4. NADH protects the enzyme more strongly than NAD^+ from inactivation by iodoacetamide and iodoacetate. The binding constants for the coenzymes have been calculated from protection data.

5. Acetaldehyde enhances, and ethanol has no effect on, the inactivation of the enzyme by iodoacetamide and iodoacetate.

6. The results are consistent with the theory proposed by Rabin & Whitehead (1962) for the mode of action of this enzyme.

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The Reaction of Yeast Alcohol Dehydrogenase with Iodoacetamide as Determined with a Silver–Silver Iodide Electrode

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In the preceding paper (Whitehead & Rabin, 1964) the alkylation of yeast alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1 by iodoacetamide was followed indirectly by measurements of loss of activity. It was thought desirable to check the conclusions by measuring the reaction directly.

The silver–silver iodide electrode (Watts, Rabin & Crook, 1961) is very suitable for this purpose because of its sensitivity and because a continuous record of the production of I^- ions can be obtained. The kinetics of the reaction have been followed by this means and are described in the present paper.

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MATERIALS AND METHODS

Enzyme. This was prepared as described by Whitehead & Rabin (1964). It was dialysed overnight against phosphate buffer, pH 7·5 and I 0·01, before use. Its concentration was measured spectrophotometrically at 280 mμ by assuming an absorbancy index of 1·26 cm.²/mg. and a mol.wt. of 150000 (Hayes & Velick, 1954).

Iodoacetamide. This was recrystallized four times from 50% (v/v) ethanol. The product (m.p. 91°) was free from I^- ions and iodine.

Buffers. The pH values of the buffer solutions used were measured at the final dilutions employed in the experiments. Except in the experiments in which the effects of ionic strength were investigated, stock buffer solutions of ionic strength 0·1 were prepared by using HCl (AnalaR-grade, redistilled) and the following salts: sodium acetate

[AnalaR material (Hopkin and Williams, Chadwell Heath, Essex), used without further purification]; potassium phenol-*p*-sulphonate (highly purified anhydrous salt, given by Dr S. P. Datta); disodium malonate (anhydrous salt, recrystallized from aq. ethanol, given by Dr D. G. Herries); disodium pentane-3,3-dicarboxylate (anhydrous salt, recrystallized from aq. ethanol, given by Dr D. G. Herries).

Measurement of iodide concentration and initial rate of iodide production. A silver–silver iodide electrode coupled to a calomel reference electrode by a saturated potassium nitrate bridge was employed in the manner described by Watts *et al.* (1961). The temperature of the cell was controlled at 25°. The volume of the experimental solution was 4 ml. and the experiments were conducted in the presence of 0·02% Tween 80 and 1 μM-KI.

The recorder gave a continuous plot of log[I^-] as a function of time. The results were replotted as progress curves for the production of I^- ions, and the initial velocities obtained by drawing tangents to the curves at zero time.

Measurement of activity. This was carried out in pyrophosphate buffer, pH 9·0 and *I* 0·01, as described by Whitehead & Rabin (1964).

RESULTS

Some control experiments were first run to test the effects of Tween 80, potassium iodide and Ag^+ ions on the catalytic activity of the enzyme. No effects of 0·08% Tween 80 or 4 μM-potassium iodide could be detected on the progress curve for the production of NADH from NAD^+ (5 μM) and ethanol (0·06 M) at pH 9·0. Silver ions are known to inhibit the enzyme (Snodgrass, Vallee & Hoch, 1960) and, as shown in Fig. 1, the enzymic activity is lost when slightly more than one Ag^+ ion/mole of enzyme is present at pH 9·0. This very powerful interaction of the enzyme with Ag^+ ions restricts the application of the silver–silver iodide electrode. There is little difficulty in the region below pH 8·0, but above this pH interactions between the enzyme and the electrode could easily be detected by an apparent increase in the concentration of I^- ions on adding the enzyme to the electrode in the absence of iodoacetamide. The effect is simply due to dissolution of silver iodide from the electrode; the Ag^+ ions are bound to the protein and I^- ions appear in solution. Below pH 8·0 this effect is not observed, and reliable interpretable results can be obtained.

The effect of Ag^+ ions on the reaction of the enzyme with iodoacetamide at pH 9·0 was investigated by measuring the loss of catalytic activity. It is possible to do this because the inhibition by Ag^+ ions is instantaneous, in contrast with the inhibition by iodoacetamide. It was found that Ag^+ ions (1·67/mole of enzyme) increased the rate of destruction of the enzyme by iodoacetamide. No effect was observed when the Ag^+ ion:enzyme molar ratio was 0·62.

A progress curve for the reaction of the enzyme with iodoacetamide at pH 6·4 is shown in Fig. 2. The reaction is biphasic and becomes linear after 120 min. Extrapolation of the linear portion back to zero time gives 4·1 g.ions of I^- ion/mole of enzyme for the stoicheiometry of the initial reaction. The second-order rate constant for the production of I^- ions is 0·63 l.mole^{-1}sec.$^{-1}$ at pH 7·0. An identical value (0·65 l.mole^{-1}sec.$^{-1}$) was determined from measurements of loss of activity.

