Isotope Effects and Structure–Reactivity Correlations in the Yeast Alcohol Dehydrogenase Reaction. A Study of the Enzyme-Catalyzed Oxidation of Aromatic Alcohols†

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ABSTRACT: Steady-state kinetic parameters for the yeast alcohol dehydrogenase catalyzed oxidation of a series of para-substituted benzyl alcohols-*1,1*-h_2 and -*1,1*-d_2 by NAD^+ are reported. Catalytic constants have been found to be characterized by large deuterium isotope effects: k_H/k_D = 4.8, *p*-Br; 4.2, *p*-Cl; 3.4, *p*-H; 4.2, *p*-CH_3; 3.2, *p*-CH_3O. The observed isotope effects on k_{cat}/K_A, K_A, and K_B, where K_A and K_B are Michaelis constants for NAD^+ and alcohol, indicate a borderline rapid equilibrium–steady-state kinetic mechanism involving the random addition of substrate and coenzyme to enzyme. With the exception of *p*-CH_3 and possibly *p*-CH_3O substituted benzyl alcohol, k_{cat} is concluded to represent a single, rate-limiting hydrogen transfer step. A multiple linear regression analysis of the combined data for benzaldehyde reduction (Klinman, J. P. (1972), *J. Biol. Chem. 247*, 7977–7987, expanded to include *p*-$CH(CH_3)_2$-substituted benzaldehyde) and benzyl alcohol oxidation has been carried out to determine the contribution of electronic, hydrophobic, and steric effects to k_{cat} and substrate binding. Benzaldehyde binding is concluded to depend on electronic substituent effects as previously reported [$\log 1/K_{ald} = (-0.92 \pm 0.18)\sigma^+ - (0.80 \pm 0.067)$], whereas benzyl alcohol binding correlates with substrate hydrophobicity [$(\log 1/K_{alc} = (0.60 \pm 0.14) \log P - (1.2 \pm 0.12)$]. In the case of benzyl alcohol oxidation, k_{cat} is independent of electronic and steric effects; the best of seven equations indicates a small negative dependence of k_{cat} on hydrophobicity, which is within experimental error of zero [$\log k_0 = (-0.075 \pm 0.25) \log P - (0.65 \pm 0.19)$]. Data for benzaldehyde reduction are correlated at the 99% significance level by a single variable equation [$(\log k_R = (2.1 \pm 0.37)\sigma^+ - (0.093 \pm 0.14)$] and a two variable equation [$(\log k_R = (1.9 \pm 0.33)\sigma^+ + (0.46 \pm 0.20) \log P - (0.46 \pm 0.20)$]; these equations indicate (a) a large dependence on electronic substituent as reported previously and (b) a possible role for hydrophobic factors in facilitating catalysis. As the result of the observed hydrophobic substituent effects, different ground-state interactions are suggested for the binding of benzaldehydes and benzyl alcohols. Electronic substituent effects lead to the conclusion that there is little or no change in charge at C-1 of substrate at the transition state, relative to alcohol in the ground state. The significance of these effects to the detailed properties of the hydrogen transfer step is discussed.

Structure–reactivity correlations can provide insight into the nature of both ground-state and transition-state interactions between enzymes and their substrates (Jencks, 1971; Kirsch, 1972). Attempts to extract such correlations from kinetic studies of enzyme systems are often hampered by the complexity of the kinetics (Cleland, 1975). For example, the rate-limiting step in the liver alcohol dehydrogenase reaction is the release of coenzyme from an enzyme–coenzyme complex in the steady state (Sund and Theorell, 1963), and information concerning the interconversion of ternary complex has relied on rapid kinetic methods (Shore and Gutfreund, 1970). In the case of yeast alcohol dehydrogenase, a study of the chemical interconversion step was facilitated by the observation that aromatic aldehydes are turned over slowly; from a study of the enzyme-catalyzed reduction of substituted benzaldehydes by reduced nicotinamide adenine dinucleotide, NADH, and reduced nicotinamide adenine dinucleotide with deuterium in the 4-A position, NADD, the transfer of hydrogen from coenzyme to aromatic aldehydes was concluded to be rate limiting under steady-state conditions (Klinman, 1972). The observed electronic substituent effects on k_{cat} and aldehyde binding, together with substituent effects on the equilibrium constant for benzaldehyde–benzyl alcohol interconversion, led to the suggestion that there is relatively little net charge at C-1 of aldehyde or alcohol in the transition state of this reaction (Klinman, 1972).

A study of substituent effects in the enzyme-catalyzed oxidation of benzyl alcohols-*1,1*-h_2 and -*1,1*-d_2 by NAD^+ was undertaken in an effort to confirm the proposed transition-state structure in the yeast alcohol dehydrogenase reaction. The enzyme-catalyzed oxidation of a series of para-substituted benzyl alcohols is shown here to be characterized by large deuterium isotope effects, suggesting a rate-limiting hydrogen transfer step in both directions for the yeast alcohol dehydrogenase catalyzed interconversion of aromatic substrates. A comparison of isotope and substituent effects for benzaldehyde reduction and benzyl alcohol oxidation is presented in this paper, where multiple linear regression analyses have been carried out to determine the contribution of electronic, steric, and hydrophobic interactions to the formation of enzyme–aldehyde, –alcohol, and –transition state complexes. The relationship of the observed substituent effects to the detailed properties of the hydrogen transfer step is discussed.

Experimental Procedures

All chemicals were obtained commercially and were reagent grade, unless otherwise indicated. Liver alcohol dehydrogenase was obtained in its lyophilized form from Worthington; yeast alcohol dehydrogenase was obtained as an ammonium sulfate

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Table I: Kinetic Parameters for Reduction of *p*-Methylbenzaldehyde, ±20% Glycerol.[a]

	s^{-1}		mM					
Solvent	k_H	k_D	$K_{A',H}$	$K_{A',D}$	$K_{B',H}$	$K_{B',D}$	K_{NADH}	K_{ald}
+ Glycerol	0.39	0.073	0.48	0.17	8.7	4.1	0.056	2.6
− Glycerol	0.51	0.10	0.11	0.050	7.7	4.3	0.016	1.7

[a] Determined at pH 8.5, 25 °C, as described in the Experimental Section. k_H and k_D refer to catalytic constants for reduction by NADH and NADD, respectively. $K_{A'}$ and $K_{B'}$ are Michaelis constants for coenzyme and substrate, respectively. Dissociation constants for the release of coenzyme, K_{NADH}, and substrate, K_{ald}, from ternary complex were calculated from isotope effects on k_{cat} and K_m, as described previously (Klinman, 1972).

suspension from Boehringer. Determinations of pH were carried out on a Radiometer (type TTT1c) equipped with an expanded scale attachment. Kinetic studies were carried out on a Cary 118B recording spectrophotometer, maintained at constant temperature. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian HA-100-15 spectrometer. Regression analyses were carried out on a PDP-11 computer, using a computer program written by Drs. E. Lustbader and S. Litwin of The Institute for Cancer Research.

