Mechanism of Inhibition of Aldehyde Dehydrogenase by Cyclopropanone Hydrate and the Mushroom Toxin Coprine†

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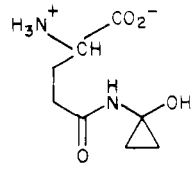
ABSTRACT: Coprine (I) is a novel natural product derived from cyclopropanone [Lindberg, P., Bergman, R., & Wickberg, B. (1977) *J. Chem. Soc., Perkin Trans. 1*, 684; Hatfield, G. M., & Schaumberg, J. P. (1975) *Lloydia 38*, 489]. Coprine inhibits mouse liver aldehyde dehydrogenase in vivo but not in vitro [Hatfield, G. M., & Schaumberg, J. P. (1975) *Lloydia 38*, 489; Tottmar, O., & Lindberg, P. (1977) *Acta Pharmacol. Toxicol. 40*, 476]. Cyclopropanone hydrate (III), which can be derived from coprine by hydrolysis, inhibits mouse liver aldehyde dehydrogenase both in vivo and in vitro. We conclude that coprine is activated in vivo by hydrolysis to cyclopropanone hydrate. The mechanism of inhibition by cyclopropanone hydrate was investigated with yeast aldehyde dehydrogenase. Inhibition of yeast aldehyde dehydrogenase by cyclopropanone hydrate is reversible, involves the active-site thiol, and shows saturation kinetics with respect to inhibitor concentration. We propose that the enzyme catalyzes the dehydration of cyclopropanone hydrate to cyclopropanone which then forms a kinetically stable thiohemiketal with the active-site thiol. This thiohemiketal would be analogous to the thiohemiacetal which is thought to be an obligatory intermediate in the enzyme-catalyzed oxidation of aldehydes. Cyclopropanone hydrate inhibits a number of enzymes which are sensitive to thiol reagents and may be useful in the design of active-site-directed enzyme inhibitors.

The *Coprinus atramentarius* mushroom has long been known to induce toxic reactions to ethanol (Josserand, 1952; Reynolds & Lowe, 1965). When ingested with alcohol, the mushroom retards the rate of ethanol metabolism and induces elevated levels of acetaldehyde in the blood (Coldwell et al., 1969). The active constituent responsible for this reaction has recently been identified as a novel derivative of glutamic acid, coprine (I),



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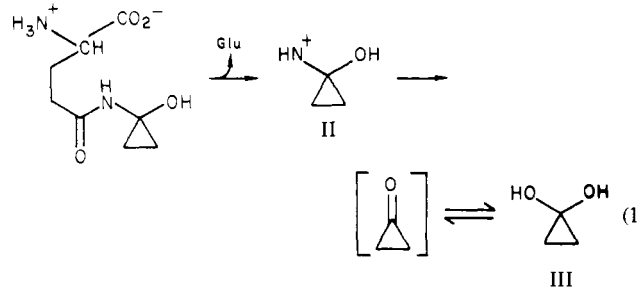
which is present at ca. 0.1% of the dry weight of the mushroom (Lindberg et al., 1977; Hatfield & Schaumberg, 1975). As far as we know, coprine is the first example of an enzyme inhibitor which contains a cyclopropyl ring at the oxidation state of cyclopropanone. Our interest in the design of active-site-directed enzyme inhibitors has led us to investigate the mechanism of action of this natural product.

It is believed that coprine inactivates aldehyde dehydrogenase (Hatfield & Schaumberg, 1975; Tottmar & Lindberg, 1977). Inhibition of aldehyde dehydrogenase would be predicted to cause all the observed effects of coprine on ethanol metabolism. A number of known inhibitors of aldehyde dehydrogenase, notably the drug antabuse [bis(diethylthiocarbonyl) disulfide], have the same effect on ethanol metabolism as coprine (Dietrich & Hellerman, 1963). Coprine, however, does not inhibit aldehyde dehydrogenase in vitro. It has been concluded on the basis of this latter result that coprine itself is not the inhibitor but that the active agent is probably a metabolite of coprine (Hatfield & Schaumberg, 1975; Tottmar & Lindberg, 1977). It is very likely that the metabolism of coprine involves hydrolysis to release cyclopropanone hemiaminal (II, eq 1). The hemiaminal eliminates



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ammonia to form cyclopropanone hydrate (III). This elimination is slow[1] (we have determined $t_{1/2}$ = 30 min at 27 °C, pH 7.4).

