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The Reactions of 1,10-Phenanthroline with Yeast Alcohol Dehydrogenase

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Freshly prepared samples of yeast alcohol dehydrogenase (EC 1.1.1.1) were inhibited by 1,10-phenanthroline at pH 7.0 and 0°C in a two-stage process. The first step appeared to be slowly established, but was rendered reversible by removal of reagent or by addition of excess Zn^{2+} ions. The second step was irreversible and was associated with the dissociation of the tetrameric enzyme. The presence of saturating concentrations of NAD^+ or NADH promoted and enhanced inhibition by the slowly established reversible process, but prevented dissociation of the enzyme. For the incubation mixtures containing NAD^+, removal of the 1,10-phenanthroline resulted in virtually complete recovery of activity, whereas, for the incubation mixtures containing NADH, removal of the reagent gave only partial re-activation. The presence of NAD^+ and pyrazole, or NADH and acetamide, in incubation mixtures with the enzyme gave rise to ternary complexes that gave protection against both forms of inactivation by 1,10-phenanthroline. The results support the view that at least some of the Zn^{2+} ions associated with yeast alcohol dehydrogenase have a catalytic, as opposed to a purely structural, role.

The inhibition of activity by metal-chelating agents has, in the past, provided evidence for the direct involvement of metal ions in the mechanism of certain enzymes. For yeast alcohol dehydrogenase (EC 1.1.1.1) it has been known for about 20 years that the enzyme contains at least four atoms of zinc/molecule and that it is inhibited by the metal-chelating agent 1,10-phenanthroline (Vallee & Hoch, 1955; Hoch *et al.*, 1958; Williams *et al.*, 1958). The work showed that the effects of 1,10-phenanthroline were of two types. There was first a rapid reversible binding of the agent, which was competitively inhibited by NAD^+ or NADH, followed by a relatively slow irreversible inactivation. The latter was associated with depolymerization of the enzyme and loss of Zn^{2+} (Kägi & Vallee, 1960), and the observation led to the view that zinc atoms played a structural role in the enzyme. Later work showed that the rapid competitive binding of 1,10-phenanthroline could be modelled even better by the poorly chelating or non-chelating compounds 1,5-phenanthroline, 5,6-benzoquinoline and 7,8-benzoquinoline (Anderson *et al.*, 1966). It was suggested that the rapid reversible binding might not be associated with metal chelation, but with binding to a hydrophobic site in the coenzyme-binding region. The fact that ADP and 1,10-phenanthroline acting as inhibitors were mutually exclusive suggested that 1,10-phenanthroline could be binding in the region of the coenzyme-binding site specific for adenine (Anderson & Reynolds, 1965). This observation is in clear contrast with those with horse liver alcohol dehydrogenase, where 1,10-phenanthroline and ADP-ribose bind independently and without mutual hindrance (Yonetani, 1963; Yonetani & Theorell, 1964), and the 1,10-phenanthroline binds to the catalytic zinc atom (Brändén *et al.*, 1975). It is also notable that the K_i for 1,10-phenanthroline with the liver enzyme, 9 μM at pH 7.0 (Yonetani & Theorell, 1964), is very much lower than the corresponding value for the yeast enzyme, 2 mM at pH 7.0 (Hoch *et al.*, 1958).

It has been suggested (Brändén *et al.*, 1975), on the basis of structural comparison with the liver enzyme, that yeast alcohol dehydrogenase should contain two atoms of zinc/subunit and therefore eight atoms of zinc/molecule. This would mean that earlier estimates of 4–5 atoms of zinc/molecule (Sund & Theorell, 1963) were too low. This question has been reinvestigated, with Veillon & Sytkowski (1975) finding four atoms of zinc/molecule and Klinman & Welsh (1976) finding eight atoms of zinc/molecule with highly purified preparations. The reason for this large difference is not clear, but Veillon & Sytkowski (1975) claim that all zinc atoms in excess of four atoms/molecule can be easily removed without loss of activity by dialysis against EDTA, whereas Klinman & Welsh (1976) find that removal of metal by EDTA results in loss of activity. The suggestion that yeast and horse liver alcohol dehydrogenases share a common structure in relation to the zinc atoms (Brändén *et al.*, 1975) suggests that the yeast enzyme contains one atom of zinc/subunit having a catalytic role. For the horse liver enzyme, 1,10-phenanthroline binds directly to the catalytic zinc atom by displacement of a water molecule. In view of this, we decided to reinvestigate the reactions of the yeast enzyme with

1,10-phenanthroline to see if evidence could be found that would support a direct catalytic role for zinc atoms. The results of these investigations are now presented.

