Estimation of Rate and Dissociation Constants Involving Ternary Complexes in Reactions Catalysed by Yeast Alcohol Dehydrogenase

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Stopped-flow studies of oxidation of butan-1-ol and propan-2-ol by NAD^+ in the presence of Phenol Red and large concentrations of yeast alcohol dehydrogenase give no evidence for the participation of a group of pK_a approx. 7.6 in alcohol binding. Such a group has been implicated in ethanol binding to horse liver alcohol dehydrogenase [Shore, Gutfreund, Brooks, Santiago & Santiago (1974) *Biochemistry* 13, 4185–4190]. The present result supports previous findings based on steady-state kinetic studies with the yeast enzyme. Stopped-flow studies of the yeast alcohol dehydrogenase-catalysed reduction of acetaldehyde by NADH in the presence of ethanol as product inhibitor indicate that the rate-limiting step is NAD^+ release from the enzyme– NAD^+ –ethanol product complex. This finding permits calculation of K_3 , the dissociation constant for ethanol from the enzyme– NAD^+ –ethanol complex, by using the product-inhibition data of Dickenson & Dickinson (1978) (*Biochem. J.* 171, 613–627). The calculations show that K_3 varies very little with pH in the range 5.95–8.9, and this agrees with the findings of the stopped-flow experiments described above. Absorption and fluorescence measurements on mixtures of substrates and coenzymes in the presence of high concentrations of alcohol dehydrogenase have been used to estimate values for the ratio $[\text{enzyme-NADH-acetaldehyde}]/[\text{enzyme-NAD}^+\text{-ethanol}]$ at equilibrium. The values obtained were in the range 0.11 ± 0.04 , and this value together with estimates of K_3 was used to provide estimates of values for rate constants and dissociation constants for steps within the catalytic mechanism.

The preceding paper (Dickenson & Dickinson, 1978) has provided estimates for the product-inhibition constants for ethanol at various pH values in the reduction of acetaldehyde by NADH catalysed by yeast alcohol dehydrogenase. To obtain estimates for the dissociation constants for ethanol from the catalytic enzyme– NAD^+ –ethanol complex by using the product-inhibition data, additional information is required (Dickenson & Dickinson, 1978). The equilibrium measurements and stopped-flow studies reported here provide this information and also allow estimation of other rate and dissociation constants in the overall catalytic mechanism. The results are discussed in relation to the role of known ionizations at the active site of the enzyme (Dickenson & Dickinson, 1975c, 1977) and permit some comparison with horse liver alcohol dehydrogenase (Brändén *et al.*, 1975).

Experimental

Materials

Glass-distilled water was used in the preparation of all solutions.

Enzymes. Yeast alcohol dehydrogenase was prepared and assayed as described by Dickinson (1970, 1972). The specific activity was 400 units ($\mu\text{mol}/\text{min}$)/mg of protein.

Coenzymes and substrates. NAD^+ (grade II) and NADH (grade I) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. Acetaldehyde (Fisons, Loughborough, Leics., U.K.) was redistilled before use. Analytical-grade alcohols (J. Burroughs, London S.E.11, U.K., or Fisons) and trifluoroethanol [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] were used without further treatment.

Stopped-flow studies

Measurements were made with a double-beam flow spectrophotometer modified from the design of

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Gibson & Milnes (1964). The principal modifications were in the use of fibre optics, the use of a ratiometer to measure the relative intensities of the two light beams, and in the provision of a stable variable backing-off potential. The ratiometer gave a nominal output of 10V to the oscilloscope when the input signals from the two photomultipliers were equal. The amplification of both input signals could be adjusted independently. The remainder of the optical and electronic equipment has been described previously (Bentley & Dickinson, 1974). The instrument was equipped with a constant-temperature water bath.

Proton production occurring in the yeast alcohol dehydrogenase-catalysed oxidation of propan-1-ol and butan-1-ol by NAD^+ was followed at 23°C and pH 7.6. A 2cm-path-length cell was fitted to the stopped-flow instrument and the dead time of the apparatus under these conditions was found to be approx. 6ms. The buffer in which all solutions were prepared was 0.5M-sodium phosphate containing 30mM- Na_2SO_4 and 20 μM -Phenol Red. Syringe 1 contained enzyme (20 μM -active sites) and syringe 2 contained 4.8mM- NAD^+ with either 600mM-propan-1-ol or 270mM-butan-1-ol. The pH of the solutions was adjusted immediately before the experiment so that the $A_{560}^{1\text{cm}}$ of each solution was the same (± 0.01). The actual $A_{560}^{1\text{cm}}$ was approx. 0.45. Reactions were followed by the decrease in transmission at 340 or 350nm (NADH production) and by the increase in transmission at 560nm (H^+ production). Comparison of the initial steady-state rates of reaction at 340 and 560nm gave a value of $A_{560} = 1.9 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ under the conditions used.

