A Study of the Kinetics and Mechanism of Yeast Alcohol Dehydrogenase with a Variety of Substrates

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(Received 16 August 1972)

1. The kinetics of oxidation of ethanol, propan-1-ol, butan-1-ol and propan-2-ol by NAD^+ and of reduction of acetaldehyde and butyraldehyde by NADH catalysed by yeast alcohol dehydrogenase were studied. 2. Results for the aldehyde- NADH reactions are consistent with a compulsory-order mechanism with the rate-limiting step being the dissociation of the product enzyme- NAD^+ complex. In contrast the results for the alcohol- NAD^+ reactions indicate that some dissociation of coenzyme from the active enzyme- NAD^+ -alcohol ternary complexes must occur and that the mechanism is not strictly compulsory-order. The rate-limiting step in ethanol oxidation is the dissociation of the product enzyme- NADH complex but with the other alcohols it is probably the catalytic interconversion of ternary complexes. 3. The rate constants describing the combination of NAD^+ and NADH with the enzyme and the dissociations of these coenzymes from binary complexes with the enzyme were measured.

Comparative kinetic studies with horse liver alcohol dehydrogenase in the oxidation of ethanol and butan-1-ol by NAD^+ and in the reduction of acetaldehyde and butyraldehyde by NADH produced extremely good evidence that the rate-limiting steps in these reactions are the dissociations of the product enzyme-coenzyme complexes (Dalziel, 1962*b*). These findings were confirmed when it was shown that the kinetic results for reactions of ethanol and acetaldehyde with purified coenzymes conformed to the requirements of a Theorell-Chance mechanism (Dalziel, 1963*b*). Later work with a variety of aldehydes and primary and secondary alcohols (Dalziel & Dickinson, 1965, 1966*a,b*) showed that in certain cases dissociation of NAD^+ from the active enzyme- NAD^+ -alcohol complexes, or in the case of aldehyde dehydrogenase reactions enzyme- NAD^+ -aldehyde complexes, could occur. The formation and dissociation of abortive enzyme- NADH -alcohol complexes were required to explain substrate-inhibition effects with primary alcohols and substrate-activation effects with cyclohexanol. These detailed studies made it possible to propose a comprehensive mechanism to account for the kinetics of all the reactions studied.

It has been reported that yeast alcohol dehydrogenase catalyses reactions involving a variety of alcohols, aldehydes and ketones (cf. Sund & Theorell, 1963). Detailed kinetic and mechanistic studies with acetaldehyde and ethanol have been undertaken (Wratten & Cleland, 1963; Silverstein & Boyer, 1964), but possible tests of mechanism such as were used with horse liver alcohol dehydrogenase, by using results for alternative substrates, do not appear

to have been exploited. Our aim was to gain insight into the mechanism of yeast alcohol dehydrogenase catalysis by the use of alternative substrates. An added benefit arising from the study was that the quantitative information gained facilitates direct comparison of the catalytic efficiencies of yeast and horse liver alcohol dehydrogenase in reactions with various substrates.

Materials and Methods

Materials

Reagent solutions were made up in glass-distilled water. EDTA at a final concentration of 0.3 mM was included in enzyme assays and in dialysed enzyme preparations.

Crystalline alcohol dehydrogenase was prepared from air-dried baker's yeast as previously described (Dickinson, 1970, 1972). The substrates were obtained from Fisons, Loughborough, U.K. and were fractionally distilled before use. Examination of samples of propan-1-ol, butan-1-ol and propan-2-ol by g.l.c. showed that these materials were free of contamination by ethanol. NAD^+ was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. and was purified by chromatography on DEAE-cellulose (Dalziel, 1963*a*). NADH was prepared as described by Dalziel (1962*a*).

Initial-rate measurements

These were performed spectrophotometrically with a Zeiss PMQ II spectrophotometer or fluorimetrically by using a recording fluorimeter of similar design to

that described by Dalziel (1962*b*). All measurements were made at 25°C and the reaction mixtures were 10.1 with respect to sodium phosphate buffer, pH 7.05. Bovine serum albumin (1 mg) was added to all assays and to dilutions of enzyme as an essential condition for enzyme stability at high dilution. Kinetic coefficients 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 primary and secondary plots of initial-rate results in the usual way (Dalziel, 1957). In the equation e is the concentration of enzyme active sites (determined by direct titration with NADH) and S_1 and S_2 are coenzyme and substrate respectively. The symbols ϕ_0 etc. are used for kinetic coefficients for alcohol-NAD⁺ reactions and ϕ'_0 , etc. for those for aldehyde-NADH reactions. This is the same convention adopted in a study of the substrate specificity and mechanism of horse liver alcohol dehydrogenase (Dalziel & Dickinson, 1966*a*).

