A Study of the Oxidation of Butan-1-ol and Propan-2-ol by Nicotinamide–Adenine Dinucleotide Catalysed by Yeast Alcohol Dehydrogenase

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1. The kinetics of oxidation of butan-1-ol and propan-2-ol by NAD^+ , catalysed by yeast alcohol dehydrogenase, were studied at 25°C from pH 5.5 to 10, and at pH 7.05 from 14° to 44°C. 2. Under all conditions studied the results are consistent with a mechanism whereby some dissociation of coenzyme from the active enzyme– NAD^+ –alcohol ternary complexes occurs, and the mechanism is therefore not strictly compulsory order. 3. A primary ^2H isotopic effect on the maximum rates of oxidation of $[1\text{-}^2\text{H}_2]\text{butan-1-ol}$ and $[^2\text{H}_7]\text{propan-2-ol}$ was found at 25°C over the pH range 5.5–10. Further, in stopped-flow experiments at pH 7.05 and 25°C, there was no transient formation of NADH in the oxidation of butan-1-ol and propan-2-ol. The principal rate-limiting step in the oxidation of these substrates appears to be the catalytic interconversion of ternary complexes. 4. The dependence on pH of the maximum rates of oxidation of butan-1-ol and propan-2-ol is consistent with the possibility that histidine and cysteine residues may affect or control catalysis.

Previous work has indicated that the maximum rate of ethanol oxidation by NAD^+ , catalysed by yeast alcohol dehydrogenase (EC 1.1.1.1), is determined chiefly by the rate of dissociation of the product enzyme–NADH complex (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975*a*). The fact that at pH 7.05 and 25°C the maximum rates of oxidation of propan-1-ol, propan-2-ol and butan-1-ol are much slower than for ethanol has indicated that with these substrates the rate-limiting step must be situated elsewhere in the mechanism (Dickinson & Monger, 1973). A likely candidate for this role is the catalytic step in which a hydride ion is transferred to the nicotinamide moiety of the oxidized coenzyme. There are, however, other possibilities. In the present work we have sought and obtained evidence that the mechanism previously proposed for these substrates (Dickinson & Monger, 1973) applies over a range of pH and temperature, and that the catalytic step is rate-limiting in the oxidation of these substrates. As a consequence of this finding we have studied the pH- and temperature-dependence of the maximum velocities of butan-1-ol and propan-2-ol oxidation, with a view of obtaining information about ionizing groups within the active site affecting or controlling catalysis.

Materials and Methods

Materials

Reagent solutions were prepared in glass-distilled water. EDTA at a final concentration of 0.3 mM was

included in enzyme assays and dialysed enzyme preparations.

Crystalline alcohol dehydrogenase was prepared from air-dried baker's yeast as described by Dickinson (1970, 1972). NAD^+ was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K., and was purified by chromatography on DEAE-cellulose before use (Dalziel, 1963*a*). LiAlH_4 and analytical-grade propan-1-ol, propan-2-ol and butan-1-ol were obtained from Fisons Ltd., Loughborough, Leics., U.K. LiAl^2H_4 and $[^2\text{H}_8]\text{propan-2-ol}$ were obtained from Prochem BOC Ltd., Deer Park Road, London S.W.19, U.K.

$[1\text{-}^2\text{H}_2]\text{Butan-1-ol}$ was prepared by reaction of *n*-butyric acid (BDH Chemicals Ltd., Poole, Dorset, U.K.) with excess of LiAl^2H_4 . The redistilled product had b.p. 120°C. Butan-1-ol prepared in a similar manner, by using LiAlH_4 , had the same boiling point and, at a concentration of 0.55 M with $685\text{ }\mu\text{M-NAD}^+$ and 1 nM-yeast alcohol dehydrogenase gave the same initial rate of reaction at pH 8.8 and 25°C, as commercial analytical-grade butan-1-ol.

Initial-rate measurements

Assays were performed fluorimetrically in duplicate as described by Dickinson & Monger (1973). Rates from duplicate assays usually agreed to within 5%. The fluorimeter was calibrated over the temperature range 15–45°C by adopting the procedures of Dalziel (1961, 1963*c*). From pH 5.5 to 8, sodium phosphate buffers, 1 0.1 mol/l, were used. Above

pH 8, 5 mM-sodium pyrophosphate buffers, containing sufficient Na_2HPO_4 to give a final I of 0.1 mol/l, were used.

