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; Dickinson & 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 (μmol/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 NaHCO3/Na2CO3 (10mM-total carbonate concentration), containing sufficient Na2HPO4 to give I 0.1, were used. Bovine serum albumin (1 mg) 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 (φi) or presence (φi,app.) of inhibitor, in the initial-rate eqns. (1) or (2):

\[
\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \tag{1}
\]

\[
\frac{e}{v_0} = \frac{\phi_0}{[S_1]} + \frac{\phi_{1,app.}}{[S_1]} + \frac{\phi_{2,app.}}{[S_2]} + \frac{\phi_{12,app.}}{[S_1][S_2]} \tag{2}
\]

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). S1 and S2 are coenzyme and substrate respectively. The symbols S1, S2 and φi,app. are used for alcohol–NAD+ reactions and primed symbols S1, S2 and φi,app. for aldehyde–NADH reactions, as in previous kinetic studies (Dalzel & Dickinson, 1966; Dickinson & Monger, 1973; Dickinson & Dickenson, 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 & Dickenson, 1975a). Plots of φi,app. versus [inhibitor] were made to evaluate linear inhibition constants K1, defined by:

\[
\phi_{i,app.} = \phi_i \left(1 + \frac{[\text{inhibitor}]}{K_1}\right) \tag{3}
\]

For apparently hyperbolic inhibition, i.e.

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

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/(\(\phi_{i,app.} - \phi_i\)) versus 1/[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 concentrations.

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**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/v₀) with the reciprocal of the acetaldehyde concentration, for several constant NADH concentrations. The NADH concentrations (μM) were: ○, 600; ●, 150; △, 75; ▲, 37.5. For clarity results with 300 μM-NADH have been omitted. (b) Secondary plot showing variation of the intercepts (○) and slopes (●) in (a) with the reciprocal of the NADH concentration.

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**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/v₀) with the reciprocal of the acetaldehyde concentration, for several constant ethanol concentrations. The ethanol concentrations (mM) were: ○, 0; ●, 32.4; △, 64.8; ▲, 130; □, 270; ■, 540; ▼, 810; ▼, 1080. (b) Variation of the intercepts (○) and slopes (●) in (a) 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.
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/(increase in intercept) versus 1/[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 φ₁,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 φ₀,app is subject to hyperbolic inhibition by ethanol (Fig. 3). Plots of 1/(φ₀,app − φ₀) versus 1/[ethanol] (Fig. 3b) were linear within experimental error. The inhibition can be described by two constants (Kₙₑᵦₙₑ and Kₑₑᵦₑ), 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 φ₀,app versus [ethanol] is subject to considerable error when φ₀,app at infinite [ethanol] is only about four times the value of φ₀ 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 (φ₀,app − φ₀), which is reflected in the proportionately greater scatter in the double-reciprocal plots at low ethanol concentrations.

At all pH values the coefficient φ₁ 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 φ₂,app is linearly dependent on the concentration of ethanol. Linear inhibition constants for φ₁,app and φ₂,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 φ₁₂,app. At pH 8.0 and 9.0 the variation with [ethanol] of φ₁₂,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, φ₀,app approaches its maximum value at 1.0M-ethanol, i.e. consistent with hyperbolic inhibition as at 25°C. Also, the inhibition affecting both φ₁,app and φ₂,app is roughly linear, with inhibition constants given in Table 2.

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

\[
\frac{e}{v_0} = \phi_0 + \phi_{1,\text{app}} + \phi_{2,\text{app}} + \phi_{12,\text{app}}
\]

where

\[
\phi_0 = \phi_0 \left( \frac{1 + \frac{[S_2]}{K_{\text{num.}}}}{1 + \frac{[S_2]}{K_{\text{denom.}}}} \right)
\]

\[
\phi_{1,\text{app}} = \phi_1 \left( \frac{1 + \frac{[S_2]}{K_{11}}}{1 + \frac{[S_2]}{K_{12}}} \right)
\]

\[
\phi_{2,\text{app}} = \phi_2 \left( \frac{1 + \frac{[S_2]}{K_{12}}}{1 + \frac{[S_2]}{K_{11}}} \right)
\]

S_2, S_1, S_2', represent ethanol, NADH and acetaldehyde respectively. \(\phi_i\) 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 ±15%.