It was recently reported that cyclopropanone hemiaminal mimics the effects of coprine on ethanol metabolism and, in addition, inhibits aldehyde dehydrogenase in vitro (Tottmar & Lindberg, 1977). It was our experience, however, that cyclopropanone hemiaminal, which is prepared and stored as the hydrochloride salt, is unstable and slowly decomposes to chloroacetone. This impurity is a potent inhibitor of aldehyde dehydrogenase and is very difficult to remove completely from the hemiaminal. On the other hand, we have found that cyclopropanone hydrate also inhibits aldehyde dehydrogenase and has the advantage of stability. Accordingly, the inhibition of aldehyde dehydrogenase by cyclopropanone hydrate and its effect on ethanol metabolism are investigated herein.

Materials and Methods

Chemicals. NAD, NADH, iodoacetamide, phosphoenolpyruvate, ethyl benzoyltyrosinate, *N*-acetyl-L-cysteine, sodium pyruvate, and glucose were purchased from Sigma Chemical Co. NADP was purchased from P-L Biochemicals and creatine phosphate from Calbiochem. ATP, ADP, and sodium

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[1] Although cyclopropanone hydrate would be in equilibrium with cyclopropanone, the ketone is unstable due to the strain energy associated with constraining an unsaturated carbon atom into the geometry of a three-membered ring. This effect is reflected in the relatively slow rate of hydrolysis of cyclopropanone hemiaminal and in the fact that cyclopropanone forms stable adducts with nucleophiles; for example, only the hydrated species has been detected in aqueous solution (Wasserman et al., 1974).

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3-phosphoglycerate were obtained from Boehringer. Acetaldehyde, pyrazole, and 2-mercaptoethanol were purchased from Eastman Organic Chemicals and benzaldehyde from Aldrich. Glycerol was certified ACS grade from Fisher. *p*-Nitrophenyl carbobenzoxyglycinate was prepared as described by Bodanszky (1955) and coprine as described by Lindberg et al. (1977).

Cyclopropanone ethyl hemiketal (1-ethoxycyclopropanol) was a generous gift from Professor H. Wasserman. The ethyl hemiketal was hydrolyzed quantitatively to cyclopropanone hydrate by heating in water (unbuffered, pH <7) at 100 °C for 4 min. The conversion of the hemiketal to the hydrate could be monitored by NMR. The signal for the ring protons changes from a characteristic aa'bb' signal for the hemiketal to a singlet (δ 0.80 ppm) for the hydrate. Hydrate formation can also be monitored by high-pressure liquid chromatography. The hydrate elutes at 2.0 void volumes (see Figure 4) and the ethyl hemiketal at 5.1 void volumes on μC_{18}-Porasil (Waters Associates, Milford, MA, 0.4 × 30 cm) in water. The lack of impurities in cyclopropanone hydrate that might inhibit aldehyde dehydrogenase was demonstrated by a number of criteria. Cyclopropanone ethyl hemiketal inactivates aldehyde dehydrogenase. When the hemiketal is chromatographed by high-pressure liquid chromatography on Corasil II (Waters Associates, Milford, MA, 0.2 × 120 cm) in 1:1 chloroform–petroleum ether (bp 35–50 °C), the inhibitory activity comigrates with the peak for the ethyl hemiketal (detected by refractive index). When the ethyl hemiketal is hydrolyzed to cyclopropanone hydrate, the inhibitory activity which originally comigrated with the hemiketal on μC_{18}-Porasil/water now migrates with the hydrate. In addition, cyclopropanone hydrate decomposes in a base-catalyzed ring-opening reaction to give propionic acid (Wasserman et al., 1974); when incubated at pH 7.0 at 75 °C, the rate at which cyclopropanone hydrate loses inhibitory activity ($t_{1/2}$ = 28 min) is the same as the rate at which propionic acid is formed (as determined by NMR).

2-[^{3}H]Cyclopropanone ethyl hemiketal was prepared by the method of Turro and Hammond (1966). 2-[^{3}H]Cyclopropanone was prepared from ketene (Andreades & Carlson, 1973) and [^{3}H]diazomethane (Fales et al., 1973) of known specific activity (1.8 Ci/mol) and was converted to the ethyl hemiketal by addition of ethanol. The ethyl hemiketal, 4 mg, was purified by chromatography on silica gel (0.7 g, 0.3 × 10 cm, Brockman activity II) in 1:10 ether–methylene chloride. The ethyl hemiketal was hydrolyzed to cyclopropanone hydrate as described above and stored frozen in aqueous solution. This cyclopropanone hydrate was radiochemically pure by chromatography on μC_{18}-Porasil/water.

