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pH Variation of Isotope Effects in Enzyme-Catalyzed Reactions. 2. Isotope-Dependent Step Not pH Dependent. Kinetic Mechanism of Alcohol Dehydrogenase†

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ABSTRACT: Theory is developed for the pH dependence of isotope effects in a mechanism where a pH-dependent step precedes the isotope-sensitive bond-breaking step, and the rate of the latter varies only slightly with the state of protonation of the acid–base catalytic group on the enzyme. In such a mechanism, the isotope effects fall to 1.0 in the forward direction and to the equilibrium isotope effect in the reverse direction at pH values where the pH-sensitive step becomes totally rate limiting in the reverse direction. This model accurately describes the kinetics of yeast alcohol dehydrogenase, where $V/K_{acetone}$ and the isotope effects on $V_{2\text{-propanol}}$ and $V/K_{2\text{-propanol}}$ decrease above a pK of 8.8 (both isotope effects becoming 1.0 at pH 10). The model also fits the kinetics of liver alcohol dehydrogenase, where $V_{cyclohexanol}$ and $V/K_{cyclohexanol}$ decrease below pKs of 6.2 and 7.1, and above pKs of 9.5 and 10.3. $pK_{i\ trifluoroethanol}$ decreases below a pK of 7.2, and above pK of 10.1, while $pK_{i\ isobutyramide}$ drops above a pK of 10.0. $V_{cyclohexanone}$ decreases above a pK of 8.4 while $V/K_{cyclohexanone}$ decreases above pKs of 8.8 and 9.7. Isotope effects on $V/K_{cyclohexanol}$ and $V/K_{cyclohexanone}$ decrease above identical pKs of 9.4 to values of 1 and 0.88, respectively, at pH 11. Comparison of a value of 2.5 for $^{D}(V/K_{cyclohexanol})$ with an average value of 5.53 for $^{T}(V/K_{cyclohexanol})$ allowed calculation of 6.3 as the intrinsic deuterium isotope effect. These data suggest that E–DPN–alcohol undergoes a proton transfer to the enzyme to give an EH–DPN–alkoxide complex which can lose its proton at high pH to give E–DPN–alkoxide and that both of these alkoxide complexes undergo hydride transfer to give DPNH and ketone. The alkoxide intermediate is not free to dissociate until it is protonated, either because it is coordinated to Zn or because the enzyme is in a closed catalytic configuration.

In the previous paper (Cook & Cleland, 1981b), we presented theory and data for an enzymatic mechanism in which the isotope-dependent bond-breaking step was part of a pH-dependent portion of the reaction mechanism. That model predicts that as V and V/K decrease as a function of pH and the chemical reaction becomes rate limiting the isotope effects on V and V/K will become equal. Isotope effects either may be pH independent or may increase as V or V/K decreases. Yeast and liver alcohol dehydrogenases conform to this model at low pH, but above pH 8 the isotope effects *decrease* at the same time that V and V/K for the ketone are decreasing, instead of increasing or staying the same as the model predicts. In this paper, we show that such behavior results when hydride transfer does not require a specific protonation state of the group on the enzyme which acts as the auxiliary acid–base catalyst, so that the pH-sensitive and isotope-dependent steps are different. With the alcohol dehydrogenases, the reduction of a ketone thus produces an intermediate with the redox properties and protonation state of an alkoxide, and this intermediate then accepts a proton in a pH-sensitive step from a group on the enzyme to give the product alcohol.

Materials and Methods

Chemicals. Cyclohexanol-*1*-(*h,d,t*) was prepared by reduction of cyclohexanone with either $NaBH_4$, $NaBD_4$, or tritiated $NaBH_4$ (Amersham Searle) followed by distillation, collecting the fraction distilling at 97 °C. The specific activity of the cyclohexanol-*1*-*t* was 0.1 mCi/mL with a cyclohexanol concentration of 63.4 mM. A-side DPND was prepared by the procedure of Viola et al (1979). 2-Propanol-*2*-(*h,d*) was prepared by the procedure described by Cook & Cleland (1981b). Ethanol-d_6 and $NaBD_4$ were from Merck. Isobutyramide and 2,2,2-trifluoroethanol were from Aldrich.

Initial Velocity Studies. All initial velocity studies were carried out by using a Beckman DU monochromator and a Gilford OD converter to monitor the change in absorbance at 340 nm. Conditions are listed where applicable in the figure legends. Isotope effects on initial velocities for substrate oxidation were obtained at saturating nucleotide concentration by comparing deuterated and nondeuterated substrates. For substrate reduction, saturating deuterated and nondeuterated

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reduced nucleotides were compared at varying substrate concentrations. Initial velocity patterns in the absence of products were obtained in the direction of alcohol oxidation at pH values from 8 to 10.5. At high pH values, DPN was added to the reaction mix just prior to addition of enzyme since it is not stable in alkaline solution. All time courses were linear for at least 5 min under all conditions employed. Studies of V and V/K as a function of pH were carried out at saturating nucleotide concentration by varying the substrate concentration. Values were obtained as a function of pH for $K_{i\ 2,2,2\text{-trifluoroethanol}}$ or $K_{i\ \text{isobutyramide}}$ (2,2,2-trifluoroethanol is a competitive inhibitor of cyclohexanol while isobutyramide is a competitive inhibitor of cyclohexanone), and also for $K_{i\ \text{DPN}}$ and $K_{i\ \text{DPNH}}$ (DPN and DPNH were used as product inhibitors competitive against DPNH and DPN, respectively). Since the kinetic mechanism for liver alcohol dehydrogenase is at least partly random, ketone and alcohol concentrations must be maintained at $0.1K_m$ when DPNH and DPN are used as substrates, respectively, to be sure that the K_i values obtained are for complex formation with free enzyme and not with a binary complex with alcohol or ketone. pH studies were carried out by using the pH jump method, in which enzyme was maintained at pH 8 in 10 mM Tris-HCl and added to the assay mix which contained 100 mM buffer at the pH studied. Buffers used in the pH studies (K^+ or Cl^- as the counterion) and their pHs were the following: acetate, 4.5–5.0; Mes,[1] 5.0–6.0; Pipes, 6.5–7.5; Tris, 7.5–8.0; Taps, 8.3–9.1; Ches, 8.8–9.5; Caps, 9.5–11.0. In all cases, overlaps were obtained when changing from one buffer to another to rule out buffer effects.

Equilibrium Perturbation. Primary isotope effects were obtained by using 2-propanol-*2-d* as a substrate for yeast alcohol dehydrogenase at pH 8.2, and cyclohexanol-*1-d* or A-side DPND as a substrate for liver alcohol dehydrogenase from pH 5.0 to 11.0. The average K_{eq} value obtained from all perturbations was $(5.2 \pm 1) \times 10^{-9}$ M for yeast alcohol dehydrogenase where $K_{eq} = (H^+)(\text{acetone})(\text{DPNH})/[(2\text{-propanol})(\text{DPN})]$ and $(3.0 \pm 0.7) \times 10^{-9}$ M for liver alcohol dehydrogenase where $K_{eq} = (H^+)(\text{cyclohexanone})(\text{DPNH})/[(\text{cyclohexanol})(\text{DPN})]$.

Tritium Discrimination. Experiments to determine $^T(V/K_{\text{cyclohexanol}})$ were carried out as described for determination of $^T(V/K_{2\text{-propanol}})$ in the previous paper (Cook & Cleland, 1981b) by using 100 mM phosphate, pH 8.0, 1 mM DPN, 10 mM semicarbazide hydrochloride, pH 8.2, and 0.01 unit of liver alcohol dehydrogenase. The reaction was initiated by addition of 0.28 mM cyclohexanol-*1-t*.

Data Processing. Reciprocal initial velocities were plotted against reciprocal substrate concentrations. Data were fitted to appropriate equations by using the FORTRAN programs of Cleland (1979). The individual saturation curves used to obtain pH profiles and K_i values for DPN and DPNH (when cyclohexanol and cyclohexanone concentrations, respectively, were maintained at $0.1K_m$ levels, K_m for the nucleotide is approximately equal to K_i) were fitted to eq 1. Data for pH

$$v = VA/(K + A) \tag{1}$$

profiles showing a decrease in log V or log V/K with a slope of 1 as the pH is lowered or a slope of −1 as the pH is raised were fitted to eq 2 and 3. When log V or log V/K decreased

$$\log y = \log [C/(1 + H/K_1)] \tag{2}$$

$$\log y = \log [C/(1 + K_2/H)] \tag{3}$$

at both high and low pH, the data were fitted to eq 4. Data exhibiting a decrease above two pKs to a final slope of −2 were fitted to eq 5. In these equations, K_1 and K_2 are dissociation

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \tag{4}$$

$$\log y = \log [C/(1 + K_1/H + K_1K_2/H^2)] \tag{5}$$

constants for groups on the enzyme, y is V or V/K, and C is the pH-independent value of y. The data for direct comparison of hydrogen- and deuterium-containing substrates at saturating levels of nucleotide were fitted to eq 6–8, which assume isotope

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \tag{6}$$

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A} \tag{7}$$

$$v = \frac{VA}{K + A(1 + F_iE_V)} \tag{8}$$

effects, respectively, on both V and V/K, V/K only, and V only. In eq 6–8, F_i is the fraction of deuterium in the substrate, while E_V and $E_{V/K}$ are the isotope effects minus one for the respective parameters. The data for $K_{i\ \text{DPN}}$ and $K_{\text{cyclohexanol}}$ as a function of pH are described by a curve with a constant value at both high and low pH. Data for these parameters were fitted to eq 9, where y is the observed value of the parameter, YL is

$$\log y = \log \left[\frac{\text{YL} + \text{YH}(K_1/H)}{1 + K_1/H} \right] \tag{9}$$

the value of y at low pH, YH is the value of y at high pH, and K_1 represents the dissociation constant of the group whose protonation decreases y. Data for linear competitive inhibition were fitted to eq 10. Isotope effects were calculated from

$$v = VA/[K(1 + I/K_{is}) + A] \tag{10}$$

equilibrium perturbation data by a FORTRAN program which gives an exact solution to the equations of Schimerlik et al. (1975), and limits on the intrinsic isotope effect for liver alcohol dehydrogenase were obtained by a FORTRAN program based on the method of Northrop (1975), as modified by Schimerlik et al. (1977) to account for the equilibrium isotope effect.