Initial-rate measurements were made in duplicate with a reproducibility of 5% in general and at worst 10%, with the smallest concentrations of both substrate and coenzyme. Two or three complete experiments were performed with each substrate. The initial-rate parameters were generally reproducible to within 15%.

Results

Alcohol-NAD⁺ reactions

The results of initial-rate measurements at 25°C and pH 7.05 with ethanol as substrate are shown in the primary and secondary plots of Figs. 1 and 2. Both primary and secondary plots are linear within experimental error over the ranges of substrate and coenzyme concentration used here (ethanol 0.01–1.0M, NAD⁺ 31–1470 μM). We have not observed the substrate-activation effect at ethanol concentrations greater than 0.1M with high NAD⁺ concentrations (770 μM) which was reported by Nygaard & Theorell (1955). The intercepts and slopes of Fig. 2 give values for the kinetic coefficients in eqn. (1). The estimated values for the coefficients are listed in Table 1.

The results of initial-rate studies at 25°C, pH 7.05, with propan-1-ol, butan-1-ol and propan-2-ol as substrates yielded linear primary plots similar to those for ethanol. Secondary plots of the slopes and intercepts of the primary plots were again linear over the concentration ranges used. Estimated values for the kinetic coefficients for these alcohols are shown in Table 1. The ranges of concentration of coenzyme and substrate used in the experiments were: NAD⁺, 30–1700 μM; propan-1-ol, 0.012–0.24M; butan-1-ol, 0.013–0.135M; propan-2-ol, 0.025–0.5M. The lack of substrate inhibition even at the highest alcohol con-

Fig. 1. Primary plots showing variation of the reciprocal of the specific initial rate at pH 7.05, 25°C, with the reciprocal of the ethanol concentration for several constant NAD⁺ concentrations

The NAD⁺ concentrations (μM) were: ○, 1470; ●, 490; △, 160; ▲, 62; □, 31.

Fig. 2. Secondary plot showing the variation of the intercepts (○) and slopes (●) of the Lineweaver-Burk plot in Fig. 1 with the reciprocal of the NAD⁺ concentration

centrations used is noteworthy and is in contrast with the situation obtaining with horse liver alcohol dehydrogenase in the oxidation of primary alcohols (Dalziel & Dickinson, 1966*a*).

Table 1. *Kinetic coefficients for the oxidation of alcohols by NAD⁺ with yeast alcohol dehydrogenase at 25°C and pH7.05*

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. ϕ_1/ϕ_0 is the Michaelis constant for NAD⁺ and ϕ_2/ϕ_0 that for the substrate.

Substrate	ϕ_0 (s)	ϕ_1 ($\mu\text{M}\cdot\text{s}$)	ϕ_2 ($\mu\text{M}\cdot\text{s}$)	ϕ_{12} ($\mu\text{M}^2\cdot\text{s}$)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (mm)	ϕ_{12}/ϕ_2 (μM)
Ethanol	0.0022	0.24	48	15 600	109	21.7	325
Propan-1-ol	0.015	2.26	440	103 000	150	29.2	235
Butan-1-ol	0.040	10	1280	200 000	250	32	160
Propan-2-ol	0.074	50	19 000	6 400 000	680	256	340

Fig. 3. *Primary plot showing variation of the reciprocal of the specific initial rate at pH7.05, 25°C, with the reciprocal of the acetaldehyde concentration for several constant NADH concentrations*

The NADH concentrations (μM) were: $\circ$, 277; $\bullet$, 37; Δ , 13.8; $\blacktriangle$, 6.9; $\square$, 3.5. For clarity results with 171 μM -NADH have been omitted.

Fig. 4. *Secondary plot showing variation of the intercepts ($\circ$) and slopes ($\bullet$) of the Lineweaver-Burk plot in Fig. 3 with the reciprocal of the NADH concentration*

Aldehyde-NADH reactions

The results of initial-rate measurements at 25°C, pH7.05, with acetaldehyde as substrate are shown in the primary and secondary plots of Figs. 3 and 4. The plots are evidently linear within the experimental error and the values of the kinetic coefficients calculated from Fig. 4 are included in Table 2. For butyraldehyde as substrate under the same conditions linear primary and secondary reciprocal plots were also obtained. Estimated values for the initial rate

parameters for butyraldehyde appear in Table 2. The ranges of coenzyme and substrate concentrations used in these experiments were: NADH, 3–330 μM ; acetaldehyde, 0.027–5.5 mm; butyraldehyde, 1.9–37 mm.