Radioactivity was measured in ACS scintillation fluid (Amersham) or in Bray's solution (New England Nuclear) in a Beckman LS-100C scintillation counter at 25 °C. Counting efficiency was determined relative to [^{14}C]- or [^{3}H]toluene standard (New England Nuclear).

Enzymes. Aldehyde dehydrogenase (yeast) was purified to homogeneity (Bradbury et al., 1975), specific activity 4.6 IU/mg. Aldehyde dehydrogenase (horse liver p*I* = 5 isozyme) was a generous gift from Professor H. Weiner. Alcohol dehydrogenase (yeast), pyruvate decarboxylase (yeast), glucose-6-phosphate dehydrogenase (yeast), papain, pyruvate kinase (rabbit muscle), and lactic dehydrogenase (rabbit muscle) were obtained from Sigma Chemical Co. Chymotrypsin was purchased from Worthington Biochemicals. Hexokinase (yeast), creatine kinase (rabbit muscle), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle), and 3-phosphoglycerate kinase (yeast) were purchased from Boehringer.

Mouse liver aldehyde dehydrogenase was prepared as follows. Livers were excised and homogenized in a glass homogenizer in (for each gram of liver) 5 mL of 0.25 M sucrose, 1 mM Tris-HCl, pH 7.3, 0.1 mM EDTA. The homogenate was centrifuged at 500*g* for 10 min at 0 °C and the pellet washed twice with 5 mL of the sucrose–Tris buffer. The combined supernatants were made up to 15 mL/g of liver and centrifuged first at 15000*g* for 5 min and then at 31000*g* for 20 min at 0 °C. The final supernatant contains 0.07 IU/mL of aldehyde dehydrogenase per milliliter.

Enzyme Assays and Inhibitions

Yeast aldehyde dehydrogenase, 0.01 IU or less, was assayed in 1 mL of buffer containing 30% glycerol, 0.1 M Tris-HCl, pH 8.0, 0.2 M KCl, 1 mM EDTA, 0.5 mM NAD, and 0.6 mM benzaldehyde by measuring the increase in absorbance at 340 nm (Bradbury et al., 1975).

Horse liver aldehyde dehydrogenase was inactivated by incubating 0.2 mL of enzyme, 0.003 IU, with 0.8 mL of an assay mixture containing 0.1 M sodium pyrophosphate, pH 9.3, 1 mM EDTA, and 0.5 mM NAD plus either 20 μL of 0.5 M cyclopropanone hydrate or 50 μL of 20 mM iodoacetamide at 25 °C. Aliquots, 0.2 mL, were added to 1 mL of buffer containing 0.1 M sodium pyrophosphate, pH 9.3, 1 mM EDTA, and 0.5 mM each NAD and benzaldehyde. Enzyme activity was determined as ΔA_{340}/min at ca. 5 min after mixing (there was a lag period before the enzyme reached full activity). Control enzyme was treated similarly but with inhibitor omitted.

Mouse liver aldehyde dehydrogenase was assayed by adding 0.1 mL of enzyme (ca. 0.007 IU) to 1 mL of assay buffer containing 0.1 M sodium pyrophosphate, pH 9.3, 1 mM EDTA, 0.5 mM NAD, and 20 mM pyrazole (to inhibit alcohol dehydrogenase, which interferes). A background rate of NADH formation was recorded, and 20 μL of 25 mM benzaldehyde was added to determine aldehyde dehydrogenase activity. The rate of formation of NADH determined by the increase in absorbance at 340 nm was shown to correspond to the rate of loss of benzaldehyde determined by a color reaction of aldehyde with 2,4-dinitrophenylhydrazine (Bohme & Winkler, 1954).

Alcohol dehydrogenase (yeast), 0.24 IU, was inhibited at 25 °C in 0.1 mL of 0.1 M Tris-HCl, pH 8.0, by addition of either 2 μL of 0.5 M cyclopropanone hydrate or 5 μL of 20 mM iodoacetamide (no inhibitor was added for control enzyme). Enzyme activity was determined (Bernt & Bergmeyer, 1974) periodically by adding 18 μL aliquots to 1 mL of assay buffer containing 0.1 M potassium phosphate, pH 7.0, 0.2 mM NADH, and 0.5 mM acetaldehyde and by following the change in absorbance at 340 nm.

