# Solvent Isotope Effects on the Reaction Catalyzed by Alcohol Dehydrogenase from Equine Liver†

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ABSTRACT: The pH profiles for the steady-state kinetic parameters in the reaction catalyzed by alcohol dehydrogenase from horse liver were determined in $H_2O$ and $D_2O$. The pH-independent solvent isotope effect on the kinetic parameters and the solvent isotope effects on dissociation constants for several substrates and substrate analogues were measured. In addition the parameters ($V_{cyclohexanol}$ and $V_{cyclohexanone}$) with the largest solvent isotope effects (3.7 and 3.8, respectively) were determined in mixtures of $H_2O$ and $D_2O$ (proton inventory experiments). The results are interpreted in terms of rate-determining coenzyme dissociation and a conformation change, respectively, at saturating substrate concentrations. The pH-dependent solvent isotope effect, determined from the change in observed p$K$ in the isotopic solvent, is interpreted as the solvent isotope effect on the intrinsic p$K$, the solvent isotope effect on the pH-dependent process, or both. The pH-dependent solvent isotope effects associated with the $V/K_{cyclohexanol}$ and the $V/K_{cyclohexanone}$ are interpreted in terms of a constellation of features associated with the zinc-bound water and the zinc-bound alcohol.

Alcohol dehydrogenase (EC 1.1.1.1) from equine liver catalyzes the reversible oxidation of cyclohexanol (Merritt & Tompkins, 1959). Efforts to characterize the intermediates and transition states in this reaction have been carried out with investigations of steady-state kinetics (Dalziel & Dickenson, 1966), isotopic exchange at equilibrium (Ainslie & Cleland, 1972), pH effects (Cook & Cleland, 1981b), primary covalent isotope effects (Cook & Cleland, 1981a,b), and secondary covalent isotope effects (Cook et al., 1981). Results of investigations of the solvent isotope effects on the steady-state parameters for this reaction are reported in the present paper.

The investigation of solvent isotope effects on enzymatic reactions is beset by unique experimental and theoretical problems. One experimental problem arises from the fact that there is a deuterium isotope effect on the p$K$s of dissociable groups and these must be separated from the effects on the rate constants. For example, the inverse solvent isotope effect reported by Schmidt et al. (1979) on the slow transient in the reduction of aromatic aldehyde by liver alcohol dehydrogenase was observed at a pH above the p$K$ of an inactive conjugate base of one of the enzyme intermediates. Therefore, it is difficult to tell whether the observed isotope effect is due to the isotope effect on the p$K$, an isotope effect on a rate constant, or both. For this reason it is necessary to compare the complete pH profile of the parameters with the pD profile.

The theoretical problem arising from the fact that multiple protons may contribute to the solvent isotope effect in enzyme-catalyzed reactions may be approached by proton inventory experiments in which the solvent isotope effect is related to the mole fraction of exchangeable deuterium in the reaction mixture (Schowen, 1978), as a means for estimation of the number of protons involved. O'Leary et al. (1981) have employed this method effectively to estimate the number of protons involved in the reaction catalyzed by glutamate decarboxylase.

Another theoretical problem arises from the fact that enzymatic reactions are made up of multiple steps, binding steps, conformation changes, catalytic steps, and product release steps, and the observed solvent isotope effect may originate in any one or more of them. In the present study these problems are dealt with by a comparison of the solvent isotope effects and the pH effects as well as by a comparison of the solvent isotope effects with the primary, covalent isotope effects (Cook & Cleland, 1981a,b).

## Materials and Methods

*Materials.* Alcohol dehydrogenase (EC 1.1.1.1) from equine liver was purchased from Sigma Chemical Co., and deuterium oxide (99.8 atom%) was purchased from Aldrich Chemical Co. Cyclohexanol and cyclohexanone (Eastman) were redistilled before use. All other chemicals were from commercial suppliers.

*Initial Velocity Measurements.* The initial velocity of the reaction was determined by measurements of the change in the $A_{340}$ of the reaction mixture with a Beckman monochromator connected to a Gilford optical density converter. The results were displayed on a strip chart recorder. Each cuvette contained, in 1.0 mL, buffer (0.05 M) and either cyclohexanol plus NAD or cyclohexanone plus NADH. For the experiments in $H_2O$ the buffer was 2-(*N*-morpholino)ethanesulfonic acid (Mes) for the pH range 5–6.8, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) for the pH range 6.7–7.4, *N*-(2-hydroxyethyl)piperazine-*N*′-2-propanesulfonic acid (HEPPS) for the pH range 7.8–8.3, or 2-(*N*-cyclohexylamino)ethanesulfonic acid (Ches) for the pH range 8.2–10.0.

