ionized thiohemiacetal (see footnote 7). A late transition state should give a value close to 4%, as with glucose-6-P dehydrogenase (Hermes et al., 1982), where the carbon is also bonded to heavy atoms in addition to the hydrogen.

(2) $^{\alpha\text{-}D}k_3$ must be at least 1.22; if it is to be less than 1.3, $^{13}k_1 \leq 1.007$, $^{13}k_5 \leq 1.003$, and $^{D}k_3 = 2.8$–3.5. This value of ^{D}k is consistent with an early transition state for hydride transfer.

(3) If $^{\alpha\text{-}D}k$ can be as large as 1.4, $^{13}k_1$ can be up to 1.012, $^{13}k_5$ can be up to 1.005, and $^{D}k_3$ can be as large as 4.0.

(4) In general, if $^{13}k_1$ is larger, $^{13}k_5$ must be smaller, and vice versa.

(5) The partition ratios k_3/k_2 and k_4/k_5 are close to unity for realistic possible solutions.

We can thus conclude that sizable primary ^{13}C and deuterium isotope effects occur on the hydride transfer step, as is the case with other dehydrogenases, but that the ^{13}C isotope effects on heavy atom addition to C-1 of benzaldehyde or the thio ester are small. Note that $^{\alpha\text{-}D}k_3$ is clearly normal, showing the effect of coupled motions of the α-secondary and primary hydrogens during hydride transfer (the bending motion of the α-secondary hydrogen becomes part of the reaction coordinate motion). Such normal α-secondary deuterium isotope effects have been seen for all dehydrogenases tested to date [formate dehydrogenase (Hermes et al., 1984); LADH (Cook et al., 1981); glucose-6-P dehydrogenase (Hermes et al., 1982)].

Data for nucleotides other than NAD are too preliminary to deduce much about transition-state structure (Table IV). In particular, we have not measured ^{13}C isotope effects with deuterated benzaldehyde. Nevertheless, it is clear that rate-limiting steps are not always the same as with NAD but that primary ^{13}C isotope effects of at least 1% are seen in all cases. Note that in no case is a significant deuterium isotope effect seen on V; it is not clear whether thio ester hydrolysis or product release is rate limiting here. The deuterium isotope effects with deamino-NAD appear inverse, suggesting that thiohemiacetal formation has come to equilibrium, and the $^{13}(V/K)$ value is only slightly higher than $^{13}K_{eq}$ for this step, but which step then limits the reaction is not clear. Perhaps a conformation change prior to hydride transfer has become slow, or hydride transfer has also come to equilibrium, so that k_5 is now rate limiting.

Size of ^{13}k Values for Hydride Transfer. Intrinsic ^{13}C isotope effects have now been determined for hydride transfer for several dehydrogenases. With NAD the highest values are 1.041 with glucose-6-P dehydrogenase (Hermes et al., 1982) and 1.043 with formate dehydrogenase (Blanchard & Cleland, 1980), while the present work with LADH and YADH gives values around 1.025, and the value for ALDH is also 1.014. We believe that the size of these values shows that some carbon motion is involved in the reaction coordinate. With formate dehydrogenase, the carbon must move to become collinear with the two oxygens in the CO_2 product, and since the oxygens have a combined mass of 32, while that of carbon is 12, most

[7] Bennett et al. (1982) have found that with fast substrates for the sheep enzyme one proton is released at pH 6 or 7.6, but not at 9.0, prior to hydride transfer, and the other only later (presumably from ionization of the acid product). This raises the possibility that the active site initially contains a His–Cys ion pair, with a pK of ~8.5 for the protonated His, and that the addition of substrate gives an ionized thiohemiacetal (with N-1 of NAD acting as the counterion) and lowers the pK of the His to ~5 (its normal pK) so that it loses its proton. This His would then act as the base to assist attack of water on the thio ester intermediate (the tetrahedral intermediate would have one less proton than shown in mechanism 9). In this mechanism, the species undergoing hydride transfer would be an ionized thiohemiacetal, which would be easily oxidized. Although there is considerable evidence for a sulfhydryl group in the active site (Weiner, 1979), there is no information concerning the presence of histidine.

[8] If k_5 involves an irreversible conformation change prior to attack of water on the acyl-enzyme, there will be no isotope effect on k_5. We have no way to evaluate this possibility.

of the motion will be by the carbon. With glucose-6-P dehydrogenase, carbon 1 changes from tetrahedral to trigonal as the hydride is removed, and this carbon is attached to one carbon and two oxygens. Again, most of the motion must be by the carbon from which hydrogen is removed.[9] With LADH and YADH with benzyl alcohol as substrate, however, there is an α-secondary hydrogen that can move to bring about the change from tetrahedral to trigonal geometry, and thus less carbon motion is required. This explanation predicts that during oxidation of secondary alcohols by LADH the ^{13}k value would be higher and might approach 4%. The acquisition of such values must await suitable methods for measurement.

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[9] The same should be true for ALDH, but the lower observed value of 1.014 may represent an early transition state.

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