Attempts were made to measure the kinetic coefficients describing the oxidation of NADH by acetone. However, even with the purest acetone available to us, the progress curves for reactions were biphasic. Apparently the acetone contained some small contamination of a more-rapidly-reacting component. Because of the uncertainty introduced by the impurity, the experiments were not continued.

Table 2. *Kinetic coefficients for the reduction of aldehydes by NADH with yeast alcohol dehydrogenase at 25°C and pH 7.05*

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 NADH and S'_2 the substrate aldehyde. ϕ'_1/ϕ'_0 is the Michaelis constant for NADH and ϕ'_2/ϕ'_0 that for the substrate.

Substrate	ϕ'_0 (s)	ϕ'_1 ($\mu\text{M}\cdot\text{s}$)	ϕ'_2 ($\mu\text{M}\cdot\text{s}$)	ϕ'_{12} ($\mu\text{M}^2\cdot\text{s}$)	ϕ'_1/ϕ'_0 (μM)	ϕ'_2/ϕ'_0 (mm)	ϕ'_{12}/ϕ'_2 (μM)
Acetaldehyde	0.00026	0.025	0.24	3.0	96	0.93	12.5
Butyraldehyde	0.00029	0.028	8	56	97	27.5	7

A very rough estimate of $\phi'_{2\text{app.}} = 49\text{mm}\cdot\text{s}$ with $80\mu\text{M}$ -NADH was obtained. Comparison with ϕ'_2 values for acetaldehyde and butyraldehyde in Table 2 shows that acetone is an extremely poor substrate for the enzyme.

Discussion

The results obtained in the oxidation of ethanol, propan-1-ol, butan-1-ol and propan-2-ol show that alcohols are increasingly less effective as substrates for yeast alcohol dehydrogenase with increasing chain length and on moving from primary to secondary alcohol. As shown in Table 1 all four kinetic coefficients in eqn. (1) increase substantially on passing from ethanol through propan-1-ol to butan-1-ol and on changing from propan-1-ol to propan-2-ol. The Michaelis constants for NAD^+ (ϕ_1/ϕ_0) increase somewhat with increasing chain length and on passing from primary to secondary alcohol, but for the primary alcohols at least the Michaelis constants (ϕ_2/ϕ_0) are similar. There is a tenfold increase in the value of the Michaelis constant for propan-2-ol over those for the primary alcohols.

The results obtained in the reduction of acetaldehyde and butyraldehyde by NADH shown in Table 2 are interesting by comparison with those found in the oxidation of the corresponding alcohols. Two parameters, ϕ'_0 and ϕ'_1 , are insensitive to change in substrate, although the others (ϕ'_2 and ϕ'_{12}) increase dramatically with increasing chain length as with the alcohols. The Michaelis constants for substrate (ϕ'_2/ϕ'_0) increase with chain length of substrate but the Michaelis constants for coenzyme (ϕ'_1/ϕ'_0) are unchanged.

Wratten & Cleland (1963) claimed on the basis of their product-inhibition studies that the mechanism of yeast alcohol dehydrogenase catalysis, with ethanol and acetaldehyde as substrates, could best be described as a strict compulsory-order mechanism in which the enzyme reacted first with the coenzymes to form enzyme-coenzyme complexes.

The studies of Wratten & Cleland (1963) effectively ruled out the possibility that the reaction proceeded by a rapid-equilibrium random-order mechanism, as suggested by Mahler & Douglas (1957). Studies by Silverstein & Boyer (1964) measuring the rates of isotope exchange at equilibrium also eliminated the rapid-equilibrium random-order mechanism for the enzyme. However, the persistence of a low NAD^+ -NADH exchange rate at saturating substrate concentrations raised the possibility that the compulsory-order mechanism suggested by Wratten & Cleland (1963) might not be totally satisfactory. The NAD^+ -NADH exchange suggested the possibility of a general non-equilibrium random-order mechanism (Scheme 1) in which the upper pathway involving enzyme-coenzyme complexes is kinetically preferred (Silverstein & Boyer, 1964). The ordered mechanism of Wratten & Cleland (1963) corresponds to the situation where the upper pathway in the random mechanism of Scheme 1, with possible isomerization of the $\text{E}\cdot\text{NAD}^+$ complex (ES_1), is the only kinetically significant pathway. Alternative possible explanations of the NAD^+ -NADH exchange were that there was some dissociation of coenzyme from ternary complexes leading to dead-end enzyme-substrate complexes (Wong & Hanes, 1964), or that there was formation of abortive complexes of the type enzyme-NADH-ethanol from which coenzyme could dissociate.

