# pH Variation of Isotope Effects in Enzyme-Catalyzed Reactions. 1. Isotope- and pH-Dependent Steps the Same†

Paul F. Cook‡ and W. W. Cleland*

ABSTRACT: Theory is developed for the pH dependence of isotope effects in enzyme-catalyzed reactions, assuming that the bond-breaking (and thus isotope-dependent) step is part of a portion of the reaction mechanism in which a group on the enzyme (or the substrate) must be in a given protonation state, so that the flux through this part of the reaction mechanism is pH dependent. This model predicts that at high or low pH, where $V$ and $V/K$ decrease and the portion of the reaction mechanism that includes the bond-breaking step becomes rate determining, the deuterium isotope effects on $V$ and $V/K$ either will be pH independent or will increase, but the two isotope effects will become equal. An increase in the $V/K$ isotope effect shows that the substrate is sticky (that is, reacts to give products as fast as or faster than it dissociates) at the pH optimum, while an increase in the $V$ isotope effect shows that some other pH-independent step, such as release of the second product, at least partly limits $V$ at the pH optimum. This theory has been verified with three dehydrogenases. With yeast alcohol dehydrogenase and 2-propanol as substrate, the intrinsic isotope effect on the bond-breaking step was determined by comparison of deuterium and tritium isotope effects on $V/K$ to be 5.7. Both $V$ and $V/K_{2\text{-propanol}}$ decrease below p$K$s of 7.6, and deuterium isotope effects on $V/K$ and $V$, which are 2.7 and 3.0, respectively, at pH 8.2, increase to 3.8 and 3.5 at pH 6. Thus, 2-propanol is somewhat sticky (that is, dissociates from the ternary complex about half the rate at which acetone is produced), and DPNH release partly limits $V$. For TPN isocitrate dehydrogenase, where $V$ decreases below a p$K$ of 6.0, and $V/K_{\text{isocitrate}}$ decreases below a p$K$ of 6.7 and above a p$K$ of 9.5, no isotope effects are seen on either $V$ or $V/K$ at pH 7.45, but equal isotope effects of 1.1 are seen at pH 4.5, and of 1.05 at pH 9.5, on both parameters. Isocitrate is thus very sticky at neutral pH, and TPNH release limits $V$. In an equilibrium perturbation experiment at pH 8, however, an isotope effect from the isocitrate side of 1.15 (essentially equal to the equilibrium isotope effect) results from a higher commitment for TPNH than for isocitrate. With malic enzymes from chicken or pigeon liver, pH-independent isotope effects on $V/K_{\text{malate}}$ of 1.5 were seen, showing that malate is not sticky. The isotope effect on $V$ was 1.0 at pH 7 (showing that TPNH release limits $V$) but became 1.4 at pH 4, and 1.26 at pH 9.5 (the p$K$ for the $V$ profile). The three enzymes studied thus fit the predictions derived from the model described above in the pH regions investigated.

Isotope effects on enzyme-catalyzed reactions are only rarely fully expressed, since bond-breaking steps are often faster than other steps such as release of reactants. To enhance observed isotope effects and obtain more useful information on the degree of rate limitation of the bond-breaking step, and the location of other rate-limiting steps along the reaction path, the pH can be raised or lowered so that the chemical reaction or the pH-dependent pathway including the chemical reaction becomes rate limiting. In this paper, we deal with the simple case where the isotope-dependent step is a part of a pH-dependent portion of the reaction mechanism. Theory is developed for several cases pertaining to this simple model, and data are presented for several dehydrogenases which adhere to this model.

## Nomenclature

The nomenclature used to express isotope effects is described in considerable detail in the previous paper (Cook & Cleland, 1981a), and readers should familiarize themselves with this material before reading the present paper.

## Materials and Methods

*Chemicals.* 2-Propanol-*2-d* (98 atom %) and sodium borodeuteride (98 atom %) were obtained from Merck. Sodium borohydride-*t* was obtained from Amersham Searle. DL-Isocitric-*2-d* lactone was the generous gift of Dr. M. H. O'Leary. All enzymes [except pigeon liver malic enzyme prepared by the method of Hsu & Lardy (1967)] and other reagents were obtained from commercially available sources.

2-Propanol-*2*-(*h*,*d*,*t*) was prepared by reduction of acetone with sodium borohydride, sodium borodeuteride, or sodium borohydride-*t*, followed by distillation of the final mixture after destroying the excess borohydride. The fraction distilling at 81 °C was collected. DL-Isocitrate-*2*-(*h*,*d*) was prepared by hydrolyzing *threo*-DL-isocitric-*2-d* lactone in 1 N NaOH for 1 h at 100 °C. The resulting mixture was adjusted to pH 8.0 and used without further treatment. L-Malate-*2-d* was synthesized enzymatically according to the procedure of Viola et al. (1979), using ethanol-$d_6$, DPN, and oxalacetate with liver alcohol dehydrogenase, yeast aldehyde dehydrogenase, and malate dehydrogenase. A 90-MHz NMR spectrum of the product in $D_2O$ showed only the $\beta$ protons of malate and no formic acid. Since the DPN concentration was 10% of the oxalacetate concentration, if there were a difference in the reduced nucleotide side specificity of alcohol and aldehyde dehydrogenases, the 2 position of malate would contain 10% protons. Since malate showed no protons in the 2 position, the stereospecificity of yeast aldehyde dehydrogenase for reduced nucleotide must be identical with that of malate de-

† From the Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received January 14, 1980; revised manuscript received October 24, 1980.* This work was supported by a National Institutes of Health postdoctoral fellowship (IF 32GM 05940-01) to P.F.C. and grants from the National Institutes of Health (GM 18938) and the National Science Foundation (BMS 16134) to W.W.C.

‡ Present address: Department of Biochemistry, Louisiana State University Medical Center, New Orleans, LA 70112.

0006-2960/81/0420-1797$01.25/0 

hydrogenase, i.e., A side.

*Initial Velocity Studies.* All initial velocity studies were performed at 25 °C by using a Beckman DU monochromator and a Gilford OD converter to monitor the change in absorbance at 340 nm. Conditions used are listed where applicable in the figure legends. Isotope effects on initial velocities were obtained at saturating nucleotide concentration by comparing deuterated and nondeuterated substrates. All pH studies were carried out at saturating nucleotide concentration by varying the substrate concentration. The pH jump technique was used in which enzyme was kept in a dilute buffer solution and reaction was initiated by addition of enzyme to the reaction mix containing 100 mM buffer at the pH being studied. Buffers were used at the following pH values with $K^+$ or $Cl^-$ as the counterion: 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. Sufficient overlaps were obtained to be sure buffers had no effect on the rate.

*Equilibrium Perturbation.* Primary isotope effects were obtained by using 2-propanol-*2-d* as a substrate for yeast alcohol dehydrogenase at pH 6.0 and 8.2 and by using isocitrate-*2-d* as a substrate for isocitrate dehydrogenase at pH 8.0. With isocitrate dehydrogenase, reaction mixtures contained 100 mM Tris-HCl, pH 8.0, 0.5 mM TPN, 0.16 mM isocitrate-*2-d*, 8.6 mM $CO_2$, 40 mM $\alpha$-ketoglutarate, 0.2 mM TPNH, and 2 mM $MgSO_4$. With yeast alcohol dehydrogenase, reaction mixtures contained 100 mM buffer (Tris-HCl, pH 8.2, or Mes-K, pH 6.0), DPN (13 $\mu$M at pH 8.2; 1.3 mM at pH 6.0), 32.8 mM 2-propanol-*2-d*, 0.24 mM DPNH, and 1.34 mM acetone. All perturbations were obtained by using the "two-pot" method, which consists of making two solutions, one for substrates and one for products, and adding an aliquot of each to a cuvette. For the first trial, aliquots were chosen so that the system would be at equilibrium, and enzyme was added. For the second trial, the aliquots were adjusted on the basis of the net extent of reaction in the first trial so that the reaction mixture was exactly at equilibrium, i.e., the perturbation returned exactly to the starting point. Identical experiments were run at each pH value by using the hydrogen-containing substrate to be sure that none of the perturbations obtained were artifactual.

