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A Study of the Ionic Properties of the Essential Histidine Residue of Yeast Alcohol Dehydrogenase in Complexes of the Enzyme with its Coenzymes and Substrates

By CHRISTOPHER J. DICKENSON and F. MARK DICKINSON
Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

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1. Initial-rate studies of the reduction of acetaldehyde by NADH, catalysed by yeast alcohol dehydrogenase, were performed at pH4.9 and 9.9, in various buffers, at 25°C. The results are discussed in terms of the mechanism previously proposed for the pH range 5.9–8.9 [Dickenson & Dickinson (1975) *Biochem. J.* **147**, 303–311]. 2. Acetaldehyde forms a u.v.-absorbing complex with glycine. This was shown not to affect the results of kinetic experiments under the conditions used in this and earlier work. 3. The variation with pH of the dissociation constant for the enzyme–NADH complex, calculated from the initial-rate data, indicates that the enzyme possesses a group with pK7.1 in the free enzyme and pK8.7 in the complex. 4. The pH-dependences of the second-order rate constants for inactivation of the enzyme by diethyl pyrocarbonate were determined for the free enzyme (pK7.1), the enzyme–NAD^+ complex (pK approx. 7.1) and the enzyme–NADH complex (pK approx. 8.4). The essential histidine residue may therefore be the group involved in formation and dissociation of the enzyme–NADH complex. 5. Estimates of the rate constant for reaction of acetaldehyde with the enzyme–NADH complex indicate that acetaldehyde may combine only when the essential histidine residue is protonated. The dissociation constants for butan-1-ol and propan-2-ol, calculated on the basis of earlier kinetic data, are, however, independent of pH. 6. The results obtained are discussed in relation to the role of the essential histidine residue in the mechanism of formation of binary and ternary complexes of the enzyme with its coenzymes and substrates.

Detailed initial-rate studies, at various pH values, of reactions catalysed by yeast alcohol dehydrogenase (EC 1.1.1.1) have provided information about the absolute values and pH-dependence of rate and equilibrium constants for certain steps in the mechanism, namely coenzyme binding and dissociation, and the catalytic step in alcohol oxidation (Dickenson & Dickinson, 1975*a*,*b*). In addition, chemical modification of certain amino acid residues, i.e. cysteine (Whitehead & Rabin, 1964; Harris, 1964; Twu & Wold, 1973) and histidine (Dickenson & Dickinson, 1973, 1975*c*; Leskovac & Pavkov-Peričin, 1975), leads to a loss of activity and of substrate binding (Dickinson, 1972; Twu *et al.*, 1973; Leskovac & Pavkov-Peričin, 1975). Coenzyme binding is largely unaffected by these modifications.

It is desirable to relate the pH-dependences of rate and equilibrium constants for various steps in the mechanism to the ionic properties of known active-site residues. Accordingly we have attempted to measure the pK of the essential histidine residue in the enzyme–coenzyme complexes. Previous studies provided an estimate of the pK of this residue in the free enzyme (Dickenson & Dickinson, 1975*c*). The enzyme possesses four histidine residues (one per subunit), which are highly reactive with diethyl pyrocarbonate, but only two active sites/molecule (Dickinson, 1974; Dickenson & Dickinson, 1975*c*; Leskovac & Pavkov-Peričin, 1975). It was therefore not possible to follow the rate of acylation of the enzyme–NADH (E·NADH) complex by direct spectrophotometric measurement of the formation of modified enzyme, as has been done with the free enzyme (Dickenson & Dickinson, 1975*c*) and for the lactate dehydrogenase–NADH complex (Holbrook & Ingram, 1973). Instead we have followed the rate of inactivation of enzyme by excess of diethyl pyrocarbonate under pseudo-first-order conditions. We have also extended the initial-rate studies of the ethanol–acetaldehyde system in an attempt to obtain more precise pK values for the variation with pH of certain kinetic coefficients.

The information obtained strengthens the proposals of Dickenson & Dickinson (1975*a*) concerning the identity and role of a basic group at the active site. Further, it allows interesting comparisons to be made between the mechanisms of catalysis of yeast and horse liver alcohol dehydrogenases.

Experimental

Materials

All solutions were prepared in glass-distilled water. Phosphate and pyrophosphate/HCl buffers were sodium salts.

Enzyme. Yeast alcohol dehydrogenase was prepared as described by Dickinson (1972). The specific activity in the standard assay at pH8.8 (Dickinson, 1970) was 400 units (μmol/min)/mg.

Coenzymes and substrates. NAD^+ (grade II) and NADH (grade I) were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. For initial-rate studies, NAD^+ was purified as described by Dalziel (1963*a*). Acetaldehyde (Fisons Ltd., Loughborough, Leics., U.K.) was redistilled before use. Ethanol (analytical-grade) was obtained from J. Burroughs Ltd., London S.E.11, U.K., and used without further treatment.

Acetamide (Fisons Ltd.) was recrystallized from methanol/ether. Diethyl pyrocarbonate, from Sigma (London) Chemical Co. Ltd., Kingston-on-Thames, Surrey, U.K., was assayed by using imidazole as described by Dickenson & Dickinson (1975*c*). Stock solutions were made in propan-2-ol so that the final concentration of propan-2-ol in reaction mixtures was less than 1% (v/v). The diethyl pyrocarbonate was approx. 60% pure by weight.

Methods

Initial-rate measurements. Assays were performed fluorimetrically for ethanol oxidation by NAD^+, and fluorimetrically and spectrophotometrically for acetaldehyde reduction by NADH, as described previously (Dickinson & Monger, 1973). Measurements were made at 25°C in buffer of *I* 0.1 mol/litre. At pH4.9, acetic acid/sodium acetate buffer (10mM total acetate concentration) containing sufficient NaH_2PO_4 to give *I* 0.1 mol/litre was used. At pH9.9, 10mM-glycine/NaOH, 10mM-pyrophosphate, or $NaHCO_3/Na_2CO_3$ (10mM total carbonate concentration) was used, and in each case sufficient Na_2HPO_4 was added to give *I* 0.1 mol/litre. Bovine serum albumin (1 mg) was added to all assays and dilutions of enzyme.

