SUMMARY

1. 3-Acetoxyoestra-1,3,5(10),6-tetraen-17-one was catalytically reduced in an atmosphere of tritium–hydrogen gas to yield [6,7-$^3H_2$]oestrone acetate which, on hydrolysis, gave [6,7-$^3H_2$]-oestrone. In two experiments the radiochemical yields were 34·2 and 15·5% respectively. [6,7-$^3H_2$]-Oestrone of high specific activity (20·2 mc/mg. or 5·45 c/m-mole) was obtained in the second (3 c of tritium gas) run.

2. [6,7-$^3H_2$]Oestradiol-17β was prepared from [6,7-$^3H_2$]oestrone by reduction with sodium borohydride.

3. [6,7-$^3H_2$]Oestriol-16α,17β and [6,7-$^3H_2$]oestriol-16α,17α were prepared from [6,7-$^3H_2$]oestrone; the intermediate products of this reaction sequence, 3,17β-diacetoxy[6,7-$^3H_2$]oestra-1,3,5(10),16-tetraene and 3,17β-diacetoxy-16α,17α-epoxy[6,7-$^3H_2$]-oestra-1,3,5(10)-triene, were isolated and characterized.

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The Thiol Groups of Yeast Alcohol Dehydrogenase

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This and the next paper (Rabin, Ruiz Cruz, Watts & Whitehead, 1964) are concerned with attempts to delineate the role of thiol groups in the catalytic action of yeast alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1). Much of the existing knowledge of the thiol chemistry of this enzyme has been summarized by Wallenfels, Sund, Zarnitz, Malhotra & Fischer (1959) and Hoch & Vallee (1959). The enzyme molecule contains 36 cysteine residues (Wallenfels & Sund, 1959) and has four active sites (Hayes & Velick, 1954). It is inactivated by common agents that react with thiols and thus satisfies the operational criteria for a thiol enzyme. These criteria do not, of course, indicate whether the thiol groups have any role, direct or indirect, in catalysis.

Attempts to titrate the thiol groups of the enzyme in the presence and absence of substrates have given contradictory results. Barron & Levine (1952) found that the number of groups/molecule titratable with Ag^+ ions decreased in the presence of NAD^+ and ethanol. Wallenfels *et al.* (1959) found that all substrates decreased the number of such groups, but Hoch & Vallee (1959) found that the presence or absence of substrates made no difference to titrations with Ag^+ ions or *p*-chloromercuribenzoate.

The reactions of yeast alcohol dehydrogenase with alkylating reagents have not been systematically investigated. Since these reagents are generally more selective than Ag^+ ions or mercurials, an investigation of the effects of iodoacetamide and iodoacetate was undertaken.

MATERIALS AND METHODS

Enzyme. Alcohol dehydrogenase was extracted from baker's yeast (The Distillers Co. Ltd.) and purified by the method of Racker (1950). It was recrystallized several times with ammonium sulphate. The crystals were either

suspended in water at a concentration of 0·07 g./ml. and frozen, or dissolved in water at a concentration of about 0·03 g./ml. and frozen. The samples were stored frozen at −5°. The enzyme was stable for some months under these conditions and there was no diminution in enzyme activity as a result of alternate freezing and thawing. When required, a quantity of enzyme preparation was diluted to about 0·01 g./ml. in water and dialysed for 24 hr. against several changes of water. Any precipitate that formed was removed by centrifuging. The dialysed enzyme solution was not significantly inactivated in 24 hr. at room temperature and was stored at 0°. Sometimes, however, a fine white precipitate, which could be removed by centrifuging, did form after overnight storage of concentrated solutions at 0°.

The ammonium sulphate used in the enzyme purification was recrystallized from 1 mM-EDTA (disodium salt) and then from water. The acetone used was redistilled from potassium permanganate. Ion-free water (specific conductance 4 μmhos/cm.^{3} at 20°) was used throughout. It was obtained by passing distilled water through successive columns of De-Acidite FF, Zeo-Karb 225 and finally Bio-Deminrolit mixed-red resin.

Coenzymes. NAD^+ and NADH were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. They were assayed to be 99% pure by the manufacturers and used without further purification.

Ethanol. This was several times redistilled through a fractionating column.

Acetaldehyde. This was similarly redistilled. In some trial experiments unpurified commercial material was used.

