Secondary Deuterium and Nitrogen-15 Isotope Effects in Enzyme-Catalyzed Reactions. Chemical Mechanism of Liver Alcohol Dehydrogenase†

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ABSTRACT: Theory is presented for the use of secondary isotope effects to study the mechanisms of enzyme-catalyzed reactions. The actual secondary isotope effects on the various steps will be seen only when commitments are small, while if either forward or reverse commitments are large the observed secondary isotope effect will be unity in the direction with the large commitment, and the equilibrium isotope effect in the other direction. If both commitments are large, one sees a portion of the equilibrium isotope effect determined by the ratio of forward and reverse commitments. β-Secondary isotope effects for liver alcohol dehydrogenase with cyclohexanone-*2,2,6,6*-d_4 and yeast alcohol dehydrogenase with acetone-d_6 show that hyperconjugation does not occur in the transition state, in which there apparently is little charge on the carbonyl carbon of the substrate. With lactate dehydrogenase, combination of primary and secondary (with pyruvate-d_3) isotope effects allows calculation of commitments for pyruvate and lactate of 12–13 and 0.4–1.4, respectively. With DPN labeled with ^{15}N at N-1 of the nicotinamide ring, the isotope effect with liver alcohol dehydrogenase and cyclohexanol and cyclohexanone as substrates was 1.062 from the DPN side, and 1.018 from the DPNH side, and the equilibrium isotope effect (determined by running perturbations with differing ratios of DPNH and DPN by a method described here) was 1.044. Analysis of these data suggests that during the step prior to hydride transfer in which a proton is transferred from the bound alcohol to give an alkoxide DPN is distorted so that N-1 becomes pyramidal, and the nicotinamide ring develops increased carbonium ion character at C-4. α-Secondary deuterium isotope effects with DPN-*4-d* and benzaldehyde-*1-d* give values more normal than expected, suggesting that the motion of the primary hydrogen being transferred as a hydride and the α-secondary hydrogens which swing through an arc of 54° during the reaction is tightly coupled so that reaction coordinate motion is contributing to the observed secondary isotope effect.

In the three preceding papers in this issue (Cook & Cleland, 1981a–c), we have developed the theory for using isotope effects and their pH dependence to deduce the kinetic mechanisms of enzyme-catalyzed reactions and to tell the degree to which the isotope-sensitive bond-breaking steps are rate limiting for V/K or V. We have illustrated this theory with data for several enzymatic reactions, but most particularly for yeast and liver alcohol dehydrogenases, and in the previous article (Cook & Cleland, 1981c) we have shown that hydride transfer to an aldehyde or ketone from DPNH to give an alkoxide is isotope sensitive, but largely pH independent, while the subsequent protonation of the alkoxide requires that an enzyme group (presumably His-51 in the liver enzyme) be protonated.

The isotope effects we have presented in these papers have all been primary ones in which the isotopic substitution was in the atom transferred during the reaction. Secondary isotope effects result from isotopic substitution in atoms which do not undergo bond cleavage but change the stiffness of their bonding (that is, one or more of the force constants for vibration of the molecule) during the reaction. Unlike primary isotope effects, they are never very large, but use of the equilibrium perturbation method of Schimerlik et al. (1975) allows isotope effects of 1.003 to be measured for reversible reactions in which one reactant is a suitably colored molecule, and careful comparison of reciprocal plots for deuterated and nondeuterated substrates can determine isotope effects of 1.05 or, if equal isotope effects are seen on both V and V/K, of 1.02. In this paper, we will discuss the application of secondary isotope effects to enzymatic reactions and illustrate the theory with data for several enzymes. In the course of these studies, we have established that for liver alcohol dehydrogenase, and probably for other dehydrogenases as well, DPN develops carbonium ion character at C-4 as the result of geometric distortion prior to hydride transfer. We have also found that because of coupled motion between the primary hydrogen being transferred and the α-secondary hydrogen at C-4 of DPN or C-1 of the alcohol deuterium substitution at the α position leads to kinetic secondary isotope effects which are normal as the result of reaction coordinate motion.

Nomenclature

The nomenclature used to describe isotope effects and commitments is presented fully in the first paper of this series (Cook & Cleland, 1981a), and the reader should familiarize himself with this nomenclature before reading this paper. We should emphasize one point; by "isotope-sensitive step", we mean one for which the rate constants in the forward or reverse direction are altered by isotopic substitution. If the rate constants do not change, the step is isotope insensitive.

Materials and Methods

Chemicals. Cyclohexanone-*2,2,6,6*-d_4 and acetone-d_6 (both 98 atom %) were from Merck. Pyruvate-d_3, DPN-*4-d*, and

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benzaldehyde-*1-d* were prepared as described by Cook et al. (1980). DPN-*2-d* was prepared by the method of San Pietro (1955) by incubating 10 mM DPN at room temperature in D_2O at pH 11 (K_2CO_3) for 90 min, at which time the deuterium content was over 98% at C-2 (the rate of exchange was 0.087 min^{-1}, and the rate of degradation to nicotinamide and ADPR was 0.0035 min^{-1}). No deuterium was found at C-4, and the product was isolated in 60% yield by absorption at 4 °C on a column of DEAE-cellulose previously equilibrated with 0.5 M ammonium bicarbonate, pH 10, and then washed with water, and (after washing with 5 column volumes of water) elution with 25 mM ammonium bicarbonate, pH 10, and adjustment of the pH to 7. [^{15}N]DPN was prepared by pig brain DPN glycohydrolase catalyzed exchange of [1-^{15}N]nicotinamide (99% label) (Oppenheimer et al., 1978b) with the thionicotinamide analogue of DPN (Oppenheimer & Davidson, 1980).

Equilibrium Perturbation. All isotope effects were determined with the equilibrium perturbation technique of Schimerlik et al. (1975). Typical perturbation conditions for the determination of $^{D}(Eq.P.)_{DPN}$ were the following: 100 mM Tris-HCl, pH 7.9; 0.1 mM DPN-*4-d*; 0.128 mM DPNH; 0.197 mM cyclohexanol; 0.015 mM cyclohexanone; 0.06 unit of liver alcohol dehydrogenase. Conditions for the determination of $^{D}(Eq.P.)_{cyclohexanone}$ were the following: 100 mM Tris-HCl, pH 7.8; 5 mM DPN; 0.198 mM DPNH; 1.12 mM cyclohexanone-*2,2,6,6-d*$_4$; 1.03 mM cyclohexanol; 0.03 unit of liver alcohol dehydrogenase. Conditions for the determination of $^{D}(Eq.P.)_{acetone}$ were the following: 100 mM Tris-HCl, pH 7.9; 0.643 mM DPN; 0.366 mM DPNH; 183 mM acetone-d_6; 17.4 mM 2-propanol; 20 units of yeast alcohol dehydrogenase. Conditions for the determination of $^{D}(Eq.P.)_{pyruvate}$ were the following: 100 mM Ches,[1] pH 9; 3.33 mM DPN; 0.184 mM DPNH; 0.196 mM pyruvate-d_3; 3.33 mM lactate; 2 units of lactate dehydrogenase. Conditions for the determination of $^{15}(Eq.P.)_{DPN}$ were the following: 100 mM Tris-HCl, pH 8; 0.1 mM [^{15}N]DPN (^{15}N is in the ring nitrogen of the nicotinamide ring); 0.054 mM DPNH; 0.2 mM cyclohexanol; 0.033 mM cyclohexanone; 0.5 unit of liver alcohol dehydrogenase. All reactant concentrations were determined enzymatically as described by Cook et al. (1980). Isotope effects were obtained from equilibrium perturbation data with a FORTRAN program which makes an exact solution to the equations of Schimerlik et al. (1975).