Under the conditions used, with all substrates, Lineweaver-Burk plots were linear, and the kinetic coefficients (ϕ_0 etc.) in the initial-rate equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (1)$$

were obtained from the slopes and intercepts of the double-reciprocal plots as described by Dalziel (1957). In this equation e is the concentration of enzyme active sites and S_1 and S_2 are coenzyme and substrate respectively. The concentration of active sites (e) is calculated on the basis of two active sites/molecule (Dickinson, 1974) of molecular weight 141 000 (Buhner & Sund, 1969), which is the same basis as that used in other kinetic studies (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975a). The kinetic coefficients obtained from experiments at pH 7.05 and 25°C in this work generally agreed to within 10% with those obtained by Dickinson & Monger (1973). The coefficients in Tables 1-3 are therefore probably accurate to within $\pm 10\%$.

Transient studies

The reactions of NAD^+ with propan-1-ol, butan-1-ol and propan-2-ol, under approximately maxi-

mum-rate conditions, were studied by using a stopped-flow spectrophotometer based on the design of Gibson & Milnes (1964). This instrument was equipped with a Bausch and Lomb high-intensity monochromator and a constant-temperature water bath. The light source was a 50 W tungsten halide lamp, screened by a Corning 9863 filter to absorb stray light of long wavelengths. Solutions of enzyme, and of substrate alcohol + NAD^+ , were mixed, and the reaction was followed for up to 50 or 1000 ms, for the slower reactions, by the decrease in transmission at 340 nm in a 2 cm-path-length cell. The dead-time of the apparatus was 7 ms.

Results

The initial-rate parameters describing the oxidation of butan-1-ol and propan-2-ol by NAD^+ with yeast alcohol dehydrogenase, at 25°C and in the pH range 5.5-10, are shown in Table 1. The initial-rate parameters for the same reactions at pH 7.05 and in the temperature range 14-44°C are shown in Table 2. The ranges of concentration of coenzyme and substrates used in these experiments were: NAD^+ , 50-2000 μM ; butan-1-ol, 27-135 mM; propan-2-ol, 25-500 mM.

Table 3 shows the values of the reciprocal of the maximum specific rate of oxidation (ϕ_0) of butan-1-ol, $[\text{}^2\text{H}_2]\text{butan-1-ol}$, propan-2-ol and $[\text{}^2\text{H}_7]\text{propan-2-ol}$,

Table 1. Variation with pH of the kinetic coefficients for the oxidation of butan-1-ol and propan-2-ol by NAD^+ with yeast alcohol dehydrogenase at 25°C

The kinetic coefficients are those in the reciprocal initial-rate equation:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}$$

where S_1 is NAD^+ and S_2 the substrate alcohol. The Michaelis constant for NAD^+ is ϕ_1/ϕ_0 and that for the substrate is ϕ_2/ϕ_0 .

Substrate ...		Butan-1-ol				Propan-2-ol			
		ϕ_0	ϕ_1	ϕ_2	ϕ_{12}		ϕ_0	ϕ_1	ϕ_2
pH	(s)	($\mu\text{M}\cdot\text{s}$)	($\mu\text{M}\cdot\text{s}$)	($\text{mm}^2\cdot\text{s}$)		pH	(s)	($\mu\text{M}\cdot\text{s}$)	($\mu\text{M}\cdot\text{s}$)
5.55	0.18	70	7000	1.56		5.65	0.395	360	97000
5.9	0.087	46	5000	1.12		6.05	0.245	230	66000
						6.35	0.165	160	49000
						6.85	0.118	98	27000
7.05*	0.040	9.8	1280	0.20		7.05*	0.074	50	19000
7.05	0.038	10.0	1630	0.37		7.05	0.079	74	19000
						7.45	0.050	30	10000
7.75	0.017	4.9	720	0.15		7.85	0.032	20	6900
8.0	0.012	3.4	660	0.12		8.1	0.030	18	6000
8.3	0.010	2.8	480	0.18		8.45	0.025	19	5900
8.6	0.0076	2.7	280	0.20		8.65	0.017	21	4800
8.9	0.0064	3.0	300	0.25					
9.45	0.0053	2.3	220	0.16		9.2	0.013	14	2700
9.9	0.0055	2.9	230	0.13		9.6	0.012	16	3500

* Results of Dickinson & Monger (1973).

