Biochem. J. (1975) **147**, 303–311
Printed in Great Britain

A Study of the pH- and Temperature-Dependence of the Reactions of Yeast Alcohol Dehydrogenase with Ethanol, Acetaldehyde and Butyraldehyde as Substrates

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(*Received* 4 *October* 1974)

The kinetics of ethanol oxidation by NAD^+, and acetaldehyde and butyraldehyde reduction by NADH, catalysed by yeast alcohol dehydrogenase, were studied in the pH range 4.9–9.9 at 25°C and in the temperature range 14.8–43.5°C at pH 7.05. The kinetics of reduction of acetaldehyde by [4*A*-^{2}H]NADH at pH 7.05 and pH 8.9 at 25°C were also studied. The results of the kinetic experiments indicate that the mechanism of catalysis, previously proposed on the basis of studies at pH 7.05 and 25°C (Dickinson & Monger, 1973), applies over the wide range of conditions now tested. Values of some of the initial-rate parameters obtained were used to deduce information about the pH- and temperature-dependence of the specific rates of combination of enzyme and coenzymes and of the dissociation of the enzyme–coenzyme compounds. Primary and secondary plots of initial-rate data are deposited as Supplementary Publication SUP 50043 (20 pages) with the British Library (Lending Division), Boston Spa, Wetherby, Yorks. LS23 7BQ, U.K., from whom copies may be obtained under the terms indicated in *Biochem. J.* (1975) **145**, 5.

Recent detailed initial-rate studies at pH 7.05 and 25°C with yeast alcohol dehydrogenase (EC 1.1.1.1) have provided evidence that the reduction of acetaldehyde by NADH is brought about by a compulsory mechanism in which NADH binds first to the enzyme, and the NAD^+ produced is the last product to leave (Dickinson & Monger, 1973). The work also indicated that the oxidation of ethanol proceeds by a reversal of the above mechanism. However, in this case there is some dissociation of the reactant ternary complex so that significant steady-state concentrations of a complex of the type enzyme–ethanol are encountered. In addition to the above it appeared that values for the rate constants of certain steps in the mechanism, namely coenzyme binding and dissociation, could be obtained from the initial-rate data (Dickinson & Monger, 1973). This possibility provided the stimulus for the present work for, provided that the mechanism remains unchanged, a detailed study of the pH- and temperature-dependence of the coenzyme-binding and dissociation reactions would facilitate comparison with the horse liver enzyme, for which such information is already available (Dalziel, 1963*b*,*c*).

Experimental

Materials

Reagent solutions were prepared 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 and assayed as previously described (Dickinson, 1970, 1972). The specific activity of the enzyme was 400 units (μmol/min)/mg. The substrates were obtained from Fisons Ltd., Loughborough, Leics., U.K. Acetaldehyde and butyraldehyde were freshly distilled before use. NAD^+ and NADH (fluorimetric grade) were purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. The NAD^+ was purified by chromatography on DEAE-cellulose before use (Dalziel, 1963*a*).

[4*A*-^{2}H]NADH was prepared by the yeast alcohol dehydrogenase-catalysed reduction of NAD^+ with [$^2H_6$]ethanol (containing 99 atom % deuterium), obtained from Prochem BOC Ltd., Deer Park Road, London S.W.19, U.K. The method used was that of Dalziel (1962) modified in that after the enzymic reduction the mixture was placed in a boiling-water bath for 90 s. This step served to inactivate the enzyme totally before the addition of non-labelled ethanol to the mixture. The product exhibited the following properties: $E_{260}/E_{340} = 2.6$, residual $E_{340} = 3.4\%$ on enzymic oxidation with excess of acetaldehyde and yeast alcohol dehydrogenase. A sample of NADH prepared in the same way had virtually identical characteristics and, within experimental error, gave exactly the same initial rates as commercial fluorimetric-grade NADH in assays with acetaldehyde and yeast alcohol dehydrogenase over a wide range of substrate concentrations. Comparison of the n.m.r. spectrum of the product [4*A*-^{2}H]NADH with that of NADH indicated that approx. 90% deuterium had been introduced into the *A* side of the nicotinamide ring at the C-4 position.

Initial-rate measurements

These were performed fluorimetrically for ethanol oxidation and fluorimetrically and spectrophotometrically for aldehyde reduction as described previously (Dickinson & Monger, 1973). Initial-rate measurements were made in duplicate with a reproducibility of 5% in general and at worst 10% with the smallest substrate and coenzyme concentrations. The fluorimeter was calibrated over the range of temperature 15–45°C by adopting the procedures of Dalziel (1961, 1963*b*). At pH5.9, 7.05 and 8.1 sodium phosphate buffers, *I* 0.1 (mol/l), were used. At pH4.9, acetic acid–sodium acetate buffer (10mM total acetate concentration) containing Na_2HPO_4 was used, and at pH8.9 and 9.9 10mM-glycine–NaOH buffer containing Na_2HPO_4 was used. In each case the sodium phosphate was added in sufficient quantity to give *I* 0.1 (mol/l). Bovine serum albumin (1mg) was added to all assays.

Under the conditions used with all substrates and coenzymes Lineweaver–Burk plots were linear within experimental error and the data fitted the equation

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

In eqn. (1) e is the concentration of active sites and S_1 and S_2 are coenzyme and substrate respectively. The symbols ϕ_0 etc. are used for the ethanol–NAD^+ reactions, and ϕ'_0 etc. for the aldehyde–NADH reactions. This is the convention adopted previously (Dickinson & Monger, 1973). The concentration of active sites, e, is calculated on the basis of two active sites/molecule (Dickinson, 1974). This is the same basis of calculation as was used in the earlier work (Dickinson & Monger, 1973).