The reduction of acetaldehyde by NADH, catalysed by yeast alcohol dehydrogenase, in the presence of ethanol as product inhibitor was followed at pH 7.05 and pH 9.0 at 13.5°C. A 5mm-path-length cell was fitted to the stopped-flow apparatus and the measured dead time of the instrument was 3ms. Reactions were followed at 340nm. The buffers were the same as those used for steady-state kinetic studies at the same pH (Dickenson & Dickinson, 1978). Two sets of conditions were used; (a) syringe 1 contained enzyme (37 μM -active sites) and syringe 2 contained 650 μM -NADH, 50mM-acetaldehyde and 2.16M-ethanol; (b) syringe 1 contained enzyme (37 μM -active sites) premixed with 2.16M-ethanol, syringe 2 contained 650 μM -NADH and 50mM-acetaldehyde.

Measurement of ternary-complex concentrations in equilibrium mixtures

Yeast alcohol dehydrogenase in 5mM-sodium phosphate buffer, pH 7.0, was added to 1cm-path-length spectrofluorimetric cuvettes containing 0.7mM-acetaldehyde and 14mM-ethanol in buffers ($I = 0.1$) of various pH values at room temperature (17°C). The final enzyme concentration was 270 μM in active

sites and the phosphate and bicarbonate buffers were the same as those used in kinetic studies at the same pH (Dickenson & Dickinson, 1978). The A_{340} of the solution was first measured in a Zeiss PMQII spectrophotometer and the fluorescence in a Farrand Mark I spectrofluorimeter with excitation at 350nm, emission at 470nm and 10nm slits. Approx. 160 μM -NADH was then added and the readings were repeated. (The fluorescence standard was 6.6 μM -NADH and the sensitivity of the instrument was adjusted to give a signal of 0.1 μA with this solution.) The concentrations of acetaldehyde and ethanol were then increased stepwise in the ratio of 1:20 to reach final concentrations of 28mM and 560mM respectively. Absorbance and fluorescence readings were made immediately after each addition.

The final mixture (approx. 3ml) was filtered at 0.2–0.4 MPa (30–35 lb/in²) with an Amicon PM 10 ultrafilter. The first 0.3 ml of ultrafiltrate was collected and assayed for the combined concentration of NAD^+ and NADH. The assay was performed in a filter fluorimeter based on the design of Dalziel (1962). NAD^+ was converted into NADH by alcohol dehydrogenase in the presence of excess ethanol and the total fluorescence gave a measure of the total coenzyme concentration. Specifically the increase in fluorescence was noted after 0.1 ml of ultrafiltrate was added to 4.0 ml of 0.2M-glycine/NaOH buffer, pH 10.0, containing 0.8M-ethanol and approx. 10nM-yeast alcohol dehydrogenase. In some experiments equilibrium dialysis was used as an alternative to ultrafiltration for determining the free coenzyme concentration. The equilibrium mixture (1 ml) was dialysed for 1½ h against 1 ml of the same buffer containing the same acetaldehyde and ethanol concentrations. The dialyses were performed at room temperature in small test tubes attached to a slowly rotating wheel and the mixtures were thus inverted once in each revolution. Trial experiments showed that equilibrium was reached within 1½ h. At equilibrium, the solutions from inside and outside the dialysis sacs were assayed fluorimetrically for the total concentration of NAD^+ and NADH as described above. The total concentration of enzyme-bound coenzyme was calculated from the difference in total concentration in solutions from the inside and outside of the dialysis sacs.

Results and Discussion

Proton production during oxidation of propan-1-ol and butan-1-ol

The oxidation of 300mM-propan-1-ol, and of 135mM-butan-1-ol, by 2.4mM- NAD^+ , with a 20 μM -active-site concentration of yeast alcohol dehydrogenase, was followed at 23°C in the stopped-flow spectrophotometer. The buffer was 0.5mM-phosphate,

pH 7.6, including 30 mM- Na_2SO_4 and 20 μM -Phenol Red. The substrate concentrations were approximately saturating (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975b). When the reaction was followed at 350 nm, neither alcohol gave a significant pre-steady-state burst of NADH production. The measured specific rates for propan-1-ol and butan-1-ol (50 and 20 s^{-1} respectively, are consistent with previously determined kinetic parameters (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975b). Under the conditions used, a burst of 0.2 mol of NADH/mol of enzyme would have been readily detected. The absence of burst confirms the result found previously at pH 7.05 (Dickenson & Dickinson, 1975b), which was attributed to the maximum specific rate being determined by the interconversion of ternary complexes. When the same reactions were followed by the decrease in A_{560} of Phenol Red, again no burst (of H^+ production) was observed, 0.2 mol of H^+ /mol of enzyme being detectable. The result shows that, in the region of pH 7.6, no proton uptake or release takes place when the enzyme combines with NAD^+ and alcohol. Protons are evidently released at some later stage in the reaction mechanism and at the same rate as NADH production. These results indicate that on formation of the ternary complex enzyme- NAD^+ -alcohol there are no marked changes in pK_a of ionizing groups at the active site. Previous work has shown that there are no marked changes in pK_a when NAD^+ combines with enzyme in the range pH 6–8. The present result indicates that the same is true for the combination of propan-1-ol or butan-1-ol with the enzyme- NAD^+ complex, and the latter result confirms the earlier conclusion that the dissociation constant for butan-1-ol from enzyme- NAD^+ -butan-1-ol is independent of pH (Dickenson & Dickinson, 1977). The same is probably true for propan-2-ol (Dickenson & Dickinson, 1977).