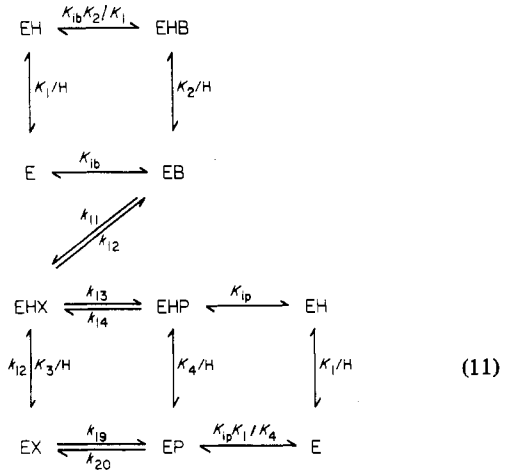
Nomenclature

The nomenclature for describing isotope effects on enzymatic reactions is described in detail in the first of the four papers in this issue (Cook & Cleland, 1981a), and the reader should familiarize himself with this nomenclature before reading the present paper.

Theory

In this paper, we deal with the case in which the isotope-dependent bond-breaking step is not totally pH dependent, but other steps in the mechanism are. Consider the model in mechanism 11, where B and P represent alcohol and ketone (since we will be discussing this mechanism with respect to the alcohol dehydrogenases), and k_{13}, k_{14}, k_{19}, and k_{20} represent isotope-dependent (hydride-transfer) steps. Rate constants k_{19} and k_{20} represent the same process as do k_{13} and k_{14}, but

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



(11)

involve the unprotonated path; k_{19} need not be equal to k_{13}, or k_{20} to k_{14}, but they must be finite. X is an intermediate with the stoichiometry of an alkoxide, and k_{11} and k_{12} are the steps in which a proton is transferred from B to a group on the enzyme to give X, and for the reverse process.[2]

In order to simplify the mechanism, we will assume that steps represented by K_1, K_2, K_3, and K_4 and binding of B and P are rapidly equilibrated. Steps represented by k_{11} and k_{12} are pH dependent, as is the overall apparent equilibrium constant, that is, $(P_{eq})/(B_{eq})$. We have assumed that the apparent equilbrium constant is pH dependent so that the model matches the alcohol dehydrogenase mechanisms discussed below.[3]

Mechanism 11 is not a complete description of the alcohol dehydrogenase mechanism in which B combines with E–DPN, and P with E–DPNH, and release of DPN or DPNH may partly limit V in either direction, and, thus, E in mechanism 11 represents a binary enzyme–nucleotide complex. Mechanism 11 correctly expresses isotope and pH effects on the V/K effects for B and P, however, and since we will be mainly concerned with V/K effects, it is an adequate model. We will comment below in connection with V profiles concerning the extra complexities introduced by a more complete kinetic model which includes the additional steps that may partly limit V.

Mechanism 11 predicts quite different pH behavior for the isotope effects than was seen in the simple model discussed in the previous paper (Cook & Cleland, 1981b), where the state of protonation of intermediates such as EHX was locked so that EX did not exist, and k_{20} was zero. In mechanism 11 at a pH above pK_3, EHX will be rapidly converted to EX, which cannot be reconverted directly to EB, but which can undergo hydride transfer to give EP. The forward commitment for the hydride-transfer step thus becomes very large, and $^D(V/K_b)$ is reduced to 1.0 at a high enough pH for the ratio of EHX/EX to become essentially zero. At a pH below pK_3, however, the forward commitment will be k_{13}/k_{12}, and if this is not large, a sizable isotope effect on V/K_b will be seen. In the back-reaction at a pH above pK_3, hydride transfer between EP and EX will come to equilibrium, since protonation of EX is very slow, and $^D(V/K_p)$ will be the equilibrium isotope effect in the reverse direction [an inverse effect of 0.85 for alcohol dehydrogenase with a ketone as substrate (Cook et al., 1980)]. At a pH below pK_3, however, a reverse commitment of k_{13}/k_{12} is seen, and, thus, an isotope effect on V/K_p is seen which is equal to that seen in the forward direction divided by the equilibrium isotope effect.

In order to analyze the kinetics of mechanism 11 more quantitatively, we will consider the rate equations involved. The rate equation derived from mechanism 11 when B is zero is given in eq 12. If we let P approach zero and substitute

$$v/E_t = k_{14}k_{12}[1 + k_{20}K_4/(k_{14}H)]P/\{K_{ip}(1 + K_1/H) \times (k_{13} + k_{12} + k_{19}K_3/H) + P[(1 + K_3/H)(k_{14} + k_{20}K_4/H) + (1 + K_4/H)(k_{13} + k_{12} + k_{19}K_3/H)]\} \quad (12)$$

K_3k_{19}/k_{13} for K_4k_{20}/k_{14}, eq 12 becomes

$$V/(K_pE_t) = k_{12}k_{14}[1 + k_{19}K_3/(k_{13}H)]/\{K_{ip}(1 + K_1/H) \times (k_{12} + k_{13})[1 + k_{19}K_3/[(k_{12} + k_{13})H]]\} \quad (13)$$

V/K_p decreases above an apparent pK given by the following:

$$pK_{app} = pK_1 + \log (1 + k_{12}/k_{13}) \quad (14)$$

As P approaches infinity, eq 12 becomes

$$V_2/E_t = k_{12}k_{14}[1 + k_{19}K_3/(k_{13}H)]/\{(k_{12} + k_{13}) \times (1 + K_4/H)[1 + k_{19}K_3/[(k_{12} + k_{13})H]] + k_{14}(1 + K_3/H)[1 + k_{19}K_3/(k_{13}H)]\} \quad (15)$$

V_2 decreases above an apparent pK given by the following:

$$pK_{app} = pK_3 + \log \left[\frac{1 + (k_{12} + k_{13})/k_{14}}{1 + k_{19}/k_{20}} \right] \quad (16)$$

The equations for $^DV_2$ and $^D(V/K_p)$ derived from eq 13 and 15 are

$$^DV_2 = \frac{^Dk_{14} + c_{Vp} + c_b/^DK_{eq\,b}}{1 + c_{Vp} + c_b} \quad (17)$$

$$^D(V/K_p) = \frac{^Dk_{14} + c_b/^DK_{eq\,b}}{1 + c_b} \quad (18)$$

where

$$^DK_{eq\,b} = {}^Dk_{13}/{}^Dk_{14} = {}^Dk_{19}/{}^Dk_{20} \quad (19)$$

[2] While proton transfers between bound reactant and enzyme occur in the interconversion of EB and EHX, the sizes of k_{11} and k_{12} are probably not limited by these proton transfers, but by the rates of the conformation changes which permit the proton transfers. We will discuss the chemistry of the reaction under Discussion.

[3] We have assumed in mechanism 11 that a proton is lost from B in going from EB to EHX, since this corresponds to what happens with alcohol dehydrogenases. It is also possible for a proton to be taken up by B (or a hydroxyl ion lost) in going from EB to the central complex which then undergoes the isotope-sensitive, but not strongly pH-dependent, step. In such a mechanism, the V/K_b profile drops at low pH, and the V/K_p one at high pH, and isotope effects are seen in both directions at high pH. At low pH, no isotope effect is seen on V/K_b, since protonation of the central complex commits it to undergo the isotope-sensitive step, while the equilibrium isotope effect is seen on V/K_p, because the isotope-sensitive step comes to equilibrium, with the acid–base catalyst in the central complex largely protonated and thus able to accept a proton from the bound intermediate to give B at only a very slow rate. It is also possible to have a mechanism with a pH-independent K_{eq} where the isotope-dependent and pH-dependent steps are different. In such a mechanism, the V/K profiles in both directions decrease above or below the same pKs, and the isotope effects likewise decrease along with the V/K profiles, one of them reaching the limiting value of 1 (in the direction where the pH-dependent step precedes the isotope-dependent one), and the other reaching a limiting value of $^DK_{eq}$ (where the isotope-dependent step precedes the pH-dependent one). Kvassman & Pettersson (1980b) have suggested an analogous mechanism for liver alcohol dehydrogenase in which the proton removed from B during the step corresponding to k_{11} is transferred to the solvent, rather than to a group on the enzyme with a finite pK, and hydride transfer then follows. In this mechanism, log (k_{12}/k_{13}) becomes the apparent pK of the ternary E–DPN–alcohol complex. The V/K_b profile will drop at both low and high pH with pK_1 and log (k_{12}/k_{13}) as the two pKs, while the V/K_p profile and both $^D(V/K)$ profiles will decrease above a pK which will be the highest of pK_1 or log (k_{12}/k_{13}). This model does not match the data from the present work, in which the pKs for the decrease of V/K_p, both $^D(V/K)$ profiles, and V/K_b at high pH are 8.8, 9.4, and 10.3 (see below).

$$c_{Vp} = \frac{\frac{k_{14}}{k_{12}}\left(1 + \frac{K_3}{H}\right)\left(1 + \frac{k_{19}K_3}{k_{13}H}\right)}{1 + \frac{k_{14}k_{19}K_3}{k_{13}k_{20}H}} \quad (20)$$

$$c_b = \frac{k_{13}}{k_{12}}\left(1 + \frac{k_{19}K_3}{k_{13}H}\right) \quad (21)$$

There is no forward commitment (i.e., c_p) in eq 18, since we have assumed rapid equilibrium binding of B and allowed for no isotope-independent steps prior to k_{14}. This simplification is a valid one for the alcohol dehydrogenases, as the data in this paper indicate, but a more general mechanism would have a c_p term in both the numerator and denominator of eq 18 (Schimerlik et al., 1977).

