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Some Properties of an Alcohol Dehydrogenase Partially Purified from Baker's Yeast Grown without Added Zinc

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Alcohol dehydrogenase was partially purified from yeast (*Saccharomyces cerevisiae*) grown in the presence of 20 μM-$MnSO_4$ without added Zn^{2+} and from yeast grown in the presence of 1.8 μM-$MnSO_4$ and 15 μM-$ZnSO_4$. The enzyme from yeast grown with added Zn^{2+} has the same properties as the crystalline enzyme from commercial supplies of baker's yeast. The enzyme from yeast grown without added Zn^{2+} has quite different properties. It has a mol.wt. in the region of 72000 and an $s_{20,w}$ of 5.8S. The values can be compared with a mol.wt. of 141000 and an $s_{20,w}$ of 7.6S for the crystalline enzyme. ADP-ribose, a common impurity in commercial samples of NAD^+, is a potent competitive inhibitor of the new enzyme ($K_i = 0.5\,\mu$M), but is not so for the crystalline enzyme. The observed maximum rate of ethanol oxidation at pH 7.05 and 25°C was decreased 12-fold by the presence of 0.06 mol of inhibitor/mol of NAD^+ when using the enzyme from Zn^{2+}-deficient yeast, but with crystalline enzyme the maximum rate was essentially unchanged by this concentration of inhibitor. The kinetic characteristics for the two enzymes with ethanol, butan-1-ol, acetaldehyde and butyraldehyde as substrates are markedly different. These kinetic differences are discussed in relation to the mechanism of catalysis for the enzyme from Zn^{2+}-deficient yeast.

Yeast alcohol dehydrogenase is an enzyme of mol.wt. 141000 (Bühner & Sund, 1969) comprised of four, apparently identical, subunits (Harris, 1964; Jörnvall, 1973) and containing, by various estimates, 4–5 zinc atoms/tetramer (Sund & Theorell, 1963; Dickinson, 1974). Investigations into the catalytic mechanism of the enzyme have reached a fairly advanced stage (Dickenson & Dickinson, 1975*b*,*c*). Further, chemical modification of certain unique amino acid residues, such as cysteine (Rabin *et al.*, 1964; Harris, 1964) and histidine (Dickenson & Dickinson, 1973, 1975*a*), leads to a loss of activity and, apparently, of substrate binding (Dickinson, 1972; Dickenson & Dickinson, 1975*a*), whereas coenzyme binding is largely unaffected. It is important to find out if the zinc atoms are involved in catalysis or act as structural supports. In horse liver alcohol dehydrogenase, for example (4 zinc atoms/molecule of mol.wt. 84000), two atoms function in catalysis, whereas the other two appear to have a structural role (Brändén *et al.*, 1973). For the yeast enzyme it has been found that incubation with metal-chelating agents, such as 1,10-*o*-phenanthroline or 8-hydroxyquinoline, results in removal of the zinc and dissociation into subunits (Kägi & Vallee, 1960). This indicates that the metal has a structural role. However, Hoch *et al.* (1958) found that 1,10-*o*-phenanthroline is a competitive inhibitor of the enzyme, which suggests that at least part of the metal is in active centres. Creighton & Sigman (1971) have provided a model for alcohol dehydrogenase which involves participation of a Zn^{2+} ion.

It has been claimed by Curdel & Iwatsubo (1968) and by Coleman & Weiner (1973) that Co^{2+} and Mn^{2+} can be incorporated into alcohol dehydrogenase when yeast is grown anaerobically in the presence of these metals. Detailed comparison of the kinetic properties of such modified enzymes with those of the zinc-containing enzyme offers the possibility of determining whether the metal has a catalytic role. We have sought to follow this approach by studying the properties of an alcohol dehydrogenase from yeast grown in the absence of added Zn^{2+}, but with added Mn^{2+}.

Experimental

Materials

Glass-distilled water was used in the preparation of all reagent solutions and growth media. EDTA was omitted from buffer solutions and assays except where otherwise stated. Reagents used in buffers and growth media were of analytical grade wherever possible and obtained mainly from Fisons Ltd., Loughborough, Leics., U.K., and BDH Chemicals Ltd., Poole, Dorset, U.K. Phosphate and pyrophosphate buffers were sodium salts unless stated otherwise. DEAE-celluloses, Whatman DE11 and DE32, were from Fisons Ltd.

Coenzymes and substrates. NAD^+ (grades I and II) and NADH (grade I) were generally obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. One sample of NAD^+ (grade III) was, however, obtained from Sigma (London) Chemical Co. Ltd., Kingston-on-Thames, Surrey, U.K., and ADP-ribose was also obtained from this source. For most of the kinetic experiments NAD^+ was purified and assayed as described by Dalziel (1963) and Dalziel & Dickinson (1966). NADH was also prepared and assayed by the method of Dalziel (1962*a*). The concentrations of solutions of ADP-ribose were determined spectrophotometrically by using $\varepsilon_{260} = 15.4 \times 10^3$ litre·mol^{-1}·cm^{-1} (Burton, 1959).

Chromatography of all the commercial samples of NAD^+ yielded the same pattern of impurities reported by Dalziel & Dickinson (1966). Component C, thought to be ADP-ribose, was present to the extent of 0.03 and 0.06 of the NAD^+ (mol/mol) respectively in two samples from Boehringer and to the extent of 0.02 of the NAD^+ (mol/mol) in the one sample from Sigma. For initial-rate measurements, the substrates acetaldehyde and butyraldehyde (Fisons Ltd.) were redistilled before use, and ethanol (J. Burroughs Ltd., London S.E.11, U.K.) and butan-1-ol (Fisons Ltd.), both analytical grade, were used without further treatment. Acetamide (Fisons Ltd.) was recrystallized from methanol/ether.

Crystalline alcohol dehydrogenase was prepared from baker's yeast (*Saccharomyces cerevisiae*) as described by Dickinson (1972). Baker's yeast obtained under the trade name of N.G. & S.F., and supplied by British Fermentation Products Ltd., Hull, U.K., was obtained locally on the day on which it was required to inoculate a culture.

Methods

Enzyme activity and protein concentrations. Alcohol dehydrogenase activity was assayed as described previously (Dickinson, 1970) but by using chromatographically purified NAD^+. During the purification procedure, protein concentrations were determined by the measurement of E_{280} and E_{280}/E_{260}, as described by Warburg & Christian (1941). The concentrations of solutions with E_{280}/E_{260} greater than 1.3 were obtained by using an arbitrary value of $E^{1\%}_{1cm} = 12.6$ at 280nm. This is the value found for pure crystalline yeast alcohol dehydrogenase (Hayes & Velick, 1954; Dickinson, 1970).

Growth of baker's yeast. The glass vessels and pipettes used were soaked in 10% (v/v) HNO_3 for 24h and rinsed several times with glass-distilled water before use. The medium used was that of Phaff *et al.* (1966) with the following modifications and additions: KH_2PO_4, 2g/l; glucose, 10g/l (aerobic growth) or 100g/l (anaerobic growth). Following Andreasen & Stier (1953, 1954), oleic acid (280mg/l) and ergosterol (7mg/l) were included for anaerobic growth. The pH of the medium was adjusted to 6.0 with NaOH. For growth in the absence of Zn^{2+}, 20μM-$MnSO_4$ was present. In control experiments with 15μM-$ZnSO_4$ present, 1.8μM-$MnSO_4$ was also included. A solution of the vitamins required (Phaff *et al.*, 1966) was passed through a sterile Millipore filter (0.45μm) before addition to the remaining constituents of the medium which had been autoclaved.