In addition to the possibilities outlined there was one other explanation of the persistence of a low NAD^+ -NADH exchange rate at saturating substrate concentrations that might have been considered. The occurrence of an aldehyde dehydrogenase reaction, such as is observed for horse liver alcohol dehydrogenase (Dalziel & Dickinson, 1965), with involvement of an enzyme-NAD⁺-aldehyde complex from which NAD^+ could dissociate could lead to such an observation.

It appears now that some of the alternative possibilities mentioned to explain the NAD^+ -NADH exchange may be eliminated. Yeast alcohol dehydro-

Scheme 1. Non-equilibrium random-order mechanism for yeast alcohol dehydrogenase

Abbreviations: alc, alcohol; ald, aldehyde. For further details see the Discussion section.

Table 3. Kinetic coefficients in the initial-rate equation for certain mechanisms for two-substrate reactions (Dalziel, 1957)

The initial-rate equation is:

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

The mechanisms are special cases of the general mechanism shown in Scheme 1 where the lower pathways occur to a negligible extent. $A = \frac{k' + k'_{-3}}{k'_{-3}}$

Mechanism	ϕ_0	ϕ_1	ϕ_2	ϕ_{12}
1. General compulsory order	$\frac{1}{k'_{-1}} + \frac{1}{k'_{-3}} + \frac{A}{k}$	$\frac{1}{k_{+1}}$	$\frac{Ak_{-3} + k}{kk_{+3}}$	$\frac{k_{-1}(Ak_{-3} + k)}{k_{+1}kk_{+3}}$
2. Theorell-Chance	$\frac{1}{k'_{-1}}$	$\frac{1}{k_{+1}}$	$\frac{Ak_{-3} + k}{kk_{+3}}$	$\frac{k_{-1}(Ak_{-3} + k)}{k_{+1}kk_{+3}}$

genase does not bring about an acetaldehyde dehydrogenase reaction and does not form abortive complexes as evidenced by the unchanged fluorescence yield of the enzyme-NADH complex in the presence of large concentrations of ethanol (F. M. Dickinson, unpublished work). The lack of substrate-inhibition or -activation effects with ethanol and acetaldehyde at high coenzyme concentrations, noted in the Results section, further supports the view that abortive complexes are not readily formed. The persistent NAD^+ -NADH exchange observed by Silverstein & Boyer (1964) is thus best explained in terms of a general non-equilibrium random mechanism or by dissociation of coenzyme from ternary complexes to form dead-end enzyme-substrate complexes.

One important consequence of a strict compulsory-order mechanism or a variant with isomerization of enzyme-coenzyme complexes is that the initial-rate parameters ϕ_1 and ϕ'_1 are expected to be independent

of the nature of the substrate. In the case of the compulsory-order mechanism this expectation arises from the fact, as shown in Table 3, that ϕ_1 and ϕ'_1 are related only to steps involving combination of enzyme and coenzyme. The situation for the variant mechanism with isomerization of enzyme-coenzyme complexes is similar in that only the rate constants describing the combination of enzyme and coenzyme and the interconversion of the isomeric enzyme-coenzyme complexes are involved (Dalziel, 1963b). Table 1 shows that for yeast alcohol dehydrogenase values of ϕ_1 change substantially with change of the substrate alcohol. Evidently both compulsory-order mechanisms mentioned are inadequate to describe the oxidation of propan-1-ol, butan-1-ol and propan-2-ol. The situation on the mechanism for the oxidation of ethanol is not immediately clear, since the ϕ_1 value for ethanol is used as the reference. However, there is good evidence, which will be discussed below, that

even with ethanol as substrate $\phi_1 > 1/k_{+1}$. The characteristics of the kinetic results for ethanol oxidation are in this regard similar to those for the oxidation of the other alcohols.

In contrast to the situation with the alcohols the results for the reduction of acetaldehyde and butyraldehyde by NADH show that ϕ'_1 is independent of the nature of the substrate aldehyde. On this point at least the aldehyde-NADH results are consistent with a compulsory-order mechanism. It is likely that ϕ'_1 is related to the rate constant describing the combination of NADH with enzyme by the expression $\phi'_1 = 1/k'_{+1}$. This is the simplest interpretation. No evidence has been produced in support of the isomerization of the enzyme-NADH complex (ES'_1). Our contention that $\phi'_1 = 1/k'_{+1}$ is supported by other evidence, which will become apparent as the discussion proceeds.