The rate equation derived from mechanism 11 when P is zero is shown in eq 22.

$$v/E_t = [k_{11}k_{13}BH/(K_{ib}K_3)][1 + k_{19}K_3/(k_{13}H)]/\{(H/K_3)(1 + H/K_1) \times (k_{13} + k_{12} + k_{19}K_3/H) + [HB/(K_3K_{ib})][(1 + H/K_2)(k_{13} + k_{12} + k_{19}K_3/H) + k_{11}(1 + K_3/H)]\} \quad (22)$$

As B approaches zero:

$$\frac{V}{K_bE_t} = \frac{k_{11}\left(1 + \frac{k_{13}H}{k_{19}K_3}\right)}{K_{ib}\left(1 + \frac{H}{K_1}\right)\left[1 + \frac{(k_{13} + k_{12})H}{k_{19}K_3}\right]} \quad (23)$$

and V/K_b decreases with an apparent pK of

$$pK_{app} = pK_1 + \log(1 + k_{12}/k_{13}) \quad (24)$$

Note that both V/K_p and V/K_b show the same pK, since in mechanism 11 for simplification we have let B and P both combine with E. For alcohol dehydrogenases, alcohol binds to E–DPN, and ketone binds to E–DPNH, and the pKs observed for $V/K_{alcohol}$ and V/K_{ketone} need not be identical. As B approaches infinity in eq 22:

$$\frac{V_1}{E_t} = \frac{k_{11}\left(1 + \frac{k_{13}H}{k_{19}K_3}\right)}{\left(1 + \frac{H}{K_2}\right)\left[1 + \frac{(k_{13} + k_{12})H}{k_{19}K_3}\right] + \frac{k_{11}}{k_{19}}\left(1 + \frac{H}{K_3}\right)} \quad (25)$$

and V_1 decreases with an apparent pK of

$$pK_{app} = pK_2 + \log\left(\frac{1 + k_{12}/k_{13}}{1 + k_{11}/k_{19}}\right) \quad (26)$$

Expressions for ${}^DV_1$ and ${}^D(V/K_b)$ are given by eq 27 and 28:

$${}^DV_1 = \frac{{}^Dk_{13} + c_{Vb}}{1 + c_{Vb}} \quad (27)$$

$${}^D(V/K_b) = \frac{{}^Dk_{13} + c_b}{1 + c_b} \quad (28)$$

where

$$c_{Vb} = \frac{k_{19}\left(1 + \frac{k_{13}H}{k_{19}K_3}\right)\left(1 + \frac{H}{K_2}\right)}{\frac{k_{12}H}{K_3}\left(1 + \frac{H}{K_2}\right) + k_{11}\left(1 + \frac{H}{K_3}\right)} \quad (29)$$

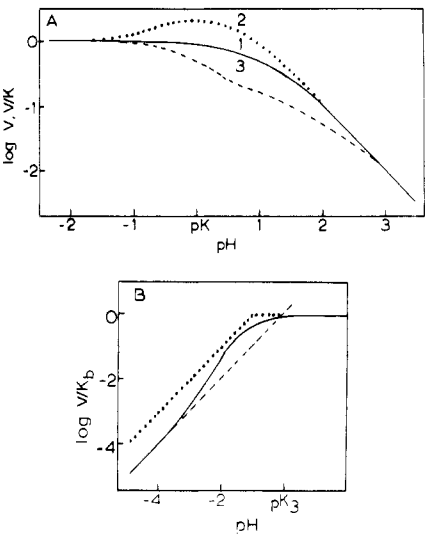


FIGURE 1: Theoretical curves for the pH dependence of V and V/K described in mechanism 11. (A) (1) Simple pH dependence obtained when $K_1 = k_{19}/(K_3k_{13})$. (2) "Hump" obtained when the wave defined by $[1 + k_{19}K_3/(k_{13}H)]/[1 + k_{19}K_3/[(k_{12} + k_{13})H]]$ in V/K occurs below pK_1. (3) "Hollow" obtained when the wave defined above occurs above pK_1 for V/K_p. (B) "Bulge" obtained when the wave defined above occurs below pK_1 for V/K_b.

and c_b is given by eq 21. There is no term in c_p in eq 27 and 28 for the reason given above.

As the pH is increased, ${}^D(V/K_p)$ as given by eq 18 decreases to a limiting value of $1/{}^DK_{eq\,b}$. If $\log[{}^D(V/K_p) - 1/{}^DK_{eq\,b}]$ is plotted vs. pH, the function decreases with a slope of -1 above an apparent pK of

$$pK_{app} = pK_3 + \log\left(\frac{k_{13} + k_{12}}{k_{19}}\right) \quad (30)$$

${}^D(V/K_b)$ as given by eq 28 also decreases as the pH is increased to a limiting value of 1. If $\log[{}^D(V/K_b) - 1]$ is plotted vs. pH, the function also decreases with a slope of -1 above the apparent pK given by eq 30. The isotope effects on both V_1 and V_2, however, go from a constant value at high pH to another constant value at low pH. ${}^DV_2$ at low and high pH is given by eq 31 and 32.

$$({}^DV_2)_{low\,pH} = \frac{{}^Dk_{14} + k_{14}/k_{12} + (k_{13}/k_{12})/{}^DK_{eq\,b}}{1 + k_{14}/k_{12} + k_{13}/k_{12}} \quad (31)$$

$$({}^DV_2)_{high\,pH} = \frac{1 + (k_{19}/k_{20})/{}^DK_{eq\,b}}{1 + k_{19}/k_{20}} \quad (32)$$

${}^DV_1$ at low and high pH is given by eq 33 and 34.

$$({}^DV_1)_{low\,pH} = \frac{{}^Dk_{13} + k_{13}/k_{12}}{1 + k_{13}/k_{12}} \quad (33)$$

$$({}^DV_1)_{high\,pH} = \frac{{}^Dk_{13} + k_{19}/k_{11}}{1 + k_{19}/k_{11}} \quad (34)$$

It should be noted that while the isotope effects drop above a single pK, expressions for V and V/K contain at least three pH-dependent terms, and, thus, more complex shapes may be manifested in the pH profiles in the vicinity of the apparent pK. For V/K_p, the term $1 + K_1/H$ in the denominator of eq 13 causes a normal decrease at high pH such as that shown as curve 1 in Figure 1A (that is, the pH profile will exhibit a slope of -1 above pK_1). The expression $[1 + k_{19}K_3/(k_{13}H)]/[1 + k_{19}K_3/[(k_{12} + k_{13})H]]$ from eq 13, however, is

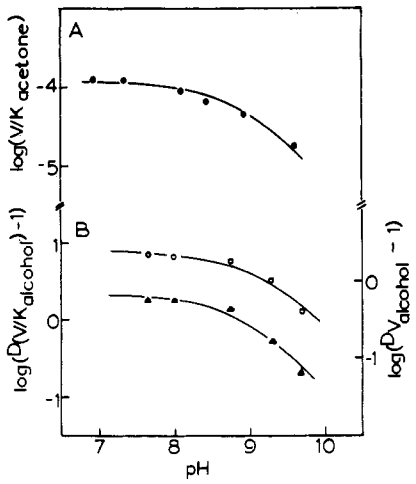


FIGURE 2: (A) pH dependence of $V/K_{acetone}$ (●) for yeast alcohol dehydrogenase. DPNH was saturating at 0.4 mM. The solid line is a fit to eq 3. (B) pH dependence of $^{D}V_{2\text{-propanol}}$ (○) and $^{D}(V/K_{2\text{-propanol}})$ (▲). Both isotope effects decrease to a value of 1 at high pH; they are plotted as isotope effect minus 1 as discussed under Theory. DPN was saturating (5–10 mM) for all assays. Solid lines are fits to eq 3.

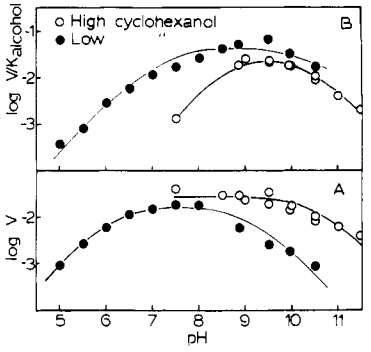


FIGURE 3: pH dependence of the kinetic parameters for cyclohexanol oxidation for liver alcohol dehydrogenase. (A) $V_{cyclohexanol}$ for the low-affinity (high cyclohexanol concentration) region (○) where the solid line is a fit to eq 3 and high-affinity (low cyclohexanol concentration) region (●) where the solid line is a fit to eq 4. DPN concentration, 1–5 mM. (B) $V/K_{cyclohexanol}$ for the low-affinity (high cyclohexanol concentration) region (○) and high-affinity (low cyclohexanol concentration) region (●). The solid lines are fits to eq 4. DPN concentration, 1–5 mM.

constant at high pH with a value of $1 + k_{12}/k_{13}$ and decreases to a constant value of 1 at low pH. When log (V/K_p) is plotted vs. pH, if the wave defined by $[1 + k_{19}K_3/(k_{13}H)]/[1 + k_{19}K_3/[(k_{12} + k_{13})H]]$ occurs below pK_1, a hump as seen in curve 2 in Figure 1A, is obtained, while if the wave occurs above pK_1, a hollow, as seen in curve 3 in Figure 1A, is obtained. If the wave occurs close to pK_1 (that is, $K_1 = k_{19}K_3/k_{13}$), a normal curve similar to curve 1 in Figure 1A will result. The log (V/K_b) vs. pH profile is also the sum of two curves. However, the profile drops at low pH instead of high pH so that if the wave occurs above pK_1 a hollow will result which will produce a curve the mirror image of curve 3 in Figure 1A, but if the wave occurs below pK_1 a bulge will result, Figure 1B. Again, when $K_1 = k_{19}K_3/k_{13}$, the curve is the mirror image of curve 1 in Figure 1A.

Similar behavior is predicted for V_1/E_t and V_2/E_t as a function of pH, and because K_b and K_p are the ratios of V_1/E_t and $V_1/(K_bE_t)$ or V_2/E_t and $V_2/(K_pE_t)$, respectively, the pH behavior of the Michaelis constants is also complex, going from one constant value at high pH to another constant value at low pH, with either a hump, a hollow, or a smooth transition in between.

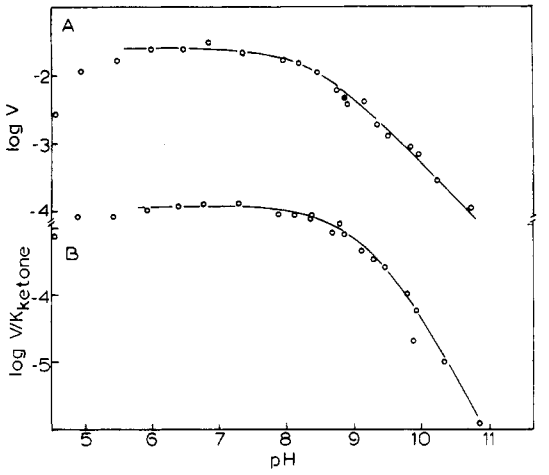


FIGURE 4: pH dependence of the kinetic parameters for cyclohexanone reduction for liver alcohol dehydrogenase. (A) $V_{cyclohexanone}$; DPNH, 0.2–0.4 mM. The solid line is a fit to eq 3. (B) $V/K_{cyclohexanone}$; DPNH, 0.2–0.4 mM. The solid line is a fit to eq 5.