Experimental

Materials

Solutions were routinely prepared in glass-distilled water, but for experiments requiring zinc analyses, enzyme solutions and reagents were prepared with deionized water obtained by passing glass-distilled water through an Elgastat deionizer (Elga Products, Lane End, Bucks., U.K.). Phosphate and pyrophosphate buffers were obtained as the sodium salts of analytical grade from Fisons, Loughborough, Leics., U.K. Tris/HCl buffers were prepared from Trizma base obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Other preparations of Tris were found to contain significant quantities of heavy metals and were unsuitable for the work. EDTA was omitted from buffer solutions and assays unless otherwise stated. 1,10-Phenanthroline was of AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. 1,5-Phenanthroline was from ICN Pharmaceuticals, Plain View, NY, U.S.A.

Enzyme. Yeast alcohol dehydrogenase was prepared as described by Dickinson (1970, 1972). Samples of enzyme were also obtained from Boehringer Corp. (London), London W.5, U.K., as a freeze-dried powder. EDTA was removed from the fresh crystalline enzyme preparation by exhaustive dialysis against 0.1 M-sodium phosphate buffer, pH 7.0, before experiments. This precaution is important, because, although the enzyme is normally insensitive to EDTA at pH 7.0, the inhibition by 1,10-phenanthroline is considerably enhanced when EDTA is present.

Coenzymes and substrates. NAD^+ (grade II) and NADH (grade I) were from Boehringer. For initial-rate studies, NAD^+ was purified as described by Dalziel & Dickinson (1966). Acetaldehyde (Fisons) was redistilled before use. Ethanol (analytical grade) was from J. Burroughs, London S.E.11, U.K., and used without further treatment.

Acetamide and pyrazole. Acetamide (Fisons) was recrystallized from methanol/diethyl ether, and pyrazole was from Aldrich Chemical Co., Gillingham, Dorset, U.K., and was used without further purification.

Methods

Enzyme assay. Alcohol dehydrogenase activity was measured at pH 8.8 and 25°C as described by Dickinson (1970). A unit of enzyme activity is defined as that catalysing the oxidation of 1 μmol of ethanol/min under the conditions of the assay. Protein concentrations were determined spectrophotometrically at 280 nm by using $A^{1\%}_{1cm} = 12.6$ (Hayes & Velick, 1954).

Inactivation experiments. Enzyme at a final concentration of 5 mg/ml was incubated with 3.3 mM-1,10-phenanthroline at 0°C in 0.1 M-sodium phosphate buffer, pH 7.0. The mixtures were protected from light by aluminium foil. For mixtures containing coenzyme, pyrazole or acetamide these agents were added to the enzyme before the 1,10-phenanthroline. At selected times samples were withdrawn for assay. In some experiments samples were withdrawn and diluted 100-fold in ice-cold buffer [either 0.1 M-sodium phosphate buffer, pH 7.2, or 0.1 M-Tris/HCl buffer (pH 7.2)/0.5 mM-$ZnSO_4$] (Tris/HCl buffers were used because of the insolubility of Zn^{2+} ions in phosphate buffer; 0.1 M-Tris/HCl buffer, pH 7.2, in the absence of added metal gave the same results as 0.1 M-phosphate buffer, pH 7.2.) The dilutions were assayed immediately and again at later times by adding 10 μl portions to assay mixtures. The overall dilution of the enzyme involved here was so large (30000-fold) that the components of the incubation mixture, apart from the enzyme, were diluted to concentrations at which they exerted no influence on assays. In other experiments 1 ml samples were withdrawn from incubation mixtures at selected times and passed through a column (1.5 cm × 21 cm) of Sephadex G-25 equilibrated at 0°C with 0.1 M-phosphate buffer, pH 7.0. This procedure separated the enzyme from the other components of the incubation mixture. The enzyme was collected in approx. 5 ml at a concentration of approx. 1 mg/ml, and was assayed immediately for activity and later for zinc. For activity assay a 20-fold dilution in 0.1 M-phosphate buffer, pH 7.2, or 0.1 M-Tris/HCl (pH 7.2)/0.5 mM-$ZnSO_4$, was prepared immediately and 10 μl portions were used per assay. 1,10-Phenanthroline is retarded by Sephadex G-25 considerably more than would be expected on the basis of its molecular weight. It was important to wash the column with about 70 ml of 0.1 M-phosphate buffer, pH 7.0, after each separation to ensure that the column was free of 1,10-phenanthroline and could be used again.