Iodoacetamide. The commercial material was recrystallized twice from light petroleum (b.p. 80–100°).

Iodoacetic acid. This was a gift from Dr D. C. Watts. It had been recrystallized several times from water and once from light petroleum. It was white and the content of free iodide had been estimated to be less than 0·001%.

Buffers. Stock buffer solutions of ionic strength 0·1 were made for the purpose of studying the rates of inactivation of the enzyme as a function of pH at a constant ionic strength of 0·01. The stock solutions were diluted tenfold with water and the pH values measured at 25° with a Radiometer TTTlc pH-meter.

Pyrophosphate buffer was made with AnalaR $Na_4P_2O_7$ adjusted to the required pH with the calculated quantity of HCl. For amine buffers, commercial pyridine, piperidine and ethylenediamine were twice redistilled and added to the calculated amounts of HCl. Commercial *N*-ethylmorpholine was redistilled and crystallized as the hydrochloride; NaOH was added to the calculated amount of hydrochloride. Phosphate buffer was made from AnalaR KH_2PO_4 and Na_2HPO_4 as described by Datta & Grzybowski (1961). Phthalate buffer was made by adding standard NaOH to an AnalaR potassium hydrogen phthalate solution. Citrate buffers were prepared from AnalaR trisodium citrate and HCl.

The HCl used in the preparation of buffers was prepared from the redistilled acid.

Method of assay. Alcohol-dehydrogenase activity was assayed spectrophotometrically at 25° with a Cary model 14 recording spectrophotometer. Into a 3 ml. silica cell were pipetted 0·1 M-pyrophosphate buffer, pH 8·8, (2·5 ml.), 5 mM-NAD^+ (0·1 ml.) and 0·3 M-ethanol (0·1 ml.). An appropriate quantity of enzyme was pipetted into this solution to start the reaction and the increase of extinction at 340 mμ was recorded, the blank cell containing buffer only. The extinction increases at a nearly constan rate.

When assayed under the conditions of Racker (1950) the best enzyme preparations had activities of 160000–170000 Racker units/mg. A preparation of this activity was used in all kinetic experiments except for the series at pH 7·6. The enzyme could not be activated by preincubation with, or assay in the presence of, cysteine. The preparation used in the series of experiments at pH 7·6 had an activity of about 60000 units/mg. Titrations with *p*-chloromercuribenzoate were performed on a sample of enzyme several months old whose specific activity was not determined.

Kinetic experiments. Time-courses of enzyme inactivation under a variety of conditions were followed at 25°. Enzyme, buffer, etc. (total volume 1 ml.) were incubated in a glass stoppered tube. No precautions were taken to exclude air. The mixtures were assayed at predetermined times by pipetting small samples (0·01 or 0·025 ml.) directly into the assay cells containing buffer, ethanol and NAD^+. The dilution stops inactivation by alkylating agents.

Titrations with p-*chloromercuribenzoate.* These were carried out by a modification of the method of Boyer (1954). The experiments were performed by adding successive increments of enzyme solution (approx. 0·5 mg./ml.) to a solution of *p*-chloromercuribenzoate in 0·1 M-pyrophosphate buffer, pH 8·8. This enabled the number of thiol groups/molecule to be determined after the enzyme had reacted with iodoacetamide at concentrations comparable with those used in the kinetic experiments (0·1 mg./ml.). A Cary model 14 spectrophotometer was used, with a slide-wire giving full-scale deflexion for an extinction of 0·1. Each titration required about 40 min.

RESULTS

Kinetics of the reaction. The inactivation by 1·46 mM-iodoacetamide at pH 9·5 and 0·1 *I* (ethylenediamine buffer) was followed at several concentrations of enzyme. The absolute concentrations of enzyme in these experiments was not determined. The relative concentrations were known from the volume of the stock enzyme solution used in the final incubation mixture. From the progress curves of inactivations the initial rates of enzyme inactivation were determined. These are plotted as functions of enzyme concentration in Fig. 1: the relation is linear.

In most experiments the progress curves were plotted as the logarithm of remaining activity against time. In all cases the plots were linear, confirming that the reactions are first-order with respect to enzyme concentration (Figs. 6–9).

The effect of iodoacetamide concentration on the apparent first-order rate constants for inactivation of the enzyme, under similar conditions to the above-mentioned experiments, is shown in Fig. 2: the reaction is first-order with respect to iodoacetamide concentration over the range of concentration studied.