Results

Yeast Alcohol Dehydrogenase. $V/K_{acetone}$ decreases above a pK of 8.76 ± 0.05 as shown in Figure 2A. Since acetone has a K_m of approximately 500 mM at pH 8, it was not possible to determine accurately the pH dependence of V_{max} for ketone reduction. The pH dependence of $^{D}(V/K_{2\text{-propanol}})$ and $^{D}V_{2\text{-propanol}}$ was also determined by using both direct comparison of initial velocities at saturating nucleotide concentration and the equilibrium perturbation technique. A summary of all the data obtained is shown in Figure 2B, plotted as the isotope effect minus one since both ^{D}V and $^{D}(V/K)$ decrease to a value of 1 at pH 10. $^{D}V_{2\text{-propanol}}$ decreases above a pK of 9.0 ± 0.05 while $^{D}(V/K_{2\text{-propanol}})$ decreases above a pK of 8.8 ± 0.04.

pH Profiles of V and V/K with Horse Liver Alcohol Dehydrogenase. When cyclohexanol combines with E–DPNH, it accelerates the rate of dissociation of DPNH, while the V/K for combination of DPN with the resulting E–cyclohexanol complex is less than that for combination of DPN with free enzyme. As a result, cyclohexanol gives nonlinear reciprocal plots with liver alcohol dehydrogenase, with the K_m being lower at low cyclohexanol concentration (the high-affinity region) than at high cyclohexanol concentration (the low-affinity region) (Dalziel & Dickinson, 1966). $V_{cyclohexanol}$ for both the high- and low-affinity regions (Figure 3A) decreased at high pH, while $V_{cyclohexanol}$ for the low-affinity region also decreased at low pH. $V/K_{cyclohexanol}$ for the high-affinity region (Figure 3B) drops at both low and high pH and appears to show a hollow on the low pH side. This hollow probably results from experimental errors, however, since a complete quantitative analysis in terms of the theory given above suggests that the expected hollow should be no more than 0.14 log unit (see Discussion). As a result, the data were fitted to eq 4. $V/K_{cyclohexanol}$ for the low-affinity region decreases at both low and high pH. $V_{cyclohexanone}$ (Figure 4A) decreases at high pH, while $V/K_{cyclohexanone}$ (Figure 4B) shows two pKs above which activity drops. The pK values are summarized in Table I.

Isotope Effects. Isotope effects were determined from pH 5 to 11 by equilibrium perturbation, which in all cases presented in this paper gives an isotope effect equal to the V/K one, since cyclohexanone concentration was held at low levels (Cook & Cleland, 1981a). They were also determined by direct comparison of initial velocities in the forward (cyclohexanol-*1-d*) and reverse (A-side DPND) reactions, varying

Table I: Summary of pK Values Obtained for Liver Alcohol Dehydrogenase

kinetic parameter[a]	pK ± SE[b] acidic	basic
$V_{cyclohexanol}$ (high affinity)[c]	6.20 ± 0.06	9.02 ± 0.06
$V/K_{cyclohexanol}$ (high affinity)[c]	7.1 ± 0.1	10.3 ± 0.1
$V_{cyclohexanol}$ (low affinity)[c]		10.5 ± 0.06
$V/K_{cyclohexanol}$ (low affinity)[c]	8.81 ± 0.05	10.18 ± 0.04
$V_{cyclohexanone}$		8.38 ± 0.07
$V/K_{cyclohexanone}$		8.76 ± 0.03, 9.67 ± 0.04
$1/K_{i\ trifluoroethanol}$[d]	7.20 ± 0.02	10.1 ± 0.02
$1/K_{i\ isobutyramide}$[d]		10.0 ± 0.02
$^{D}(V/K_{cyclohexanol}) - 1$		9.43 ± 0.04
$^{D}(V/K_{cyclohexanone}) - 1/^{D}K_{eq}$		9.34 ± 0.03
$1/K_{i\ DPN}$[d]	8.18 ± 0.08, (6.80 ± 0.05)[e]	
$1/K_{i\ DPNH}$[d]		8.65 ± 0.05

[a] The kinetic parameter which exhibits the pK value(s) listed when the logarithm is plotted vs. the pH. [b] An acidic pK indicates that the kinetic parameter decreases below the pK listed while a basic pK indicates the parameter decreases above the pK. [c] High affinity indicates the non-substrate-activating pathway at low cyclohexanol concentration while low affinity indicates the substrate-activating pathway at high cyclohexanol concentration. [d] Shown as the reciprocal so that when plotted as log vs. pH a decrease in pK_i indicates a decrease in binding affinity. [e] pH value at which the profile levels out.

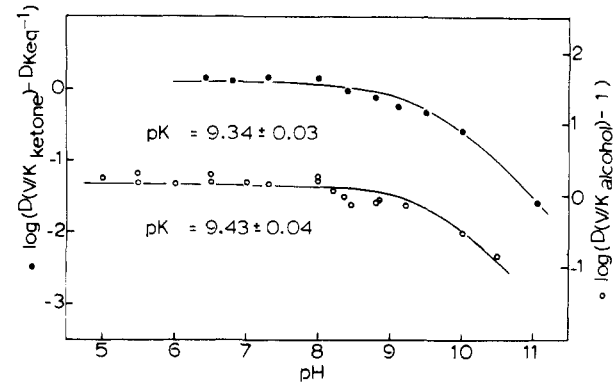


FIGURE 5: pH dependence of $^{D}(V/K_{cyclohexanol})$ (○) [plotted as $^{D}(V/K_{cyclohexanol}) - 1$ since it reaches a value of 1 at high pH] and $^{D}(V/K_{cyclohexanone})$ (●) [plotted as $[^{D}(V/K_{cyclohexanone}) - 1/^{D}K_{eq}]$ since it reaches a value of $1/^{D}K_{eq}$ at high pH] for horse liver alcohol dehydrogenase. DPN, 1–5 mM; DPNH, 0.2–0.4 mM. The solid lines are fits to eq 3.

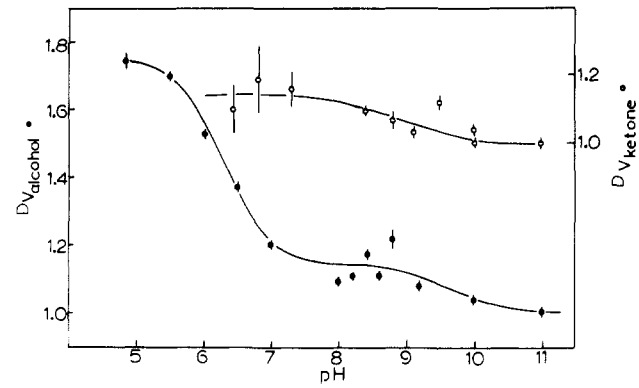


FIGURE 6: pH dependence of $^{D}V_{cyclohexanol}$ (●) and $^{D}V_{cyclohexanone}$ (○) for horse liver alcohol dehydrogenase. Nucleotide concentrations are given in the legend of Figure 5.

cyclohexanol and cyclohexanone concentrations, respectively, at saturating nucleotide concentrations. All $^{D}(V/K)$ data in the direction of cyclohexanol oxidation are plotted as log $[^{D}(V/K_{cyclohexanol}) - 1]$ vs. pH in Figure 5, since at pH 11 $^{D}(V/K_{cyclohexanol})$ decreases to a value of 1. In the direction of cyclohexanone reduction, $^{D}(V/K)$ data are plotted as log $[[^{D}(V/K_{cyclohexanone}) - 1]/^{D}K_{eq\ cyclohexanol}]$ vs. pH, since at pH 11 $^{D}(V/K_{cyclohexanone})$ decreases to a value of 0.88 (approximately equal to $1/^{D}K_{eq\ cyclohexanol}$, which is 0.85). Essentially equal pKs are obtained from these plots [9.43 ± 0.04 for $^{D}(V/K_{cyclohexanone})$ and 9.34 ± 0.03 for $^{D}(V/K_{cyclohexanol})$] as predicted under Theory. $^{D}V_{cyclohexanol}$ and $^{D}V_{cyclohexanone}$ are shown as a function of pH in Figure 6. Both appear to change from a value of 1.15 at around pH 8 to a value of 1 at pH 11. In the forward direction, however, $^{D}V_{cyclohexanol}$ increases to a new plateau value of 1.75 at pH 4.85 with a pK of 6.4, which is the same as that seen in the $V_{cyclohexanol}$ profile.

When tritium discrimination experiments were carried out at pH 8 for liver alcohol dehydrogenase by the same techniques described in the previous paper (Cook & Cleland, 1981b) for the yeast enzyme, but with cyclohexanol-*1-t* as substrate, an average value of 5.53 was obtained for $^{T}(V/K_{cyclohexanol})$. Limits on the intrinsic deuterium and tritium isotope effects in the forward and reverse directions (corresponding to $^{D}k_{13}$ and $^{D}k_{14}$ in mechanism 11) were obtained by the method of Schimerlik et al. (1977) and are shown in Table II. Since there appears to be no commitment for cyclohexanone, but a finite one for cyclohexanol, the upper limits in Table II should be the correct values, and we will adopt 6.3 as the intrinsic deuterium isotope effect [see footnote 5 of the previous paper (Cook & Cleland, 1981b)].

pH Dependence of K_i Values for Competitive Inhibitors. pH profiles for 2,2,2-trifluoroethanol and isobutyramide inhibition are shown in Figure 7, and the pK values are listed in Table I. The dissociation constants for DPN and DPNH were determined by varying DPNH concentration (in 10-cm cells because of the low K_m for DPNH) with cyclohexanone concentration at $0.1K_{cyclohexanone}$, and using DPN as a competitive inhibitor. Because of the low cyclohexanone level used, the apparent K_m for DPNH should be its K_i value, and the K_{is} for DPN from a fit to eq 10 is its K_i value. Similar experiments in the other direction (varying DPN concentration with cyclohexanol concentration at $0.1K_{cyclohexanol}$, and inhibiting with DPNH) gave a second set of values for $K_{i\ DPN}$ and $K_{i\ DPNH}$. The K_i values for DPN and DPNH as a function of pH are compared in Figure 8 with values from the literature (Theorell et al., 1955; Theorell, 1958; Theorell & Winer, 1959; Dalziel, 1963; Taniguchi et al., 1967; Iweibo & Weiner, 1972; Coleman et al., 1972; DeTraglia et al., 1977). At high pH, $K_{cyclohexanol}$ is too low to run experiments at $0.1K_{cyclohexanol}$, so initial velocity patterns were obtained by varying cyclohexanol and DPN concentrations at pH values from 8 to 10.5. Substrate activation by cyclohexanol is observed at pH 8.85 on the intercepts but not on the slopes when DPN concentration is varied. At pH values above 9, activation is observed for both slopes and intercepts. The apparent $K_{cyclohexanol}$ for the high-affinity region decreases from 600 μM at pH 8 to 12 μM at pH 10.5, and from a plot of p$K_{cyclohexanol}$ vs. pH (Figure 9), the pK is 9.77 ± 0.15 from a fit to eq 9. (The pH at which the curve levels out is 7.65 ± 0.17.)