For each complete experiment, duplicate assays were performed with four to six different concentrations of coenzyme and substrate, in all possible combinations. The duplicate measured initial rates agreed to within 5% in general, and at worst 10% with the smallest substrate and coenzyme concentrations. The ranges of substrate and coenzyme concentrations used were: at pH4.9, NADH, 0.73–280μM, and acetaldehyde, 34μM–14mM; at pH9.9, NADH, 40–1955μM, acetaldehyde, 73–9480μM, NAD^+, 46–4485μM, ethanol, 1.25–405mM.

The kinetic coefficients (ϕ_0 etc.) in the initial-rate equation:

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

were obtained from the slopes and intercepts of the double-reciprocal plots as described by Dalziel (1957). In eqn. (1) *e* is the concentration of enzyme active sites [two per molecule (Dickinson, 1974)], and S_1 and S_2 are coenzyme and substrate respectively. The symbols S_1, S_2 and ϕ_0 etc. are used for the ethanol–NAD^+ reaction, and primed symbols S_1', S_2', ϕ_0' etc. are used for the acetaldehyde–NADH reaction. This is the convention adopted in previous kinetic studies (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975*a*,*b*). ϕ_2' values are defined in terms of the total acetaldehyde concentration, even though it is known that the true substrate for the enzyme is the unhydrated form (Müller-Hill & Wallenfels, 1964). This is done to facilitate comparison with earlier kinetic data with both yeast and horse liver alcohol dehydrogenases, where the same convention was adopted.

Measurement of H^+ uptake. A Radiometer TTT1c pH-stat was used to measure the uptake of protons occurring when NADH or NAD^+ was added to yeast alcohol dehydrogenase at 25°C. The enzyme was first dialysed against 0.4mM-phosphate buffer, pH7.0, including 30mM-Na_2SO_4. Approx. 80mg of enzyme, in 7ml of this buffer, was adjusted to the required pH. A solution of the coenzyme, in the same buffer, was adjusted to the same pH and 1ml was added to the enzyme solution. The pH was maintained by titration with 2mM-HCl. NaOH (0.5μmol) was then added for calibration. In some experiments a further addition of 2ml of 5M-acetamide was then made. The concentrations of coenzyme solutions were such that the final concentrations in the reaction mixtures were 10 times the dissociation constant of the enzyme–coenzyme complex at the pH used (Dickenson & Dickinson, 1975*a*). Diethyl pyrocarbonate-modified enzyme was prepared as described previously (Dickenson & Dickinson, 1975*c*) and iodoacetamide-inactivated enzyme was prepared as described by Dickinson (1972).

Rate of inactivation by diethyl pyrocarbonate. Enzyme (0.1ml) in 2.5mM-phosphate buffer, pH7.0, was added to 1.0ml of buffer (*I* 0.1 mol/litre) at 25°C, containing NAD^+ or NADH where required, and assayed immediately by using the standard assay at pH8.8 (Dickinson, 1970). Diethyl pyrocarbonate (10μl of a solution in propan-2-ol) was then added. Samples (10μl) were withdrawn for assay at intervals of approx. 30s. Alternate samples were assayed by using two different recording spectrophotometers so that progress curves could be recorded for at least 30s for each sample. With the final concentrations of

enzyme used in all reaction mixtures (0.2–0.4 μM) no dilution of the assay samples was necessary. The time of sampling was taken to be the time of addition to the assay mixture. The presence of 8 μM-diethyl pyrocarbonate in the standard assay had no effect on the measured initial rate. In the inactivation experiments the highest concentration of diethyl pyrocarbonate used (less than 2 mM) would therefore have no effect on initial rates when diluted into 3 ml assay mixtures.

Below pH 8.8 phosphate buffers were used, and above pH 8.8 $NaHCO_3/Na_2CO_3$ buffers (10 mM total carbonate concentration), each containing sufficient Na_2HPO_4 to give a total I 0.1 mol/litre, were used. The pH of NAD^+ solutions was adjusted to that required immediately before use. Above pH 8.5, in the absence of coenzyme, the enzyme was slightly unstable, losing activity in a first-order manner with a rate constant of approx. 0.001 s^{-1}. This was always much less than the measured rate constant in the presence of diethyl pyrocarbonate. Under all other conditions used the enzyme was completely stable for at least 10 min in the absence of diethyl pyrocarbonate.

Results

Hydration of acetaldehyde

Identical u.v.-absorption spectra were obtained with redistilled acetaldehyde in all of the phosphate and pyrophosphate/phosphate buffers used for the initial-rate studies. These spectra showed a peak at 278 nm corresponding to the carbonyl function, and with the value of $\varepsilon_{278} = 17$ litre·mol^{-1}·cm^{-1} (Bell & Clunie, 1952) indicate that the extent of hydration of acetaldehyde (58%) does not vary over the range pH 5–10 at 25°C. It was shown by Müller-Hill & Wallenfels (1964) that the unhydrated form of acetaldehyde is the true substrate for yeast alcohol dehydrogenase. Our observations show that changes in initial-rate parameters for acetaldehyde reduction with pH cannot be due to changes in the effective concentration of the substrate.

