Inhibition by Ethanol, Acetaldehyde and Trifluoroethanol of Reactions Catalysed by Yeast and Horse Liver Alcohol Dehydrogenases

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1. Product inhibition by ethanol of the acetaldehyde–NADH reaction, catalysed by the alcohol dehydrogenases from yeast and horse liver, was studied at 25°C and pH 6–9. 2. The results with yeast alcohol dehydrogenase are generally consistent with the preferred-pathway mechanism proposed previously [Dickenson & Dickinson (1975) *Biochem. J.* **147**, 303–311]. The observed hyperbolic inhibition by ethanol of the maximum rate of acetaldehyde reduction confirms the existence of the alternative pathway involving an enzyme–ethanol complex. 3. The maximum rate of acetaldehyde reduction with horse liver alcohol dehydrogenase is also subject to hyperbolic inhibition by ethanol. 4. The measured inhibition constants for ethanol provide some of the information required in the determination of the dissociation constant for ethanol from the active ternary complex. 5. Product inhibition by acetaldehyde of the ethanol–NAD⁺ reaction with yeast alcohol dehydrogenase was examined briefly. The results are consistent with the proposed mechanism. However, the nature of the inhibition of the maximum rate cannot be determined within the accessible range of experimental conditions. 6. Inhibition of yeast alcohol dehydrogenase by trifluoroethanol was studied at 25°C and pH 6–10. The inhibition was competitive with respect to ethanol in the ethanol–NAD⁺ reaction. Estimates were made of the dissociation constant for trifluoroethanol from the enzyme–NAD⁺–trifluoroethanol complex in the range pH 6–10.

Recent detailed kinetic studies with yeast alcohol dehydrogenase in the absence of products have indicated that the enzyme utilizes a preferred-pathway mechanism (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975a). Since it should be possible, in principle, to demonstrate the occurrence of a less-favoured pathway by product-inhibition studies, and, perhaps also be possible to estimate values for the dissociation constant of substrate from catalytic ternary complexes, we have tried this approach with the yeast enzyme. The measurement of dissociation constants for substrate from ternary complexes at different pH values raises the possibility of being able to identify the group(s) on the enzyme that binds the substrate or that affects the binding. The same end might also be gained by using substrate analogues as inhibitors and measuring the pH-dependence of inhibitor constants. In this connection, we have studied the inhibition of the enzyme by trifluoroethanol. Shore *et al.* (1974) used this compound successfully in their investigations with the horse liver enzyme.

We have also used product inhibition by ethanol to study the acetaldehyde–NADH reaction catalysed

by horse liver alcohol dehydrogenase. Although there is evidence for a preferred-order mechanism with this enzyme (Silverstein & Boyer, 1964; Dalziel & Dickinson, 1966) in the ethanol–acetaldehyde inter-conversions, the favoured pathway is so predominant that the alternative pathway escaped detection in detailed initial-rate studies in the pH range 6–9 (Dalziel, 1963b). Hanes *et al.* (1972) did obtain some evidence for the preferred-pathway mechanism at pH 8.6 from initial-rate measurements and confirmed this by observing hyperbolic product inhibition by ethanol of the acetaldehyde–NADH reaction. It seemed worth while to extend these product-inhibition experiments over a wider pH range.

The product-inhibition studies of Wratten & Cleland (1963), using rather limited concentrations of product inhibitor, did not provide evidence for alternative pathways of coenzyme and substrate addition with either yeast or horse liver alcohol dehydrogenase. Further, the work did not provide estimates of dissociation constants of substrates from ternary complexes. Attempts have been made more recently to obtain these dissociation constants for the horse liver enzyme from product-inhibitor constants on the assumption of a compulsory mechanism (Shore & Theorell, 1966a; Ainslie & Cleland, 1972), but it has been pointed out that information in ad-

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dition to product-inhibitor constants is required to do this (Plapp, 1973). Since it now appears that a preferred-pathway mechanism is a more accurate description of catalysis for both yeast and horse liver enzymes, it seemed important to determine how product-inhibition data can be used in this case to estimate dissociation constants for substrate from catalytic ternary complexes.

A preliminary report of some of this work has appeared (Dickinson & Dickenson, 1977).

Experimental

Materials

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

Enzymes. Yeast alcohol dehydrogenase was prepared and assayed as described by Dickinson (1970, 1972). The specific activity was 400 units ($\mu\text{mol}/\text{min}$) per mg of protein. Horse liver alcohol dehydrogenase was obtained from Boehringer Corp. (London) Ltd., London W.5, U.K., as a suspension in 10% (v/v) ethanol, and was exhaustively dialysed against sodium phosphate buffer, pH 7.0, I 0.1, before use. The enzyme was shown to be 100% active by the assay of Dalziel (1961).

Coenzymes and substrates. NAD^+ (grade II) and NADH (grade I) were obtained from Boehringer Corp. For initial-rate studies NAD^+ was purified as described by Dalziel (1963a). Aldehydes (Fisons, Loughborough, Leics., U.K.) were redistilled before use. Analytical-grade alcohols (J. Burroughs, London S.E.11, U.K., or Fisons) and trifluoroethanol [Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.] were used without further treatment.

Initial-rate measurements

Assays were performed fluorimetrically for ethanol oxidation by NAD^+ and spectrophotometrically for aldehyde reduction by NADH, as described previously (Dickinson & Monger, 1973). Except where stated otherwise, measurements were made at 25°C in buffers of I 0.1. Below pH 8.5 sodium phosphate buffers were used. Above pH 8.5 $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ (10mm-total carbonate concentration), containing sufficient Na_2HPO_4 to give I 0.1, were used. Bovine serum albumin (1mg) was added to all assays and dilutions involving yeast alcohol dehydrogenase.

Initial-rate measurements were performed in duplicate, with a reproducibility of 5% in general, and at worst 10% with the smallest concentrations of both coenzyme and substrate. Progress curves were generally linear for at least 30s. The ranges of substrate, coenzyme and inhibitor concentrations were somewhat different in each experiment and are detailed in the Results section. For ethanol inhibition of yeast alcohol dehydrogenase, complete

experiments with four or five different concentrations of both substrate and coenzyme (in all possible combinations) were performed in the absence of product inhibitor and at the highest inhibitor concentrations. At the lower inhibitor concentrations, usually three different concentrations of both substrate and coenzyme (in all possible combinations) were used, the principal object being the determination of the maximum specific rate.

Kinetic coefficients in the absence (ϕ_i) or presence ($\phi_{i, \text{app.}}$) of inhibitor, in the initial-rate eqns. (1) or (2):

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

$$\frac{e}{v_0} = \phi_{0, \text{app.}} + \frac{\phi_{1, \text{app.}}}{[S_1]} + \frac{\phi_{2, \text{app.}}}{[S_2]} + \frac{\phi_{12, \text{app.}}}{[S_1][S_2]} \quad (2)$$

were obtained from the slopes and intercepts of double-reciprocal plots (at each fixed concentration of inhibitor) as described by Dalziel (1957). In eqns. (1) and (2) e is the concentration of enzyme active sites, for both enzymes two per molecule (Theorell & Bonnichsen, 1951; Dickinson, 1974). S_1 and S_2 are coenzyme and substrate respectively. The symbols S_1 , S_2 and $\phi_{i, \text{app.}}$ are used for alcohol-NAD⁺ reactions and primed symbols S'_1 , S'_2 and $\phi'_{i, \text{app.}}$ for aldehyde-NADH reactions, as in previous kinetic studies (Dalziel & Dickinson, 1966; Dickinson & Monger, 1973; Dickenson & Dickinson, 1975a,b, 1977).