The kinetic results obtained here in the oxidation of the various alcohols are very similar to those obtained in the oxidation of secondary alcohols by NAD^+ catalysed by horse liver alcohol dehydrogenase (Dalziel & Dickinson, 1966a). In that work the increasing values of ϕ_1 with change of substrate were remarkable because in the oxidation of primary alcohols by NAD^+ with that enzyme, values of ϕ_1 were constant, as was consistent with the Theorell-Chance mechanism established for the enzyme (Dalziel, 1963b). The results for the oxidation of secondary alcohols were explained on the basis of a mechanism which allowed for a significant dissociation of NAD^+ from the enzyme-NAD⁺-alcohol complex. It has been pointed out that the findings of Silverstein & Boyer (1964) show that dissociation of coenzyme from the catalytic ternary complexes can occur with

yeast alcohol dehydrogenase. It appears, therefore, that the mechanism proposed to describe the behaviour of horse liver alcohol dehydrogenase with secondary alcohols may also be applicable here.

The ensuing discussion will be made in terms of the mechanism shown in Scheme 2. The alternative pathways of association on the left correspond to the reactions of NAD^+ and alcohol with the enzyme. In the oxidation of alcohols no provision is made for a random dissociation of products. Instead a compulsory pathway is followed proceeding through the binary enzyme-NADH complex (ES'_1). The neglect of an alternative pathway of dissociation is justified with ethanol as substrate by the finding of Silverstein & Boyer (1964) that in Scheme 1, $k'_{-3} \gg k'_{-4}$. The same inequality probably applies with all other alcohols as substrates, since the corresponding products are much worse substrates for the reverse reaction than is acetaldehyde. For reactions starting from the NADH side, random association of reactants is not suggested because the constancy of ϕ'_1 in changing from acetaldehyde to butyraldehyde satisfies the requirements of a strictly compulsory mechanism. In the dissociation of products after aldehyde reduction a compulsory pathway is followed proceeding through the enzyme-NAD⁺ complex (ES_1). This arises with acetaldehyde as substrate from the fact that in Scheme 1, $k_{-3} \gg k_{-4}$ (Silverstein & Boyer 1964). For butyraldehyde as substrate the same condition probably applies since butan-1-ol is a much poorer substrate than ethanol in the reverse reaction.

In summary, the proposals made here are for a compulsory-order mechanism for aldehyde reduction and a partly random-order one for alcohol oxidation with compulsory order of product dissociation. The

Scheme 2. *Proposed mechanism for yeast alcohol dehydrogenase*

Abbreviations: alc, alcohol; ald, aldehyde. For further details see the Discussion section.

exclusion of binary complexes of the type enzyme-aldehyde from Scheme 2 does not imply that they cannot be formed. The NAD^+ -NADH exchange observed at saturating substrate concentrations in isotope-exchange experiments (Silverstein & Boyer, 1964) suggests that they may exist. Scheme 2 implies that such complexes are not kinetically significant, and therefore that no appreciable proportion of the total conversion into products in either direction proceeds through them.

The initial-rate equation for the mechanism in Scheme 2 in the forward direction is given by the expression:

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

with $A = \frac{k' + k'_{-3}}{k'_{-3}}$

Eqn. (2) may be obtained from that given by Dalziel (1958) for a general random-order mechanism (Scheme 1) or from that derived for a modified random-order mechanism with abortive complex

product aldehyde in the enzyme-NADH-aldehyde complex ($\text{ES}'_1\text{S}_2$).

The initial rate-equation applicable to the reverse of Scheme 2 with compulsory order of product dissociation is obtained from that for the general compulsory-order mechanism in Table 3 by insertion and deletion of primes. It is:

$$\frac{e}{v_0} = \left\{ \frac{1}{k_{-1}} + \frac{1}{k_{-3}} + \frac{A'}{k'} \right\} + \frac{1}{k'_{+1}[S'_1]} + \frac{A'k'_{-3} + k'}{k'k'_{+3}} \frac{1}{[S'_2]} + \frac{k'_{-1}(A'k'_{-3} + k')}{k'_{+1}k'k'_{+3}[S'_1][S'_2]} \quad (4)$$