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<th>(K_{\text{num.}}) (mM)</th>
<th>(K_{\text{denom.}}) (mM)</th>
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<th>(K_{11}) (mM)</th>
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* 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 15 mM 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 0.1–2.0 mM-acetaldehyde on the oxidation of 40–1215 mM-ethanol by 1250 μM-NAD⁺, with yeast alcohol dehydrogenase at pH 7.05 and 25°C. The concentration of NAD⁺ is about 10 times its Michaelis constant \((\phi_1/\phi_0)\) and about 4 times its dissociation constant \((\phi_{12}/\phi_2)\) in the absence of product (Dickinson & Monger, 1973). The intercepts and slopes of Fig. 7 therefore, at least in the absence of acetaldehyde, approximate to the coefficients \(\phi_0\) and \(\phi_2\) 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 \((K_1=110 \mu M)\). The intercept, however, is increased at the most only by about 25% with 2 mM-acetaldehyde. Clearly no conclusion can be drawn from Fig. 7 about the pattern of inhibition on \(\phi_0\) (i.e. linear or hyperbolic), nor may any useful value be obtained for the inhibition constant. When concentrations of acetaldehyde higher than 2 mM 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 pH 8.0.

Preliminary experiments were also conducted to
measure the effect of acetaldehyde on the coefficient \( \phi_1 \) 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 \( \phi_1 \). This result is not unexpected if \( \phi_{12} \) increases with increasing [acetaldehyde] in the same way as \( \phi_2 \) and if \( \phi_1 \) is affected much less. The ethanol concentration must be much higher than \( \phi_{12}/\phi_1 \) in order to obtain \( \phi_2 \) 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 \( \mu \)M-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.

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<th>pH</th>
<th>[Ethanol] (M)</th>
<th>$\phi_{0, \text{app}}$ (s)</th>
<th>$\phi_{1, \text{app}}'$ (mM·s)</th>
<th>$\phi_{1, \text{app}}^\prime$ (mM·s)</th>
<th>$\phi_{12, \text{app}}$ (mM·s)</th>
<th>$K_{11}$ (mM)</th>
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<td>~680</td>
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<td>1.08</td>
<td>0.0021</td>
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<tr>
<td>9.0</td>
<td>0.54</td>
<td>0.00157</td>
<td>0.16</td>
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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 $\phi_2$ ($K_1$, 3.0 mM), with a much smaller effect on $\phi_{12}$ ($K_1$, 55 mM) and no effect on $\phi_0$ or $\phi_1$ (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 $\phi_{12}/\phi_2$) approximates to the inhibition constant for $\phi_2$.

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 $\phi_{12}/\phi_2$ given by Dickinson & Dickinson (1975a), and assuming that the inhibition effect on $\phi_{12}$ 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 ($\phi_0'$) of the acetaldehyde–NADH reaction was examined at pH 7.05. The values of $\phi_0'$, 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, $\phi_0'$, app, varied linearly with trifluoroethanol, with $K_1$ 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/[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 $\phi_1'/\phi_2$ 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 (v/v₀) 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 153 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 (v/v₀) 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.

ϕ₁₂/ϕ₂ (Dalziel, 1963b), so that the slopes and intercepts (at [ethanol]=0) approximate to ϕ₂ and ϕ₀ respectively. The variation of v/v₀ with 1/[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 ϕ₀,app. Also at pH 5.5, it appears that the slopes approximate to the coefficient ϕ₂,app, though this is not so at pH 9.0.
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 $\phi_{o, app}$ was determined (using 0 and 2.7–1080mM-ethanol). Two high concentrations of both NADH (154 and 77 $\mu$M) and acetaldehyde (3.3 and 6.7 $\mu$M for lower ethanol concentrations; 6.7 and 13.3 $\mu$M 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 $\phi_{o, app}$ 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/($\phi_{o, app} - \phi_o$) 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 $\phi_{o, app}$ (at infinite [ethanol]) is many times greater than $\phi_o$. The inhibition therefore appears linear within experimental error until much higher relative values of $\phi_{o, app}$.