Under all the conditions used, primary and secondary double-reciprocal plots were linear within experimental error. Coefficients obtained in the absence of inhibitor agreed to within 10% with published values (Dalziel, 1963b; Dickinson & Dickinson, 1975a). Plots of $\phi_{i, \text{app.}}$ versus [inhibitor] were made to evaluate linear inhibition constants K_i , defined by:

$$\phi_{i, \text{app.}} = \phi_i \left(1 + \frac{[\text{inhibitor}]}{K_i} \right) \quad (3)$$

For apparently hyperbolic inhibition, i.e.

$$\phi_{i, \text{app.}} = \frac{A + B [\text{inhibitor}]}{C + D [\text{inhibitor}]} \quad (4)$$

the constants $K^{\text{num.}} = A/B$ and $K^{\text{denom.}} = C/D$ (i.e. constants for the numerator and denominator respectively) were evaluated from plots of $1/(\phi_{i, \text{app.}} - \phi_i)$ versus $1/[\text{inhibitor}]$ from which

$$\text{Intercept} = CD/(BC - AD)$$

and

$$\text{Slope} = C^2/(BC - AD)$$

so that:

$$B/D = \phi_i + (1/\text{Intercept}) = (A/C) + (1/\text{Intercept})$$

and

$$C/D = \text{Slope}/\text{Intercept}$$

Hence A/B can be calculated.

Results

Yeast alcohol dehydrogenase

Product inhibition by ethanol of the acetaldehyde-NADH reaction. Primary and secondary double-reciprocal plots of initial-rate data, at pH 8.0 and 25°C, and at a fixed concentration of product inhibitor

(1.08 M-ethanol) are shown in Fig. 1. Similarly, linear plots were obtained at the other concentrations of ethanol used, and at the other pH values. The variation of the reciprocal specific rate (at pH 8.0 and 25°C) with the reciprocal of the acetaldehyde concentration, at a fixed high concentration of NADH (600 μM), is shown in Fig. 2(a) for several ethanol

Fig. 1. Reduction of acetaldehyde by NADH, in the presence of 1.08 M-ethanol, with yeast alcohol dehydrogenase at pH 8.0 and 25°C

(a) Primary plot showing variation of the reciprocal of the specific initial rate (e/v_0) with the reciprocal of the acetaldehyde concentration, for several constant NADH concentrations. The NADH concentrations (μM) were: $\circ$, 600; $\bullet$, 150; Δ , 75; $\blacktriangle$, 37.5. For clarity results with 300 μM -NADH have been omitted. (b) Secondary plot showing variation of the intercepts ($\circ$) and slopes ($\bullet$) in (a) with the reciprocal of the NADH concentration.

Fig. 2. Product inhibition by ethanol of the reduction of acetaldehyde by 600 μM -NADH, with yeast alcohol dehydrogenase at pH 8.0 and 25°C

(a) Variation of the reciprocal of the specific initial rate (e/v_0) with the reciprocal of the acetaldehyde concentration, for several constant ethanol concentrations. The ethanol concentrations (mM) were: $\circ$, 0; $\bullet$, 32.4; Δ , 64.8; $\blacktriangle$, 130; $\square$, 270; $\blacksquare$, 540; ∇ , 810; $\blacktriangledown$, 1080. (b) Variation of the intercepts ($\circ$) and slopes ($\bullet$) in (a) with the ethanol concentration. Δ , Variation of the reciprocal of the increase in intercept [(intercept at a particular ethanol concentration) - (intercept in absence of ethanol)] with the reciprocal of the ethanol concentration.

concentrations. The secondary plots of slopes and intercepts versus [ethanol] (Fig. 2b) indicate that the slopes are affected by linear inhibition and the intercepts by hyperbolic inhibition. The latter is further demonstrated by the linear double-reciprocal plot of $1/(\text{increase in intercept})$ versus $1/[\text{ethanol}]$ (Fig. 2b). Results similar to those of Fig. 2 were obtained at all the pH values used.

The range of coenzyme and substrate concentrations used in the following experiments was: at pH 5.95, NADH 18.5–351 μM , acetaldehyde 2.3–36.4 mM; at pH 7.05 and pH 8.0, NADH 37–675 μM , acetaldehyde 0.42–37 mM; at pH 9.0, NADH 59–1180 μM , acetaldehyde 0.45–36 mM. Ethanol concentration was varied from 27 to 1080 mM (also including zero) in all these experiments.

The variation with [ethanol] of the coefficients $\phi'_{1, \text{app.}}$ in eqn. (2), obtained from plots such as in Fig. 1, is shown in Figs. 3–6. It is apparent that at all pH values the reciprocal maximum specific rate $\phi'_{0, \text{app.}}$ is subject to hyperbolic inhibition by ethanol (Fig. 3). Plots of $1/(\phi'_{0, \text{app.}} - \phi'_0)$ versus $1/[\text{ethanol}]$ (Fig. 3b) were linear within experimental error. The inhibition can be described by two constants ($K^{\text{num.}}$ and $K^{\text{denom.}}$), calculated as described in the Experimental section; their values are given in Table 1, along with the estimates for the maximum specific rate at infinite ethanol concentration $[D/B]$ in eqn. (4). Fig. 3(a) shows that estimation of inhibition constants by the conventional method of (intercept)/(initial slope) of plots of $\phi'_{0, \text{app.}}$ versus [ethanol] is subject to considerable error when $\phi'_{0, \text{app.}}$ at infinite [ethanol] is only about four times the value of ϕ'_0 (at [ethanol]=0). This is the procedure used by Wratten & Cleland (1963). The most important

points in a plot of this type have the highest error in $(\phi'_{0, \text{app.}} - \phi'_0)$, which is reflected in the proportionately greater scatter in the double-reciprocal plots at low ethanol concentrations.

At all pH values the coefficient ϕ'_1 is increased only slightly in the presence of the product inhibitor (Fig. 4); the inhibition appears to be linear within experimental error. Fig. 5 shows that $\phi'_{2, \text{app.}}$ is linearly dependent on the concentration of ethanol. Linear inhibition constants for $\phi'_{1, \text{app.}}$ and $\phi'_{2, \text{app.}}$ are given in Table 1. The range of substrate and coenzyme concentrations used at pH 5.95 and 7.05 was inadequate for the determination of the coefficient $\phi'_{12, \text{app.}}$. At pH 8.0 and 9.0 the variation with [ethanol] of $\phi'_{12, \text{app.}}$ is shown in Fig. 6. The inhibition does not appear to be linear with respect to [ethanol], nor can the initial slopes of the plots be determined precisely in the absence of additional data at low ethanol concentrations.