The experiments described here are not possible with ethanol as substrate, because the principal rate-limiting step is at a later stage of the reaction (Dickenson & Dickinson, 1975a). Although a pre-steady-state burst of NADH production would be expected, the rate at 25°C is too high ($>450 \text{ s}^{-1}$) to be measured with our stopped-flow spectrophotometer.

When yeast alcohol dehydrogenase (final concn. 20 μM -active sites) was mixed with NAD^+ and 30 or 300 mM-trifluoroethanol in the stopped-flow spectrophotometer (conditions as for propan-1-ol and butan-1-ol) there was proton production equivalent to about 0.5 mol of H^+ /mol of active site, occurring with a half-time of approx. 5 ms. There was no observable steady-state rate. The observation of a proton burst is consistent with perturbation of the pK_a of a group resulting from the combination of trifluoroethanol with enzyme- NAD^+ . Dickenson & Dickinson (1978) showed that

the dissociation constant for trifluoroethanol from the ternary enzyme- NAD^+ -trifluoroethanol complex varies with pH, though not in the manner expected from dependence on a single ionizing group.

Reduction of acetaldehyde by NADH in the presence of ethanol

The reaction of yeast alcohol dehydrogenase with saturating concentrations of acetaldehyde and NADH, in the presence of 1.08 M-ethanol, was examined in the stopped-flow spectrophotometer at 13.5°C. It was not possible to study this reaction at 25°C, since, even with this high concentration of product inhibitor, the maximum rate of acetaldehyde reduction is over 500 s^{-1} (Dickenson & Dickinson, 1978). When enzyme (37 μM -active sites) was mixed with an equal volume of a solution containing 50 mM-acetaldehyde, 650 μM -NADH and 2.16 M-ethanol, a burst of NADH disappearance equivalent to 1.0 ± 0.2 mol of NADH/mol of enzyme was observed at both pH 7.05 and 9.0, occurring within the dead time of the instrument. The result confirms that the rate-limiting step occurs after hydride transfer. The results in the preceding paper (Table 2 of Dickenson & Dickinson, 1978) indicate that, under the conditions used, the enzyme is saturated with respect to substrate, coenzyme and product inhibitor and that the measured rate is governed by the rate of product dissociation by the pathway routed through the binary enzyme-ethanol complex. According to Dickenson & Dickinson (1978) the inhibited reciprocal maximum rate at saturating ethanol concentrations is given by:

$$\phi'_{0, \text{app.}} = \frac{1}{k_{-2}} + \frac{1}{k_{-4}} \left(1 + \frac{k}{k'} \right) + \frac{1}{k'} \quad (1)$$

with the rate constants being defined in the mechanism shown in Scheme 1. Eqn. (1) simplifies to:

$$\phi'_{0, \text{app.}} = \frac{1}{k_{-2}} + \frac{1}{k_{-4}} \quad (2)$$

because, as shown earlier (Dickenson & Dickinson, 1975a) $k \ll k'$ and $k' \gg 1/\phi'_0$ (i.e. k' is much greater than the uninhibited maximum rate). The results of Dickenson & Dickinson (1978) also suggest that, at pH 7.05 and 13.5°C, the dissociation constant (K_2) for the enzyme-ethanol complex is approx. 0.7 M. Therefore, when a solution of enzyme and approx. 2 M-ethanol is mixed with a solution of acetaldehyde and NADH, then at the moment of mixing over 50% of the enzyme will be in the form of enzyme-ethanol complex. If the overall reaction is limited by k_{-2} , the rate of dissociation of this complex [which must occur before enzyme can combine with more substrate (NADH and acetaldehyde)], then over 50%

Scheme 1. *Proposed mechanism for yeast alcohol dehydrogenase*

Detailed initial-rate studies (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975a, 1978) have indicated that the mechanism is actually a 'preferred-order mechanism' in the direction of ethanol oxidation with the main flux going through the upper pathways. The E·ethanol complex is kinetically significant in the forward direction and for acetaldehyde reduction in the presence of high ethanol concentrations (Dickenson & Dickinson, 1978). For acetaldehyde reduction in the absence of product inhibitor, the reaction takes place almost exclusively through the upper pathways and the mechanism is compulsory (Dickenson & Dickinson, 1975a). Conventional steady-state studies provide no evidence for the formation and dissociation of the E·NADH·acetaldehyde complex via the binary E·acetaldehyde complex. Though this limb of the mechanism is not normally kinetically significant, isotope-exchange studies at equilibrium have provided some evidence for it (Silverstein & Boyer, 1964).