Determination of the Equilibrium Isotope Effect during Equilibrium Perturbation Experiments. In an equilibrium perturbation experiment (Schimerlik et al., 1975), the apparent isotope effect determined from the size of the perturbation is

$$app^{D}(Eq.P.) = \frac{^{D}(Eq.P.)_r(1 + K)}{1 + K/^{D}K_{eq}} \quad (1)$$

where $^{D}K_{eq}$ is the equilibrium isotope effect in the forward direction, and $^{D}(Eq.P.)_r$ is the isotope effect in the reverse direction.[2] K is the equilibrium ratio of unlabeled R (the initially unlabeled reactant) and unlabeled B (the initially labeled reactant) and is given by

$$K = \{-(1/^{D}K_{eq})(1 - R_0/B_0) + [[(1/^{D}K_{eq}) \times (1 - R_0/B_0)]^2 + (4/^{D}K_{eq})(R_0/B_0)]^{1/2}\}/(2/^{D}K_{eq}) \quad (2)$$

where R_0 and B_0 are the initial concentrations of unlabeled and labeled perturbants, respectively. When K is near zero, $app^{D}(Eq.P.) = {}^{D}(Eq.P.)_r$, while if K is very large, $app^{D}(Eq.P.) = {}^{D}(Eq.P.)_r{}^{D}K_{eq} = {}^{D}(Eq.P.)_f$. When $K = {}^{D}K_{eq}$, $app^{D}(Eq.P.)$ becomes the average of $^{D}(Eq.P.)_f$ and $^{D}(Eq.P.)_r$. Thus, if $app^{D}(Eq.P.)$ is obtained as a function of K, the parameters $^{D}K_{eq}$, $^{D}(Eq.P.)_r$, and $^{D}(Eq.P.)_f$ can be determined without a prior knowledge of $^{D}K_{eq}$. [The usual analysis of equilibrium perturbation data as described by Schimerlik et al. (1975) involves calculation of $^{D}(Eq.P.)_f$ and $^{D}(Eq.P.)_r$ from $app^{D}(Eq.P.)$ with the knowledge of $^{D}K_{eq}$.] Rearrangement of eq 1 gives

$$\frac{1 + K}{app^{D}(Eq.P.)} = \frac{K}{^{D}(Eq.P.)_f} + \frac{1}{^{D}(Eq.P.)_r} \quad (3)$$

Thus, when $(1 + K)/[app^{D}(Eq.P.)]$ is plotted against K, a straight line should be obtained with a slope of $1/^{D}(Eq.P.)_f$ and an intercept of $1/^{D}(Eq.P.)_r$, with $^{D}K_{eq}$ as the ratio of the two. Since an estimate of $^{D}K_{eq}$ is needed to evaluate K from the known values of R_0 and B_0 with eq 2, the $^{D}K_{eq}$ value calculated by the procedure must be used to calculate a new set of K values, and the analysis must be repeated. Several cycles of iteration result in rapid convergence on a final value of $^{D}K_{eq}$, and we have written a FORTRAN program to make these calculations.

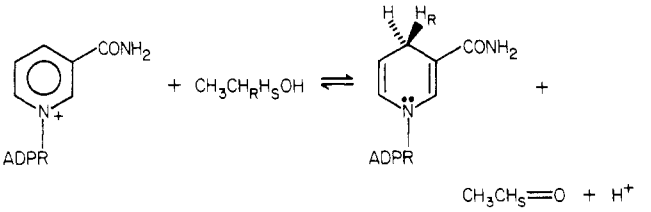
When no perturbation is obtained and, thus, $app^{D}(Eq.P.) = 1$, we can solve for K in eq 1:

$$K = \frac{^{D}K_{eq}[1 - ^{D}(Eq.P.)_r]}{^{D}(Eq.P.)_f - 1} \quad (4)$$

For the case where c_f and c_r are large, substitution of the value of $^{D}(Eq.P.)_f$ given by eq 6 shows that the K value which produces no perturbation is equal to c_f/c_r.

Theory

Primary isotope effects involve isotopic substitution of an atom which undergoes bond breaking or making during a chemical reaction, while secondary isotope effects involve atoms that do not undergo bond breaking or making but change the nature of their bonding during the reaction. Thus, in the reaction catalyzed by alcohol dehydrogenase



primary isotope effects are seen when deuterium replaces the *pro-R* hydrogen on C-1 of ethanol, or the A-side (*pro-R*) hydrogen at C-4 of DPNH, or when ^{13}C is placed at C-1 of ethanol or C-4 of DPN. An α-secondary isotope effect results from deuterium substitution at the *pro-S* hydrogen at C-1 of ethanol, or the aldehydic hydrogen of acetaldehyde, and also the four position of DPN or the B-side (*pro-S*) position at C-4 of DPNH. A β-secondary isotope effect would result from deuterium or ^{13}C substitution at C-2 of ethanol, and a secondary isotope effect is also expected at N-1 of DPN and DPNH, since the bonding to the nitrogen is different in DPN and DPNH. The other atoms in DPN do not appear to un-

[1] Abbreviations used: Taps, 3-[[tris(hydroxymethyl)methyl]amino]-propanesulfonic acid; Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid.

[2] What we are calling $app^{D}(Eq.P.)$ was called app α by Schimerlik et al. (1975) and is a parameter determined by the size of the perturbation. The isotope effect in the forward direction [$^{D}(Eq.P.)_f$ in our nomenclature] was called α, and that in the reverse direction, $^{D}(Eq.P.)_r$, was called $\alpha\beta$, where $\beta = 1/^{D}K_{eq}$.

dergo bonding changes during the reaction, although, if in an intermediate or transition state the bonding were altered, a secondary isotope effect would be seen.

Isotope effects can be on rates [most importantly for our purposes, on V/K values; see Cook & Cleland (1981a) for the mechanism dependence of isotope effects], in which case they are called kinetic isotope effects, and may be either normal (slower reaction with the heavier isotope) or inverse (faster reaction with the heavier isotope), although intrinsic primary isotope effects will almost always be normal [an exception is the inverse ^{37}Cl isotope effect on reactions of unsolvated Cl^- acting as a nucleophile (Cromartie & Swain, 1976); the chloride is more tightly bonded in the transition state since it is not bonded at all as the free ion]. Isotope effects are also seen on equilibrium constants [see Cook et al. (1980) and Cleland (1980) for tabulations of these for reactions of interest to biochemists], and the ratio of the observed kinetic isotope effects on the appropriate V/K values in forward and reverse reactions equals the equilibrium isotope effect. The equilibrium isotope effect is normal when the bonding of the atom involved is stiffer (higher vibrational frequencies) in the substrate than in the corresponding product, and inverse if the product shows stiffer bonding. The heavier isotope thus becomes enriched in the more stiffly bonded reactant. Equilibrium isotope effects depend only on the differences in structures (more specifically, in the vibrational frequencies) of the corresponding substrate and product, and it makes no difference whether one is considering primary or secondary isotope effects.