Number of thiol groups/molecule that react with iodoacetamide. The number of thiol groups in the unmodified enzyme molecule reacting with *p*-chloromercuribenzoate varies between 7 and 12.

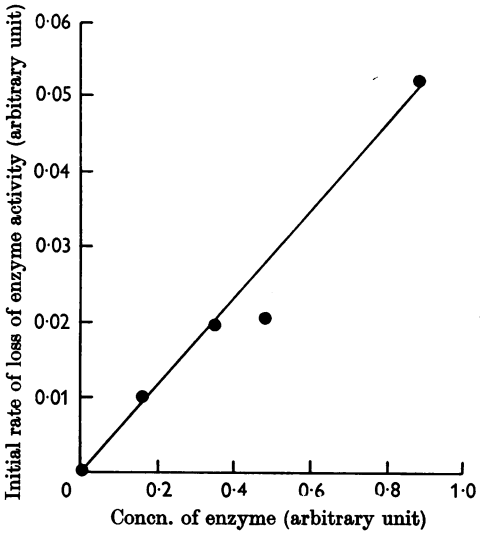

Fig. 1. Kinetics of reaction of yeast alcohol dehydrogenase with iodoacetamide (1·46 mM) at pH 9·5 and *I* 0·01 (ethylenediamine buffer).

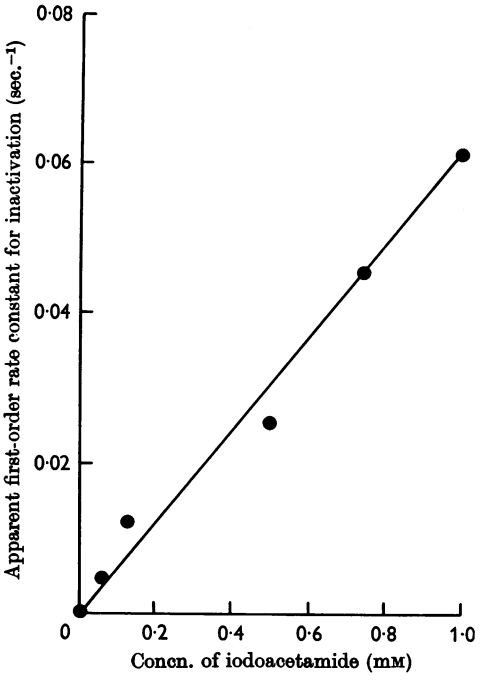

Fig. 2. Kinetics of reaction of yeast alcohol dehydrogenase with iodoacetamide at pH 9·5 and *I* 0·01 (ethylenediamine buffer).

However, the number of thiol groups/molecule that disappear after reaction with iodoacetamide is roughly constant. Enzyme was allowed to react with 1·5 mM-iodoacetamide at pH 9·0 and *I* 0·1 (pyrophosphate buffer) for 40 min., after which time over 95% of the enzyme activity was lost. Typical titrations of enzyme before and after reaction with iodoacetamide are shown in Figs. 3 and 4. The difference in slopes of the lines before and

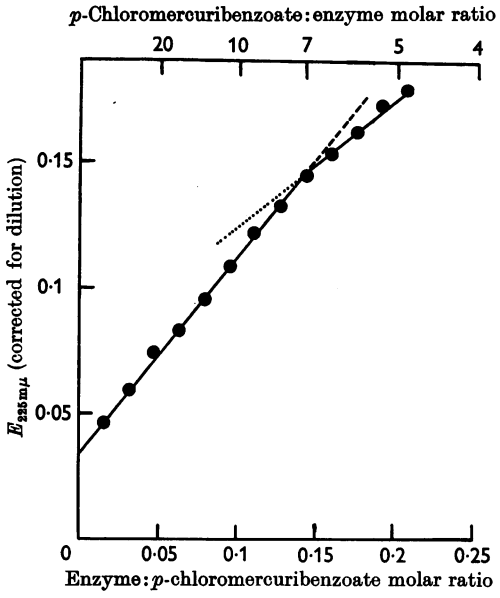

Fig. 3. Titration of thiol groups of yeast alcohol dehydrogenase with *p*-chloromercuribenzoate.