of the burst should be abolished because the slowest step now precedes the formation of enzyme-bound NAD⁺. If, however, $k_{-2} \gg k_{-4}$, i.e. $\phi'_{0, \text{app.}} \gg 1/k_{-2}$, then the prior dissociation of enzyme-ethanol complex will not limit the rate of the overall reaction and the time course of NADH disappearance will be the same as in the experiment where enzyme was not pre-mixed with ethanol. The analysis here assumes that there will be no complication involved by the formation of an abortive complex enzyme-NADH-ethanol. Previous studies (Dickenson & Dickinson, 1978) have not found evidence for the existence of this complex with yeast alcohol dehydrogenase.

At pH 7.05 and 13.5°C mixing of a solution of enzyme (37 μM-active site) and 2.18 M-ethanol with a solution of 650 μM-NADH and 50 mM-acetaldehyde resulted in a burst of NADH oxidation of 1 ± 0.2 mol enzyme/mol of active site within the dead time of the stopped-flow instrument. This result is the same as that recorded above when enzyme was mixed with the NADH/acetaldehyde/ethanol mixture at the same concentrations. It seems therefore that in this system $k_{-2} \gg k_{-4}$. The same inequality probably applies at 25°C too, since the reaction mechanism does not change with temperature (Dickenson & Dickinson, 1975a, 1978).

The experiments suggest that for yeast alcohol dehydrogenase at pH 7.05 the maximum rate of

acetaldehyde reduction in the presence of saturating ethanol concentrations simplifies to:

$$\frac{1}{\phi'_{0, \text{app.}}} = k_{-4}$$

This finding, together with the knowledge that $k_{-1} = 1/\phi'_0$ (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975a) allows calculation of a value for K_3 , the dissociation constant of ethanol from the catalytic enzyme-NAD⁺-ethanol ternary complex from the product inhibition constant $K^{\text{denom.}} = k_{-1} K_3/k_{-4}$ (Dickenson & Dickinson, 1978).

At pH 9.0, the stopped-flow experiment produced a result similar to that obtained at pH 7.05. However, at pH 9.0 the value for the dissociation constant of the enzyme-ethanol complex, K_2 , is not known (but is greater than 0.6 M), because at 25°C the overall reaction approximates to a compulsory-order mechanism. It is possible, therefore, that a mixture of enzyme and 2.16 M-ethanol does not contain a significant proportion of enzyme in the form of the enzyme-ethanol complex. However, for a compulsory mechanism, Petterson (1972) has shown that $k_{-4} \ll k$ and k_{-3} . At pH 9.0 and 25°C, k probably does not exceed approx. 4000 s^{-1} , and at saturating ethanol concentrations

$$\phi'_{0, \text{app.}} = \frac{1}{k_{-4}} + \frac{1}{k_{-2}} = 400 \text{ s}^{-1}$$

(Dickenson & Dickinson, 1975*a*, 1978). It seems likely that if $k_{-4} \ll k$ then $k_{-4} \approx 1/\phi_{0, \text{app.}}$ at saturating ethanol concentrations, so that K_3 may be obtained as at pH 7.05.

Estimates of K_3 , calculated from $K^{\text{denom.}} = K_3(k_{-1}/k_{-4})$ on the basis of $\phi'_0 = 1/k_{-1}$ and $\phi'_{0, \text{app.}}([\text{ethanol}] \rightarrow \infty) = 1/k_{-4}$ are given in Table 1 for the range pH 6–9 at 25°C.

Equilibrium measurements

The effect of increasing the concentrations of acetaldehyde and ethanol (in the ratio 1:20) on the A_{340} and fluorescence of a mixture of enzyme (270 μM -active sites) and 160 μM -NADH at pH 7.05 is shown in Fig. 1. Very similar results were obtained at pH 7.8 and 8.9. It is expected that as the concentrations of acetaldehyde and ethanol increase, the equilibrium mixture of NADH and NAD^+ will be forced increasingly into the ternary complexes enzyme– NAD^+ –ethanol and enzyme–NADH–acetaldehyde, with very little coenzyme free or in the binary complexes (see Scheme 1). The constant A_{340} obtained with high substrate concentrations is consistent with the perturbation of the equilibrium mixture by the high enzyme concentration [cf. the value of A_{340} reached with very low enzyme concentrations (1 μM); Fig. 1]. The fluorescence of the bound NADH decreased to a constant value, suggesting that a ternary enzyme–NADH–substrate complex was formed with a specific fluorescence much less than that of E–NADH. The data of Fig. 1 also suggest that the absorption coefficient for NADH in the complex might be slightly decreased, but are not sufficiently detailed to establish the point. Since the absorption coefficient of the bound NADH is similar to that of free NADH at 340 nm (Dickenson, 1975), the results of Fig. 1 indicate that the enzyme-bound NADH has a fluorescence roughly equivalent to that of free NADH under our conditions. The complex giving rise to the A_{340} values is therefore not the binary E–NADH complex, for which the coenzyme fluorescence is enhanced (Duyens & Kronenberg, 1957). The observed quenching in Fig. 1 is most