Table 2. Variation with temperature of the kinetic coefficients for the oxidation of butan-1-ol and propan-2-ol by NAD⁺ with yeast alcohol dehydrogenase at pH 7.05

The kinetic coefficients are those in the reciprocal initial-rate equation shown in Table 1, where again S₁ is NAD⁺ and S₂ the substrate alcohol. The Michaelis constant for NAD⁺ is ϕ_1/ϕ_0 and that for the substrate is ϕ_2/ϕ_0 .

Substrate	Temperature (°C)	ϕ_0 (s)	ϕ_1 ($\mu\text{M}\cdot\text{s}$)	ϕ_2 ($\mu\text{M}\cdot\text{s}$)	ϕ_{12} ($\text{mm}^2\cdot\text{s}$)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (mm)	ϕ_{12}/ϕ_2 (μM)
Butan-1-ol	15.6	0.083	12	2900	0.42	140	34	150
	36.8	0.018	15	820	0.46	800	45	570
	43.0	0.006	24	430	0.55	3860	70	1280
Propan-2-ol	14.8	0.264	76	58000	6.6	290	220	115
	35.0	0.034	63	10000	6.2	1830	290	620
	42.5	0.017	74	5900	7.7	4300	350	1290

Table 3. Reciprocal maximum specific rates of oxidation (ϕ_0) of butan-1-ol, [1-²H₂]butan-1-ol, propan-2-ol and [2-H₇]propan-2-ol by NAD⁺ with yeast alcohol dehydrogenase at 25°C and at various pH values

The isotope effects shown are calculated from the ratio of ϕ_0 for the [²H]alcohol to ϕ_0 for the [¹H]alcohol.

pH	ϕ_0 for butan-1-ol (s)	ϕ_0 for [1- ² H ₂]butan-1-ol (s)	Isotope effect on ϕ_0 for [1- ² H ₂]butan-1-ol	ϕ_0 for propan-2-ol (s)	ϕ_0 for [2-H ₇]propan-2-ol (s)	Isotope effect on ϕ_0 for [2-H ₇]propan-2-ol
5.55	0.18	0.84	4.7			
5.65				0.42	2.9	6.9
7.05	0.039	0.145	3.7	0.096	0.208	2.2
7.9				0.027	0.074	2.7
9.45	0.0053	0.0215	4.1			
9.55				0.0126	0.0215	1.7

Table 4. Oxidation of alcohols by 1.95 mM-NAD⁺ with 3.6 μM -yeast alcohol dehydrogenase at pH 7.05 and 25°C, studied by using a stopped-flow spectrophotometer

v_0/e is the specific rate of the reaction. Each measured v_0/e value is the mean of two to four determinations which agreed to within 10%. The calculated v_0/e was obtained by using eqn. (1) with the kinetic coefficients in Table 1, or those of Dickinson & Monger (1973) (indicated by *). The contribution of 3.6 μM -alcohol dehydrogenase to the blank E_{340} was 0.03. The values of E_{340} at the time of mixing equal volumes of 7.2 μM -alcohol dehydrogenase with 3.9 mM-NAD⁺+substrate alcohol were obtained by extrapolation of the plots of E_{340} versus time. All E_{340} values quoted are those in a cell of 2 cm path-length.

Substrate	v_0/e (s ⁻¹)		Sum of blank E_{340} of each reagent separately	E_{340} at time of mixing (by extrapolation)
	Measured	Calculated		
0.25 M-Propan-1-ol	46	56*	0.234	0.240
0.135 M-Butan-1-ol	20	18	0.256	0.254
0.5 M-Propan-2-ol	8.4	6.7	0.234	0.236

at 25°C and various pH values. Also shown for each of these pH values are the values of the kinetic isotope effect on the maximum specific rates of oxidation of butan-1-ol compared with [1-²H₂]butan-1-ol, and of propan-2-ol compared with [2-H₇]propan-2-ol. With [²H]alcohols a restricted range of coenzyme and substrate concentrations was used, because of the small quantities of substrates available. The concentrations were: NAD⁺, 440–1800 μM ; butan-1-ol and [1-²H₂]butan-1-ol, 27–135 mM; propan-2-ol and [2-H₇]propan-2-ol, 150–500 mM. The absence of low concentrations of coenzyme and substrate prevented accurate determination of parameters other than ϕ_0 .