Coenzymes. NAD^+, grade III, was purchased from Sigma and was used without further purification; solutions of NAD^+ were assayed enzymatically (Klinman, 1974, 1975). NADH and NADD were prepared and assayed as previously described (Klinman, 1972).

Substrates. Solutions of fractionally distilled *p*-methylbenzaldehyde (Eastman) and *p*-isopropylbenzaldehyde (Eastman) were made immediately following the distillation, and were assayed enzymatically (Klinman, 1972). The synthesis of a series of para-substituted benzyl alcohols-*1,1-h*$_2$ and -*1,1-d*$_2$ was carried out in parallel experiments by reduction of the appropriate benzoyl chloride with either lithium aluminum hydride (Calbiochem) or lithium aluminum deuteride (Merck Sharp and Dohme of Canada), 99 atom % deuterium (Feiser and Feiser, 1967). In a typical synthesis 13 mmol of the appropriate benzoyl chloride in 25 ml of dry tetrahydrofuran was added dropwise to a suspension of 13 mmol of lithium aluminum hydride or lithium aluminum deuteride in 100 ml of dry tetrahydrofuran. The reaction was carried out under N_2. Following the addition of benzoyl chloride, the reaction mixture was stirred with gentle heating for 30 min. Excess hydride was destroyed by the addition of base. The reaction mixture was filtered, adjusted to pH 11, and refluxed for 2 h. This solution was extracted two times with 100 ml of diethyl ether. Product was obtained after evaporation of ether, and either recrystallized to constant melting point from heptane, or fractionally distilled under vacuum, *p*-hydrobenzyl alcohol (bp 70 °C (2.5 mm)) and *p*-methoxybenzyl alcohol (bp 75 °C (0.095 mm)). The isotopic purity of deuterated benzyl alcohols was confirmed by the absence of methylene protons at C-1 of product, as ascertained by NMR. Commercially available benzyl alcohols, *p*-Cl, *p*-H, *p*-$CH(CH_3)_2$, and *p*-CH_3O (Eastman), were either recrystallized or distilled prior to use. Solutions of alcohol were assayed with liver alcohol dehydrogenase at 25 °C, pH 8.5, in PP_i buffer (40 mM KPP_i–140 mM glycine–5 mM KCl) containing 1 mg/ml of acetylpyridine NAD^+. Assays were initiated by the addition of alcohol and monitored at 340 nm (E_{340nm} = 6.3).

Kinetic Measurements. Kinetic measurements were carried out at 25 °C in PP_i buffer (pH 8.5, μ = 0.22) by measuring either the disappearance or appearance of NADH or NADD at 340 nm. The enzyme used for kinetic studies was dialyzed overnight, 4 °C, against PP_i buffer (containing 0.2 mM dithiothreitol and 0.2 mM EDTA) to remove ammonium sulfate; enzyme dialyzed in this way is routinely characterized by a specific activity of approximately 100 U/mg (assayed in the direction of ethanol oxidation, pH 9, 1 M ethanol, and 1 mM NAD^+). Rate constants reported in this paper are calculated assuming four active sites per mole and normalized to a specific activity of 100 U/mg as originally described (Klinman, 1972).

The general form of the rate equation for a two-substrate enzyme is given by eq 1, where according to the nomenclature of Cleland (1963) V = maximum velocity, K_A = limiting Michaelis constant for A, K_B = limiting Michaelis constant for B, and K_{IA} = inhibition constant. Primary reciprocal plots have been analyzed for $1/v$ and K/v by a weighted least-squares computer program described by Cleland (1967). Secondary reciprocal plots were analyzed by an unweighted least-squares fit for $1/V$, K_A, K_B, and K_{IA}.

$$v = \frac{VAB}{K_{IA}K_B + K_BA + K_AB + AB} \quad (1)$$

Results

Kinetic Parameters for p-Methylbenzaldehyde Reduction ±20% Glycerol. As a result of the low solubility of substituted benzaldehydes in water, kinetic studies on the yeast alcohol dehydrogenase catalyzed reduction of aromatic aldehydes were carried out in the presence of 20% glycerol (Klinman, 1972). The observation that glycerol is slowly oxidized by yeast alcohol dehydrogenase, together with the greater solubility of substituted benzyl alcohols in water, indicated that glycerol was both unsuitable and unnecessary as a solvent for studies on aromatic alcohol oxidation. In order to compare kinetic parameters for alcohol oxidation with those obtained for aldehyde reduction, the enzyme-catalyzed reduction of *p*-methylbenzaldehyde by NADH and NADD was studied, ±20% glycerol. As indicated in Table I, catalytic constants in 20% glycerol are approximately 70% of values obtained in water. The Michaelis constant for aldehyde, K_B, is essentially unchanged, whereas the Michaelis constant for coenzyme, K_A, is increased approximately fourfold. Dissociation constants, calculated from the observed isotope effects on K_A and K_B as previously described (Klinman, 1972), indicate that NADH binds more weakly to form ternary complex in the presence of glycerol. Myers and Jakoby (1975) have reported a decrease in both turnover number and Michaelis constants for the yeast alcohol dehydrogenase catalyzed oxidation of ethanol by NAD^+ at pH 8.8 in the presence of 20% glycerol, whereas 30% glycerol appeared to have no effect on the turnover number or Michaelis constant for acetaldehyde reduction by NADH.

Kinetic Parameters for the Oxidation of Para-Substituted Benzyl Alcohols-1,1-h$_2$ *and -1,1-d*$_2$. Linear Lineweaver–Burk plots have been observed in the yeast alcohol dehydrogenase catalyzed oxidation of benzyl alcohols. The oxidation of *p*-

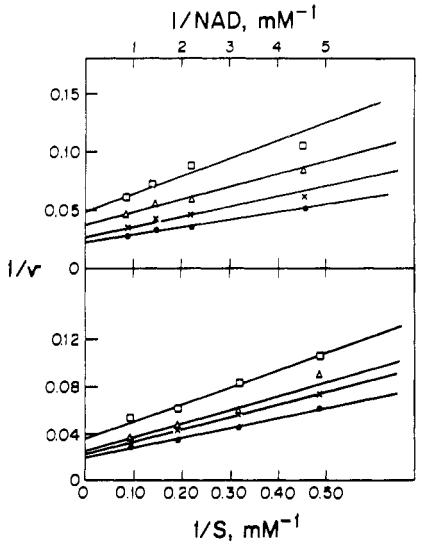


FIGURE 1: Lineweaver-Burk plots for the oxidation of *p*-chlorobenzyl alcohol-*1,1*-d_2 by NAD^+, catalyzed by yeast alcohol dehydrogenase, where *p*-chlorobenzyl alcohol is designated as S. For $1/v$ vs. $1/[NAD^+]$ plots: [S] = 2.1 mM (□); 3.1 mM (△); 5.1 mM (×); 10 mM (●). For $1/v$ vs. $1/[S]$ plots: $[NAD^+]$ = 0.22 mM (□); 0.45 mM (△); 0.67 mM (×); 1.11 mM (●).