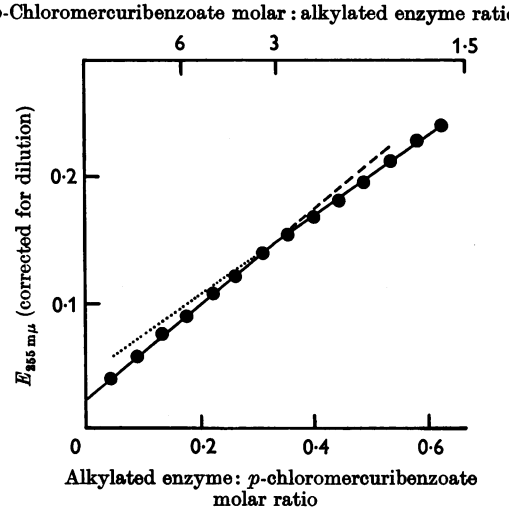

Fig. 4. Titration of thiol groups of alkylated yeast alcohol dehydrogenase with *p*-chloromercuribenzoate.

after the end points of the titration is less for the alkylated enzyme than for the unmodified enzyme because of light-absorption by I^- ions. In three series of experiments, one of which is illustrated in Figs. 3 and 4, the numbers of thiol groups/molecule that disappear on reaction with iodoacetamide were 4·0, 3·8 and 4·3. This is effectively 1/active site.

Effect of pH *on alkylation of the enzyme.* The enzyme was incubated with 1 mM-iodoacetamide in buffers with different pH values but a constant ionic strength of 0·01, and the disappearance of enzymic activity followed kinetically. At each pH a control experiment for spontaneous inactivation was carried out. Velocity constants for inactivation were determined from the slopes of logarithmic plots. Where the rate of inactivation of the enzyme in the absence of iodoacetamide was measurable, the velocity constants for this spontaneous process was subtracted from that obtained in the corresponding experiments with iodoacetamide.

It was felt that a possible cause of spontaneous inactivation was the presence in some of the buffers of trace metal ions, particularly Fe^{3+}, which are likely contaminants in commercial sodium hydroxide. Iron is known to be a catalyst of the oxidation of thiol groups, and inhibits the enzyme in low concentrations (0·1 μM-Fe^{3+} ion totally inhibited in one of the authors' experiments). The rates of inactivation in sodium glycinate and piperidine–glycinate buffers, both pH 10·0 and I 0·01, were accordingly determined. The same rates of inactivation were observed in these two buffers for both spontaneous and iodoacetamide-induced inactivation. The rates observed with ethylenediamine–hydrochloric acid buffer, pH 9·9 and I 0·01, were also very similar. It can be concluded that if trace-metal ions are present in this system they do not influence the results.

The rates of inactivation are shown as a function of pH in Fig. 5. The notable feature of the curve is that reactivity does not vary with pH over the entire range in which the enzyme is stable. At about pH 4 the rate of spontaneous inactivation of the enzyme increases extremely sharply as the pH decreases. Spontaneous rates are so high and so sensitive to pH that estimations of the rate of inactivation by iodoacetamide are unreliable. For this reason the estimates published by Rabin & Whitehead (1962) have no significance.

Effects of substrates and coenzymes. The progress curves for inactivation of the enzyme by 1·5 mM-iodoacetamide and 1·5 mM-iodoacetate, alone and in the presence of NAD^+, NADH, ethanol and acetaldehyde, are shown in Figs. 6–9, where the activity remaining is plotted logarithmically against time. The negative slopes of the straight lines drawn through the experimental points are proportional to the velocity constants. The rates of