The kinetic coefficients in eqn. (1) were obtained from primary and secondary plots as described by Dalziel (1957). The plots are deposited as Supplementary Publication SUP 50043. The concentrations of substrate and coenzyme were within the following ranges: NAD^+, 30–1500μM, ethanol, 20–400mM, for the ethanol–NAD reactions; NADH, 3–450μM, acetaldehyde, 0.05–8mM for the NADH–acetaldehyde reactions; NADH, 45–300μM, butyraldehyde, 1.5–34mM, for the NADH–butyraldehyde reactions; [4*A*-^{2}H]NADH, 70–500μM, acetaldehyde 1.15–8.9mM for the [4*A*-^{2}H]NADH–acetaldehyde reactions.

Results

The initial-rate parameters describing the reduction of acetaldehyde by NADH or [4*A*-^{2}H]NADH and the reduction of butyraldehyde by NADH at 25°C with yeast alcohol dehydrogenase in the pH range 5.9–8.9 are shown in Table 1. The initial-rate parameters for ethanol oxidation by NAD^+ at 25°C in the pH range 4.9–9.9 are shown in Table 2. It is noted that certain of the parameters, notably ϕ_2 at pH8.9 and 9.9 and ϕ'_{12} for the reduction of acetaldehyde by [4*A*-^{2}H]NADH at pH7.05 and 8.9, could not be determined with the usual precision. The difficulty in the case of ϕ'_{12} arose because of the limited quantity of [4*A*-^{2}H]NADH available, with the consequence that only a limited range of concentrations could be used.

Certain points from Table 1 are of immediate interest. Within the limits of experimental error

Table 1. *Kinetic coefficients for the reduction of acetaldehyde and butyraldehyde by NADH at 25°C and for the reduction of acetaldehyde by [4A-^{2}H]NADH at 25°C catalysed by yeast alcohol dehydrogenase*

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 $[S'_1]$ and $[S'_2]$ are the concentrations of coenzyme and substrates respectively. ϕ'_1/ϕ'_0 is the Michaelis constant for coenzyme and ϕ'_2/ϕ'_0 that for the substrate. Where complete duplicate experiments were performed kinetic coefficients generally agreed to within 15%.

pH	Coenzyme	Substrate	ϕ'_0 (s)	ϕ'_1 (μM·s)	ϕ'_2 (μM·s)	ϕ'_{12} (μM^2·s)	ϕ'_1/ϕ'_0 (μM)	ϕ'_2/ϕ'_0 (μM)	ϕ'_{12}/ϕ'_2 (μM)
5.95	NADH	Acetaldehyde	0.00027	0.014	0.188	1.1	51.5	690	5.9
7.05*	NADH	Acetaldehyde	0.00026	0.025	0.24	3.0	96	930	12.5
7.05†	NADH	Acetaldehyde	0.00026	0.023	0.23	3.0	84	840	13.0
7.05	[4*A*-^{2}H]NADH	Acetaldehyde	0.000254	0.021	0.52	~12	86	~2050	~20
7.05*	NADH	Butyraldehyde	0.00029	0.028	8	56	97	27500	7
8.1	NADH	Acetaldehyde	0.000375	0.049	0.29	13.6	130	770	47
8.9	NADH	Acetaldehyde	0.00042	0.16	0.52	70	370	1200	135
8.9	[4*A*-^{2}H]NADH	Acetaldehyde	0.00046	0.15	1.22	~130	325	~2700	~110
8.9	NADH	Butyraldehyde	0.0004	0.185	13.9	1250	460	35000	90

* Values taken from Dickinson & Monger (1973).

† Best values now available based on this work and earlier work of Dickinson & Monger (1973).

Table 2. *pH-dependence of the kinetic coefficients for the oxidation of ethanol 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 $[S_1]$ and $[S_2]$ are NAD$^+$ and ethanol concentrations respectively. ϕ_1/ϕ_0 is the Michaelis constant for NAD$^+$ and ϕ_2/ϕ_0 that for substrate. Where complete duplicate experiments were performed kinetic coefficients generally agreed to within 15%.

pH	ϕ_0 (s)	ϕ_1 (μM·s)	ϕ_2 (μM·s)	ϕ_{12} (μM^2·s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (mM)	ϕ_{12}/ϕ_2 (μM)
4.9	0.0184	4.1	1950	770000	224	107	390
5.95	0.0057	0.6	245	83000	106	43	340
7.05*	0.0022	0.24	48	15600	109	21.7	325
7.05†	0.0024	0.26	62	16500	108	26	270
8.1	0.0018	0.215	33.5	12900	118	18.5	385
8.9	0.0019	0.28	~23	19700	150	~10	~860
9.9	0.00195	0.395	~10	24400	200	~5	~2400

* Values taken from Dickinson & Monger (1973).

† Best values now available based on the earlier work of Dickinson & Monger (1973) and more recent work.

Table 3. *Kinetic coefficients at various temperatures for the ethanol–acetaldehyde reaction at pH 7.05*

Where complete duplicate experiments were performed kinetic coefficients generally agreed to within 15%.