chlorobenzyl alcohol-*1,1*-d_2 by NAD^+ is illustrated in Figure 1. Steady-state kinetic parameters were obtained from secondary plots of slopes and intercepts. Catalytic constants have been summarized in Table II. The constants in parentheses in Table II were obtained from the oxidation of commercially available alcohols, and are similar to catalytic constants for the oxidation of benzyl alcohols-*1,1*-h_2 prepared by $LiAlH_4$ reduction, as described in the Experimental Section. In contrast to the large effect of electronic substitution on k_{cat} for benzaldehyde reduction, the rate of oxidation of benzyl alcohols appears to be essentially independent of electronic substituent. Despite the apparent insensitivity of k_{cat} to substrate structure, large deuterium isotope effects are observed in the oxidation of these alcohols, suggesting a rate-limiting hydrogen transfer step.

In Table III, Michaelis constants for NAD^+ (K_A) and benzyl alcohols (K_B) are summarized. Isotope effects on K_A and K_B are seen to be small relative to isotope effects on k_{cat} and in some instances the observed isotope effects are within experimental error of 1.0 ± 0.2. For an ordered kinetic mechanism with coenzyme binding first the ratio of K_A to k_{cat} is a measure of the reciprocal of the rate constant for the addition of coenzyme to enzyme (Frieden, 1957). In the direction of benzaldehyde reduction, the variation in K_A/k_{cat} with changes in substrate structure was used as evidence for a random kinetic mechanism (Klinman, 1972). The data reported here for benzyl alcohol oxidation (Tables II and III) indicate that both K_A and k_{cat} are fairly insensitive to substrate structure for para-substituted alcohols, so that the invariance of K_A/k_{cat} cannot be used as a test of kinetic mechanism. However, an examination of K_A/k_{cat} for para-substituted substrates indicates values which are significantly larger in the case of deuterated alcohol oxidation, e.g., $K_{A,H}/k_H$ = 0.43 mM s^{-1} vs. $K_{A,D}/k_D$ = 1.6 mM s^{-1} for *p*-chlorobenzyl alcohol. Since substrates rather than coenzymes are isotopically labeled in these studies, the observation of an isotope effect on K_A/k_{cat} provides direct evidence that this ratio is a complex kinetic expression, and that the order of addition of aromatic alcohols and NAD^+ to enzyme does not occur in an obligatory ordered fashion with coenzyme binding first.

Table II: Rate Constants for the Oxidation of Aromatic Alcohols by NAD^+, Catalyzed by Yeast Alcohol Dehydrogenase.[a]

Alcohol	k_H (s^{-1})	k_D (s^{-1})	k_H/k_D
p-Bromobenzyl alcohol	0.34 ± 0.06	0.071 ± 0.02	4.8
p-Cl	0.23 ± 0.01 (0.31)[b]	0.05 ± 0.003	4.2
p-H	0.83 (0.53)[b]	0.25 ± 0.12	3.4
p-CH_3	0.32 ± 0.04	0.076	4.2
p-$CH(CH_3)_2$	(0.26)[b]		
p-CH_3O	0.31 ± 0.05 (0.42)[b]	0.094 ± 0.01	3.2

[a] Rate constants calculated and normalized as described in detail in the Experimental Section. [b] Values in parentheses represent catalytic constants for the oxidation of commercially available alcohols for comparison with constants for the oxidation of substrates synthesized by $LiAlH_4$ reduction, as described in the Experimental Section.

Table III: Michaelis Constants and Isotope Effects on Michaelis Constants.[a]

Alcohol	$K_{A,H}$ (mM)	$K_{A,H}/K_{A,D}$	$K_{B,H}$ (mM)	$K_{B,H}/K_{B,D}$
p-Bromobenzyl alcohol	(0.093)[b]	(0.28)	4.0	1.5
p-Cl	0.30	1.1	4.8	1.3
p-H	5.0	1.3	300	0.80
p-CH_3	0.48	1.6	11	0.84
p-$CH(CH_3)_2$	0.44		2.5	
p-CH_3O	0.55	1.4	20	0.91

[a] K_A is the Michaelis constant for NAD^+, and K_B is the Michaelis constant for alcohol. [b] Since $K_{A,D}$ = 0.31 mM for *p*-bromobenzyl alcohol is the same as the average value observed for the other para-substituted benzyl alcohols, 0.32 ± 0.07 mM, the low observed isotope effect on K_A is concluded to reflect an anomalously low $K_{A,H}$ for this substrate.

Under conditions of rate-limiting interconversion of ternary complex, limiting Michaelis constants are equal to $(k_{-1} + k_{cat})/k_1$, where k_{-1} and k_1 are dissociation and binding rate constants for the formation of ternary complex. If one neglects isotope effects on binding steps, the magnitudes of the observed isotope effects on K_A and K_B are a direct reflection of the relative rate of k_{-1} and k_{cat}, and indicate the extent to which the Michaelis constant is a kinetic constant as opposed to a dissociation constant. Previously, ternary dissociation constants for the binding of benzaldehydes and NADH to yeast alcohol dehydrogenase were calculated from the observed isotope effects on Michaelis constants and catalytic constants (Klinman, 1972). In Table IV, the relative rate of substrate dissociation vs. turnover is summarized for isotope effects of 1-2 on K_A and K_B and an isotope effect of 4 on k_{cat}. For the range of isotope effects on K_A and K_B observed in these studies, the kinetic mechanism in the case of benzyl alcohol-*1,1*-h_2 oxidation is seen to be borderline between a steady state vs. rapid equilibrium mechanism, i.e., k_{-1}/k_{cat} = 1.3-7.3. The fourfold decrease in k_{cat}, which is brought about by deuterium substitution, is sufficient to convert the reaction mechanism to a rapid equilibrium mechanism, i.e., k_{-1}/k_{cat} = 4-29. These considerations indicate that dissociation constants can be obtained directly from kinetic data for benzyl alcohol-*1,1*-d_2 oxidation, and binary and ternary dissociation constants for NAD^+ and alcohol are summarized in Table V. With the exception of

Table IV: Relationship between the Observed Isotope Effect on the Limiting Michaelis Constant and the Relative Rate at Which the Substrate Dissociates from Ternary Complex (k_{-1}) Compared to Turnover (k_{cat}).