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; Dickinson & Dickinson, 1975a). In the oxidation of ethanol by NAD$,^+$, although the net reaction takes place principally through the upper pathway (via ES$1$ and ES$1'_2$), a significant steady-state concentration of ES$2$ exists in the range pH6–8. In the reduction of acetaldehyde by NADH, no significant steady-state concentration of the corresponding complex ES$2'_2$ (Scheme 1b) is indicated at pH6–9, although the possible existence of ES$2'_2$ is suggested by isotope-exchange experiments (Silverstein & Boyer, 1964). Acetaldehyde reduction in the absence of
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 $S_1'(\text{NADH})$ and $S_2' (\text{acetaldehyde})$ in the presence of the product $S_2 (\text{ethanol})$ yields an expression of the form of eqn. (2). The coefficients are given by:

\[
\phi_{0,\text{app.}} = \frac{k_{-1}k_{-2}(k+k_{-3}+k_{-4})+k'(k_{-1}k_{-2}+k_{-1}k_{-4}+k_{-2}k_{-3})+[k_{-2}(k'+k_{-4})+k'k_{-4}]k_{+3}[S_2]}{k_{-2}k'[k_{-1}(k_{-3}+k_{-4})+k_{-4}k_{+3}[S_2]]}
\]

\[
\phi'_{1,\text{app.}} = \frac{1}{k'_{+1}} \left( 1 + \frac{[S_2]}{K_2} \right) = \frac{\phi_{1}}{1 + \frac{[S_2]}{K_2}}
\]

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

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

Expressions for $\phi_{0,\text{app.}}$, $\phi'_{1,\text{app.}}$, $\phi'_{2,\text{app.}}$ and $\phi'_{12,\text{app.}}$ for the mechanism in the absence of products are given by Dickinson & Monger (1973). One may note that identical expressions for $\phi'_{1,\text{app.}}$, $\phi'_{2,\text{app.}}$ and $\phi'_{12,\text{app.}}$ may be 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 $k'_{-3} > k_{-4}$, $\phi_1 > 1/k_{+1}$, $\phi_1 = 1/k'_{+1}$, with (for $\phi_{1,\text{app.}}$) product inhibitor being kept at concentrations where apparently linear inhibition is observed. The general asymptote expression for $\phi_{0,\text{app.}}$, even with these simplifications, is considerably more complex than that given above.

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).
PRODUCT INHIBITION OF ALCOHOL DEHYDROGENASES

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 ES₁, 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 ES₂. 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:

$$\phi'_0, \text{app.} = \frac{1}{k_{-2}} + \frac{1}{k_{-4}} \left(1 + \frac{k}{k'}\right)$$  \hspace{1cm} (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 ES₂ complex from ES₁S₂, 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 (ES₁S₂), formed from ES₂, and from which ethanol could dissociate. Although liver alcohol dehydrogenase will form an ES₁S₂ 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 mM) of ethanol in the ethanol-NAD⁺ reaction (Dickinson & Monger, 1973); (2) the unchanged fluorescence yield of enzyme-NADH in the presence of 0.54M- and 1.08M-ethanol at 25°C and pH 7.05 (F. M. Dickinson & C. J. Dickenson, 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 ES₁ to that via ES₂. Since $k_{-3} \gg k_{-4}$ (Silverstein & Boyer, 1964; Dickinson & Dickinson, 1975a), the expression for $\phi'_0$, app. (eqn. 5) simplifies to:

$$\phi'_0, \text{app.} = \frac{k_{-1} k_{-3} (k + k_{-3}) + k'(k_{-1} k_{-2} + k_{-1} k_{-4} + k_{-2} k_{-3}) + [k_{-2} (k + k' + k_{-4}) + k' k_{-4}] k_{+3} [S_2]}{k_{-2} k' [k_{-1} k_{-3} + k_{-4} k_{+3} [S_2]]}$$  \hspace{1cm} (10)