Over the experimental pH range the cyclohexanol concentration was 0.072–36 mM as the variable substrate and 9.6–130 mM as the constant substrate; NAD was 0.0066–3.3 mM as the variable substrate and 4.9–7.3 mM as the constant substrate. Cyclohexanone concentration was 6.4–160 mM as the variable substrate, and NADH was 0.2 mM. Over the pH range 7.0–8.5 the cyclohexanol concentration was less than 10 mM, the concentration above which substrate activation and inhibition were shown by Dalziel & Dickenson (1966). Furthermore, the linearity of the double-reciprocal plots at all pH values substantiates the absence of significant substrate activation under these conditions.

The reaction was initiated by the addition of 0.004–0.03 unit of alcohol dehydrogenase. The nonenzymatic rate of change in the $A_{340}$ was, in all cases, less than 1% of the enzymatic rate. After sufficient data had been collected (less than 10 min), the pH of the reaction mixture was measured, and initial velocity was determined from the chart.

The experiments in $D_2O$ were performed in the same way except that all solutions were made up in that solvent. The

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Table I: Solvent Isotope Effects on the Reaction Catalyzed by Liver Alcohol Dehydrogenase

| parameter | isotope effect,[d] pH independent | apparent $pK_{H_2O}$ | apparent $pK_{D_2O}$ | $\Delta pK$ |
|---|---|---|---|---|
| $V_{cyclohexanol}$[a] | 3.72 ± 0.08[b] | | | |
| $V/K_{cyclohexanol}$ | 1.75 ± 0.15 | 6.98 ± 0.03 | 8.24 ± 0.05 | 1.26 ± 0.07 |
| | | 9.45 ± 0.04 | 10.84 ± 0.17 | 1.39 ± 0.21 |
| $V/K_{NAD}$ | 1.30 ± 0.19 | 6.89 ± 0.04 | 7.89 ± 0.09 | 1.00 ± 0.1 |
| | | 9.48 ± 0.07 | 9.89 ± 0.1 | 0.41 ± 0.2 |
| $V_{cyclohexanone}$ | 3.78 ± 0.13 | 8.58 ± 0.06 | 9.62 ± 0.001 | 1.04 ± 0.06 |
| $V/K_{cyclohexanone}$ | 1.16 ± 0.03 | 8.60 ± 0.0003 | 9.60 ± 0.0001 | 1.00 ± 0.0004 |
| | | 8.82 ± 4.0 | 9.12 ± 2.5 | *c* |
| $1/K_{i,trifluoroethanol}$ | 0.58 ± 0.09 | | | |
| $1/K_{i,isobutyramide}$ | 0.64 ± 0.10 | | | |
| $1/K_{i,NADH}$ | 0.80 ± 0.12 | | | |

[a] Because of the complex nature of the profile the isotope effect is calculated from the points at pH 9 instead of the horizontal asymptotes, and the values for p$K$ are not given. [b] Errors are standard error. [c] Because of the large standard error, the difference is not calculated. [d] The isotope effect is the ratio of the pH independent parameter value in $H_2O$ to that in $D_2O$.

enzyme was equilibrated at least 18 h at 4 °C in $D_2O$ before use, and the buffer ranges were approximately 0.5 pD unit higher than the corresponding pH ranges. The pD was measured with a pH electrode that was equilibrated for at least 1 h in $D_2O$, and compensation was made for the isotope effect on the pH electrode by the addition of 0.4 to the meter reading (Schowen, 1978). With each set of stock solutions of enzyme and substrate the parameters were determined at a reference pH, and the parameters were determined in $H_2O$ with enzyme equilibrated in $D_2O$. The difference in the $H_2O$ parameters determined with enzyme equilibrated in the two different solvents was within experimental error.

The dissociation constants for trifluoroethanol, isobutyramide, and NADH were measured as competitive inhibitors vs. cyclohexanol, cyclohexanone, and NAD, respectively. The initial velocity was determined as a function of substrate concentration with no inhibitor and as a function of inhibitor concentration at constant substrate concentration (approximately equal to its $K_m$). The pH (pD) for each measurement was on the pH-independent portion of the $V/K$ profile for that particular substrate. The experiments with NADH as an inhibitor were performed with the cyclohexanol concentration at half of its $K_m$.

*Data Processing.* The data for initial velocity and substrate concentration were fitted to eq 1, and the values for $V$ and $V/K$ at different pHs (pDs) were fitted to eq 2 or 3 with the computer programs of Cleland (1979). The profiles for $V_{cyclohexanol}$ and $V/K_{cyclohexanone}$ were fitted to eq 4 or eq 5 with a generalized nonlinear regression program (SAS Institute Inc., Cary, NC). Convergence of the nonlinear regression programs was achieved when the sum of the square of the residuals changed by less than $10^{-6}$% in successive iterations.