Table 2 shows the results of experiments at 13.5°C and pH 7.05 and 9.0, using 0, 0.54M- and 1.08M-ethanol. These results were required to assist in interpreting the stopped-flow kinetic experiments described in the following paper (Dickinson & Dickenson, 1978). It appears from Table 2 that under both sets of conditions, $\phi'_{0, \text{app.}}$ approximates to its maximum value at 1.0M-ethanol, i.e. consistent with hyperbolic inhibition as at 25°C. Also, the inhibition affecting both $\phi'_{1, \text{app.}}$ and $\phi'_{2, \text{app.}}$ is roughly linear, with inhibition constants given in Table 2.

Product inhibition by butan-1-ol of the butyraldehyde-NADH reaction. Attempts were made to obtain inhibition constants for the inhibition by 0–364 mM-butan-1-ol of the butyraldehyde-NADH reaction with yeast alcohol dehydrogenase at pH 7.05

Fig. 3. Product inhibition by ethanol of the reciprocal maximum specific rate ($\phi'_{0, \text{app.}}$) of reduction of acetaldehyde by NADH with yeast alcohol dehydrogenase at 25°C and at various pH values

(a) Variation of $\phi'_{0, \text{app.}}$ with the ethanol concentration at pH 5.95 (○), 7.05 (●), 8.0 (△) and 9.0 (▲). (b) Variation of $1/(\phi'_{0, \text{app.}} - \phi'_0)$ with the reciprocal of the ethanol concentration at pH 5.95 (○), 7.05 (●), 8.0 (△) and 9.0 (▲).

Table 1. Inhibition constants for product inhibition by ethanol of the reduction of acetaldehyde by NADH, with alcohol dehydrogenases from yeast and horse liver at 25°C and at various pH values

The inhibition constants are defined by the initial-rate equation:

$$\frac{e}{v_0} = \phi'_{0, \text{app.}} + \frac{\phi'_{1, \text{app.}}}{[S'_1]} + \frac{\phi'_{2, \text{app.}}}{[S'_2]} + \frac{\phi'_{12, \text{app.}}}{[S'_1][S'_2]} \quad (2)$$

where

$$\phi'_{0, \text{app.}} = \phi'_0 \left(1 + \frac{[S_2]}{K_{\text{num.}}} \right) \left(1 + \frac{[S_2]}{K_{\text{denom.}}} \right)$$

$$\phi'_{1, \text{app.}} = \phi'_1 \left(1 + \frac{[S_2]}{K_{11}} \right)$$

$$\phi'_{2, \text{app.}} = \phi'_2 \left(1 + \frac{[S_2]}{K_{12}} \right)$$

S_2 , S'_1 , S'_2 , represent ethanol, NADH and acetaldehyde respectively. ϕ'_i are the coefficients obtained in the absence of ethanol. Complete duplicate experiments were performed with yeast alcohol dehydrogenase, and inhibition constants were reproducible to within $\pm 15\%$.

pH	$K_{10}^\dagger$ (mM)	$K_{\text{num.}}$ (mM)	$K_{\text{denom.}}$ (mM)	$1/(\phi'_{0, \text{app.}})$ (at infinite [ethanol]), (s ⁻¹)	K_{11} (mM)	K_{12} (mM)	$\phi_{12}/\phi_1^\ddagger$ (mM)	$k_{-1}\phi_2^\ddagger$ (mM)
Yeast alcohol dehydrogenase								
5.95	—	80	225	1300	750	170	140	900
7.05	—	52	200	1000	840	75	63	240
8.0	—	32	165	520	700	40	60	90
9.0	—	21	140	350	580	30	70	55
Horse liver alcohol dehydrogenase								
5.5*	40	32	290	8.9	—	22	—	—
5.95	35	31	240	14.7	—	—	—	—
7.05	20	22	220	9.1	—	—	—	—
8.0	14	11	220	2.9	—	—	—	—
9.0*	9.5	—	—	—	—	~2	—	—

* Apparent values obtained at high fixed [NADH].

† Inhibition constant from initial slope of plot of $\phi'_{0, \text{app.}}$ versus [ethanol].

‡ Values taken from Dickenson & Dickinson (1975a).

and 25°C. However, the occurrence of substrate inhibition by butyraldehyde at concentrations higher than 15mM prevented reasonably precise determination of the coefficients $\phi'_{0, \text{app.}}$ and $\phi'_{1, \text{app.}}$ (which require saturating butyraldehyde concentrations) and the experiments were not continued.

Product inhibition by acetaldehyde of the ethanol-NAD⁺ reaction. Fig. 7 shows the effect of 0.1–2.0mM-acetaldehyde on the oxidation of 40–1215mM-ethanol by 1250 μ M-NAD⁺, with yeast alcohol dehydrogenase at pH7.05 and 25°C. The concentration of NAD⁺ is about 10 times its Michaelis constant (ϕ_1/ϕ_0) and about 4 times its dissociation constant (ϕ_{12}/ϕ_2) in the absence of product (Dickinson & Monger, 1973). The intercepts and slopes of Fig. 7 therefore, at least in the absence of acetalde-

hyde, approximate to the coefficients ϕ_0 and ϕ_2 respectively. The slope of a plot of e/v_0 versus $1/[\text{ethanol}]$ increases rapidly, in a linear manner, with increasing concentration of acetaldehyde ($K_1=110\mu\text{M}$). The intercept, however, is increased at the most only by about 25% with 2mM-acetaldehyde. Clearly no conclusion can be drawn from Fig. 7 about the pattern of inhibition on ϕ_0 (i.e. linear or hyperbolic), nor may any useful value be obtained for the inhibition constant. When concentrations of acetaldehyde higher than 2mM were used, although the slopes increased linearly, the resulting very high apparent K_m for ethanol meant that the intercepts could not be determined. Results similar to those in Fig. 7 were also obtained at pH8.0.

Preliminary experiments were also conducted to

Fig. 4. Product inhibition by ethanol of the coefficient $\phi'_{1, app.}$ in the reduction of acetaldehyde by NADH with yeast alcohol dehydrogenase at 25°C and at various pH values (○, 5.95; ●, 7.05; △, 8.0; ▲, 9.0)

Fig. 5. Product inhibition by ethanol of the coefficient $\phi'_{2, app.}$ in the reduction of acetaldehyde by NADH with yeast alcohol dehydrogenase at 25°C and at various pH values (○, 5.95; ●, 7.05; △, 8.0; ▲, 9.0)

measure the effect of acetaldehyde on the coefficient ϕ_1 at pH 7.05. It was found impossible to make precise extrapolations in secondary plots of the slopes of e/v_0 versus $1/[NAD^+]$ when acetaldehyde concentrations were high enough to give a significant increase in ϕ_1 . This result is not unexpected if ϕ_{12} increases with increasing [acetaldehyde] in the same

Fig. 6. Product inhibition by ethanol of the coefficient $\phi'_{12, app.}$ in the reduction of acetaldehyde by NADH with yeast alcohol dehydrogenase at 25°C and at pH 8.0 (△) and pH 9.0 (▲)

Fig. 7. Product inhibition by acetaldehyde of the oxidation of ethanol by 1250 μM -NAD⁺, with yeast alcohol dehydrogenase at pH 7.05 and 25°C

The acetaldehyde concentrations (mM) were: ○, 0; ●, 0.1; △, 0.2; ▲, 0.4; □, 1.0; ■, 2.0.

way as ϕ_2 and if ϕ_1 is affected much less. The ethanol concentration must be much higher than ϕ_{12}/ϕ_1 in order to obtain ϕ_1 precisely.

