The Enzymic Reduction of Nicotinamide-Adenine Dinucleotide by 2-Mercaptoethanol

BY R. F. LAMBE* AND D. C. WILLIAMS

The Research Department, The Marie Curie Memorial Foundation, Caterham, Surrey

(Received 22 March 1965)

1. The reduction of NAD⁺, by an enzyme preparation from rat liver, in the presence of 2-mercaptoethanol is reported. 2. It is suggested that the NAD⁺-linked alcohol dehydrogenase in the extract transfers hydrogen from 2-mercaptoethanol to NAD⁺. 3. Both yeast and horse-liver alcohol dehydrogenases were observed to reduce NAD⁺ in the presence of 2-mercaptoethanol. 4. Some interactions of 2-mercaptoethanol, cysteine or hydroxylamine with the alcohol dehydrogenases from rat liver, horse liver and yeast are discussed.

Thiols have been extensively used as stabilizing agents during the isolation and purification of enzymes that are dependent on the integrity of thiol groups for their activity. For the assay of many such enzymes *in vitro* the presence of a thiol in the assay medium is a requirement for maximum activity. Cysteine, reduced glutathione and 2-mercaptoethanol produce a marked activation of yeast aldehyde dehydrogenase (Black, 1955), and similar compounds are required for maximum activity of cathepsin C (de la Haba, Cammarata & Fruton, 1955). Tryptophanase is stabilized by the presence of thiols, and after spontaneous inactivation it may be reactivated by similar substances (Gunsalus, Galeener & Stamer, 1955).

Cutolo (1956) observed that in the presence of a yeast preparation NAD⁺ was quantitatively reduced by reduced lipoic acid. Cysteine and reduced glutathione were less effective. This effect was attributed to the presence in the yeast extract of a lipoate dehydrogenase. Macleod, Fridovich & Handler (1957) also observed these effects but suggested that they reflected the influence of thiols on the equilibrium of alcohol dehydrogenase. These authors demonstrated that with a preparation of crystalline alcohol dehydrogenase from yeast, which was devoid of lipoate-dehydrogenase activity, there was an increased reduction of NAD⁺ in the presence of dimercaptolipoate, cysteine and hydroxylamine.

During a study of the NAD⁺-linked 3 α -hydroxy steroid dehydrogenase of normal rat liver (Lambe & Williams, 1965) it was observed that the addition of 2-mercaptoethanol to the assay medium caused an increase in the rate of reduction of NAD⁺ by the partly purified extract. Because of the widespread use of 2-mercaptoethanol in enzyme preparations and in view of the results of Cutolo

(1956) and Macleod *et al.* (1957), it was decided to examine this effect in more detail. In the present work we have studied the effect of 2-mercaptoethanol and a number of related compounds on the reduction of NAD⁺ by a partly purified rat-liver preparation and by crystalline preparations of alcohol dehydrogenase from both yeast and horse liver.

MATERIALS AND METHODS

Materials. NAD⁺ and NADP⁺ (monosodium salt) were obtained from C. F. Boehringer and Soehne G.m.b.H., Mannheim, Germany. Androsterone was from Mann Research Laboratories, New York, N.Y., U.S.A. 2,3-Dimercaptopropanol (BAL), sodium thioglycollate, cysteine, 2-chloroethanol, 2-ethoxyethanol and ethanolamine were from British Drug Houses Ltd., Poole, Dorset. Homocysteine was prepared from homocysteine thiolactone hydrochloride (obtained from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.) by dissolving it in 10 mM-tris-HCl buffer, pH 7.5. Hydroxylamine hydrochloride was from May and Baker Ltd., Dagenham, Essex; it was neutralized before addition to the enzyme solutions. 2-Mercaptoethanol was obtained from B. Newton Maine Ltd., North Walsham, Norfolk, and was redistilled before use. α -Thioglycerol, glutathione (reduced), 2-mercaptoethylamine and crystalline alcohol dehydrogenase (from both yeast and horse liver) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Preparation of enzyme extract. A partially purified enzyme extract was obtained from a homogenate of normal rat liver essentially as described by Lambe & Williams (1965). The protein fraction that was precipitated between 50% and 80% saturation with (NH₄)₂SO₄ was dialysed against 10 mM-tris-HCl buffer, pH 7.5, and applied to a column of Sephadex G-25. Elution was effected with the same buffer and the enzyme solution was stored at -10°.