The effect of ionic strength on the reaction of the enzyme with iodoacetamide at pH 6·8 is shown in Fig. 3. The reaction is independent of ionic strength in the absence or presence of NADH.

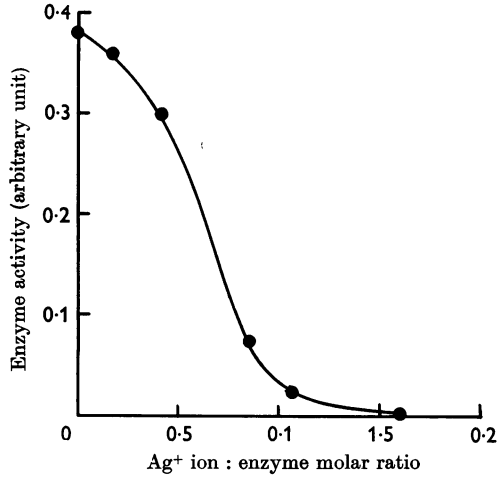

Fig. 1. Effect of Ag^+ ions on the activity of yeast alcohol dehydrogenase (3·26 μg./ml.) in 0·1 M-pyrophosphate buffer, pH 9·0, in the presence of ethanol (0·01 M) and NAD^+ (16·6 μM).

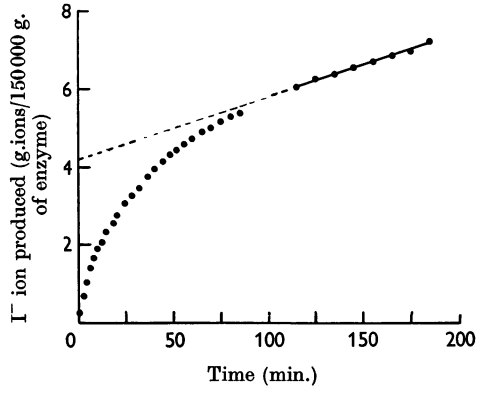

Fig. 2. Progress curve for the reaction of yeast alcohol dehydrogenase (2·5 mg.) with iodoacetamide (2 mM) in malonate buffer, pH 6·4 and *I* 0·05.

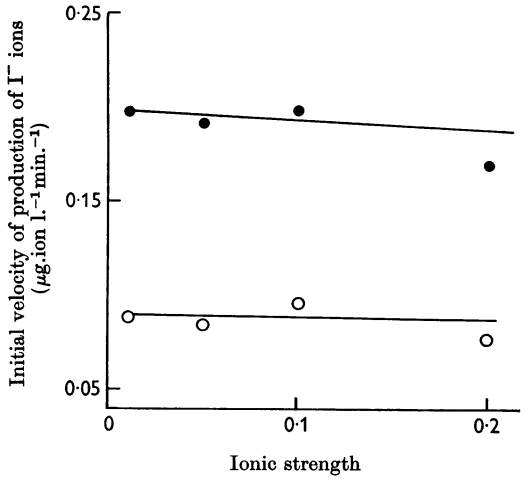

Fig. 3. Effect of ionic strength on the reaction of yeast alcohol dehydrogenase (1·74 mg.) with iodoacetamide (2 mM) in pentane-3,3-dicarboxylate buffer, pH 6·8. ●, No NADH added; ○, in the presence of 1 mM-NADH.

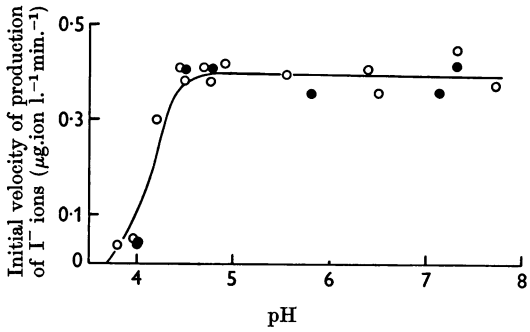

Fig. 4. Effect of pH on the reaction of yeast alcohol dehydrogenase (1·25 mg.) with iodoacetamide (2 mM) in the absence (○) and presence (●) of ethanol (0·33 M) at I 0·05. The following buffers were used in the pH ranges stated: pH 3·7–4·8, acetate; pH 5·5–6·4, malonate; pH 6·8–7·8, pentane-3,3-dicarboxylate. The pH values were measured immediately after the first 15 min. of the progress curves.

The effect of pH on the reaction of the enzyme with iodoacetamide in the absence and presence of ethanol is shown in Fig. 4. The reaction is independent of pH between 4·5 and 8. Between pH 8 and 9 I^- ions are produced by interaction of the enzyme with the electrode. It was possible to correct for this, and it was shown that the rate of reaction of the enzyme with iodoacetamide did not vary between these pH values.