Ethanol oxidation by NAD$^+$

Temp. (°C)	ϕ_0 (s)	ϕ_1 (μM·s)	ϕ_2 (μM·s)	ϕ_{12} (μM^2·s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (mM)	ϕ_{12}/ϕ_2 (μM)
14.8	0.0069	0.295	172	19000	43	25	110
35.5	0.0010	0.255	48.6	27000	258	49	555
42.8	0.0007	0.345	38.4	64500	484	55	1670

Acetaldehyde reduction by NADH

Temp. (°C)	ϕ'_0 (s)	ϕ'_1 (μM·s)	ϕ'_2 (μM·s)	ϕ'_{12} (μM^2·s)	ϕ'_1/ϕ'_0 (μM)	ϕ'_2/ϕ'_0 (mM)	ϕ'_{12}/ϕ'_2 (μM)
14.8*	0.000525	0.031	0.42	1.26	59	0.8	3.0
16.5	0.0004	0.026	0.22	1.7	65	0.55	7.73
35.5*	0.000125	0.0204	0.302	7.24	164	2.4	24
37	0.0001	0.0185	0.27	6.85	183	2.7	25.3
42.8*	0.0000785	0.0178	0.343	12.7	230	4.4	37
43.5	0.000074	0.0163	0.38	15.2	215	5.1	40

* Values obtained by interpolation from plots of $\log(1/\phi'_0)$ etc. versus $1/T$ by using data obtained at 16.5°, 25°, 37° and 43.5°C.

ϕ'_0 and ϕ'_1 and hence the Michaelis constant for coenzyme (ϕ'_1/ϕ'_0) are the same when acetaldehyde and butyraldehyde are substrates or when [4*A*-^{2}H]-NADH replaces NADH as coenzyme. These observations are in marked contrast with those found in the oxidation of ethanol and butan-1-ol by NAD$^+$. At pH 7.05 and 25°C ϕ_0 and ϕ_1 change 20- and 40-fold respectively when ethanol is replaced by butan-1-ol (Dickinson & Monger, 1973) and similar variations have been found at other pH values (Dickenson & Dickinson, 1975*b*). It is further to be noted from Table 1 that the value of the ratio ϕ'_{12}/ϕ'_2 is independent of the nature of the substrate aldehyde used or whether [4*A*-^{2}H]NADH replaces NADH, and this despite the fact that ϕ'_2 and ϕ'_{12} change quite markedly on changing coenzyme or substrate.

The initial-rate parameters for the reduction of acetaldehyde by NADH at pH 7.05 in the temperature range 16.5–43.5° and for the oxidation of ethanol by NAD$^+$ at pH 7.05 in the temperature range 14.8–42.8°C are shown in Table 3. In order to apply the usual tests of mechanism (Dalziel, 1957) it is necessary to have values for the initial-rate parameters for forward and reverse reactions under the same conditions. Values for the initial-rate

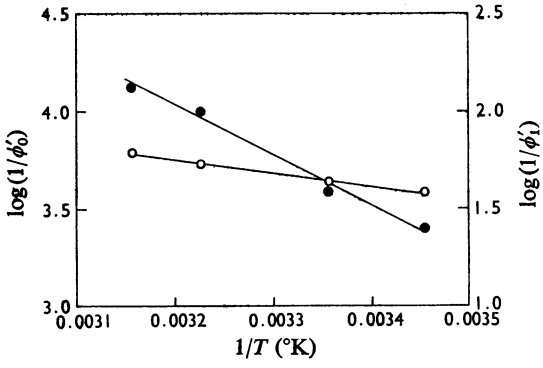


Fig. 1. *Plot showing the temperature dependence of the initial-rate parameters* ϕ'_0 (●) and ϕ'_1 (○) *for acetaldehyde reduction by NADH at pH*7.05

For experimental details see the text.

parameters for acetaldehyde reduction at 14.8°C, 35.5°C and 42.8°C which also appear in Table 3 have been obtained by interpolation from Arrhenius plots of $\log(1/\phi'_0)$, $\log(1/\phi'_1)$ etc. versus $1/T$. Such plots (see Fig. 1) were linear within the limits of experimental error.

Discussion

Previous initial-rate studies of the oxidation of ethanol by NAD^+ and of the reduction of acetaldehyde by NADH at pH 7.05 and 25°C (Dickinson & Monger, 1973) together with the studies of isotope exchange at equilibrium by Silverstein & Boyer (1964) have indicated that these reactions are brought about by yeast alcohol dehydrogenase using the mechanism shown in Scheme 1 operating under the conditions:

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

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

(iii) $k_{-3} \gg k_{-4}$

Conditions (i) and (ii) ensure that the mechanism predicts strictly linear Lineweaver–Burk plots such as are observed in initial-rate studies over wide ranges of NAD^+ and ethanol concentrations. The general solution to Scheme 1 predicts non-linear Lineweaver–Burk plots (Dalziel, 1958).