Enzyme assay. Cuvettes were prepared by adding the following ingredients: cuvette 1, 1.9 ml. of tris-HCl buffer, pH 8 (0.1M); cuvette 2, 1.8 ml. of tris-HCl buffer, pH 8 (0.1M), and 0.1 ml. of a solution of 1 μ mole of NAD⁺ in the same buffer; cuvette 3, 1.7 ml. of tris-HCl buffer, pH 8 (0.1M), 0.1 ml. of NAD⁺ solution and 1.0 μ mole of the test

* Present address: Department of Social Medicine, Trinity College, Dublin, Irish Republic.

compound in 0.1ml. of the same buffer. The reaction was started by the addition of 0.1ml. of enzyme solution to each cuvette and rapidly mixing the contents. Cuvette 3 was duplicated in all experiments and the reduction rate of NAD^+ was estimated spectrophotometrically by measuring the initial linear increase in extinction at $340\text{ m}\mu$ in a Hilger-Gilford recording spectrophotometer. Each cuvette was scanned for 5 sec. at 20 sec. intervals. One unit of enzyme activity represents a change of extinction of 0.001/min.

In experiments involving the partially purified rat-liver extract the reaction was initiated by the addition of 0.1ml. of the enzyme preparation (containing 2.5–3.0 mg. of protein) immediately before assay. Cuvettes 1 and 2 served as blank and control respectively. Crystalline alcohol dehydrogenase, from yeast or horse liver, was dissolved in 10 mM-tris-HCl buffer, pH 7.5, dialysed against two changes of the same buffer, and diluted to give the required concentration. Protein concentrations (mg./ml.) were calculated from the formula: $1.5 E_{280} - 0.75 E_{260}$.

RESULTS

The following compounds were tested in the above test system: 2-mercaptoethanol, 2,3-dimercaptopropanol, 2-mercaptoethylamine, α -thioglycerol, thioglycollate, cysteine, homocysteine, reduced glutathione, 2-chloroethanol, 2-ethoxyethanol and ethanolamine. Of these compounds only 2-mercaptoethanol, and to a smaller extent 2-chloroethanol, stimulated the reduction of NAD^+ by the liver extract. Results obtained with the two active compounds are shown in Fig. 1. The

Fig. 1. Reduction of coenzymes by the rat-liver preparation in the presence of 2-mercaptoethanol and 2-chloroethanol. Each cuvette contained tris-HCl buffer, pH 8.0 (200 μ moles), and enzyme solution (0.2 ml., containing approx. 3.0 mg. of protein) in a total volume of 2.0 ml. The following ingredients were also present (1.0 μ mole of each): A, NAD^+ + 2-mercaptoethanol; B, NAD^+ + 2-chloroethanol; C, NADP^+ + 2-mercaptoethanol; D, NAD^+ + 2-mercaptoethanol, with enzyme solution previously heated at 70° for 5 min.

result of substitution of NADP^+ for NAD^+ in the reactions mixture is shown in Fig. 1, as is also the effect of heating the enzyme extract at 70° for 5 min. Neither curve differs from that of the control reaction.