Zinc analysis. Samples (4 ml) of eluates from the Sephadex G-25 column, containing about 4 mg of enzyme, were collected in glass tubes that had been soaked in 10% (v/v) HNO_3 for 12 h. They were dried at 90°C and the contents wet-ashed in 2 ml of conc. HNO_3 plus 0.3 ml of 36% (v/v) $HClO_4$ before being made up to 25 ml with water. A Pye–Unicam SP. 1950 atomic absorption spectrophotometer was used to determine the zinc content of the samples. The standards used were samples (containing 12–25 μg of Zn) taken from solutions of analytical-grade ZnO (BDH) to which were added 4 ml of 0.1 M-phosphate buffer, pH 7.0, so that the phosphate concentration in the standards was the same as that in the enzyme

samples. The standards were then dried and wet-ashed as described above and made up to 25ml with water. Preliminary experiments indicated that significant errors could result if standards were not treated in exactly the same way as the test samples. Duplicate determinations of zinc content generally agreed to within ±5%.

Sedimentation experiments. Ultracentrifugal analysis was performed in an MSE analytical ultracentrifuge fitted with a photoelectric scanning system. The protein solutions were dissolved in 0.1M-phosphate, pH7.0, and runs were performed at 4°C with a rotor speed of 50000rev./min (180000*g*) and scanning at 280nm.

Results and Discussion

Previous studies (Hoch *et al.*, 1958; Williams *et al.*, 1958) with 1,10-phenanthroline as an inhibitor of yeast alcohol dehydrogenase have shown that at least two types of inhibition occur. When the enzyme was assayed in the presence of this reagent, instantaneous reversible inhibition was observed, which was relieved by increasing concentrations of NAD^+ or NADH. 1,10-Phenanthroline behaved as a competitive inhibitor with a K_i value of about 2mM at 23°C. Experiments in which the enzyme was preincubated with 1,10-phenanthroline before assay showed that prolonged incubation at 0.2°C resulted in a slow loss of activity. This loss was only partly reversed by adding $ZnCl_2$ to the incubation mixture and was not apparently reversed by dilution of the incubation mixture (Vallee & Hoch, 1955; Williams *et al.*, 1958). Because of the metal-chelating properties of 1,10-phenanthroline and because the enzyme is known to contain zinc atoms, it was supposed that the inhibitions were the result of the binding of reagent to the metal component. Although this may well be true for the slow process, there is evidence which suggests that the rapid reversible inhibition could be due to binding of 1,10-phenanthroline to a hydrophobic region of the coenzyme-binding site (Anderson & Reynolds, 1965; Anderson *et al.*, 1966).

It is important to remember that 1,10-phenanthroline may bind to at least two different types of site on yeast alcohol dehydrogenase. The present paper is concerned principally with the slow time-dependent and sometimes irreversible effects of 1,10-phenanthroline on the enzyme and the influence of coenzymes on these processes. The rapid reversible binding of 1,10-phenanthroline will also occur in simple mixtures of enzyme and reagent, but will be effectively abolished by the presence of saturating coenzyme concentrations. It may be that some changes observed in behaviour in the presence and absence of coenzyme are due in part to the failure to form the rapidly formed and fully reversible enzyme–1,10-phenanthroline complex in the presence of excess coenzyme.

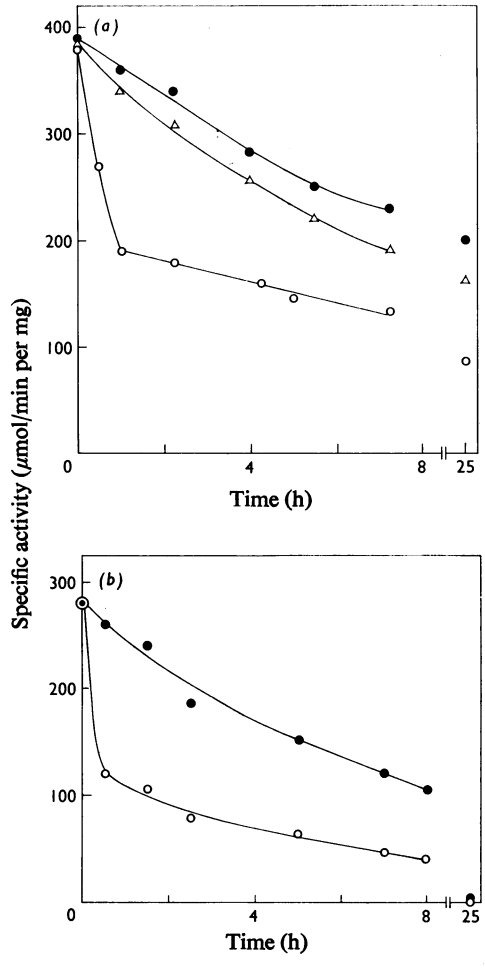

Fig. 1. *Changes in the activity of preparations of yeast alcohol dehydrogenase on incubation with* 3.3mM-1,10-*phenanthroline at pH*7.0 *and* 0°*C*

(*a*) Experiments were performed with freshly prepared enzyme as described in the text. The results were obtained from immediate assay after dilution 100-fold in 0.1M-phosphate buffer, pH7.2 (○), dilution in 0.1M-Tris/HCl buffer (pH7.2)/0.5mM-$ZnSO_4$ (△), or after passage of mixture through Sephadex G-25 and dilution of enzyme 20-fold in 0.1M-phosphate buffer, pH7.2 (●). (*b*) Experiments were performed with commercially obtained enzyme. ○, ● Same conditions as in (*a*).

