Inhibition of Alcohol Dehydrogenase from Yeast by Pyridine

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1. Inhibition by pyridine of reduction of NAD by ethanol in the presence of yeast alcohol dehydrogenase was studied at 25° in 60mm-glycine buffer (K⁺, pH 9.3). 2. The apparent Michaelis constant for ethanol increases linearly and that for NAD increases non-linearly with pyridine concentration. 3. Rates, *v*, observed in the presence of pyridine are lower than the values calculated from the effect of pyridine on the two apparent Michaelis constants and are described by the expression $V/v = \{1 + 5.8[\text{pyridine}]\} \times \{1 + 0.016(1 + 124[\text{pyridine}])/[\text{EtOH}]\} \times \{1 + 0.00019(1 + 3.3[\text{pyridine}] + 110[\text{pyridine}]^2)/[\text{NAD}]\}$. 4. Mixed inhibitor studies with pyridine and *N*¹-methylnicotinamide chloride in 40mm-pyrophosphate buffer (Na⁺, pH 8.2) indicated little interaction of pyridine with the 'pyridinium site' of the dehydrogenase (interaction constant, α , 2.1). 5. The possible competition of ethanol and pyridine for a zinc atom in the active centre of yeast alcohol dehydrogenase is discussed.

Alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) from yeast is inhibited by pyridine and by pyridine derivatives substituted at C₍₃₎ or C₍₄₎. Inhibitory activity increases with increasing *pK_a* in this set of bases, and inhibition has therefore been attributed to interaction of the corresponding pyridinium ion with the dehydrogenase (Van Eys & Kaplan, 1957). The stability constants of zinc complexes with aromatic bases also increase with increasing basicity of the ligand (Rossotti, 1960), and as the postulated inhibition by pyridinium ions requires relative inhibitory activities 10³–10⁵ times greater than those of the corresponding *N*¹-methylpyridinium iodides (Van Eys & Kaplan, 1957) the alternative possibility, that unprotonated pyridine inhibits alcohol dehydrogenase by binding to the catalytic centre, has been examined.

The bidentate ligand 1,10-phenanthroline inhibits yeast alcohol dehydrogenase and increases the Michaelis constant for NAD but not that for ethanol (Hoch, Williams & Vallee, 1958). From this observation it has been suggested that ethanol is not bound to zinc in the active centre of the dehydrogenase (Vallee, 1960). Co-ordination of the monodentate ligand pyridine may be less subject to steric hindrance than that of polycyclic ligands used by other investigators (for references to earlier work see Anderson, Reynolds & Anderson, 1966), and it has now been shown that the Michaelis constant for ethanol increases linearly with pyridine concentration and that this effect of pyridine is

only slightly modified in the presence of *N*¹-methylnicotinamide chloride.

As with recent studies of inhibition of yeast alcohol dehydrogenase by imidazole (McKinley-McKee, 1964) our results are consistent with models in which ethanol and inhibitor compete for a binding site, possibly a zinc atom, in the active centre of the dehydrogenase.

MATERIALS AND METHODS

Ethanol. A sample that had been boiled under reflux with zinc dust and KOH and fractionally distilled was provided by Dr D. H. Treble. The extinction in a 1 cm. cell was 0.24 at 220mμ and the absorption curve had no maxima or inflexions between 210mμ and 340mμ. Spectroscopic ethanol (British Drug Houses Ltd., Poole, Dorset) was used in later experiments.

NAD. The β-NAD content of the coenzyme ('reinst'; C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) was measured with 0.5M-ethanol and alcohol dehydrogenase at pH 9.3 (Found: 88%. Calc. for C₂₁H₂₇N₇O₁₄P₂·4H₂O: 90%). Solutions were brought to pH 5.5 with KOH before addition to reaction mixtures. Where indicated below, NAD purified by the method of Dalziel & Dickinson (1965) was used.

Pyridine. A.R. grade pyridine (British Drug Houses Ltd.) was distilled in glass apparatus; the inhibitory activity was not changed by a further distillation. The i.r. spectrum in the range 4–15μ was identical with that reported by Biddiscombe, Coulson, Handley & Herington (1954) apart from bands due to a trace of water (<0.4%).

Alcohol dehydrogenase. Recrystallized preparations from yeast (Sigma Chemical Co., St Louis, Mo., U.S.A., or C. F.