The stoicheiometry of the initial reaction was the same at pH 9·0 as at pH 6·4, provided that corrections were made for the binding of Ag^+ ions

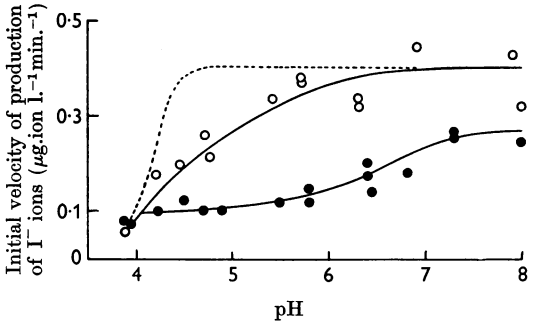

Fig. 5. Effect of coenzymes (1 mM) on the reaction of yeast alcohol dehydrogenase (1·25 mg.) with iodoacetamide (2 mM) at I 0·05. - - - - -, In the absence of coenzymes; ●, in the presence of NADH; ○, in the presence of NAD^+. Where different quantities of enzyme were used, controls without coenzyme were run and the velocities adjusted by simple proportion.

at the higher pH value. The effects of NAD^+ and NADH on the reaction of the enzyme with iodoacetamide are shown in Fig. 5.

In the presence of relatively high concentrations of sodium lauryl sulphate, a large number of groups on the alkylated protein molecule react with iodoacetamide, as shown in Fig. 6. Control experiments showed that the detergent had no direct effects on the electrode performance.

DISCUSSION

Yeast alcohol dehydrogenase is not particularly suitable for investigation with the silver–silver iodide electrode because it binds Ag^+ ions above pH 8·0. Nevertheless, valuable information can be obtained which is complementary to the results obtained by Whitehead & Rabin (1964).

Fig. 2 shows that one thiol group/active site (reactive thiol group) has unique properties as a nucleophilic reagent towards iodoacetamide, thus confirming the conclusions of Whitehead & Rabin (1964). Most of the thiol groups are unreactive but, as shown in Fig. 6, they become reactive in the presence of anionic detergents. The nature of the masked groups is not known but masking clearly depends on the integrity of the secondary and tertiary structure of the protein.

The results confirm that the rate of alkylation is independent of pH over the range 4–9. The identity of the values for the rate constants obtained by measurements of I^- ions produced and activity loss shows that the two methods measure the same chemical event. At pH values below 4 the rate of alkylation falls off. Spontaneous denaturation occurs in this region and loss of secondary and tertiary structure causes a decrease in nucleophilic

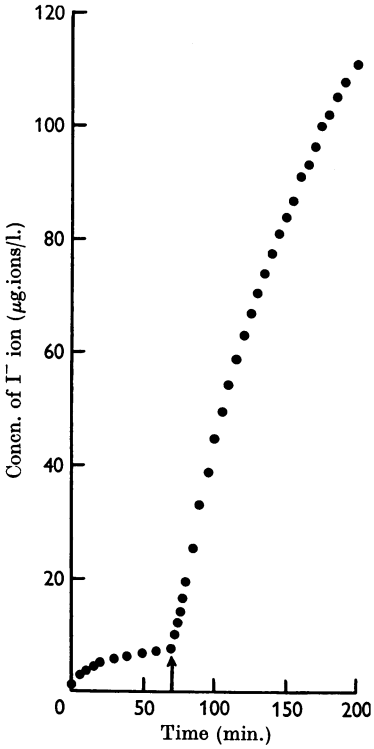

Fig. 6. Effect of anionic detergent on the reaction of yeast alcohol dehydrogenase (1·45 mg.) with iodoacetamide (4 mM) in malonate buffer, pH 6·37 and I 0·05. After 70 min. (denoted by the arrow) 20% (w/v) sodium lauryl sulphate (0·2 ml.) was added (final concn. approx. 1%).

reactivity of the reactive thiol groups. This is entirely consistent with the idea that the reactive thiol group consists of a mercaptan hydrogen-bonded to a base species and that this hydrogen-bonded pair dissociates on denaturation. At low pH values a free thiol group is produced which is unreactive towards the reagent. The absence of any effect of ionic strength is also consistent with the hydrogen-bond hypothesis.

In agreement with the experiments on activity loss, ethanol has no effect on the initial rate of the production of I^- ions. The effect of acetaldehyde could not be investigated because aldehydes reduce the silver iodide of the electrode. The small protection by NAD^+ and the greater protection by NADH confirm the results previously obtained. The fall-off in the protection by coenzymes with increasing pH above 5·0 needs to be interpreted with caution, owing to the possibility of artifacts associated with the binding of Ag^+ ions, which appears to interfere with the interaction between the reactive thiol groups and the coenzymes.

SUMMARY

1. The reaction of yeast alcohol dehydrogenase with iodoacetamide has been investigated by measuring the I^- ions produced by means of a silver–silver iodide electrode.

2. One thiol group/active site reacts rapidly with iodoacetamide. A large number of other groups react in the presence of high concentrations of anionic detergent.

3. The initial rate of reaction of the enzyme with iodoacetamide is independent of pH over the range 4–9. The rate falls off below pH 4.

4. NADH, and to a lesser extent NAD^+, protects the enzyme against iodoacetamide. Ethanol is without effect.

5. The enzyme is inhibited by very low concentrations of Ag^+ ions which labilize the protein towards iodoacetamide and interfere with the binding of coenzymes.

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