Glyceraldehyde-3-phosphate dehydrogenase, 1.7 IU, was incubated at 25 °C in 0.1 mL of 0.1 M imidazole hydrochloride, pH 7.6, containing 1 mM EDTA and either 10 mM cyclopropanone hydrate, 1 mM iodoacetamide, or, as a control, no inhibitor. Aliquots, 18 μL, were assayed (Bergmeyer, 1974a) for loss of enzyme activity by addition to 1 mL of assay buffer containing 0.1 M imidazole hydrochloride, pH 7.6, 1 mM EDTA, 2 mM $MgCl_2$, 6 mM 3-phosphoglyceric acid, 1 mM ATP, and 0.2 mM NADH. 3-Phosphoglycerate kinase, 0.8 IU, was added to initiate the assay which was followed by a decrease in absorbance at 340 nm.

Pyruvate decarboxylase, 1.8 IU, was incubated with 10 mM cyclopropanone hydrate in 0.1 M potassium phosphate, pH 7.0, at 25 °C. Enzyme activity was assayed (Bergmeyer,

1974b) by addition of 18 μL of this solution to 1 mL of 0.2 M sodium citrate, pH 6.1, containing 30 mM pyruvate, 0.3 mM NADH, and 0.7 IU of yeast alcohol dehydrogenase. Activity was determined as the decrease in absorbance at 340 nm.

Creatine kinase, 0.4 IU, was incubated at 25 °C in 0.1 mL of 0.1 M sodium pyrophosphate, 1 mM EDTA, pH 9.3, without inhibitor (control) or with 10 mM cyclopropanone hydrate or 1 mM iodoacetamide. Aliquots, 18 μL, were assayed (Bergmeyer, 1974c) by addition to 1 mL of buffer containing 0.1 M imidazoleacetic acid, pH 7.0, 20 mM glucose, 10 mM magnesium acetate, 1 mM ADP, 0.8 mM NADP, 10 mM *N*-acetylcysteine, 20 mM creatine phosphate, and hexokinase (7.0 IU). The rate of inhibition by cyclopropanone hydrate was too fast to measure directly but was estimated from the equilibrium concentration of active enzyme and the rate of recovery of enzyme activity on dilution of inhibited enzyme into the assay buffer.

Pyruvate Kinase. Enzyme, 4 IU, was incubated at 25 °C in 20 μL of buffer containing 0.1 M sodium pyrophosphate, pH 8.85, 1 mM EDTA, and 10 mM cyclopropanone hydrate or 1 mM iodoacetamide or with inhibitor omitted (control); 2-μL aliquots were assayed (Gutmann & Bernt, 1974) by addition to 1 mL of 0.1 M Tris-HCl, pH 7.75, containing 0.1 M KCl, 1 mM EDTA, 0.2 mM NADH, 3 mM ADP, 1 mM phosphoenolpyruvate, 10 mM $MgCl_2$, and 36 IU of lactic dehydrogenase. The decrease in absorbance at 340 nm was followed.

Papain. Cyclopropanone hydrate (2 μL of a 0.5 M solution) or iodoacetamide (5 μL of a 20 mM solution) was added to 0.1 mL of 0.08 IU of papain in assay buffer (0.1 M potassium acetate, pH 5.5, 0.3 M KCl, 1 mM EDTA, and 0.5 mM 2-mercaptoethanol). Enzyme activity was measured (Westerik & Wolfenden, 1972) periodically by addition of 20-μL aliquots to 1 mL of assay buffer containing 0.2 mM *p*-nitrophenyl carbobenzoxyglycinate. The change in absorbance at 340 nm was followed. Control enzyme was incubated in assay buffer without inhibitor.

Chymotrypsin, 0.03 IU, in 50 μL of assay buffer (0.1 M Tris-HCl, 1 mM EDTA, pH 7.8) containing 10 mM cyclopropanone hydrate was incubated at 25 °C, and enzyme activity was measured (Hummel, 1959) after 40 min by addition of 0.95 mL of assay buffer and 40 μL of 10 mM ethyl benzoyltryosinate in 50% ethanol (change in absorbance at 256 nm was measured). The activity was compared to that of the control enzyme treated similarly but without inhibitor in the preincubation buffer.