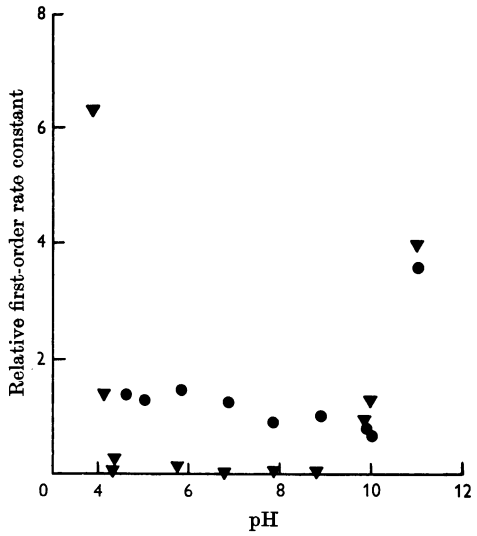

Fig. 5. Effect of pH on the inactivation of yeast alcohol dehydrogenase: ●, in the presence of iodoacetamide (1·5 mM); ▼, spontaneously. The rate constants are relative to that at pH 9·0 and I 0·01 in the presence of iodoacetamide which is taken as unity. The buffers used were: pH 3·88, citrate; pH 4·0, 4·35 and 4·39, phthalate; pH 5·05, pyridine; pH 5·8, phosphate; pH 6·85, ethylenediamine; pH 7·9, *N*-ethylmorpholine; pH 8·9, pyrophosphate; pH 9·9, ethylenediamine; pH 10·0, glycine; pH 11·0, piperidine.

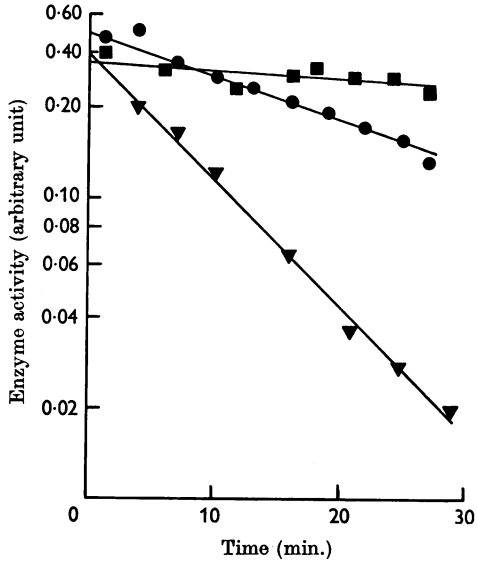

Fig. 6. Effect of coenzyme on the inactivation of yeast alcohol dehydrogenase by iodoacetamide (1·5 mM) at pH 7·9 and I 0·01 (*N*-ethylmorpholine buffer): ▼, no addition; ●, NAD^+ (1·35 mM); ■, NADH (1·12 mM).

spontaneous inactivation of enzyme in the presence of substrates have also been measured. The rate of spontaneous inactivation of the enzyme in the absence of added substrates was too low to be detected in the 30 min. period over which these kinetic experiments were performed.

Some other results obtained at pH 9·0 are given in Table 1. Both NAD^+ and NADH protect the enzyme against inactivation by iodoacetamide, NADH being more effective than NAD^+. Ethanol does not effect the rate of inactivation by iodoacetamide whereas acetaldehyde enhances it. When ethanol is added to the enzyme in the presence of NAD^+ at pH 9·0 the protection is

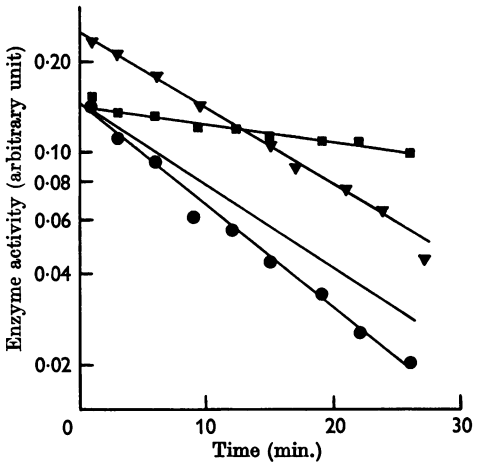

Fig. 7. Effect of ethanol on the inactivation of yeast alcohol dehydrogenase by iodoacetamide (1·5 mM) at pH 9·0 and I 0·01 (pyrophosphate buffer): ▼, 1·5 mM-iodoacetamide, no ethanol; ■, 0·16M-ethanol, no iodoacetamide; ●, 1·5 mM-iodoacetamide and 0·16M-ethanol. The line without experimental points corresponds to the inactivation by 1·5 mM-iodoacetamide in the presence of 0·16M-ethanol after correction for spontaneous inactivation due to the ethanol.