From the initial velocity patterns in the low cyclohexanol concentration region, values for $K_{i\ DPN}$ at pH values of 8.85, 9.52, and 9.95 were 20.5, 15, and 11 μM, respectively, while values of 2 and 6.3 μM were obtained for $K_{i\ DPNH}$ at pH 9.52

Table II: Limits on the Intrinsic Isotope Effect at pH 8 for Liver Alcohol Dehydrogenase with Cyclohexanol as Substrate[a]

isotope effect	direction of reaction	symbol	lower limit	upper limit
deuterium	forward	^{D}k	$5.91 \pm 0.18 = {}^{D}K_{eq}[app(^{D}k/^{D}K_{eq})]$	$6.34 \pm 0.19 = app(^{D}k)$
tritium	forward	^{T}k	$12.95 \pm 0.40 = {}^{T}K_{eq}[app(^{T}k/^{T}K_{eq})]$	$14.36 \pm 0.44 = app(^{T}k)$
deuterium	reverse	$^{D}k/^{D}K_{eq}$	$5.03 \pm 0.16 = app(^{D}k/^{D}K_{eq})$	$5.40 \pm 0.17 = [app(^{D}k)]/^{D}K_{eq}$
tritium	reverse	$^{T}k/^{T}K_{eq}$	$10.26 \pm 0.32 = app(^{T}k/^{T}K_{eq})$	$11.38 \pm 0.35 = [app(^{T}k)]/^{T}K_{eq}$

[a] The value for $^{D}K_{eq}$ has been determined as 1.18 (Cook et al., 1980), where K_{eq} = (cyclohexanone)(DPNH)(H^+)/[(cyclohexanol)(DPN)]. ^{D}k is the intrinsic deuterium isotope effect for the hydride-transfer step in the direction of cyclohexanol oxidation. $^{T}K_{eq} = (^{D}K_{eq})^{1.442}$. $^{T}k = (^{D}k)^{1.442}$ (the intrinsic tritium isotope effect in the direction of cyclohexanol oxidation). $^{D}k/^{D}K_{eq}$ and $^{T}k/^{T}K_{eq}$ are the intrinsic deuterium and tritium isotope effects, respectively, in the direction of cyclohexanone reduction. The apparent value of ^{D}k is the one calculated from the average observed $^{D}(V/K_{cyclohexanol})$ and $^{T}(V/K_{cyclohexanol})$ at pH 8 with the expression (Northrop, 1975) $[^{D}(V/K) - 1]/[^{T}(V/K) - 1] = [[app(^{D}k)] - 1]/[[app(^{D}k)]^{1.442} - 1]$ and the apparent value of $^{D}k/^{D}K_{eq}$ is the one calculated by dividing $^{D}(V/K_{cyclohexanol})$ by $^{D}K_{eq}$ and $^{T}(V/K_{cyclohexanol})$ by $^{T}K_{eq}$, and using these values in the above equation. The apparent ^{T}k and $^{T}k/^{T}K_{eq}$ values are the 1.442 power of the deuterium values.

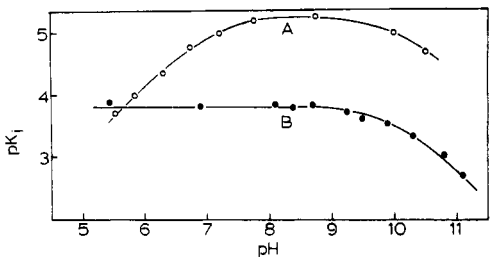


FIGURE 7: (A) pH dependence of $pK_{i\ trifluoroethanol}$ for competitive inhibition vs. cyclohexanol. DPN was saturating (2–5 mM, depending on pH). The solid line is a fit to eq 4. (B) pH dependence of $pK_{i\ isobutyramide}$ for competitive inhibition vs. cyclohexanone. DPNH was saturating (0.2–0.4 mM, depending on pH). The solid line is a fit to eq 3.

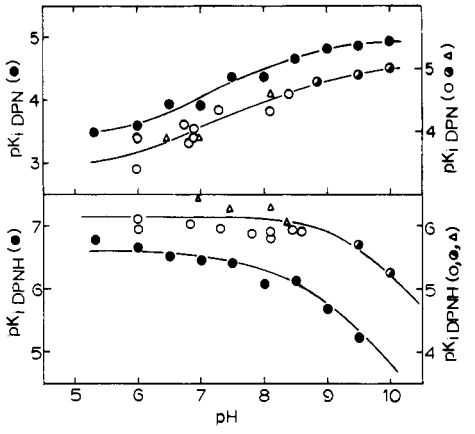


FIGURE 8: (Top) pH dependence of $pK_{i\ DPN}$. (●) Average literature values as discussed under Results; (○) determined as K_{DPN} (cyclohexanol concentration maintained at $0.1K_{cyclohexanol}$); (△) $K_{i\ DPN}$ from product inhibition vs. DPNH (cyclohexanone concentration maintained at $0.1K_{cyclohexanone}$); (◑) $K_{i\ DPN}$ determined from initial velocity patterns for cyclohexanol and DPN. The solid line is a fit to eq 9. (Bottom) pH dependence of $pK_{i\ DPNH}$. (●) Average literature values as discussed under Results; (○) $K_{i\ DPNH}$ from product inhibition vs. DPN (cyclohexanol concentration maintained at $0.1K_{cyclohexanol}$); (△) determined as K_{DPNH} (cyclohexanone concentration maintained at $0.1K_{cyclohexanone}$); (◑) $K_{i\ DPNH}$ as a product inhibitor for DPN (cyclohexanol concentration equal to $0.1K_m$). The solid line is a fit to eq 3.

and 9.95. These $K_{i\ DPN}$ and $K_{i\ DPNH}$ values agree with those in the literature. The pK values derived from the data presented in Figure 8 are listed in Table I.

Haldane Relationship. The kinetic Haldane relationship is the ratio of the apparent first-order rate constants in the forward and reverse directions when all substrates are at low concentrations. For a bireactant mechanism with substrates adding in a definite order, the apparent rate constant in the forward direction is the product of V/K_b (pseudo-first-order rate constant for reaction of EA with B to give products) and $1/K_{ia}$ (A/K_{ia} representing the proportion of enzyme that is EA), while that for the reverse direction is $(V/K_p)/K_{iq}$. For liver alcohol dehydrogenase, this relationship is[4]

$$K_{eq'} = \frac{(V/K_{cyclohexanol})K_{i\ DPNH}}{(V/K_{cyclohexanone})K_{i\ DPN}} \qquad (35)$$

In order to fit the above relationship, the K_i values for DPN and DPNH from Figure 8, represented by the solid lines, were used with $V/K_{cyclohexanol}$ from the high-affinity region (Figure 3) and $V/K_{cyclohexanone}$ (Figure 4). The pH dependence of the Haldane relationship calculated by using these values is shown in Figure 10 along with the pH dependence of $(V/K_{cyclohexanol})/(V/K_{cyclohexanone})$ and $K_{i\ DPNH}/K_{i\ DPN}$. The straight line in Figure 10 corresponds to a K_{eq} of 3×10^{-9} M (the average value observed in equilibrium perturbation experiments), and the fact that the Haldane relationship fits this line from pH 5 to 11 indicates that the kinetic constants have been correctly determined.

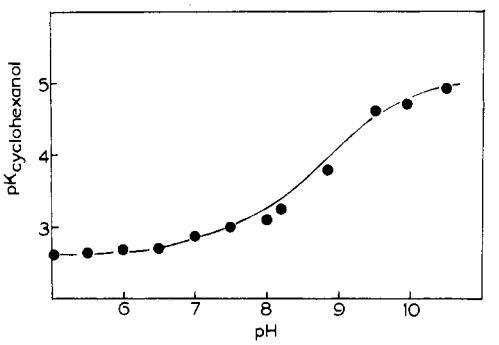


FIGURE 9: pH dependence of $pK_{cyclohexanol}$ from initial velocity patterns in the absence of products at the pH values indicated. The solid line is a fit to eq 9.

Discussion

Yeast Alcohol Dehydrogenase. $V_{2\text{-propanol}}$ and $V/K_{2\text{-propanol}}$ decrease below a pK of 7.6 (Cook & Cleland, 1981b) while $V/K_{acetone}$ decreases above a pK of 8.8. Since 2-propanol and acetone bind to E–DPN and E–DPNH, respectively, the pKs in their V/K profiles need not be the same. Mechanism 11 predicts that $V/K_{acetone}$ and $^{D}(V/K_{2\text{-propanol}})$ should decrease at high pH above identical pKs, as they do. Although no experimental data were obtained by using DPND and acetone,

[4] Since we have not included (H^+), the Haldane relationship gives the apparent equilibrium constant, K_{eq}', which equals $K_{eq}/(H^+)$. While K_{eq} is, of course, pH independent, K_{eq}' increases a factor of 10 per pH unit.

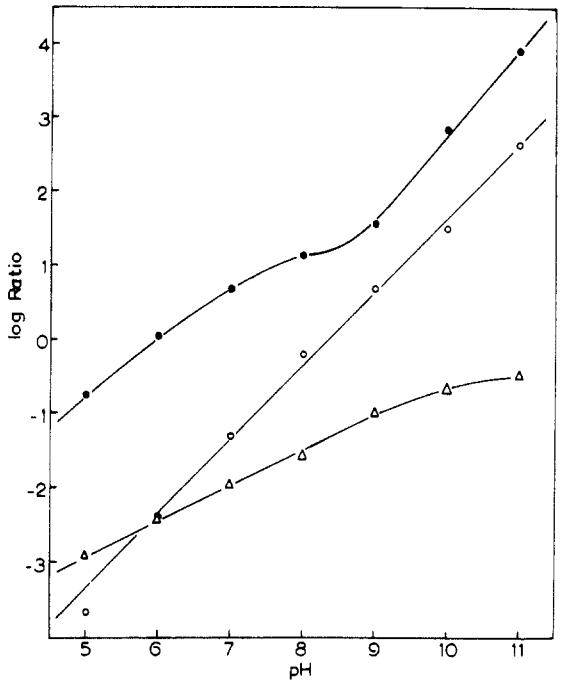


FIGURE 10: pH dependence of the Haldane relationship for liver alcohol dehydrogenase. (○) Haldane relationship, where the solid line represents a K_{eq} of 3×10^{-9} M, an average value for all equilibrium constants obtained experimentally in these studies. (●) $(V/K_{alcohol})/(V/K_{ketone})$ from data in Figures 4 and 5 (substrate-activating pathway data not used). (▲) $K_{i\,DPNH}/K_{i\,DPN}$ from experimental data in Figure 8 (not including literature values).

mechanism 11 predicts that ${}^{D}(V/K_{acetone})$ will decrease with a pK identical with that for ${}^{D}(V/K_{2\text{-propanol}})$ and go to a limiting value of 0.85 $(1/{}^{D}K_{eq\ 2\text{-propanol}})$ above pH 10.