Determination of Enzyme-Bound [^{14}C]Iodoacetamide. Reaction of radioactive [^{14}C]iodoacetamide with yeast aldehyde dehydrogenase was determined by a modification of the filter-binding assay for proteins described by Schaffner and Weissman (1973). Enzyme, 0.24 mg/mL, in 40 μL of 1.4 mM [^{14}C]iodoacetamide was added to 150 μL of quenching solution containing 0.2 M Tris-HCl, pH 7.5, 0.2% $NaDodSO_4$, 10% trichloroacetic acid, and 0.3 M 2-mercaptoethanol. After 10–30 min at 25 °C, the precipitated protein was applied to a millipore filter (GSWP, 0.22 μm pore size) by pipet such that the spot containing filter-bound protein was ca. 0.5 cm in diameter. The filter was washed twice with 10 mL of 5% trichloroacetic acid and then soaked, first for 10 min in 25 mL of acetic acid–methanol–water (2:2:1), then for 1 min in 100 mL of acetic acid–methanol–water (1:45:4), and finally for 0.5 min in 100 mL of water. Disks 1 cm in diameter containing filter-bound protein were cut out, dissolved in 1 mL of ethylene glycol monomethyl ether by swirling for 5 min, and then counted in 10 mL of Bray's solution. A control in which enzyme-bound [^{14}C]iodoacetamide was isolated by chromatography on Sephadex G-25 (medium) indicated that 100% of the protein was bound to the millipore filters.

Table I: Effects of Benzaldehyde on Rates of Inhibition of Yeast Aldehyde Dehydrogenase

reaction examined	NAD (mM)	K_m^{PhCHO} (μM)	rel rate[a] of inhibn
inhibn by cyclopropanone hydrate	0[b]	>25 000	1
	1.0[c]	9.8	0.057
oxidn of benzaldehyde[d]	1.0	9.0	

[a] Rate of inhibition at saturating concentrations of benzaldehyde relative to the rate in the absence of benzaldehyde. [b] Enzyme, 0.2 IU, was incubated at 25 °C in 100 μL of "low aldehyde" buffer (see Materials and Methods) containing 5 mM cyclopropanone hydrate and varying benzaldehyde concentrations. Aliquots, 15 μL, were assayed periodically for enzyme activity by addition to 1 mL of assay buffer (see Materials and Methods). [c] Enzyme, 0.007 IU, was incubated at 25 °C in 1 mL of buffer containing 30% glycerol, 0.1 M Tris-HCl, pH 8.0, 0.2 M KCl, 1 mM EDTA, 1 mM NAD, 10 mM cyclopropanone hydrate, and varying concentrations of benzaldehyde. The rate of inhibition was determined from the change in ΔA_{340}/min with time. [d] Enzyme was assayed as described under Materials and Methods.

Determination of Ethanol and Acetaldehyde in Blood. Mice were bled, 75–100 μL, from behind the eye (three to four blood samples per mouse could be collected). The blood was cooled to 0 °C in a stoppered tube and centrifuged for 2 min to pellet cells. Acetonitrile, 5 μL of 0.2 M solution, was added to 30 μL of the supernatant as an internal standard, and 5 μL of the resulting solution was assayed by gas chromatography (Baker et al., 1969) on Porapak Q (Waters Associates, 60–80 mesh, 0.6 × 130 cm) at 110 °C.

Low Aldehyde Buffer. Glycerol contains an impurity that is present at ca. 0.3 mM and is a substrate for yeast aldehyde dehydrogenase. This problem has been discussed in detail by Bradbury and Jakoby (1972). For those experiments in which it was necessary to determine the effects of NAD and benzaldehyde without interference from the impurity, "low aldehyde buffer" was used. Glycerol, 50 mL, was stirred with 50 mg of sodium borohydride at 25 °C until the borohydride dissolved and then distilled under vacuum (bp 140 °C). Low aldehyde buffer contained 0.1 M potassium phosphate, pH 8.0, 1 mM EDTA, and 30% by volume of this glycerol. The concentration of oxidizable impurities in this buffer was 8.5 μM. This buffer was used to determine the kinetic parameters for benzaldehyde (Table I). For those experiments in which NAD was also present (Table II), the concentration of impurities was further reduced. Addition of 4 μL of 2.5 mM NAD, 1 μL of 1 M 2-mercaptoethanol, and 0.22 IU of yeast aldehyde dehydrogenase (in 20 μL) to 1 mL of this buffer resulted in the formation of 8.5 nmol of NADH (determined by absorbance at 340 nm) after 15 h. The NADH generated in this assay was reoxidized to NAD within 30 min by the addition of 2 μL of 5.6 mM pyruvate and 0.7 IU of lactic dehydrogenase (in 2 μL).