Table 4 shows the results of experiments to determine the time-course of the decrease in transmission at 340 nm on mixing NAD⁺+propan-1-ol, butan-1-ol or propan-2-ol with yeast alcohol dehydrogenase in the stopped-flow spectrophotometer. The final concentrations of enzyme, coenzyme and substrates in the reaction mixtures were: enzyme, 3.6 μM ; NAD⁺, 1.95 mM; propan-1-ol, 0.25 M; butan-1-ol, 0.135 M; propan-2-ol, 0.5 M. Reactions were performed at pH 7.05 and 25°C, and proceeded under approximately maximum-rate conditions, coenzyme and substrate concentrations being at least twice the respective Michaelis constants (Table 1; Dickinson

& Monger, 1973). Table 4 shows, for each substrate, the measured specific steady-state rate (v_0/e), the extrapolated value of E_{340} at the time of mixing of the reactants, and the total blank value of E_{340} owing to the absorption of the reactants before mixing. Also shown is the expected value of the specific rate, calculated from eqn. (1) by using the initial-rate parameters obtained from steady-state kinetics (Table 1; Dickinson & Monger, 1973). Table 4 shows that the measured initial rate was, within experimental error, the same as that expected from the parameters ϕ_0 etc., determined previously. Also there was no transient formation of NADH before the steady-state rate was established, for any of the substrates tested. With $3.6 \mu\text{M}$ -alcohol dehydrogenase, used in these experiments, the transient formation of 1 mol of NADH/mol of enzyme would have given an E_{340} increase of 0.05.

Discussion

Dickinson & Monger (1973) showed that the kinetics of oxidation of ethanol, propan-1-ol, butan-1-ol and propan-2-ol by NAD^+ , catalysed by yeast alcohol dehydrogenase at pH 7.05 and 25°C , could be described by the mechanism shown in Scheme 1 and by eqn. (2) below:

$$\frac{e}{v_0} = \frac{1}{k'_{-1}} + \frac{1}{k'_{-3}} + \frac{A}{k} + \left\{ \left(\frac{Ak_{-4}}{kk_{+4}} + \frac{1}{k_{+1}} \right) \left(\frac{1}{[S_1]} \right) \right\} + \left\{ \left(\frac{Ak_{-3} + k}{kk_{+3}} \right) \left(\frac{1}{[S_2]} \right) \right\} + \left\{ \left[\frac{k_{-1}(Ak_{-3} + k)}{k_{+1}kk_{+3}} \right] \left(\frac{1}{[S_1][S_2]} \right) \right\} \quad (2)$$

with $A = (k' + k'_{-3})/k'_{-3}$.

Eqn. (2) may be derived for Scheme 1 provided that conditions are chosen so that the flux through the lower pathway (via ES_2) is negligible (Dalziel & Dickinson, 1966). The conditions mean that ES_2 arises principally by dissociation of NAD^+ from the reactant ternary complex.

Dickenson & Dickinson (1975a) showed that the above mechanism describes the oxidation of ethanol and the reduction of acetaldehyde and butyraldehyde over the range pH 6–9 at 25°C and over the range 15 – 44°C at pH 7.05. The present work indicates that the mechanism also provides a satisfactory description of events in the oxidation of butan-1-ol and propan-2-ol over a similar range of pH and temperature. The important points are as follows.

(1) As required by eqn. (2), both substrates yielded Lineweaver–Burk plots, and associated secondary plots of slopes and intercepts, which were strictly linear over all our experimental conditions.

(2) Arrhenius plots of $\log(1/\phi_0)$, $\log(1/\phi_1)$ etc. versus $1/T$ were generally linear, within experimental error (see Fig. 1 for example), suggesting that the physical significance of the parameters did not alter over the range of temperature used.