Portions (1ml) of a suspension of yeast (one loopful from the centre of the block, in 30ml of sterile water) were used to inoculate two 100ml cultures, which were allowed to grow aerobically at 29°C, with shaking, for 18–24h. These cultures were then transferred to two 1-litre aspirators, each containing 500ml of medium, and these were subsequently used to inoculate a further 5 litres of medium. The final growth was in two 20-litre aspirators, each inoculated with 3 litres from the previous culture. In each of the last three growth stages growth was continued at 28°C for 24h, and N_2 gas was bubbled through the medium to keep the suspension agitated and to ensure anaerobic growth. In the absence of Zn^{2+}, approx. 140g of cells was harvested, by centrifugation, from the final 40-litre culture. The cells were washed at 4°C, first with 40mM-phosphate buffer, pH7.0, and then with 5mM-Na_2HPO_4 before being resuspended in about 200ml of 5mM-Na_2HPO_4.

Partial purification of alcohol dehydrogenase. A typical purification procedure is shown in Table 1. From cell breakage to final preparation took about 15h. The temperature throughout was maintained at 0–4°C. Yeast cells were disrupted by one passage of the suspension through a French Pressure Cell, and the cell debris was then removed by centrifugation. The supernatant was adjusted from about pH6.3 to pH7.0 with 1M-NaOH, and the solution was then applied to a 30cm×5cm column of DE11-DEAE-cellulose equilibrated with 5mM-phosphate buffer, pH7.0. The enzyme was washed straight through this column and much nucleic acid was retained. The enzyme solution was then dialysed for 4h against 2×4 litres of 5mM-phosphate buffer, pH7.0, and applied to a 30cm×2cm column of DE32 DEAE-cellulose equilibrated with the same buffer. The column was washed with the 5mM buffer until the E_{280} of the eluate was less than 0.4 and then a linear gradient made from 100ml of 20mM- and 100ml of 80mM-phosphate, pH7.0, was applied. The enzyme was eluted at a concentration of 2–3mg/ml in 35–40mM-phosphate. Initial-rate studies were performed with this material. For other experiments, the eluent fractions with the highest specific activity were combined and dialysed for 6–8h against $(NH_4)_2SO_4$ (460g/l) in 44mM-phosphate buffer, pH7.0. The precipitate formed was dissolved in a small volume of 44mM-phosphate, pH7.0, and dialysed for 3h

Table 1. *Partial purifications of alcohol dehydrogenases from yeast grown under N_2 in the presence and absence of added Zn^{2+}*

For details see the text.

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)
Yeast (140g) grown in the presence of 20μM-$MnSO_4$ and no added Zn^{2+}					
Extract	100	104000	9170	11.4	100
DE11 DEAE-cellulose	88	67000	1750	39	67
DE32 DEAE-cellulose	22	12200	51	240	12
Yeast (145g) grown in the presence of 1.8μM-$MnSO_4$ and 15μM-$ZnSO_4$					
Extract	75	73500	11400	6.7	100
DE11 DEAE-cellulose	60	45000	2350	19	75
DE32 DEAE-cellulose	32	8600	130	66	12

against 1 litre of the same buffer. In some purifications of the enzyme from Zn^{2+}-deficient yeast, N_2 was bubbled through the buffers during dialysis, and the columns were equilibrated and eluted with buffers which had previously been bubbled with N_2. The only improvement noted was in the dialyses under N_2, when about 20% more activity was recovered. This modification was then included in the routine purification procedure.

Metal analysis. Samples of eluates from the DE32 DEAE-cellulose column [collected in glass tubes which had been soaked in 10% (v/v) HNO_3 for 12h] were dried at 90°C and the contents wet-ashed in 2ml of conc. HNO_3 plus 0.3ml of 36% (v/v) $HClO_4$ before being made up to 5ml with water. A Pye Unicam SP.1950 atomic absorption spectrophotometer was used to estimate the zinc and manganese contents of the samples. The standards used were samples taken from solutions of analytical-grade $ZnSO_4,7H_2O$ and $MnSO_4,4H_2O$ (BDH Chemicals Ltd.) which had been similarly dried and wet-ashed.

Molecular-weight determinations. Molecular weights were estimated by using a 60cm×0.9cm column of Sephadex G-200, equilibrated at 0–4°C with 44mM-phosphate buffer, pH7.0. The standards used, with their mol.wt., were: ox liver catalase, 250000 (Schroeder *et al.*, 1964); crystalline yeast alcohol dehydrogenase, 141000 (Bühner & Sund, 1969); horse liver alcohol dehydrogenase, 84000 (Ehrenberg & Dalziel, 1958); ovalbumin, 45000 (Warner, 1954); soya-bean trypsin inhibitor, 21500 (Wu & Scheraga, 1962). The void volume (V_0) of the column was obtained by using Blue Dextran 2000 [Pharmacia (G.B.) Ltd.]. The elution volume (V_e) of enzymic activity or E_{280} was determined as the peak volume from an elution profile for each of the proteins used; fractions (1.3ml) were collected. The molecular weights of the alcohol dehydrogenases from yeast grown with or without added Zn^{2+} were estimated from the plot of log molecular weight versus V_e/V_0 (Andrews, 1964).

The sedimentation behaviour of alcohol dehydrogenase extracted from Mn^{2+}-grown yeast was compared with that of the crystalline yeast enzyme by centrifugation in a sucrose gradient (Martin & Ames, 1961; Noll, 1967). An isokinetic gradient was prepared, as described by Noll (1967), by pumping 5.5ml of sucrose (32.86g/100ml of 44mM-phosphate buffer, pH7.0) into a mixing chamber (vol. 6.5ml) filled with another sucrose solution (10.41g/100ml of buffer). The resulting logarithmic gradient is isokinetic for molecules with partial specific volume of approx. 0.71. Samples of crystalline yeast enzyme and the enzyme from Mn^{2+}-grown yeast (in 0.2ml of 44mM-phosphate buffer, pH7.0) were layered on to the gradients. A third gradient was layered with a mixture of the two enzymes (in 0.2ml). The samples were centrifuged for 20h at 5°C at 52000rev./min in the 3×6ml swing-out rotor of an MSE 65 preparative ultracentrifuge. The tubes were then pierced and 0.18ml fractions were collected and assayed for enzymic activity.