K_H/K_D	$k_{-1}/k_{cat,H}$[a]	$k_{-1}/k_{cat,D}$
1.1	7.3	29
1.2	3.5	14
1.3	2.3	9
1.4	1.6	6.5
1.5	1.3	5.0
1.6	1.0	4.0
1.8	0.69	2.8
2.0	0.50	2.0

[a] Calculated from $k_{-1}/k_{cat,H} = [1 - (k_{cat,D}/k_{cat,H} \times K_H/K_D)]/(K_H/K_D - 1)$ where $k_{cat,H}/k_{cat,D} = 4$.

Table V: Binary and Ternary Dissociation Constants.[a]

	mM			
Alcohol	K_{NAD^+}	$K_{alc \cdot NAD^+}$	K_{alc}	$K_{NAD^+ \cdot alc}$
p-Bromobenzyl alcohol	0.31	0.31	2.7	2.7
p-Cl	0.30	0.30	3.7	3.7
p-H	0.28	3.9	27	380
p-CH_3	0.20	0.30	8.8	13
p-CH_3O	0.17	0.41	9.2	22

[a] Calculated from data for the oxidation of deuterated benzyl alcohols for a preequilibrium kinetic mechanism: $K_{NAD^+} = K_{IA}K_B/K_B$, $K_{alc \cdot NAD^+} = K_A$, $K_{alc} = K_{IA}K_B/K_A$, $K_{NAD^+ \cdot alc} = K_B$, where for a random equilibrium kinetic mechanism $K_{IA}K_B = K_AK_{IB}$ (Cleland, 1963).

unsubstituted benzyl alcohol, binary and ternary complexes are characterized by similar dissociation constants. The absence of a substituent in the para position of the phenyl ring results in ternary dissociation constants for both coenzyme and substrate which are approximately tenfold larger than values observed for para-substituted benzyl alcohols, analogous with observations in the direction of benzaldehyde reduction (Klinman, 1972).

Haldane Equations. The available dissociation and catalytic constants for benzaldehyde reduction and benzyl alcohol oxidation have been compared to equilibrium constants using the Haldane equation for a random, rapid equilibrium kinetic mechanism in which binary and ternary dissociation constants are the same:

$$K_{EQ}[H^+] = \frac{k_R K_{NAD^+} K_{alc}}{k_O K_{NADH} K_{ald}} \quad (2)$$

where k_R and k_O are rate constants for aldehyde reduction and alcohol oxidation and K_{NAD^+}, K_{alc}, K_{NADH}, and K_{ald} are dissociation constants. Previously reported dissociation constants for NADH and aldehyde were calculated from isotope effects on Michaelis constants so that these values represent ternary dissociation constants (Klinman, 1972), whereas binary dissociation constants for NAD^+ and alcohol, Table V, are used in eq 2. Values for $K_{EQ}[H]$ calculated from eq 2 are compared to measured equilibrium constants in Table VI; despite the cumulative errors which are present in a calculation of $K_{EQ}[H^+]$, there is reasonable agreement between these values, indicating the internal consistency of the data for aldehyde reduction and alcohol oxidation.

Multiple Linear Regression Analyses. As discussed by Hansch et al. (1972), substituent effects on kinetic and equilibrium processes in biochemical systems may be a complex reflection of hydrophobic, steric, and electronic factors. In an effort to clarify the various factors contributing to catalysis in the yeast alcohol dehydrogenase reaction, a multiple linear regression analysis of the contribution of electronic, hydrophobic, and steric effects to substrate binding and k_{cat} was carried out:

$$\log K,k = a\sigma^+ + b \log P + cR + d \quad (3)$$

According to eq 3, k represents rate constants for the enzyme-catalyzed reduction of aldehydes or oxidation of alcohols and K represents association constants for the binding of aldehyde or alcohol to enzyme. Parameters a, b, and c represent the contribution of electronic, hydrophobic, and steric variables to a given rate or equilibrium process, respectively. Electronic substituent constants (σ^+ or σ), hydrophobic constants (log P), and steric constants (R) for a series of benzaldehydes and benzyl alcohols are summarized in Table VII.

The data for substrate binding have been analyzed in a stepwise fashion using single and two parameter equations, where association constants are the reciprocal of K_{alc} and K_{ald} (Table VI). In the case of k_O (Table II) and k_R (Table VI and Klinman, 1972) there were sufficient data points for a three-parameter analysis. The best single, two, and where appropriate, three variable parameter equations are summarized below. The number of data points is represented by n and r is the correlation coefficient; F relates the variance of the null hypothesis to the variance of each correlation and $F_{0.99}$, obtained from statistical tables, represents a lower limit of F for the correlation to be significant at the 99% level (Anderson and Bancroft, 1952).

Benzaldehyde binding

$$\log 1/K_{ald} = -(0.92 \pm 0.18)\sigma^+ - (0.80 \pm 0.067) \quad (4)$$

$$n = 6, r = 0.99, F_{1,4} = 26.6, F_{1,4(0.99)} = 21.2$$

$$\log 1/K_{ald} = -(0.96 \pm 0.20)\sigma^+ + (0.082 \pm 0.13) \log P - (0.87 \pm 0.12) \quad (5)$$

$$n = 6, r = 0.99, F_{2,3} = 11.6, F_{2,3(0.99)} = 30.8$$

Benzaldehyde reduction

$$\log k_R = (2.1 \pm 0.37)\sigma^+ - (0.093 \pm 0.14) \quad (6)$$

$$n = 12, r = 0.91, F_{1,10} = 31.7, F_{1,10(0.99)} = 10.0$$

$$\log k_R = (1.9 \pm 0.33)\sigma^+ + (0.46 \pm 0.20) \log P - (0.46 \pm 0.20) \quad (7)$$

$$n = 12, r = 0.94, F_{2,9} = 24.8, F_{2,9(0.99)} = 8.02$$

$$\log k_R = (1.6 \pm 1.7)\sigma^+ + (0.57 \pm 0.84) \log P - (0.12 \pm 0.84)R - (0.33 \pm 0.96) \quad (8)$$

$$n = 12, r = 0.94, F_{3,8} = 14.7, F_{3,8(0.99)} = 7.59$$

Benzyl alcohol oxidation

$$\log k_O = (-0.073 \pm 0.25) \log P - (0.65 \pm 0.19) \quad (9)$$

$$n = 11, r = 0.88 F_{1,9} = 0.085, F_{1,9(0.99)} = 10.6$$

$$\log k_O = (0.014 \pm 0.45)\sigma^+ - (0.078 \pm 0.31) \log P - (0.64 \pm 0.26) \quad (10)$$

$$n = 11, r = 0.88, F_{2,8} = 0.038, F_{2,8(0.99)} = 8.65$$

$$\log k_O = (0.43 \pm 2.1)\sigma^+ - (0.26 \pm 0.95) \log P + (0.19 \pm 0.93)R - (0.87 \pm 0.11) \quad (11)$$

Table VI: Haldane Equations.