Results

Effect of Cyclopropanone Hydrate on Ethanol Metabolism and on Mouse Liver Aldehyde Dehydrogenase. It has been shown (Hatfield & Schaumberg, 1975; Tottmar & Lindberg, 1977) that administration of coprine to mice decreases the rate of ethanol metabolism and increases blood acetaldehyde levels. We have confirmed this effect (results not shown) and have demonstrated that cyclopropanone hydrate produces similar results (Figure 1). When mice are injected with cyclo-

Table II: Effects of NAD on Rates of Inhibition of Yeast Aldehyde Dehydrogenase

reaction examined	benzaldehyde (mM)	K_m^{NAD} (μM)	rel rate[a] of inhibn
inhibn by cyclopropanone hydrate[b]	0	110	30
inhibn by iodoacetamide[c]	0	10	0.15
oxidn of benzaldehyde[d]	0.6	8.3	

[a] Rate of inhibition at saturating concentrations of NAD relative to the rate in the absence of NAD. [b] Enzyme, 0.2 IU, was incubated at 25 °C in 100 μL of "low aldehyde" buffer (see Materials and Methods) containing 1 mM cyclopropanone hydrate and varying NAD concentrations. Aliquots, 15 μL, were assayed periodically for enzyme activity by addition to 1 mL of assay buffer (see Materials and Methods). [c] As in footnote *b* but with 1 mM iodoacetamide instead of cyclopropanone hydrate. [d] Enzyme was assayed as described under Materials and Methods.

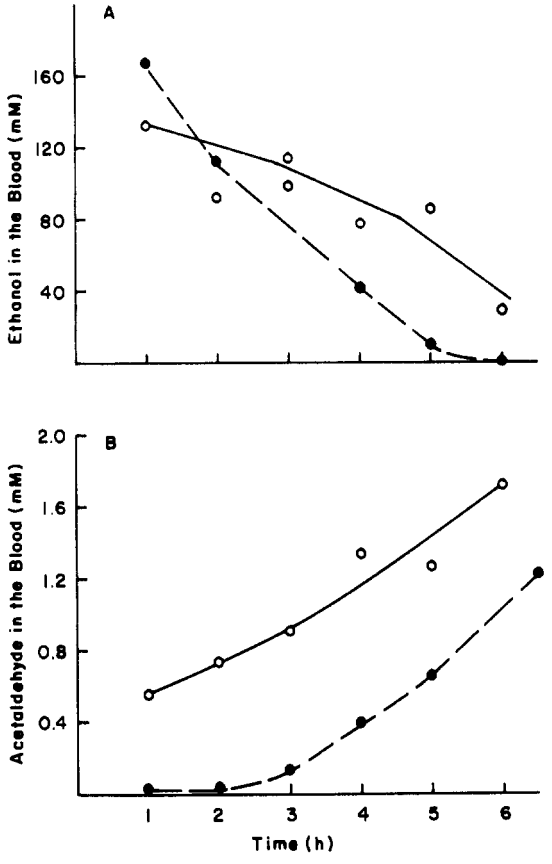


FIGURE 1: Inhibition of ethanol metabolism by cyclopropanone hydrate. Male Swiss albino mice, 12–18 g, were injected interperitoneally with 0.06 M cyclopropanone hydrate in water (17 mL, 1.0 mmol per kg mouse) and 3 h later with 4 M ethanol (17 mL, 67 mmol per kg mouse). Blood was withdrawn and assayed for ethanol and acetaldehyde (see Materials and Methods). (A) Decrease in blood ethanol levels. (B) Appearance of acetaldehyde in the blood. (○—○) Cyclopropanone hydrate treated mice; (●—●) control mice, injected only with ethanol.

propanone hydrate, 0.25 mmol/kg, and then ethanol, the rate at which ethanol is lost from the blood is decreased by a factor of about three, and the levels of acetaldehyde in the blood are increased by 0.8 mM relative to control mice. Although the time dependence of the action of this inhibitor was not extensively investigated, the effects persisted for at least 20 h.

Tottmar and Lindberg (1977) have shown that liver aldehyde dehydrogenase is inhibited in rats injected with coprine

Table III: Inhibition of Mouse Liver Aldehyde Dehydrogenase in Vivo[a]

	mmol per kg mouse	rel aldehyde dehydrogenase act.
control		100 ± 1
coprine	0.8	83
cyclopropanone hydrate	0.8	64 ± 4

[a] Male Swiss albino mice (22–33 g) were injected interperitoneally with 0.1 M coprine in 0.01 M sodium phosphate buffer (pH 7.3) or with 0.1 M cyclopropanone hydrate in the same buffer; 8 mL was injected per kilogram of body weight. Control mice received no injection. Four hours after injection livers were removed and assayed for aldehyde dehydrogenase activity (see Materials and Methods).