Primary isotope effects on bond-breaking steps usually show large values because at the transition state one of the stretching frequencies (corresponding to the asymmetrical stretch along the reaction coordinate) is lost, and, thus, the vibrational bonding is much looser in the transition state than in either substrate or product. The kinetic primary isotope effect can thus be thought of as the equilibrium isotope effect for going from the substrate to the transition-state structure. Secondary isotope effects are normally thought of in the same way, except that it is usually assumed that the vibrational frequency lost in the transition state does not involve the isotopic atom (we will present evidence that this may not always be true) but rather that there is a continuous, although not necessarily linear, change in the stiffness of bonding in going from the substrate through the transition state to the product. Organic chemists thus commonly refer to "early" or "late" transition states where the kinetic secondary isotope effect is, respectively, either near unity or nearly as large as the equilbrium isotope effect. Thus, if $^{D}K_{eq} = 0.80$, a $^{D}(V/K)$ value of 0.95 would be thought of as representing an early transition state (a structure only slightly different from the substrate) and a value of 0.85 as showing a late transition state (resembling the product). It is possible, however, for the transition state (or for an intermediate in the reaction) to show looser vibrational bonding than in either substrate or product, and in this case the kinetic secondary effect will be normal in both directions (we will give several examples in the present work). If the transition state involved stiffer bonding than in either substrate or product, the kinetic isotope effect would be inverse in both directions [see Gray et al. (1979) for a number of examples]. These possibilities are illustrated in Table I in a diagrammatic manner. Exactly the same considerations apply for tritium and heavier atom isotope effects, except that the latter are closer to unity and are harder to measure.

Extent to Which Secondary Effects Are Expressed. In the three previous papers in this issue (Cook & Cleland, 1981a–c), we have discussed the degree to which primary isotope effects

Table I: Observed Secondary Isotope Effect as a Function of Transition-State Structure[a]

transition-state structure (in forward direction)	isotope effect 0.7	0.8	0.9	1.0	1.1	1.2	1.3
more loosely bonded than substrate				0			●
substratelike				0			●
early			0	|		●
symmetrical			0	|	●	⋮
late		0	|	|	●	⋮
productlike		0	|	●	⋮	⋮	⋮
more stiffly bonded than product	0	|	●	|	⋮	⋮	⋮

[a] Isotope effects in forward (0) or reverse (●) directions are shown. In each case, these values must have the same ratio as the equilibrium isotope effect, which is 0.8 (shown by the dashed vertical line) in the forward direction and 1.25 (dotted line) in the reverse direction (that is, each pair of symbols must have the same spacing as that between the solid line at 1.0 and one of the other vertical lines).

are actually observed in enzymatic reactions. There is usually an appreciable primary isotope effect only on the bond-breaking step itself,[3] and, thus, we were able to write the simple equation:

$$^{D}(V/K) = {}^{D}(\text{Eq.P.})_f = \frac{{}^{D}k + c_f + {}^{D}K_{eq}c_r}{1 + c_f + c_r} \qquad (5)$$

where c_f and c_r are commitments in forward and reverse directions. When $^{D}(V/K)$ is being determined, these represent the ratio of the rate constant for the bond-breaking step to the net rate constant[4] for release of the varied substrate (for c_f) or first product (for c_r). For isotope effects measured by the closely related equilbrium perturbation method of Schimerlik et al. (1975), c_f and c_r are for the molecules between which label is exchanged during the experiment, and eq 5 yields $^{D}(\text{Eq.P.})_f$. Since ^{D}k values are normally from 3 to 8, while $^{D}K_{eq}$ values are not far from unity [ranging, for example, from 1.07 for ethanol to 1.28 for glucose for oxidation by DPN (Cook et al., 1980)], a normal isotope effect will be seen unless c_r is really quite large and $^{D}K_{eq}$ less than 1.0.

If only one step in an enzymatic reaction shows a secondary isotope effect, eq 5 can be used for secondary isotope effects as well as for primary ones. However, since ^{D}k is now the same order of magnitude as (and, in fact, usually closer to unity than) $^{D}K_{eq}$, one sees the intrinsic isotope effect ^{D}k only when c_f and c_r are very small indeed. If c_f is large and c_r is small, $^{D}(V/K)$ or $^{D}(\text{Eq.P.})_f$ will be 1.0, regardless of where the transition state lies, while if c_r is large and c_f is small, one sees $^{D}K_{eq}$, regardless of the nature of the transition state. If both c_f and c_r are large, eq 5 reduces to

$$^{D}(V/K) = {}^{D}(\text{Eq.P.})_f = \frac{{}^{D}K_{eq} + c_f/c_r}{1 + c_f/c_r} \qquad (6)$$

[3] We have implicitly assumed that there is only one bond-breaking step involving the isotopic atom in the mechanism, which is true for the dehydrogenases we have been studying. However, in mechanisms such as that of chymotrypsin, a primary isotope effect is expected for the carbonyl carbon on both formation and breakdown of the tetrahedral intermediate in both the acylation and deacylation portions of the mechanism, and a more complete analysis similar to that given in eq 8 is required. This has not been done for an enzyme-catalyzed case, but O'Leary & Marlier (1979) have measured ^{13}C isotope effects for the carbonyl carbon in the hydrolysis or hydrazinolysis of methyl benzoate.

[4] A net rate constant (Cleland, 1975) for a series of reversible steps is the rate constant that would result in the same overall rate through this series of steps.

Table II: β-Secondary Isotope Effects for Dehydrogenases[a]

liver alcohol dehydrogenase		
$^{D}(Eq.P.)_{cyclohexanone}$	0.80	0.801
$^{D}(Eq.P.)_{cyclohexanol}$	0.997	0.997
pH	6.0	8.0
yeast alcohol dehydrogenase		
$^{D}(Eq.P.)_{acetone}$	0.78	
$^{D}(Eq.P.)_{2\text{-propanol}}$	0.998	
pH	8.0	
lactate dehydrogenase		
$^{D}(Eq.P.)_{pyruvate}$	0.984	
$^{D}(Eq.P.)_{lactate}$	1.19	
pH	9.0	

[a] For experimental conditions, see Materials and Methods.

and it is clear that the nature of the transition state has nothing to do with the observed isotope effect, which can now be used to determine c_f/c_r from eq 6. If the same step in the mechanism shows both a primary and a secondary isotope effect, and the observed primary isotope effect is large enough to allow the method of Northrop (1975) to be used to determine the intrinsic primary ^{D}k value, one thus has a method for determining separate c_f and c_r values, since eq 5 for the secondary isotope effect can be solved simultaneously with eq 5 for the primary isotope effect. We will give an example of such an analysis, and we will also demonstrate how by raising c_f and c_r by manipulating reactant concentrations (Cook & Cleland, 1981a) it is possible to alter the observed secondary isotope effect from the one given by eq 5 to the one between unity and $^{D}K_{eq}$ predicted by eq 6.