*Tritium Discrimination.* The specific activity of the final 2-propanol–water mixture was 15.2 mCi/mL with a 2-propanol concentration of 29.2 mM. To six 3-mL cuvettes containing 1 mM DPN, 100 mM Tris-HCl, pH 8, 10 mM semicarbazide hydrochloride, pH 8, and 0.01 unit of yeast alcohol dehydrogenase was added 0.25 mM 2-propanol-*2-t*. DPNH production was monitored until the reaction was approximately 40% complete, at which time $OD_{340}$ was recorded and 0.06 mL of saturated $AgNO_3$ was added to stop the reaction. Six additional cuvettes were prepared, identical with those above, the reaction was allowed to proceed to completion, $OD_{340}$ was recorded, and $AgNO_3$ was added. From each of the 12 cuvettes, 0.3 mL was applied to circles of DEAE paper (Whatman DE 81), which were dried, washed with 500 mL of water, and then counted by using a scintillation counter. Specific activities for the initial substrate (determined as the product at 100% reaction) and product at 40% reaction were obtained by dividing the counts per minute on the paper disks by the concentration of DPNH (calculated from the $OD_{340}$ recorded when the reaction was stopped). The isotope effect was calculated by the method of Northrop (1975) according to eq 1, where $f$ is the fractional reaction, $R$ is the specific

$$^{T}(V/K_{2\text{-propanol}}) = \frac{\log (1-f)}{\log [1-f(R/R_0)]} \quad (1)$$

activity of the product at $f$, and $R_0$ is the specific activity at 100% reaction. Experiments were repeated at $f = 0.55$ and 0.76.

*Data Processing.* Reciprocal initial velocities were plotted against reciprocal substrate concentrations, and all plots were linear. All data were fitted to appropriate equations by using the FORTRAN programs of Cleland (1979). The individual saturation curves used to obtain pH profiles and saturation curves for L- and DL-malate were fitted to eq 2. The data

$$v = \frac{VA}{K + A} \quad (2)$$

for direct comparison of hydrogen- and deuterium-containing substrates at saturating levels of nucleotide were fitted to eq 3–5, which respectively assume isotope effects on both $V$ and

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (3)$$

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A} \quad (4)$$

$$v = \frac{VA}{K + A(1 + F_iE_V)} \quad (5)$$

$V/K$, $V/K$ only, and $V$ only. In these equations, $F_i$ is the fraction of deuterium label in the substrate, while $E_V$ and $E_{V/K}$ are the isotope effects minus one for the respective parameters. Data for linear competitive inhibition were fitted to eq 6, where

$$v = \frac{VA}{K[1 + (I/K_i)] + A} \quad (6)$$

$I$ represents the inhibitor concentration. Data for pH profiles, showing a drop in log $V$ or log $V/K$ with a slope of 1 as the pH is lowered, were fitted to eq 7, while when log $V$ or log

$$\log y = \log [C/(1 + H/K_1)] \quad (7)$$

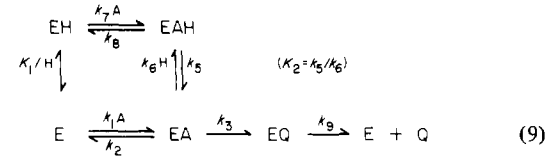
$V/K$ decreased at both high and low pH, the data were fitted to eq 8. In these equations, $K_1$ and $K_2$ are the dissociation

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (8)$$

constants for groups on the enzyme, $y$ is $V$ or $V/K$, and $C$ is the pH-independent value of $y$. Isotope effects were extracted from equilibrium perturbation data by the method of Schimerlik et al. (1975) by using a FORTRAN program which achieves an exact solution to the equations, and limits on the intrinsic isotope effect for yeast alcohol dehydrogenase were obtained by using a FORTRAN program which is based on the method of Northrop (1975).

## Theory

In this paper, we shall deal with the case where the isotope-dependent bond-breaking step is a part of a pH-dependent portion of the reaction mechanism. Consider the simple model shown in mechanism 9, where $k_3$ includes the bond-breaking




(9)

[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; BSA, bovine serum albumin.

step and is thus isotope dependent. While $k_3$ itself is pH independent, the fact that the distribution between EA and EAH varies with pH makes the rate of formation of EQ pH dependent. We assume $k_9$ is both pH and isotope independent. The other rate constants shown are also not isotope dependent. The conversion of EA to EQ will probably consist of several steps, only one of which is isotope dependent, but this simple model should suffice, as long as protons may not come and go from the catalytic groups on the enzyme during the conversion of EA to EQ.[2]

Expressions for $V$, $V/K$, and the isotope effects on these two parameters for mechanism 9 are listed in Table I. The model is symmetrical, so that if EH and EAH are the active species, all conclusions concerning the pH variation of isotope effects at low pH become applicable to high pH.

For case I, when the entire mechanism is used, the full isotope effect ($^Dk_3$) on $V$ and $V/K$ will be observed below the p$K$s seen in the $V$ and $V/K$ profiles, but not above the p$K$s in the profiles unless $k_2 \gg k_3$ (for $V/K$) or $k_9 \gg k_3$ (for $V$). In any event, the $V$ and $V/K$ effects will become equal as the pH is lowered.[3] For case II ($k_5 = k_6 = 0$), the $V/K$ isotope effect will be pH independent and will not be the full effect on $k_3$ unless $k_2 \gg k_3$. The $V$ and $V/K$ isotope effects will be equal at low pH but may not be at neutral pH. The $V/K$ isotope effect is also pH independent for case III ($k_7 = k_8 = 0$), but again is not the full effect on $k_3$ unless $k_2 \gg k_3$. The $V$ isotope effect, in this case, will approach the full effect on $k_3$ at low pH, but again may not be equal to the $V/K$ effect at neutral pH. This is the only case with a pH-dependent $V/E_t$ in which the $V$ isotope effect can be larger than the $V/K$ effect at low pH. However, this case is very unlikely since it calls for substrate binding only to the correctly protonated form of the enzyme but allows a proton to lock the substrate on the enzyme to form a dead-end EAH complex. Finally, for case IV ($k_5 = k_6 = k_7 = k_8 = 0$), both $V$ and $V/K$ effects are pH independent, are not the full effect on $k_3$, and may or may not be equal. Case IV is readily identified by the fact that $V/E_t$ is pH independent; in all other cases, both $V/E_t$ and $V/KE_t$ decrease at low pH, although the apparent p$K$ values for the two profiles do not have to be identical (Cleland, 1977).

Table I: Kinetic Parameters and Isotope Effects for Equation 9 as a Function of pH[a]