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:

$$C/D = \frac{k_{-1} k_{-3} k_{+3}}{k_{-4}}$$  \hspace{1cm} (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 ES₁S₂ 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. $k < k'$ and $k_{-4} \ll k_{-2}$, then $K_3$ could be calculated. It seems that $k < k'$ (Dickenson & Dickinson, 1975a), and the fact that the rate of dissociation of NAD⁺ from ES₁ ($k_{-1}$) is much smaller, at all pH values, than that of ethanol from ES₁S₂ ($k_{-3}$) (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 $\phi_0/k_0$ (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 43mM for the linear inhibition constant for the apparent maximum rate of acetaldehyde reduction at pH 7.15. They used 208$\mu$M-NADH (twice the Michaelis constant) and the inhibitor concentrations were 0.23mM and 50mM-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:

$$\phi'_0, \text{app.} = \phi_0 + \frac{[S_2]}{K_3} \left(1 + \frac{k}{k'} + \frac{k_{-4}}{k_{-2}} - \frac{k_{-4}}{k_{-1}}\right) \frac{1}{k_{-1}}$$  \hspace{1cm} (12)

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

\[
\phi_{0, \text{app}} = \phi_0 + \frac{[S_2]}{K_3} \left( 1 + k' \right) \frac{1}{k_{-1}} \tag{13}
\]

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 ES2) 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 ES2 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 ES2 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 \( \phi_{1, \text{app}} (K_{11}, \text{Table 1}) \) over the range pH 6-8 are equal to the respective dissociation constants for the binary ES2 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 ES2 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 \( \phi_1 \) is not significantly greater than \( 1/k_{+1} \). Expressions for \( \phi_1 \) and \( \phi_{12} \) 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 \( K_2 = \phi_{12}/\phi_1 = 190 \text{ mm} \). The discrepancy between these calculated values of \( K_2 \) and those obtained as inhibition constants affecting \( \phi_{1, \text{app}} (K_{11}, \text{Table 1}) \) 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. \( \phi_1, \phi_2, \phi_{12} \) and \( \phi_0 \)) 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

\[
\frac{k_{-3}(k+k_{-2})+k'k_{-4}}{k_{-3}k'k_{-4}}
\]

which may be compared with the expression for \( \phi_2 \) (when \([S_2]=0\)), which is

\[
\frac{k_{-3}(k+k_{-2})+k'k_{-4}}{k_{-3}k'k_{-3}}
\]

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 mm 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 \( k_{-1}\phi_2 = k_{+1}\phi_{12}/\phi_1 \) (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 ES2 (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 \( \phi_1 \) and thus whether there is evidence for the formation of ES2. There is evidence for the formation of ES2 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 μM was derived from the secondary plot of intercepts versus [acetaldehyde]. However, the highest concentration of acetaldehyde used was only 250 μM, and extrapolations to the intercepts of plots of c/v0 versus 1/[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 μM) 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 $k_{-1} = \frac{k_{-1} \phi_2}{k_{11} \phi_{12}}$. For yeast alcohol dehydrogenase at pH 7.05 this latter expression is equal to $\phi_{12} \phi_1$ and has the value of 120 μM (Dickinson & Monger, 1973).