When glycine/NaOH buffer was used, the 278 nm peak was diminished and a new peak appeared at approx. 230 nm. The effect was more marked at pH 10.0 than at pH 9.0, and with high glycine and acetaldehyde concentrations. At pH 10.0, with 83 mM-acetaldehyde in 100 mM-glycine, the 278 nm shoulder was absent and a value of ε_{230} approx. 73 litre·mol^{-1}·cm^{-1} was obtained. With 10 mM-glycine and 2 mM- or 10 mM-acetaldehyde, at pH 10.0, there was only a small decrease (approx. 10%) in the 278 nm peak. The results indicate that, under the conditions used in the initial-rate experiments (34 μM–14 mM-acetaldehyde, 10 mM-glycine/NaOH), very little of the acetaldehyde will be in the form of the complex absorbing at 230 nm. In particular, there should be no significant error in the coefficients ϕ_2' and ϕ_{12}', which depend on measurements with low substrate concentrations. This conclusion is supported by comparison of the kinetic coefficients obtained in experiments using pyrophosphate and $NaHCO_3/Na_2CO_3$ buffers in place of glycine/NaOH, as described below. Similarly, there should be no error in the coefficients reported by Dalziel (1963*b*) for horse liver alcohol dehydrogenase. In that study low concentrations of glycine (3.6 and 2.1 mM) were used in the buffers at pH 9 and pH 9.9. In contrast, the high (60–140 mM) glycine concentrations used by Klinman (1972, 1975) with the yeast enzyme may be expected to give some uncertainty in the concentrations of free acetaldehyde and hence in the values of some of the kinetic coefficients. It is apparent that high concentrations of glycine should be avoided in kinetic studies with carbonyl-containing substrates. It is notable that the carbonyl absorption of acetaldehyde disappears completely in excess of Tris/HCl buffer at pH 10.0, without the formation of a complex absorbing at 230 nm (Dickinson & Dalziel, 1967).

Initial-rate studies

The kinetic coefficients for the oxidation of ethanol by NAD^+ at pH 9.9, and the reduction of acetaldehyde by NADH at pH 4.9 and 9.9, are shown in Table 1. In all experiments linear primary and secondary double-reciprocal plots were obtained, and the data fitted eqn. (1).

The coefficients for the ethanol–NAD^+ reaction at pH 9.9 (in $Na_2CO_3/NaHCO_3$/phosphate buffer) are the same, within experimental error, as those obtained previously (Dickenson & Dickinson, 1975*a*) when glycine/NaOH/phosphate buffer was used. In addition a more precise value for ϕ_2 has been obtained by using a much wider range of ethanol concentrations.

For the acetaldehyde–NADH reaction at pH 9.9, there is no significant difference between values of each of the kinetic coefficients determined by using glycine/NaOH, pyrophosphate or $NaHCO_3/Na_2CO_3$ buffers. The values of ϕ_0' at pH 9.9, shown in Table 1, are only approximate because of the high K_m for NADH (ϕ_1'/ϕ_0') under these conditions.

Fig. 1 shows the variation with pH of $\log(1/\phi_2')$, $\log(\phi_{12}'/\phi_2')$ and $\log(\phi_{12}/\phi_2)$ obtained from the results of Table 1 and the work of Dickenson & Dickinson (1975*a*).

Measurements of H^+ uptake

Fig. 2 shows the results of experiments to determine the extent of uptake of H^+ (at various pH values) occurring when yeast alcohol dehydrogenase was mixed with saturating concentrations of NAD^+ or NADH. The results are expressed as protons taken up per active site. The curve shown in Fig. 2 represents

Table 1. *Kinetic coefficients for the oxidation of ethanol by NAD$^+$ and the reduction of acetaldehyde by NADH with yeast alcohol dehydrogenase at 25°C and in buffers of I 0.1 mol/litre*

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

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

where S_1 and S_2 are coenzyme and substrate respectively, ϕ_1/ϕ_0 is the Michaelis constant for coenzyme and ϕ_2/ϕ_0 is that for the substrate. The symbols without primes refer to ethanol oxidation and with primes to aldehyde reduction. Detailed buffer compositions are given in the Experimental section. Where complete duplicate experiments were performed kinetic coefficients generally agreed to ±15%.

Ethanol–NAD$^+$ reaction

Buffer	pH	ϕ_0 (s)	ϕ_1 (μM·s)	ϕ_2 (μM·s)	ϕ_{12} (μM^2·s)	ϕ_{12}/ϕ_2 (μM)
$NaHCO_3/Na_2CO_3$	9.9	0.0017	0.63	8.0	16000	2000

Acetaldehyde–NADH reaction

Buffer	pH	ϕ_0' (s)	ϕ_1' (μM·s)	ϕ_2' (μM·s)	ϕ_{12}' (μM^2·s)	ϕ_{12}'/ϕ_2' (μM)
Acetate	4.9 *	0.00027	0.0115	0.18	1.1	6.4
		0.00030	0.013	0.27	1.2	4.5
$NaHCO_3/Na_2CO_3$	9.9	~0.0012	1.0	2.6	700	270
Glycine/NaOH	9.9	~0.0011	0.58	3.0	460	153
Pyrophosphate	9.9	~0.0005	1.1	2.5	630	250

* Results of two complete experiments.

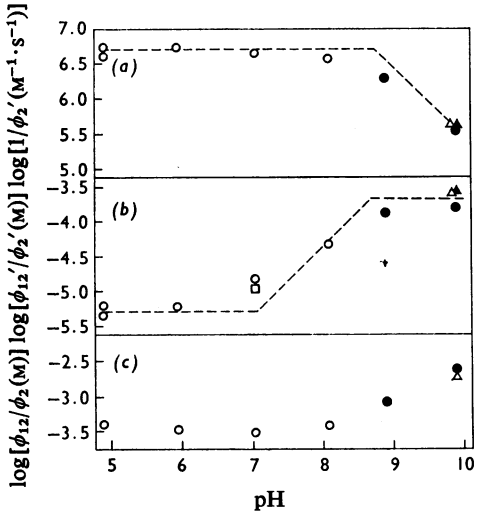


Fig. 1. *Variation with pH of the logarithm of certain kinetic coefficients and ratios of kinetic coefficients in the ethanol–acetaldehyde system with yeast alcohol dehydrogenase at 25°C*

The kinetic coefficients are from Table 1 and the data of Dickenson & Dickinson (1975*a*). The broken lines are of zero or unit slope according to Dixon (1953). The detailed composition of the buffers (*I* 0.1 mol/litre) used at each pH value is given in the Experimental section. Plots against pH are shown of logs of the following: (*a*) $1/\phi_2'$ (M^{-1}·s^{-1}) for acetaldehyde reduction; (*b*) ϕ_{12}'/ϕ_2' (M) for acetaldehyde reduction; □, $K_{E\cdot NADH}$ from Dickinson (1970); (*c*) ϕ_{12}/ϕ_2 (M) for ethanol oxidation. Buffers: ○, phosphate (including also acetate at pH4.9); ●, glycine/phosphate; △, carbonate/phosphate: ▲, pyrophosphate/phosphate.