The data from the inhibition experiments were fitted to eq 6 with the generalized, nonlinear regression program indicated above. The symbols in eq 1–6 are the following: $v$, initial velocity; $V$, maximum velocity; $A$, substrate concentration; $K$, Michaelis constant; $y$, either $V$ or $V/K$; $C$ and $C'$, pH-independent value of the parameter; $K_1$ and $K_2$, proton dissociation constants; $I$, inhibitor concentration; $K_I$, inhibitor dissociation constant.

$$v = VA/(K + A) \quad (1)$$

$$\log y = \log [C/(1 + K_2/H)] \quad (2)$$

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \quad (3)$$

$$y = [C/(1 + K_2/H + K_2K_2'/H^2)] \quad (4)$$

$$y = [C/[1 + H/K_1 + C'/(1 + K_2/H)]] \quad (5)$$

$$v = VA/(A + K + KI/K_I) \quad (6)$$

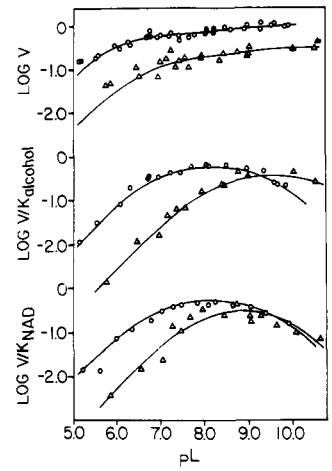


FIGURE 1: pH (circles) and pD (triangles) profiles for the steady-state parameters in the oxidation of cyclohexanol catalyzed by alcohol dehydrogenase. The $V$ profile was fitted to eq 5 whereas both of the $V/K$ profiles were fitted to eq 3.

*Measurement of $D_2O$.* The mole fraction of D in $H_2O$–$D_2O$ mixtures was estimated for the proton inventory experiments in an infrared spectrophotometer by measurement of the absorbance at 3.0 μm (H–O stretch) and at 4.0 μm (D–O stretch) of a thin film (0.001 mL) between CaF plates. The mole fraction of D was determined by interpolation of the ratio of the two absorbances on a standard curve constructed the same way with samples that were a mixture of known amounts of $D_2O$ (99.8 atom %) and $H_2O$.

## Results

The pH profiles for the steady-state kinetic parameters in the oxidation of cyclohexanol in $H_2O$ and the pD profiles for the same parameters in the reaction in $D_2O$ are shown in Figure 1. The analogous profiles for the reverse reaction are shown in Figure 2. The solvent isotope effect in the pH-independent region of each curve and the isotope effects on the p$K$s are presented in Table I. Significant normal solvent isotope effects are seen on $V_{cyclohexanol}$, $V/K_{cyclohexanol}$, and $V_{cyclohexanone}$. In addition the solvent isotope effects were determined on the binding constants for trifluoroethanol and isobutyramide, as competitive inhibitors, and for NADH, as a product inhibitor (Table I). The binding equilibrium of trifluoroethanol and of isobutyramide has significant inverse solvent isotope effects, whereas the effect on the binding of NADH is questionably significant.

In an effort to determine the number of protons involved in the apparent solvent isotope effect on $V_{cyclohexanol}$ and

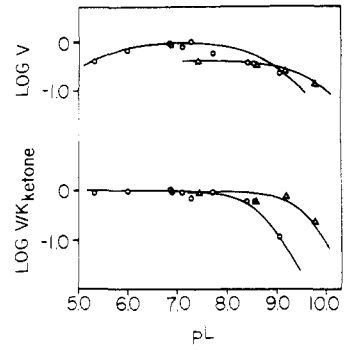


FIGURE 2: pH (circles) and pD (triangles) profiles for the steady-state parameters in the reduction of cyclohexanone catalyzed by alcohol dehydrogenase. The $V$ profile in $H_2O$ was fitted to eq 3, whereas the $V$ profile in $D_2O$ was fitted to eq 2. The $V/K$ profiles were fitted to eq 4.