Inhibition by trifluoroethanol. Pettersson (1974) has examined the general case of an inhibitor competing with a substrate (S1 or S2) in the fully random mechanism of Scheme 1b. For an inhibitor, I, competing with S2, the additional reactions are:

$$E + I \stackrel{k_{-11}}{\rightleftharpoons} EI \stackrel{k_{+2}}{\rightarrow} ES_I \stackrel{k_{+2}}{\rightarrow} ESI \quad (14)$$

and $K_1 K_9 = K_7 K_8$. For the preferred-pathway mechanism considered in this paper (Scheme 1a, with the conditions $k_{-3} \gg k_{-4}$), the kinetic coefficients in the presence of inhibitor become:

$$\phi_{0, app.} = \phi_0, \phi_{12, app.} = \phi_{12} \left(1 + \frac{[I]}{K_7}\right),$$

$$\phi_{2, app.} = \phi_2 \left(1 + \frac{[I]}{K_9}\right)$$

$$\phi_{1, app.} = \left(\phi_1 - \frac{1}{k_{+1}}\right) + \frac{1 + [I]/K_7}{k_{+1} k_{-1} k_{-8} + [I]}$$

The expressions for $\phi_0$, $\phi_1$, $\phi_2$ and $\phi_{12}$ 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 $\phi_0$ (Fig. 8) is as expected and it indicates that the alternative ES$I$ complex does not form, or else ES$I$ dissociates from ES$I$ at the same rate as from ES$I$. 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 (ES$I$S) complex was discussed.

The absence of an inhibition term affecting $\phi_1$ implies the following relationship between the rate constants in Scheme 1(a) and eqn. (14), i.e.

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

Linear inhibition factors affecting $\phi_2$ and $\phi_{12}$ are expected, the factors being

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

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$I$ (K9 = 3 mM). If this applies at the other pH values, then the competitive inhibition constants $K_i$ (Fig. 9) approximate to $K_9$. The pH-dependence of $K_i$ (Fig. 9) implies that the binding of trifluoroethanol in the ternary enzyme–NAD$^+$–trifluoroethanol (ES$I$I) 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$I$ was dependent on the unprotonated form of a group with $pK_a$ 7.6.

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

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

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$I$I via ES$I$ [(1/k_{-1} + 1/k_{-9})] is faster than the rate of breakdown of EI (1/k_{-7}). If $K_i'$ were equal to $K_9$, this would imply either that $k_{-8} \ll k_{-1}$, $k_{-7}$, and $k_{-9}$, or that

$$\frac{1}{k_{-1}} + \frac{1}{k_{-7}} = \frac{1}{k_{-7}}$$
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, $\phi^*_{\text{app}}$, is therefore dependent on the rate of an alternative pathway involving ES$_2$. This pathway may be either:

\[
\frac{k'}{k} \quad \text{ES}_1 S_2 \xrightleftharpoons{k-4} \text{ES}_2 \xrightleftharpoons{k-2} E \quad (15)
\]

as was shown above for the yeast enzyme, or via the abortive complex enzyme–NAD–ethanol (ES$_1$S$_2$), i.e.:

\[
\frac{k'}{k} \quad \text{ES}_1 S_2 \xrightarrow{k-4} \text{ES}_2 \xrightarrow{k-6} \text{ES}_1 S_2 \quad (16)
\]

Shore & Theorell (1966b) obtained a value of 50 mM for the dissociation constant of ethanol from the abortive complex ES$_1$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

\[
\phi^*_{\text{app}} = \frac{1}{k'} + \frac{k}{k'k_{-4}} + \frac{1}{k_{-4}} + \frac{1}{k_{-6}} \quad (17)
\]

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

\[
\phi^*_{\text{app}} = \frac{1}{k_{-4}} + \frac{1}{k_{-6}} \quad (18)
\]

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 $\phi^*_{\text{app}} = 1/k_{-4}$, and $\phi^*_{\text{app}} = 1/k_{-1}$ (Dalziel, 1963b) at zero ethanol concentration. Analysis shows that the dissociation constant for ethanol from the ternary complex ES$_1$S$_2$ ($K_2^*$) 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 $K_2$ at pH 7.05 is 20 mM. At the other pH values, however, we have no estimate of the ratio $k/k'$ and so $K_2$ cannot yet be obtained.

It seems that at pH 7 ethanol binds much more weakly to the enzyme–NAD$^+$ complex than does trifluoroethanol ($K_t \approx 10 \mu M$ 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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