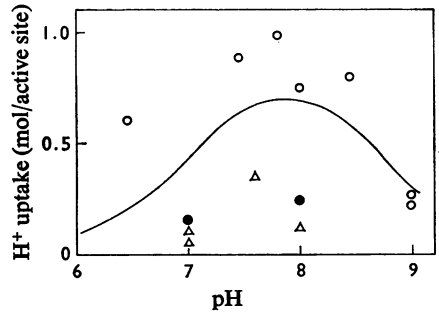


Fig. 2. *Uptake of H$^+$ at various pH values occurring when yeast alcohol dehydrogenase is mixed with saturating concentrations of coenzyme at 25°C*

○, Native enzyme + NADH; ●, native enzyme + NAD$^+$; △, diethyl pyrocarbonate-modified enzyme (<7% active) + NADH, The line represents the H$^+$ uptake expected if the combination of enzyme with coenzyme perturbed the p*K* of a group from 7.1 in the free enzyme to 8.7 in the enzyme–coenzyme complex.

the H$^+$ uptake expected if the combination of enzyme with NADH perturbed the p*K* of an ionizing group from 7.1 in the free enzyme to 8.7 in the E·NADH complex. Also shown in Fig. 2 is the uptake of H$^+$ at various pH values, resulting from saturation by NADH of diethyl pyrocarbonate-modified enzyme (<7% active). In a similar experiment at pH7.0, the addition of a saturating concentration of NADH to iodoacetamide-inactivated enzyme (<1% active)

produced an uptake of 1.0 proton per coenzyme-binding site. For each of the experiments with native enzyme shown in Fig. 2, the further addition of acetamide (final concentration 0.5–1.0M) produced no further proton uptake.

Inactivation by diethyl pyrocarbonate

The inactivation of yeast alcohol dehydrogenase by various concentrations of diethyl pyrocarbonate at pH7.05 is shown in Fig. 3. In the presence of a large excess of the reagent, the inactivation followed pseudo-first-order kinetics for about 2min, after which activity was lost more slowly, as expected from the stability of the reagent in aqueous solution (Dickenson & Dickinson, 1975*c*). The first-order rate constants for inactivation, determined from the initial slopes of Fig. 3, were proportional to the concentration of diethyl pyrocarbonate, indicating a second-order reaction over the range of concentrations used. Similar results were obtained throughout the pH range 5.6–9.4. The concentration ranges of diethyl pyrocarbonate varied from 200–800μM at pH5.6 to 50–200μM at pH9.4. When the highest concentrations of reagent were used at any particular pH value, the loss of enzyme activity followed first-order kinetics, at least until 95% of the initial activity had been lost. At the lower concentrations of diethyl pyrocarbonate, significant hydrolysis of the reagent had occurred before this extent of inactivation was reached. The variation with pH of the second-order rate constant (calculated from the pseudo-first-order rate constant and the concentration of diethyl pyrocarbonate) is shown in Fig. 4. At the alkaline pH values the results were less precise, since the half-life of diethyl pyrocarbonate is only 12min (Dickenson & Dickinson, 1975*c*), and consequently only two or three samples could be taken in the period where activity was lost according to first-order kinetics.

The protection against inactivation by increasing concentrations of NADH at pH5.8 is shown in Fig. 5(*a*). If inactivation of the free enzyme and the E·NADH complex are characterized by rate constants k_0 and k_1 respectively, it is expected that:

$$\frac{1}{k_0-k}=\frac{K_{E\cdot NADH}}{(k_0-k_1)[NADH]}+\frac{1}{k_0-k_1} \qquad (2)$$

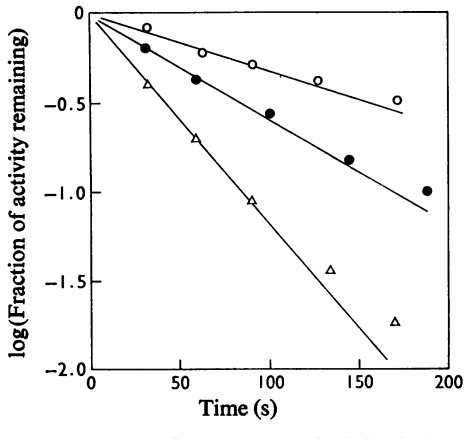


Fig. 3. *Inactivation of* 0.4μM*-yeast alcohol dehydrogenase by diethyl pyrocarbonate at pH* 7.05 *and* 25°C

The diethyl pyrocarbonate concentrations (μM) were: ○, 100; ●, 200; △, 400. The second-order inactivation rate constants, calculated from the pseudo-first-order rate constants (obtained from the initial slopes) and the diethyl pyrocarbonate concentrations, were 78, 72 and 67 $M^{-1}\cdot s^{-1}$ respectively.

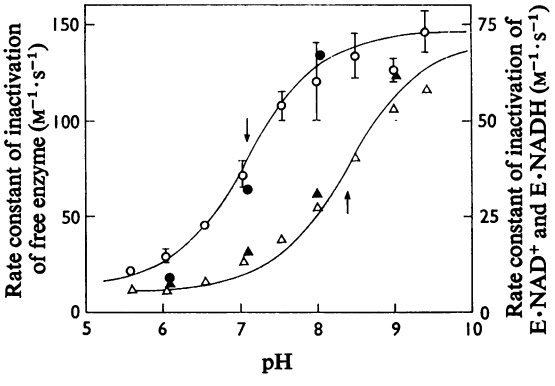


Fig. 4. *Variation with pH of the rate of diethyl pyrocarbonate-dependent inactivation of yeast alcohol dehydrogenase and its enzyme–coenzyme complexes at* 25°C