More Than One Step Isotope Sensitive. For secondary isotope effects, it is possible for more than one step in a mechanism to show a secondary isotope effect. For example, in the fumarase mechanism studied by Blanchard & Cleland (1980), the use of dideuteriofumarate (or the corresponding dideuteriomalate made from it by the enzyme) gives rise to secondary isotope effects both on the formation of the intermediate carbanion (which is tetrahedral at C-2 and trigonal at C-3) and on its subsequent protonation to give malate (which makes C-3 tetrahedral). With [^{15}N]DPN and liver alcohol dehydrogenase, we will show in the present paper that ^{15}N-sensitive steps both precede and follow the hydride-transfer step, which probably does not show a secondary ^{15}N effect. We can illustrate all useful cases with mechanism 7, in which

$$E_1 \underset{k_2}{\overset{k_1B}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E_4 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_5 \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} E_6 \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E_7 \overset{k_{13}}{\rightarrow} E_8 \quad (7)$$

all of k_5–k_{10} show a secondary isotope effect, but k_1–k_4 and k_{11}–k_{13} are isotope independent. The isotope effect will be[5]

$$^{D}(V/K) = \{^{D}K_{eq\,5}\,^{D}k_7 + (k_7/k_6)[^{D}k_5 + (k_5/k_4) \times (1 + k_3/k_2)] + ^{D}K_{eq}(k_8/k_9)[^{D}k_{10} + (k_{10}/k_{11})(1 + k_{12}/k_{13})]\}/\{1 + (k_7/k_6)[1 + (k_5/k_4) \times (1 + k_3/k_2)] + (k_8/k_9)[1 + (k_{10}/k_{11})(1 + k_{12}/k_{13})]\} \quad (8)$$

where $^{D}K_{eq\,5} = {}^{D}k_5/{}^{D}k_6$ and $^{D}K_{eq} = {}^{D}k_5{}^{D}k_7{}^{D}k_9/({}^{D}k_6{}^{D}k_8{}^{D}k_{10})$. The difference from eq 5 is that $^{D}k_5$ and $^{D}k_{10}$ occur as part of what would be c_f and c_r in eq 5, and the "intrinsic" isotope effect term is $^{D}K_{eq\,5}{}^{D}k_7$, rather than a single ^{D}k. We will illustrate the use of eq 8 later in the paper.

[5] If the other molecule besides B between which label is exchanged in an equilibrium perturbation experiment adds during the conversion of E_8 to E_7 in eq 7, eq 8 also gives $^{D}(Eq.P.)_f$. Equation 5 can be factored differently; for instance, if the numerator and denominator are multiplied by k_6/k_7, eq 5 becomes $^{D}(V/K) = \{^{D}k_5 + (k_5/k_4)(1 + k_3/k_2) + {}^{D}K_{eq}(k_6/k_7)[^{D}k_8/{}^{D}K_{eq\,9} + (k_8/k_9)[^{D}k_{10} + (k_{10}/k_{11})(1 + k_{11}/k_{13})]]\}/\{1 + (k_5/k_4)(1 + k_3/k_2) + (k_6/k_7)[1 + (k_8/k_9)[1 + (k_{10}/k_{11})(1 + k_{12}/k_{13})]]\}$.

Table III: α-Secondary Isotope Effects at pH 8 for Liver Alcohol Dehydrogenase with DPN-*4-d*[a]

cyclohexanol (mM)	cyclohexanone (mM)	$^{D}(Eq.P.)_{DPN}$	$^{D}(Eq.P.)_{DPNH}$
1.97	0.282	0.973	1.098
1.97	0.212	0.955	1.08
12.6	0.026	0.996	1.125
0.197	0.026	1.099	1.242
0.197	0.015	1.095	1.237

[a] For experimental conditions, see Materials and Methods.

Results

β-Secondary Deuterium Isotope Effects. Table II shows kinetic isotope effects for liver and yeast alcohol dehydrogenases and for lactate dehydrogenase determined by equilibrium perturbation. In each case, the ketone substrates were perdeuterated in the β position. The equilibrium isotope effects for these cases have been reported by Cook et al. (1980).

α-Secondary Deuterium Isotope Effects. Table III shows the results of equilibrium perturbations by using DPN-*4-d* as the labeled reactant with liver alcohol dehydrogenase. With yeast alcohol dehydrogenase, an isotope effect of 1.08 ± 0.01 on V/K was determined at pH 8 (100 mM Taps) with 1 mM 2-propanol (K_m = 38 mM; Cook & Cleland, 1981a) by varying the concentration of DPN or DPN-*4-d*. Because the concentration level of 2-propanol was so far below its K_m, this isotope effect corresponds to a $V/K_{2\text{-propanol}}$ one (Cook & Cleland, 1981a). With formate dehydrogenase, formate concentration was varied at pH 8 (100 mM Tris) in the presence of saturating (2.5 mM) DPN or DPN-*4-d*. The $^{D}(V/K_{formate})$ value observed was 1.22. By contrast, no isotope effects (≤1.002; ≥0.998) could be found by equilibrium perturbation at pH 8 with DPN-*2-d* and liver alcohol dehydrogenase at the same concentration levels of cyclohexanol, cyclohexanone, DPN, and DPNH which gave large perturbations with [^{15}N]DPN.

α-Secondary deuterium isotope effects were also measured with benzaldehyde-*1-d* by varying the concentration of labeled and unlabeled aldehyde at fixed DPNH concentration (400 μM at pH 8; 200 μM at higher pH). With yeast alcohol dehydrogenase at pH 8 (100 mM Tris), $^{D}(V/K_{benzaldehyde})$ was 0.97–1.00, while at pH 9 (100 mM Taps), it was 0.843 ± 0.015. With the liver enzyme at pH 10.5 (100 mM Caps), it was about 0.8 [$^{D}K_{eq}$ was reported by Cook et al. (1980) to be 0.79 ± 0.01].

Acetaldehyde-*1-d* was also used in an equilibrium perturbation experiment with yeast alcohol dehydrogenase at pH 8 (0.34 mM acetaldehyde-*1-d*, 6.8 mM ethanol, 0.18 mM DPNH, 1.4 mM DPN, and 100 mM Tris) and gave $^{D}(Eq.P.)_{acetaldehyde}$ = 0.957 ± 0.008 and $^{D}(Ep.P.)_{ethanol}$ = 1.004 ± 0.007. $^{D}K_{eq}$ here is only 0.953 (Cook et al., 1980) because acetaldehyde is 60% hydrated, and acetaldehyde-*1-d* is 67% hydrated [$^{D}K_{eq}$ for the free aldehyde is 0.78, and for the hydrate is 1.07 (Cook et al., 1980)]. Because the commitments for ethanol and acetaldehyde are not known, we cannot make a detailed analysis of these data at this time.