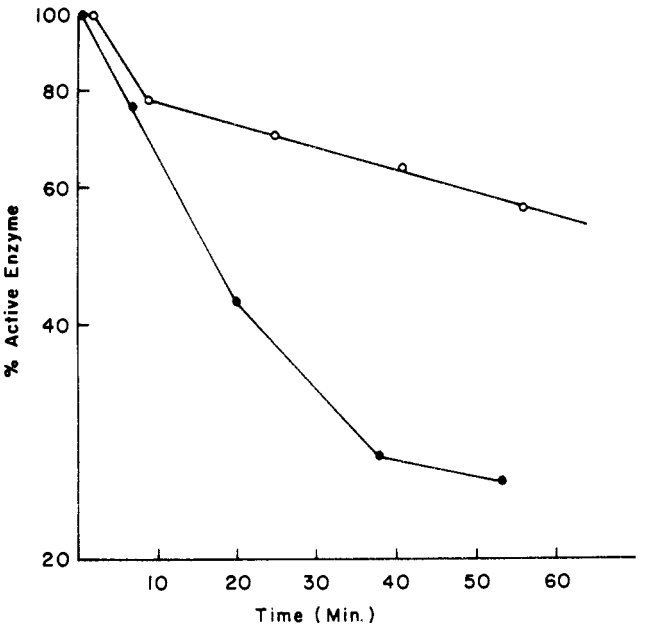


FIGURE 2: Inhibition of mouse liver aldehyde dehydrogenase in vitro. Aldehyde dehydrogenase was prepared from the livers of two 20-g Swiss albino mice (see Materials and Methods). The crude enzyme, 0.25 mL (0.11 IU/mL) in 0.25 M sucrose, 1 mM Tris-HCl, pH 7.4, was added to 0.25 mL of 0.1 M sodium pyrophosphate, 1 mM EDTA pH 9.3, containing 0.25 mM NAD and 10 mM cyclopropanone hydrate and incubated at 25 °C. Aliquots, 0.1 mL, were assayed for enzyme activity by dilution into 1 mL of assay buffer (see Materials and Methods). (●—●) Inhibition by cyclopropanone hydrate; (○—○) control, no cyclopropanone hydrate.

and suggest that this result accounts for the effects of coprine on ethanol metabolism. We also find that injection of mice with coprine results in partial inhibition of the enzyme in vivo. The same result, but to a greater extent, was obtained on injection with cyclopropanone hydrate (Table III). Cyclopropanone hydrate differs from coprine, however, by the fact that cyclopropanone hydrate inhibits mouse liver aldehyde dehydrogenase in vitro (Figure 2) while coprine does not.[2] These results are in accord with the belief that the toxic effects of coprine are due to a metabolic product of coprine and allow that cyclopropanone hydrate be this metabolite.

These results led us to a study of the mechanism of the inhibition of aldehyde dehydrogenase by cyclopropanone

[2] In addition to data in the literature which indicate that mouse liver aldehyde dehydrogenase is not inhibited by coprine in vitro (Hatfield & Schaumberg, 1975; Tottmar & Lindberg, 1977), we find that 10 mM coprine gave no inhibition of yeast aldehyde dehydrogenase after 20 h. The same concentration of cyclopropanone hydrate inhibited with a half-life of 3 min under the same conditions (Figure 3A).