In the proposed mechanism the conversion of reactants into products proceeds principally through the upper pathway (Scheme 1) where coenzyme binds first followed by substrate and where the product coenzyme leaves the enzyme last. In acetaldehyde reduction a compulsory pathway is followed because $k_{-3} \gg k_{-4}$ (Silverstein & Boyer, 1964). In ethanol oxidation the enzyme–ethanol complex (ES_2) arises principally by dissociation of NAD^+ from the enzyme–NAD–ethanol ternary complex (ES_1S_2). The postulation of the ES_2 complex was necessary because the isotope-exchange data of Silverstein & Boyer (1964) indicate that NAD^+ dissociates from the ternary complex and because of the observed relationship at pH 7.05 that $\phi_1\phi_2/\phi_{12}\phi'_0 > 1$ (Dickinson & Monger, 1973). Such an inequality is incompatible with a strictly compulsory mechanism (Dalziel, 1957) which would arise from Scheme 1 if the formation of ES_2 was forbidden. A rapid-equilibrium random-order mechanism was not permitted as an explanation of the initial-rate data because of the widely differing rates of $NAD^+ \rightleftharpoons$ NADH and ethanol $\rightleftharpoons$ acetaldehyde exchange at equilibrium (Silverstein & Boyer, 1964).

The initial-rate equation for ethanol oxidation according to Scheme 1 with conditions (i) and (ii) above is

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

(Dickinson & Monger, 1973). This is of the form of eqn. (1) and the physical significance of the kinetic coefficients may be obtained by comparison of eqns. (1) and (2). The initial-rate equation for acetaldehyde reduction by Scheme 1 with condition (iii) above is given by Dalziel (1957) and may be obtained from eqn. (2) by insertion or deletion of primes (k_{+1} becomes k'_{+1}, k'_{-1} becomes k_{-1} etc.) and by putting $k'_{-4} = 0$. The lack of the complex of type ES'_2 results in the simplification that $\phi'_1 = 1/k'_{+1}$.

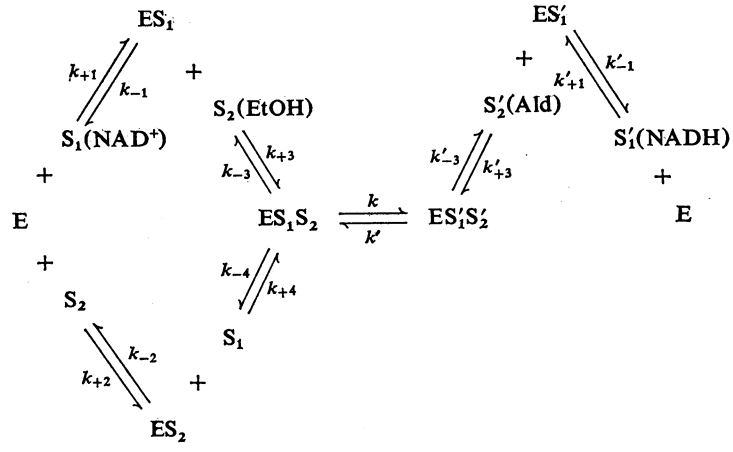
In addition to showing that the initial-rate data at pH 7.05 and 25°C were compatible with Scheme 1, Dickinson & Monger (1973) also obtained information about the rate-limiting steps in the reactions with ethanol and acetaldehyde. Thus the fact that the maximum rates of acetaldehyde and butyraldehyde reduction ($1/\phi'_0$) were, within the limits of error, the same, suggested that NAD^+ dissociation from E·NAD^+ was the rate-limiting step. That is $\phi'_0 = 1/k_{-1}$, with

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

Again, the observation that $\phi'_1\phi'_2/\phi'_{12}\phi_0 = 0.9$ was taken to indicate that in ethanol oxidation, dissociation of the terminal E·NADH complex is the rate-limiting step. That is $\phi_0 = 1/k'_{-1}$ with

$$\frac{1}{k'_{-1}} \gg \frac{1}{k'_{-3}} + \frac{(k'+k'_{-3})}{kk'_{-3}}$$

It is now possible as a result of the present investigation to examine the applicability of Scheme 1



Scheme 1. *Proposed mechanism for yeast alcohol dehydrogenase*

EtOH represents ethanol and Ald represents either acetaldehyde or butyraldehyde.

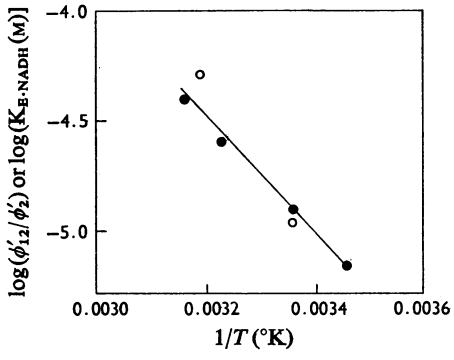


Fig. 2. *Temperature-dependence of the ratio of kinetic parameters* ϕ'_{12}/ϕ'_2 (●) *for acetaldehyde reduction by NADH and of the dissociation constant* $K_{E\cdot NADH}$ (○) *at pH7.05*

The value of $K_{E\cdot NADH}$ at 25°C is from Dickinson (1970) and the value at 40°C was obtained by using the same experimental procedures.