Initial-rate studies. Duplicate assays with enzyme from Zn^{2+}-deficient yeast were performed fluorimetrically for alcohol oxidation by NAD^+, and fluorimetrically and spectrophotometrically for aldehyde reduction by NADH, as described previously (Dickinson & Monger, 1973). Bovine serum albumin (1mg) was added to all assays and dilutions of enzyme. Measurements were made at 25°C in phosphate buffer, pH7.05, I 0.1mol/l. Dilutions of enzyme from the 2–3mg/ml stock were renewed every 30–60min and were assayed under the standard conditions at pH8.8 (Dickinson, 1970) at the beginning and end of this period. The loss of activity of dilutions in 1h was no more than 15%. The duplicate measured initial rates, when corrected to the same specific activity, generally agreed to within 5%. Two complete experiments were performed with each

substrate, purified NAD^+ being used for the study of the alcohol–NAD^+ reactions. In addition, the oxidations of ethanol and butan-1-ol were also studied by using NAD^+ (grade I, Boehringer Corp.). The coenzyme and substrate concentrations used were within the following ranges: NAD^+, 6.5–1840 μM; ethanol, 10–500 mM; butan-1-ol, 6.8–270 mM; NADH, 4.9–343 μM; acetaldehyde, 23–5620 μM; butyraldehyde, 1.8–74 mM.

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

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

were obtained from the slopes and intercepts of the double-reciprocal plots as described by Dalziel (1957). In eqn. (1) e is the concentration of enzyme active sites, and S_1 and S_2 are coenzyme and substrate, respectively. The symbols S_1, S_2 and ϕ_0 etc. are used for alcohol–NAD^+ reactions, and primed symbols, S_1', S_2', ϕ_0' etc., are used for aldehyde–NADH reactions. This is the convention adopted in previous kinetic studies (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975*b*,*c*). In complete duplicate experiments, estimates of initial-rate parameters generally agreed to within ±10%.

Results

Crude cell extracts from yeast grown anaerobically in the absence of added Zn^{2+}, but with about ten times the normal concentration of $MnSO_4$, contain substantial quantities of alcohol dehydrogenase activity. When assayed in the standard assay, but with chromatographically purified NAD^+ and not commercial NAD^+ [which contains the inhibitor ADP-ribose (Dalziel, 1963; Dalziel & Dickinson, 1966)], the extracts from 140g of cells contained about 100000 units (μmol/min) of activity. This is a little more than that found in extracts of a similar weight of yeast grown in the presence of added Zn^{2+} (Table 1). The specific activities of extracts from Zn^{2+}-deficient yeast were also somewhat higher than those from the controls.

In contrast with the partially purified enzyme from yeast grown with added Zn^{2+}, or the purified enzyme from commercial yeast, both of which are quite stable, the enzyme from Zn^{2+}-deficient cells is unstable. At 4°C in 0.045M-phosphate buffer, pH 7.0, the activity (1000 units/ml; 14.4 units/mg) of a crude cell extract declined by 45% in 8h. Addition of 20% (w/v) sucrose did not affect the stability of the enzyme, but addition of 0.1% (v/v) mercaptoethanol, with or without 20% sucrose, resulted in the loss of 70% of the activity in 8h. The enzyme was more unstable when 3mM-$MnSO_4$ or 3mM-EDTA was added, only 9 and 3% respectively of the initial activity being recovered after 8h. The partially purified enzyme was also labile, but not quite as unstable as in the crude extract. A sample recovered from the DE32 DEAE-cellulose column (490 units/ml; 240 units/mg) lost 60% of its activity in 16h, and the stability was not increased by the inclusion of 20% sucrose plus 0.1% mercaptoethanol. With 3mM-EDTA present, 85% of the activity was lost in 16h. Addition of 120μM-ADP-ribose caused detectable stabilization, 60% of the initial activity remaining after 16h, and anaerobic conditions provided greater stabilization, with 70% of the activity remaining over the same period. The addition of 120μM-ADP-ribose under anaerobic conditions gave virtually complete protection: 90% of the initial activity was detected after 16 and 40h and 50% after 6 days. Our findings on the stability of the enzyme are in general agreement with those of Coleman & Weiner (1973) with the Mn^{2+}-containing alcohol dehydrogenase from yeast YU 1001. However, as noted above, we did not find protection of the enzyme by 20% sucrose plus 0.1% mercaptoethanol under anaerobic conditions.

In purifications of alcohol dehydrogenase from Zn^{2+}-deficient cells carried out so far (Table 1) we have achieved about a 20-fold purification, with yields of about 12% in the fractions of highest specific activity, and final specific activities of 240–260 units/mg. The procedure uses mild methods which can be performed quickly. We have not used the methods of acetone precipitation or heat denaturation, which are used in our normal preparations of alcohol dehydrogenase (Dickinson, 1970). These may be expected to cause drastic losses of activity with the more labile enzyme (Coleman & Weiner, 1973). We have not used ADP-ribose as a stabilizing agent in purifications. Much of our work has been concerned with the kinetic properties of the enzyme and since, as shown below, this agent is a powerful inhibitor of the enzyme, we have thought it best to exclude it from preparations.

When the same purification procedure was applied to cell extracts from yeast grown in the presence of Zn^{2+}, overall purifications of about tenfold were obtained, with yields of 12–20% in the best fractions and specific activities of 60–70 units/mg (Table 1). Crystalline enzyme has a specific activity of 400 units/mg (Dickinson, 1970) and so it seems that the product here is roughly 20% pure. In one of the preparations from yeast grown in the presence of Zn^{2+}, the product from the final column was further purified by heat treatment at 55°C for 10min followed, over a period of 2 days, by two $(NH_4)_2SO_4$ precipitations at about 45% saturation. The product of this extended procedure had a specific activity of 380 units/mg, confirming the validity of the purity calculation. The degree of purity of the modified enzyme obtained under Zn^{2+}-deficient conditions is, of course, unknown, but since the degree of purification with each enzyme is similar (Table 1) and the final specific

activity of the new enzyme is some 260 units/mg, it may be moderately pure.

We have attempted to estimate the specific activity of the pure, modified enzyme by fluorimetric titration of the partly pure preparation with NADH, in the manner described by Dickinson (1970). In contrast with the findings of Coleman & Weiner (1973) with alcohol dehydrogenase from both Zn^{2+}- and Mn^{2+}-grown yeast YU 1001, we found that our preparation enhanced the fluorescence of NADH about fourfold at pH6 and twofold at pH7. In the presence of 0.5M-acetamide there was no further enhancement of NADH fluorescence, in contrast with the 13-fold enhancement obtained with pure, crystalline alcohol dehydrogenase from commercial baker's yeast (Dickinson, 1970). In the presence of 0.5M-acetamide, the binding of NADH to our Zn^{2+}-deficient preparation was slightly weaker than in the absence of acetamide. For the normal enzyme the affinity is about 20-fold greater. In the presence of this new enzyme there was a significant blank decrease of NADH fluorescence, with or without acetamide, at pH6, and, to a lesser extent, at pH7. Since the enzyme preparation may contain components, other than alcohol dehydrogenase, which bind NADH and since there was no further enhancement of NADH fluorescence in the presence of the substrate analogue acetamide, the experiments were not continued.

As an alternative, we have tried to estimate the specific activity of pure enzyme by studying the binding of ADP-ribose to the enzyme at pH7.0 and 20°C with the method of Hummel & Dreyer (1962). Inhibitor, at a concentration of 29μM, was used in the experiments and this should have been sufficient to saturate the enzyme ($K_i = 0.5\mu$M at pH7.0; see below). There were real technical difficulties with these experiments owing to the presence in dialysed preparations of low-molecular-weight u.v.-absorbing compounds, presumably arising from proteinase activity. However, we found that a sample containing 1080 units of activity (specific activity 70 units/mg) bound about 50nmol of inhibitor. Assuming two catalytic sites/molecule of mol.wt. 141000 the results indicate a minimum specific activity of 300 units/mg for pure enzyme. This value may be revised upwards to 1100 units/mg if allowance is made for the fact that the enzyme preparation had declined in specific activity from 260 to 70 units/mg since it was first prepared, and it is assumed that inactive enzyme still bound inhibitor. The calculations suggest that our best preparations are reasonably pure.