Substituent	$K_{eq}[H^+]^a$	$K_{eq}[H^+]_{calcd}$ =	$\frac{K_{NAD^+}\ (mM)^b}{K_{NADH}\ (mM)^c}$	×	$\frac{K_{alc}\ (mM)^d}{K_{ald}\ (mM)^e}$	×	$\frac{k_R\ (s^{-1})^f}{k_O\ (s^{-1})}$
p-Br	96	84	$\frac{0.25}{0.016}$	×	$\frac{2.7}{6.0}$	×	$\frac{0.85}{0.071}$
p-Cl	92	75		×	$\frac{3.7}{8.4}$	×	$\frac{0.60}{0.055}$
p-H	64	63		×	$\frac{27}{7.2}$	×	$\frac{0.27}{0.25}$
p-CH_3	24	56		×	$\frac{8.8}{2.4}$	×	$\frac{0.073}{0.076}$
p-$CH(CH_3)_2$	24	29		×	$\frac{2.5}{4.0}$	×	$\frac{1.3\ (1.3)}{0.56}$
p-CH_3O	4.4	7.6		×	$\frac{9.2}{1.2}$	×	$\frac{0.006}{0.094}$

[a] Obtained from previously reported, pH-independent equilibrium constants (Klinman, 1972). [b] K_{NAD^+} is the average of five values in Table V. [c] The value for K_{NADH} in the absence of 20% glycerol, Table I. [d] Binary dissociation constants, Table V. The value for *p*-$CH(CH_3)_2$ is a Michaelis constant, Table III. [e] Aldehyde dissociation constants have been calculated from isotope effects on Michaelis constants and represent ternary dissociation constants. In the case of unsubstituted benzyl alcohol, dissociation constants for both NAD^+ and alcohol are seen to increase approximately tenfold in going from binary to ternary complexes (Table V), indicating that binary and ternary dissociation constants cannot be compared to one another. For the calculations summarized in this table, the observed ternary dissociation constant for benzaldehyde has been divided by 10. [f] With the exception of *p*-$CH(CH_3)_2$, k_R represents catalytic constants for the reduction of benzaldehydes by NADD (Klinman, 1972) and k_O represents catalytic constants for the oxidation of benzyl alcohols-*1,1-d*$_2$ by NAD^+ (Table II). The presence of deuterium in the pro-*S* position at C-1 of alcohol should reduce the magnitude of k_O by a factor of approximately 1–1.3 (do Amaral et al., 1973), relative to alcohols stereospecifically deuterated in the pro-*R* position. This effect should be roughly cancelled by the small reduction in k_R due to the presence of 20% glycerol, Table I. In the case of $CH(CH_3)_2$, k_R and k_O are catalytic constants for the interconversion of protonated substrates; k_R has been corrected for a 30% inhibition by 20% glycerol.

Table VII: Electronic and Hydrophobic Substituent Constants and Van der Waals Radii.

Substituent	σ^a	σ^{+a}	log *P*(ald)[b]	log *P*(alc)[b]	*R* (Å)[c]
p-Br	0.30	0.14	1.12	1.06	1.95
p-Cl	0.29	0.11	0.91	0.86	1.8
p-H	0	0	0	0	1.2
p-CH_3	−0.10	−0.32	0.54	0.48	2.0
p-$CH(CH_3)_2$	−0.14	−0.29	1.50	1.43	3.2
p-OCH_3	−0.09	−0.79	0.22	0	3.0

[a] Hoefnagel and Wepster (1973); σ is based on the ionization of benzoic acids and σ^+ is based on the SN1 reaction of dimethyl phenyl methyl chloride. [b] Leo et al. (1971); derived from octanol–water partition studies. [c] Pauling (1945); *R* represents van der Waals radii.

$$n = 11,\ r = 0.88,\ F_{3,7} = 0.037,\ F_{3,7(0.99)} = 8.45$$

Benzyl alcohol binding

$$\log 1/K_{alc} = (0.62 \pm 0.14) \log P - (1.2 \pm 0.12) \quad (12)$$

$$n = 6,\ r = 0.99,\ F_{1,4} = 18.6,\ F_{1,4(0.99)} = 21.2$$

$$\log 1/K_{alc} = (0.57 \pm 0.16) \log P + (0.11 \pm 0.12)R - (0.14 \pm 0.26) \quad (13)$$

$$n = 6,\ r = 0.99,\ F_{2,3} = 9.28,\ F_{2,3(0.99)} = 30.8$$

For benzaldehyde binding, the data correlate with σ^+ at the 99% level, eq 4, whereas neither log *P* nor *R* was significant at the 95% level. The best two-parameter equation, eq 5, did not produce a statistically significant improvement over eq 4, $F_{1,3} = 0.43$ compared to $F_{1,3(0.99)} = 34.1$. For benzaldehyde reduction, the best single variable equation was eq 6; neither log *P* nor *R* was significant at the 95% level. Although both a two-parameter (eq 7) and a three-parameter equation (8) were significant by themselves at the 99% level, they were not statistically better than eq 6. A comparison of eq 7 to eq 6 indicates $F_{1,9} = 5.06$ vs. $F_{1,9(0.99)} = 10.9$; similarly, a comparison of eq 8 to eq 6 indicates $F_{2,8} = 4.53$ vs. $F_{2,8(0.99)} = 8.65$. These findings are consistent with previous conclusions concerning the importance of electronic substituent effects on benzaldehyde binding and reduction in the yeast alcohol dehydrogenase reaction.

In the case of benzyl alcohol oxidation, the data did not correlate well with σ^+, log *P* or *R* alone, nor were the data fit by two or three variable equations. The best correlation among seven equations was to log *P*, eq 9, $F_{1,9} = 0.086$ vs. $F_{1,9(0.99)} = 10.6$. Thus, k_{cat} for benzyl alcohol oxidation is concluded to be independent of electronic, steric, and probably hydrophobic effects. For benzyl alcohol binding, the best single variable equation was for log *P*, eq 12; neither σ^+ nor *R* was significant by themselves at the 95% level. A comparison of eq 13 to eq 12 indicates that two parameters do not produce a statistically significant improvement over a single parameter, $F_{1,3} = 0.815$ vs. $F_{1,3(0.99)} = 34.1$. The significant substituent effect in benzyl alcohol binding to yeast alcohol dehydrogenase is concluded to be hydrophobic.

Discussion

Rate Limiting Steps. Kinetic studies on the yeast alcohol dehydrogenase catalyzed reduction of substituted benzaldehydes by NADH and NADD indicated a mechanism in which (a) hydrogen transfer is the rate-limiting step under conditions of substrate saturation and (b) the release of substrates from ternary complex is not infinitely fast, relative to the chemical interconversion step (Klinman, 1972). The enzyme-catalyzed oxidation of aromatic alcohols is reported in this paper to be characterized by large deuterium isotope effects, suggesting a rate-limiting hydrogen transfer step. Since slow substrate release from ternary complex in the direction of aldehyde reduction should result in a partially rate-limiting release of product in the case of alcohol oxidation, the conclusions concerning alcohol oxidation require further explanation.