(Dalziel & Dickinson, 1966a) by deletion of the appropriate steps. Eqn. (2) is not of the linear reciprocal form of eqn. (1) and is not directly applicable to the results obtained here. However, if the conditions:

(i) $(k_{+4}/k_{+1})k_{+2}[S_2] \ll k_{-2} + k_{+4}[S_1]$ and

(ii) $k_{+2}[S_2] \ll k_{-2} + k_{+4}[S_1]$

apply an equation of the form of eqn. (1) is obtained, namely:

$$\frac{e}{v_0} = \phi_0 + \left\{ \frac{Ak_{-4}}{kk_{+4}} + \frac{1}{k_{+1}} \right\} \frac{1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (3)$$

The parameters ϕ_0 , ϕ_2 and ϕ_{12} in eqn. (3) are the same functions of velocity constants as for the general compulsory-order mechanism and are given in Table 3. A more restrictive condition than either (i) or (ii) above which also leads to eqn. (3) is if $k_{+2} = k_{-2} = 0$. This situation corresponds to a dead-end complex mechanism where ES_2 cannot dissociate and is only formed via ES_1S_2 . Eqn. (3) was originally derived to explain the kinetic data observed in the oxidation of secondary alcohols by NAD^+ with horse liver alcohol

dehydrogenase (Dalziel & Dickinson, 1966a). As pointed out there, conditions (i) and (ii) mean that the rate of reaction through the bottom pathway (via ES_2) is negligible compared with that through the upper pathway.

The applicability of eqn. (3) to alcohol oxidation with yeast alcohol dehydrogenase is readily apparent. The equation is of a linear reciprocal form, as is required. In addition the variation in ϕ_1 with substrate is expected, since apart from k_{+1} the rate constants involved will depend on the nature of the alcohol in enzyme-NAD⁺-alcohol and enzyme-alcohol complexes (ES_1S_2 and ES_2) and on the nature of the

with $A' = \frac{k + k_{-3}}{k_{-3}}$

Eqn. (4) satisfies the results obtained for reduction of acetaldehyde and butyraldehyde with yeast alcohol dehydrogenase in predicting a linear reciprocal equation of the form of eqn. (1) and that ϕ'_1 should be independent of the nature of the substrate aldehyde (see Table 2).

The rate equations (3) and (4) for the forward and reverse reaction in Scheme 2 lead to the expectation that the kinetic results for yeast alcohol dehydrogenase should satisfy all relationships between the coefficients for a compulsory-order mechanism in both directions except for those involving ϕ_1 . Thus, it is expected that the initial-rate parameters should be related to the overall equilibrium constant for the oxidation of alcohol by NAD^+ to the corresponding products by the expression $\phi'_{12}[\text{H}^+]/\phi_{12} = K_{\text{eq}}$. (Dalziel, 1957). Table 4 shows that with ethanol and acetaldehyde as substrates the relationship is reasonably well satisfied. Inspection of eqn. (4) shows that

Table 4. *Summary of some thermodynamic values for yeast alcohol dehydrogenase and some tests of possible relationships between kinetic coefficients at 25°C and pH 7.05*

$$K_{E \cdot NAD^+} = 3.5 \times 10^{-4} \text{ M (Dickinson, 1972)}$$

$$K_{E \cdot NADH} = 1.1 \times 10^{-5} \text{ M (Dickinson, 1970)}$$

$$= 2.6 \times 10^{-4} \text{ M (0°C, pH 7.8; Hayes & Velick, 1954)}$$

Ethanol-acetaldehyde reaction

$$K_{eq.} = 0.98 \times 10^{-11} \text{ M (Bäcklin, 1958)}$$

$$\text{cf. } \frac{\phi'_{12}[\text{H}^+]}{\phi_{12}} = 1.7 \times 10^{-11} \text{ M}$$

$$\text{cf. } \frac{\phi'_0 \phi'_1 \phi'_2 k_{+1} [\text{H}^+]}{\phi_0 \phi_2} = 1.4 \times 10^{-11} \text{ M}$$

$$\phi'_1 \phi'_2 / \phi'_{12} = 0.002 \text{ s, cf. } \phi_0 = 0.0022 \text{ s}$$

$$\phi_1 \phi_2 / \phi_{12} = 0.00074 \text{ s, cf. } \phi'_0 = 0.00026 \text{ s}$$