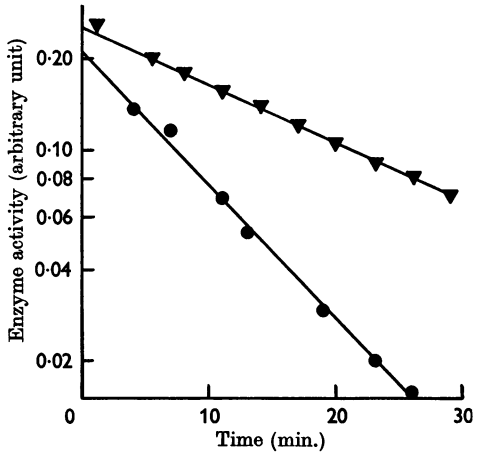

Fig. 8. Effect of acetaldehyde on the inactivation of yeast alcohol dehydrogenase by iodoacetamide (1·5 mM) at pH 9·0 and I 0·01 (pyrophosphate buffer): ▼, no acetaldehyde; ●, in the presence of 83·5 mM-acetaldehyde.

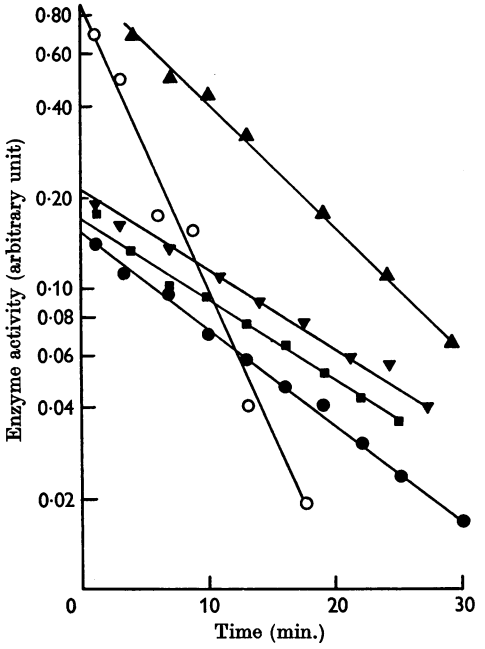

Fig. 9. Effect of acetaldehyde on the inactivation of yeast alcohol dehydrogenase by iodoacetate (1·5 mM) at pH 9·0 and I 0·01 (pyrophosphate buffer): ■, no acetaldehyde; ▼, 1·5 mM-acetaldehyde; ●, 7·5 mM-acetaldehyde; ▲, 15 mM-acetaldehyde; ○, 0·15M-acetaldehyde. The activities have been multiplied by 10 in the last two experiments for convenience of presentation.

Table 1. *Effect of substrates and coenzymes on the reaction of yeast alcohol dehydrogenase with iodoacetamide* (1·5 mM) *and iodoacetate* (1·5 mM) *at* pH 9·0 *and* I 0·01 (*pyrophosphate buffer*) *at* 25°

The rates are relative to the rate of alkylation in the absence of modifiers, which is taken as unity.

Alkylating agent	Addition	Relative rate of alkylation
Iodoacetamide	Ethanol (0·16M)	0·93
	Acetaldehyde (83·5 mM)	2·4
	NAD^+ (1·35 mM)	0·73
	NADH (1·12 mM)	0·21
	NADH (1·35 mM) (produced *in situ*)	0·40
Iodoacetate	Acetaldehyde (0·15M)	1·8
	NAD^+ (1·35 mM)	0·94
	NADH (1·12 mM)	0·01

greater than in the presence of NAD^+ alone, and the rate of inactivation is about the same as that in the presence of a solution of commercial NADH at the same concentration. The protection observed in these circumstances is, therefore, almost certainly due to the NADH produced *in situ*.

Impure acetaldehyde is more potent than purified acetaldehyde in enhancing the reactivity. When trial experiments were performed with impure commercial acetaldehyde the enzyme reactivity was enhanced at concentrations corresponding to 6 mM-acetaldehyde. When the acetaldehyde was purified by redistillation it did not affect the reactivity at these concentrations. It may be, however, that the effect observed with high concentrations of acetaldehyde is due to traces of these substances still present.

The effects of substrates on the reactivity of the enzyme towards iodoacetate have also been investigated and some results are given in Table 1. Protection of the thiol groups by NADH and enhancement of the reactivity of the enzyme by acetaldehyde are also observed in this reaction. NAD^+ in a concentration that gives some protection against inactivation by iodoacetamide has little or no effect on inactivation by iodoacetate.