Inhibition by trifluoroethanol. Study of the effect of 0–100 mM-trifluoroethanol on the variation of the reciprocal specific rate e/v_0 with $1/[ethanol]$ with 1080 μM -NAD⁺ and yeast alcohol dehydrogenase at pH 7.05 and 25°C showed that inhibition is strictly

Table 2. *Product inhibition by ethanol of the reduction of acetaldehyde by NADH with yeast alcohol dehydrogenase at 13.5°C*
The coefficients $\phi_{i, \text{app.}}$ are those in eqn. (2). Inhibition constants are defined in Table 1.

pH	[Ethanol] (M)	$\phi'_{0, \text{app.}}$ (s)	$\phi'_{1, \text{app.}}$ ($\mu\text{M} \cdot \text{s}$)	$\phi'_{2, \text{app.}}$ ($\mu\text{M} \cdot \text{s}$)	$\phi'_{12, \text{app.}}$ ($\mu\text{M}^2 \cdot \text{s}$)	K_{11} (mM)	K_{12} (mM)	K_{112} (mM)
7.05	0	0.00054	0.026	0.25	—	~680	~66	—
	0.54	0.0019	~0.05	1.84	—			
	1.08	0.0021	~0.064	4.6	—			
9.0	0	0.00157	0.16	0.88	~35	~550	~11	~6
	0.54	0.012	~0.3	30	~2800			
	1.08	0.012	~0.6	90	~4700

Fig. 8. *Variation with trifluoroethanol concentration of kinetic coefficients describing the oxidation of ethanol by NAD⁺ with yeast alcohol dehydrogenase at pH 7.05 and 25°C*

The coefficients were determined as described in the text. $\circ$, $\phi_{0, \text{app.}}$; $\bullet$, $\phi_{1, \text{app.}}$; $\triangle$, $\phi_{2, \text{app.}}$; $\blacktriangle$, $\phi_{12, \text{app.}}$.

Fig. 9. *pH-dependence of the inhibition constant for trifluoroethanol (K_i) in the inhibition of the oxidation of ethanol by NAD⁺ with yeast alcohol dehydrogenase at 25°C*
Variation of K_i ($\circ$) and $\log K_i$ ($\bullet$) with pH is shown.

competitive and that the slopes of Lineweaver–Burk plots vary linearly with the concentration of trifluoroethanol. Complete initial-rate experiments at pH 7.05, 25°C (11.2–1720 μM , NAD⁺; 25–500 mM, ethanol) with 0, 12.5 mM- and 25 mM-trifluoroethanol showed that the inhibitor affects principally the coefficient ϕ_2 (K_i 3.0 mM), with a much smaller effect on ϕ_{12} (K_i 55 mM) and no effect on ϕ_0 or ϕ_1 (Fig. 8). Thus the inhibition constant derived from the slopes of Lineweaver–Burk plots in the presence of trifluoroethanol at high NAD⁺ concentrations (i.e. several times ϕ_{12}/ϕ_2) approximates to the inhibition constant for ϕ_2 .

At other pH values competitive inhibition was again obtained. Concentrations were in the ranges ethanol 3.3–670 mM and trifluoroethanol 6.5–52 mM. The fixed NAD⁺ concentrations were 1.8 mM (pH 6.0 and 8.0), 2.9 mM (pH 9.0) and 2.3 mM (pH 10.0). The slope inhibition constants were extrapolated to infinite [NAD⁺] by using the values of ϕ_{12}/ϕ_2 given by Dickenson & Dickinson (1975a), and assuming that the inhibition effect on ϕ_{12} is negligible, as at pH 7.05. The variation with pH of the corrected inhibition constants is shown in Fig. 9.

The effect of 4.3–133 mM-trifluoroethanol on the reciprocal maximum specific rate (ϕ'_0) of the acetaldehyde–NADH reaction was examined at pH 7.05. The values of $\phi'_{0, \text{app.}}$ were obtained by using 161 μM - and 323 μM -NADH, and 2.5 mM and 5.0 mM-acetaldehyde, and assuming linear primary and secondary double-reciprocal plots. Large extrapolations were not necessary. Under the conditions used, within experimental error, $\phi'_{0, \text{app.}}$ varied linearly with [trifluoroethanol], with K_i approx. 7 mM.

Horse liver alcohol dehydrogenase

Product inhibition by ethanol of the acetaldehyde–NADH reaction. The variation of the reciprocal specific rate of acetaldehyde reduction with $1/[\text{acetaldehyde}]$, with horse liver alcohol dehydrogenase at 25°C and pH 5.5 and 9.0, is shown in Figs. 10(a) and 11(a) for various concentrations of ethanol as product inhibitor. The NADH concentration was 153 μM , which is much higher than either ϕ'_1/ϕ'_2 or

Fig. 10. Product inhibition by ethanol of the reduction of acetaldehyde by 153 μM -NADH, with horse liver alcohol dehydrogenase at pH 5.5 and 25°C

(a) Variation of the reciprocal of the specific initial rate (e/v_0) with the reciprocal of the acetaldehyde concentration, for several constant ethanol concentrations. The ethanol concentrations (mm) were: ○, 0; ●, 21.6; △, 43.2; ▲, 64.8; □, 81; ■, 135 (with 153 μM -NADH). ▽, 77 μM -NADH and 135 mm-ethanol. (b) Variation of the intercepts (○) and slopes (●) in (a) (at 153 μM -NADH) with the ethanol concentration. △, Variation of the reciprocal of the increase in intercept [(intercept at a particular ethanol concentration) – (intercept in absence of ethanol)] with the reciprocal of the ethanol concentration (at 153 μM -NADH).

Fig. 11. Product inhibition by ethanol of the reduction of acetaldehyde by 153 μM -NADH, with horse liver alcohol dehydrogenase at pH 9.0 and 25°C

(a) Variation of the reciprocal of the specific initial rate (e/v_0) with the reciprocal of the acetaldehyde concentration, for several constant ethanol concentrations. The ethanol concentrations (mm) were: ○, 0; ●, 5.4; △, 10.8; ▲, 21.6; □, 32.4; ■, 43.2 (with 153 μM -NADH). ▽, 77 μM -NADH and 43.2 mm-ethanol. (b) Variation of the intercepts (○) and slopes (●) in (a) (at 153 μM -NADH) with the ethanol concentration.

ϕ'_{12}/ϕ'_2 (Dalziel, 1963b), so that the slopes and intercepts (at [ethanol]=0) approximate to ϕ'_2 and ϕ'_0 respectively. The variation of e/v_0 with $1/[\text{acetaldehyde}]$ at 77 μM -NADH at the highest concentration of ethanol is also shown in Figs. 10(a) and 11(a). At both pH 5.5 and 9.0 the intercept of this plot is

affected very little by the NADH concentration, indicating that, over the range of ethanol concentrations used, the intercepts in Figs. 10(a) and 11(a) approximate to the coefficient $\phi'_{0, \text{app.}}$. Also at pH 5.5, it appears that the slopes approximate to the coefficient $\phi'_{2, \text{app.}}$, though this is not so at pH 9.0.