| | neutral pH | low pH |
|---|---|---|
| | Case I (Full Mechanism) | |
| $V/KE_t$ | $\frac{k_1k_3}{k_2 + k_3}$ | $\frac{k_1k_3}{k_2}\left(\frac{K_1}{H}\right)$ |
| $^D(V/K)$ | $\frac{^Dk_3 + k_3/k_2}{1 + k_3/k_2}$ | $^Dk_3$ |
| $V/E_t$ | $\frac{k_3k_9}{k_3 + k_9}$ | $k_3\left(\frac{K_2}{H}\right)$ |
| $^DV$ | $\frac{^Dk_3 + k_3/k_9}{1 + k_3/k_9}$ | $^Dk_3$ |
| | Case II ($k_5 = k_6 = 0$) | |
| $V/KE_t$ | [b] | $\frac{k_1k_3}{k_2 + k_3}\left(\frac{K_1}{H}\right)$ |
| $^D(V/K)$ | [b] | $\frac{^Dk_3 + k_3/k_2}{1 + k_3/k_2}$ |
| $V/E_t$ | [b] | $\left(\frac{k_1k_3}{k_2 + k_3}\right)\frac{k_8}{k_7}\left(\frac{K_1}{H}\right)$ |
| $^DV$ | [b] | $\frac{^Dk_3 + k_3/k_2}{1 + k_3/k_2}$ |
| | Case III ($k_7 = k_8 = 0$) | |
| $V/KE_t$ | [b] | $\frac{k_1k_3}{k_2 + k_3}\left(\frac{K_1}{H}\right)$ |
| $^D(V/K)$ | [b] | $\frac{^Dk_3 + k_3/k_2}{1 + k_3/k_2}$ |
| $V/E_t$ | [b] | $k_3\left(\frac{K_2}{H}\right)$ |
| $^DV$ | [b] | $^Dk_3$ |
| | Case IV ($k_5 = k_6 = k_7 = k_8 = 0$) | |
| $V/KE_t$ | [b] | $\frac{k_1k_3}{k_2 + k_3}\left(\frac{K_1}{H}\right)$ |
| $^D(V/K)$ | [b] | $\frac{^Dk_3 + k_3/k_2}{1 + k_3/k_2}$ |
| $V/E_t$ | [b] | $\frac{k_3k_9}{k_3 + k_9}$ |
| $^DV$ | [b] | $\frac{^Dk_3 + k_3/k_9}{1 + k_3/k_9}$ |

[a] Neutral pH means at least a pH unit above the p$K$ in the $V$ or $V/K$ profile; low pH means at least a pH unit below the p$K$.[3] [b] Same as in case I.

As noted above, the isotope effect on $k_3$ is not necessarily an intrinsic one but may be reduced by internal commitments. If the EA to EQ conversion in mechanism 9 is expanded as shown in eq 10, where $k_{13}$ and $k_{14}$ are for the isotope-dependent

$$\mathrm{EA} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{EA'} \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} \mathrm{EPQ'} \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} \mathrm{EPQ} \overset{k_{17}}{\rightharpoonup} \mathrm{EQ} \overset{k_9}{\rightharpoonup} \mathrm{E} \quad (10)$$

(bond-breaking) steps, the steps represented by $k_{11}$, $k_{12}$, $k_{15}$, and $k_{16}$ involve the non-isotope-dependent conformation

[2] In this model, we show the conversion of EA to EAH to involve reaction with $H^+$, and the reverse reaction to be pH independent (that is, involve reaction with water to give $H_3O^+$). In practice, these proton transfers are likely to be mediated by buffers, which because they can transfer protons through one or two water molecules can react with even partially buried groups at rates up to $10^9$ $M^{-1}$ $s^{-1}$ in the favored direction. If a catalytic group is really isolated from the solvent in the EA complex, however, buffers may not be able to mediate the reaction, and if p$K_2$ is above 7, one must consider the conversion of EAH to EA by reaction with $OH^-$ and the protonation of EA to EAH by reaction with water. Since the bimolecular rate constants for reaction of $H^+$ and $OH^-$ with groups such as carboxyl, imidazole, or amino are nearly the same because they are limited by diffusion (Eigen, 1964), the p$K$ at which one switches from reaction with $H^+$ and water to reaction with water and $OH^-$ is 7. In the case of thiol groups, however, reaction of $H^+$ with $RS^-$ is diffusion limited, but reaction of RSH with $OH^-$ is slower by a factor of about 50, so that the cross-over p$K$ between the two processes is 7.85 (elevated from 7 by half-log 50). When one is considering the loss of activity at high pH (that is, when EAH is active, and EA is not), we will normally be dealing with a case where if buffers do not mediate the proton transfers EAH is converted to EA by $OH^-$, and EA to EAH by reaction with water. For a more complete description of the pH profiles expected for this model and data which show slow proton transfers to and from EA, see Cook et al. (1981).

[3] The p$K$s for the shift of the $V/K$ and $V$ isotope effects from their low to high pH values will be the same as the corresponding p$K$s in the $V/K$ and $V$ profiles, except in those cases where humps or hollows are seen in the vicinity of the apparent p$K$s in the profiles, as discussed by Cleland (1977). In those cases, the p$K$ for the change in the isotope effect corresponds to the intersection of the low pH asymptote with the floor of the hollow (lower than the apparent p$K$ for the $V$ or $V/K$ profile), or for the hump pattern, which can occur only in a $V/K$ profile, to a position near the top of the hump (higher than the p$K$ in the $V/K$ pattern). In practice, the isotope effect has reached its limiting value by the time the $V$ or $V/K$ profile has become coincident with its low or high pH asymptote.

changes which convert the collision complexes EA and EPQ into the catalytically competent ones EA′ and EPQ′, and $k_{17}$ is for release of P from EPQ. We will assume that the state of protonation of EA′ and EPQ′ is locked so that the rate constants shown are pH independent.[4] For consideration of $^{D}(V/K)$, we can simply substitute into the equations in Table I the following expressions for $k_3$ and $^{D}k_3$:

$$k_3 = \frac{k_{11}k_{13}/k_{12}}{1 + k_{13}/k_{12} + (k_{14}/k_{15})(1 + k_{16}/k_{17})} \quad (11)$$

$$^{D}k_3 = \frac{^{D}k_{13} + k_{13}/k_{12} + {}^{D}K_{eq}(k_{14}/k_{15})(1 + k_{16}/k_{17})}{1 + k_{13}/k_{12} + (k_{14}/k_{15})(1 + k_{16}/k_{17})} \quad (12)$$

where

$$^{D}K_{eq} = {}^{D}k_{13}/{}^{D}k_{14} \quad (13)$$

The expressions $k_{13}/k_{12}$ and $(k_{14}/k_{15})(1 + k_{16}/k_{17})$ are commitments which are pH independent in this model. These commitments make $^{D}k_3$ less than the intrinsic isotope effect, $^{D}k_{13}$, which can be determined experimentally by the method of Northrop (1975).

For consideration of $^{D}V$ in eq 9, one can substitute the values of $k_3$ and $^{D}k_3$ from eq 11 and 12 into the expressions in Table I for low pH in cases I, II, and III. For the expressions at neutral pH, and for case IV at any pH, however, the numerator of the expression in eq 11 is $k_{11}k_{13}/(k_{11} + k_{12})$, and $k_{13}/k_{12}$ in the denominator is replaced by $c_V$:

$$c_V = \frac{k_{13}}{(1 + k_{12}/k_{11})}[1/k_{11} + 1/k_{17} + 1/k_{15}(1 + k_{16}/k_{17})] \quad (14)$$

The ratio $k_{13}/k_{12}$ in both the numerator and denominator of eq 12 is also replaced by $c_V$ in these cases. Thus, while the reverse commitment $[(k_{14}/k_{15})(1 + k_{16}/k_{17})]$ is identical at neutral pH for $^{D}(V/K)$ and $^{D}V$, $k_{13}/k_{12}$ and $c_V$ are not identical unless $k_{11}$ is smaller than $k_{12}$, $k_{15}$, and $k_{17}$. The fact that these terms in the equations for $^{D}V$ and $^{D}(V/K)$ are not identical at neutral pH will cause little trouble in practice, since the isotope effects at low pH are of greatest interest, and for cases I, II, and III the internal commitments at low pH are identical for $^{D}V$ and $^{D}(V/K)$.

## Results

*Yeast Alcohol Dehydrogenase.* The pH dependence of $V$ and $V/K_{2\text{-propanol}}$ below pH 8 is shown in Figure 1. Both $V$ and $V/K$ decrease below a p$K$ of 7.6, which suggests that the binding of 2-propanol to E and EH occurs with equal affinity (see mechanism 9). 2-Propanol was used as a substrate in an attempt to make the hydride-transfer step more rate limiting [its $V/E_t$ value is 39% that of ethanol (Sund & Theorell, 1963), with which DPNH release is largely rate limiting].