To find if the different protective efficiencies of NAD^+ and NADH are due to differences in their binding constants, a series of experiments were performed in phosphate buffer, pH 7·6 and I 0·05, under which conditions the binding constants for the two coenzyme complexes have previously been determined (Hayes & Velick, 1954). Rate constants of inactivation at various concentrations of coenzyme were determined and the effects of concentration are shown in Figs. 10 and 11. If an enzyme–coenzyme complex is unreactive towards the reagent and the rate constant for reaction of the free enzyme with the reagent is $k_{max.}$, then the rate constant, k, for reaction at coenzyme concentration, s, will be given by:

$$k = \frac{k_{max.}}{1+s/K}$$

where K is the dissociation constant for the enzyme–coenzyme complex. Therefore:

$$\frac{1}{k} = \frac{1}{k_{max.}} + \frac{s}{k_{max.}K}$$

Plots of $1/k$ against coenzyme concentration gave straight lines, enabling dissociation constants to be calculated. The dissociation constants obtained were: for NADH, 55 μM; for NAD^+, 0·7 mM. These values are somewhat higher than, but comparable with, those reported by Hayes & Velick (1954). Their ratio is similar to that of the constants reported. It is concluded that the functional thiol groups of both the enzyme–NAD^+ and enzyme–NADH

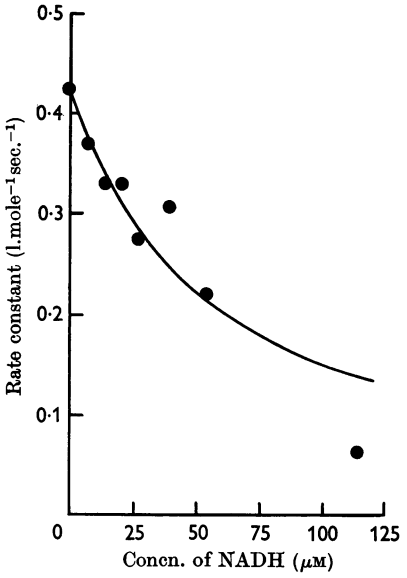

Fig. 10. Effect of NADH concentration on the second-order rate constant for the inactivation of yeast alcohol dehydrogenase by iodoacetamide (1·5 mM) at pH 7·6 and I 0·05 (phosphate buffer). The curve is theoretical for a dissociation constant of 55 μM.

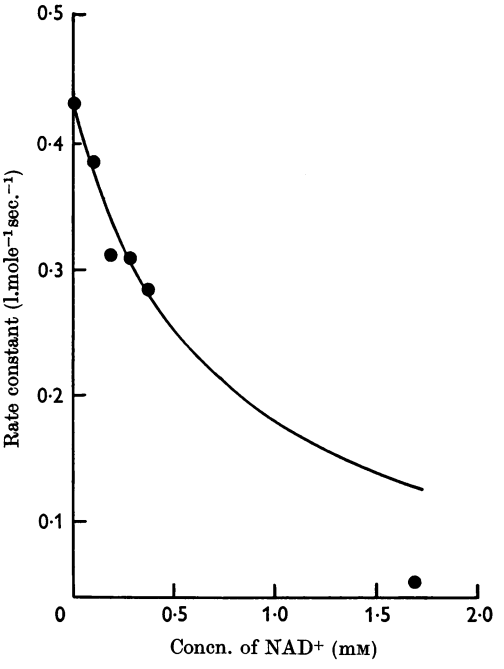

Fig. 11. Effect of NAD^+ concentration on the second-order rate constant for the inactivation of yeast alcohol dehydrogenase by iodoacetamide (1·5 mM) at pH 7·6 and I 0·05 (phosphate buffer). The curve is theoretical for a dissociation constant of 0·7 mM.

Table 2. *Effect of substrates on spontaneous inactivation at* pH 9·0 *and I* 0·01 (*pyrophosphate buffer*) *at* 25°

Substrate	First-order rate constant (sec.$^{-1}$)
None	0
NAD^+ (1·35 mM)	0
Acetaldehyde (all concentrations)	0
Ethanol (0·16 M)	$2·2 \times 10^{-4}$
NADH (1·12 mM)	$1·3 \times 10^{-4}$

complexes have little reactivity towards iodoacetamide.