to the initial-rate data over a fairly wide range of pH and temperature. Consideration of Table 1 shows that at pH8.9 and 25°C, as well as at pH7.05, ϕ'_0 and ϕ'_1 are essentially independent of the nature of the substrate aldehyde. These observations are consistent with a compulsory-order mechanism with $\phi'_1 = 1/k'_{+1}$ and with ϕ'_0 determined principally by k_{-1}. It is also apparent that for the two aldehyde substrates ϕ'_{12}/ϕ'_2 is reasonably constant at each pH satisfying the expectation that $\phi'_{12}/\phi'_2 = K_{E\cdot NADH}$. At pH7.05 $\phi'_{12}/\phi'_2 = 13\,\mu M$, and $K_{E\cdot NADH} = 11\,\mu M$ (Dickinson, 1970). At pH8.6 an approximate value of $K_{E\cdot NADH} = 90\,\mu M$ has been obtained (F. M. Dickinson, unpublished work) and this may be compared with $\phi'_{12}/\phi'_2 = 136\,\mu M$ at pH8.9. Confirmation of the above conclusions is obtained by examination of the results from acetaldehyde reduction by [4*A*-^{2}H]NADH at pH7.05 and 8.9. Within experimental error ϕ'_0, ϕ'_1 and ϕ'_{12}/ϕ'_2 are independent of whether the reduced coenzyme contains hydrogen or deuterium as the atom to be transferred in catalysis. The change in ϕ'_2 and ϕ'_{12} in changing from NADH to [4*A*-^{2}H]NADH presumably arises because of the expected primary isotope effect on k and k'. Although the initial-rate equation in general predicts a change in ϕ'_0 for the same reason, the effect is not observed, because as the butyraldehyde results indicate, the maximum velocity is determined by the rate of dissociation of NAD^+ from the terminal $E\cdot NAD^+$ complex. The insensitivity of ϕ'_1 and ϕ'_{12}/ϕ'_2 to the change from NADH to [4*A*-^{2}H]NADH is not unexpected if, as indicated, $\phi'_1 = 1/k'_{+1}$ and $\phi'_{12}/\phi'_2 = k'_{-1}/k'_{+1}$.

Although the information is much less detailed it seems likely that the above mechanistic features are preserved over the temperature range 16–43°C at pH7.05. Fig. 1 shows that plots of $\log(1/\phi'_0)$ and $\log(1/\phi'_1)$ versus $1/T$ are linear within experimental error and there is thus no indication that these parameters obtain a differences significance as the temperature is altered. The ratio ϕ'_{12}/ϕ'_2 varies with temperature in the manner shown in Fig. 2 and it seems from the very limited data available that $K_{E\cdot NADH}$ varies in the same way. The slope of Fig. 2 provides an estimate of $\Delta H = 50\,kJ\cdot mol^{-1}$ as the heat of dissociation of the E·NADH complex.

It should be noted that our results with acetaldehyde and NADH and [4*A*-^{2}H]NADH are at variance with

Table 4. *Relationships between the kinetic coefficients in the ethanol–acetaldehyde reactions*

Values of the equilibrium constant for the reaction ($K_{eq.}$) taken from Bäcklin (1958). The values of $K_{eq.}$ at other temperatures were obtained by calculation from the value at 25°C by using $\Delta H^0 = 30 kJ \cdot mol^{-1}$ (Bäcklin, 1958).

pH	$\phi_1\phi_2/\phi_{12}\phi'_0$	$\phi'_1\phi'_2/\phi'_{12}\phi_0$	$10^{11} \times \phi'_{12}[H^+]/\phi_{12}$ (M)	$10^{11} \times K_{eq.}$ (M)
At 25°C				
5.95	6.5	0.45	1.5	0.98
7.05*	2.9	0.9	1.7	0.98
7.05†	3.8	0.75	1.5	0.98
8.1	1.5	0.6	0.8	0.98
8.9	~0.8	0.65	0.5	0.98
At pH7.05				
Temp. (°C)	$\phi_1\phi_2/\phi_{12}\phi'_0$	$\phi'_1\phi'_2/\phi'_{12}\phi_0$	$10^{11} \times \phi'_{12}[H^+]/\phi_{12}$ (M)	$10^{11} \times K_{eq.}$ (M)
14.8	5.1	0.7	0.4	0.64
35.5	1.85	0.85	1.8	1.48
42.8	2.6	0.7	1.1	1.93

* Values taken from Dickinson & Monger (1973).

† Best results available based on this work and the earlier work of Dickinson & Monger (1973).

those of Klinman (1972). This author observed that at pH8.5, ϕ'_0 decreased by a factor of about 4.6 on changing from NADH to [4*A*-^{2}H]NADH, ϕ'_1 and ϕ'_2 varied much as observed here, but ϕ'_{12} was essentially constant, in contrast with the two- to four-fold increase observed here. The differences, particularly with respect to ϕ'_0, are not easily explained, but certain points should be noted. In the work of Klinman (1972) the commercial enzyme preparations were dialysed at pH8.5 under conditions where the enzyme was acknowledged to be unstable. The quoted specific activity of the enzyme of about 100units (μmol/min)/mg is roughly one-quarter of that used here. Further, the observed $V_{max.}$ for acetaldehyde reduction of $88 s^{-1}$ at pH8.5 ($176 s^{-1}$ if calculated on the basis of two active sites/molecule as here) is extremely low compared with the values of $2650 s^{-1}$ at pH8.1 and $2400 s^{-1}$ at pH8.9 which we are now reporting.