Isotope effects determined by direct comparison of initial velocities were obtained at pH 8.2, which is on the plateau of both pH profiles, and at pH 6, well below the p$K$ [the behavior of the isotope effects above pH 8 will be discussed in the following paper (Cook & Cleland, 1981b)]. A summary of these data is shown in Table II. The best fits were obtained by assuming isotope effects on both $V$ and $V/K_{2\text{-propanol}}$. $^{D}(V/K_{2\text{-propanol}})$ increases from 2.7 at pH 8.2 to 4.0 at pH 6.0, and $^{D}V_{2\text{-propanol}}$ increases from 3.0 at pH 8.2 [in agreement with the value obtained at pH 7.9 by Dickenson & Dickinson (1975)] to 3.5 at pH 6.0. The effects on $V/K$ were confirmed by using the equilibrium perturbation technique (Schimerlik et al., 1975), and these data are also shown in Table II.

Table II: Isotope Effects for Yeast Alcohol Dehydrogenase at 25 °C

| Direct Comparison of Initial Velocities[a] | | | | |
|---|---|---|---|---|
| pH | $^{D}V_{2\text{-propanol}}$ | $^{D}(V/K_{2\text{-propanol}})$ | eq fitted | $\sigma^b$ |
| 6.0 | 3.51 ± 0.24 | 3.99 ± 0.30 | 3 | 0.128[c] |
| | | 9.11 ± 1.49 | 4 | 0.687 |
| | 6.84 ± 2.54 | | 5 | 0.890 |
| 8.2 | 2.96 ± 0.17 | 2.72 ± 0.29 | 3 | 0.155[c] |
| | | 7.45 ± 1.66 | 4 | 0.699 |
| | 4.18 ± 0.34 | | 5 | 0.429 |

| Equilibrium Perturbation | |
|---|---|
| pH | $^{D}(\text{Eq.P.})_{2\text{-propanol}}$ |
| 6.0 | 3.77 ± 0.07 |
| 8.2 | 3.16 ± 0.06 |

[a] Data were fitted to eq 3, 4, or 5. The best fit is the one which gives the lowest $\sigma$ and the smallest standard errors. [b] $\sigma$ is equal to (sum of squares of residuals)/(degrees of freedom) where degrees of freedom are defined as (number of points) minus (number of parameters). [c] Best fit.

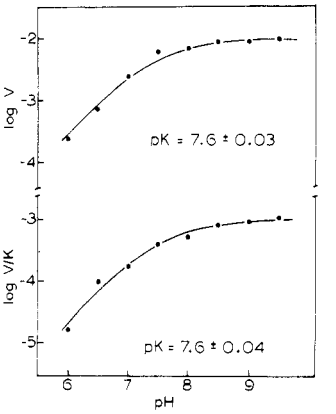



FIGURE 1: pH dependence of $V$ and $V/K_{2\text{-propanol}}$ for yeast alcohol dehydrogenase at 25 °C. DPN concentration was maintained at saturating levels (5–10 mM) for all assays. The solid lines are fits to eq 7 with a p$K$ of 7.6 in each case.

The large value of $^{D}(V/K_{2\text{-propanol}})$ makes it practical to determine the intrinsic isotope effect by comparison of $^{D}(V/K)$ and $^{T}(V/K)$ according to the method of Northrop (1975). The values of $^{T}(V/K_{2\text{-propanol}})$ in three experiments at pH 8 (see Materials and Methods for experimental details) were 5.28, 5.28, and 4.85 (all tritium isotope effects are $V/K$ ones, since they are determined by internal competition between the few molecules containing tritium and the many molecules containing hydrogen in the labeled position). The equilibrium isotope effect is 1.175 ± 0.010 (Cook et al., 1980), so the simplified equation of Northrop (1975), which assumes a value of 1.00 for $^{D}K_{eq}$, could not be used. The limits on the intrinsic isotope effects for deuterium and tritium in the forward and reverse directions were obtained from the average $^{D}(V/K_{2\text{-propanol}})$ and $^{T}(V/K_{2\text{-propanol}})$ values observed at pH 8 as discussed by Schimerlik et al. (1977) and are shown in Table III.[5]

[4] Locked protonation states of EA′ and EPQ′ make only $k_{12}$–$k_{15}$ pH independent. However, we are considering EA separately from EAH, so that $k_{11}$ is also a pH-independent rate constant. To be rigorous, we should consider possible protonation states for EPQ, or $k_{16}$ will probably be pH dependent ($k_{17}$ is likely to be pH independent, or nearly so, in any case), but we will not add further complexities at this point.

Table III: Limits on the Intrinsic Isotope Effect at pH 8 for Yeast Alcohol Dehydrogenase with 2-Propanol as Substrate[a]

| isotope effect | direction of reaction | symbol | lower limit | upper limit |
|---|---|---|---|---|
| deuterium | forward | $^{D}k$ | $5.32 \pm 0.60 = {}^{D}K_{eq}[app(^{D}k/^{D}K_{eq})]$ | $5.70 \pm 0.60 = app(^{D}k)$ |
| tritium | forward | $^{T}k$ | $11.2 \pm 1.23 = {}^{T}K_{eq}[app(^{T}k/^{T}K_{eq})]$ | $12.4 \pm 1.36 = app(^{T}k)$ |
| deuterium | reverse | $^{D}k/^{D}K_{eq}$ | $4.53 \pm 0.48 = app(^{D}k/^{D}K_{eq})$ | $4.85 \pm 0.52 = [app(^{D}k)]/^{D}K_{eq}$ |
| tritium | reverse | $^{T}k/^{T}K_{eq}$ | $8.88 \pm 0.98 = app(^{T}k/^{T}K_{eq})$ | $9.83 \pm 1.10 = [app(^{T}k)]/^{T}K_{eq}$ |

[a] The value for $^{D}K_{eq}$ has been determined as 1.18 (Cook et al., 1980) where $K_{eq}$ = (acetone)(DPNH)($H^+$)/[(2-propanol)(DPN)]. $^{D}k$ is the intrinsic deuterium isotope effect for the hydride-transfer step in the direction of 2-propanol oxidation. $^{T}K_{eq} = (^{D}K_{eq})^{1.442}$. $^{T}k = (^{D}k)^{1.442}$ (the intrinsic tritium isotope effect in the direction of 2-propanol oxidation). $^{D}k/^{D}K_{eq}$ and $^{T}k/^{T}K_{eq}$ are the intrinsic deuterium and tritium effects, respectively, in the direction of acetone reduction. The apparent value of $^{D}k$ is the one calculated from the average observed $^{D}(V/K_{2\text{-propanol}})$ and $^{T}(V/K_{2\text{-propanol}})$ 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 $^{D}(V/K_{2\text{-propanol}})$ by $^{D}K_{eq}$ and $^{T}(V/K_{2\text{-propanol}})$ by $^{T}K_{eq}$, and using these values in the above equation. The apparent $^{T}k/^{T}K_{eq}$ values are the 1.442 power of the deuterium values.

*Isocitrate Dehydrogenase.* The pH dependence of $V$ and $V/K_{isocitrate}$ is shown in Figure 2. $V$ decreases below a p$K$ of 6.0, in agreement with Colman & Chu (1969), while $V/K_{isocitrate}$ decreases below a p$K$ of 6.7 and above a p$K$ of 9.5. A comparison of initial velocities obtained by using deuterated and nondeuterated isocitrate as substrates at pH 7.45, which is on the plateau of both profiles, is shown in Figure 3. Isotope effects were also obtained at pH 4.5, which is below the acid p$K$ in both profiles, and at pH 9.5, which is the $V/K$ p$K$. These data are also shown in Figure 3. A summary of the data fitted to equations by assuming isotope effects on either $V$, $V/K$, or both parameters is shown in Table IV. No significant isotope effect is observed on either $V$ or $V/K_{isocitrate}$ at pH 7.45. At pH 4.5, an isotope effect of about 10% is seen on both parameters while at pH 9.5 an isotope effect of about 5% is observed on both parameters. Although no significant isotope effect is observed by direct comparison on $V/K_{isocitrate}$ at neutral pH, by using the equilibrium perturbation method with 200 $\mu$M TPNH as the limiting perturbant, a perturbation of 1.7 $\mu$M is obtained at pH 8, which gives an isotope effect of 1.15.