Fig. 1. Effect of increasing substrate concentrations on the absorption and fluorescence of equilibrium mixtures of substrates and coenzymes in the presence of yeast alcohol dehydrogenase at pH 7.0 and 17°C

The [ethanol]/[acetaldehyde] ratio was maintained at 1:20 while the ethanol concentration was increased stepwise from 0.015 to 0.52 M. The Figure records values for A_{340} with enzyme concentrations of (●) 1 μM - and (○) 270 μM -active sites and values for the fluorescence of the mixture at 460 nm (Δ) with an enzyme concentration of 270 μM -active sites. The measurements were carried out as described in the text.

probably due to the formation of the catalytic enzyme–NADH–acetaldehyde complex. The quenching contrasts with the much enhanced fluorescence of the inactive enzyme–NADH–acetamide complex (Dickinson, 1970), but it is noteworthy that in the lactate dehydrogenase–NADH–pyruvate complex and the glutamate dehydrogenase–NADH–2-oxoglutarate complexes the coenzyme fluorescence is severely quenched (Whitaker *et al.*, 1974; di Franco & Iwatsubo, 1972).

From the above it seems that in the equilibrium mixture at high enzyme concentration practically all the NADH will be present as the enzyme–NADH–acetaldehyde complex. The free coenzyme concentration ($\text{NAD}^+ + \text{NADH}$) in the mixture was obtained from measurements on the ultrafiltrate or by equilibrium dialysis. The equilibrium constant for ternary-complex interconversion [k/k'] (see Scheme 1) was calculated as follows. The species present in the mixture would be enzyme, NAD^+ , NADH, enzyme– NAD^+ , enzyme–NADH, enzyme– NAD^+ –ethanol, enzyme–NADH–acetaldehyde, enzyme–ethanol and possibly enzyme–acetaldehyde.

Table 1. Values for the dissociation constants of substrate from the enzyme– NAD^+ –ethanol complex (K_3) and the enzyme–NADH–acetaldehyde complex (K'_3) with yeast alcohol dehydrogenase at 25°C

Values for K_3 were obtained from $K^{\text{denom.}} = K_3 k_{-1}/k_{-4}$ and of K'_3 from $\phi'_2/\phi_2 = K'_3 k/K_3 k'$, as described in the text.

pH	K_3 (mM)	K'_3 (mM)
5.95	80	—
7.05	52	1.8
8.1	32	2.5
8.9	21	4.3

The concentrations of substrate greatly exceeded all others and were taken as the concentrations added initially. The ratio $[NAD^+]/[NADH]$ was obtained from the overall equilibrium constant for the reaction (Bäcklin, 1958), and $[NAD^+]+[NADH]$ was measured in the ultrafiltrate, so that $[NAD^+]$ and $[NADH]$ could be determined. The latter was negligible. Since the total coenzyme concentration was known and as the total NADH concentration ($[NADH]+[enzyme-NADH]+[enzyme-NADH-acetaldehyde]$) could be obtained from the limiting value of A_{340} at high substrate concentration (Fig. 1), so the total NAD^+ concentration ($[NAD^+]+[enzyme-NAD^+]+[enzyme-NAD^+-ethanol]$) was obtained. Using the estimate of $[NAD^+]$ we now had a value for the bound NAD^+ concentration ($[enzyme-NAD^+]+[enzyme-NAD^+-ethanol]$). For the above calculations the absorption coefficient at 340 nm for NADH bound or free, was taken to be $6.22 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Horecker & Kornberg, 1948; van Eys & Kaplan, 1958; Dickenson, 1975), although an estimate for the enzyme-NADH-acetaldehyde complex is not available. Taking the value for the dissociation constant of ethanol from the enzyme- NAD^+ -ethanol complex, K_3 (Table 1) and the ethanol concentration, the ratio $[enzyme-NAD^+]/[enzyme-NAD^+-ethanol]$ was obtained, giving values for $[enzyme-NAD^+]$ and $[enzyme-NAD^+-ethanol]$. The free enzyme concentration, $[E]$, was then calculated by using $[NAD^+]$, $[enzyme-NAD^+]$ and the dissociation constant K_1 of the enzyme- NAD^+ complex (Dickenson & Dickinson, 1975a). The use of $[E]$, $[NADH]$ and K_1' , the dissociation constant of the enzyme-NADH complex (Dickenson & Dickinson, 1975a) showed that $[enzyme-NADH]$ was small as predicted above, and hence $[enzyme-NADH-acetaldehyde]$ was ob-

tained. Values for $[enzyme-NADH-acetaldehyde]$ and $[enzyme-NAD^+-ethanol]$ lead to the estimate of k/k' , the equilibrium constant for the interconversion of ternary complexes. The values obtained in the pH range 7.05–8.9 are shown in Table 2 together with the estimated concentrations of all the species present in equilibrium mixtures. Similar experiments were also performed at pH 6.0, but were abandoned because of precipitation of the enzyme.