The second-order rate constants for inactivation were obtained from the concentration of diethyl pyrocarbonate and the pseudo-first-order rate constants as in Fig. 3. Complete duplicate experiments with free enzyme, enzyme·NAD^+ and enzyme·NADH complexes gave results the same, within experimental error, as those shown here. ○, Inactivation of free enzyme. Each individual point shows the measured rate constant at one concentration of diethyl pyrocarbonate. The bars at certain pH values represent the range of values obtained with three or more different concentrations of diethyl pyrocarbonate. The line is drawn assuming that inactivation depends on a group with pK7.1 with rate constants of 15 $M^{-1}\cdot s^{-1}$ and 145 $M^{-1}\cdot s^{-1}$ in the protonated and unprotonated forms respectively. ●, Inactivation of enzyme·NAD^+ complex. Each point represents the mean of two determinations with different concentrations of diethyl pyrocarbonate. △, ▲, Inactivation of enzyme·NADH complex. The △ points show the rate constant obtained with a single concentration of diethyl pyrocarbonate. The ▲ points represent the means of two or more determinations with different reagent concentrations. The line is drawn assuming that inactivation depends on a group with pK8.4 with rate constants of 6 $M^{-1}\cdot s^{-1}$ and 70 $M^{-1}\cdot s^{-1}$ in the protonated and unprotonated forms respectively.

where $K_{E\cdot NADH}$ is the dissociation constant of the E·NADH complex and k is the measured rate constant of inactivation of enzyme in the presence of non-saturating concentrations of NADH. Fig. 5(*b*) shows that the expected relationship is fulfilled and gives an estimate of $K_{E\cdot NADH} = 6.5\,\mu M$, which may be compared with the value of $5.9\,\mu M$ obtained from steady-state kinetics at pH5.95 (Dickenson & Dickinson, 1975*a*). Less detailed but similar results were obtained at pH7.05 and 8.7, and with NAD^+ at pH5.8. Approximate values obtained for $K_{E\cdot NADH}$ were 15 and $120\,\mu M$ respectively, and for $K_{E\cdot NAD+}$ $\sim 500\,\mu M$, which may be compared with the kinetic values found by Dickenson & Dickinson (1975*a*).

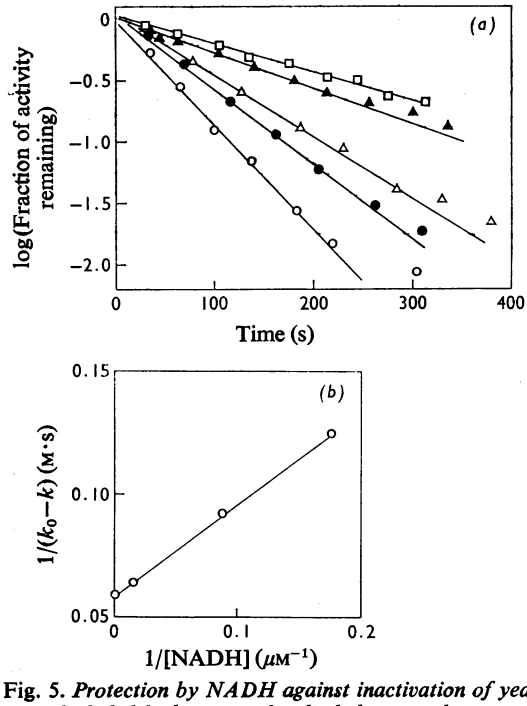


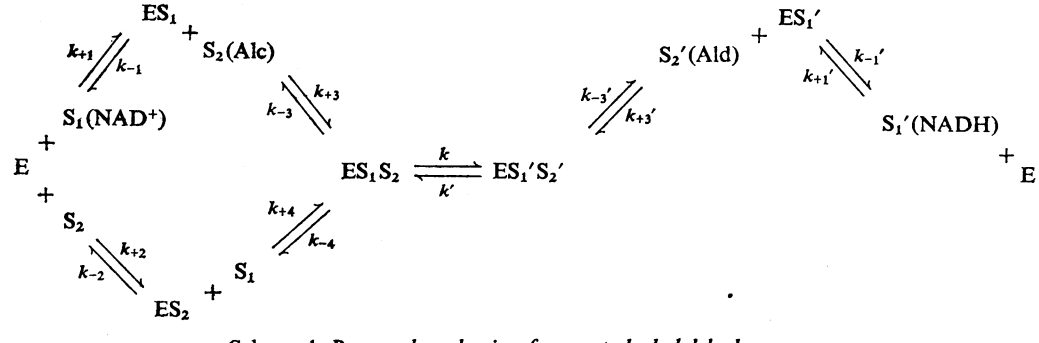
Fig. 5. *Protection by NADH against inactivation of yeast alcohol dehydrogenase by diethyl pyrocarbonate*
(*a*) Inactivation of 0.23 μM-yeast alcohol dehydrogenase by 910 μM-diethyl pyrocarbonate at pH5.8 and 25°C, in the presence and absence of NADH. The NADH concentrations (μM) were: ○, 0; ●, 5.9; △, 11.8; ▲, 62; □, 620. (*b*) Variation of the inactivation rate constants in (*a*) with the NADH concentration. k_0 and k are the rate constants obtained in the absence and presence of NADH respectively.

Less detailed measurements were made of the inactivation of the $E\cdot NAD^+$ complex because of the large quantities of coenzyme required to give concentrations of $100K_{E\cdot NAD+}$ (Dickenson & Dickinson, 1975*a*,*b*). The results obtained at three pH values, with concentrations of diethyl pyrocarbonate in the range 210–1676 μM, are also shown in Fig. 4. At pH9.0 and 9.9 the plots of log(residual activity) against time showed no initial linear portion, which suggests that diethyl pyrocarbonate is more unstable in the presence of the very high NAD^+ concentrations used. It was not possible to confirm this conclusion by using the assay system of Dickenson & Dickinson (1975*c*), because of the u.v. absorption of the NAD^+ solutions.

Discussion

Binary complexes

Dickenson & Dickinson (1975*a*) showed that, in the range pH5.9–8.9, the reduction of acetaldehyde by NADH, catalysed by yeast alcohol dehydrogenase,



Scheme 1. *Proposed mechanism for yeast alcohol dehydrogenase*
Alc represents primary or secondary alcohol and Ald represents acetaldehyde or butyraldehyde.