*Malic Enzyme.* The isotope effects obtained for malic enzymes from chicken liver and pigeon liver at pH values 4.0, 7.0, and 9.5 by using L-malate-*2-d* are summarized in Table V. At pH 7.0, both enzymes exhibit an isotope effect of 1.5 on $V/K_{malate}$ which is in very good agreement with the work of Schimerlik et al. (1977). At pH 4.0, however, the pigeon liver enzyme has isotope effects on $V$ and $V/K_{malate}$ of about 1.5, while at pH 9.5 (the p$K$ for $V$), a value of 1.5 is obtained for $^{D}(V/K_{malate})$ and about 1.3 for $^{D}V$.

The presence of a competitive inhibitor in the variable substrate increases the intercept but has no effect on the slope of a double-reciprocal plot (Cleland et al., 1973). Since DL-malate was used in some of the studies by Schimerlik et al. (1977), and D-malate could be a competitive inhibitor at the extremes of pH, comparisons of DL-malate and L-malate were run at pH 4.0 and 9.5 and are shown in Figure 4. The presence of the D isomer increased the intercept by a factor of just over 2 at pH 9.5, but not at pH 4.0, and caused no change in the slope at either pH.

A summary of $K_m$ values for L-malate and $K_i$ values for D-malate is shown in Table VI. The reason the $K_m$ for malate increases more rapidly on the basic side than on the acidic side of the profile is, as shown by Schimerlik & Cleland (1977), that log $(V/K_{malate})$ drops with a slope greater than 1 at high pH. At pH 9.5, the $K_m$ for L-malate and the $K_i$ for D-malate are about equal, while at pH 7.0, the $K_m$ is about $K_i/60$. At pH 4.0, not only the $K_m$ for L-malate increases 10-fold but also the $K_i$ for D-malate increases about 5-fold so that $K_m$ is still about 30-fold less than $K_i$. Thus, D-malate present in L-malate would cause inhibition at pH 9.5, but not at pH 4.0.

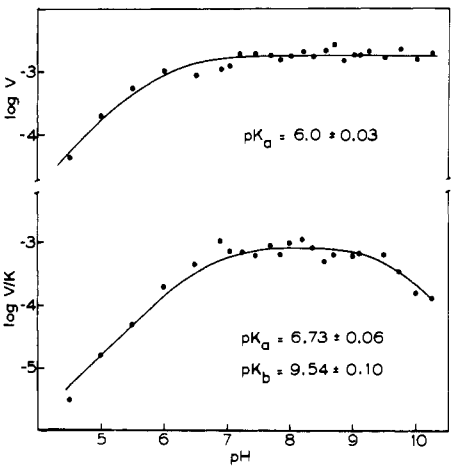



FIGURE 2: pH dependence of $V$ and $V/K_{isocitrate}$ for TPN isocitrate dehydrogenase at 25 °C. TPN concentration was maintained at saturating levels (0.2–1 mM). Due to the low value of $K_{isocitrate}$, 10-cm path-length cuvettes were used. Solid lines represent fits to eq 7 for $V$ and eq 8 for $V/K$ with the p$K$s indicated.

[5] Northrop (1975) assumed that any observed deuterium isotope effect is given by an equation of the form $(^{D}k + a)/(1 + a)$, where $a$ is a constant made up of rate constants for unlabeled reactants in the mechanism being considered, and $^{D}k$ is the intrinsic deuterium isotope effect for the bond-breaking step (that is, $k_H/k_D$). The corresponding tritium isotope effect is given by a similar equation in which $^{D}k$ is replaced by $^{T}k$, the intrinsic tritium isotope effect. Since tritium is normally used as a trace label and one obtains only $^{T}(V/K)$ values, Northrop (1975) compared $^{T}(V/K)$ and $^{D}(V/K)$ values by subtracting 1 from each parameter and taking their ratio. This eliminates the constant $a$ from the equation, and since $^{T}k = (^{D}k)^{1.442}$, one can solve for $^{D}k$. When $^{D}K_{eq}$ is not unity, however, the constant $a$ in the denominator of the expression for the isotope effect is the sum of forward and reverse commitments ($c_f + c_r$), but in the numerator $c_r$ is multiplied by $^{D}K_{eq}$. Subtracting 1 from $^{D}(V/K)$ no longer eliminates $c_r$ completely from the numerator, and the resulting apparent $^{D}k$ value obtained by Northrop's method is not correct. However, if the calculation is also performed with $^{D}(V/K)$ and $^{T}(V/K)$ values for the reverse reaction [most easily obtained by dividing $^{D}(V/K)$ for the forward direction by $^{D}K_{eq}$, and $^{T}(V/K)$ for the forward direction by $^{T}K_{eq}$, which is $(^{D}K_{eq})^{1.442}$], the true value of $^{D}k$ lies between the limits set by the two calculations, as shown in Table III. Since in the present case the evidence from the isotope effects suggests that the commitment for acetone is very small, while that for 2-propanol is about 1.8, Northrop's method should give the correct answer when applied to isotope effects from the 2-propanol side [$c_r = 0$, and $c_f$ *will* be eliminated from the equation by subtracting 1 from $^{D}(V/K)$ or $^{T}(V/K)$]. Thus, the upper limits shown in Table III are more likely the correct values. It should be noted that as long as only one step in the mechanism is isotope sensitive this procedure gives the intrinsic isotope effects on the forward and reverse rate constants for this step alone, regardless of how many other steps there are in the mechanism, or which ones are rate limiting for $V/K$ (the bond-breaking step must only be sufficiently rate limiting that a measurable isotope effect is observed).

Table IV: Isotope Effects for Isocitrate Dehydrogenase at 25 °C[a]

| pH | $^{D}V_{isocitrate}$ | $^{D}(V/K_{isocitrate})$ | eq fitted | $\sigma^{b}$ |
|---|---|---|---|---|
| 4.50 | 1.10 ± 0.05 | 1.08 ± 0.02 | 3 | 1.19[c] |
| | | 1.13 ± 0.01 | 4 | 1.57 |
| | 1.26 ± 0.04 | | 5 | 2.16 |
| 7.45 | 0.95 ± 0.06 | 1.07 ± 0.16 | 3 | 0.44 |
| | | 0.99 ± 0.09 | 4 | 0.42 |
| | 0.98 ± 0.04 | | 5 | 0.40 |
| 9.50 | 1.041 ± 0.005 | 1.063 ± 0.008 | 3 | 0.47[c] |
| | | 1.12 ± 0.02 | 4 | 1.90 |
| | 1.07 ± 0.01 | | 5 | 1.48 |

[a] Data were fitted to eq 3, 4, or 5. [b] $\sigma$ defined as in Table II. [c] Best fit on the basis of lowest $\sigma$.