Spontaneous inactivation is noticeable in the presence of NADH or ethanol, but is negligible in the presence of NAD^+ or acetaldehyde (Table 2).

DISCUSSION

Previous work on the thiol chemistry of yeast alcohol dehydrogenase has failed to yield much in the way of an understanding of the role of the thiol groups because the reagents reacted with a large number of these groups/molecule. It is clear from the work reported in the present paper that iodoacetamide has a high degree of selectivity, and it is a reasonable hypothesis that it reacts with only one thiol group/catalytic site. Complete proof of this requires analytical determination of the *S*-carboxymethylcysteine after the protein has reacted with iodoacetamide. The presence of a single thiol group/molecule essential for enzyme activity and distinguished from all others by its reactivity has been demonstrated for liver alcohol dehydrogenase (van Eys, Kretszychmar, Tseng & Cunningham, 1962), lactate dehydrogenase (Velick, 1958; Pfleiderer, Jeckel & Wieland, 1959), glyceraldehyde phosphate dehydrogenase (Szabolcsi & Elodi, 1958; Pihl & Lange, 1962) and microsomal cytochrome b_5 reductase (Strittmatter, 1959). This property may well be a general feature of the chemistry of the dehydrogenases.

The significance of the invariance of the reactivity of the thiol group with pH has been discussed elsewhere (Rabin & Watts, 1960; Watts & Rabin, 1962; Rabin & Whitehead, 1962). A plausible explanation is that the reactive thiol group is hydrogen-bonded, probably to an imidazole group. This would also account for the fact that the rate constant for the alkylation of the enzyme is very much lower than the constant for the reaction between mercaptide ions and iodoacetamide. Although the rate constant for the latter reaction has not been measured directly, it probably has a value of about 800 l.mole^{-1}sec.$^{-1}$ [estimated from data of Lindley (1960), Hellström (1931) and Dickens (1933)] compared with 0·4–0·65 l.mole^{-1}sec.$^{-1}$ for the reaction of yeast alcohol dehydrogenase with iodoacetamide. The reaction of the protonated form, the mercaptan species, with iodoacetamide is too slow to measure under the conditions used. Thus the enzyme thiol group has a reactivity between that expected for SH and S^-, as would be expected if it is hydrogen-bonded to the base form of another group on the protein.

It has been suggested (Rabin & Whitehead, 1962) that the reactive thiol group interacts with the nicotinamide or dihydronicotinamide ring of the coenzyme. This type of interaction has been convincingly demonstrated in liver alcohol dehydrogenase, where an analogue of NAD^+, with 5-hydroxyethyl-4-methylthiazole replacing nicotinamide, was covalently bound to the enzyme through a disulphide bond when the enzyme–coenzyme complex was oxidized with ferricyanide (van Eys *et al.* 1962). Electrostatic interaction between the thiol group and the nicotinamide (or dihydronicotinamide) ring would be greater for NAD^+ than NADH, whereas the latter is the more strongly bound. This possibility can therefore be discarded and the formation of a covalent adduct seems more likely. The only plausible sites for the addition of nucleophiles to the ring are positions 2 and 6. Addition to position 4 is possible for NAD^+ but cannot occur for NADH. Addition at the 2 position would be similar in some respects to the familiar reaction of thiols with the activated double bond of *N*-ethylmaleimide (Roberts & Rouser, 1958; Alexander, 1958; Dixon & Webb, 1958). It would also explain the specificity of the enzyme (and dehydrogenases in general) with respect to the substituent in the 3 position of the pyridine ring. All of the active coenzyme analogues (Kaplan, 1961; Kaplan & Ciotti, 1961) contain a 3-substituent with an electronegative atom double-bonded to the carbon atom attached to the 3 position. This double bond is conjugated to, and thus activates, the 2–3 double bond of the nicotinamide ring.

The results presented in the present paper are entirely consistent with the hypothesis already advanced for the mode of action of this enzyme (Rabin & Whitehead, 1962) and which provides a satisfactory basis for the design of further experiments.

SUMMARY

1. The reactions of yeast alcohol dehydrogenase with iodoacetamide, iodoacetate and *p*-chloromercuribenzoate have been investigated.

2. One thiol group/catalytic site reacts much faster than all the others with iodoacetamide.

3. The reaction of the enzyme with iodoacetamide is first-order with respect to the concentration of each component and independent of pH between pH 4 and 10.

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