If mechanism 11 adequately described the oxidation of 2-propanol, ${}^{D}V_{2\text{-propanol}}$ at high pH should be given by eq 34. Since ${}^{D}k_{13}$ is 5.7 (Cook & Cleland, 1981b), k_{19} would have to be much larger than k_{11} in order for the isotope effect to be as near 1.0 as is observed. However, mechanism 11 does not include such steps as the release of DPNH or the isomerization of E–DPN to a form capable of combining with 2-propanol, either of which could partially limit $V_{2\text{-propanol}}$, and if such steps are combined and given the rate constant k_{21}, the numerator and denominator of eq 34 include the term k_{19}/k_{21}. It is clear that k_{19} could be larger than k_{21}, rather than k_{11}, and this is probably the case in the present example [in the previous paper (Cook & Cleland, 1981b), we estimated that at pH 8.2 DPNH release from E–DPNH was 2.5 times the rate of the catalytic sequence which involves k_{11}, k_{12}, and either k_{13} or k_{19}, but we do not know whether DPNH release slows down sufficiently at higher pH for k_{21} to correspond to this step]. ${}^{D}V_{2\text{-propanol}}$ at low pH is given by eq 33 (the addition of k_{21} to the mechanism has no effect), which is identical with the equation for ${}^{D}(V/K_{2\text{-propanol}})$ discussed in the previous paper (Cook & Cleland, 1981b). As shown there, at low pH, ${}^{D}(V/K_{2\text{-propanol}})$ and ${}^{D}V_{2\text{-propanol}}$ are equal, as expected.

Horse Liver Alcohol Dehydrogenase. Predictions made from mechanism 11 are also upheld with liver alcohol dehydrogenase. $V_{cyclohexanol}$ and $V/K_{cyclohexanol}$ decrease at low pH while $V_{cyclohexanone}$, $V/K_{cyclohexanone}$, ${}^{D}(V/K_{cyclohexanol})$, and ${}^{D}(V/K_{cyclohexanone})$ all decrease at high pH with identical pKs for ${}^{D}(V/K_{cyclohexanol})$ and ${}^{D}(V/K_{cyclohexanone})$. As with yeast alcohol dehydrogenase, $V/K_{cyclohexanol}$ and $V/K_{cyclohexanone}$ show different pKs since alcohol and ketone bind to E–DPN and E–DPNH, respectively. At low pH, ${}^{D}(V/K_{cyclohexanol})$ given by eq 28 reduces to $({}^{D}k_{13} + k_{13}/k_{12})/(1 + k_{13}/k_{12})$. The observed isotope effect is 2.5, and ${}^{D}k_{13}$ has a value of 6.3 (the upper limit from Table II, which should be the correct value because there is a finite commitment only for cyclohexanol), so that k_{13}/k_{12} is 2.5. Since $1 + k_{12}/k_{13}$ equals 1.4, and its log is 0.14, the changes in the log V/K profiles caused by the expression $(1 + k_{13}H/k_{19}K_3)/[1 + [(k_{12} + k_{13})H/(k_{19}K_3)]]$ as described under Theory will be only 0.14, and should hardly be noticed. The pKs of the groups responsible for the decrease in the V/K profiles can now be assigned from eq 14 and 24 as 6.96 in E–DPN and 8.62 in E–DPNH.

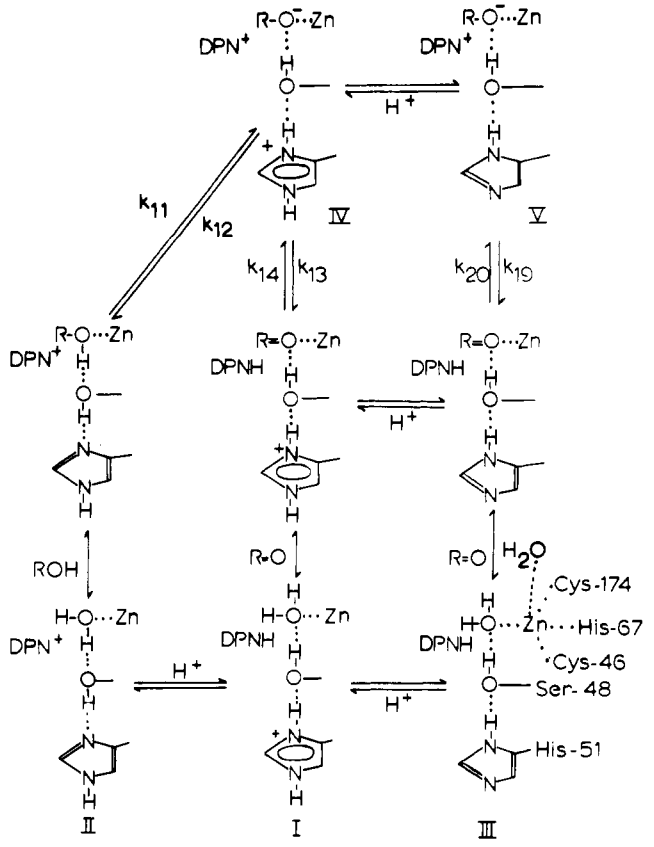
We have no way to evaluate pK_3 in mechanism 11 exactly, but because the net charge in the active site for the EHX intermediate will more nearly resemble that in E–DPNH than that in E–DPN, we can assume the value is approximately 8.6. From eq 30 and the apparent pKs of 9.4 for the decrease of ${}^{D}(V/K_{cyclohexanol})$ and ${}^{D}(V/K_{cyclohexanone})$ at high pH, we can then estimate that k_{13}/k_{19} is about 4.5. Loss of a proton from the auxiliary acid–base catalytic group on the enzyme thus appears to slow hydride transfer only slightly.

As noted above, mechanism 11 does not allow for steps such as release of DPN or DPNH from their binary complexes, or the isomerization of these binary complexes between forms which allow dissociation of nucleotides or addition of alcohol or ketone substrates. We can see evidence of such steps and in some cases their pH dependence from differences in the pH bahavior of $V_{cyclohexanol}$, $V_{cyclohexanone}$, ${}^{D}V_{cyclohexanol}$, ${}^{D}V_{cyclohexanone}$, and $K_{cyclohexanol}$ from that predicted by mechanism 11. For example, $K_{cyclohexanol}$ is predicted to be $K_{ib}K_2/K_1$ at low pH and $K_{ib}/(1 + k_{11}/k_{19})$ at high pH. However, if another rate-limiting step is present and given the rate constant k_{21}, the value at high pH is $K_{ib}(1 + k_{11}/k_{19} + k_{11}/k_{21})$. The 130-fold change in $K_{cyclohexanol}$ from low to high pH (Figure 9) shows that either k_{11}/k_{19} or k_{11}/k_{21} becomes much larger at high pH, since K_1 and K_2 are probably nearly the same. Probably a lower value of k_{21} is responsible; this is supported by the decrease in $V_{cyclohexanol}$ at high pH, which is not predicted by mechanism 11 (see Figure 3A), and the decrease in ${}^{D}V_{cyclohexanol}$ to 1.0 at high pH, which is not predicted by eq 34 unless k_{19}/k_{11} is very large. In a mechanism with k_{21} included, eq 34 includes k_{19}/k_{21} in both numerator and denominator, and this term presumably is the one responsible for decreasing ${}^{D}V_{cyclohexanol}$ to 1.0 at high pH. Thus, the step represented by k_{21} appears to become largely rate limiting at pH 10.

${}^{D}V_{cyclohexanol}$ shows a plateau around pH 8 with an average value of about 1.15. At this pH, k_{21} does not appear to be totally rate limiting, and the sum of k_{19}/k_{11} and k_{19}/k_{21} is about 32. At low pH, ${}^{D}V_{cyclohexanol}$ approaches a limiting value of 1.75, rather than the value of 2.5 predicted by eq 33 [that is, a value equal to ${}^{D}(V/K_{cyclohexanol})$]. This requires that some step other than those in mechanism 11 be partly rate limiting and also decrease as the pH is decreased so that the ratio of it and the chemical reaction remains constant. For alanine dehydrogenase, Grimshaw et al. (1981) (C. E. Grimshaw, P. F. Cook, and W. W. Cleland, unpublished experiments) have concluded that isomerization of E–DPN is responsible for a similar situation where ${}^{D}V$ and ${}^{D}(V/K_{alanine})$ do not become equal at low pH, but it is not clear whether this is also true for liver alcohol dehydrogenase.

A similar inconsistency is seen between the observed value of ${}^{D}V_{cyclohexanone}$ at low pH and that predicted by eq 31. The value of ${}^{D}V_{cyclohexanone}$ of about 1.15 at low pH requires that $k_{14}/k_{12} + k_{13}/k_{12}$ be about 30, and since $k_{13}/k_{12} = 2.5$, k_{13}/k_{14} would have to be 0.09. It is probable that the true value is higher than this (0.3–0.5), since pre-steady-state bursts of hydride transfer in single-turnover experiments with aromatic aldehydes are 50–70% of the enzyme level (Dunn et al., 1979). But if a step other than those shown in mechanism 11 is partly

Scheme I



rate limiting (and represented by k_{22}), eq 31 contains k_{14}/k_{22} in both numerator and denominator. A value of 20–22 for k_{14}/k_{22} is consistent with the data, but we cannot specify what step k_{22} corresponds to. The value of ${}^{D}V_{\text{cyclohexanone}}$ near unity seen at high pH is consistent with eq 32.