The changes in specific activity resulting from incubation of freshly prepared enzyme at a concentration of 5mg/ml with 3.3mM-1,10-phenanthroline in 0.1M-phosphate buffer, pH7.0, at 0°C in the dark are shown in Fig. 1(*a*). Samples were withdrawn at the times indicated and treated in different ways. Small samples (0.05ml) were diluted 100-fold in either 0.1M-phosphate buffer, pH7.2, or 0.1M-Tris/HCl

buffer (pH7.2)/0.5mM-$ZnSO_4$, and 10μl portions were taken from the dilution and used immediately to initiate assays. Larger samples (1.0ml) were passed through a column (1.5cm × 21cm) of Sephadex G-25 to separate the 1,10-phenanthroline from the enzyme. This process, taking about 10min to complete, also produced a 5-fold dilution of the enzyme. Portions (0.05ml) of the eluted enzyme solution were diluted 20-fold in 0.1M-phosphate buffer, pH7.2, and 10μl portions of the dilutions were used immediately to initiate assays.

The results of Fig. 1(*a*) show that incubation with 1,10-phenanthroline produced a time-dependent loss of activity that was apparently reversed on dilution in buffer containing 0.5mM-$ZnSO_4$ or by removal of the reagent. The early stages of the inhibition appeared to be completely reversible by these procedures, and it was only in the later stages that there was a clear irreversible loss of activity. The activity recorded in the 25h samples do not represent the final activities attained. Over the next 50h the activity in the incubation mixture declined to about one-third of that at 25h. At the same time the re-activating effect of Zn^{2+} ions was virtually lost and the incubation mixture developed a slight precipitate of denatured protein. When a higher concentration of 1,10-phenanthroline was used (7.5mM), the activity of the mixture was undetectable after 48h and a very substantial protein precipitate had developed. Clearly additional effects came into play over these long incubation periods, and because of this we restricted our work to the period where no precipitation occurred.

The present results are in some important respects different from those of Hoch *et al.* (1958) and Williams *et al.* (1958). These authors found that activity was lost in a first-order manner, whereas the kinetics of Fig. 1(*a*), curve (○), are clearly complex. They also found that activity was not recovered on simple dilution in phosphate buffer, and Fig. 1(*a*) seems to confirm this. However, when the 100-fold dilutions in 0.1M-phosphate buffer, pH7.2, were re-assayed after long periods, evidence for re-activation was obtained. After 24h at 0°C re-activation was complete, and the dilutions exhibited the same activity as dilutions in Tris/HCl buffer (pH7.2)/0.5mM-$ZnSO_4$ which were prepared 24h earlier and assayed immediately. The reason for this slow re-activation is obscure, but it could be simply that dissociation of the complex is very slow. There would be 33μM-1,10-phenanthroline in these dilutions, and this might have influenced the process. In the experiments involving gel filtration the reagent was, of course, completely removed. It is noteworthy that the irreversible losses of activity recorded in Fig. 1(*a*) also obeyed rather complex kinetics. The slope of a first-order plot decreased 3–4-fold over the first 25h of incubation. Repeat experiments with different enzyme preparations and also using a higher 1,10-phenanthroline concentration showed the same pattern of behaviour.

The experiments of Fig. 1(*a*), like most studies of yeast alcohol dehydrogenase performed in this laboratory, involved freshly prepared enzyme of high specific activity. Some of the earlier work on 1,10-phenanthroline inhibition was performed with commercial preparations of the enzyme (Kägi & Vallee, 1960; Leskovac *et al.*, 1976). Since it is well known that preparations of the enzyme can deteriorate on storage (Bühner & Sund, 1969), it was decided to repeat the experiments of Fig. 1(*a*) with commercial preparations of enzyme. The results of those experiments are shown in Fig. 1(*b*), and show that the broad features of 1,10-phenanthroline inhibition were as in Fig. 1(*a*). Progressive loss of activity was found on assay of dilutions in phosphate buffer which was partly reversed on dilution in Tris/HCl buffer/0.5mM-$ZnSO_4$ or by removal of the 1,10-phenanthroline by gel filtration. There are, however, two important differences between the results of Figs. 1(*a*) and 1(*b*). The commercial preparations used had a considerably lower initial specific activity and were less resistant to inactivation by 1,10-phenanthroline.