Fig. 12. Product inhibition by ethanol of the reciprocal maximum specific rate ($\phi'_{0, app.}$) of reduction of acetaldehyde by NADH with horse liver alcohol dehydrogenase at 25°C and at various pH values

(a) Variation of $\phi'_{0, app.}$ with the ethanol concentration at pH 5.95 (○), 7.05 (●), 8.0 (Δ) and 9.0 (▲). (b) Variation of $1/(\phi'_{0, app.} - \phi'_0)$ with the reciprocal of the ethanol concentration at pH 5.95 (○), 7.05 (●) and 8.0 (Δ).

Secondary plots of slopes and intercepts versus [ethanol] are shown in Figs. 10(b) and 11(b). At both pH values the inhibition of the slopes is linear, with inhibition constants as given in Table 1. At pH 5.5 the intercepts do not vary linearly with [ethanol]; the linear plot of 1/(increase in intercept) versus 1/[ethanol] (Fig. 10b) indicates that the inhibition is hyperbolic.

At the intermediate pH values only the reciprocal maximum specific rate $\phi'_{0, app.}$ was determined (using 0 and 2.7–1080 mM-ethanol). Two high concentrations of both NADH (154 and 77 μ M) and acetaldehyde (3.3 and 6.7 mM for lower ethanol concentrations; 6.7 and 13.3 mM for the highest ethanol concentrations) were used and linear primary and secondary double-reciprocal plots were assumed. Figs. 10(a) and 11(a) indicate that this is justified with the ranges of substrate concentrations used.

The variation of $\phi'_{0, app.}$ with [ethanol], at various pH values, is shown in Fig. 12(a). Except at pH 9.0, where the highest ethanol concentration used was only 43.2 mM, the inhibition appears to be hyperbolic. Plots of $1/(\phi'_{0, app.} - \phi'_0)$ versus 1/[ethanol] are shown in Fig. 12(b). These plots are linear within experimental error, and the measured inhibition constants are shown in Table 1. Also given in Table 1 are estimates of the apparent linear inhibition constants, derived from the initial slopes of the plots in Fig.

12(a). These constants could be determined much more precisely than those for the corresponding plots with the yeast enzyme (Fig. 3a), since for the liver enzyme $\phi'_{0, app.}$ (at infinite [ethanol]) is many times greater than ϕ'_0 . The inhibition therefore appears linear within experimental error until much higher relative values of $\phi'_{0, app.}$.

Discussion

Yeast alcohol dehydrogenase

With ethanol and acetaldehyde as substrates, the reaction catalysed by yeast alcohol dehydrogenase (at 25°C and pH 6–9) has been shown to utilize the preferred-pathway mechanism shown in Scheme 1(a) (Silverstein & Boyer, 1964; Dickinson & Monger, 1973; Dickenson & Dickinson, 1975a). In the oxidation of ethanol by NAD⁺, although the net reaction takes place principally through the upper pathway (via ES₁ and ES'₁), a significant steady-state concentration of ES₂ exists in the range pH 6–8. In the reduction of acetaldehyde by NADH, no significant steady-state concentration of the corresponding complex ES'₂ (Scheme 1b) is indicated at pH 6–9, although the possible existence of ES'₂ is suggested by isotope-exchange experiments (Silverstein & Boyer, 1964). Acetaldehyde reduction in the absence of

Scheme 1. *Proposed mechanism for yeast alcohol dehydrogenase*

Abbreviations: E, enzyme; alc, alcohol; ald, aldehyde. The mechanism enclosed by the dotted line is referred to as Scheme 1(a). The complete mechanism is referred to as Scheme 1(b). For further details see the Discussion section. $K_i = k_{-i}/k_{+i}$.

products therefore takes place by an effectively compulsory-order mechanism. The discussion will therefore be in terms of the mechanism in Scheme 1(a).

Product inhibition by ethanol of the acetaldehyde-NADH reaction. Steady-state treatment of Scheme 1(a) for the reaction of S'_1 (NADH) and S'_2 (acetaldehyde) in the presence of the product S_2 (ethanol) yields an expression of the form of eqn. (2). The coefficients are given by:

obtained from the general product-inhibition equations of Pettersson (1972) for Scheme 1(b) if conditions are chosen so that the special case, Scheme 1(a), is an accurate description of the mechanism. The conditions are $k'_{-3} \gg k'_{-4}$, $\phi_1 > 1/k_{+1}$, $\phi'_1 = 1/k'_{+1}$, with (for $\phi'_{1,app.}$) product inhibitor being kept at concentrations where apparently linear inhibition is observed. The general asymptote expression for $\phi'_{0,app.}$, even with these simplifications, is considerably more complex than that given above.

$$\phi'_{0,app.} = \frac{k_{-1}k_{-2}(k+k_{-3}+k_{-4}) + k'(k_{-1}k_{-2} + k_{-1}k_{-4} + k_{-2}k_{-3}) + [k_{-2}(k' + k + k_{-4}) + k'k_{-4}]k_{+3}[S_2]}{k_{-2}k'[k_{-1}(k_{-3} + k_{-4}) + k_{-4}k_{+3}[S_2]]} \quad (5)$$

$$\phi'_{1,app.} = \frac{1}{k'_{+1}} \left(1 + \frac{[S_2]}{K_2} \right) = \phi'_1 \left(1 + \frac{[S_2]}{K_2} \right) \quad (6)$$

$$\phi'_{2,app.} = \frac{k_{-1}[k'_{-3}(k+k_{-3}+k_{-4}) + k'(k_{-3}+k_{-4})] + [k'_{-3}(k+k_{-4}) + k'k_{-4}]k_{+3}[S_2]}{k'_{+3}k'[k_{-1}(k_{-3}+k_{-4}) + k_{-4}k_{+3}[S_2]]} \quad (7)$$

$$\phi'_{12,app.} = K'_1 \left(1 + \frac{[S_2]}{K_2} \right) \times \phi'_{2,app.} \quad (8)$$

Expressions for ϕ'_0 , ϕ'_1 , ϕ'_2 and ϕ'_{12} for the mechanism in the absence of products are given by Dickinson & Monger (1973). One may note that identical expressions for $\phi'_{1,app.}$, $\phi'_{2,app.}$ and $\phi'_{12,app.}$ may be

The effect of ethanol on the reduction of acetaldehyde by NADH will be considered for each coefficient, in eqn. (2), in the light of eqns. (5–8).