Initial-rate studies

The results of detailed initial-rate studies at pH7.05 and 25°C with the enzyme from zinc-deficient yeast are shown in Figs. 1(*a*) and 1(*b*) for ethanol oxidation and in Figs. 2(*a*) and 2(*b*) for acetaldehyde reduction. The primary and secondary plots are linear, within experimental error, over the ranges of substrate and coenzyme concentrations used, and the data conform to eqn. (1). Similar plots were obtained with butan-1-ol and butyraldehyde as substrates and when commercial NAD^+ was used as coenzyme in alcohol

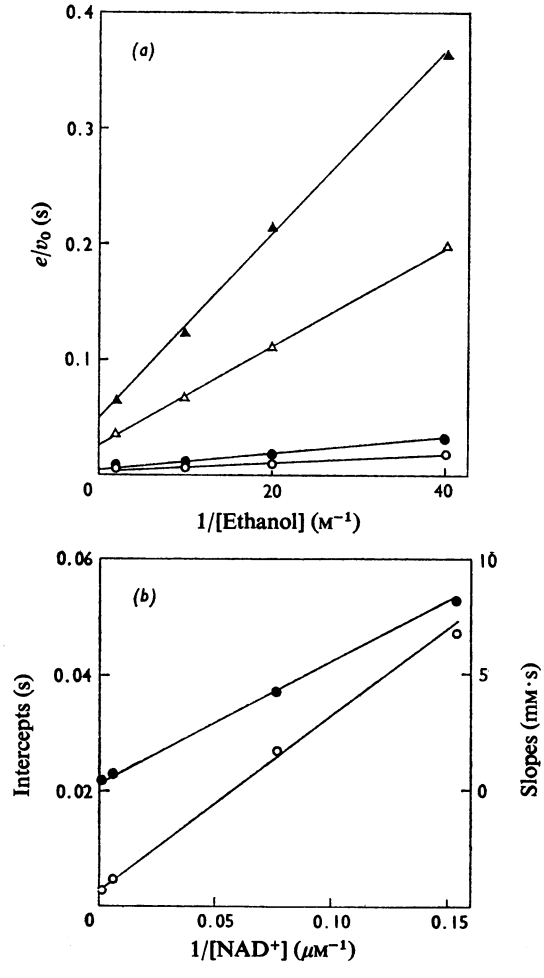

Fig. 1. *Kinetics of alcohol dehydrogenase from Zn^{2+}-deficient yeast with ethanol and NAD^+ as substrates*

(*a*) Primary plot showing variation of the reciprocal of the specific initial rate at pH7.05 and 25°C with the reciprocal of the ethanol concentration for several NAD^+ concentrations. The NAD^+ was chromatographically purified and the concentrations (μM) were: ○, 1000; ●, 167; △, 13; ▲, 6.5. (*b*) Secondary plot showing the variation of the intercepts (○) and slopes (●) of the Lineweaver–Burk plots in Fig. 1(*a*) with the reciprocal of the NAD^+ concentration.

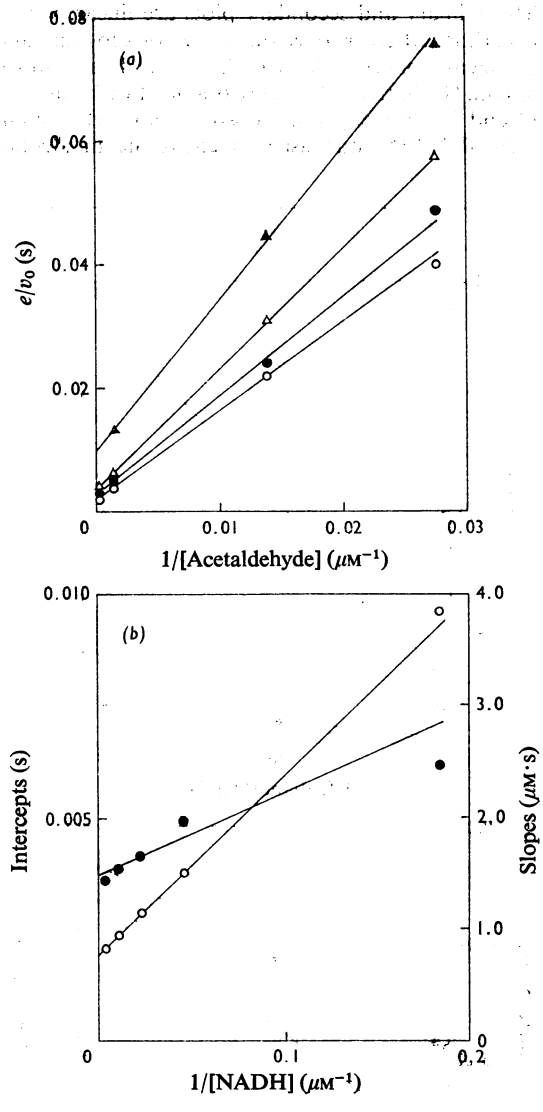

Fig. 2. *Kinetics of alcohol dehydrogenase from Zn^{2+}-deficient yeast with acetaldehyde and NADH as substrates*

(*a*) Primary plot showing variation of the reciprocal of the specific initial rate at pH 7.05 and 25°C with the reciprocal of the acetaldehyde concentration for several NADH concentrations. The NADH concentrations (μM) were: ○, 280; ●, 44; △, 21.7; ▲, 5.45. For clarity, results at 89 μM have been omitted. (*b*) Secondary plot showing the variation of intercepts (○) and slopes (●) of the Lineweaver–Burk plots in Fig. 2(*a*) with the reciprocal of the NADH concentration.

oxidations. The experiments described were performed with preparations of enzyme having specific activities in the range 100–260 units/mg. When complete duplicate experiments were performed with different preparations, the results generally agreed to within ±10% when corrected to the same specific activity.

The kinetic coefficients derived from the initial-rate studies are shown in Table 2 together with those for the enzyme obtained from commercial yeast and given by Dickenson & Dickinson (1975*b*,*c*). Studies performed with enzyme purified from yeast grown in the presence of Zn^{2+} with ethanol, butan-1-ol and acetaldehyde showed that the coefficients for the crystalline enzyme are, within experimental error, applicable to these preparations also. The basis of comparison in Table 2 is that the pure enzymes have a specific activity of 400 units/mg and that they contain two active sites/molecule of mol.wt. 141000. These values apply to our crystalline enzyme preparations (Dickinson, 1970, 1974) but may not apply to the enzyme from Zn^{2+}-deficient yeast. The basis of comparison is thus arbitrary. Nevertheless it is clear from Table 2 that the kinetic characteristics of the two enzymes are quite different. For example, when pure coenzymes are used the ratio of the maximum rate of aldehyde reduction to the maximum rate of alcohol oxidation (ϕ_0/ϕ_0') is about eight times smaller for the enzyme from Zn^{2+}-deficient yeast. Also the K_m values for alcohol and NAD^+ in butan-1-ol oxidation are substantially larger, the K_m for NAD^+ in ethanol oxidation and the K_m values for the aldehydes are rather smaller and the K_m values for NADH much smaller. Coleman & Weiner (1973) have observed similar trends in apparent K_m values for ethanol and NAD^+ between the Zn^{2+}- and Mn^{2+}-containing alcohol dehydrogenases from yeast YU 1001.