Although the above procedure is somewhat lengthy, it is emphasized that, in the equilibrium mixture, the principal coenzyme forms are enzyme- NAD^+ -ethanol and enzyme-NADH-acetaldehyde, with the latter being considerably less than the former. The precision of the experimental calculations depends on a significant measured A_{340} , and at all pH values that was at least 0.05. If it is assumed that there was no significant concentration of enzyme-acetaldehyde under the conditions used (Dickenson & Dickinson, 1975b) then an estimate may be made for K_2 , the dissociation constant of the enzyme-ethanol complex. The estimate arises from the values for $[E]$, $[ethanol]$ and $[enzyme-ethanol]$, i.e. total $[enzyme]-[E]-[coenzyme-containing complexes]$. The values obtained for K_2 are of the same order as those found earlier by kinetic methods (Dickenson & Dickinson, 1978).

The estimations of free coenzyme concentrations in equilibrium mixtures were obtained by ultrafiltration and by equilibrium dialysis. For the experiments using the ultrafiltration results, corrections have been applied to the data to take into account coenzyme binding to the filter. It was found that at the concentrations involved the filter bound approx. 50% of the NAD^+ passing through it. Coenzyme was not adsorbed to the side of the filter next to the equilibrium mixture. According-

Table 2. *Equilibrium concentrations (μM) of enzyme forms in equilibrium mixtures at high concentrations of yeast alcohol dehydrogenase at pH 7.05 and 25°C*

The total active site concentration was $270 \mu\text{M}$, total coenzyme $164 \mu\text{M}$ and the ethanol and acetaldehyde concentrations were 560 and 28 mM respectively. The calculations were made by using the available data as described in the text. k and k' are defined in Scheme 1.

Enzyme species	Free [coenzyme] estimated by using ultrafiltration			Free [coenzyme] estimated by using equilibrium dialysis	
	pH 7.05	pH 7.8	pH 8.9	pH 7.05	pH 8.0
E	75	26	104	108	71
E·NAD ⁺	10	5	4	10	6.4
E·NAD ⁺ ·ethanol	106	85	99	89	103
E·NADH	<1	0.5	5	0.5	0.6
E·NADH·acetaldehyde	8	13	15	11	11
E·ethanol	75	14	4	13	~60
NAD ⁺	40	55	34.5	27	27
NADH	<0.1	0.7	5.5	0.05	0.4
$\frac{[E \cdot NADH \cdot acetaldehyde]}{[E \cdot NAD^+ \cdot ethanol]} = \frac{k}{k'}$	0.075	0.15	0.15	0.12	0.11

ly the measured concentration of NAD^+ in the ultrafiltrate were corrected to account for that. Since the concentrations of free NAD^+ were in any case much lower than the total concentration of bound coenzyme, there was only a small change (less than 20%) in the estimated value of k/k' when the correction was applied. The results based on equilibrium dialysis in Table 2 did not require any correction for adsorption of coenzyme to membrane, and the values of k/k' obtained are in reasonable agreement with those based on the ultrafiltration results.

The calculations of the value for the ratio of [enzyme-NADH-acetaldehyde]/[enzyme-NAD $^+$ -ethanol] at equilibrium lead to an interesting point in connection with the site of proton liberation within the mechanism of alcohol oxidation. The relative constancy of this ratio over the pH range 7.05–8.9 indicates that, within this range, proton liberation is not coupled with hydride transfer. The results indicate that ternary-complex interconversion may be formulated as

with proton liberation at a later stage in the mechanism. Essentially the same proposal has been made on the basis of the variation of $\phi'_2[\text{H}^+]/\phi_2$ with pH (Dickenson & Dickinson, 1975a, 1977). Results obtained by two independent methods thus support the same conclusion. There is evidence that the group involved in proton binding to the enzyme is the essential histidine residue (Dickenson & Dickinson, 1977).

Estimation of other constants

The rate constants and dissociation constant discussed in this section are defined in Scheme 1.