The rat-liver extract used in these experiments contained considerable NAD^+ -specific alcohol-dehydrogenase activity. A direct comparison between this activity and the reduction of NAD^+ in the presence of 2-mercaptoethanol by the same extract at various pH values is shown in Fig. 2. The pH-activity curves are similar, although ethanol produced a more rapid reduction of NAD^+ than did an equivalent concentration of 2-mercaptoethanol at all indicated pH values. The rat-liver alcohol dehydrogenase was inhibited by 2-mercaptoethanol: under the standard conditions the presence of 0.5 mM-, 0.1 mM- and 0.05 mM-2-mercaptoethanol gave rise to inhibitions of 85, 55 and 35% respectively. 2-Mercaptoethanol produced a similar inhibition of crystalline horse-liver alcohol dehydrogenase under similar experimental conditions, but yeast alcohol dehydrogenase was not inhibited by this substance (Fig. 3). However, when 2-mercapto-

Fig. 2. pH-activity curves for the reduction of NAD^+ by the rat-liver preparation in the presence of ethanol and 2-mercaptoethanol. Reaction cuvettes contained tris-HCl buffer of the specified pH (200 μ moles), NAD^+ (1.0 μ mole), enzyme solution (0.2 ml., containing approx. 3.0 mg. of protein) and either ethanol (■) or 2-mercaptoethanol (○) (1.0 μ mole) in a total volume of 2.0 ml.

ethanol was substituted for ethanol in the assay of horse-liver alcohol dehydrogenase a slow reduction of NAD⁺ was observed.

The effects of hydroxylamine on the rate of reduction of NAD⁺ by the rat-liver extract in the presence of both ethanol and 2-mercaptoethanol are illustrated in Fig. 4. Both reactions were inhibited, hydroxylamine being a more effective inhibitor of the alcohol-dehydrogenase system. We have confirmed the observations of Kaplan & Ciotti (1953) that hydroxylamine is a potent inhibitor of horse-liver alcohol dehydrogenase (Fig. 5). These workers reported that hydroxylamine also inhibits yeast alcohol dehydrogenase but, in agreement with the findings of Macleod *et al.* (1957), we find that the activity of this enzyme is enhanced by hydroxylamine (Fig. 5). The addition of cysteine to the yeast alcohol-dehydrogenase system produced a marked increase in the rate of reduction of NAD⁺, which agrees with the observations of Macleod *et al.* (1957). However, when this substance was added to horse-liver alcohol dehydrogenase there was no significant change in the reaction rate.

Fig. 3. Effect of 2-mercaptoethanol on yeast and horse-liver alcohol dehydrogenases. Each reaction mixture contained tris-HCl buffer, pH 8.0 (200 μ moles), and NAD⁺ (0.5 μ mole) in a total volume of 2.0 ml. The following ingredients were also present: A, horse-liver alcohol dehydrogenase (100 μ g.) + 2-mercaptoethanol (0.80 μ mole); B, yeast alcohol dehydrogenase (25 μ g.) + 2-mercaptoethanol (0.80 μ mole); C, horse-liver alcohol dehydrogenase (100 μ g.) + ethanol (0.80 μ mole); D, as for C, but also containing 2-mercaptoethanol (0.80 μ mole); E, yeast alcohol dehydrogenase (25 μ g.) + ethanol (0.80 μ mole); F, as for E, but also containing 2-mercaptoethanol (0.80 μ mole).

DISCUSSION

The enzymic nature of the reaction is indicated by the fact that the activity can be destroyed by heating the rat-liver extract. 2-Mercaptoethanol appears to be specific for the reduction of NAD⁺, and has no effect on NADP⁺; in this respect the reaction resembles the NAD-linked alcohol dehydrogenase that is also present in the crude extract. Since the pH-activity curves for both systems over the pH range 6.0–9.0 are similar, it seems likely that 2-mercaptoethanol was being oxidized by the liver alcohol dehydrogenase. The fact that 2-mercaptoethanol inhibits the oxidation of ethanol by the rat-liver preparation indicates a strong binding of the less reactive thiol to the active centres of the enzyme. This appears to be so also with the horse-liver alcohol dehydrogenase, where 2-mercaptoethanol, which is a strong inhibitor of enzyme, is itself slowly oxidized by the enzyme in the presence of NAD⁺. It is unlikely that this oxidation was due to contaminating amounts of

Fig. 4. Inhibitory effect of hydroxylamine on the reduction of NAD⁺ by the rat-liver preparation. Each cuvette contained tris-HCl buffer, pH 8.0 (200 μ moles), NAD⁺ (0.8 μ mole) and enzyme solution (0.2 ml., containing approx. 3.0 mg. of protein) in a total volume of 2.0 ml. The following ingredients were also present (1.0 μ mole of each): A, ethanol; B, ethanol + hydroxylamine; C, 2-mercaptoethanol; D, 2-mercaptoethanol + hydroxylamine.