(3) Comparison of the results of Tables 1 and 2 with the results of previous work on ethanol oxidation (Dickenson & Dickinson, 1975a) shows that, under all conditions, the initial-rate parameters increase substantially on moving from ethanol through butan-1-ol to propan-2-ol. The large increases in ϕ_1 are particularly important, since they demonstrate that neither a strict simple compulsory-order mechanism, or a variant involving isomeric enzyme–coenzyme complexes, can explain the oxidation of butan-1-ol or propan-2-ol. Both of these mechanisms require ϕ_1 to be independent of the nature of the substrate (Dalziel, 1957, 1963b). On the other hand, eqn. (2) predicts the variation in ϕ_1 with change of substrate. According to eqn. (2) $\phi_1 > 1/k_{+1}$, and it is this inequality that results in the observed ratios $\phi_1\phi_2/\phi_{12}\phi_0' = 175$ and 9 , at pH 7.05 and 8.9 respectively, for the butan-1-ol–butyraldehyde reaction at 25°C . [ϕ_0' is the reciprocal maximum velocity of butyraldehyde reduction, obtained from Dickinson & Dickinson (1975a).] Such large values of this ratio are quite incompatible with a simple compulsory-order mechanism (Dalziel, 1957).

(4) Although the values of ϕ_2 and ϕ_{12} may change considerably at any pH and temperature on changing from ethanol to butan-1-ol or propan-2-ol, the ratio ϕ_{12}/ϕ_2 is essentially constant. This may be observed in both Figs. 1 and 2. According to eqn. (2) $\phi_{12}/\phi_2 = K_{\text{E}\cdot\text{NAD}^+}$, the dissociation constant of the enzyme– NAD^+ binary complex, and so the insensitivity of this ratio to change in substrate is expected. At present the supposition that $\phi_{12}/\phi_2 = K_{\text{E}\cdot\text{NAD}^+}$ has only been confirmed by indirect measurement of $K_{\text{E}\cdot\text{NAD}^+}$ at pH 7.05 and 25°C (Dickinson, 1972).

(5) Scheme 1 predicts the Haldane relationship $\phi_{12}'[\text{H}^+]/\phi_{12} = K_{\text{eq}}$. (Dickinson & Monger, 1973). The relationship was reasonably well satisfied with ethanol and acetaldehyde as substrates (Dickenson & Dickinson, 1975a). Taking the present results for butan-1-ol with those for butyraldehyde (Dickenson & Dickinson, 1975a) we obtain $\phi_{12}'[\text{H}^+]/\phi_{12} = 1.8 \times 10^{-11} \text{ M}$ and $0.6 \times 10^{-11} \text{ M}$ at pH 7.05 and 8.9 respectively. These values can be compared with the equilibrium constant for butan-1-ol oxidation by NAD^+ , $K_{\text{eq}} = 0.9 \times 10^{-11} \text{ M}$ at 23.5°C (Dalziel, 1962).

We have shown that the results presented here are compatible with Scheme 1 and eqn. (2). It must be admitted, however, that the results could also be accommodated by a rapid-equilibrium, random-order mechanism. With ethanol as substrate such an alternative is not possible. Isotope-exchange experiments eliminate it even though they also indicate that a complex of the type enzyme–ethanol exists (Silverstein & Boyer, 1964). Isotope-exchange data are, unfortunately, not available with the present substrates. However, Hollis (1967), using nuclear-magnetic-resonance techniques, was unable to detect any significant interaction of ethanol with yeast alcohol

Scheme 1. Proposed mechanism for yeast alcohol dehydrogenase

Fig. 1. Variation with temperature of certain initial-rate parameters and ratios of initial-rate parameters describing the oxidation of butan-1-ol and propan-2-ol by NAD^+ with yeast alcohol dehydrogenase

Maximum specific rate ($1/\phi_0$) of butan-1-ol ($\circ$) and propan-2-ol ($\bullet$) oxidation at pH 7.05; variation of the ratio ϕ_{12}/ϕ_2 for the oxidation of ethanol ($\square$), butan-1-ol ($\triangle$) and propan-2-ol ($\blacktriangle$) at pH 7.05. The enthalpies of activation for the maximum specific rates of oxidation of butan-1-ol and propan-2-ol are 62 and 58 $\text{kJ}\cdot\text{mol}^{-1}$ respectively.