^{15}N Isotope Effects. Equilibrium perturbation data obtained with DPN labeled with ^{15}N in the nicotinamide ring are shown in Figure 1. As discussed under Materials and Methods, when K [approximately equal to (DPNH)/(DPN) in this case] is varied, app^{15}(Eq.P.) will also change. From this variation, isotope effects for the forward and reverse reactions as well

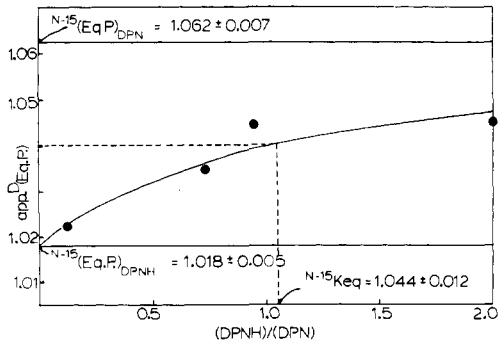


FIGURE 1: Dependence of app^{15}(Eq.P.) from equilibrium perturbation with [^{15}N]DPN on (DPNH)/(DPN), which is approximately equal to K in eq 1. The solid line is a fit to eq 1. Liver alcohol dehydrogenase was used with cyclohexanol and cyclohexanone at pH 8 (see Materials an ' Methods for details).

as the equilibrium isotope effect can be obtained. A fit of the data in Figure 1 to eq 1 by the iterative procedure discussed under Materials and Methods gave $^{15}(\text{Eq.P.})_{\text{DPN}} = 1.062 \pm 0.007$, $^{15}(\text{Eq.P.})_{\text{DPNH}} = 1.018 \pm 0.005$, and $^{15}K_{\text{eq DPN}} = 1.044 \pm 0.012$. As was done in Table III, when the forward and reverse commitments were made large by increasing the concentrations of alcohol and ketone to 44 and 17.45 mM, respectively, the isotope effects were brought back within the limits of 1 and the equilibrium isotope effect, and $^{15}(\text{Eq.P.})_{\text{DPN}} = 1.014 \pm 0.001$ and $^{15}(\text{Eq.P.})_{\text{DPNH}} = 0.970 \pm 0.002$.

Discussion

β-Secondary Deuterium Isotope Effects. These isotope effects arise almost entirely from the presence of hyperconjugation in aldehydes and ketones, and its absence in alcohols, and the observed isotope effects reflect the degree of hyperconjugation which can occur in the transition state (Melander & Saunders, 1980). It seems very unlikely that there could be major changes in hyperconjugation for any step other than the one in which the carbonyl group is reduced by hydride transfer to give an alkoxide, but there might be small isotope effects in alcohol dehydrogenases associated with establishment of inner-sphere Zn–carbonyl coordination. If we thus assume hydride transfer is the only isotope-sensitive step, eq 5 and the data in Table II for alcohol dehydrogenases yield a value near unity for the intrinsic isotope effect from the alcohol side, since c_r is very small for both cyclohexanone and acetone, and c_f is 2.5 for cyclohexanol (Cook & Cleland, 1981c) and 1.8 for 2-propanol (Cook & Cleland, 1981b). Such a value argues that at the transition state hyperconjugation is almost totally absent. Klinman (1972, 1976) has also concluded from structure–reactivity relationships in a series of aromatic substrates for the yeast enzyme that this carbon has no appreciable charge in the transition state. This does not mean, however, that the primary hydrogen being transferred is not symmetrically placed between C-4 of DPN and the carbonyl carbon of the ketone in the transition state, as the large size of the primary deuterium isotope effects suggests [5.7 for 2-propanol-*2-d* (Cook & Cleland, 1981b); 6.3 for cyclohexanol-*1-d* (Cook & Cleland, 1981c)]. A bond order of 0.5 between the primary hydrogen and the carbonyl carbon, and a bond order of 1.5 between the carbonyl oxygen and carbon in the transition state, represents a structure which would not show hyperconjugation and has little charge at the carbonyl carbon.

Unlike the situation with the alcohol dehydrogenases, finite isotope effects are obtained for both $^{D}(\text{Eq.P.})_{\text{pyruvate}}$ and $^{D}(\text{Eq.P.})_{\text{lactate}}$ with lactate dehydrogenase, although the isotope effect is larger from the lactate side. If the commitments for pyruvate and lactate were both small, intrinsic isotope effects would be observed. However, $^{D}(V/K_{\text{lactate}}) = 1.5$ (Cantwell & Dennis, 1970) so that hydride transfer is not very rate limiting [assuming the intrinsic isotope effect is greater than 1.5, since the intrinsic isotope effects for alcohol dehydrogenases are near 6 (Cook & Cleland, 1981b,c)]. This rules out small commitments, unless the isotope-dependent step for the secondary isotope effect is not the same as that for the primary effect (that is, not hydride transfer), which is unlikely.

This is an excellent case with which to illustrate the simultaneous solution of equations similar to eq 5 for both primary and secondary isotope effects to obtain separate values for c_f and c_r. The intrinsic primary isotope effect has not been measured by the method of Northrop (1975), but we will assume a value of 6 in analogy with the observed values for alcohol dehydrogenases (Cook & Cleland, 1981b,c). We must also assume an intrinsic secondary isotope effect, and we will use the two reasonable limits for a β-secondary isotope effect, 1.0 and the equilibrium isotope effect. Since $^{D}K_{\text{eq}} = 1.18$ for the primary effect and 1.21 for the secondary effect (Cook et al., 1980), we can write equations similar to eq 5 for the primary (eq 9) and secondary (eq 10) isotope effects. Solution

$$1.5 = \frac{6 + c_{\text{lactate}} + 1.18c_{\text{pyruvate}}}{1 + c_{\text{lactate}} + c_{\text{pyruvate}}} \quad (9)$$

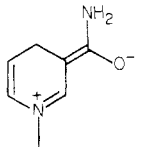
$$1.19 = \frac{(1.0 \text{ or } 1.21) + c_{\text{lactate}} + 1.21c_{\text{pyruvate}}}{1 + c_{\text{lactate}} + c_{\text{pyruvate}}} \quad (10)$$

of these equations gives 0.4 and 13.4 for c_{lactate} and c_{pyruvate} when the assumed intrinsic secondary isotope effect was 1.0, and 1.4 and 11.9 when it was 1.21. These calculations illustrate the way in which separate forward and reverse commitments can be estimated in a favorable case by conbining the results of primary and secondary isotope effect experiments.

Secondary Isotope Effects with [^{15}N]DPN. We had anticipated that isotope effects with DPN labeled with ^{15}N at N-1 of the nicotinamide ring would be small and would indicate a transition-state intermediate between the structures of DPN and DPNH. It is clear from the results in Figure 1, however, that the values are normal in both directions and, thus, that in some intermediate or transition state N-1 is bonded more loosely than in either DPN or DPNH. In DPN, N-1 has a bond order of 4, while in DPNH, the bond order is greater than 3, since in unfolded DPNH in solution[6] and in the X-ray structures of dihydronicotinamides (Karle, 1961; Koyama, 1963) the dihydronicotinamide ring is planar and there clearly is resonance between the lone pair in the p orbital of N-1 and the π electrons of the double bonds. Thus, in the X-ray structure of *N*-benzyl-1,4-dihydronicotinamide (Karle, 1961), the N-1 to C-6 and N-1 to C-2 distances are 1.43 and 1.38 Å, respectively, compared to the expected single-bond distance of 1.47 Å. In contrast, the C-4 to C-5 and C-4 to C-3 distances of 1.53 and 1.51 Å (with a 109° C-3 to C-4 to C-5 bond angle) are the expected single-bond length, and the C-2 to C-3 and C-5 to C-6 distances of 1.32 Å are normal double-bond lengths. The N-1 to C-2 distance is shorter than

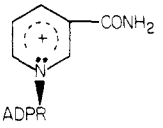
[6] Oppenheimer et al. (1978a) have shown that 64% of DPNH in solution is in an unfolded form with a planar dihydronicotinamide ring (the form adsorbed by dehydrogenases), but that the 36% in the folded form has the B side of the dihydronicotinamide ring stacked with adenine, so that C-4 is puckered. The facility of this distortion (which makes H_B equatorial and H_A axial at C-4) suggests that distortion of the dihydronicotinamide ring of DPNH on the enzyme surface, driven by conformation changes in the enzyme, is not unreasonable.

the N-1 to C-6 one because of enhanced resonance with the side chain (which is coplanar with the ring) as indicated by the structure

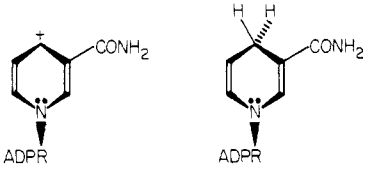


This resonance form, which requires a planar nitrogen in the ring, is presumably responsible for the 340-nm band in the UV spectrum, since the 3-halopyridine analogues of DPN which are substrates for liver alcohol dehydrogenase lack this band (Abdallah et al., 1976).