As discussed in detail in an earlier paper (Klinman, 1972) and the Results section, the magnitude of isotope effects on

Michaelis constants for aromatic substrate interconversion is considered to be a measure of the rate of substrate release from ternary complex compared to ternary complex interconversion. By extension, the relative magnitude of isotope effects on Michaelis constants for NADH-NAD^+ vs. benzaldehyde-benzyl alcohol is a measure of the relative rate of coenzyme vs. aldehyde or alcohol release from ternary complex. With the exception of *p*-methylbenzaldehyde reduction, and possibly *p*-bromobenzyl alcohol oxidation, the observed isotope effects on at least one of the two Michaelis constants are within experimental error of 1.0 ± 0.2 (Klinman, 1972, and Table III). Thus, interpretation of the observed isotope effects on Michaelis constants in terms of a preferential release of NADH-NAD^+ or benzaldehyde-benzyl alcohol from ternary complex, followed by the rapid release of the second substrate from a binary complex, is consistent with a rate-limiting hydrogen transfer step in the direction of both benzaldehyde reduction and benzyl alcohol oxidation. In the case of *p*-methylbenzaldehyde reduction, the isotope effects on K_A' and K_B' are 2.2–2.8 and 1.8–2.1, respectively (Table I). The magnitude of isotope effects on both K_A' and K_B' suggests a partially rate-limiting release of product for *p*-methyl benzyl alcohol oxidation.

Cleland (1975) has reported a primary equilibrium isotope effect for the reaction, $H^+ + CO_2^-CH_2C(=O)CO_2^- + NADH(D) \rightleftharpoons CO_2^-CH_2CH(D)OHCO_2^- + NAD^+$. The measured value is $K_{EQ,H}/K_{EQ,D} = 0.77$–0.83, consistent with theoretical calculations of Hartshorn and Shiner (1972). Neglecting secondary isotope effects, the magnitudes of isotope effects for benzaldehyde reduction and benzyl alcohol oxidation are interrelated by an equilibrium isotope effect:

$$(k_{R,H}/k_{R,D})/(k_{O,H}/k_{O,D}) = K_{EQ,H}/K_{EQ,D} \quad (14)$$

A recent determination of the equilibrium isotope effect for the process, $H^+ + CH_3CHO + NADH(D) \rightleftharpoons CH_3CH,H(D)OH + NAD^+$, indicates $K_{EQ,H}/K_{EQ,D} = 0.89 \pm 0.03$ (J. P. Klinman, unpublished data). Since benzyl alcohols, dideuterated at C-1, were used in this study, eq 14 can be expanded to include a secondary kinetic deuterium isotope effect:

$$(k_{R,H}/k_{R,D})/(k_{O,H'}/k_{O,D'}) \times (k_{\alpha,H}/k_{\alpha,D}) = (K_{EQ,H}/K_{EQ,D})\,(k_{\alpha,D}/k_{\alpha,H}) \quad (15)$$

The observed isotope effect for alcohol oxidation is $k_{O,H}/k_{O,D} = k_{O,H'}/k_{O,D'} \times k_{\alpha,H}/k_{\alpha,D}$. The magnitude of $k_{\alpha,H}/k_{\alpha,D}$ is expected to vary from 1 to 1.3; the upper limit of $k_{\alpha,H}/k_{\alpha,D}$ is approximated from values in the literature (do Amaral et al., 1973) for the conversion of a tetrahedral (-C(-H(D))OH) to a trigonal carbon (-C(-H(D))=O). The observed ratios[1] of $(k_{R,H}/k_{R,D})/(k_{O,H}/k_{O,D})$ are *p*-Br = 0.73, *p*-Cl = 0.78, *p*-H = 0.88, *p*-CH_3 = 1.21, and *p*-CH_3O = 1.06. The expected range, from the right-hand side of eq 15, is 0.69–0.89. The ratio of 1.21 for *p*-CH_3 is concluded to reflect a partially rate-limiting product release step for the oxidation of the alcohol, i.e., the observed isotope effect for *p*-methylbenzyl alcohol oxidation is smaller than the intrinsic isotope effect. The ratio of 1.06 for *p*-CH_3O most probably reflects experimental error, but may indicate that hydrogen transfer is not fully rate limiting for *p*-methoxybenzyl alcohol oxidation. A comparison of isotope effects on k_R and k_O indicates that the hydrogen transfer step is rate limiting in both directions for the interconversion of *p*-Br, *p*-Cl, *p*-H, and most likely, *p*-CH_3O substrates. In the case of *p*-methylbenzyl alcohol oxidation, k_O is concluded to be an underestimate of the intrinsic rate constant for the chemical interconversion step.[2]

Table VIII: Contribution of Electronic,[a] Hydrophobic,[b] and Steric Factors[c] to Substrate Binding and Turnover.

Process	Kinetic Parameter	$\log K(k) = a\sigma^+ + b \log P + cR + d$		
		a	*b*	*c*
(1) Benzaldehyde binding	$1/K_{ald}$[a]	−0.92 ± 0.18[d]	0	0
(2) Benzaldehyde reduction	k_R[b]	2.1 ± 0.37[e]	0	0
		[1.9 ± 0.33	0.46 ± 0.21	0[f]
(3) Benzyl alcohol oxidation	k_O[c]	0	0	0
(4) Benzyl alcohol binding	$1/K_{alc}$[a]	0	0.62 ± 0.14[g]	0

[a] Table VI. [b] Table VI and Klinman (1972). [c] Table II. [d] Equation 4 in text. [e] Equation 6 in text. [f] Equation 7 in text. [g] Equation 12 in text.

Structure–Reactivity Correlations. In order to dissociate electronic from hydrophobic and steric factors in substrate binding and turnover, multiple regression analyses were carried out (eq 4–13). A stepwise analysis of the data indicates that the data correlate best to single variable equations. The contributions of electronic, hydrophobic, and steric factors to aldehyde and alcohol binding and k_{cat} are summarized in Table VIII. Since the *p*-$CH(CH_3)_2$ substituent has the same electronic but different hydrophobicity and steric constants from *p*-CH_3 (Table VII), the series of para-substituted substrates was expanded to include *p*-$CH(CH_3)_2$ in this study. The binding of benzaldehydes is concluded to depend on the electronic properties of para substituents, $a = -0.92 \pm 0.18$, consistent with previous reports of $a = -0.85$. Hansch et al. (1973) have analyzed binding constants for the interaction of substituted benzamides with liver alcohol dehydrogenase and conclude that both apolar and electronic releasing substituents facilitate binding, $a = -0.80 \pm 0.30(\sigma)$ and $b = 0.45 \pm 0.28$. It appears that a feature common to both yeast and liver alcohol dehydrogenase is the interaction of an active-site electrophile with the oxygen of the carbonyl of bound substrate.