Fig. 2. Variation with pH of the kinetic coefficient ratio ϕ_{12}/ϕ_2 for the oxidation of ethanol ($\triangle$), butan-1-ol ($\circ$) and propan-2-ol ($\bullet$) by NAD^+ with yeast alcohol dehydrogenase at 25°C

The kinetic coefficients ϕ_{12} and ϕ_2 for the oxidation of butan-1-ol and propan-2-ol are those in Table 1. The coefficients for the oxidation of ethanol are from Dickinson & Dickinson (1975a).

dehydrogenase in the absence of NAD^+ . If the conclusion that ethanol does not interact with the enzyme in the absence of NAD^+ is reliable, then it is unlikely that butan-1-ol or propan-2-ol interacts with the free enzyme either, for they are poorer substrates than ethanol. We therefore conclude that Scheme 1, operating under the given conditions, offers the most likely description of events with these alcohols.

Fig. 3. Variation with pH of the maximum specific rates of oxidation ($1/\phi_0$) of ethanol (Δ), butan-1-ol ($\circ$) and propan-2-ol ($\bullet$) by NAD^+ with yeast alcohol dehydrogenase at 25°C

The values of ϕ_0 for the oxidation of butan-1-ol and propan-2-ol are those in Table 1. The values of ϕ_0 for the oxidation of ethanol are from Dickenson & Dickinson (1975a). ----, Dissociation curve for an ionizing group of $\text{pK}_a 8.2$.

The results of earlier work (Dickenson & Dickinson, 1975a) have suggested that at pH 7.05 the principal rate-limiting step in ethanol oxidation is the dissociation of the product enzyme-NADH complex. The fact that ϕ_0 values for butan-1-ol and propan-2-ol are much larger than for ethanol under all conditions shows that for these two substrates the maximum rate is determined by some other step(s) in the mechanism. This is confirmed for the butan-1-ol-butyraldehyde system by the observed ratios $\phi_1'\phi_2'/\phi_{12}'\phi_0 = 0.1$ and 0.35 at pH 7.05 and 8.9 respectively [values of ϕ_1' , ϕ_2' and ϕ_{12}' are from Dickenson & Dickinson (1975a)]. It is interesting that, on changing from ethanol to butan-1-ol or propan-2-ol, a larger value of ϕ_0 is accompanied by a larger value of ϕ_1 . This is understandable in terms of eqn. (2), which shows that a smaller value of k leads to a larger value of ϕ_1 . Table 4 shows that there is no detectable 'burst' of NADH formation on mixing enzyme with NAD^+ and the substrates propan-1-ol, butan-1-ol and propan-2-ol under approximately maximum-rate conditions. Approximate calculations based on initial-rate studies (Dickenson & Dickinson, 1975a) with acetaldehyde and NADH or $[4A\text{-}^2\text{H}]\text{NADH}$, and with reasonable values for k and k_{-3} and a minimum value for k' based on the lack of isotope effect on ϕ_0' , have indicated that in the ethanol-acetaldehyde reactions $k_{-3}' \gg k$. It seems likely that the same applies with butan-1-ol and propan-2-ol as substrates, and the results of Table 4 are entirely consistent with this view. The results of Table 4 also suggest that the maximum rates of oxidation of these alcohols are not limited significantly by a step involving the isomerization of the complex $\text{ES}_1'\text{S}_2'$ before dissociation of S_2' . These results, together with the observation that $1/k_{-1}'$ is substantially smaller than ϕ_0 for butan-1-ol and propan-2-ol at all pH values [from comparison

with the results of Dickenson & Dickinson (1975a)] suggest that for these substrates $\phi_0 \approx (k' + k_{-3}')/kk_{-3}'$ which if $k'/k_{-3}' \ll 1$ becomes $\phi_0 \approx 1/k$. There is no direct evidence whether the latter simplification is valid in the present case. However, acetone is such an exceedingly poor substrate for the enzyme (Dickinson & Monger, 1973) that it seems likely that $k_{-3}' \gg k'$ for the propan-2-ol-acetone system. The fact that the pH-dependences of $1/\phi_0$ for butan-1-ol and propan-2-ol at 25°C are so similar (Fig. 3) suggests that the same step(s) are rate-limiting with both alcohols.