We interpret the normal isotope effects of 1.062 from the DPN side and 1.018 from the DPNH side as evidence that N-1 becomes pyramidal during the reaction, so that the bond order is reduced to 3. We thus propose that the enzyme distorts the bound DPN or DPNH molecule so that the bond from N-1 to C-1 of the ribose is no longer coplanar with the nicotinamide ring but is bent far enough out of plane to destroy any resonance between a lone pair on N-1 and the remaining double bonds in the ring. One can imagine two structures for a bent form of DPN. In the first, the rest of the ring would remain planar as in the benzenium cation (Olah et al., 1972), and the positive charge would be delocalized over the planar ring so that carbonium ion character would be shown by C-2, C-4, and C-6:

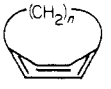


A more likely structure, however, would be one in which the ring did not remain planar but assumed a boat form in response to the deformation brought to bear by the enzyme and adsorbed substrate:

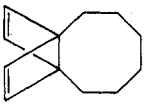


bent DPN bent DPNH

Such a structure for bent DPN would show carbonium ion character only at C-4 and not at C-2 or C-6. A good model for such a bent form of DPN would be the paracyclophanes, in which the benzene ring has a boat shape and the bridgehead carbons are not planar:



The lowest member of this series which has been prepared has six methylene groups (Kane et al., 1974), and has an angle of 22° between the CH carbons of the ring and the bridgehead carbons and an angle of 18° between the side chain and the plane of the bridgehead carbons (Allinger et al., 1974). It is still aromatic (although the UV absorption is red shifted by 28 nm from that of *p*-xylene) but is converted by UV irradiation into 1,4-hexamethylene(Dewar benzene):



which reverts smoothly to 6-paracyclophane on mild heating (Kammula et al., 1977). This degree of distortion from planarity thus leads to greatly enhanced reactivity of what is otherwise a stable aromatic ring, and it seems clear that a similar degree of distortion of a nicotinamide ring by conformation changes in the enzyme would certainly increase greatly the carbonium ion character of C-4 and decrease the bond order at N-1.[7]

If we assume that the only step which shows an ^{15}N isotope effect is the hydride-transfer one, eq 5 can be written as

$$1.062 = (^{15}k + 2.5)/(1 + 2.5) \qquad (11)$$

since cyclohexanone has no commitment, and that for cyclohexanol is 2.5 (Cook & Cleland, 1981c). This equation yields 1.22 as the value of ^{15}k, which is an unbelievably large ^{15}N isotope effect. However, if we assume that the step prior to hydride transfer in which an alkoxide intermediate is generated is ^{15}N sensitive (that is, the deformation of DPN to the bent carbonium ions occurs during this step),[8] we can calculate a much more reasonable intrinsic ^{15}N isotope effect. If the carbonium ion is an actual intermediate (that is, if DPN stays bent during hydride transfer), the hydride-transfer step will not be ^{15}N sensitive, and the bending of DPNH will occur in the reverse direction prior to hydride transfer. We will use eq 8 for this model and assume that k_5 is for alkoxide formation and DPN bending, k_7 is for hydride transfer, and k_9 is the step in which DPNH becomes planar again at N-1. Since only k_7/k_6 of the various partition ratios in eq 8 is finite, we have

$$1.062 = \frac{^{15}K_{eq\,5}{}^{15}k_7 + 2.5{}^{15}k_5}{1 + 2.5} \qquad (12)$$

Let us first assume $^{15}k_7 = 1.0$ (that is, that hydride transfer shows no ^{15}N isotope effect). Values for $^{15}K_{eq\,5}$ and $^{15}k_5$, respectively, that fit eq 12 are the following: 1.062, 1.062; 1.067, 1.060; 1.079, 1.055; 1.091, 1.050; 1.22, 1.00. If we let $^{15}k_7$ have a value other than 1.0, the solutions to eq 12 are for $^{15}K_{eq\,5}{}^{15}k_7$ and $^{15}k_5$. If $^{15}k_7$ is less than 1.0, this only makes the values of $^{15}K_{eq\,5}$ even higher and less believable. For $^{15}k_7$ to be greater than 1.0, the value of $^{15}K_{eq\,5}{}^{15}k_7$ must then exceed $^{15}k_5$, and the overall ^{15}N equilibrium isotope effect in going from DPN to the intermediate or transition state with lowest bond order at N-1 becomes unbelievably high. We feel that the data are best explained by assuming $^{15}k_7 = 1.0$, with a value of 1.07–1.08 for $^{15}K_{eq\,5}$, and a corresponding value for $^{15}k_5$ of 1.06–1.055, and, thus, it appears that the transition state

[7] It is intriguing that in the 1.75-Å energy-minimization refined structure of the *L. casei* dihydrofolate reductase–TPNH–methotrexate complex C-1 of the ribose lies 11° out of the plane of the nicotinamide ring, and away from the side of the ring on which the *pro-R* hydrogen (the one transferred during the reaction) lies. The nicotinamide ring itself is slightly bent into a twist boat, the root mean square deviation of the atoms from the plane being 0.17 Å. The bending in this case appears to come from anchoring of the 2′-P-ADPR part of the TPNH molecule and contacts from methotrexate on one side and Ile-13 on the other side of the nicotinamide ring. We thank Dr. Robin Spencer for making these calculations, Dr. G. A. Petsko for supplying the computer programs used in the calculations, and David Filman and Dr. D. A. Matthews for supplying the unpublished coordinates.

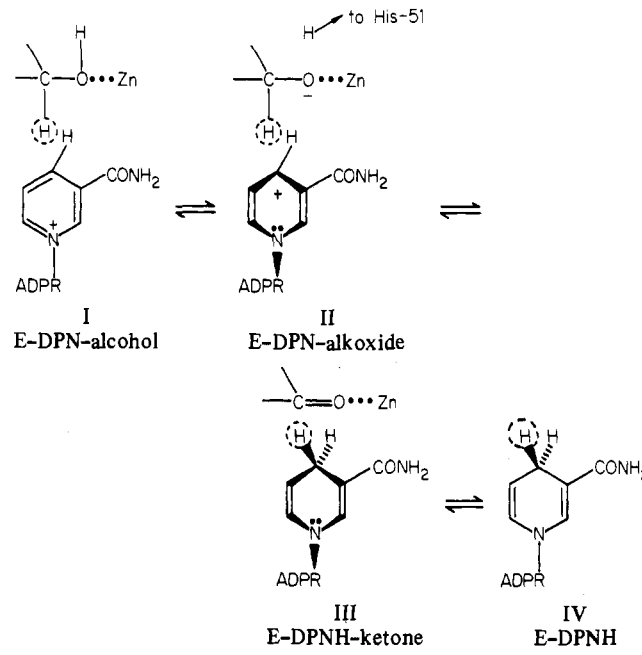
[8] We are assuming that the only kinetically important steps in the mechanism involve alkoxide formation and hydride transfer (Cook & Cleland, 1981c) and, thus, that deformation of DPN must accompany one or the other of these steps. It is possible (although no evidence for it exists) that DPN bending precedes the proton transfer that generates the alkoxide intermediate. In this case, the observed isotope effect (1.062) is the equilibrium isotope effect for the step in which DPN is bent, and we cannot calculate the kinetic isotope effects in either direction on this step.

for the step represented by k_5 is rather late. This is reasonable, since the resonance of the nicotinamide ring would only be eliminated by a large angular distortion of the N-1 to ribose bond.