Boehringer und Soehne G.m.b.H.) were diluted to 0.1 mg. of protein/ml. in water or, where indicated, in 1 mM-dithiothreitol and were measured from a micrometer syringe kept at 0–1°. Protein concentration was calculated from the extinction at 280 m μ (Hayes & Velick, 1954). The specific activity, V , calculated from the extrapolated rate at saturating concentrations of ethanol and NAD at pH 9.3 and 25° (cf. Fig. 5) was about 100 μ moles of NAD reduced/min./mg. of protein.

Measurement of enzyme activity. A solution of glycine (180 μ moles, adjusted with KOH to pH 9.3) or KH₂PO₄ (150 μ moles, adjusted with KOH to pH 7.5) containing ethanol, NAD and, where appropriate, pyridine (for details see the Results section) was brought to 25.0 $\pm$ 0.5° in the thermostatic cell holder of a Beckman DK-2 spectrophotometer. For experiments at pH 7.5 the pyridine was adjusted to this pH with HCl. For experiments at pH 9.3 unbuffered pyridine was added to the other reactants and the pH, after addition of dehydrogenase, was in the range 9.25–9.35. Measurements at pH 8.2 were carried out as described by Anderson & Reynolds (1965).

Enzyme (0.01–0.14 ml.) was added to give a final volume of 3.0 ml. in a 1 cm. cell. The extinction was recorded at 340 m μ and the rate of reduction of NAD between 15 sec. and 20 sec. after the addition of enzyme was calculated. Similar results were obtained when rates were measured between 10 sec. and 15 sec. after the addition of enzyme, but instrumental errors were then greater. Reduction of NAD was also measured at 334 m μ with an Eppendorf photometer with extinction accessory (Netheler und Hinz G.m.b.H., Hamburg-Wellingsbuttel, Germany) and recorder (EPR-2TB; TOA Electronics Ltd., Tokyo, Japan). The volume of enzyme was chosen to give an extinction change of 0.05–0.30/min. The rate of reduction of NAD was proportional to enzyme concentration in this range. To allow for inactivation of the enzyme in the syringe during a series of assays, dehydrogenase activity was measured under standard conditions at frequent intervals and activities were corrected for this inactivation. When the syringe had been rinsed with 1% HNO₃ to remove metals inactivation of the enzyme was less than 2%/hr.; no inactivation was observed when the enzyme was kept in 1 mM-dithiothreitol, but in this case no more than 0.02 ml. of enzyme solution was added to assays.

RESULTS

At pH 9.3 and at pyridine concentrations in the range 0–133 mM plots of $[NAD]V/v$ against $[NAD]$ (Fig. 1) or of V/v against $1/[EtOH]$ (Fig. 2) were linear. Here v is the observed rate (μ moles of NAD reduced/min./mg. of protein) and V is the extrapolated maximum rate (see the Materials and Methods section). At each concentration of pyridine, the apparent Michaelis constants were calculated as the intercepts in Fig. 1 and as the reciprocals of these intercepts in Fig. 2, and are defined by the equations:

$$[NAD]V/v = K_{NAD}V/V_{NAD} + (V/V_{NAD})[NAD]$$

and

$$V/v = V/V_{EtOH} + (VK_{EtOH}/V_{EtOH})/[EtOH]$$

Fig. 1. Effect of pyridine and NAD concentrations on the activity of alcohol dehydrogenase at pH 9.3 in the presence of 0.5 M-ethanol. The numbers by the lines indicate the molarity of pyridine used.

Fig. 2. Effect of pyridine and ethanol concentrations on the activity of alcohol dehydrogenase at pH 9.3 in the presence of 0.95 mM-NAD. The numbers by the lines indicate the molarity of pyridine used. Values in parentheses refer to ethanol concentrations for experiments in presence of pyridine.