Scheme 1 and the associated initial-rate equation predicts the Haldane relationship $\phi'_{12}[H^+]/\phi_{12} = K_{eq.}$ between the initial-rate parameters and the equilibrium constant for the overall reaction. Table 4 shows that this relationship is reasonably well satisfied over the pH and temperature range studied. However, such a relationship is expected for a number of two-substrate mechanisms (Dalziel, 1957) and this observation is only of limited value. Of more interest are the tests of the maximum-rate relationships (Dalziel, 1957) $\phi'_1\phi'_2/\phi'_{12}\phi_0 \leqslant 1$ and $\phi_1\phi_2/\phi_{12}\phi'_0 \leqslant 1$. Over the range of pH and temperature studied $\phi'_1\phi'_2/\phi'_{12}\phi_0$ is fairly constant, with a value just less than unity. In previous work (Dickinson & Monger, 1973) at pH7.05 and 25°C the observed value of $\phi'_1\phi'_2/\phi'_{12}\phi_0 = 0.9$ was thought to be within experimental error equal to unity. The same may now be said, with some justification, for a number of the individual values of this ratio of parameters appearing in Table 4. However, the fact that all the values fall below unity suggests that, within the range of pH and temperature studied, $\phi'_1\phi'_2/\phi'_{12}\phi_0 < 1$, but only slightly. On this basis the dissociation of NAD^+ from the enzyme is not the sole rate-limiting step, as was suggested previously (Dickinson & Monger, 1973), but rather it is the principal rate-limiting step. The other steps which might be partially rate-limiting in a simple mechanism are product aldehyde release (k'_{-3}) or the catalytic step (k) of the reaction. Mahler & Douglas (1957) observed a small isotope effect of 1.8 on the maximum rate of oxidation of ethanol and [1-$^2H_2$]ethanol at 22°C, pH7.6. Similarly Levy *et al.* (1957) observed an isotope effect of 1.45 on $V_{max.}$ at pH9.5, 25°C. These results suggest that hydride transfer may be partially rate-limiting with ethanol as substrate.

It is of interest that, as with liver alcohol dehydrogenase (Dalziel & Dickinson, 1966), the isotope-exchange data of Silverstein & Boyer (1964) provide confirmatory evidence that in the oxidation of ethanol the principal rate-limiting step is the dissociation of the terminal E·NADH complex. At pH7.9 the maximum rate of acetaldehyde reduction, V', was 1.75 times greater than the maximum acetaldehyde $\rightleftharpoons$ ethanol exchange rate, R' (Silverstein & Boyer, 1964). If we interpolate from our results we find that at this pH the maximum rate of ethanol oxidation, V, is about one-twentieth of V' and therefore about one-twelfth of R'. From the relationship given by

Silverstein & Boyer (1964) for R' it is clear that V ($1/\phi_0$ in the present work) is determined principally by k'_{-1}.

Examination of the values for the alternate maximum-rate relationship in Table 4 reveals that in all cases other than at pH8.9 and 25°C, $\phi_1\phi_2/\phi_{12}\phi'_0 > 1$. It was the observation of this fact at pH7.05 which fostered the proposal of Dickinson & Monger (1973) that an enzyme–ethanol complex is involved in the mechanism. Table 4 indicates that under all our conditions of pH and temperature except pH8.9 this complex is kinetically important. At pH8.9 the maximum-rate relationships are in accordance with the requirements of a strict compulsory-order mechanism, and the formation of significant concentrations of ES_2 may not be important. In this case possibly $\phi_1 = 1/k_{+1}$. It may be noted that the deviations from a compulsory mechanism in ethanol oxidation and hence the contribution of ES_2 become less as the temperature is increased and as the pH becomes more alkaline. At higher temperatures the value of k will increase, and experiments with butan-1-ol as substrate (Dickenson & Dickinson, 1975*b*) indicate that values of k will increase about tenfold over the pH range 6–9. It seems that $\phi_1 > 1/k_{+1}$ when smaller values of k are encountered. This makes sense in relation to the second term of eqn. (2). If ES_2 arises principally by dissociation from ES_1S_2 then this process is likely to be more marked if the rate of conversion into ES_1S_2 is lower. The mechanism shown in Scheme 1 has also been found to describe the oxidation of primary and secondary alcohols by horse liver alcohol dehydrogenase (Dalziel & Dickinson, 1966) and the oxidation of glycerol 3-phosphate by rabbit muscle glycerol 3-phosphate dehydrogenase (Bentley & Dickinson, 1974). In those cases also the formation of the complex ES_2 is manifest when the rate of the catalytic step is relatively low.

There is one further test of eqn. (2) which requires mention and this is that the mechanism requires $\phi_{12}/\phi_2 = K_{E\cdot NAD+}$. Reasonable agreement has been observed at pH7.05 (Dickinson & Monger, 1973), but there are no data available for other conditions. We have observed (Dickenson & Dickinson, 1975*b*) that the ratio ϕ_{12}/ϕ_2 is fairly constant at any selected pH and temperature on changing from ethanol to butan-1-ol or propan-2-ol as substrate, despite large variations in the values of individual parameters. These observations are consistent with the requirement that $\phi_{12}/\phi_2 = K_{E\cdot NAD+}$.

The evidence presented suggests that Scheme 1 and the associated initial-rate equation provide an adequate description of the mechanism of catalysis with ethanol and acetaldehyde as substrates over a broad range of pH and temperature. If this is accepted then one may get some idea of how the velocity constants for the combination of enzyme and coenzymes k_{+1} ($\phi_2/\phi_{12}\phi'_0$) and k'_{+1} ($1/\phi'_1$) and those for the dissociation of the enzyme–coenzyme compounds k'_{-1} ($\phi'_{12}/\phi'_2\phi'_1$) and k_{-1} ($1/\phi'_0$) vary with pH (Fig. 3) and temperature. Table 5 gives the values for the heats, entropies and free energies of activation for these processes at pH7.05 and 25°C.