A mechanism for liver alcohol dehydrogenase consistent with these data is shown in Scheme I.[9] In this mechanism, structures I and IV represent the structures of DPN and DPNH in solution, and presumably also in the binary complexes as shown. Structure II is the form of DPN in which the sugar–base bond is bent out of the plane of the nicotinamide ring, so that N-1 is no longer trigonal, and carbonium ion character is developed at C-4. This change in the geometry of DPN is caused by a conformation change in the enzyme which at the same time causes (or allows) the proton on the Zn-coordinated alcohol to be transferred to His-51 to form an alkoxide. Development of carbonium ion character at C-4 of DPN at the same time as, and in immediate proximity to, the alkoxide clearly decreases the energy required for both processes. Hydride transfer from alkoxide to C-4 of DPN then produces III, and the conversion of bent DPNH to planar DPNH occurs in the subsequent fast steps of the mechanism.

The mechanism for liver alcohol dehydrogenase discussed above illustrates the principle in enzymology that geometry may dictate chemistry. We are all used to the concept that the chemistry of a molecule determines its geometry, but the converse must also be true, and provides an easy mechanism for enzymatic catalysis. When a substrate is adsorbed in such a way that it is forced to undergo suitable geometry changes during a conformation change of the enzyme, it will undergo the corresponding chemical changes at the same rate as the conformation change. As further enzymatic mechanisms become known in the detail we now have for the alcohol dehydrogenase mechanism, we will undoubtedly see many more examples of the application of this principle.

Scheme I



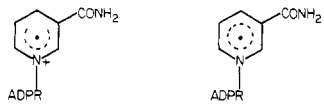
α-Secondary Isotope Effects with DPN-4-d. As shown in Table III, these isotope effects with liver alcohol dehydrogenase are normal in both directions at low levels of cyclohexanol and cyclohexanone where the commitments of the nucleotides have been reduced to those of the alcohol and ketone (Cook & Cleland, 1981a), but become inverse one way and normal the other at high levels of alcohol and ketone where the commitments of the nucleotides are greatly elevated, and eq 6 describes the observed isotope effects. When cyclohexanol concentration was 2 mM and cyclohexanone concentration was 0.2–0.3 mM, the c_f/c_r ratio calculated from eq 6 was 1.4–3.1, while when cyclohexanol concentration was 13 mM and cyclohexanone concentration was low enough to make the commitment of DPNH very small, the isotope effect was near unity from the DPN side and the equilibrium value from the DPNH side, as predicted when c_f is large and c_r is very small in eq 5.

Under conditions where cyclohexanol and cyclohexanone concentrations are low, Cook & Cleland (1981c) in the previous paper have shown that the only important internal partition ratio is that for the EH–DPN–alkoxide complex and that the ratio of rates of hydride transfer to give ketone and proton transfer to give E–DPN–alcohol is 2.5. Equation 8 can thus be simplified to give

$$1.097 = \frac{{}^{D}K_{eq\,5}{}^{D}k_7 + 2.5{}^{D}k_5}{1 + 2.5} \qquad (13)$$

As we did with the ^{15}N experiments, we will assume that k_5 is for conversion of DPN to the bent form with carbonium ion character at C-4, and k_7 is for hydride transfer. All of the evidence accumulated by organic chemists suggests that carbonium ions are normally sp^2 hybridized and that the fractionation factor of a hydrogen attached to a carbonium ion is nearly the same as that on a aromatic CH carbon, or

[9] Welsh et al. (1980) have recently proposed radical intermediates in the reduction of aromatic aldehydes with yeast alcohol dehydrogenase in order to explain the observed α-secondary isotope effects. However, in either a DPNH radical formed by loss of an electron to the aldehyde or a DPN radical formed by subsequent proton transfer (or original hydrogen atom transfer), there will be extensive delocalization of the electron, and the various canonical structures for these radicals indicate that as long as N-1 is trigonal that its bond order will be at least as high as that in DPNH:



DPNH radical DPN radical

Neither of these radicals as intermediates will explain the large normal ^{15}N isotope effects, and, thus, we are still forced to conclude that N-1 becomes pyramidal in the alkoxide intermediate, regardless of whether the hydride-transfer step involves actual simultaneous movement of hydrogen and an electron pair, or separate movement of an electron and a hydrogen atom, or an electron, a proton, and then another electron. DPN radicals do exist and can be formed by oxidation of DPNH with radicals (Chan & Bielski, 1975), or by reduction of DPN by hydrated electrons (Land & Swallow, 1968). Their absorption spectrum (Land & Swallow, 1968) shows a long-wavelength band at 400 nm (ϵ = 2400) in addition to the band at 260 nm (ϵ = 19 000). Since benzene is protonated to benzenium ion only by superacids such as $HF–SbF_5$ in SO_2ClF (Olah et al., 1972), it is likely that DPN radicals will require similar conditions for protonation to DPNH radicals, which are simply protonated DPN radicals [the *N*-methylnicotinamide radical, which should be quite similar to the DPN radical, is protonated with a p*K* of 1.3, but this is thought to occur on the oxygen of the side chain (Bruhlmann & Hayon, 1974)]. In agreement with this, malate radicals (formed by attack of hydroxyl radicals on fumarate) react with DPNH to remove a hydrogen atom, either when in solution or when DPNH is present as a complex with lactate dehydrogenase [in which case, A-side-specific removal of hydrogen is seen (Chan & Bielski, 1975)]. However, the measured redox potential at pH 7 for reduction of DPN to the radical is −0.73 V (Blankenhorn, 1976), which is so much lower than the value (−0.32 V) for two-electron reduction to DPNH that it is extremely unlikely that the radical is involved in normal dehydrogenase reactions. We will discuss the α-secondary isotope effects which led Welsh et al. (1980) to suggest the radical mechanism and show that they are more easily explained by coupled reaction coordinate motion.

a =CH– carbon.[10] We can thus assume that $^{D}K_{eq\,5}$ is near unity and that $^{D}k_5$ will also be 1.0, since there is no equilibrium isotope effect on this step and no actual motion of the C-4 hydrogen. With these simplifications, eq 13 gives 1.34 for $^{D}k_7$, and $^{D}k_8$ must then be this value divided by $^{D}K_{eq}$ (0.89; Cook et al., 1980), or 1.50.