The values of k/k' (Table 2) are consistent, within experimental error, with the earlier suggestion that k is very small compared with k' (Dickenson & Dickinson, 1975a, 1977). It was pointed out in the preceding paper (Dickenson & Dickinson, 1978) that the study of product inhibition by acetaldehyde could not provide a value for K'_3k_{-1}/k_{-4} , corresponding to the inhibition constant K_3k_{-1}/k_{-4} found from product inhibition by ethanol. This avenue for the possible determination of K'_3 , the dissociation constant of acetaldehyde from the enzyme-NADH-acetaldehyde complex, was therefore closed. However, for the reaction of substrates with the enzyme-bound coenzyme, the equilibrium constant is:

$$K = \frac{[\text{E} \cdot \text{NADH}][\text{acetaldehyde}]}{[\text{E} \cdot \text{NAD}^+][\text{ethanol}]} = \phi'_2/\phi_2 = K'_3k/K_3k'$$

from which K'_3 may be estimated by using the known values of ϕ'_2/ϕ_2 (Dickenson & Dickinson, 1975a), k/k' (Table 2) and K_3 (Table 1). Values of K'_3 obtained by this means are included in Table 1. From the results it appears that acetaldehyde binds more firmly to the enzyme-NADH complex when a group of pK approx. 8 is protonated. This suggestion has been made previously on the basis of the variation of ϕ'_2 with pH (Dickenson & Dickinson, 1977), and it was suggested that the group involved was the essential histidine.

Table 3 shows estimates of, or limits for, additional rate and equilibrium constants in the reaction mechanism with ethanol and acetaldehyde as substrates at pH 7.05 and 25°C. Some values were obtained from the literature as indicated and others were obtained as follows: similar calculations can obviously be made at other pH values in the range pH 6–9 where data are also available.

Table 3. Estimated values of rate constants and dissociation constants in the mechanism of yeast alcohol dehydrogenase at pH 7.05 and 25°C

The rate and dissociation constants are defined in Scheme 1. ^a Values obtained from Dickenson & Dickinson (1975a); ^b value obtained from product inhibition effects on $\phi_{1,\text{app}}$ as described by Dickenson & Dickinson (1978). Values of other coefficients were obtained as described in the text. Alternative estimates of $k_{+2} \gg 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{+4} = 7 \times 10^6 \text{ M}^{-1}$ and $K_4 = 140 \mu\text{M}$ are obtained if a lower value of $K_2 = 100 \text{ mM}$ is taken. The latter alternative estimate was obtained by calculation from values of the initial rate parameters ϕ_1 , ϕ_2 , ϕ_{12} and ϕ_0 as described by Dickenson & Dickinson (1978). At present we are unable to say which set of values is the more accurate, but it seems likely that a decision could be made by fitting the initial-rate equation for Scheme 1 to initial-rate data obtained for the enzyme-catalysed oxidation of ethanol by NAD^+ .

$^a K_1 = 270 \mu\text{M}$	$^a k_{+1} = 1.1 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$	$^a K'_1 = 11 \mu\text{M}$	$^a k'_{+1} = 4.4 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$
	$^a k_{-1} = 3900 \text{ s}^{-1}$		$^a k'_{-1} = 480 \text{ s}^{-1}$
$^b K_2 = 840 \text{ mM}$	$k_{+2} \gg 1.2 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$		
	$k_{-2} \gg 1000 \text{ s}^{-1}$		
$K_3 = 52 \text{ mM}$	$k_{+3} \gg 7.5 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$	$K'_3 = 1.8 \text{ mM}$	$k'_{+3} \approx 4.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$
	$k_{-3} \gg 3900 \text{ s}^{-1}$		$k'_{-3} \approx 11000 \text{ s}^{-1}$
$K_4 = 17 \mu\text{M}$	$k_{+4} = 5.9 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$		$k'_{-4} \approx 360 \text{ s}^{-1}$ (pH 7.9)
	$k_{-4} = 1000 \text{ s}^{-1}$		
	$^a k \approx 4000 \text{ s}^{-1}$		
	$k' \approx 35000 \text{ s}^{-1}$		

(1) Since $K_1 K_3 = K_2 K_4$, K_4 may be obtained and thence k_{+4} , since k_{-4} is known (see above).

(2) Since $k_{-2} \gg k_{-4}$ (see above) and K_2 is known (Dickenson & Dickinson, 1978), approximate lower limits for k_{+2} and k_{-2} are provided.

(3) The existence of a small isotope effect on ϕ_0 (Mahler & Douglas, 1957) allows a rough estimate of k , and hence a rough value for k' is obtained from k/k' .

(4) The procedure used by Dickenson & Dickinson (1975a) to obtain k'_{+3} also provides a value for k'/k'_{-3} (Dickenson, 1975), from which an estimate of k'_{-3} can be obtained. The value of K'_3 calculated from k'_{-3}/k'_{+3} may be compared with the value in Table 1.