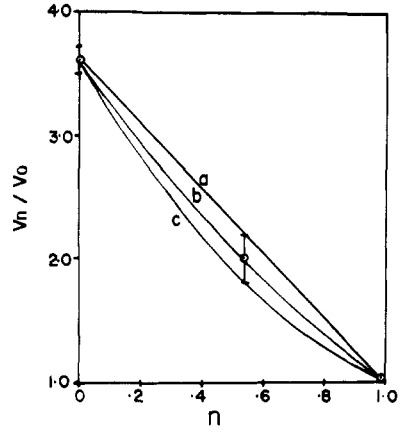


FIGURE 3: Solvent isotope effect on $V_{cyclohexanol}$ as a function of the mole fraction of deuterium ($n$). (a) Theoretical curve for a reaction involving a single proton; (b) theoretical curve for a reaction involving two protons; (c) theoretical curve for a reaction involving an infinite number of protons each having a partition factor very near unity. Open circles represent the experimental points, and the error bars are standard error.

$V_{cyclohexanone}$, they were determined in approximately in 0.5 mole fraction of $D_2O$ (pH 9 and pH 7.2, respectively), and the corresponding data are shown on the diagrams in Figure 3, where curve a relates the mole fraction of deuterium in the solvent to the magnitude of the proton inventory parameter (Schowen, 1978) for a single proton in the transition state, curve b relates the same quantities for a reaction involving two protons, and curve c relates the same quantities for a reaction involving an infinite number of protons. It can be seen from Figure 3 that the transition state (or states) that determine the value of $V_{cyclohexanol}$ involves bonding changes in a number of protons greater than one but less than infinity, whereas that (those) for the $V_{cyclohexanone}$ involves an infinite number of protons (Figure 4).

## Discussion

The interpretation of solvent isotope effects on enzyme-catalyzed reactions is complicated by two principal problems, the possible multiplicity of rate-determining steps and the possible multiplicity of protons involved in a single intermediate or transition state. The latter difficulty can be approached with proton inventory experiments (Schowen, 1978), in which the number of protons involved can be estimated, whereas the former difficulty could heretofore be approached quantitatively only with methods in which the rate of individual steps can be measured, e.g., transient state kinetics and isotope trapping. The attempt will be made here to deal with the possible

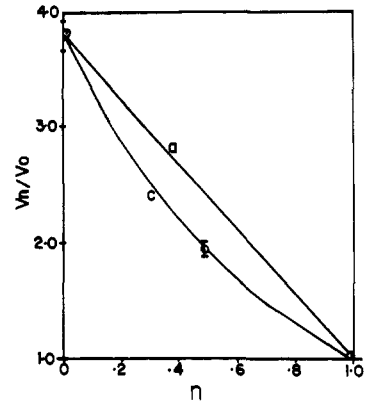


FIGURE 4: Solvent isotope effect on $V_{cyclohexanone}$ as a function of the mole fraction of deuterium ($n$). (a) Theoretical curve for a reaction involving a single proton; (c) theoretical curve for a reaction involving an infinite number of protons each having a partition factor very near unity. Open circles represent the experimental points, and the error bars are standard error.

multiplicity of rate-determining steps from consideration of a combination of effects on the steady-state kinetic parameters, e.g., solvent isotope effects, primary covalent isotope effects, and pH effects.

The catalytic cycle consists of an overall sequential arrangement of reaction steps between intermediates. In the case of a relatively slow step preceded by one or more contiguous, reversible steps the critical energy transition for the rate of the reaction may be from one of the intermediates in the reversible sequence to the transition state of the slow step. So that such a transition could be distuinguished from those of each individual step, the former is called a "process", which may consist of a single step or several sequential steps. However, if the process consists of more than one step, the penultimate and preceding steps (if any) must be reversible.

Experimentally the value of $V$ and $V/K$ is the rate of the reaction with saturating substrate concentrations ($V$ conditions) and the normalized rate (normalized to substrate concentration) as the substrate concentration approaches zero ($V/K$ conditions), respectively, and these rates are determined by somewhat different processes. For a simple reaction, in which one process is slower than the others (one energy transition greater than the others), the value of the appropriate parameter ($V$ or $V/K$) is determined by a single process. However, in a more generalized and realistic reaction the experimental value for a given kinetic parameter may be partially determined by the rate of each of several processes. The concept of a partially rate-determining process has been instrumental in the interpretation of primary, covalent isotope effects, and equations have been published to deal with this fact (Northrop, 1975; Northrop, 1977; Cook & Cleland, 1981a; Albery & Knowles, 1976). Furthermore, these several rate-determining processes may be at least partially overlapping, since a sequence of reversible steps may have more than one slow forward step.