A similar analysis can be made of the data with yeast alcohol dehydrogenase and DPN-*4-d*. The c_f value for 2-propanol in this case is 1.8 (Cook & Cleland, 1981b), and the calculated intrinsic value is 1.22 for $^{D}k_7$, corresponding to 1.38 from the DPNH side. With formate dehydrogenase, where there are no commitments and one sees the intrinsic isotope effect directly (Blanchard & Cleland, 1980), ^{D}k with DPN-*4-d* was 1.22, and, thus, normal α-secondary isotope effects of this size appear to be a general phenomenon with dehydrogenases, and not simply to occur with alcohol dehydrogenases alone.

Such large normal intrinsic α-secondary isotope effects are unprecedented and require that the hydrogen at C-4 of DPN be much more loosely bonded than in DPN at the transition state for hydride transfer. Clearly, something unusual is occurring to the hydrogen which is not happening to the hydrogen at C-2, which shows no secondary isotope effect and remains sp^2 hybridized in the carbonium ion intermediate and in DPNH. We believe these normal isotope effects result from the coupled motion of the primary and α-secondary hydrogens during the hydride-transfer step. An intuitive explanation is that because of the coupled motion (the α-secondary hydrogen swings through a 54° arc during the reaction) the motion of the primary hydrogen is slowed somewhat by a heavier atom in the α-secondary position. A more rigorous explanation is that the out of plane C–H bending vibration is lost in the transition state in the same way that the asymmetrical stretching vibration is lost for the primary hydrogen (that is, in each case there is no restoring force, and motion of this nature corresponds to that along the reaction coordinate). The effect is considerably smaller for th α-secondary hydrogen than for the primary one because it is a bending rather than a stretching vibration that is lost, but it still constitutes a normal kinetic isotope effect resulting from reaction coordinate motion. A similar proposal has recently been made by Kurz & Frieden (1980) to explain their α-secondary isotope effects in the reduction of 4-cyano-2,6-dinitrobenzenesulfonate by DPNH. This phenomenon has not been previously recognized by organic chemists because α-secondary deuterium isotope effects have been determined almost entirely for reactions where the primary atom undergoing bond cleavage is not hydrogen, but a heavy atom such as oxygen or nitrogen. Deuterium substitution on the α carbon in such a case will have only a small direct effect on the rate of the primary bond cleavage because of the great difference in masses of the atoms involved.[11] It would be of great interest to see calculations of the expected size of these α-secondary isotope effects!

α-Secondary Isotope Effects with Deuterated Aldehydes and Alcohol Dehydrogenases. At first glance, the α-secondary isotope effects with benzaldehyde-*1-d* at pH 8 of near unity from the aldehyde side, and nearly full equilibrium isotope effect from the alcohol side, do not appear consistent with our conclusions that the transition state is closer to the alcohol structure but would argue that the transition state resembles the aldehyde. [Hydride transfer is solely rate limiting in this system (Klinman, 1976), so commitments are very small, and the intrinsic secondary isotope effect should be seen.] Welsh et al. (1980) also noted this inconsistency in their β- and α-secondary isotope effects with aromatic substrates for yeast alcohol dehydrogenase. However, if we assume that there is a normal kinetic component of the observed isotope effect that results from coupling of the motion of the primary and α-secondary hydrogens during the reaction as described above for the hydrogen at C-4 of DPN, the data are readily explained. For primary alcohols and aldehydes, therefore, the motion of three hydrogens (the primary one and the α-secondary ones at C-4 of DPN and C-1 of the alcohol) is coupled during the hydride transfer.

Consistent with this explanation, the observed $^{D}(V/K)$ with benzaldehyde-*1-d*, which is near unity at pH 8 with the yeast enzyme, becomes 0.84 at pH 9, and about 0.8 at pH 10 with the liver enzyme ($^{D}K_{eq}$ = 0.79; Cook et al., 1980). This change shows that as protonation of the alkoxide intermediate becomes rate limiting at high pH the hydride-transfer step comes to equilibrium, and we see only the equlibrium isotope effect and lose the component resulting from reaction coordinate motion.

On the basis of the studies in this paper, we conclude that secondary isotope effects are equally as useful as primary ones in determining chemical mechanisms of enzyme-catalyzed reactions, since they are sensitive to the chemical structure of the reactants in the transition state, in addition to what is happening along the primary reaction coordinate. In cases where the primary isotope effects are large and the commitments are small enough to determine their values, intrinsic secondary effects can be determined, and, thus, as we have shown in the present work, chemical structures for intermediates and transition states in the reaction can be deduced. When primary isotope effects are small, and commitments are large, the size of the secondary isotope effect then determines the c_f/c_r ratio, which can be used to calculate separate values

[10] The fact that the p orbital of a carbonium ion that is perpendicular to the plane of the ion is empty, while that for an aromatic or unsaturated =CH– carbon is occupied by π electrons, might suggest slightly looser bonding in the carbonium ion. In the 2-propyl cation the fractionation factor of the 2 hydrogen relative to the 1 and 3 hydrogens is 0.92 ± 0.08 (H. U. Siehl and M. Saunders, unpublished experiments), and since the fractionation factor of methyl protons adjacent to a carbonium ion relative to those of a normal methyl group is 0.92 at 25 °C in the dimethylcyclopentyl cation (Saunders et al., 1977), we conclude that the value for the α hydrogen of a carbonium ion is 0.85 ± 0.08 relative to the hydrogens of a CH_2 group. This value is not signicantly different from the value of 0.89 for =CH— relative to —CH_2— (Cook et al., 1980). On the other hand, the maximum size of α-secondary deuterium isotope effects in solvolytic reactions such as the elimination of sulfonates from 2-adamantyl esters is only 1.23 (Shiner & Fischer, 1971), while the equilibrium isotope effect for formation of an sp^2 carbon from the ester of a secondary alcohol is 1.33 [on the basis of fractionation factors measured by Cook et al. (1980)]. Because attack from the rear is impossible, solvolysis of 2-adamantyl sulfonates can only be an S_N1 process, and because of the insensitivity of the isotope effects to the nature of the solvent, it is thought to involve equilibrium formation of a tight ion pair between the carbonium ion and the sulfonate, followed by rate-limiting insertion of a solvent molecule between the ions (Shiner & Fisher, 1971). If this hypothesis is correct, the fractionation factor of the α hydrogen on the carbonium ion in the tight ion pair must be higher than that of the usual sp^2 carbon by at least 1.33/1.23 = 1.08. However, the $^{D}K_{eq}$ values for ionization of diphenylcarbinol and bis(*p*-chlorophenyl)carbinol to give carbonium ions in sulfuric acid are 1.29 and 1.35, respectively (Mocek & Stewart, 1963), which are similar to the value of 1.33 expected if the carbonium ion has the same fractionation factor as other sp^2 carbons. We thus conclude that the fractionation factor of the α hydrogen on a free carbonium ion is not significantly different from that on an unsaturated sp^2 carbon, but that in a tight ion pair the bonding is somewhat stiffer, with the transition state for separation of the ion pair being early, so that an appreciable kinetic isotope effect on the separation is not multiplied times the equilibrium isotope effect for ion pair formation.

[11] In recent unpublished experiments in this laboratory, J. Hermes has shown that the secondary isotope effect with DPN-*4-d* and formate dehydrogenase is reduced from 1.22 to 1.07 when formate-*d* is used as the substrate. These data strongly support the explanation given here.

for c_f and c_r when a value for the primary intrinsic deuterium isotope effect can be determined by the method of Northrop (1975).

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