The results shown in Figs. 1(*a*) and 1(*b*) probably arise from a combination of at least two processes. The initial step may be reversible (though slowly established) binding to zinc atoms in the active centre of the enzyme. The fact that the enzyme is completely stable when incubated for up to 20h with 3.3mM-1,5-phenanthroline under the conditions of Fig. 1(*a*) supports the view that metal chelation could be involved in inactivation by 1,10-phenanthroline. The work of Kägi & Vallee (1960) indicates that the irreversible losses of activity in Figs. 1(*a*) and 1(*b*) are probably associated in some way with the dissociation of the tetrameric enzyme into subunits. Ultracentrifugation at 4°C of samples obtained after incubation of enzyme for 25h with 1,10-phenanthroline at 0°C and removal of reagent by gel filtration confirmed this expectation. With the freshly prepared enzyme two boundaries were observed, with 48% of the material sedimenting with a corrected sedimentation coefficient of $s_{20,w} = 2.5$S and 52% sedimenting with $s_{20,w} = 7.5$S. For the commercial preparation over 95% of the material sedimented with $s_{20,w} = 2.3$S. Both preparations of enzyme showed only one sedimenting boundary with $s_{20,w} = 7.6$S before treatment with 1,10-phenanthroline. Bühner & Sund (1969) showed that the dissociated subunit of yeast alcohol dehydrogenase and the native enzyme have sedimentation coefficients of 2.8S and 7.6S respectively. There is thus a connexion between the irreversible losses of activity recorded in Figs. 1(*a*) and 1(*b*) at 25h and the extent of depolymerization of the enzyme.

It is interesting to consider what effects coenzymes have on the processes described in Fig. 1(*a*). The

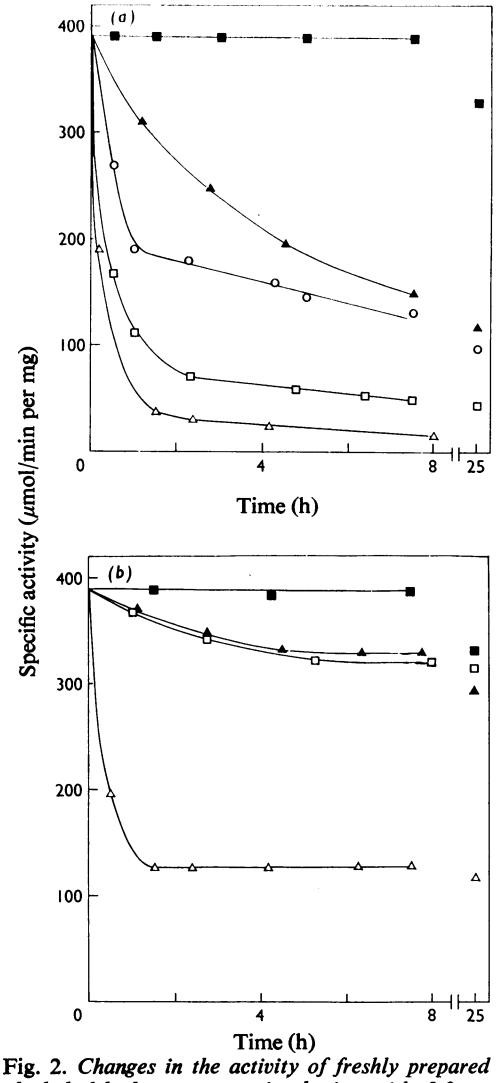

Fig. 2. *Changes in the activity of freshly prepared yeast alcohol dehydrogenase on incubation with* 3.3mM-1,10-*phenanthroline at pH* 7.0 *and* 0°C *in the presence of coenzyme and substrate analogues*

(*a*) The results were obtained from immediate assay of 100-fold dilutions of the incubation mixtures in 0.1M-phosphate buffer, pH7.2. The incubation mixtures were prepared as described in the text and contained no addition (○), 180μM-NADH (△), 180μM-NADH+0.5M-acetamide (▲), 5.5mM-NAD^+ (□) and 5.5mM-NAD^++10mM-pyrazole (■). (*b*) The results were obtained from immediate assay of 20-fold dilutions of the enzyme solution obtained after passage of incubation mixtures through Sephadex G-25. (△, ▲, □, ■) Same incubation conditions as in (*a*).