The observed values for the p$K$s from the profiles for the steady-state kinetic parameters are determined by two factors: (1) the intrinsic p$K$s of important acidic or basic groups (proton donor or proton acceptor, respectively) on some intermediate in the catalytic cycle and (2) the extent to which the forward processes away from that intermediate determine the value of that particular parameter at the pH-independent region of the profile. If the pH-dependent processes exclusively determine the parameter value, the observed p$K$ will be the same as the intrinsic p$K$. If not, the observed p$K$s will be displaced toward the pH extremes relative to the intrinsic p$K$s, since the

pH-dependent processes must be made rate-determining, under the conditions for that particular parameter, in order for the inflection to be observed.

Since in $D_2O$ the p$K$ of the great majority of acids will increase approximately 0.5 pH units (Schowen, 1978) due to the solvent isotope effect on their dissociation constants, the intrinsic p$K$s for the kinetic profiles are expected to increase by the same amount. Therefore, any deviation from this expectation is interpreted in one of three ways: (1) as an abnormal solvent isotope effect on the dissociation constant, (2) as a change in the extent to which the pH-sensitive process determines the experimental value of the kinetic parameter, or (3) as a combination of both. For example if the pH-sensitive process exclusively determines the parameter value at the pH-independent region of the profile, any deviation of the log of the solvent isotope effect on the apparent dissociation constants ($pK_{D_2O}$–$pK_{H_2O}$) from +0.5 would be caused by an abnormal solvent isotope effect on the intrinsic p$K$. However, if the pH-dependent process is not rate determining at the pH-independent region, the log of the solvent isotope effect on the apparent dissociation constants will depend upon the relative magnitude of the intrinsic, solvent isotope effects on the pH-dependent processes to that on the other significant processes, as well as upon the solvent isotope effect on the intrinsic p$K$s. Then, the three possible cases that can be distinguished are those in which the intrinsic, solvent isotope effect on the pH-dependent processes is the same as, greater than, or less than the effect on the other significant processes. When the pH-dependent and the other significant processes have the same intrinsic solvent isotope effect, the log of the solvent isotope effect on the apparent p$K$s should be +0.5 in the absence of an abnormal effect on the intrinsic p$K$s. Alternatively, when the pH-dependent processes have a greater normal intrinsic, solvent isotope effect than the other significant processes, then the apparent p$K$ in $D_2O$ will tend to approach the intrinsic p$K$ in $D_2O$, because a pH-dependent process becomes more rate determining in the latter solvent. Thus, the log of the solvent isotope effect on the apparent p$K$s will be greater than +0.5 for an essential basic group and less than +0.5 for an essential acidic group. Finally, when the pH-dependent processes have a lower normal, intrinsic isotope effect than the other significant processes, the apparent p$K$ in $D_2O$ will be displaced further toward the pH extremes relative to the intrinsic p$K$ in $D_2O$. Thus, the log of the solvent isotope effect on the apparent p$K$s will be less than +0.5 for an essential basic group and greater than +0.5 for an essential acidic group, the opposite effect from that in the previous case.

The experimental pH (pD) profiles for $V_{cyclohexanol}$ decreases with a rather shallow slope (less than 1.0) as the pH decreases. Since the experimental error is rather modest compared with the magnitude of the observed decrease, a mechanistic explanation must be considered. Dalziel (1963) reported a very similar profile for $V_{ethanol}$ with the same enzyme and attributed it to the effects of multiple basic groups whose conjugate acids resulted in somewhat slower dissociation of NADH. Equation 5 was derived from a mechanism embodying two basic groups, but more may actually exist. The parallel nature of the curves indicates that the pH-sensitive and the isotopically sensitive processes are the same and determine the parameter value over the range of the pH profile. Futhermore, the results of the proton inventory experiments indicate that a moderate number of protons are involved in the critical transition state. Since $V$ is not sensitive to the substrate-binding process per se (although it is sensitive to any preceding and subsequent conformation changes) and since the small primary covalent isotope effect on this parameter (Cook & Cleland, 1981a,b) indicates that hydride transfer is not determining, the critical process for $V_{cyclohexanol}$ must involve a conformation change, product release, or both. The similarity of the $V_{cyclohexanol}$ profile to the pH profile for the rate of dissociation of NADH from liver alcohol dehydrogenase, derived from the data of Kvassman & Pettersson (1979), and to that of DeTraglia et al. (1977) suggests that the rate-limiting process for the steady-state rate at saturating substrate concentration is the same. This hypothesis is consistent with previous hypotheses by Dalziel & Dickenson (1966) and by Ainslie & Cleland (1972) to explain the activation by high concentrations of cyclohexanol and with that by Cook & Cleland (1981b) to explain the small primary covalent isotope effect on $V_{cyclohexanol}$. The small normal solvent isotope effect on the dissociation rate constant of NADH measured by Schmidt et al. (1979) with transient-state methods suggests that this rate and the steady-state rate at saturating substrate concentration are determined by somewhat different processes, even though they may be controlled by the same basic groups. The fact that the critical transition state for the steady-state parameter involves changes in only a modest number of protons suggests that the dissociation per se may be more important than a conformation change in this process, because the transition state for a conformation change conceivably should involve changes in a large number of protons, e.g., associated with hydrogen bonds of the protein and of the bulk solvent. However, the importance of a conformation change cannot be excluded.