Fig. 5. Effect of hydroxylamine on yeast and horse-liver alcohol dehydrogenases. Each reaction mixture contained tris-HCl buffer, pH 8.0 (200 μ moles), and NAD⁺ (0.6 μ mole) in a total volume of 2.0 ml. The following ingredients were also present: A, yeast alcohol dehydrogenase (10 μ g.) + ethanol (0.8 μ mole); B, as for A, but also containing hydroxylamine (10 μ moles); C, horse-liver alcohol dehydrogenase (100 μ g.) + ethanol (0.8 μ mole); D, as for C, but also containing hydroxylamine (10 μ moles).

ethanol in the 2-mercaptoethanol (which had been redistilled before use), since 2-mercaptoethanol is a potent inhibitor of the oxidation of ethanol by this enzyme. The relative inhibitions by hydroxylamine of the reduction of NAD⁺ by the rat-liver extract in the presence of either ethanol or 2-mercaptoethanol is consistent with these observations. The concentration of 2-mercaptoethanol appears to decrease as the reduction of NAD⁺ proceeds, but we have been unable to establish a quantitative relationship between these facts. The chemical determination of thiol groups with *p*-chloromercuribenzoate (Boyer, 1954) is not sufficiently sensitive, owing presumably to the slow reaction rate of the system. A polarographic method (Trkal, 1956) showed some decrease in thiol groups during the reaction, but no quantitative results could be obtained because monomercapto alcohols give a polarographic wave which is masked by that of NH₄⁺.

The differing behaviour of yeast and horse-liver alcohol dehydrogenases when they are treated with cysteine and hydroxylamine is of interest in the light of the results of Kaplan & Ciotti (1953) and Macleod *et al.* (1957). The former workers report that the inhibition of yeast alcohol dehydrogenase by hydroxylamine is overcome by the addition of excess of ethanol, whereas the latter report that

both hydroxylamine and cysteine increase the activity of this enzyme. We have confirmed the stimulation of yeast alcohol dehydrogenase by both substances. It was suggested by Macleod *et al.* (1957) that hydroxylamine and cysteine disturb the equilibrium of this enzyme by reacting with acetaldehyde to form acetaldoxime and 2-methylthiazolidine-4-carboxylic acid respectively. This now appears unlikely since such a mechanism would require that hydroxylamine and cysteine produce similar shifts in the equilibrium of horse-liver alcohol dehydrogenase. In fact, hydroxylamine is a potent inhibitor whereas cysteine has a negligible effect on activity.

Yeast alcohol dehydrogenase is considerably more sensitive than the horse-liver enzyme to substances that react with thiol groups (Kaplan & Ciotti, 1953). It has been observed by Wagner-Jauregg & Möller (1935) that the enzyme is readily oxidized by shaking in air and that it may be reactivated by reduced glutathione. The activity of yeast alcohol dehydrogenase has been reported to be directly proportional to the number of free thiol groups on the enzyme, aging resulting in a loss of those groups and a proportional loss of activity (Wallenfels & Lund, 1957). It appears likely therefore that the enhanced reduction of NAD⁺ by yeast alcohol dehydrogenase observed by Macleod *et al.* (1957) in the presence of cysteine is due to the liberation of essential thiol groups in the enzyme rather than to a non-enzymic reaction between cysteine and acetaldehyde. The fact that hydroxylamine stimulates yeast alcohol dehydrogenase activity, but is a strong inhibitor of horse-liver alcohol dehydrogenase, also suggests an interaction between this compound and the enzymes rather than a reaction with the acetaldehyde.

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