The results in Fig. 3, although very limited, suggest that the combination of enzyme and NADH and the dissociations of the enzyme–coenzyme compounds are not controlled by the state of ionization of single groups within the active centre. The variation of k_{+1} with pH in the alkaline region, however, indicates that the combination of enzyme and NAD^+ may be controlled by a group with a pK in the region of pH8.0. The same group may also partly affect the combination of enzyme and NADH, since this process does vary markedly with pH in this region and in the same direction as the reaction with NAD^+. What the group might be is open to question, and the essential thiol of the enzyme (Whitehead & Rabin, 1964) comes to mind. However, the fact that this group can be alkylated without affecting the dissociation constant for NADH at pH7.0 and 25°C (Dickinson, 1972) does not lend support to the idea.

The values of Fig. 3 and Table 5 may be compared with those obtained for the reactions of horse liver alcohol dehydrogenase with NAD^+ and NADH by Dalziel (1963*b*,*c*). One might hope to observe similarities between the two enzymes because

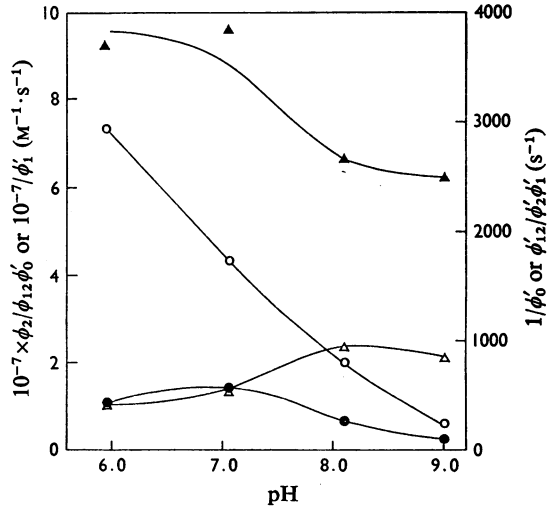


Fig. 3. *Plot showing the pH-dependence of certain initial-rate parameters or ratios of initial-rate parameters from the ethanol–acetaldehyde reactions at 25°C*

$1/\phi'_1 = k'_{+1}$, ○; $\phi_2/\phi_{12}\phi'_0 = k_{+1}$, ●; $\phi'_{12}/\phi'_2\phi'_1 = k'_{-1}$, △; $1/\phi'_0 = k_{-1}$, ▲. For experimental details see the text.

Table 5. *Free energies, heats and entropies of activation for the reactions of yeast alcohol dehydrogenase with coenzymes at pH 7.05 and 25°C*

Reaction	ΔG^* (kJ·mol^{-1})	ΔH^* (kJ·mol^{-1})	ΔS^* (J·mol^{-1}·deg^{-1})
E+NAD$^+$ → E·NAD$^+$	32	~0	−107
E·NAD$^+$ → E+NAD$^+$	52.5	49.5	−10.1
E+NADH → E·NADH	29.4	11.3	−61
E·NADH → E+NADH	57	52.8	−14.3

of evidence that there are similarities of primary sequence (Jornvall, 1973). The specific rates of combination of enzyme with NAD$^+$ and of dissociation of E·NAD$^+$ and E·NADH are much higher (in the range 10–400-fold) with the yeast enzyme over the whole range of pH and temperature. On the other hand the velocity constants for the combination of enzyme with NADH are of comparable magnitude, although the reaction with the yeast enzyme is again significantly faster. The velocity constants show similar trends with pH with both enzymes, but the variations with the yeast enzyme are much less pronounced. One cannot identify a group with a pK in the range 6.4–7.0 controlling the reaction of enzyme and NAD$^+$ or groups with pK values of approx. 8.0 and 9.5 controlling NAD$^+$ and NADH dissociation which were apparent in the data for the liver enzyme (Dalziel, 1963*c*). It is possible that the group with pK 8.0–8.5 that we tentatively identify as controlling the combination of enzyme and NAD$^+$ may be compared with the group with pH9.0–9.6 influencing this reaction in the liver enzyme.

The data in Table 5 may be compared with those for the reactions of the liver enzyme with NAD$^+$ and NADH (Dalziel, 1963*c*). The insensitivity to temperature of the reactions of enzyme with NADH, and particularly with NAD$^+$, seen with yeast alcohol dehydrogenase, is not observed with the liver enzyme. On the other hand the dissociation reactions of coenzyme from enzyme show a stronger temperature-dependence with the yeast enzyme. The comparisons that we have been able to make so far emphasize differences between the two alcohol dehydrogenases rather than helping to pick out common features.