The substantial solvent isotope effect on the $V_{cyclohexanone}$ at its pH-independent region also is a reflection of the effect on product dissociation, a conformation change, or both; since $V$ is not sensitive to substrate binding per se and since the primary covalent isotope effect on this parameter is small (Cook & Cleland, 1981a,b). The results of the proton inventory experiments suggest that the rate-determining process at the pH-independent region of the profile is a conformation change, in which there would be changes in a large number of protons, e.g., associated with hydrogen bonds of the protein and of the bulk solvent. There are two recognized conformation changes in the catalytic cycle (Bränden et al., 1975): one following the binding of NADH and one preceding the release of NAD. Although it is attractive to associate the observed solvent isotope effect with one or the other of these, the present evidence does not permit it.

The fact that the log of the solvent isotope effect on the apparent proton-dissociation constant for an acidic group essential for the $V_{cyclohexanone}$ is greater than +0.5 suggests that the pH-sensitive process has a lower normal intrinsic isotope effect than the other rate-determining processes at saturating substrate concentrations. Therefore, the pH-sensitive process would be different from the process that is determining at the pH-independent region of the profile. In addition since the excess log-isotope effect on the p$K$ ($pK_{D_2O} - pK_{H_2O} - 0.5$) is nearly the same as the log of the observed isotope effect at the pH-independent region of the profile, the simplest model to explain the effects would be one in which isotopically sensitive processes are rate determining with saturating substrates at the pH-independent region of the profile, and the pH-sensitive process has a very small solvent isotope effect. For example, if the pH-dependent process were the rate of dissociation of NAD (or at least controlled by the same basic group) as suggested by Dalziel (1963) and DeTraglia et al. (1977), it should be controlled by a basic group with an intrinsic p$K$ of 7.6–8.1 (DeTraglia et al., 1977; Kvassman & Pettersson,

1979). The observed p$K$ for $V_{cyclohexanone}$ is 8.58, because a different process (e.g., a conformation change) is critical at the pH-independent region of the profile. The observed p$K$ in $D_2O$ will move even further toward the pH extreme due to the solvent isotope effect on the p$K$ (0.5 pH unit) and a small isotope effect on the pH-dependent process relative to that on the pH-independent process. Although the small, inverse solvent isotope effect on the rate of NAD dissociation reported by Schmidt et al. (1979) would support this model, the usefulness of these measurements is limited by the fact that they were only made above the p$K$ of the essential acidic group. An alternative model to that presented above will be described later.

In contrast, the $V/K_{NAD}$ has no significant solvent isotope effect at the pH-independent region of the profile, presumably because no transition state that is critical in this region involves substantial changes in proton binding. Furthermore, since the log of the solvent isotope effect on the proton dissociation constant of the essential acidic group is nearly +0.5, the process for which this group is essential must be the same as the one that determines the value of the parameter at the pH-independent region or at least have the same intrinsic isotope effect. This interpretation is supported by the fact that the apparent p$K$ in $H_2O$ (9.48) corresponds closely to the intrinsic p$K$ (9.5–9.6) of an acidic group on the free enzyme known to be essential for the rate of NAD binding (DeTraglia et al., 1977; Kvassman & Pettersson, 1979).

However, since the log of the solvent isotope effect on the proton-dissociation constant of the essential basic group is greater than +0.5, either the process for which this group is essential must have a substantial normal solvent isotope effect or the intrinsic p$K$ must have an abnormally large change in $D_2O$. Since NAD and cyclohexanol bind to the enzyme predominately in that order (Ainslie & Cleland, 1972; Cook & Cleland, 1981a), the $V/K_{NAD}$ will be sensitive to the rate of binding of NAD and the forward rate of any other contiguous reversible steps prior to the binding of cyclohexanol. Therefore, the simplest hypothesis to explain the data is that the value of the $V/K_{NAD}$ is determined by the rate of binding of NAD at the pH-independent region of the profile and above and by some other process, probably a conformation change, below the pH-independent region. However, it may be determined by the rate of binding below the pH-independent region, if the p$K$ of the basic group has an abnormally large solvent isotope effect.