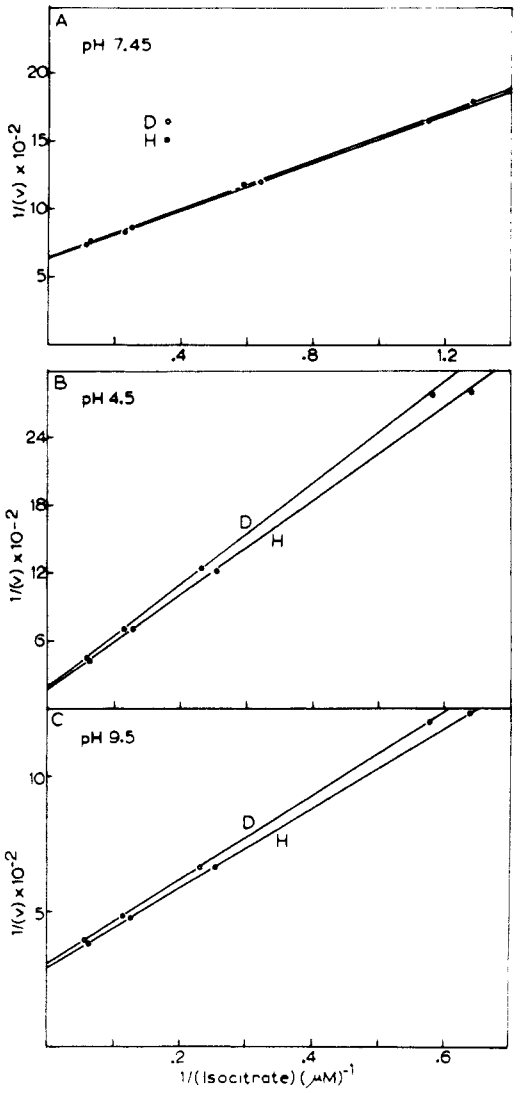



FIGURE 3: Initial velocity for isocitrate dehydrogenase as a function of the concentration of isocitrate-2-(*h*,*d*) at 25 °C. Conditions were the following: (A) pH 7.45, 0.002 unit/mL isocitrate dehydrogenase, 0.2 mM TPN, 2 mM $MgSO_4$, and 0.1 mM dithiothreitol; (B) pH 4.5, 0.4 unit/mL isocitrate dehydrogenase, 2 mM TPN, 2 mM $MgSO_4$, and 0.1 mM dithiothreitol; (C) pH 9.5, 0.005 unit/mL isocitrate dehydrogenase, 1 mM TPN, 2 mM $MgSO_4$, and 0.1 mM dithiothreitol. All velocities are in μmol/min.

Table V: Isotope Effects for Malic Enzymes at 25 °C[a]

| pH | $^{D}V_{malate}$ | $^{D}(V/K_{malate})$ | eq fitted | $\sigma^{b}$ |
|---|---|---|---|---|
| | Chicken Liver Malic Enzyme | | | |
| 7.0 | 1.046 ± 0.045 | 1.45 ± 0.09 | 3 | 0.09[c] |
| | | 1.54 ± 0.05 | 4 | 0.09[c] |
| | 1.29 ± 0.07 | | 5 | 0.24 |
| 9.5 | 1.23 ± 0.13 | 1.53 ± 0.17 | 3 | 0.05[c] |
| | | 1.84 ± 0.09 | 4 | 0.06 |
| | 1.69 ± 0.11 | | 5 | 0.08 |
| | Pigeon Liver Malic Enzyme | | | |
| 4.0 | 1.40 ± 0.02 | 1.46 ± 0.01 | 3 | 0.02[c] |
| | | 1.68 ± 0.05 | 4 | 0.20 |
| | 2.52 ± 0.40 | | 5 | 0.46 |
| 7.0 | 1.02 ± 0.03 | 1.44 ± 0.06 | 3 | 0.03[c] |
| | | 1.49 ± 0.03 | 4 | 0.03[c] |
| | 1.25 ± 0.06 | | 5 | 0.12 |
| 9.5 | 1.29 ± 0.06 | 1.51 ± 0.17 | 3 | 0.12[c] |
| | | 2.56 ± 0.37 | 4 | 0.31 |
| | 1.48 ± 0.37 | | 5 | 0.22 |

[a] Data were fitted to eq 3, 4, or 5. [b] $\sigma$ defined as in Table II. [c] Best fit on the basis of lowest $\sigma$ and smallest standard errors.

Table VI: Summary of Kinetic Parameters for Malic Enzymes at 25 °C[a]

| pH | $K_{L\text{-malate}}$ (mM) | $K_{i\ D\text{-malate}}$ (mM) |
|---|---|---|
| | Chicken Liver Malic Enzyme | |
| 4.0 | 1.4 ± 0.1 | 25 ± 3 |
| 7.0 | 0.096 ± 0.007 | 3.7 ± 0.1 |
| 9.5 | 11.1 ± 0.3 | 6.5 ± 0.7 |
| | | 5.72[b] |
| | Pigeon Liver Malic Enzyme | |
| 4.0 | 1.11 ± 0.08 | 36 |
| 7.0 | 0.094 ± 0.005 | ~1.5 |
| 9.5 | 4.50 ± 0.30 | ~1.0 |

[a] Calculated from fits to eq 6, unless noted otherwise. [b] Calculated from the intercept effect obtained when L- and DL-malate were compared.

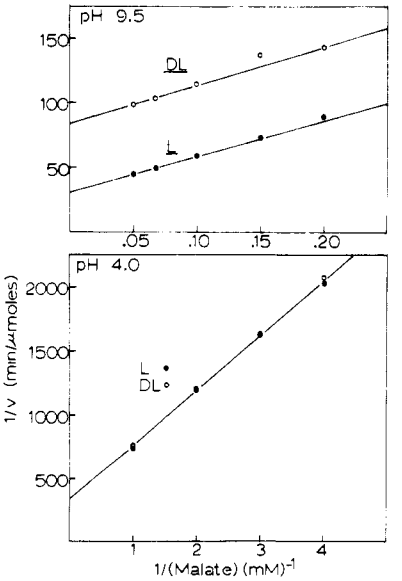



FIGURE 4: Comparison of initial velocities for pigeon liver malic enzyme by using L-malate and DL-malate at 25 °C. Conditions were the following: (top) pH 9.5, 0.005 unit/mL malic enzyme, 1 mg/mL bovine serum albumin, 2 mM $MgSO_4$, 1 mM TPN, and 0.1 mM dithiothreitol; (bottom) pH 4.0, 0.02 unit/mL malic enzyme, 1 mg/mL bovine serum albumin, 2 mM $MgSO_4$, 1 mM TPN, and 0.1 mM dithiothreitol. Reciprocal substrate concentrations are in terms of L-malate concentrations. All velocities are in μmol/min.

## Discussion

*Yeast Alcohol Dehydrogenase.* The magnitude of the isotope effect on $V$ for 2-propanol indicates that hydride transfer is to a large extent rate determining at pH values below 8. This is similar to the findings of Klinman (1975, 1976) for the

oxidation of benzyl alcohols and the reduction of substituted benzaldehydes (Klinman, 1972, 1975), although the isotope effects were somewhat larger for the aromatic substrates. At saturating DPN concentration, the kinetic mechanism can be considered ordered:

$$\mathrm{EA} \underset{k_2}{\overset{k_1\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} \mathrm{EAB'} \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} \mathrm{EPQ'} \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} \mathrm{EPQ} \xrightarrow{k_{17}} \mathrm{EQ} \xrightarrow{k_9} \mathrm{E} \quad (15)$$

where A, B, P, and Q are DPN, 2-propanol, acetone, and DPNH, and the rate constants are similar to those used in eq 9 and 10, with $k_{13}$ and $k_{14}$ being the isotope-dependent steps.

At pH 6, which is well below the p$K$s obtained from the $V$ and $V/K$ profiles, ${}^{D}V_{\text{2-propanol}}$, ${}^{D}(V/K_{\text{2-propanol}})$, and ${}^{D}(\text{Eq.P.})_{\text{2-propanol}}$ are equal within experimental error as predicted by the theory for case I in Table I, with the average value of 3.75 corresponding to ${}^{D}k_3$ in eq 12. Because acetone is such a loosely bound substrate, it is likely that $k_{17} > k_{16}$, and it is also probable that $k_{15} > k_{14}$ [see the discussion concerning the mechanism in the following paper (Cook & Cleland, 1981b)]. If this is true, eq 12 reduces to

$$3.75 = \frac{5.7 + k_{13}/k_{12}}{1 + k_{13}/k_{12}} \quad (16)$$

where 5.7 is the intrinsic isotope effect determined by Northrop's method.[5] Equation 16 yields $k_{13}/k_{12} = 0.71$.