(5) The reciprocal maximum rate of isotope exchange ($\text{NAD}^+ \rightleftharpoons \text{NADH}$) at equilibrium at pH 7.9 (Silverstein & Boyer, 1964) is, since $k/k_{-4} \gg 1$, given by:

$$\frac{1}{k_{-4}} \left(1 + \frac{k}{k'} \right) + \frac{1}{k'_{-4}} \left(1 + \frac{k'}{k} \right) = 0.028$$

[Value calculated from maximum rate of acetaldehyde reduction and maximum rate of $\text{NAD}^+ \rightleftharpoons \text{NADH}$ exchange (Dickenson & Dickinson, 1975a; Silverstein & Boyer, 1964).] The results in Table 3 show that the first term is relatively insignificant; from the second term a value of $k'_{-4} \approx 360 \text{ s}^{-1}$ is obtained.

(6) If it is assumed that NADH binding is not influenced by the presence of acetaldehyde, then $k'_{+4} \approx k'_{+1}$ and K'_4 may be estimated, and hence K'_2 , since $K'_1 K'_3 = K'_2 K'_4$.

Comparison of aspects of the mechanisms of yeast and horse liver alcohol dehydrogenase

Our detailed investigations with yeast alcohol dehydrogenase described in this and the preceding paper (Dickenson & Dickinson, 1978) have provided estimates of K_3 , the dissociation constant for ethanol from the enzyme-NAD⁺-ethanol complex. In the pH range 6.0–9.0 the value varies little. The stopped-flow experiments with butan-1-ol and propan-2-ol with Phenol Red at pH 7.6 support this conclusion and indeed indicate that the dissociation constant for alcohol from the enzyme-NAD⁺-alcohol ternary complex should be pH-independent in this region. It is true that the determination of K_3 from product-inhibition data depends on certain assumptions, but again with butan-1-ol and propan-1-ol as substrates the Michaelis constant for alcohol is independent of pH over the range 5.5–10.0 (Dickenson & Dickinson, 1975b). With these substrates hydride transfer is probably rate-limiting, and for butan-1-ol, at least, the Michaelis constant is probably equal to the dissociation constant of alcohol from the enzyme-NAD⁺-alcohol ternary complex (Dickenson & Dickinson, 1977). The weight of evidence with authentic substrates is against the idea that

substrate alcohol binds preferentially to the alkaline form of an acid group of pK_a approx. 7.6. For horse liver alcohol dehydrogenase, studies with the substrate analogue trifluoroethanol (Shore *et al.*, 1974) have implicated a group of pK_a approx. 7.6 (as yet unidentified, but possibly a water molecule bound to zinc) in ethanol binding. The results then indicate a clear contrast for the two alcohol dehydrogenases in the binding of alcohol substrate. It is true that our studies (Dickenson & Dickinson, 1978) with trifluoroethanol as an inhibitor indicated a fairly strong pH-dependence of the dissociation constant for trifluoroethanol from the ternary complex (50-fold increase from pH 6.0 to 10.0), but there was no clear indication of the involvement of a particular group. Further, trifluoroethanol binds much more strongly to the enzyme-NAD⁺ complex than does ethanol. At pH 7.05 $K_{\text{E} \cdot \text{NAD}^+ \cdot \text{trifluoroethanol}} = 3 \text{ mM}$ and $K_{\text{E} \cdot \text{NAD}^+ \cdot \text{ethanol}} = 52 \text{ mM}$ and the disparity is somewhat greater at more alkaline pH. It appears that there may be additional factors controlling the binding of trifluoroethanol, and these could be responsible for the more marked pH variation of $K_{\text{E} \cdot \text{NAD}^+ \cdot \text{trifluoroethanol}}$. One cannot assume that the binding of trifluoroethanol will necessarily give a reliable view of what happens in the binding of the true substrate when the affinities of the enzyme for the two compounds are quite different. This criticism could also be brought against conclusions drawn from trifluoroethanol binding to horse liver alcohol dehydrogenase. However, in that case the conclusions were supported by stopped-flow experiments in the presence of Phenol Red and the use of an alternative inhibitor, decanoate. Further, the group, identified as being implicated in ethanol binding, had already been identified in the enzyme-NAD⁺ complex by kinetic studies (Shore *et al.*, 1974; Dalziel, 1963). Our conclusion then is that there must be a real difference in the mechanism of binding of alcohol substrate to yeast and horse liver alcohol dehydrogenases. The difference is probably connected with differences in the binding of NAD⁺ by these enzymes. For the liver enzyme, binding of NAD⁺ results in perturbation of the pK_a of an enzyme group from 9.6 in the free enzyme to 7.6–8.0 in the enzyme-NAD⁺ complex (Dalziel, 1963; Shore *et al.*, 1974). It is this group that is implicated in alcohol binding. For the yeast enzyme the dissociation constant of the enzyme-NAD⁺ complex is practically constant over the pH range 6.0–8.5 (Dickenson & Dickinson, 1975b), and there is no evidence for the participation of a similar functional group with this enzyme.

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