(a) $\phi'_{0,app.}$. The hyperbolic inhibition of the reci-

procal maximum rate $\phi'_{0,app.}$ (Fig. 3) demonstrates the existence of an alternative, less effective, pathway of product dissociation as provided for in Scheme 1(a). In the absence of added ethanol, product dissociation is via ES_1 , since $k_{-3} \gg k_{-4}$ and $\phi'_0 = 1/k_{-1}$ (Dickenson & Dickinson, 1975a). In the presence of ethanol Scheme 1(a) predicts that the reaction will be diverted via ES_2 . With ethanol present at saturating concentrations and because the maximum rate in the absence of product ($1/\phi'_0$) is not limited by k' (Dickenson & Dickinson, 1975a), the reciprocal maximum rate $\phi'_{0,app.}$ (eqn. 5) will simplify to:

$$\phi'_{0,app.} = \frac{1}{k_{-2}} + \frac{1}{k_{-4}} \left(1 + \frac{k}{k'} \right) \quad (9)$$

The results obtained here in the range pH 6–9 are thus entirely consistent with the mechanism proposed previously. The results of earlier initial-rate studies of ethanol oxidation in the absence of products could also be explained by the formation of a dead-end ES_2 complex from ES_1S_2 , i.e. Scheme 1(a) with $k_{+2} = k_{-2} = 0$ (Dickinson & Monger, 1973), but such a dead-end complex could not explain hyperbolic inhibition of the reciprocal maximum rate $\phi'_{0,app.}$.

Another possible explanation of the hyperbolic inhibition of $\phi'_{0,app.}$, however, would be the existence of a pathway involving an abortive complex enzyme–NADH–ethanol (ES'_2), formed from ES_2 , and from which ethanol could dissociate. Although liver alcohol dehydrogenase will form an ES_1S_2 complex, there is no evidence that such a complex can exist with the yeast enzyme. The evidence against abortive complex-formation by the yeast enzyme is: (1) the lack of substrate activation or inhibition by high concentrations (up to 1 M) of ethanol in the ethanol–NAD⁺ reaction (Dickinson & Monger, 1973); (2) the unchanged fluorescence yield of enzyme–NADH in the presence of 0.54 M- and 1.08 M-ethanol at 25°C and pH 7.05 (F. M. Dickinson & C. J. Dickinson, unpublished work). One may note that the fluorescence of the enzyme–NADH–acetamide complex is strongly enhanced relative to that of enzyme–NADH (Dickinson, 1970). The observations described under (1) and (2) above also indicate that the hyperbolic plots are not due to a trivial cause such as solvent effects causing a change in the enzyme structure.

It is concluded that the hyperbolic inhibition of $\phi'_{0,app.}$ with yeast alcohol dehydrogenase is caused by the diversion of product dissociation from the pathway via ES_1 to that via ES_2 . Since $k_{-3} \gg k_{-4}$ (Silverstein & Boyer, 1964; Dickinson & Dickinson, 1975a), the expression for $\phi'_{0,app.}$ (eqn. 5) simplifies to

Comparing this equation with eqn. (4) shows that the reciprocal maximum rate at infinite ethanol concentration (B/D) is given by eqn. (9) and that:

$$C/D = \frac{k_{-1}K_3}{k_{-4}} \quad (11)$$

Values of D/B and C/D at various pH values are given in Table 1. To determine the dissociation constant (K_3) for ethanol from the ternary complex ES_1S_2 it is necessary to know the value of k_{-4} , k_{-1} being given by $1/\phi'_0$ (Dickenson & Dickinson, 1975a). If it could be shown that B/D is given by $1/k_{-4}$, i.e. $k \ll k'$ and $k_{-4} \ll k_{-2}$, then K_3 could be calculated. It seems that $k \ll k'$ (Dickenson & Dickinson, 1975a), and the fact that the rate of dissociation of NAD⁺ from ES_1 (k_{-1}) is much smaller, at all pH values, than that of ethanol from ES_1S_2 (k_{-3}) (Dickenson & Dickinson, 1975a) suggests that k_{-4} may be much less than k_{-2} . Clearly, additional evidence is required, and further discussion is given in the following paper, in which are reported additional experiments bearing on this question (Dickinson & Dickinson, 1978).

The inhibition by butan-1-ol of the maximum rate of reduction of butyraldehyde by NADH was examined in an attempt to obtain values of B/D and C/D for this system. These could then have been compared with the known value of K_3 for butan-1-ol and possibly provide information on the relative values of k_{-4} and k_{-2} with this substrate. For butan-1-ol the dissociation constant K_3 is equal to the Michaelis constant ϕ_2/ϕ_0 (Dickenson & Dickinson, 1977). Unfortunately, as explained in the Results section, it was not possible to obtain precise values for $\phi'_{0,app.}$.

Wratten & Cleland (1963) obtained a value of 43 mM for the linear inhibition constant for the apparent maximum rate of acetaldehyde reduction at pH 7.15. They used 208 μ M-NADH (twice the Michaelis constant) and the inhibitor concentrations were 0.23 mM and 50 mM-ethanol. Their value of the inhibition constant is consistent with the variation of $\phi'_{0,app.}$ with ethanol at low inhibitor concentrations [Fig. 3(a), pH 7.05]. There is no further information to be obtained from an examination of the apparent linear inhibition constant for $\phi'_{0,app.}$, since, for the mechanism of Scheme 1(a), the initial variation of $\phi'_{0,app.}$ versus [ethanol] is given by:

$$\phi'_{0,app.} = \phi'_0 + \frac{[S_2]}{K_3} \left(1 + \frac{k}{k'} + \frac{k_{-4}}{k_{-2}} - \frac{k_{-4}}{k_{-1}} \right) \frac{1}{k_{-1}} \quad (12)$$

To obtain K_3 from this expression the same additional information is required as in the use of

$$\phi'_{0,app.} = \frac{k_{-1}k_{-2}(k+k_{-3}) + k'(k_{-1}k_{-2} + k_{-1}k_{-4} + k_{-2}k_{-3}) + [k_{-2}(k+k'+k_{-4}) + k'k_{-4}]k_{+3}[S_2]}{k_{-2}k'[k_{-1}k_{-3} + k_{-4}k_{+3}[S_2]]} \quad (10)$$

eqns. (9) and (11). It may be noted that eqn. (12) allows for the possibility of product activation (if $k_{-2} \gg k_{-1}$, $k_{-4} > k_{-1}$) which is inherent in Scheme 1(a). In the cases where inhibition is observed, as here, it may also be noted that eqn. (12) cannot be readily distinguished from the corresponding expression for product inhibition of a strict compulsory-order mechanism (upper pathway, Scheme 1). In this case

$$\phi'_{0, \text{app.}} = \phi'_0 + \frac{[S_2]}{K_3} \left(1 + \frac{k}{k'}\right) \frac{1}{k_{-1}} \quad (13)$$

Thus if linear inhibition is observed in the accessible range of S_2 concentrations, it does not necessarily follow that the lower pathway of product dissociation (via ES_2) cannot occur. Nor can eqn. (13) be assumed rather than eqn. (12). Conversely the finding of non-linear product inhibition by S_2 indicates that the pathway via ES_2 can exist even if it is kinetically insignificant when S_2 is absent.

(b) $\phi'_{1, \text{app.}}$. The observation of a linear inhibition effect on the coefficient $\phi'_{1, \text{app.}}$ (Fig. 4) is consistent with the existence of an ES_2 complex in a preferred-pathway mechanism and with the above expression for $\phi'_{1, \text{app.}}$ (eqn. 6). On this basis, therefore, the linear inhibition constants for $\phi'_{1, \text{app.}}$ (K_{11} , Table 1) over the range pH 6–8 are equal to the respective dissociation constants for the binary ES_2 complex (K_2). At pH 9.0 where the reaction in both directions is effectively compulsory-order in the absence of added products (Dickenson & Dickinson, 1975a) only a lower limit for K_2 is available, since $K_2 > K_{11}$. The observation of inhibition, however, indicates that the route through ES_2 is used at this pH value. The above inequality is indicated by the equations of Pettersson (1972) when the mechanism is effectively compulsory-order and where inhibition is of the linear type.