Interpretation of the pKs in Table I. True pKs for those groups in either the enzyme or the inhibitor whose state of ionization affects binding are obtained from the pH dependence of the K_i for a competitive inhibitor (Cleland, 1977). Since $pK_{i\ \text{trifluoroethanol}}$ decreases below a pK of 7.2 [a similar profile has been reported by Shore et al. (1974)], a group with this pK in the E–DPN complex must be unprotonated for binding alcohol, since trifluoroethanol has no ionizable groups in this pH region. [Kvassman & Pettersson (1980a) have suggested that trifluoroethanol actually binds to protonated enzyme, followed by loss of a proton; this is thermodynamically equivalent to binding to the unprotonated enzyme.] A similar corrected pK of 7 is also observed in the $V/K_{\text{cyclohexanol}}$ profile and seen at pH 6.2 in the $V_{\text{cyclohexanol}}$ profile. Since both V and V/K decrease below this pK, cyclohexanol binds to both E–DPN and EH–DPN, although more tightly to the former. However, trifluoroethanol only binds very weakly to EH–DPN, since the K_i does not level off in the pH range covered in Figure 7[Kvassman & Pettersson (1980a) report the pK to be 4.3]. We believe this group with a pK of 7 in E–DPN is His-51 (Eklund et al., 1976); see also Scheme I.[5]

The pK of His-51 appears to be higher in E–DPNH than in E–DPN, which is a reasonable result of the presence of the positive charge at N-1 of the nicotinamide ring of DPN. Thus, $V/K_{\text{cyclohexanol}}$ in the substrate-activating region where cyclohexanol adds to E–DPNH (Dalziel & Dickinson, 1966) shows a pK of 8.8 instead of 7, and this pK is the same as one of these seen in the $V/K_{\text{cyclohexanone}}$ profile, except that His-51 must be protonated for reduction of cyclohexanone. Note that this pK does not show in the $K_{i\ \text{isobutyramide}}$ profile; in terms of Scheme I, which we will discuss below, aldehydes, ketones, and isobutyramide can all combine with either form I or form III of E–DPNH because there is no difference in the arrangement of reactants around the Zn–H_2O complex.

pKs of 9.7–10.4 occur in the profiles for $V/K_{\text{cyclohexanol}}$ at both high and low cyclohexanol levels, $V/K_{\text{cyclohexanone}}$, $K_{i\ \text{trifluoroethanol}}$, and $K_{i\ \text{isobutyramide}}$, and in every case the group must be protonated for binding or activity. This pK may represent ionization of $ZnOH_2$ to ZnOH. Such an ionization is expected to affect the V/K and pK_i profiles, but for this pK to represent formation of ZnOH in the V profiles, water would have to remain in the coordination sphere after substrate binding, as would be the case if the Zn were pentacoordinate during the reaction (see below). Alternatively, steps involving release of nucleotides from their binary complexes, or isomerization between binary complexes, may become rate limiting at high pH. In that case, formation of ZnOH would have to alter the protein conformation so that one of these steps becomes much slower. A pK of at least 10 for $ZnOH_2$ is expected, since Zn^{2+} is coordinated to two S^- groups from cysteines, and, thus, there is no overall charge present before ionization of $ZnOH_2$.

The pK_i profiles for DPN and DPNH present a problem. If $K_{i\ \text{DPN}}$ increases below the pK of His-51, the pK is then perturbed from 8.2 in free enzyme to 6.8 in the binary complex, in fair agreement with the value near 7 from the $V/K_{\text{cyclohexanol}}$ and $pK_{i\ \text{trifluoroethanol}}$ profiles. However, $K_{i,\text{DPNH}}$ also increases above a pK of 8.65. If this pK is that of His-51, the pK would be displaced to above 10 in the E–DPNH complex, the enzyme would be in the correct protonation state for reduction of ketone until the pH was above 10, and there should be no change in $V_{\text{cyclohexanone}}$ at high pH. Thus, either DPN is sensitive to the protonation state of His-51 and DPNH is not (but is sensitive to the protonation state of some other group on the enzyme) or neither DPN nor DPNH is sensitive to the protonation state of His-51, and the pKs seen are for other groups. Since the nicotinamide ring of DPN is positively charged and binds in the proximity of His-51, it is likely that the protonation state of the group to which DPN binding is sensitive is His-51, while that which affects the binding of DPNH is some other group.[6]

Mechanistic Conclusions. The data for both yeast and horse liver alcohol dehydrogenases can be interpreted in terms of Scheme I (Dunn et al., 1977; Kvassman & Pettersson, 1978),

[5] Kvassman & Pettersson (1980b) have postulated that the group is water coordinated to Zn and that it becomes hydroxide above the pK of 7. The complex shown as II in Scheme I could indeed exist partly with His-51 protonated and the coordinated water as OH, since this is what happens with alcohol replacing water in the coordination sphere. The observed pK is thus a global one for protonation of all forms of II and III (as DPN complexes) to give I (as a DPN complex) in Scheme I.

[6] DPN appears to bind selectively to a form of enzyme in which the fluorescence of Trp-314 is quenched by interaction with what is thought to be ionized Tyr-286 (Laws & Shore, 1978), so that the pK of Tyr-286 is shifted from 9.8 to about 8 (Wolfe et al., 1977). The binding of trifluoroethanol to E–DPN has been shown (Wolfe et al., 1977; Parker et al., 1978) to decrease the pK of a group from 7.6 to a value below 4. Parker et al. (1978) have also shown with acetimidylated enzyme that DPN binding shifts the pK of some group from 9.8 to 9.0, and subsequent trifluoroethanol binding then shifts the pK to 5. These data were interpreted by Parker et al. (1978) to indicate weak trifluoroethanol binding to a protonated form of E–DPN. If, as Laws & Shore (1978) suggest, this group is Tyr-286, the pK_i profile for DPN should show the Tyr-286 pK of 9.8, and it does not. Further work is needed on this problem, but we prefer to interpret the pK seen in the pK_i profiles of DPN and trifluoroethanol as that of His-51, rather than that of Tyr-286.

which shows direct coordination of alcohol to the active-site Zn, as suggested by X-ray studies of liver alcohol dehydrogenase ternary complexes (Plapp et al., 1978). In free enzyme, the Zn is apparently tetracoordinate (Eklund et al., 1976), with a bound water hydrogen bonded to Ser-48 and His-51 as shown. However, it has recently been demonstrated on the basis of EPR studies of the active site specific Co^{2+}-reconstituted enzyme (Maret et al., 1979) in the presence of $H_2{}^{17}O$ that DPN binding changes the metal ion from tetra- to pentacoordinate, and that in the E–DPN–pyrazole and E–DPN–trifluoroethanol complexes a water molecule remains in the inner coordination sphere of the metal (M. B. Yim and M. W. Makinen, unpublished experiments). In Scheme I, we thus show alcohol or ketone displacing one bound water and taking its place in the proton relay with Ser-48 and His-51.[7] The key feature of Scheme I is that His-51 lies at the surface of the proton (Eklund et al., 1976), and thus can be deprotonated as in the conversion of I to III while reactants are bound and the state of protonation of molecules in the substrate binding site is locked. Thus, in mechanism 11, EHX (which correspondonds to IV in Scheme I) can be deprotonated to EX (V in Scheme I) without preventing subsequent hydride transfer.

In the mechanism shown in Scheme I, ketone binds productively to protonated forms I or III of E–DPNH, displacing bound water and forming part of what we presume by analogy with the results of Yim & Makinen on DPN complexes to be a pentacoordinate inner-sphere complex with Zn in which the carbonyl oxygen is hydrogen bonded to Ser-48. Hydride transfer (k_{14} or k_{20}) can now take place regardless of whether His-51 is protonated on the nitrogen which faces the solvent, since the internal geometry of the active site is the same (the rate is 4.5-fold faster with His-51 protonated). At neutral or low pH, the Zn-bound alkoxide IV which results from hydride transfer is protonated by His-51 through the proton relay system (k_{12}), and alcohol is released to give II.[8] In the high-pH pathway which leads to V, His-51 must be protonated from solution to give IV before alcohol release can occur, and at sufficiently high pH so little His-51 is protonated that alcohol release becomes very slow and hydride transfer comes to equilibrium, with the resulting change in the primary isotope effect from 2.25 to 0.85. In the forward direction, alcohol binds productively only to protonation form II of E–DPN, displacing bound water so that the alcohol may donate its proton to His-51 to form the alkoxide IV (k_{11}). At high pH, His-51 in IV is rapidly deprotonated to V, thus giving an infinite commitment for alcohol, and a decrease in the primary isotope effect from 2.5 to 1.0.

Morris et al. (1980) have recently postulated a very similar mechanism for liver alcohol dehydrogenase with *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde as a substrate and have shown that (1) the rate of disappearance of the highly colored E–DPNH–aldehyde intermediate which is throught to be an inner-sphere complex with Zn (Dunn et al., 1975) decreases above a p*K* near 6, while the rate of formation of the intermediate is pH independent, and (2) the isotope effect on the rate of disappearance of the intermediate is 2.8 at low pH, but decreases to 1.0 above pH 7. These results from rapid single-turnover experiments are very similar to the steady-state data in the present paper and lend additional support to Scheme I as the mechanism of liver alcohol dehydrogenase.

In further support of Scheme I, Dunn (1974) has shown that at pH 8.8 proton uptake does not occur in the pre-steady-state burst of aldehyde reduction, but does in the subsequent slow step which is limited by alcohol dissociation from the site (Bernhard et al., 1970; Dunn & Bernhard, 1971; McFarland & Bernhard, 1972). As the pH is lowered, however, the fast (hydride transfer) and slow (alcohol release) transients become more difficult to separate from each other (Schmidt et al., 1979).

Dunn et al. (1979) have argued that since the size of the pre-steady-state burst is not sensitive to substituent effects when a variety of para-substituted aromatic aldehydes are used in which the solution equilibrium constant changes by 3 orders of magnitude mechanisms similar to that in Scheme I cannot be correct. To explain their data, these authors postulate site–site interactions with half of the enzyme sites loaded with substrates and half with products at any time during the steady state. However, such a mechanism only explains the pre-steady-state burst amplitudes observed for several of the aldehydes of 60 or 70%, as opposed to 50%, of the sites if only a portion of the enzyme exhibits the site–site interactions, while the remainder is noncooperative.[9] For a number of kinases, Nageswara Rao et al. (1978, 1979) have shown that the equilibrium constant on the enzyme is maintained at unity, even with a range of substrates where the solution equilibrium constants differ by over 4 orders of magnitude, and even with such different substrates as glycolate and pyruvate with pyruvate kinase. These data have been explained by postulating that the enzyme exists in two conformations, one matching the geometry of MgATP and the other matching MgADP, with the equilibrium constant for the conformation change determined by the thermodynamic properties of the enzyme. Similarly, the data of Dunn et al. (1979) suggest that for alcohol dehydrogenases the equilibrium constant for hydride

[7] Kvassman & Pettersson (1980b) have suggested that alcohols bind only to EH–DPN and that a proton is subsequently lost to solution to give a Zn-coordinated alkoxide prior to hydride transfer. This mechanism predicts that the $V/K_{\text{cyclohexanol}}$, $V/K_{\text{cyclohexanone}}$, and $^{D}(V/K)$ profiles will all decrease at high pH with the same p*K* and does not predict the two p*K*s in the $V/K_{\text{cyclohexanone}}$ profile. We thus feel that alkoxide formation involves proton transfer to His-51, which has a finite p*K*. The suggestion by Kvassman & Pettersson (1980b) that alcohols bind only to EH–DPN, and that a proton is then lost, can be explained by postulating that direct replacement of water by alcohol in form II of Scheme I does not occur, but rather that alcohol replaces water in a form of EH–DPN similar to I in Scheme I, and that this complex then loses a proton to give the E–DPN–alcohol complex which subsequently forms an alkoxide by proton transfer to His-51, as shown in Scheme I.