There is, however, one area in which there is some agreement between the results for yeast and horse liver alcohol dehydrogenases and this concerns the reaction of substrates with the bound coenzymes in E·NAD$^+$ and E·NADH. The ratio $\phi'_2[H^+]/\phi_2$ for the ethanol–acetaldehyde reaction decreases some 30-fold between pH5.9 and 8.9. A plot of $\log \phi'_2[H^+]/\phi_2$ versus pH is approximately linear between pH7.05 and 8.9 with a slope of −0.7. $\phi'_2[H^+]/\phi_2$ for the butan-1-ol–butyraldehyde reaction indicates a similar variation. For horse liver alcohol dehydrogenase with ethanol and acetaldehyde as substrates the same plot exhibited a slope of approx. −1.0 (Dalziel, 1963*b*) and this was interpreted as evidence that the oxidation of ethanol by enzyme-bound NAD$^+$ proceeds via the following mechanism:

$$\text{E·NAD}^+ + \text{ethanol} \rightleftharpoons \text{E·[H}^+\text{]·NADH} + \text{acetaldehyde}$$

Thus the proton from the hydroxyl group is not released into free solution but remains bound to a basic group within the active centre. It is released on binding NAD$^+$ in the next catalytic cycle (Dalziel, 1963*b*). In the present case inspection of Scheme 1 and the associated initial-rate equations indicates that the ratio $\phi'_2[H^+]/\phi_2$ may also be equated with the equilibrium constant for the reaction of substrates with the bound coenzymes. The marked variation of this ratio with pH, though less than for the liver enzyme, indicates that the proton released in the catalytic step may also remain bound to a basic group within the active centre.

The substantial increase in $\phi'_{12}/\phi'_2 = K_{\text{E·NADH}}$ over the pH range 5.9–8.9 (Table 1) whereas $\phi_{12}/\phi_2 = K_{\text{E·NAD+}}$ remains fairly constant over the pH range 5.9–8.5 [Table 2 and data from butan-1-ol and propan-2-ol oxidation (Dickenson & Dickinson, 1975*b*)] indicates that there are group(s) in the enzyme whose pK values are shifted to the alkaline side on binding NADH, but are largely unaffected by binding NAD$^+$. This evidence provides some support for the above proposal. In addition we observe that $1/\phi'_1 = k'_{+1}$ changes in such a manner as to suggest that NADH combines with the enzyme when certain group(s) with pK values in the region 6.0–7.0 are protonated. The reaction with NAD$^+$ is not similarly affected. Further, it has been shown from experiments with butan-1-ol and propan-2-ol that hydride transfer is promoted when group(s) with pK values in the region 7.0–8.0 in the E·NAD$^+$–alcohol complex are in the unprotonated form Dickenson & Dickinson, 1975*b*).

It is reasonable to suppose on the basis of the above evidence that the transition state for

alcohol oxidation is favoured by the presence of a basic group in the vicinity of the hydroxyl group of the alcohol substrate. This group would become protonated when hydride transfer occurs. The proton could be liberated into solution when NADH dissociates from the enzyme and the pK of the group(s) shifts to the lower value characteristic of the free enzyme. The present data do not provide evidence that subsequent combination of NAD^+ with the enzyme results in a deprotonation of the group, which was suggested for the liver enzyme on the basis of steady-state experiments (Dalziel, 1963*b*) and which has now been confirmed by stopped-flow methods (Shore *et al.*, 1974).

One group which might be involved in the above mechanism is histidine. Like lactate dehydrogenase (Holbrook & Ingram, 1973) yeast alcohol dehydrogenase contains one histidine/subunit with an abnormally high reactivity towards diethyl pyrocarbonate but with a pK very similar to that of free histidine. In addition, reaction of one histidine/subunit with diethyl pyrocarbonate yields an enzyme able to bind coenzyme but which cannot form ternary complexes (Dickenson & Dickinson, 1973, 1975*a*). In lactate dehydrogenase the histidine (histidine-195) accepts the proton liberated from lactate in the catalytic step of the reaction (Holbrook & Stinson, 1973). It seems reasonable to suppose that the reactive histidine of yeast alcohol dehydrogenase functions in a similar manner.

There is one final point of interest which may be obtained from the data for acetaldehyde reduction by NADH and [4A-^{2}H]NADH in Table 1. According to eqns. (1) and (2)

$$\phi'_2 = \frac{1}{k'_{+3}} + \frac{k'_{-3}}{k'k'_{+3}} + \frac{kk'_{-3}}{k_{-3}k'k'_{+3}}$$

If it is assumed that k and k' change sevenfold on changing from normal to deuterated compounds while the other velocity constants remain essentially unchanged, and if reasonable values are assumed of $k_{-3} = 45000\,s^{-1}(10 \times 1/\phi'_0)$ and $k = 4500\,s^{-1}(10 \times 1/\phi_0)$ then the values of ϕ'_2 with NADH and [4A-^{2}H]NADH indicate that for acetaldehyde $k'_{+3} = 5.8 \times 10^6\,M^{-1}\cdot s^{-1}$. This may be compared with $1/\phi'_2 = 4.4 \times 10^6\,M^{-1}\cdot s^{-1}$. Obviously this calculation is very approximate but it indicates that $\phi'_2 \sim 1/k'_{+3}$. Much larger values for k'_{-3} do not alter the above conclusion, and significantly larger values of k are unlikely, in view of arguments given above. Similar calculations at pH 8.9 indicate that for acetaldehyde $k'_{+3} = 2.7 \times 10^6\,M^{-1}\cdot s^{-1}$, which may be compated with $1/\phi'_2 = 1.9 \times 10^6\,M^{-1}\cdot s^{-1}$. It may be noted here that values of ϕ'_2 for the yeast enzyme are much smaller than for the liver enzyme (Dalziel, 1963*b*), so that on the simplest interpretation the reaction of acetaldehyde with the yeast enzyme–NADH complex is much faster than for the liver enzyme–NADH complex.

We thank Mr. M. Girling for technical assistance and the Science Research Council for support. C. J. D. acknowledges the receipt of a Ferens' Schools Scholarship from the University of Hull.

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