At pH 8.2, if eq 11 and 12 are substituted into the equation for ${}^{D}(V/K)$ for case I in Table I (assuming again that $k_{17} \gg k_{16}$; $k_{15} \gg k_{14}$), we get

$${}^{D}(V/K_{\text{2-propanol}}) = \frac{{}^{D}k_{13} + (k_{13}/k_{12})(1 + k_{11}/k_2)}{1 + (k_{13}/k_{12})(1 + k_{11}/k_2)} \quad (17)$$

With 2.7 as ${}^{D}(V/K_{\text{2-propanol}})$, 5.7 as ${}^{D}k_{13}$, and 0.71 as $k_{13}/k_{12}$, eq 17 gives $k_{11}/k_2 = 1.49$. If the simple equation from Table I is used in place of eq 17 with 3.75 from the pH 6 experiments as ${}^{D}k_3$, the value of $k_3/k_2$ is 0.62. 2-Propanol is thus a somewhat sticky substrate, being released from the enzyme just 60% faster than the entire catalytic sequence represented by $k_3$.

The comparable equation for ${}^{D}V$ at pH 8.2 from Table I is

$${}^{D}V = \frac{{}^{D}k_3 + k_3/k_9}{1 + k_3/k_9} \quad (18)$$

and with 2.96 as ${}^{D}V$, and 3.75 from the pH 6 experiments as ${}^{D}k_3$, eq 18 gives 0.40 for $k_3/k_9$. Thus, DPNH is released 2.5 times faster than the entire catalytic sequence represented by $k_3$. We can roughly confirm this value by considering that $V/E_t$ for 2-propanol is 0.39 that for ethanol, where DPNH release is believed to be totally rate limiting. We thus have

$$(V/E_t)_{\text{2-propanol}} = \frac{k_3k_9}{k_3 + k_9} = 0.39[(V/E_t)_{\text{ethanol}} = k_9] \quad (19)$$

from which $k_3/k_9 = 0.64$, in fair agreement with the value from eq 18.

The apparent rate constant $k_3$ in eq 18 and 19 can be expanded in terms of the rate constants in eq 15 by using eq 11 modified as described under Theory. For the assumptions $k_{17} \gg k_{16}$ and $k_{15} \gg k_{11}$, $k_{14}$, we get

$$k_3/k_9 = \frac{k_{11}k_{13}}{k_9(k_{11} + k_{12} + k_{13})} = 0.4 \quad (20)$$

from which, with $k_{13}/k_{12} = 0.71$, we get

$$2.5/k_9 = 1/k_{13} + 2.41/k_{11} \quad (21)$$

Equation 21 allows both $k_9$ and $k_{11}$ to be much smaller than $k_{13}$, in which case they must be nearly equal. When $k_{11} \gg k_{13}$, however, $k_9$ has an upper limit of $2.5k_{13}$. If $k_{11} = k_{13}$, $k_9 = 0.7k_{13}$, while if $k_9 = k_{13}$, $k_{11} = 1.6k_{13}$.

In summary, the increase in the $V$ and $V/K$ isotope effects for yeast alcohol dehydrogenase as the pH is decreased from 8.2 to below the p$K$s in the pH profiles appears fully consistent with the predictions shown for case I in Table I and allows us to conclude that 2-propanol is released from the enzyme at 0.67 the rate of the first pH-dependent but not isotope-dependent step in the mechanism, and that hydride transfer to DPN occurs with 0.64 the rate of the reversal of the first step. While the exact rate constants for hydride transfer and the step that precedes it cannot be determined, limits can be set for the ratios of all of these rate constants and the rate of release of DPNH from the enzyme.

*Isocitrate Dehydrogenase.* The pH variation of the isotope effects on $V$ and $V/K$ indicates that isocitrate can bind to either the protonated or the unprotonated form of the enzyme, but only the unprotonated ternary complex can react to give products. Even at the pH extremes, the isotope effects on both parameters are small, which suggests that slowing catalysis does not make hydride transfer totally rate limiting.

At pH 7.45, no significant isotope effects are observed on either $V$ or $V/K$ [previous reports by Ramachandran et al. (1974) and O'Leary & Limburg (1977) of inverse $V/K$ effects at neutral pH were clearly caused by experimental errors]. Thus, TPNH release must completely limit the maximum velocity, as suggested by Uhr et al. (1974), while isocitrate must have a high commitment for there to be no $V/K$ effect. The slow step cannot be accounted for by $c_{CO_2}$, the reverse commitment for $CO_2$, which is present in the equations for both ${}^{D}V_{\text{isocitrate}}$ and ${}^{D}(V/K_{\text{isocitrate}})$, since both isotope effects would be at least equal to the equilibrium isotope effect of 1.17 (Cook et al., 1980):

$${}^{D}(V/K_{\text{isocitrate}}) = \frac{{}^{D}k + c_{\text{isocitrate}} + {}^{D}K_{\text{eq}}(c_{CO_2})}{1 + c_{\text{isocitrate}} + c_{CO_2}} \quad (22)$$

$${}^{D}V_{\text{isocitrate}} = \frac{{}^{D}k + c_{V\,\text{isocitrate}} + c_{CO_2}}{1 + c_{V\,\text{isocitrate}} + c_{CO_2}} \quad (23)$$

It is thus clear that $c_{\text{isocitrate}}$ is the large term for ${}^{D}(V/K_{\text{isocitrate}})$, and $c_{V\,\text{isocitrate}}$ for ${}^{D}V_{\text{isocitrate}}$.

The preferred pathway for isocitrate dehydrogenase involves the ordered release of $CO_2$, $\alpha$-ketoglutarate, and TPNH (Uhr et al., 1974). Since TPN was maintained at a saturating level in these studies, the mechanism can be written

$$\mathrm{EA} \underset{k_2}{\overset{k_1\mathrm{B}}{\rightleftharpoons}} \mathrm{EAB} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{EPQR} \xrightarrow{k_5} \mathrm{EQR} \xrightarrow{k_7} \mathrm{ER} \xrightarrow{k_9} \mathrm{E} \quad (24)$$

where A, B, P, Q, and R are TPN, isocitrate, $CO_2$, $\alpha$-ketoglutarate, and TPNH. In the above mechanism, $k_3$ and $k_4$ are rate constants for the isotope-dependent step; $c_{CO_2} = k_4/k_5$, $c_{\text{isocitrate}} = k_3/k_2$, and $c_{V\,\text{isocitrate}} = k_3(1/k_5 + 1/k_7 + 1/k_9)$. Since it has been shown previously (Uhr et al., 1974) that $CO_2$ and $\alpha$-ketoglutarate are loosely bound, the commitments for ${}^{D}(V/K)$ and ${}^{D}V$ in this simple model reduce to $k_3/k_2$ and $k_3/k_9$, respectively. Thus, as stated above, isocitrate is sticky (that is, reacts to give products more rapidly than it dissociates from the enzyme), and the TPNH off rate is slow with respect to the chemical reaction on the enzyme.

The observation of an equilibrium perturbation isotope effect at pH 8 at first appears to contradict the lack of a $V/K$ effect obtained by the direct comparison method. Since the per-

turbants are isocitrate and TPNH, however, eq 24 must be rewritten as

$$EA \underset{k_2}{\overset{k_1B}{\rightleftharpoons}} EAB \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EPQR \underset{k_6P}{\overset{k_5}{\rightleftharpoons}} EQR \underset{k_8Q}{\overset{k_7}{\rightleftharpoons}} ER \underset{k_{10}R}{\overset{k_9}{\rightleftharpoons}} E \quad (25)$$

where A, B, P, Q, and R have the same meaning as above. For this scheme, the isotope effect from the isocitrate side is given by eq 22 in which $c_{isocitrate}$ is still $k_3/k_2$, but $c_{CO_2}$, the reverse commitment factor, is replaced by

$$c_{TPNH} = \frac{k_4}{k_5}\left[1 + \frac{k_6P}{k_7}\left(1 + \frac{k_8Q}{k_9}\right)\right] \quad (26)$$

Thus, if $k_9 \ll k_2 \ll k_3$ and the concentrations of P and Q are fairly high, $c_{TPNH}$ will be much larger than $c_{isocitrate}$, and the observed isotope effect will become $^DK_{eq}$, the equilibrium isotope effect of 1.17. The isotope effect of 1.15 obtained by equilibrium perturbation is within experimental error of this value. O'Leary & Limburg (1977) have reached similar conclusions concerning the mechanism of isocitrate dehydrogenase.