Table 2. *Relationships between kinetic coefficients in the ethanol–acetaldehyde reaction at pH 4.9 and pH 9.9*
The coefficients are taken from Table 1 and from Dickenson & Dickinson (1975*a*, Table 2). At 25°C and in phosphate buffer, *I* 0.1 mol/litre, the equilibrium constant for the overall reaction is 0.98×10^{-11} M (Bäcklin, 1958).

pH	$\phi_1\phi_2/\phi_{12}\phi_0'$	$\phi_1'\phi_2'/\phi_{12}'\phi_0$	$\phi_{12}'[H^+]/\phi_{12}$ (M)
4.9	29	0.12	1.95×10^{-11}
9.9	~0.26	2.25	0.38×10^{-11}

follows a compulsory-order mechanism, with NADH combining first with the enzyme and the rate-limiting step being the dissociation of the terminal E·NAD$^+$ complex. The oxidation of ethanol by NAD$^+$ follows a 'preferred-order' mechanism (Dalziel, 1975) in which there is significant dissociation of NAD$^+$ from the ternary E·NAD$^+$–ethanol complex. The initial-rate equations for the mechanism (Scheme 1), when compared with eqn. (1), indicate that the dissociation constants for the enzyme–coenzyme complexes are given by $K_{E\cdot NAD^+}=\phi_{12}/\phi_2$ and $K_{E\cdot NADH}=\phi_{12}'/\phi_2'$ (Dalziel, 1957, 1975).

To define more precisely the pH-dependence of these dissociation constants, we have determined the kinetic coefficients at pH 4.9 and 9.9 (Table 1). The results are generally consistent with the mechanism previously proposed. Some of the tests of mechanism of two-substrate reactions (Dalziel, 1957) used in the earlier work (Dickenson & Dickinson, 1975*a*) are shown in Table 2. The relationship $\phi_{12}'[H^+]/\phi_{12}=K_{eq.}$ is reasonably well satisfied at both pH values. At pH 4.9 the maximum-rate relationships are in agreement with the preferred-order mechanism previously proposed (Scheme 1). The finding of $\phi_1'\phi_2'/\phi_{12}'\phi_0<1$ indicates that, at pH 4.9, the dissociation of NADH from the terminal E·NADH complex has ceased to be the principal rate-limiting step in ethanol oxidation. At pH 9.9, the maximum-rate relationships are consistent with a compulsory order of ethanol oxidation and a preferred order for acetaldehyde reduction. At this pH value therefore it is possible that there is no significant formation of ES_2 (Scheme 1), as was found at pH 8.9 (Dickenson & Dickinson, 1975*a*). However, there is a significant contribution from a complex of the type enzyme–acetaldehyde (since $\phi_1'\phi_2'/\phi_{12}'\phi_0>1$). The existence, though not necessarily the kinetic significance, of a binary ES_2'-type complex is implied by the isotope-exchange experiments of Silverstein & Boyer (1964), which were conducted at pH 7.9.

The above considerations suggest that the relationships $K_{E\cdot NAD^+}=\phi_{12}/\phi_2$ and $K_{E\cdot NADH}=\phi_{12}'/\phi_2'$ hold over the entire range pH 4.9–9.9 (at 25°C). These relationships are true for both compulsory- and preferred-order mechanisms in which the coenzyme combines first with the enzyme (with no isomerization of the ES_1 or ES_1' complexes), and are also true for a rapid-equilibrium random-order mechanism.

The variation with pH of ϕ_{12}'/ϕ_2', plotted as described by Dixon (1953) (Fig. 1), indicates that the combination of enzyme with NADH perturbs the p*K* of an ionizing group from 7.1 in the free enzyme to 8.7 in the E·NADH complex. The proton-uptake experiments with NADH (Fig. 2) are consistent with this view. In contrast, the value of $K_{E\cdot NAD^+}=\phi_{12}/\phi_2$ is constant from pH 4.9 to about pH 8.5. above which it increases (Fig. 1). Also, the uptake of protons occurring on formation of the E·NAD$^+$ complex at pH 7 or 8 is much less than that found with the E·NADH complex, and is independent of pH over this range (Fig. 2).

Dickenson & Dickinson (1975*c*) found that the enzyme possesses one histidine residue which is essential for enzymic activity and which, in the unprotonated form, is highly reactive towards diethyl pyrocarbonate. Measurement of the pH-dependence of the rate constant for acylation of the enzyme by this reagent (by using the increase in A_{250} caused by ethoxycarbonylhistidine residues) gave a value of 7.1 for the p*K* of the essential histidine residue. Measurement of the rate constant of inactivation of the enzyme by diethyl pyrocarbonate (Fig. 4) gives an identical value for the histidine p*K*, and justifies the use of this method for studying the ionization of this residue. Inactivation of the E·NADH complex (Fig. 4) shows that, in this complex, the histidine residue has a p*K* approx. 8.4, and that both ionized forms are only about half as reactive as in the free enzyme. The limited results with the E·NAD$^+$ complex suggest, however, that, although the presence of the coenzyme lowers the reactivity by about one-half, there is no significant change in the p*K* of the essential histidine residue.

The rate constant of inactivation when the essential histidine residue is in the unprotonated form (approx. $145\,M^{-1}\cdot s^{-1}$) is roughly half the rate constant measured by absorption changes caused by acylation (Dickenson & Dickinson, 1975*c*). This is consistent with there being one reactive histidine residue/subunit, but only two active sites/tetrameric molecule (Dickinson, 1974; Leskovac & Pavkov-Peričin, 1975).