Fig. 3. Variation of K_{NAD} (O, from Fig. 1) and K_{EtOH} (●, from Fig. 2) with pyridine concentration.

where V_{NAD} is the extrapolated rate at saturating NAD concentration and at the particular concentrations of ethanol and pyridine used, and V_{EtOH} is the extrapolated rate at saturating ethanol concentration and at the concentrations of NAD and pyridine used in that experiment. The variation of K_{NAD} with pyridine concentration in the presence of 0.5 M-ethanol is shown in Fig. 3. The curve is described by the equation:

$$K_{\text{NAD}} = 0.00019 (1 + 3.3[\text{pyridine}] + 110[\text{pyridine}]^2) \text{ (M)}$$

The variation of K_{EtOH} with pyridine concentration in the presence of 0.95 mM-NAD is also shown in Fig. 3. The equation of the line is:

$$K_{\text{EtOH}} = 0.016 (1 + 124[\text{pyridine}]) \text{ (M)}$$

In 0.12 M-pyridine, K_{EtOH} was 0.255 M with 1.42 mM-NAD and 0.250 M with 2.27 mM-NAD. The corresponding value with 0.95 mM-NAD (Fig. 3) was 0.245 M. Mahler & Douglas (1957) found that K_{NAD} and K_{EtOH} were independent of the ethanol and NAD concentrations respectively for this enzyme and that:

$$V/v = \left(1 + \frac{0.019}{[\text{EtOH}]}\right) \left(1 + \frac{0.00027}{[\text{NAD}]}\right)$$

at pH 7.6 in the presence of semicarbazide. The corresponding expression, from the results obtained here, is:

$$V/v = \left\{1 + \frac{0.016}{[\text{EtOH}]} (1 + 124[\text{pyridine}])\right\} \times \left\{1 + \frac{0.00019}{[\text{NAD}]} (1 + 3.3[\text{pyridine}] + 110[\text{pyridine}]^2)\right\} \quad (1)$$

Fig. 4. Variation of V_{NAD}/V (a, from Fig. 1) and V_{EtOH}/V (b, from Fig. 2) with pyridine concentration.

Fig. 5. Variation of $(V/v)^{0.5}$ in the absence of pyridine (O) and in the presence of 0.12 M-pyridine (●) when NAD and ethanol concentrations are varied simultaneously and kept in the ratio of their Michaelis constants observed in the absence of pyridine; $a = 0.016 \text{ M}/[\text{EtOH}] = 0.00019 \text{ M}/[\text{NAD}]$.

where $v \rightarrow V_{\text{NAD}}$ as $1/[\text{NAD}] \rightarrow 0$ and $v \rightarrow V_{\text{EtOH}}$ as $1/[\text{EtOH}] \rightarrow 0$.

The variation with pyridine concentration of the reciprocals of the slopes of Fig. 1, V_{NAD}/V , and the reciprocals of the intercepts of Fig. 2, V_{EtOH}/V , are shown in Fig. 4. In all cases observed values of V_{NAD} and V_{EtOH} are less than the values calculated from eqn. (1).

Fig. 6. Variation of (extrapolated maximum rate calculated from eqn. 1)/(extrapolated observed maximum rate) with pyridine concentration. Values of V_{NAD} (○) and V_{EtOH} (●) are from Fig. 4 and the extrapolated value for simultaneous variation of NAD and ethanol concentrations (■) is from Fig. 5.

Fig. 7. Partial inactivation of alcohol dehydrogenase with 0.33 mm-iodoacetate in 37 mm-phosphate buffer (K^+ , pH 7.5) at 25° . At the time indicated by arrow I enzyme was added, and at the time indicated by arrow II 3 ml. of the reaction mixture (B) was mixed with 0.75 ml. of 0.1 M-cysteine. Cysteine was added before iodoacetate in A and was not added in C. Samples (0.025 ml.) were taken for assay at pH 7.5 at the times indicated, and the partially inactivated enzyme B was used for kinetic studies described in the Results section.

To test the possibility that pyridine has an inhibitory activity that cannot be reversed by increased concentrations of ethanol and NAD the concentrations of these substrates were varied in such a way that they were kept in the ratio of their Michaelis constants (16:0.19) for a range of substrate concentrations with or without 0.12 M-pyridine. Fig. 5 shows the variation of $(V/v)^{0.5}$ with

the reciprocal of substrate concentration in this case. In the absence of pyridine:

$$V/v = \left(1 + \frac{K_{\text{EtOH}}}{[\text{EtOH}]}\right) \left(1 + \frac{K_{\text{NAD}}}{[\text{NAD}]}\right)$$

and if $K_{\text{EtOH}}/[\text{EtOH}] = K_{\text{NAD}}/[\text{NAD}] = a$, then $(V/v)^{0.5} = (1+a)$. V , the extrapolated value of v for saturating NAD and ethanol concentrations in the absence of pyridine, was greater than the value found in the presence of 0.12 M-pyridine.