Butan-1-ol-butyraldehyde reaction

$$\phi'_1 \phi'_2 / \phi'_{12} = 0.004 \text{ s,}$$

$$\text{cf. } \phi_0 = 0.04 \text{ s}$$

the dissociation constant of the enzyme-NADH complex is related to the initial-rate parameters for aldehyde reduction by the expression $\phi'_{12}/\phi'_2 = K_{E \cdot NADH}$. The dissociation constant of the enzyme-NAD⁺ complex is given by the equivalent expression $\phi_{12}/\phi_2 = K_{E \cdot NAD^+}$ (see eqn. 3). Tables 1 and 2 show that the ratios ϕ_{12}/ϕ_2 and ϕ'_{12}/ϕ'_2 are remarkably constant in view of the large changes in the absolute values of the parameters that occur on changing substrate. Estimated values of $K_{E \cdot NAD^+}$ and $K_{E \cdot NADH}$ obtained in independent titration experiments are given in Table 4. The value for $K_{E \cdot NAD^+}$ at 25°C, pH 7.0, was obtained by an indirect method and may be a slight overestimate (Dickinson, 1972). Comparison of the estimated dissociation constants with the ratios of the kinetic coefficients in Tables 1 and 2 shows that the expected relationships are satisfied.

The information given so far shows that the kinetic measurements satisfy some of the expectations based on the proposed mechanism. More detailed examination yields further support for the proposals and provides other valuable information.

It is expected (Dalziel, 1957) that for the ethanol-acetaldehyde results the relationship $\phi'_1 \phi'_2 / \phi'_{12} \leq \phi_0$ should be satisfied. The inequality applies if the rate-limiting step in ethanol oxidation is hydride transfer or dissociation of product acetaldehyde from the ternary complex. The equality applies if the rate-limiting step is the dissociation of NADH from the terminal enzyme-NADH complex. It is apparent from Table 4 that in the present case $\phi'_1 \phi'_2 / \phi'_{12} = \phi_0$. In terms of eqns. (2) and (3) this means that $\phi_0 = 1/k'_{-1}$ and therefore that

$$\frac{1}{k'_{-1}} \geq \frac{A}{k} + \frac{1}{k'_{-3}}$$

Thus with ethanol as substrate ϕ_0 , ϕ_2 and ϕ_{12} are the same functions of velocity constants as for a Theorell-Chance mechanism (see Table 3).

The conclusion that $\phi_0 = 1/k'_{-1}$ is readily confirmed by the following consideration. The constancy of ϕ'_1 suggests, as has been pointed out above, that it may be equated with $1/k'_{+1}$. The calculated value of k'_{+1} combined with the dissociation constant for the enzyme-NADH complex under the same conditions ($K_{E \cdot NADH} = 11 \mu\text{M}$, Dickinson, 1970) yields a value of $k'_{-1} = 420 \text{ s}^{-1}$. When this estimate is compared with the maximum rate of ethanol oxidation, $1/\phi_0 = 450 \text{ s}^{-1}$, it is evident that the rate-limiting step in ethanol oxidation is NADH dissociation from the terminal enzyme-NADH complex.

Comparison of ϕ_0 for ethanol with values for the other alcohols used shows that NADH dissociation cannot be rate-limiting in the oxidation of propan-1-ol, butan-1-ol and propan-2-ol. Instead, as is shown in Table 4 the results for butan-1-ol and butyraldehyde satisfy the expected relationship $\phi'_1 \phi'_2 / \phi'_{12} < \phi_0$. For propan-1-ol, butan-1-ol and propan-2-ol it is possible that in eqn. (3)

$$\frac{A}{k} \geq \frac{1}{k'_{-3}} + \frac{1}{k'_{-1}}$$

and that

$$\phi_1 = \frac{Ak_{-4}}{kk_{+4}}$$

k_{+1} being neglected. In this case $\phi_1/\phi_0 = k_{-4}/k_{+4}$, that is the Michaelis constant for NAD⁺ is equal to the dissociation constant of NAD⁺ from the enzyme-NAD⁺-alcohol complex. On this interpretation the dissociation constant of NAD⁺ from the enzyme is little affected by the presence of propan-1-ol or butan-1-ol at the active site. The presence of propan-2-ol, however, weakens the binding of NAD⁺ significantly.