results of incubation of a 5mg/ml solution of freshly made alcohol dehydrogenase with 3.3mM-1,10-phenanthroline at 0°C in 0.1M-phosphate buffer, pH7.0, in the presence of 180μM-NADH, 180μM-NADH+0.5M-acetamide, 5.5mM-NAD^+ or 5.5mM-NAD^++10mM-pyrazole are shown in Fig. 2(*a*). Samples were withdrawn at indicated times, diluted 100-fold in 0.1M-phosphate buffer, pH7.2, and then a 10μl portion of the diluted solution was used for immediate assay. The incubation conditions were chosen so that in each case the enzyme would be present largely either as an enzyme–coenzyme complex or as a ternary complex. The dissociation constants of the enzyme–NAD^+ and enzyme–NADH complexes at 0°C are probably significantly less than the respective values of 110μM and 3μM found at 15°C (Dickenson & Dickinson, 1975*a*). The dissociation constants of the enzyme–NAD^+–pyrazole and enzyme–NADH–acetamide complexes are unknown, but titrations have indicated that the conditions chosen were sufficient to fill at least 95% of the available binding sites with the appropriate ternary complex in the absence of 1,10-phenanthroline (Dickinson, 1970; F. M. Dickinson, unpublished work).

The results shown in Fig. 2(*a*), when compared with those of Fig. 1(*a*), show that in the presence of coenzyme the rate and extent of loss of activity in the presence of 1,10-phenanthroline (as measured in immediate assays of dilutions in 0.1M-phosphate, pH7.2) is much increased. The enzyme–coenzyme complexes are therefore more susceptible to attack by 1,10-phenanthroline. Similar observations were obtained with a commercial preparation of enzyme. The enzyme–NADH–acetamide complex on the other hand affords some protection from the inhibitor, whereas the enzyme–NAD^+–pyrazole complex affords almost complete protection. In the presence of pyrazole or acetamide alone no effect was observed on the inactivation by 1,10-phenanthroline. This may be because the binary enzyme–pyrazole and enzyme–acetamide complexes do not form readily under the conditions used. When the experiments with NAD^+ and NADH alone were repeated with 1,5-phenanthroline, but otherwise with the conditions of Fig. 2(*a*), no losses of activity were recorded during an 18h period of incubation. This observation again suggests that the processes depicted in Fig. 2(*a*) are associated with the binding of 1,10-phenanthroline to enzyme-bound zinc atoms. On this basis Fig. 2(*a*) indicates that formation of the enzyme–coenzyme complex makes at least some of the zinc atoms more easily accessible to 1,10-phenanthroline. Formation of the ternary complex on the other hand protects these zinc atoms. For horse liver alcohol dehydrogenase it has been suggested that the substrate binds to the catalytic zinc atoms as a vital step in the overall mechanism. There has been no direct evidence for the catalytic role of zinc atoms in yeast alcohol dehydrogenase. The results presented in Fig. 2(*a*) indicate that at least some zinc atoms may be close enough to the substrate

in ternary complexes to fulfil a catalytic role. Facilitated access to the zinc atoms in the enzyme–coenzyme complexes would support this idea, and further evidence is that there appear to be significant conformational changes in the enzyme on formation of the enzyme–coenzyme complexes (Hvidt *et al.*, 1963; Dickinson, 1971). For horse liver alcohol dehydrogenase it is thought that, in the ternary complex with pyrazole and NAD^+, the pyrazole forms a direct link with the catalytic zinc atom (Brändén *et al.*, 1975). If this also occurs with the yeast enzyme it would explain the great protection afforded by this ligand in the ternary complex.

Attempts to re-activate the enzyme–coenzyme mixtures led to further information of interest. For the mixtures containing NAD^+, NAD^++pyrazole and NADH+acetamide, removal of the 1,10-phenanthroline (and other components of the incubation mixture) resulted in substantial, and in some cases complete, re-activation when assayed by 20-fold dilution in 0.1M-phosphate buffer, pH7.2, with a 10μl portion taken for immediate assay. The data are presented in Fig. 2(*b*). The results obtained from the mixture containing NADH were rather different. There was a substantial recovery of activity on removal of the 1,10-phenanthroline, but the enzyme had only about one-quarter of the activity of the native enzyme. In the presence of NADH, 1,10-phenanthroline brought about an inactivation of the enzyme that could not be reversed simply by removing the reagent. If, as an alternative, re-activation of incubation mixtures was attempted by dilution into 0.1M-Tris/HCl buffer (pH7.2)/0.5mM-$ZnSO_4$ and immediate assay, full re-activation occurred within the period required for the manipulations (1min). The change brought about in the incubations containing NADH and recorded in Fig. 2(*b*) is clearly reversible by Zn^{2+} ions. [If $(NH_4)_2SO_4$ replaced $ZnSO_4$ in the dilutions, no detectable recovery of activity took place.]