It is apparent from Fig. 4 that, when the histidine residue is protonated, the enzyme does have some reactivity towards diethyl pyrocarbonate, about one-tenth of that of the unprotonated form. A similar result was found for rabbit liver arylsulphatase B by Lee & van Etten (1975), who found rate constants for inactivation by diethyl pyrocarbonate of $3.4\,M^{-1}\cdot s^{-1}$ in the protonated form and $13\,M^{-1}\cdot s^{-1}$ in the unprotonated form. The essential histidine residue of this enzyme is therefore much less reactive than that

of either yeast alcohol dehydrogenase or pig heart lactate dehydrogenase (Holbrook & Ingram, 1973).

Comparison of the results of Fig. 4 with those of Fig. 1 indicates that the essential histidine residue is the ionizing group responsible for the pH-dependence of $K_{E\cdot NADH}$, but does not affect the formation or dissociation of the E·NAD$^+$ complex. The ionization of some other residue(s) must therefore be either directly, or indirectly via a pH-dependent conformational change, responsible for the increase in $K_{E\cdot NAD^+}$ above pH8. It may be noted here that Rabin *et al.* (1970) found that the alkylation of the essential cysteine residue of yeast alcohol dehydrogenase by iodoacetate depended on the protonated form of a group with pK approx. 7.2. It seems possible that this group is the essential histidine, and, if so, implies that it is also located at or near the active site.

The results of Fig. 2 with yeast alcohol dehydrogenase modified by diethyl pyrocarbonate suggest that the pH-dependence of NADH binding is different in the native and modified enzymes. Also, Dickenson & Dickinson (1975*c*) found that $K_{E\cdot NADH}$ at pH7.05 was higher in the modified enzyme than in the native enzyme. These results are expected, since the pK of the ethoxycarbonylhistidine residue will be lower than that of the histidine in the unmodified enzyme (Mühlrád *et al.*, 1967).

Ternary complexes

Enzyme inactivated by diethyl pyrocarbonate is similar to enzyme inactivated by alkylation of specific thiol groups by iodoacetamide or butyl isocyanate in that all these modified enzymes have lost the ability to form ternary enzyme–coenzyme–substrate-analogue complexes (Dickinson, 1972; Twu *et al.*, 1973; Dickenson & Dickinson, 1975*c*), whereas coenzyme binding is largely unaffected. However, enzyme alkylated with iodoacetamide has the same value of $K_{E\cdot NADH}$ at pH7.05 as does native enzyme (Dickinson, 1972). In addition, the uptake of protons on forming E·NADH is unaffected by this modification, and the rate of inactivation by iodoacetamide is essentially independent of pH (Whitehead & Rabin, 1964). These considerations suggest that the essential histidine residue is the group whose pK is perturbed from 7.1 to approx. 8.7 in the E·NADH complex, and may also be the group responsible for binding the substrate in the ternary complex. It is possible that the abolition of substrate-binding capacity, which is observed when the enzyme is modified by alkylation of one or other of its essential thiol groups, is due to steric blockage of the substrate-binding site, even though the substrate does not combine with a thiol group.

Since it is the unprotonated form of the essential histidine residue which reacts preferentially with diethyl pyrocarbonate, and since, when modified, the enzyme cannot form ternary complexes (Dickenson & Dickinson, 1975*c*), it is possible that the histidine must be protonated for ternary-complex-formation to occur. An estimate of the dissociation constant for the reaction of acetaldehyde with the E·NADH complex (k'_{-3}/k'_{+3} in Scheme 1) would therefore be desirable and, in principle, could be obtained from product-inhibition studies if the ratio k/k' were known, or if $k' \ll k$ (Plapp, 1973). However, we have no estimate of the ratio k/k', and estimates of a lower limit for k' indicate that $k \ll k'$ (Dickenson, 1975; Dickenson & Dickinson, 1975*a*).

The proton-uptake experiments with the substrate analogue acetamide, which forms a ternary E·NADH–acetamide complex (Dickinson, 1970), indicate that acetamide has no preference for a particular ionized form of the enzyme. However, Dickenson & Dickinson (1975*a*) showed that, with acetaldehyde at pH7.05 and 8.9, $1/\phi_2' \approx k'_{+3}$, the rate constant for reaction of acetaldehyde with the E·NADH complex (Scheme 1). If this is also true at pH9.9, then the variation of $1/\phi_2'$ with pH (Fig. 1) implies that acetaldehyde reacts with the E·NADH complex only when a group of pK approx. 8.7 is protonated. This group could be the essential histidine residue, pK approx. 8.4 in the E.NADH complex (Fig. 4). Klinman (1975) obtained a similar variation with pH of $1/\phi_2'$, with a pK of 8.25. This lower value may be attributed to the use of rather higher concentrations of glycine in the buffer than were used in some of the experiments in the present work, thus lowering the effective concentration of acetaldehyde, as discussed above. As noted above, the variation of ϕ_2' with pH in the present work cannot be due to a change in the extent of hydration of acetaldehyde.

When the formation of the E·NAD$^+$–alcohol complex is considered, the results of Dickenson & Dickinson (1975*b*) suggest that the combination of butan-1-ol or propan-2-ol with the E·NAD$^+$ complex does not require a particular ionized form of the enzyme. In the initial-rate equation for alcohol oxidation (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975*a*,*b*),

$$\phi_2 = \frac{Ak_{-3}+k}{kk_{+3}}$$

with $A = 1+(k'/k'_{-3})$ (rate constants defined by Scheme 1). With butan-1-ol (and propan-2-ol), there is a substantial ^{2}H-isotope effect on the maximum rate of reaction, implying that hydride transfer is a significant rate-limiting step and that $\phi_0 = A/k$ (Dickenson & Dickinson, 1975*b*). Also, the maximum rate of reduction of butyraldehyde is much greater than the maximum rate of butan-1-ol oxidation, so that $k_{-3} \gg k$ (Dickenson & Dickinson, 1975*a*). Together with the fact that $A > 1$, these earlier results

indicate that, for the butan-1-ol–butyraldehyde system:

$$\phi_2 = \frac{Ak_{-3}}{kk_{+3}}$$

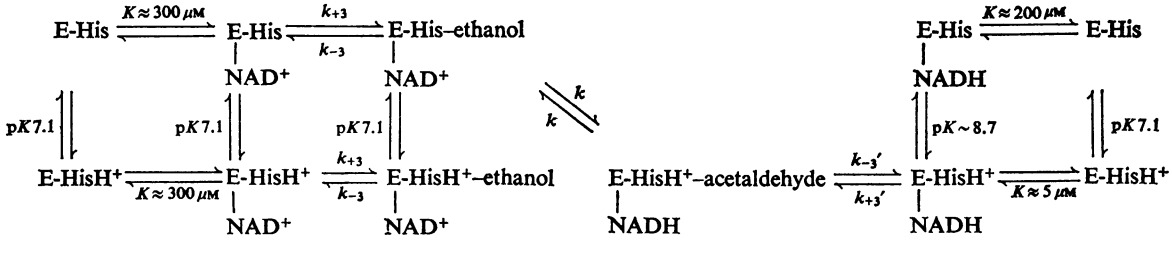
It seems therefore that the Michaelis constant for butan-1-ol (and possibly propan-2-ol), ϕ_2/ϕ_0, equals k_{-3}/k_{+3}, the dissociation constant for alcohol from the E·NAD$^+$–alcohol complex. For both butan-1-ol and propan-2-ol, ϕ_2/ϕ_0 is approximately constant from pH5.5 to 9.9, with values of about 44 and 250mM respectively (Dickenson & Dickinson, 1975*b*). It is reasonable to suppose that, with ethanol as substrate, k_{-3}/k_{+3} is also independent of pH; this might be tested by product-inhibition experiments, since, as noted above, $k \ll k'$, which would allow the estimation of k_{-3}/k_{+3} from observed inhibition constants (Plapp, 1973). It is noteworthy that the relationship $\phi_2/\phi_0 = k_{-3}/k_{+3}$, and that mentioned previously, i.e. $\phi_1/\phi_0 = k_{-4}/k_{+4}$ (Dickinson & Monger, 1973), are also characteristic of a rapid-equilibrium random-order mechanism when $\phi_0 = 1/k$. With the preferred-order mechanism, it is only necessary that $\phi_0 = A/k$.

Comparison of the mechanisms of alcohol dehydrogenases from yeast and horse liver

Scheme 2 summarizes the conclusions drawn from the results of the present work with respect to binding of coenzymes and substrates, and the ionization of the essential histidine residue. Scheme 2 implies that the hydride-transfer step (k) in ethanol oxidation depends on the unprotonated form of the essential residue. The pH-dependence of the rate of hydride transfer in the oxidation of butan-1-ol and propan-2-ol indicates the involvement of the unprotonated form of groups with pK values in the range 6.5–8.5 (Dickenson & Dickinson, 1975*b*). It seems likely that one of these groups is the essential histidine residue and is also involved in hydride transfer with ethanol. There are, however, other group(s) involved. The pH profiles of k for butan-1-ol and propan-2-ol (Dickenson & Dickinson, 1975*b*) and for *p*-methylbenzyl alcohol (Klinman, 1975) indicate also the necessity for the unprotonated form of a group with pK approx. 8.2. The identity of this group is at present unknown, but a possibility is a water molecule co-ordinated to Zn^{2+}. In the opposite direction (k'), Klinman (1975) has observed that the hydride-transfer step in the reduction of *p*-methylbenzaldehyde decreases five-fold from pH8.5 to pH9.5. This is consistent with a requirement for the protonated form of an ionizing group.

It is apparent that there are a number of differences in the overall mechanism between the alcohol dehydrogenases of yeast and horse liver. Thus, with the yeast enzyme, it is possible that the essential histidine residue may be the group responsible for acid–base catalysis of the reaction between the ternary complexes (Klinman, 1975), and could be the group which binds the substrate. In this respect it could fulfil a role similar to histidine-195 of lactate dehydrogenase (Holbrook *et al.*, 1975; Shore *et al.*, 1975). In horse liver alcohol dehydrogenase this role is taken by the 'catalytic' zinc atom (Brändén *et al.*, 1975). The great similarity between Scheme 2 and the current mechanism for lactate dehydrogenase (Shore *et al.*, 1975) emphasizes that there may yet be common mechanisms of catalysis by the dehydrogenases.

It is notable that arginine-47 in liver alcohol dehydrogenase is replaced by histidine-44 in the enzymes from yeast and *Bacillus stearothermophilus* (Brändén *et al.*, 1975). Also, liver alcohol dehydrogenase is relatively insensitive to diethyl pyrocarbonate (Morris & McKinley-McKee, 1972). Since arginine-47 in the liver enzyme binds the pyrophosphate moiety of the coenzyme, if histidine-44 is the essential reactive residue in the yeast enzyme, then this implies that the active-site structures are rather different. This is consistent with the different substrate specificities (Dickinson & Dalziel, 1967), with the lower affinity for the coenzymes shown by the yeast enzyme, and with the different orientation of



Scheme 2. *Ionizations of the essential histidine residue and the binding of coenzymes and substrates to yeast alcohol dehydrogenase*

E-His represents the enzyme and its essential histidine residue. Rate constants are defined in Scheme 1.

thiol-group modification by coenzyme analogues (Jörnvall *et al.*, 1975).

A further difference between the two alcohol dehydrogenases lies in the reaction of substrates with the enzyme–coenzyme complexes, described by the equilibrium constant $\phi_2'[H^+]/\phi_2$ (Dalziel, 1963*b*). The decrease of this ratio in the pH range 6–10 with the liver enzyme implies the participation of at least two ionizing groups. At any particular pH value one of these could become protonated when the E·NAD$^+$–ethanol complex is converted into EH$^+$·NADH–acetaldehyde (Dalziel, 1963*b*). With the yeast enzyme, although the plot of $\log(\phi_2'[H^+]/\phi_2)$ against pH has a maximum slope of about −0.7 in the range pH 7–pH 8.9 (Dickenson & Dickinson, 1975*a*), the data of Table 1 show that there is no further decrease above pH 8.9. The reduction of E·NAD$^+$ to E·NADH thus involves net proton uptake only in the pH region 7.0–9.0, and only one ionizing group (the reactive histidine) is implicated in the process.

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