Inhibition by ADP-ribose

One of the most striking differences to emerge from Table 2 is that the new enzyme is very sensitive to the presence of a small amount of inhibiting impurity, probably ADP-ribose, in commercial samples of NAD^+. The inhibition arises because two of the initial-rate parameters (ϕ_0 and ϕ_2) are enormously increased, whereas the other two (ϕ_1 and ϕ_{12}) are essentially unchanged. Less precise data were obtained with butan-1-ol as substrate, but a similar result was indicated. The inhibitor may be tentatively identified as ADP-ribose since, if impurity C (Dalziel & Dickinson, 1966) found in NAD^+ purifications is added back to purified NAD^+, severe inhibition is observed. Impurity C co-chromatographs with authentic ADP-ribose under the conditions used in NAD^+ purification (Dalziel & Dickinson, 1966).

It is clear from Table 2 that at pH 7.05 the maximum rate of ethanol oxidation with enzyme from Zn^{2+}-deficient yeast is decreased by about 12-fold when the NAD^+ contains 6% ADP-ribose. On the other hand the crystalline enzyme is almost insensitive to the

Table 2. *Kinetic coefficients for the oxidation of ethanol and butan-1-ol by NAD$^+$ and the reduction of acetaldehyde and butyraldehyde by NADH with alcohol dehydrogenase purified from Zn^{2+}-deficient yeast*

The kinetic coefficients were obtained at pH7.05 and 25°C and are defined by the initial-rate equation:

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

$[S_1]$ and $[S_2]$ are coenzyme and substrate concentrations respectively. ϕ_1/ϕ_0 is the Michaelis constant for coenzyme and ϕ_2/ϕ_0 that for the substrate. The same symbols without primes refer to alcohol oxidation and with primes to aldehyde reduction. The value of e was calculated by assuming that the pure enzyme has a specific activity of 400 units/mg in the standard assay (with purified NAD$^+$) and contains 2 sites/molecule of mol.wt. 141000. The values apply to our normal enzyme preparations (Dickenson & Dickinson, 1975*b*,*c*). Values given in parentheses are for comparative purposes and apply to crystalline alcohol dehydrogenase from baker's yeast (Dickenson & Dickinson, 1975*b*,*c*). Kinetic coefficients were generally reproducible to within ±10%. Unless otherwise stated the coenzymes were highly purified.

Substrate	ϕ_0 (s)	ϕ_1 (μM·s)	ϕ_2 (μM·s)	ϕ_{12} (μM^2·s)	ϕ_1/ϕ_0 (μM)	ϕ_2/ϕ_0 (mM)	ϕ_{12}/ϕ_2 (μM)
Alcohol oxidation							
Ethanol	0.0026	0.2	260	56000	77	100	180
	(0.0024)	(0.26)	(62)	(16500)	(108)	(26)	(270)
Butan-1-ol	0.10	52	16000	5200000	520	160	325
	(0.038)	(10.0)	(1630)	(370000)	(260)	(43)	(228)
Ethanol, with NAD$^+$ containing 6% ADP-ribose (mol/mol)	0.03	0.28	3800	60000			
	(0.0026)	(0.25)	(90)	(12000)			
	ϕ_0' (s)	ϕ_1' (μM·s)	ϕ_2' (μM·s)	ϕ_{12}' (μM^2·s)	ϕ_1'/ϕ_0' (μM)	ϕ_2'/ϕ_0' (mM)	ϕ_{12}'/ϕ_2' (μM)
Aldehyde reduction							
Acetaldehyde	0.002	0.03	1.4	~9.0	15	0.7	~6.5
	(0.00026)	(0.023)	(0.23)	(3.0)	(84)	(0.84)	(13.0)
Butyraldehyde	0.01	0.32	100	1000	32	10	10
	(0.00029)	(0.028)	(8)	(56)	(97)	(27.5)	(7.0)

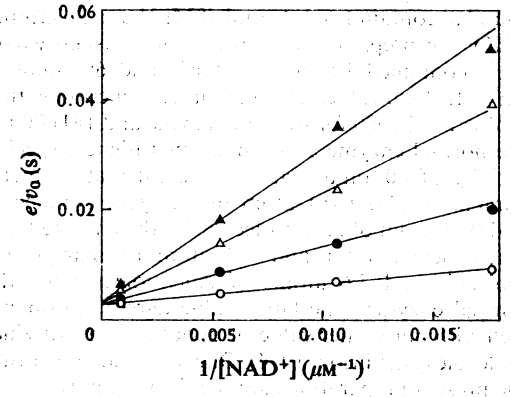

Fig. 3. *Effect of ADP-ribose on the oxidation of ethanol by NAD$^+$ catalysed by alcohol dehydrogenase from Zn^{2+}-deficient yeast*

Variation of the reciprocal specific initial rate of reaction at pH7.05, 25°C and 61 mM-ethanol with the reciprocal of the NAD$^+$ concentration in the presence of ADP-ribose. The ADP-ribose concentrations (μM) were: ○, 0; ●, 0.63; △, 1.57; ▲, 3.14.

impurity. The enzyme purified from yeast grown with added Zn^{2+} is also insensitive. The new enzyme is rather less sensitive to the inhibitor at alkaline pH. In the standard assay at pH8.8, the rate observed with NAD$^+$ containing 6% ADP-ribose is only about half of that found by using pure NAD$^+$. Once again the crystalline enzyme is unaffected by this amount of the impurity.

The behaviour of the sensitive enzyme with ADP-ribose becomes clearer when the concentrations of the inhibitor and purified NAD$^+$ are varied independently at a constant ethanol concentration. Fig. 3 shows the results of such an experiment at pH7.05 and 25°C. There is a clear indication that the inhibitor competes directly with the NAD$^+$ for the coenzyme-binding site. Such a result is, of course, expected because of the strong structural resemblance between the two compounds. A plot of the apparent K_m from Fig. 3 versus the inhibitor concentration, [i], is linear, within the experimental error, and fits an equation of the type $K_{m_{app.}} = K_m(1+[i]/K_i)$. From the slope of the secondary plot a value for the dissociation constant K_i of the enzyme–inhibitor complex of

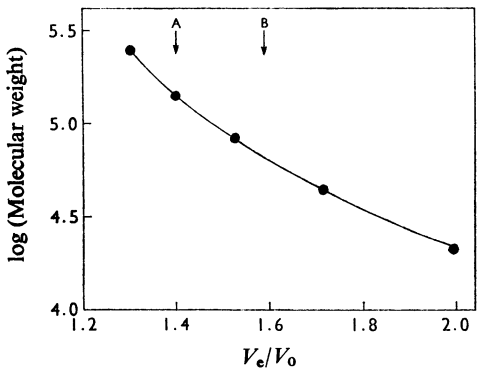

Fig. 4. *Calibration curve for Sephadex G-200 column used in molecular-weight determinations*

The plot shows the variation of the elution volume, V_e, in relation to the void volume, V_0, of the column for several calibrating proteins. The standards used, in order of increasing molecular weight, were soya-bean trypsin inhibitor, 21500; ovalbumin, 45000; horse liver alcohol dehydrogenase, 84000; crystalline yeast alcohol dehydrogenase, 141000; bovine liver catalase, 250000. Arrow A indicates the value of V_e/V_0 obtained for alcohol dehydrogenase purified from yeast grown with added Zn^{2+} and arrow B indicates the value of V_e/V_0 obtained for alcohol dehydrogenase purified from yeast grown without added Zn^{2+}.