The results of Fig. 2 confirm that the slow inactivations recorded in Fig. 1 are produced by a combination of a slowly established reversible process and an even slower irreversible change. Both processes are clearly distinguished from the rapid (on the present time-scale, instantaneous) reversible binding by 1,10-phenanthroline which is observed when assay mixtures contain the reagent (Williams *et al.*, 1958). Coenzyme effectively stops the irreversible inactivation of the enzyme. Ultracentrifugal analysis at 4°C of samples of enzyme obtained after 25h incubation at 0°C with 1,10-phenanthroline and NAD^+ or 1,10-phenanthroline and NADH and passage through Sephadex G-25 (Fig. 2*b*) showed that enzyme sedimented as a single species with $s_{20,w} = 7.6S$. The coenzymes effectively prevent the depolymerization of the enzyme that is mediated by 1,10-phenanthroline. The result with NAD^+ confirms the finding of Kägi & Vallee (1960). Ultracentrifugal analysis was not performed on the 25h samples from the incubation mixtures containing NAD^++pyrazole and NADH+acetamide, but the retention of activity of the enzyme on removal of the reagents suggests that the quaternary structure of the enzyme was unchanged.

The suggestion has been put forward that the changes in enzyme activity recorded here are due to attack of 1,10-phenanthroline on certain zinc atoms (possibly catalytic zinc atoms) in the enzyme. It is important to know if the zinc content of the enzyme varies on incubation with 1,10-phenanthroline in the presence and absence of coenzyme. Information on this point was obtained by incubating the enzyme with 1,10-phenanthroline under the conditions of Figs. 1(*a*), 1(*b*) and 2(*a*). At selected times the incubation mixtures were passed through Sephadex G-25 to remove the reagents from the enzyme, and samples of enzyme were taken for zinc analysis. As shown in Table 1, the zinc content of freshly prepared enzyme was decreased significantly over a 25h incubation period on incubation with 3.3mM-1,10-phenanthroline. On the other hand, when the enzyme was incubated with 180μM-NADH+0.5M-acetamide, 5.5mM-NAD^+ or 5.5mM-NAD^++10mM-pyrazole the zinc content remained unchanged. These results may be correlated with the fact that the enzyme became partially dissociated into subunits by treatment with 1,10-phenanthroline, but was protected by NAD^+, NAD^++pyrazole and NADH+acetamide (see above). It is clear that the slowly established but reversible inhibition by 1,10-phenanthroline in these cases is not due to removal of zinc.

The incubation mixture containing 180μM-NADH gave the surprising result (Table 1) that approx. 2 g-atom of zinc/mol was removed in the first 2h of the incubation, and thereafter the zinc content of the enzyme remained at approx. 4g-atom of zinc/mol. The loss of zinc paralleled the loss of recoverable activity noted in the incubations containing NADH in Fig. 2(*b*) on removal of 1,10-phenanthroline, but which was recoverable on adding Zn^{2+} ions. In connexion with these results it is noteworthy that our preparations of enzyme display about 2 NADH-binding sites/molecule (Dickinson, 1974) and that the removal of the 2 atoms of zinc/molecule did not result in dissociation of the enzyme, as judged by the ultracentrifugal analysis described above.

These results are consistent with a catalytic role for Zn^{2+} ions and show that treatment of the enzyme with 1,10-phenanthroline in the presence of an excess of NADH results in the formation of a modified but active enzyme. This was confirmed by the observation that the treated enzyme was inhibited by EDTA under conditions that do not affect native enzyme. Assay of dilutions in 0.1M-phosphate buffer, pH7.2, containing 0.1mM-EDTA showed a loss of 50% of

Table 1. *Changes in the zinc content of preparations of yeast alcohol dehydrogenase after incubation at pH 7.0 and 0°C with 3.3 mM-1,10-phenanthroline*

The experiments were performed as described in the text. Duplicate determinations generally agreed to within ±5%.

Source of enzyme ...	Zinc content (mol/mol)					
	Commercially obtained	Freshly prepared				
Additions to incubation mixture ...	None	None	180 μM-NADH	180 μM-NADH +0.5 M-acetamide	5.5 mM-NAD^+	5.5 mM-NAD^+ +1.0 mM-pyrazole
Time of incubation (h)						
0	6.4	6.5	6.2	6.1	5.8	6.5
1	6.0	6.0	3.8	6.4	6.0	6.5
2	4.8	5.9	3.6	6.3	6.0	—
4	4.0	5.8	4.0	6.1	6.1	6.4
6	3.5	5.3	3.9	5.8	5.6	—
25	1.8	4.0	3.8	5.6	5.9	6.5

the residual activity, with a complete recovery on addition of an excess of $ZnSO_4$. The modified enzyme was not sensitive to ADP-ribose and was thus different from the dimeric, apparently zinc-deficient, enzyme obtained from yeast grown without added zinc (Dickenson & Dickinson, 1976). The kinetic characteristics of the modified enzyme were rather similar to those of native enzyme. At pH 7.0 and 25°C the K_m values for NAD^+, NADH, ethanol and acetaldehyde were 175 μM, 60 μM, 29 mM and 0.5 mM respectively. The corresponding values for the native enzyme are 100 μM, 84 μM, 22 mM and 0.8 mM (Dickenson & Dickinson, 1975*a*).