In Fig. 6 the ratios V_{NAD}/V and V_{EtOH}/V calculated from eqn. (1) and Figs. 4 and 5 are plotted against pyridine concentration. Clearly the discrepancy increases with increasing pyridine concentrations and the ratio is approximately related to pyridine concentration by the equation of the line (Fig. 6):

$$V_{\text{calc.}}/V_{\text{obs.}} = 1 + 5.8[\text{pyridine}]$$

The most satisfactory expression describing the variation of v with pyridine, ethanol and NAD concentrations under our conditions is obtained by combining this expression with eqn. (1):

$$\begin{aligned} V/v = & (1 + 5.8[\text{pyridine}]) \\ & \times \left\{ 1 + \frac{0.016}{[\text{EtOH}]} (1 + 124[\text{pyridine}]) \right\} \\ & \times \left\{ 1 + \frac{0.00019}{[\text{NAD}]} (1 + 3.3[\text{pyridine}] \right. \\ & \left. + 110[\text{pyridine}]^2) \right\} \end{aligned} \quad (2)$$

Van Eys & Kaplan (1957) showed that 0.070 M-pyridine caused 50% inhibition of alcohol dehydrogenase with 1.3 mm-NAD-0.5 M-ethanol at pH 9.3. From eqn. (2) the calculated inhibition in these conditions is 52%.

To find if the inhibition by pyridine was reversed on dilution of the inhibitor, enzyme was kept at 0.1° in 0.133 M-pyridine-0.5 M-ethanol-60 mm-glycine buffer (K^+ , pH 9.3) and samples were assayed at a 1:29 dilution in 0.66 mm-NAD-0.5 M-ethanol-60 mm-glycine buffer (K^+ , pH 9.3). Comparison with a control sample that was kept at 0.1° in ethanol-glycine without pyridine showed no inhibition. The observed rate was four times that calculated from eqn. (2) on the basis that inhibition had not been reversed by dilution. Over a longer period the enzyme kept in the presence of pyridine lost less activity than the control.

To study the possibility that partial inactivation changed the properties of the enzyme the Michaelis constant for ethanol and the inhibitory activity of pyridine were measured with a sample of alcohol dehydrogenase that had been brought to 33% of

Fig. 8. Variation of alcohol-dehydrogenase activity with pyridine concentration at pH 9.3 in 0.5M-ethanol-0.185mm-NAD. The broken line in the plot of $\log[(v_0-v)/v]$ against $\log[\text{pyridine}]$ shows the values calculated from eqn. (2) where they deviate from observed values.

the specific activity of a control sample with iodoacetate. Further inhibition by iodoacetate was stopped by addition of cysteine. The control contained enzyme that was added to the solution of iodoacetate 10min. after the cysteine (Fig. 7). Activity was measured at pH 7.5 to permit comparison with the value of K_{EtOH} (21.4mm) found by Hoch *et al.* (1958). In 1.5mm-NAD-50mm-phosphate buffer (K^+ , pH 7.5) both the control and the enzyme that had been treated with iodoacetate had K_{EtOH} 21mm. In 0.12M-pyridine-40mm-ethanol-1.5mm-NAD-50mm-phosphate buffer (K^+ , pH 7.5) the control and inhibited preparations had rates that were 0.20 ± 0.01 (3) and 0.175 ± 0.005 (3) respectively of those in the absence of pyridine.

The variation of v with pyridine concentration in 0.5M-ethanol-0.185mm-NAD (pH 9.3) is shown in Fig. 8, where $\log[(v_0-v)/v]$ is a linear function of $\log[\text{pyridine}]$ and v_0 is the rate observed in the absence of pyridine. The deviation of values, calculated from eqn. (2), from the observed values is shown by the broken line.