The solvent isotope effect on $V/K_{cyclohexanol}$ is small, but significant, at the pH-independent region of the profile, and the effect on $V/K_{cyclohexanone}$ is even smaller. The observations that the primary covalent isotope effects on both of these parameters are significant but less than the intrinsic isotope effect at their respective pH-independent regions (Cook & Cleland, 1981b) suggest that hydride transfer determines the value of both parameters to a significant extent. Therefore, the solvent isotope effect on hydride transfer must be rather modest, and hydride transfer is not concerted with proton abstraction in the direction of cyclohexanol oxidation. These are the same conclusions reached by Welsh et al. (1980) from a study of the solvent isotope effect on the reaction catalyzed by yeast alcohol dehydrogenase of benzylic alcohol, a substrate for which hydride transfer determines the value of $V$. Furthermore, the present results indicate that the rate of proton abstraction is not limiting for the parameter value in this pH range. Either the perturbation of proton binding responsible for the inverse solvent isotope effect on the binding constants for trifluoroethanol and isobutyramide is not present in the critical transition states for the $V/K_{cyclohexanol}$ and the $V/K_{cyclohexanone}$, respectively, or their effects are canceled out by the effects of other perturbations that are not present in the principal intermediates responsible for the binding equilibrium.

The fact that the log of the solvent isotope effect on the proton dissociation constant for a basic group essential for the $V/K_{cyclohexanol}$ is greater than + 0.5 indicates that the critical process for this parameter is changing at this p$K$, that there is an abnormally large isotope effect on the proton dissociation constant, or both. However, since the primary covalent isotope effect is constant, as the pH decreases below this p$K$ (Cook & Cleland, 1981b), the rates of the critical processes must remain approximately the same, at least relative to hydride transfer, and the rate of hydride transfer is determined by the same p$K$ that determines the rate of the other critical processes. Therefore, the possibility must be examined that the solvent isotope effect on the intrinsic proton dissociation constant of this essential basic group is abnormally large, and the identity of the apparent basic group becomes of particular interest. Ordinarily the $V/K_{cyclohexanol}$ should be controlled by essential acidic or basic groups in the E–NAD complex. Kvassman et al. (1981; Kvassman & Pettersson, 1980) have shown that the rate of hydride transfer in transient-state kinetic studies is determined by an essential basic group whose p$K$ depends upon the acidity of the alcohol, and they concluded that the group is the enzyme-bound alcoholate anion whose p$K$ has been drastically reduced. Other investigators (Shore et al., 1974; Cook & Cleland, 1981b; Brooks et al., 1972; Morris et al., 1980) have hypothesized base catalysis by a group on the enzyme to explain the p$K$s of apparent basic groups essential for the binding equilibrium of trifluoroethanol, the $V/K_{cyclohexanol}$, the transient-state burst of oxidation of ethanol, and the transient-state rate of reduction of cinnamaldehyde, respectively. The abnormally large solvent isotope effect on the p$K$ of the essential basic group could be the result of a deuterium fractionation factor of the conjugate acid (Schowen, 1978) greater than 1.0 (the value for common free acidic groups), several exchangeable protons with fractionation factors less than 1.0 on the conjugate base, or both. The hypothesized charge relay complex of hydrogen bonds contributed by the Zn-bound alcohol (or water), serine-48, and histidine-51, hypothesized from the crystallographic structure (Bränden et al., 1975), might fill the requirement for the large isotope effect, because of the possibilities of stronger proton bonding and of several exchangeable protons. However, the question of the relative timing of alcohol binding and proton release to solvent cannot be decided with the evidence presented here.

The log of the solvent isotope effect on the proton dissociation constants of an essential acidic group for the $V/K_{cyclohexanol}$ and one for the $V/K_{cyclohexanone}$ is each also greater than +0.5. Therefore, in the case of each of these two parameters, either the process dependent upon the acidic group has an inverse solvent isotope effect, the proton dissociation constant of the group has an abnormally large normal solvent isotope effect, or both. The fact that the primary covalent isotope effect on each of these two parameters decreases at high pH (Cook & Cleland, 1981b) indicates that the value of both parameters is determined by different processes at high pH and at the pH-independent regions of the profiles. It is possible that the value of $V/K_{cyclohexanol}$ is determined by a p$K$ of the E–NAD complex, at high pH, since the rate of binding of other ligands, bipyridine, 1,10-phenanthroline (Evans & Shore, 1980), and benzyl alochol (Kvassman & Pettersson, 1980), is dependent upon an acidic group with a p$K$ of 7.6,

which would appear higher if the value of the steady-state parameter were not determined by the same process at its pH-independent region. Because the p$K$ of the acidic group for the binding of bipyridyl is eliminated in the presence of imidazole, which displaces zinc-bound water, Evans & Shore (1980) concluded that the p$K$ is due to the deprotonation of the latter species. The possibility exists that the charge-relay complex involving Zn-bound water may have an abnormally large isotope effect on its proton-dissociation constant and that this large effect may account for the large increase in p$K$ in $D_2O$. However, it is also possible that the same proton perturbations responsible for the inverse solvent isotope effect on the equilibrium for trifluoroethanol binding are dominant in the transition state of the pH-dependent process.