An alternative method of calculating K_2 involves the coefficients for the oxidation of ethanol in the absence of added products. Since k_{+1} is known (Dickenson & Dickinson, 1975a), K_2 may be calculated from $\phi_{12}/[\phi_1 - (1/k_{+1})]$, except at pH ≥ 8.9 , where ϕ_1 is not significantly greater than $1/k_{+1}$. Expressions for ϕ_1 and ϕ_{12} in terms of the rate constants in the mechanism are given by Dickinson & Monger (1973). The values of K_2 obtained by this method are 160, 100 and 180 mM at pH 5.95, 7.05 and 8.1 respectively. At pH 4.9, the very large value for the maximum rate relationship (Dickenson & Dickinson, 1977) suggests that $K_2 = \phi_{12}/\phi_1 = 190$ mM. The discrepancy between these calculated values of K_2 and those obtained as inhibition constants affecting $\phi'_{1, \text{app.}}$ (K_{11} , Table 1) may not be outside the combined errors of the measurements. It is clear from Fig. 4 that there may be large errors associated with the determination of K_2 from the slopes of plots of $\phi'_{1, \text{app.}}$ versus ethanol concentration. Errors associated with the parameters required for the

alternative determination of K_2 (i.e. ϕ_1 , ϕ_2 , ϕ_{12} and ϕ'_0) may well be compounded in the calculations.

(c) $\phi'_{2, \text{app.}}$. The theoretical expression for this term (eqn. 7) predicts hyperbolic effects as for $\phi'_{0, \text{app.}}$. As in the latter case, if hyperbolic inhibition is observed, a plot of $1/(\phi'_{2, \text{app.}} - \phi'_2)$ versus $1/[S_2]$ will be linear with slope/intercept given by eqn. (11) if, as before, $k_{-3} \gg k_{-4}$. However, the maximum value of $\phi'_{2, \text{app.}}$ at saturating concentrations of $[S_2]$ becomes

$$\frac{k'_{-3}(k + k_{-4}) + k'k_{-4}}{k'_{+3}k'k_{-4}}$$

which may be compared with the expression for ϕ_2 (when $[S_2] = 0$), which is

$$\frac{k'_{-3}(k + k_{-3}) + k'k_{-4}}{k'_{+3}k'k_{-3}}$$

If, as seems to be the case, $k_{-3} \gg k_{-4}$ (Silverstein & Boyer, 1964; Dickenson & Dickinson, 1975a), it may be difficult to demonstrate hyperbolic inhibition because of the very large range of values that $\phi'_{2, \text{app.}}$ can take, and apparently linear inhibition may be observed. Thus the apparently linear inhibition of the coefficient $\phi_{2, \text{app.}}$ up to 1 M-ethanol, observed at all pH values (Fig. 5), is perhaps consistent with the preferred-pathway mechanism. The preferred-pathway mechanism also predicts that the inhibition constant should be $k_{-1}\phi_2 = k_{+1}\phi_{12} \geq \phi_{12}/\phi_1$ (since $\phi_1 \geq 1/k_{+1}$). The results of Table 1 indicate that, at all pH values, the measured inhibition constant is smaller than the predicted value. The discrepancies appear to be outside the experimental error, and at present we are unable to explain them.

(d) $\phi'_{12, \text{app.}}$. The non-linear inhibition observed for this coefficient at pH 8.0 and 9.0 is predicted for the preferred-pathway mechanism by eqn. (8). Since $\phi'_{2, \text{app.}}$ is a linear function of $[S_2]$ in the range studied, $\phi'_{12, \text{app.}}$ should be a parabolic function of $[S_2]$. The plots in Fig. 6 are concave upwards, consistent with this prediction. It is clear from Fig. 6 that no further information could be obtained in the absence of more data at low concentrations of ethanol.

Product inhibition by acetaldehyde of the ethanol-NAD⁺ reaction. It cannot be ascertained from Fig. 7 whether the product inhibition of the maximum rate is linear or hyperbolic. Accordingly we cannot say whether product dissociation via ES'_2 (Scheme 1b) may occur at high acetaldehyde concentrations. It was pointed out in the Results section that we were unable to determine the effect of acetaldehyde on ϕ_1 and thus whether there is evidence for the formation of ES'_2 . There is evidence for the formation of ES'_2 from isotope exchange at equilibrium (Silverstein & Boyer, 1964), but it seems that this complex has no kinetic significance at pH values less than 9 (Dickenson & Dickinson, 1975a).

Wratten & Cleland (1963) claimed a significant inhibition affecting the intercepts of Lineweaver-Burk plots in a similar experiment to that of Fig. 7. An inhibition constant for acetaldehyde of $670\text{ }\mu\text{M}$ was derived from the secondary plot of intercepts versus [acetaldehyde]. However, the highest concentration of acetaldehyde used was only $250\text{ }\mu\text{M}$, and extrapolations to the intercept of plots of e/v_0 versus $1/[\text{ethanol}]$ were considerably greater and more inaccurate than in Fig. 7. The highest concentration of ethanol used was only 100 mM , which is one-tenth that used in the experiment of Fig. 7. Furthermore, the equilibrium of the reaction is unfavourable for precise spectrophotometric initial-rate measurements of ethanol oxidation at pH 7 (Bäcklin, 1958), particularly in the presence of acetaldehyde and with relatively low concentrations of ethanol. It is concluded that the results of Wratten & Cleland (1963) are probably compatible with those reported here. The inhibition constant for the slopes of Fig. 7 ($110\text{ }\mu\text{M}$) is in agreement with that obtained by Wratten & Cleland (1963) and is consistent with the preferred-pathway mechanism which predicts that the inhibition constant should be $k_{-1}'\phi_2' = k_{+1}'\phi_{12}'$. For yeast alcohol dehydrogenase at pH 7.05 this latter expression is equal to ϕ_{12}'/ϕ_1' and has the value of $120\text{ }\mu\text{M}$ (Dickinson & Monger, 1973).

Inhibition by trifluoroethanol. Pettersson (1974) has examined the general case of an inhibitor competing with a substrate (S_1 or S_2) in the fully random mechanism of Scheme 1b. For an inhibitor, I, competing with S_2 the additional reactions are:

and $K_1K_9 = K_7K_8$. For the preferred-pathway mechanism considered in this paper [Scheme 1(a), with the conditions $k_{-3} \gg k_{-4}$], the kinetic coefficients in the presence of inhibitor become:

$$\begin{aligned} \phi_{0, \text{app.}} &= \phi_0, \phi_{12, \text{app.}} = \phi_{12} \left(1 + \frac{[\text{I}]}{K_7}\right), \\ \phi_{2, \text{app.}} &= \phi_2 \left(1 + \frac{[\text{I}]}{K_9}\right) \\ \phi_{1, \text{app.}} &= \left(\phi_1 - \frac{1}{k_{+1}}\right) + \frac{1 + [\text{I}]/K_7}{k_{+1} \left(1 + \frac{k_{-8}k_{+9}[\text{I}]}{k_{-1}(k_{-1} + k_{-8})}\right)} \end{aligned}$$

The expressions for ϕ_0 , ϕ_1 , ϕ_2 and ϕ_{12} for this mechanism in the absence of inhibitor are given by Dickinson & Monger (1973).