[8] The rate constant k_{12} is certainly not limited by the rate of the proton transfer itself, but rather by the rate of the conformation change on the protein which accompanies it (or perhaps permits it), since k_{13} is 2.5 times k_{12}. This conclusion is supported by the analysis of the solvent isotope effects of Welsh et al. (1980) presented below, although those data are for the yeast enzyme.

[9] Theorell & Bonnichsen (1951) have shown that for liver alcohol dehydrogenase the equilibrium ratio on the enzyme of DPNH/DPN at pH 7 is about 0.5. Both E–DPN–alcohol and EH–DPN–alkoxide contain DPN, and we do not know the equilibrium constant for interconversion of these two enzyme forms. However, a value near unity [as expected from the principles of Albery & Knowles (1976)] is consistent with a 50% pre-steady-state burst (corresponding to an equilibrium constant of unity for interconversion of E–DPN–alkoxide and E–DPNH–aldehyde), and the equilibrium DPNH/DPN ratio of 0.5 at pH 7. Also consistent with a value near unity for the above equilibrium are isotope effects as a function of pH with hydroxybutyrimidylated liver alcohol dehydrogenase (Dworschack & Plapp, 1977). This modification increases the rate of dissociation of DPNH, so that it is no longer rate limiting for V. The isotope effect on V for benzyl alcohol oxidation is 2.5 at pH 6.0 and 3.5 above pH 8. From eq 29 c_{Vb} is k_{13}/k_{12} at low pH and k_{19}/k_{11} at high pH. If an intrinsic isotope effect of 6.3 is assumed (identical with that for cyclohexanol), eq 27 gives $k_{13}/k_{12} = 2.5$ and $k_{19}/k_{11} = 1.1$. Since hydride transfer is relatively insensitive to pH ($k_{13}/k_{19} = 4.5$ with cyclohexanol), K_{eq} for alkoxide formation (k_{11}/k_{12}) is expected to be close to unity.

transfer is also close to unity, regardless of the nature of the substrate, and, thus, that conformation changes in the protein are coordinate with geometry changes between DPN and DPNH.

Question of Inner-Sphere vs. Outer-Sphere Zn–Substrate Binding. In Scheme I and our description of the alcohol dehydrogenase mechanism we have assumed inner-sphere binding of alcohol and aldehyde or ketone to the catalytic Zn. This is consistent with the fact that the alkoxide resulting from reduction of the aldehyde or ketone cannot be released until it is protonated. However, for the liver enzyme, Sloan et al. (1975) have suggested, on the basis of distances measured by the paramagnetic effect of Co^{2+} (in enzyme in which Co^{2+} replaced Zn^{2+}) on the relaxation rates of protons of isobutyramide or ethanol in dead-end complexes with E–DPNH, that the normal mode of binding is a second-sphere one. Recent extended X-ray absorption fine structure studies of the Zn enzyme by Smith et al. (1980) have also been interpreted to support second-sphere binding.

However, the ^{113}Cd NMR spectrum of liver alcohol dehydrogenase in which Cd has replaced Zn at both the structural and catalytic sites (the signals for which are easily distinguished since one site involves coordination by two sulfurs and the other by four, and sulfur is much more deshielding than oxygen or nitrogen for ^{113}Cd) shows a downfield shift of the catalytic Cd signal by 36 ppm, the value expected for inner-sphere binding, when imidazole was added (Bobsein & Myers, 1980). It is also clear that aromatic aldehydes form inner-sphere complexes with Zn (Dunn et al., 1975), and, thus, we believe the preponderance of the evidence favors the inner-sphere mechanism for the liver enzyme. The results of Sloan et al. (1975) could mean that second-sphere binding is predominate in the dead-end complexes they studied.

Perhaps the best evidence for inner-sphere binding of alcohol and aldehyde or ketone for yeast alcohol dehydrogenase comes from the solvent isotope effect studies of Welsh et al. (1980) with *p*-methoxybenzaldehyde. These authors fitted the pH profiles for V in H_2O and D_2O to the appropriate rate equations and then compared the extrapolated rates at high pH for alcohol oxidation and at low pH for aldehyde reduction to determine solvent isotope effects of 1.2 ± 0.1 and 0.50 ± 0.05, respectively. They also derived apparent pK values for the drop in V at low pH for alcohol oxidation, and at high pH for aldehyde reduction, and noted that D_2O only elevated the former pK by 0.21 unit, and the latter one by 0.02 unit, although one expects a pK elevation for ionization of most groups of about 0.5 pH unit [the fractionation factor relative to water for a proton on such groups as carboxyl, imidazole, and amino is near unity, while that of H_3O^+ is 0.33 (0.69 per proton) and [$-\log 0.33$] = 0.48 (Schowen, 1972)].

These data are explainable in terms of mechanism 11 as follows. Klinman (1972, 1976) concluded that hydride transfer was solely rate limiting with *p*-methoxybenzaldehyde, and, thus, k_{14} limits V for aldehyde reduction at low pH, k_{19} limits alcohol oxidation at high pH, and $k_{12} \gg k_{13}$. Since we have shown that hydride transfer is not accompanied by any proton transfer, the chemical reaction itself should not show any solvent isotope effect (it is occurring in a pocket in the enzyme removed from the solvent). If, however, conversion of an initially formed outer-sphere complex to an inner-sphere one with displacement of a water molecule from coordination with the metal occurs as a rapid preequilibrium step prior to hydride transfer, then the observed inverse solvent isotope effect of 0.50 could result from the protons on zinc-bound water each having a fractionation factor of 0.71 relative to water [in H_3O^+, each proton has a fractionation factor of 0.69 (Schowen, 1972)]. Welsh et al. (1980) proposed a similar explanation for their results, but no experimental values are available for similar metal-bound water in a partly nonaqueous environment similar to that in the active site. A similar effect of Zn and a proton on the fractionation factor of water protons does not seem unreasonable, however. Note that this explanation is valid only if water is displaced during substrate binding, and, thus, there must not only be inner-sphere binding but the coordination number must not change (presumably, it is pentacoordinate both in E–DPNH and in E–DPNH–aldehyde complexes, in analogy with the results of Yim & Makinen mentioned previously).

In the direction of alcohol oxidation, the step represented by k_{11}, which is the only one that should show a solvent isotope effect, is irreversible at high pH because of the rapid deprotonation of EHX to EX and is faster than k_{19} (Klinman, 1972, 1976). One thus expects no solvent isotope effect at all on V, and the observed value of 1.2 ± 0.1 may not be significantly different from unity.

The apparent pK values for the V profiles are given by eq 16 for aldehyde reduction and eq 26 for alcohol oxidation. For aldehyde reduction, the ratio k_{19}/k_{20} should not be changed in D_2O, and since $k_{12} \gg k_{13}$, we have

$$\Delta pK_{app} = 0.02 = \Delta pK_3 + \Delta pk_{14} - \Delta pk_{12}$$

where Δ means the value in D_2O minus that in H_2O. Since ΔpK_3 should be 0.48, and Δpk_{14} is -0.30 (the value is twice as high in D_2O as in H_2O), Δpk_{12} is 0.16, corresponding to a normal solvent isotope effect on k_{12} of 1.45. Since k_{12} involves proton transfer to the alkoxide to give an alcohol (and also likely proton transfer from histidine to serine, if the geometry of the yeast enzyme is similar to that of the liver enzyme), it should show a normal solvent isotope effect, but the relatively small size of the effect suggests that the proton transfer itself is not solely rate limiting, and the conformation change in the protein that permits proton transfer is the major limiting factor for k_{12}.

In the direction of alcohol oxidation, $k_{12} \gg k_{13}$ and $k_{11} \gg k_{19}$, while the ratio k_{13}/k_{19} should not be altered in D_2O, so that from eq 26 we have

$$\Delta pK_{app} = 0.21 = \Delta pK_2 + \Delta pk_{11} - \Delta pk_{12}$$

Since ΔpK_2 should be 0.48, and we have estimated Δpk_{12} as 0.16, Δpk_{11} is -0.11, corresponding to an inverse solvent isotope effect on k_{11} of 0.78. If the alcohol replaces water in the inner coordination sphere as part of the step represented by k_{11}, two protons on the displaced water increase their fractionation factor, while that of the proton on the hydroxyl group of the alcohol is decreased, so that the net overall effect is on only one proton with a fractionation factor when bonded to zinc of 0.78. The value for this fractionation factor calculated from the inverse solvent isotope effect on k_{14} was 0.71 when DPNH was used (see above), but with DPND the solvent isotope effect found by Welsh et al. (1980) was 0.58 ± 0.06, which corresponds to 0.76 for each proton. The agreement among the three values for this fractionation factor is excellent in view of the complexities of the calculations and strongly suggests that inner-sphere binding occurs during the reaction catalyzed by the yeast enzyme. The isotope effects deduced for the separate steps are also internally consistent in the sense that the ratio of solvent isotope effects on k_{11} and k_{12} ($0.78/1.45 = 0.52$) matches that on k_{14}/k_{13} if we assume that k_{13} shows no solvent isotope effect.

The studies presented here demonstrate the power of the pH dependence of isotope effects to establish the relationship between the isotope-sensitive bond-breaking step and the

proton transfers which accompany the overall chemical reaction. For the alcohol dehydrogenases, these studies prove that the direct transfer of hydrogen from DPNH to the carbonyl group of the substrate (which we have referred to as hydride transfer) precedes the proton transfer which completes synthesis of the alcohol product and show that hydride transfer can take place at only a slightly reduced rate when the group on the enzyme which normally donates this proton is in its ionized form at high pH. All postulated chemical mechanisms for the reaction must fit this model, and, thus, we can rule out any mechanism that calls for protonation of the carbonyl oxygen of a ketone or aldehyde prior to or simultaneous with the transfer of hydrogen from DPNH to the carbonyl carbon. In the following paper (Cook et al., 1981), we will present studies of secondary isotope effects which give us clues concerning the detailed geometry of the reactants during the reaction, and suggest an explanation for how the enzyme catalyzes the reaction.

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