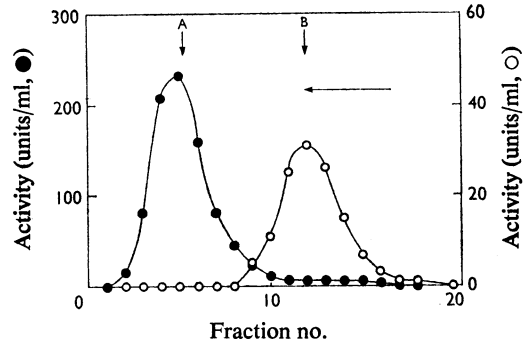

Fig. 5. *Sedimentation behaviour of alcohol dehydrogenase at 5°C in a sucrose density gradient*

The profile marked ● was found for crystalline yeast alcohol dehydrogenase and that marked ○ for the alcohol dehydrogenase from Zn^{2+}-deficient yeast. The arrows A and B mark the peaks of activity found in the gradient used to analyse a mixture of the two enzymes. The activity found in fractions 8–16 was sensitive to EDTA. The horizontal arrow indicates the direction of sedimentation.

0.5 μM was obtained. A less detailed experiment at pH 8.8 and 25°C gave a similar result with a value for K_i of 5 μM. The binding of the inhibitor is clearly pH dependent.

Molecular-weight determinations

We required information about structural differences which may be responsible for the marked kinetic and stability differences between alcohol dehydrogenase from Zn^{2+}-deficient yeast on the one hand, and normal crystalline enzyme and enzyme from yeast grown with added Zn^{2+} on the other. Since the enzyme preparations are not pure we have used gel filtration on calibrated columns of Sephadex G-200 to estimate the molecular weight and centrifugation in sucrose density gradients to study sedimentation behaviour.

Gel-filtration experiments (Fig. 4) gave values of mol.wt. 72000 ± 10000 for the Zn^{2+}-deficient enzyme and 140000 ± 10000 for the 'normal' enzyme. The $s_{20,w}$ values for the enzymes, calculated from density-gradient-centrifugation data (Fig. 5) were 5.8S and 8.0S respectively [cf. 7.6S for normal enzyme from analytical ultracentrifuge experiments (Bühner & Sund, 1969)]. These experiments show that the low value of the apparent molecular weight (from gel filtration) of the Zn^{2+}-deficient enzyme cannot be due to a more compact tetrameric molecular structure with the same molecular weight as the normal enzyme. A sedimentation coefficient of 5.8S is consistent with a mol.wt. of about 80000.

Metal analyses

Three samples containing 2.4–24mg of a preparation from yeast grown without added Zn^{2+} were found to contain 1.6–6.4nmol of Mn^{2+} and 3–32nmol of Zn^{2+}. A preparation of 7.0mg from yeast grown on Zn^{2+} contained 1.8nmol of Mn^{2+} and 66 nmol of Zn^{2+}. If these values are taken in conjunction with a mol.wt. of 72000 for the new enzyme and 141000 for the normal enzyme and a presumed highest specific activity of 400units/mg, then we calculate a metal content of about 0.1 atom of manganese/molecule and 0.25 atom of zinc/molecule for the enzyme from zinc-deficient yeast and 0.2 atom of manganese/molecule and 7.6 atoms of zinc/molecule for the normal enzyme. The latter value agrees reasonably well with our earlier value of about 5 atoms of zinc/molecule (Dickinson, 1974). One must remember that the present preparations are relatively impure.

Discussion

It is clear from the criteria that we have been able to apply that alcohol dehydrogenase from Zn^{2+}-deficient yeast grown under N_2 is quite different from the enzyme found in yeast grown with added Zn^{2+} under N_2 or the enzyme found in commercial yeast.

The enzymes from the latter sources appear to be identical, as expected. The relationship between the new enzyme and the normal alcohol dehydrogenase is not easy to resolve, because yeast apparently produces two other types of alcohol dehydrogenase which can be distinguished from the classical enzyme. These are a second type of enzyme found in the cytoplasm (Lutstorf & Megnet, 1968) and a third found in mitochondria (Wenger & Bernofsky, 1971). There are, however, certain arguments which indicate that the enzyme with which we are concerned is not of these alternative forms. The second type of cytoplasmic enzyme is repressed by high concentrations of glucose in the growth medium (Lutstorf & Megnet, 1968); further, the enzyme has a higher activity with butan-1-ol than with ethanol and it is a tetramer of mol.wt. around 140000 (Lutstorf & Megnet, 1968; Ciriacy, 1975). Our new enzyme is produced by the yeast in the presence of high glucose concentrations, has a much lower activity with butan-1-ol than with ethanol (Table 2) and is of mol.wt. about 72000. The mitochondrial enzyme is unlikely to be produced in significant amounts under our growth conditions, since the synthesis of yeast mitochondrial enzymes is severely repressed by high glucose concentrations (Utter *et al.*, 1967).

It seems possible that the new enzyme comprises the same protein as the normal alcohol dehydrogenase. This conclusion is supported by the fact that simply lowering the $MnSO_4$ concentration from 20μM to 1.8μM and including 15μM-$ZnSO_4$ results in the synthesis of the well-recognized enzyme. This conclusion can only be verified, however, when the enzyme is obtained in a pure form and can be subjected to structural analysis.

Normal alcohol dehydrogenase is a tetramer of mol.wt. 141000 composed of apparently identical subunits (Harris, 1964; Jörnvall, 1973). If our enzyme is made from the same subunits it must be a dimer. The inference is that in the face of a Zn^{2+} deficiency, the dimers are not joined together to form tetramers. This suggests that the role of at least some of the zinc atoms in the normal enzyme (4–5 atoms/molecule of mol.wt. 141000) is to stabilize the quaternary structure; the enzyme seems to be a dimer of dimers. A structural role for some of the zinc atoms would not be surprising, since Kägi & Vallee (1960) showed that, on complete removal of the metal by chelating agents, the enzyme dissociates into subunits. It is also notable that in the dimeric horse liver alcohol dehydrogenase (mol.wt. 84000, 4 atoms of zinc/molecule) half of the metal atoms have a possibly structural role and half form part of the active sites (Brändén *et al.*, 1973). The liver enzyme is also inhibited by ADP-ribose (Dalziel, 1963), like the dimeric yeast enzyme we have now encountered.