Inhibition of the ethanol-NAD⁺ reaction by trifluoroethanol (I) is consistent with the mechanism indicated above. The lack of inhibition or activation

effects on the maximum rate ϕ_0 (Fig. 8) is as expected and it indicates that the alternative $\text{ES}_1'\text{I}$ complex does not form, or else S_1' dissociates from $\text{ES}_1'\text{I}$ at the same rate as from ES_1' . If the complex does not form then the situation appears to be very similar to that referred to above when the failure of the enzyme to form the enzyme-NADH-ethanol ($\text{ES}_1'\text{S}_2$) complex was discussed.

The absence of an inhibition term affecting ϕ_1 implies the following relationship between the rate constants in Scheme 1(a) and eqn. (14), i.e.

$$\frac{k_{+1}}{k_{+8}} = \frac{k_{-9}}{k_{-1} + k_{-8}} = \frac{k_{-9}}{k_{-8} + k_{-9}}, \text{ so } k_{-1} \approx k_{-9}$$

Linear inhibition factors affecting ϕ_2 and ϕ_{12} are expected, the factors being

$$\left(1 + \frac{[\text{I}]}{K_9}\right) \text{ and } \left(1 + \frac{[\text{I}]}{K_7}\right)$$

respectively. At pH 7.05 the binding of trifluoroethanol in the binary complex EI appears to be much weaker ($K_7 = 55\text{ mM}$) than in the ternary complex ES_1I ($K_9 = 3\text{ mM}$). If this applies at the other pH values, then the competitive inhibition constants K_1 (Fig. 9) approximate to K_9 . The pH-dependence of K_1 (Fig. 9) implies that the binding of trifluoroethanol in the ternary enzyme-NAD⁺-trifluoroethanol (ES_1I) complex is not controlled by a single ionizing group at the active site. This contrasts with the finding of Shore *et al.* (1974) with horse liver alcohol dehydrogenase, that trifluoroethanol binding to ES_1 was dependent on the unprotonated form of a group with $\text{p}K_a$ 7.6.

The inhibition by trifluoroethanol of the maximum rate of acetaldehyde reduction at pH 7.05 gave an inhibition constant K_1' approx. 7 mM . Steady-state analysis of the mechanism and differentiation of the expression for $\phi_{0, \text{app.}}$ shows that the inhibition constant determined from the initial slope of a plot of $\phi_{0, \text{app.}}$ versus $[\text{I}]$ is given by:

$$K_1' = \frac{K_9 \left(1 + \frac{k_{-8}}{k_{-9}}\right)}{1 + \frac{k_{-8}}{k_{-7}} - \frac{k_{-8}}{k_{-1}}}$$

provided that $\phi_0' = 1/k_{-1}$, as appears to be the case (Dickinson & Monger, 1973). Taken with the value of $K_9 = 3\text{ mM}$, the result shows that the rate of breakdown of ES_1I via ES_1 $[(1/k_{-1}) + (1/k_{-9})]$ is faster than the rate of breakdown of EI $(1/k_{-7})$. If K_1' were equal to K_9 , this would imply either that $k_{-8} \ll k_{-1}$, k_{-7} and k_{-9} , or that

$$\frac{1}{k_{-1}} + \frac{1}{k_{-9}} = \frac{1}{k_{-7}}$$

Horse liver alcohol dehydrogenase

Detailed initial-rate studies in the absence of added product in the pH range 6–9 indicated that, with the ethanol–acetaldehyde system, horse liver alcohol dehydrogenase follows a compulsory-order mechanism with coenzymes binding before the substrates, i.e. Scheme 1, upper pathway (Dalziel, 1963b). The present results, showing hyperbolic inhibition by ethanol of the maximum rate of acetaldehyde reduction, show that an enzyme–ethanol complex can exist in the steady state in the pH range 6–8, even though it may not be important when products are absent. The present results were not detailed enough to detect hyperbolic inhibition at pH 9.0 but Hanes *et al.* (1972) did demonstrate it at pH 8.6 using fixed high concentrations of NADH and acetaldehyde and varying ethanol concentrations. In the presence of a saturating concentration of ethanol, the reciprocal maximum specific rate of acetaldehyde reduction, $\phi'_{0, \text{app.}}$ is therefore dependent on the rate of an alternative pathway involving ES_2 . This pathway may be either:

as was shown above for the yeast enzyme, or via the abortive complex enzyme–NADH–ethanol ($\text{ES}'_2\text{S}_2$), i.e.:

Shore & Theorell (1966b) obtained a value of 50 mM for the dissociation constant of ethanol from the abortive complex $\text{ES}'_2\text{S}_2$. It seems likely therefore that, in the presence of a saturating concentration of ethanol, the reaction will be diverted through the pathway represented by eqn. (16), the maximum rate of which (with saturating ethanol) is

$$\phi'_{0, \text{app.}} = \frac{1}{k'} + \frac{k}{k'k_{-4}} + \frac{1}{k_{-4}} + \frac{1}{k_{-6}} \quad (17)$$

At pH 7, present knowledge of lower limits for k (Shore & Gutfreund, 1970) allows eqn. (17) to be simplified to

$$\phi'_{0, \text{app.}} = \frac{1}{k_{-4}} + \frac{1}{k_{-6}} \quad (18)$$

It seems possible that $k_{-4} \ll k_{-6}$, in view of the earlier findings that dissociation rates of substrates are much

higher than those of coenzymes (Dalziel, 1963b; Shore & Gutfreund, 1970). If this is so, then at pH 7.05 and at infinite ethanol concentration $\phi'_{0, \text{app.}} = 1/k_{-4}$, and $\phi'_0 = 1/k_{-1}$ (Dalziel, 1963b) at zero ethanol concentration. Analysis shows that the dissociation constant for ethanol from the active ternary complex ES_1S_2 (K_3) may be estimated by using eqn. (11) if, as seems to be the case, $k_{-3} \gg k_{-4}$ (Silverstein & Boyer, 1964). The value obtained for K_3 at pH 7.05 is 20 mM. At the other pH values, however, we have no estimate of the ratio k/k' and so K_3 cannot yet be obtained.

It seems that at pH 7 ethanol binds much more weakly to the enzyme–NAD⁺ complex than does trifluoroethanol ($K_1 \approx 10 \mu\text{M}$ at pH 7; Shore *et al.*, 1974). On this basis, the behaviour of the two compounds cannot be regarded as being similar, and studies with trifluoroethanol need not necessarily give a reliable view of what might occur with the natural substrate. The comparison of the binding of ethanol and trifluoroethanol with the yeast enzyme is discussed in the following paper (Dickinson & Dickenson, 1978).

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