The small deuterium isotope effects seen at high and low pHs on both $V$ and $V/K$ show that some step other than hydride transfer is largely rate limiting for catalysis. For the closely related malic enzyme, the slow step is decarboxylation, and a large $^{13}C$ isotope effect of 1.031 together with the $^D(V/K_{malate})$ of 1.5 allowed Schimerlik et al. (1977) to conclude that reverse hydride transfer was 6–8 times faster than decarboxylation. With isocitrate dehydrogenase, O'Leary & Limburg (1977) found no $^{13}C$ isotope effect, but this was certainly because of the large value of $c_{isocitrate}$, rather than because decarboxylation is not slow. Until the $^{13}C$ isotope effect is measured at very high or very low pH, we cannot say whether the decarboxylation step, or some other step between addition of isocitrate and release of $CO_2$, is the one which is slower than hydride transfer.

*Malic Enzyme.* Malic enzymes from pigeon and chicken livers are kinetically very similar, as judged by the pH variation of isotope effects, $K_{malate}$ values, and $K_i$ values for D-malate. The $K_i$ for D-malate is relatively pH independent over the pH range 7–10, while the $K_m$ for L-malate increases over this range such that at pH 9.5 $K_{malate}$ is 4.5 mM for the pigeon liver enzyme, and the $K_i$ for D-malate is about 5 mM. The equation for a competitive inhibitor present as a contaminant in the variable substrate is

$$v = \frac{VA}{K + A + (AKr/K_i)} \quad (27)$$

where $r = I/A$ ($I$ is inhibitor concentration, and $A$ is substrate concentration), $K_i$ is the inhibitor constant for the inhibitor, and $K$ is the Michaelis constant. In a DL mixture where $K \simeq K_i$, eq 27 reduces to

$$v = \frac{VA}{K + 2A} \quad (28)$$

so a difference in the intercepts (that is, apparent $V$ values) of a factor of about 2 should be, and is, seen when L- and DL-malate are compared at pH 9.5. At pH 4, however, $K_i \simeq 40K$ so that the ratio of intercepts should be about 1.04. Thus, the value of 3.0 obtained for $^DV_{malate}$ by Schimerlik et al. (1977) at pH 10.0 could have been due to inhibition by D-malate,[6] while at pH 4.0, the observed values of 2–3 must have been caused by experimental error.

[6] The ratio of intercepts caused by the isotope effect was 1.5, and that by the presence of inhibitory D-malate was 2, so the overall effect was $2 \times 1.5 = 3$.

Since $^D(V/K_{malate})$ is pH independent, it appears that the external commitment for malate is close to zero over the entire pH range, and the $V/K$ isotope effect is therefore decreased to 1.5 by the reverse commitment, which Schimerlik et al. (1977) have calculated to result from the reverse hydride transfer being 6–8 times faster than decarboxylation and subsequent $CO_2$ release. At neutral pH, $c_{V\,malate}$ is large because TPNH is much slower than the chemical reaction, but when the pH is raised or lowered sufficiently to make the chemical reaction slower than TPNH release, the $c_{V\,malate}$ term in $^DV_{malate}$ will decrease to zero, and $^DV_{malate}$ will then also be limited by the same reverse commitment as $^D(V/K_{malate})$.

*Conclusions.* The enzymes discussed in this paper (except for yeast alcohol dehydrogenase above pH 8, which will be discussed in the following paper) all give results in close agreement with the predictions of a model where the hydride-transfer step is pH dependent. Thus, $^DV$ and $^D(V/K)$ become equal at low and high pHs beyond the point where the corresponding $V$ and $V/K$ profiles begin to decrease and the pathway including the hydride-transfer step has become rate limiting. This pattern of pH variation for the isotope effects can thus be considered diagnostic for such a model, and we may expect to see such results for a variety of dehydrogenases where the state of protonation of the auxiliary catalytic group is critical for the hydride-transfer process. In the following paper, we will describe the very interesting case where the hydride-transfer step is not dependent on correct protonation of the auxiliary catalytic group, and show the greatly different pH profiles for $^DV$ and $^D(V/K)$ which result.

## Acknowledgments

We thank Drs. William P. Jencks and Marion H. O'Leary for their very useful comments on the manuscript.

## References

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.
Cleland, W. W., Gross, M., & Folk, J. E. (1973) *J. Biol. Chem. 248*, 6541.
Colman, R. F., & Chu, R. (1969) *Biochem. Biophys. Res. Commun. 34*, 528.
Cook, P. F., & Cleland, W. W. (1981a) *Biochemistry* (first of four papers in this issue).
Cook, P. F., & Cleland, W. W. (1981b) *Biochemistry* (third of four papers in this issue).
Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4853.
Cook, P. F., Kenyon, G. L., & Cleland, W. W. (1981) *Biochemistry 20*, 1204.
Dickenson, D. J., & Dickinson, F. M. (1975) *Biochem. J. 147*, 541.
Eigen, M. (1964) *Angew. Chem., Int. Ed. Engl. 3*, 1.
Hsu, R. Y., & Lardy, H. A. (1967) *J. Biol. Chem. 242*, 520.
Klinman, J. P. (1972) *J. Biol. Chem. 247*, 7977.
Klinman, J. P. (1975) *J. Biol. Chem. 250*, 2569.
Klinman, J. P. (1976) *Biochemistry 15*, 2018.
Northrop, D. B. (1975) *Biochemistry 14*, 2644.
Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122–148, University Park Press, Baltimore, MD.
O'Leary, M. H., & Limburg, J. A. (1977) *Biochemistry 16*, 1129.
Ramachandran, N., Durbano, M., & Colman, R. F. (1974)

*FEBS Lett. 49*, 129.
Schimerlik, M. I., & Cleland, W. W. (1977) *Biochemistry 16*, 576.
Schimerlik, M. I., Rife, J. E., & Cleland, W. W. (1975) *Biochemistry 14*, 5347.
Schimerlik, M. I., Grimshaw, C. E., & Cleland, W. W. (1977) *Biochemistry 16*, 571.
Sund, H., & Theorell, H. (1963) *Enzymes, 2nd Ed. 7*, 25.
Uhr, M. L., Thompson, V. W., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2920.
Viola, R. E., Cook, P. F., & Cleland, W. W. (1979) *Anal. Biochem. 96*, 334.

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

Paul F. Cook‡ and W. W. Cleland*

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 p$K$ 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 p$K$s of 6.2 and 7.1, and above p$K$s of 9.5 and 10.3. $pK_{i\ trifluoroethanol}$ decreases below a p$K$ of 7.2, and above p$K$ of 10.1, while $pK_{i\ isobutyramide}$ drops above a p$K$ of 10.0. $V_{cyclohexanone}$ decreases above a p$K$ of 8.4 while $V/K_{cyclohexanone}$ decreases above p$K$s of 8.8 and 9.7. Isotope effects on $V/K_{cyclohexanol}$ and $V/K_{cyclohexanone}$ decrease above identical p$K$s 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

† From the Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706. *Received January 14, 1980; revised manuscript received October 24, 1980.* This work was supported by a National Institutes of Health postdoctoral fellowship (IF 32GM 05940-01) to P.F.C. and grants from the National Institutes of Health (GM 18938) and the National Science Foundation (BMS 16134) to W.W.C.

‡ Present address: Department of Biochemistry, Louisiana State University Medical Center, New Orleans, LA 70112.

0006-2960/81/0420-1805$01.25/0 