The results in Table 3 provide evidence that hydride transfer from alcohol to NAD^+ is an important rate-limiting step in the oxidation of butan-1-ol and propan-2-ol. The primary isotope effects with butan-1-ol and $[1\text{-}^2\text{H}_2]\text{butan-1-ol}$ in the range pH 5.5–9.5 and for propan-2-ol and $[2\text{H}_7]\text{propan-2-ol}$ at pH 5.6 are substantial. The smaller isotope effects with propan-2-ol and $[2\text{H}_7]\text{propan-2-ol}$ at pH 7.05, 7.9 and 9.55 suggest possibly that additional step(s) are rate-determining under these conditions. However, as there is no other evidence bearing on this point, and since primary isotope effects in the range 2–15 are thought compatible with rate-limiting breakage of a carbon-hydrogen bond (Richards, 1970), it seems reasonable to suggest that the maximum rates of oxidation of butan-1-ol, propan-2-ol (and possibly propan-1-ol) are principally determined by the rate of conversion of the enzyme-NAD⁺-alcohol complex into the corresponding product complex.

It appears from Fig. 3 that the maximum rates of butan-1-ol and propan-2-ol oxidation are controlled by more than one ionizing group with pK values in the range 6.5–9.0. One may further note that the pH-dependence of ϕ_0 for ethanol is quite different from that shown by other alcohols. This confirms the earlier conclusion (Dickenson & Dickinson, 1975a)

that for ethanol oxidation the maximum velocity is determined by a different step in the mechanism, namely dissociation of the enzyme-NADH complex. The similar pH profiles of $1/\phi_0$ for butan-1-ol and propan-2-ol suggest that a similar curve would be obtained for the pH-dependence of k for ethanol as substrate if such measurements were possible. If this is true then the process shows quite a different pH-dependence from the same step in ethanol oxidation catalysed by horse liver alcohol dehydrogenase. The latter process was found to be controlled by a single group of $pK_a = 6.4$, tentatively identified as histidine (Brooks *et al.*, 1972).

The possibility that a histidine residue is directly involved in the catalytic step of oxidations catalysed by liver alcohol dehydrogenase now seems less likely. Eklund *et al.* (1974) have demonstrated that the active-site region of this enzyme mainly comprises a hydrophobic pocket and the only hydrophilic residues in the vicinity of the catalytic site are serine and threonine. Further, Morris & McKinley-McKee (1972) found the enzyme to be fairly insensitive to ethoxycarbonylation of histidine residues by diethyl pyrocarbonate and concluded that 'accessible' histidines do not play a role in substrate or coenzyme binding or in catalysis. For yeast alcohol dehydrogenase, however, modification of 1 histidine residue/subunit with diethyl pyrocarbonate causes total inactivation, and the modified enzyme, although still reacting almost normally with coenzyme, does not form ternary complexes (Dickenson & Dickinson, 1973, 1975b). It seems likely that a histidine residue is important for catalysis with the yeast enzyme and could be partly responsible for the pH variation of the maximum rate of butan-1-ol and propan-2-ol oxidation. A second residue which might be involved in catalysis and which might affect the pH-dependence of the maximum rates of butan-1-ol and propan-2-ol oxidation is the essential thiol (Harris, 1964). This group may be blocked by reaction with iodoacetamide without affecting NADH or NAD^+ binding to the enzyme, but the modified enzyme cannot form normal ternary complexes (Dickinson, 1972). A third moiety that may be involved is zinc, which is essential for activity and structural stability (Kägi & Vallee, 1960).

Neither histidine nor cysteine seem to be residues directly involved in hydride transfer in catalysis by liver alcohol dehydrogenase (Eklund *et al.*, 1974).

However, the primary and quaternary structures of the two alcohol dehydrogenases and their catalytic properties are so different (Jornvall, 1974; Dickinson & Monger, 1973; Dickenson & Dickinson, 1975a) that it need not be assumed that the active-site regions are entirely similar.

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