In contrast to the binding of benzaldehydes, the best single variable equation for benzyl alcohol binding indicates the importance of hydrophobic interactions, $b = 0.62 \pm 0.14$. The importance of a hydrophobic pocket at the active site of horse liver alcohol dehydrogenase has been demonstrated: the mean slope of lines correlating various parameters to hydrophobicity constants was found to be 0.79 ± 0.19 (Hansch et al., 1972). In an extensive analysis by Hansch and Dunn (1972), of systems where a linear relationship between log *P* and log biochemical response obtains, 71 of 128 examples were found to have a mean slope of 0.66 ± 0.12. These results indicate the potential for comparable hydrophobic interactions between numerous enzymes and their substrates. It should be noted that a second class of enzymes was also reported by Hansch and Dunn in which the mean slope of 57 linear relationships was found to be 1.0 ± 0.13.

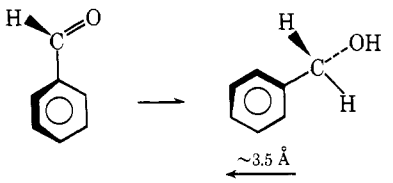
Since benzaldehyde and benzyl alcohol substrates are ex-

[1] Values of $k_{R,H}/k_{R,D}$ used for this calculation are the observed values for each benzaldehyde, rather than the value of 3.6 obtained from intercepts of Hammett plots (Klinman, 1972).

[2] An underestimate of the intrinsic rate constant for the chemical interconversion step in the direction of alcohol oxidation would give rise to an overestimate of $K_{EQ}[H^+]$ calculated from eq 2; this is, in fact, what is observed for *p*-CH_3 interconversion (Table VI).

pected to bind at the same active site, the absence of a hydrophobic contribution to benzaldehyde binding is surprising. Although the best two-variable equation for benzaldehyde binding, eq 5, indicates a role for hydrophobic factors, the observed value of $b = 0.082 \pm 0.13$ is small and within experimental error of zero. In contrast, the best two-variable equation for benzaldehyde reduction is significant at the 99% level; from eq 7 one obtains values of $a = 1.9 \pm 0.33$ and $b = 0.46 \pm 0.21$, as summarized in brackets in Table VIII. By the principle of microscopic reversibility, a positive hydrophobic contribution to k_{cat} for benzaldehyde reduction ($b > 0$) should express itself as a negative contribution to k_{cat} for benzyl alcohol oxidation ($b < 0$); the magnitude of b need not be the same in both directions and will depend on the nature of the transition state (reactant-like vs. product-like). The best of seven equations for alcohol oxidation was eq 9. The statistical significance of this equation is quite low ($F_{1,9} = 0.085$ compared to $F_{1,9(0.99)} = 10.6$) and the value of b obtained from this equation is within experimental error of zero, $b = -0.073 \pm 0.25$. Although the data do not permit an unambiguous distinction between a hydrophobic contribution to benzaldehyde binding vs. k_{cat}, the high statistical significance of eq 7 suggests a hydrophobic contribution to k_R. The low correlation between log k_O and log P may be the consequence of a transition-state structure which resembles the alcohol product, or possibly either a stepwise mechanism of hydrogen transfer or nonproductive binding of aldehyde[3]; a stepwise mechanism is discussed in more detail in the next section of this Discussion. Scheme I illustrates how the conversion of a trigonal to a tetrahedral carbon could result in an approximately 3.5 Å displacement at C-4 of the benzene ring, leading to different ground-state interactions for aldehyde and alcohol enzyme-bound substrates.

Scheme I



The sensitivity of k_R to electronic substituent is concluded to be 2.1 ± 0.37 from eq 6 and 1.9 ± 0.33 from eq 7, consistent with a previous report of $a = 2.2$ (Klinman, 1972). The value observed for benzaldehyde reduction was proposed to be the result of ground-state effects exclusively, reflecting an equilibrium electronic substituent effect of 1.5 and a substituent effect on aldehyde binding.[4] The observation that $a = 0$ for benzyl alcohol oxidation, eq 9, leads to the conclusion that there is little or no change in charge at C-1 of substrate at the transition state, relative to alcohol in the ground state.

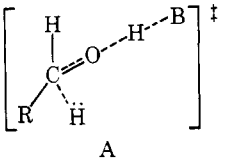
Scheme II summarizes the observed electronic substituent effects in the yeast alcohol dehydrogenase reaction, where ETX represents the enzyme transition state complex.

A study of substituent effects in the horse liver alcohol dehydrogenase catalyzed reduction of three benzaldehydes by Jacobs et al. (1974) indicated deuterium isotope effects of 2–3; the absence of a significant substituent effect on k_{cat} led these authors to suggest a highly positively charged transition state in the liver ADH catalyzed interconversion of aromatic substrates. In contrast, Hardman et al. (1974) have reported a small electronic substituent effect, $a = -0.75$, (σ), in the direction of benzyl alcohol oxidation, under conditions where the oxidation of dideuterated p-methylbenzyl alcohol is characterized by a substantial isotope effect, $k_H/k_D = 4.3$. The results of Hardman et al. suggest that charge development is much less important at the transition state of the liver alcohol dehydrogenase reaction analogous to the reaction catalyzed by yeast alcohol dehydrogenase.

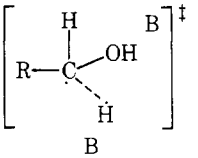
Nature of Hydrogen Transfer. The divergence of deuterium isotope effects measured from kinetic vs. isotope partitioning data in several model reactions of 1,4-dihydronicotinamide has led to the proposal that these reactions proceed through the formation of a kinetically significant intermediate (Steffens and Chipman, 1971; Creighton et al., 1973). Although the precise nature of the intermediate is unknown, the rate of intermediate formation has been estimated to be considerably lower than rates expected for the formation of stacked dimers of aromatic compounds (Creighton et al., 1973). Williams et al. (1975) have proposed radical intermediates in the reactions of flavins and dihydronicotinamides.

It was previously argued (Klinman, 1972) that the observed electronic substituent effects in the yeast alcohol dehydrogenase catalyzed reduction of benzaldehydes were unlikely to be consistent with a mechanism involving the preequilibrium formation of a substrate radical anion, followed by rate-limiting hydrogen atom transfer from $NADH^{\cdot+}$. An analogous argument would pertain to a mechanism for benzyl alcohol oxidation in which a one-electron abstraction from alcohol leads to the intermediate formation of a radical cation, followed by rate-limiting hydrogen atom abstraction by NAD·. The intermediacy of *charged* intermediates is difficult to reconcile with the observed lack of charge development at C-1 of substrate at the transition state of the enzyme-catalyzed reaction.