The finding that the maximum rates of acetaldehyde and butyraldehyde reduction ($1/\phi'_0$) are, within the limits of error, identical implies that the rate-limiting step occurs at a common step within both

Table 5. Summary of values estimated for rate constants describing the combination and dissociation of coenzymes from yeast alcohol dehydrogenase at 25°C and pH 7.05

The values given are obtained as described in the text.

reactions. The most likely place for this is the dissociation of NAD^+ from the binary enzyme- NAD^+ complex. This observation suggests that $\phi'_0 = 1/k_{-1}$ and that in eqn. (4):

$$\frac{1}{k_{-1}} \gg \frac{1}{k_{-3}} + \frac{A'}{k'}$$

Thus eqn. (4) reduces to that for a Theorell-Chance mechanism (see Table 3). The situation here is precisely that found for horse liver alcohol dehydrogenase with acetaldehyde and butyraldehyde as substrates (Dalziel, 1962b). If the relationship $\phi'_0 = 1/k_{-1}$ is accepted and the calculated value of k_{-1} is combined with the estimated dissociation constant $K_{E \cdot NAD^+} = 3.5 \times 10^{-4} M$ (Dickinson, 1972) a value of $k_{+1} = 1 \times 10^7 M^{-1} \cdot s^{-1}$ is obtained.

Comparison of the estimate of k_{+1} with ϕ_1 for ethanol indicates that even with this alcohol $\phi_1 > 1/k_{+1}$. This conclusion is readily confirmed on testing the possible relationships $\phi_1 \phi_2 / \phi_{12} = \phi'_0$ which would apply for a strictly compulsory mechanism with $\phi'_0 = 1/k_{-1}$. It is obvious from Table 4 that the relationship is not satisfied and that the left-hand term is some three times bigger than the right-hand one, which suggests that for ethanol $\phi_1 = 3/k_{+1}$. On this basis $k_{+1} = 1.25 \times 10^7 M^{-1} \cdot s^{-1}$, which agrees with the alternative calculation.

The observation of a relationship $\phi_1 \phi_2 / \phi_{12} > \phi'_0$ has in the past been used as evidence for a compulsory-order mechanism with isomerization of enzyme-coenzyme complexes (Mahler *et al.*, 1962; Wratten & Cleland, 1963). In the present situation with ethanol as substrate it seems that dissociation of NAD^+ from the ternary complex resulting in an inflated value of ϕ_1 is the most likely cause of the observed inequality. The observations with ethanol are part of a trend seen with all the alcohol substrates. Further, the isotope-exchange experiments of Silverstein & Boyer (1964) provide evidence for the existence of an enzyme-ethanol complex. There is at present no direct evidence for the occurrence of isomeric enzyme-coenzyme complexes with yeast alcohol dehydrogenase.

Calculation of an estimate for k_{+1} as described above allows a test of the relationship

$$\phi'_0 \phi'_1 \phi'_2 k_{+1} [H^+] / \phi_0 \phi_2 = K_{eq}.$$

As is shown in Table 4 the relationship is well satisfied. The expression given is a modification of the Haldane relationship $\phi'_0 \phi'_1 \phi'_2 / \phi_0 \phi_1 \phi_2 = K_{eq}$, which applies to a reversible Theorell-Chance mechanism (Dalziel, 1957). The good agreement justifies our conclusions that for the ethanol-acetaldehyde results ϕ_0 , ϕ_2 , ϕ'_0 , ϕ'_1 and ϕ'_2 are the same functions of velocity constants as for a Theorell-Chance mechanism.

In the course of the preceding discussion it has been shown that there is evidence that some of the initial-rate parameters in Tables 1 and 2 may be used to estimate values for the rate constants in Scheme 2, describing the combination and dissociation of coenzyme from the enzyme. The estimates of these rate constants are summarized in Table 5. Comparison with estimates of the rate constants for the same steps in the mechanism of horse liver alcohol dehydrogenase (Dalziel, 1963b) shows that the present values are larger in each case. Thus k_{-1} and k'_{-1} are some 30-fold and 160-fold larger at pH 7.05, 25°C. It is these substantially increased rates of dissociation that are largely responsible for the much larger maximum rates of ethanol oxidation and acetaldehyde reduction obtained with the yeast enzyme. For both enzymes using these substrates the maximum rates are determined by the rate of dissociation of product coenzyme. It appears, however, that the yeast enzyme also brings about hydride transfer from ethanol much faster than the liver enzyme. According to Brooks & Shore (1971) the hydride transfer step with liver alcohol dehydrogenase and ethanol is characterized by a rate constant of $k = 140 s^{-1}$ at pH 7.0, 25°C. This is three times slower than the maximum rate of ethanol oxidation with the yeast enzyme, which must be much less than the rate of hydride transfer.

We thank the Science Research Council for support, Mr. M. Girling for able technical assistance and Mr. N. Dixon, who constructed the recording fluorimeter.

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