If the normal yeast alcohol dehydrogenase, containing about 4 atoms of zinc/molecule, requires two atoms to maintain the tetrameric structure, this leaves about two atoms/molecule for an alternative function. If these atoms have a catalytic role, then one would expect two active sites/molecule. Highly purified preparations of the enzyme seem to have only two active sites/molecule (Dickinson, 1974; Leskovac & Pavkov-Peričin, 1975).

The fact that the new enzyme is very sensitive to EDTA suggests that a metal component is important. The findings of Hoch *et al.* (1958) that 1,10-*o*-phenanthroline competes with coenzyme for normal alcohol dehydrogenase suggests a catalytic role for at least some of the metal atoms in this enzyme. Comparison with the horse liver enzyme also suggests that the active centre might contain a metal component. The results of Coleman & Weiner (1973) indicate that the enzyme might contain Mn^{2+}. The metal analyses that we have obtained are not very helpful, since the enzyme preparations were undoubtedly impure. If the specific activity of the pure enzyme is much higher than 400 units/mg then the enzyme could contain stoicheiometric amounts of either Mn^{2+} or Zn^{2+}. The origin of the zinc in our preparations is unknown. It is possible that it is incorporated in growth and is due to contamination of the reagents. On the other hand, it might be picked up by the proteins (not necessarily the enzyme) in the course of the purification. Because of the sensitivity of the enzyme to EDTA we have not been able to use metal-chelating agents in our reagents.

We may now consider what information can be derived from the kinetic studies about the mechanism of catalysis with the enzyme from zinc-deficient yeast. Detailed studies have been performed with the normal enzyme (Dickenson & Dickinson, 1975*b*,*c*) and the results for the new enzyme are compared in Table 2. As has been pointed out, the basis for comparison of the kinetic coefficients is arbitrary because of uncertainty about the specific activity of the new enzyme. Nevertheless, the following points merit consideration.

(1) The apparently similar maximum rates of ethanol oxidation ($1/\phi_0$) at pH 7.05 indicate that the pH dependences of $V_{max.}$ for the two enzymes are similar. The activities of both enzymes were, of course, normalized at pH 8.8 with saturating coenzyme and substrate concentrations. Coleman & Weiner (1973) claim that the pH dependences of the Zn^{2+}- and Mn^{2+}-containing enzymes from yeast YU 1001 are rather different. However, the results of these workers may require some revision. The NAD^+ used in assays probably contained ADP-ribose (Dalziel, 1963; Dalziel & Dickinson, 1966) and, as shown in Table 2 and Fig. 3, this is an extremely potent inhibitor of enzyme from yeast grown in a Zn^{2+}-deficient, Mn^{2+}-rich, medium. The effect of this inhibitor is markedly pH dependent.

(2) The parameters ϕ_0' and ϕ_1' change dramatically

on passing from acetaldehyde to butyraldehyde with the new enzyme, but are essentially constant for the normal enzyme. For the latter, this invariance has been taken as evidence that a compulsory mechanism is operative and that the rate-limiting step is NAD^+ dissociation from the terminal enzyme–NAD^+ complex (Dickinson & Monger, 1973; Dickenson & Dickinson, 1975*b*). The variation of ϕ_0' with substrate indicates a different rate-limiting step (perhaps hydride transfer), whereas the variation of ϕ_1' shows that a strictly compulsory mechanism is not applicable, at least in butyraldehyde reduction. It seems that a complex of the type enzyme–aldehyde may be kinetically significant in aldehyde reduction.

(3) Despite the changes in some parameters and K_m values on changing from one enzyme to another, the ratios ϕ_{12}/ϕ_2 and ϕ_{12}'/ϕ_2' are fairly constant. For a number of possible mechanisms these ratios are a measure of the dissociation constants of the enzyme–coenzyme complexes (Dalziel, 1957) and this is found to be so for normal alcohol dehydrogenase (Dickenson & Dickinson, 1975*b*,*c*). If this relationship applies to the new enzyme then it appears that the affinities of the two types of binding site for both coenzymes are very similar, if not identical.

(4) The ratios $\phi_{12}'[H^+]/\phi_{12}$ for the ethanol–acetaldehyde and butan-1-ol–butyraldehyde reactions are essentially independent of the enzyme used (1.5×10^{-11} to 1.9×10^{-11} M) and are probably within experimental error equal to the respective equilibrium constants of 10^{-11} M and 9×10^{-12} M (Bäcklin, 1958; Dalziel, 1962*b*) for the overall reactions.

(5) Application of the maximum-rate relationships to the new data in Table 2 shows that for the ethanol–acetaldehyde system $\phi_1\phi_2/\phi_{12}\phi_0' = 0.56$ and $\phi_1'\phi_2'/\phi_{12}'\phi_0 = 1.8$; for the butan-1-ol–butyraldehyde system $\phi_1\phi_2/\phi_{12}\phi_0' = 15$ and $\phi_1'\phi_2'/\phi_{12}'\phi_0 = 0.32$. There is no consistent pattern of behaviour here and the data do not conform to the requirements of a strict compulsory-order mechanism, which are that $\phi_1\phi_2/\phi_{12}\phi_0' \leqslant 1$ and $\phi_1'\phi_2'/\phi_{12}'\phi_0 \leqslant 1$ (Dalziel, 1957).

A tentative explanation for the above results is that the reactions take place via a random-order mechanism, possibly with rapid equilibrium between the reactant complexes and with the binding of substrate affecting the affinity for coenzyme. This mechanism is not applicable to the data for the normal, Zn^{2+}-containing enzyme, for reasons which are discussed elsewhere (Silverstein & Boyer, 1964; Dickenson & Dickinson, 1975*b*,*c*).

We began this work with the idea that if an alternative metal ion, in this case Mn^{2+}, could be substituted for Zn^{2+}, then documentation of the differences in catalytic properties of the two enzymes might allow one to deduce something about the role of the metal ion in catalysis. We have clearly obtained a different enzyme, but we are not sure what the metal component is. In addition, the enzyme has a very different oligomeric structure and one cannot be confident that the conformation of the subunits in the two enzymes is identical or even similar. It is perhaps unwise, at this stage, to attempt to identify changes in the kinetic properties of the enzyme with change in a particular component, and we cannot obtain any further insight into the role of the metal in catalysis.

Apart from the kinetic differences between the two alcohol dehydrogenases discussed above, the striking difference in relation to ADP-ribose merits further consideration. Fig. 3 shows that the compound is competitive with NAD^+ for the enzyme from Zn^{2+}-deficient yeast. The behaviour noted in Table 2, where ϕ_0 and ϕ_2 are much increased and ϕ_1 and ϕ_{12} unchanged by the presence of inhibitor in constant molar proportion to the coenzyme, may be explained satisfactorily on the basis of this competitive inhibition. It has been shown (Dalziel, 1963) that for a compulsory-order two-substrate mechanism in the presence of an inhibitor, i, competing with coenzyme, S_1, and present in constant molar proportion, $r = [i]/[S_1]$, the initial rate equation for competitive inhibition:

$$\frac{e}{v_0} = \phi_0 + \frac{\phi_1}{[S_1]}\left(1+\frac{[i]}{K_i}\right) + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}\left(1+\frac{[i]}{K_i}\right) \quad (2)$$

becomes on substitution of [i] for $r[S_1]$

$$\frac{e}{v_0} = \left(\phi_0 + \frac{\phi_1 r}{K_i}\right) + \frac{\phi_1}{[S_1]} + \left(\phi_2 + \frac{\phi_{12} r}{K_i}\right)\frac{1}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]} \quad (3)$$