Interpretation of the profiles for the $V/K_{cyclohexanone}$ is analogous to that of the high pH region of the profile for $V/K_{cyclohexanol}$, except that in the present case the rate-determining process under consideration at high pH is the rate of dissociation of cyclohexanol, which must be protonated in order to dissociate from the enzyme. This hypothesis is supported by the fact that both the decay constant and its primary covalent deuterium isotope effect for the enzyme–NADH–(dimethylamino)cinnamaldehyde in transient-state kinetic experiments are controlled by an essential acidic group with a p$K$ of 6.0, which lead Morris et al. (1980) to conclude that the dissociation of the product was rate determining. The analogous p$K$ with cyclohexanone should be considerably higher because cyclohexanone is less acidic and because its release is even less rate determining at the pH-independent region of the profile. As shown above in the interpretation of the isotope effects associated with the lower p$K$ in the $V/K_{cyclohexanol}$ profile, the p$K$ of the charge-relay complex is abnormally high in $D_2O$. Therefore the abnormally large effect on the p$K$ in the $V/K_{cyclohexanone}$ profile may be explained by a similar model containing the alcohol. However, the possibility of an inverse isotope effect on the rate of dissociation cannot be eliminated. If the $V_{cyclohexanone}$ were controlled by the rate of cyclohexanol release at high pH, this same hypothesis would provide an alternative explanation for the large increase in p$K$ for that parameter also.

**Registry No.** Alcohol dehydrogenase, 9031-72-5; cyclohexanol, 108-93-0; cyclohexanone, 108-94-1; trifluoroethanol, 75-89-8; isobutyramide, 563-83-7; NADH, 58-68-4.

References


Ainslie, G. R., Jr., & Cleland, W. W. (1972) *J. Biol. Chem. 247*, 946.

Albery, W. J., & Knowles, J. R. (1976) *Biochemistry 15*, 5627.

Brañden, C. I., Jörnvall, H., Elkund, H., & Furugren, B. (1975) *Enzymes, 3rd Ed. 11*, 103.

Brooks, R. L., Shore, J. D., & Gutfreund, H. (1972) *J. Biol. Chem. 247*, 2382.

Cleland, W. W. (1979) *Methods Enzymol. 63A*, 103.

Cook, P. F., & Cleland, W. W. (1981a) *Biochemistry 20*, 1790.

Cook, P. F., & Cleland, W. W. (1981b) *Biochemistry 20*, 1805.

Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry 20*, 1817.

Dalziel, K. (1963) *J. Biol. Chem. 238*, 2580.

Dalziel, K., & Dickenson, F. M. (1966) *Biochem. J. 100*, 491.

DeTraglia, M. C., Schmidt, J., Dunn, M. F., & McFarland, J. T. (1977) *J. Biol. Chem. 252*, 3493.

Evans, S. A., & Shore, J. D. (1980) *J. Biol. Chem. 255*, 1509.

Kvassman, J., & Pettersson, G. (1979) *Eur. J. Biochem. 100*, 115.

Kvassman, J., & Pettersson, G. (1980) *Eur. J. Biochem. 103*, 565.

Kvassman, J., Larsson, A., & Pettersson, G. (1981) *Eur. J. Biochem. 114*, 555.

Merritt, A. D., & Tompkins, G. M. (1959) *J. Biol. Chem. 234*, 2778.

Morris, R. G., Saliman, G., & Dunn, M. F. (1980) *Biochemistry 19*, 725.

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.) p 122, University Park Press, Baltimore, MD.

O'Leary, M. H., Yamada, H., & Crayton, J. Y. (1981) *Biochemistry 20*, 1476.

Schmidt, J., Chen, J., DeTraglia, M., Minkel, D., & McFarland, J. T. (1979) *J. Am. Chem. Soc. 101*, 3634.

Schowen, K. B. J. (1978) in *Transition State of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) p 225, Plenum Press, New York.

Shore, J. D., Gutfreund, H., Brooks, R. L., Santiago, D., & Santiago, P. (1974) *Biochemistry 13*, 4185.

Welsh, K. M., Creighton, D. J., & Klinman, J. P. (1980) *Biochemistry 19*, 2005.