The results of the incubation of 1,10-phenanthroline with fresh preparations of enzyme may be compared with those of Kägi & Vallee (1960) and Leskovac *et al.* (1976), who found that treatment of commercial enzyme preparations with 1,10-phenanthroline led to considerable loss of zinc. A repeat of the above experiment with commercial enzyme confirmed that in these preparations greater amounts of zinc were lost during a 25 h incubation period than was the case with freshly made enzyme. The loss of about 4 atoms of zinc/molecule resulted in total loss of activity (Table 1, Fig. 1*b*) and complete dissociation of the enzyme into subunits (see above). These results are in broad agreement with those of Kägi & Vallee (1960) and Leskovac *et al.* (1976). Also in agreement with Leskovac *et al.* (1976) is the additional fact that the totally inactive enzyme retained about 2 atoms of zinc/molecule, suggesting that the enzyme has two classes of zinc atoms, one easily removed by 1,10-phenanthroline and leading to loss of activity, and the other being resistant to removal.

The results with commercial and fresh preparations of alcohol dehydrogenase show that the behaviour of the enzyme towards 1,10-phenanthroline depends to a considerable extent on the source of the enzyme used. The reason for this is not clear, but it may be due to changes occurring in preparations of enzyme that have been stored for long periods of time. Bühner & Sund (1969) showed that storage leads to loss of activity and loss of thiol groups. If some of the zinc–protein bonds are through thiol groups (Brändén *et al.*, 1975), then the oxidation of such groups could well lead to increased accessibility to 1,10-phenanthroline and easier removal of zinc by this agent. The commercial preparations were less resistant to loss of activity, loss of zinc and to dissociation than the fresh preparations. They were also less active initially. These results are not presented so as to criticize the quality of commercial preparations of enzyme. It may well be that other preparations from the same source or from other suppliers are more stable than our fresh preparations. A detailed study of this question was not part of the present investigation. The point of presenting the results is simply to show that conflicting reports on the effects of 1,10-phenanthroline on the enzyme may well arise because of basic differences in what is apparently the same material. Most of the work described here has concerned the properties of the freshly prepared enzyme because these preparations have already been studied and characterized in great detail (Dickinson, 1974; Dickenson & Dickinson, 1975*a*,*b*,*c*, 1977).

There is an additional point relating to Table 1 that deserves comment. The data indicate that both freshly made and commercial enzyme preparations contain about 6 atoms of zinc/molecule. Early work on this enzyme indicated 4–5 atoms of zinc/molecule (Sund & Theorell, 1963), but it has been suggested from comparison of the primary sequences of horse liver and yeast alcohol dehydrogenases that there

should be 8 atoms of zinc/molecule (Brändén *et al.*, 1975). This suggestion has stimulated further work, with the result that Veillon & Sytkowski (1975) found 4 atoms of zinc/molecule and Klinman & Welsh (1976) found 8 atoms of zinc/molecule. Both groups apparently used enzyme of high specific activity comparable with the material used here. The reason for the discrepancy is not clear, but in the present work, using atomic-absorption spectrophotometry, it was found that the method was sensitive to the way samples were prepared. Zinc standards gave readings approx. 20% higher if dried and wet-ashed as described under 'Methods' rather than simply being made up in water. For this reason protein samples and standards used here were treated in exactly the same way.

The present results do not resolve the problem of the zinc content of the enzyme but do indicate a zinc content of $\geq$6.0 atom ($\geq$1.5 atom/subunit). According to Veillon & Sytkowski (1975), dialysis against EDTA removes all but the four essential zinc atoms. Unfortunately these experiments were not described in sufficient detail to repeat. However, in the present work, dialysis of freshly prepared enzyme against 20mM-EDTA in 0.1M-phosphate buffer, pH7.0, at 0°C for 24h left the zinc content of the preparation quite unchanged. Passage through Chelex 100 at pH7.0 gave a similar result. Dialysis at pH5.5 at 0°C did remove zinc, but there was also loss of activity and breakdown of quaternary structure. Klinman & Welsh (1976) report that EDTA removes 1 atom of zinc/molecule with total loss of activity when employed in dialysis at pH8.5.

The results given here suggest that the enzyme preparations must have undergone some degradation if in fact there should be 8 atoms of zinc/molecule in native enzyme. Whether or not the predictions of Brändén *et al.* (1975) are borne out will depend on the outcome of further work. The fact that the preparations exhibit only approx. two coenzyme-binding sites/molecule, instead of the four expected from the quaternary structure, may be connected with the loss of 2 atoms of zinc/molecule, or with the lack of these two metal atoms.

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