K_i is the dissociation constant of the enzyme–inhibitor complex. The presence of the inhibitor causes the parameters ϕ_0 and ϕ_2 in eqn. (1) to increase while ϕ_1 and ϕ_{12} are unchanged. As explained in (5) above, the results with the enzyme from Zn^{2+}-deficient cells are apparently incompatible with a compulsory mechanism. However, equations of the same form as eqns. (2) and (3) may be deduced for a rapid-equilibrium random-order mechanism if it is assumed that the inhibitor competes with the coenzyme (S_1) and that the binding of inhibitor does not affect the affinity of the enzyme for substrate (S_2). The latter is equivalent to a statement that the affinities of the free enzyme and the enzyme–alcohol complex for inhibitor are the same. By using eqn. (3) and the results of Table 2 a value of $K_i = 0.6\,\mu$M is obtained from the variation of ϕ_0 and ϕ_2 in the presence of inhibitor. This value is obviously in good agreement with the value of $K_i = 0.5\,\mu$M obtained from the more conventional experiment of Fig. 3. Studies of normal alcohol dehydrogenase with pure and commercial NAD^+ indicate a minimum value of $K_i = 50\,\mu$M for ADP-ribose calculated from small variations in ϕ_0 and ϕ_2. However, these variations were probably within the experimental error, so the true value may

be much larger. It is interesting that the affinities of the two enzymes for ADP-ribose should be so different when the dissociation constants for both coenzymes, as measured from ϕ_{12}/ϕ_2 and ϕ_{12}'/ϕ_2' are so similar (Table 2).

The results described illustrate once again how important it can be to use highly purified coenzyme preparations in kinetic studies with NAD^+-linked dehydrogenases. This point was originally made in kinetic work with horse liver alcohol dehydrogenase (Dalziel, 1962*a*, 1963). The present work provides a particularly striking example of an enzyme being inhibited by a common contaminant of NAD^+. The example is all the more impressive since the behaviour of normal alcohol dehydrogenase would lead one to expect that the new enzyme would be insensitive to this compound.

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References

Andreasen, A. A. & Stier, T. J. B. (1953) *J. Cell. Comp. Physiol.* **41**, 23–36
Andreasen, A. A. & Stier, T. J. B. (1954) *J. Cell. Comp. Physiol.* **43**, 271–281
Andrews, P. (1964) *Biochem. J.* **91**, 222–233
Bäcklin, K.-I. (1958) *Acta Chem. Scand.* **12**, 1279–1285
Brändén, C.-I., Eklund, H., Nordström, B., Boiwe, T., Söderlund, G., Zeppezauer, E., Ohlsson, I. & Åkeson, Å. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2439–2442
Bühner, M. & Sund, H. (1969) *Eur. J. Biochem.* **11**, 73–79
Burton, K. (1959) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M., eds.), pp. 74–81, Oxford University Press, Oxford
Ciriacy, M. (1975) *Mol. Gen. Genet.* **138**, 157–164
Coleman, P. L. & Weiner, H. (1973) *Biochemistry* **12**, 3466–3472
Creighton, D. J. & Sigman, D. S. (1971) *J. Am. Chem. Soc.* **93**, 6314–6316
Curdel, A. & Iwatsubo, M. (1968) *FEBS Lett.* **1**, 133–136
Dalziel, K. (1957) *Acta Chem. Scand.* **11**, 1706–1723
Dalziel, K. (1962*a*) *Biochem. J.* **84**, 240–244
Dalziel, K. (1962*b*) *Biochem. J.* **84**, 244–254
Dalziel, K. (1963) *J. Biol. Chem.* **238**, 1538–1543
Dalziel, K. & Dickinson, F. M. (1966) *Biochem. Prep.* **11**, 84–88
Dickenson, C. J. & Dickinson, F. M. (1973) *Biochem. Soc. Trans.* **1**, 1270–1272
Dickenson, C. J. & Dickinson, F. M. (1975*a*) *Eur. J. Biochem.* **52**, 595–603
Dickenson, C. J. & Dickinson, F. M. (1975*b*) *Biochem. J.* **147**, 303–311
Dickenson, C. J & Dickinson, F. M. (1975*c*) *Biochem. J.* **147**, 541–547
Dickinson, F. M. (1970) *Biochem. J.* **120**, 821–830
Dickinson, F. M. (1972) *Biochem. J.* **126**, 133–138
Dickinson, F. M. (1974) *Eur. J. Biochem.* **41**, 31–36
Dickinson, F. M. & Monger, G. P. (1973) *Biochem. J.* **131**, 261–270
Ehrenberg, A. & Dalziel, K. (1958) *Acta Chem. Scand.* **12**, 465–469
Harris, J. I. (1964) in *Structure and Activity of Enzymes* (Goodwin, T. W., Harris, J. I. & Hartley, B. S., eds.), pp. 102–107, Academic Press, London and New York
Hayes, J. E. & Velick, S. F. (1954) *J. Biol. Chem.* **207**, 225–244
Hoch, F. L., Williams, R. J. P. & Vallee, B. L. (1958) *J. Biol. Chem.* **232**, 453–464
Hummel, J. P. & Dreyer, W. J. (1962) *Biochim. Biophys. Acta* **63**, 530–532
Jörnvall, H. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2295–2298
Kägi, J. H. R. & Vallee, B. L. (1960) *J. Biol. Chem.* **235**, 3188–3192
Leskovac, V. & Pavkov-Peričin, D. (1975) *Biochem. J.* **145**, 581–590
Lutstorf, V. & Megnet, R. (1968) *Arch. Biochem. Biophys.* **126**, 933–944
Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
Noll, H. (1967) *Nature (London)* **215**, 360–363
Phaff, H. J., Miller, M. W. & Mark, E. M. (1966) *The Life of Yeasts*, p. 85, Harvard University Press, Cambridge, Mass.
Rabin, B. R., Ruiz-Cruz, J., Watts, D. C. & Whitehead, E. P. (1964) *Biochem. J.* **90**, 539–542
Schroeder, W. A., Shelton, J. R., Shelton, J. B. & Olson, B. M. (1964) *Biochim. Biophys. Acta* **89**, 47–65
Silverstein, E. & Boyer, P. D. (1964) *J. Biol. Chem.* **239**, 3908–3914
Sund, H. & Theorell, H. (1963) *Enzymes*, 2nd edn., **7**, 25–83
Utter, M. F., Duell, E. A. & Bernofsky, C. (1967) in *Aspects of Yeast Metabolism* (Mills, A. K. & Krebs, H. A., eds.), pp. 197–215, Blackwell Scientific, Oxford
Warburg, O. & Christian, W. (1941) *Biochem. Z.* **310**, 384–421
Warner, R. C. (1954) in *The Proteins* (Neurath, H. & Bailey, K., eds.), vol. 2, part A, pp. 435–485, Academic Press, London and New York
Wenger, J. I. & Bernofsky, C. (1971) *Biochim. Biophys. Acta* **227**, 479–490
Wu, Y. V. & Scheraga, H. A. (1962) *Biochemistry* **1**, 698–705