To test for interaction between pyridine and N^1 -methylnicotinamide chloride (Anderson & Reynolds, 1965; Anderson *et al.* 1966) a number of the rates reported above were checked with NAD purified by the method of Dalziel & Dickinson (1965)

Fig. 9. Mixed inhibition of alcohol dehydrogenase by pyridine (O, none; ●, 21mm; Δ, 31mm) and N^1 -methylnicotinamide chloride. Rates were measured in the presence of 93mm-ethanol-0.31mm-NAD in 40mm-pyrophosphate buffer (Na^+ , pH 8.2). The NAD and the chloride were passed through Chelex 100 resin before use.

and with enzyme diluted from a stock in 1mm-dithiothreitol, at concentrations of the quaternary chloride up to 5.5mm. The chloride, in the absence of pyridine, caused no significant inhibition of the initial rate of NAD reduction, nor did it significantly alter the effect of pyridine on K_{EtOH} . In the presence or absence of 5.5mm- N^1 -methylnicotinamide chloride K_{EtOH} with 0.11mm-NAD at pH 9.3 was 0.014 ± 0.005 M or 0.022 ± 0.002 M respectively; the value calculated from eqn. (2) was 0.016M. In the presence of 0.016M-pyridine the corresponding values of K_{EtOH} were 0.044 ± 0.018 M and 0.052 ± 0.009 M, the calculated value being 0.048M. None of these differences are significant [$P > 0.05$ in a two-sample t test; standard errors calculated by Wilkinson's (1961) method].

At higher concentrations (25–100mm) N^1 -methylnicotinamide chloride does inhibit alcohol dehydrogenase significantly. In the absence of pyridine or in the presence of 21mm- or 31mm-pyridine v_0/v was a linear function of the concentration of N^1 -methylnicotinamide chloride concentration (Fig. 9). The lines were concurrent, and the interaction constant, α , calculated as described by Anderson & Reynolds (1965), was 2.1, a value similar to that found by these authors for the weak interaction of N^1 -methylnicotinamide chloride and ADP-ribose (α 1.8).

DISCUSSION

The slope of a line relating $\log[(v_0-v)/v]$ to $\log[\text{pyridine}]$ was 1.51 (Fig. 8). With enzymes

with multiple substrate-binding sites (Atkinson, Hathaway & Smith, 1965; Atkinson, 1966) the slopes of corresponding double-logarithmic plots are functions of the number of substrate-binding sites and of the strength of interaction between them. Roy (1964) concludes that a relationship similar to that in Fig. 8 indicates the occurrence of a number of interacting inhibitor-binding sites on an enzyme, but emphasizes the difficulty in attributing a physical significance to the slope.

From eqn. (2):

$$K_{\text{EtOH}} = 0.016(1 + [\text{pyridine}]/K_1)$$

where $K_1 = 8.1 \text{ mM}$. A linear increase of apparent Michaelis constant with inhibitor concentration is found when substrate and inhibitor compete for a single binding site in the catalytic centre of an enzyme (Dixon & Webb, 1964). The relationship found here may result from competition between pyridine and ethanol at a site in the dehydrogenase.

Although K_{NAD} is fairly well described by the quadratic:

$$K_{\text{NAD}} = 0.00019(1 + [\text{pyridine}]/K_2 + [\text{pyridine}]^2/K_2K_3)$$

where $K_2 = 0.30 \text{ M}$ and $K_3 = 0.03 \text{ M}$, the significance of these constants is not known. The relationship could result from competition of pyridine and NAD for a site in the catalytic centre that could interact with two molecules of inhibitor or one of substrate. This formulation is consistent with models in which zinc is attached to the dehydrogenase by three bonds, to NAD by two bonds and to ethanol by one bond (Mahler & Douglas, 1957; McKinley-McKee, 1964). The low concentration of pyridinium ions at pH 9.3 (4 pH units above the pK_a of pyridine) and the weak interaction with N^1 -methylnicotinamide chloride make it unlikely that pyridine is acting by binding to a 'pyridinium' site (Van Eys & Kaplan, 1957; Anderson *et al.* 1966; Anderson & Reynolds, 1966). In the absence of more direct physical methods it cannot be concluded that inhibition by pyridine is due to displacement of ethanol from a co-ordinated zinc atom in the active centre of alcohol dehydrogenase. The natural abundance of ^{67}Zn , with a spin of 5/2, is only 4%,

and its broad nuclear-magnetic-resonance (n.m.r.) signal might not be modified sufficiently by ligands to provide evidence of co-ordination of zinc in alcohol dehydrogenase by ethanol. An examination of the effect of alcohol dehydrogenase on the n.m.r. signals of protons in pyridine or ethanol might provide more direct evidence on the mechanism of binding of these compounds to the enzyme.

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