A role for acid–base catalysis in pyridine nucleotide dependent, enzyme-catalyzed oxidation–reduction reactions at carbonyl centers has been proposed by numerous investigators (Sund and Theorell, 1963; Klinman, 1975; Hollbrook and Gutfreund, 1973; Akhtar et al., 1972). As the result of x-ray crystallographic studies, a catalytic role for imidazolium–imidazole and possibly Zn or Zn–H_2O–Zn–OH has been suggested for lactate and horse liver alcohol dehydrogenase, respectively (Rossmann et al., 1971; Eklund et al., 1974). In the case of yeast alcohol dehydrogenase, the observed electronic substituent effects for benzaldehyde binding are consistent with the interaction of an acidic group at the enzyme active site with the carbonyl oxygen of bound aldehyde; from studies of the effect of pH on the enzyme-catalyzed reduction of aldehydes and oxidation of alcohol, a side chain, pK = 8.25, has been implicated in acid–base catalysis of the chemical



A

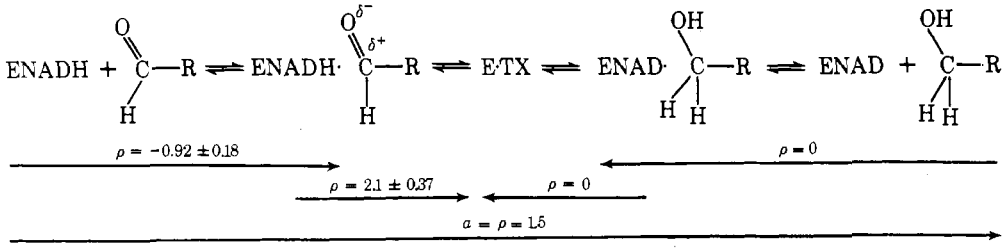


B

[3] The possibility of nonproductive binding was pointed out by Professor W. P. Jencks. A mechanism in which aldehyde binds nonproductively, moving over into a hydrophobic pocket prior to hydrogen transfer, would be consistent with the observed data. This mechanism need not imply that the observed electronic effects for aldehyde binding are "nonproductive". The observed electronic effects are consistent with the chemical mechanism and similar to the electrostatic effects reported by Hansch et al. (1973) for benzamide binding to liver alcohol dehydrogenase.

[4] This was based on the simplifying assumption that the effect of electronic substituent on the equilibrium is a reflection of the interaction of para substituents with the carbonyl of benzaldehydes, rather than any significant interaction between these substituents and the carbinol of benzyl alcohols (Klinman, 1972).

Scheme II



interconversion step[5] (Klinman, 1975). Incorporating a role for acid–base catalysis, two mechanisms can be proposed for the yeast alcohol dehydrogenase reaction which are consistent with the "uncharged" nature of the transition state. Structure A illustrates a mechanism involving concerted general acid–base catalysis of a hydride transfer; according to B a *protonated* radical intermediate is formed either prior to (aldehyde reduction) or after (alcohol oxidation) a rate-determining hydrogen atom transfer.

Jencks (1972a) has discussed stepwise vs. concerted proton transfer, concomitant with heavy atom rearrangements, for reactions at carbonyl centers. Concerted reactions are concluded to be unlikely, except for reactions which would involve the formation of highly unstable intermediates. A simple rule has been formulated, which states that "concerted acid–base catalysis of complex reactions in aqueous solution can occur only (a) at sites that undergo a large change in pK in the course of the reaction, and (b) when this change in pK converts an unfavorable to a favorable proton transfer with respect to the catalyst, i.e., the pK of the catalyst is intermediate between the initial and final pK values of the substrate site" (Jencks, 1972b). The appropriate pK values for the yeast alcohol dehydrogenase reaction can be estimated: p$K \simeq 8.3$ (Klinman, 1975) for the presumed catalytic base, p$K \simeq -3$ to -7 (Stewart et al., 1959) for para-substituted, protonated benzaldehydes, and p$K \simeq 15$ (Ballinger and Long, 1959) for benzyl alcohols. The large change in pK that occurs upon conversion of aldehyde to alcohol and the observation that the pK of the catalyst is intermediate between the pK values of substrate and product would support a role for concerted acid–base catalysis in the yeast alcohol dehydrogenase reaction. Concerted proton transfer as illustrated in A may be characterized by a transition state in which the transferring proton is in a potential energy well, as has been discussed for similar reactions in solution (Swain et al., 1965; Schowen, 1972; Choi and Thornton, 1974).

Protonated radical intermediates have been proposed for the reduction of carbonyls by FAD. The free energy for formation of $\cdot CH_2OH$ was concluded to be less than the free energy of activation for the conversion of formaldehyde and FAD_{red} to methanol and FAD_{ox}, consistent with a role for $\cdot CH_2OH$ as an intermediate. The reaction was described in terms of a rate-limiting *formation* of the protonated radical intermediate (Williams et al., 1975). A comparison of pK values for the ionization of α-hydroxy radicals [e.g., $CH_3\dot{C}H\text{-}OH \rightleftharpoons CH_3CHO^- + H_3O^+$, p$K = 11.51$ (Laroff and Fessenden, 1973)] to the pK of the presumed active-site base in yeast alcohol dehydrogenase (p$K = 8.25$) indicates that protonated radicals, such as illustrated in B, rather than radical anions would be intermediates in a stepwise enzyme reaction.

In an effort to distinguish between A and B, it is of value to consider the deuterium isotope effects observed in the yeast alcohol dehydrogenase reaction. The magnitude of these effects (Table II and Klinman, 1972) indicates a single rate-limiting hydrogen transfer step for the interconversion of a series of aromatic substrates, with the exception of p-CH_3 benzyl alcohol oxidation for which product release is partially rate limiting. For B to be operative in aldehyde reduction, the formation of protonated radical intermediate would be required to be rapid, relative to a rate-limiting hydrogen atom abstraction from $NADH^{\cdot+}$. The observed linear relationship between log k_R and σ^+ leads to the same conclusion regarding a required preequilibrium formation of protonated radical intermediates. Preliminary reports of Kurz and Frieden (1975) suggest that the glutamate dehydrogenase catalyzed reduction of para-substituted dinitrobenzenesulfonates by NADH and NADD is characterized by large deuterium isotope effects and a linear relationship between log k and σ^-, consistent with a single rate-limiting step for the conversion of substrate to product. Although A and B are both consistent with the "uncharged" nature of the transition state in the yeast alcohol dehydrogenase reaction, there is no evidence to date implicating kinetic intermediates in the reactions catalyzed by dehydrogenases. We are currently exploring the use of solvent deuterium isotope effects to distinguish these mechanisms.

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[5] Yeast alcohol dehydrogenase is a zinc-containing enzyme, and it is not possible to exclude a direct coordination of substrate to an active-site zinc in the course of catalysis. However, as discussed in detail elsewhere (Klinman, 1975), the observed titration curves for aldehyde reduction and alcohol oxidation support a role for a single active-site side chain of pK = 8.25 in acid–base catalysis of the hydrogen transfer step.

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