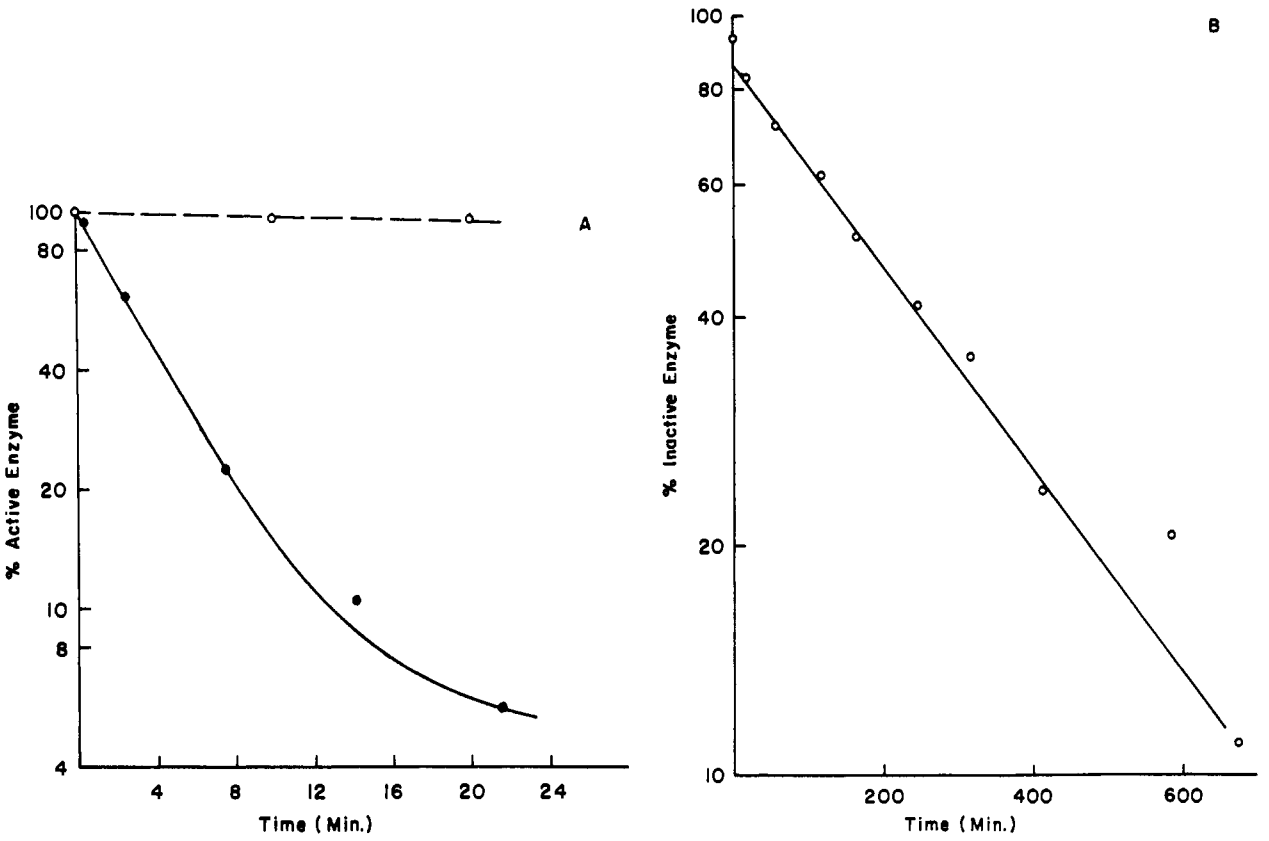


FIGURE 3: Inhibition of yeast aldehyde dehydrogenase by cyclopropanone hydrate. (A) The inhibition reaction. Enzyme, 0.22 IU, was incubated at 25 °C in 0.2 mL of buffer containing 25% glycerol, 80 mM Tris-HCl, pH 8.0, 0.16 M KCl, 0.8 mM EDTA, 1 mM 2-mercaptoethanol, and 10 mM cyclopropanone hydrate. Aliquots, 0.01 mL, were removed periodically and added to 1 mL of assay buffer to determine remaining enzyme activity (see Materials and Methods). (●—●) Percent active enzyme vs. time; line was drawn for loss of activity with a half-life of 3.1 min and 4.8% activity remaining at infinite time. (○—○) Control, no cyclopropanone hydrate present. (B) Recovery of activity on dilution. Enzyme, 0.11 IU, was incubated at 25 °C in 0.01 mL of buffer containing 30% glycerol, 10 mM Tris-HCl, pH 8.0, 0.2 M KCl, 1 mM EDTA, 30 mM 2-mercaptoethanol, and 10 mM cyclopropanone hydrate. After 70 min, this solution was diluted with 20 mL of buffer containing 0.1 M Tris-HCl, pH 8.0, 0.2 M KCl, 1 mM EDTA, and 30 mM 2-mercaptoethanol. This solution was assayed for recovery of enzyme activity by adding 20 μL each of 25 mM NAD and 20 mM benzaldehyde to 1-mL aliquots (see Materials and Methods).

hydrate. These studies were carried out with yeast aldehyde dehydrogenase.

Inhibition of Yeast Aldehyde Dehydrogenase by Cyclopropanone Hydrate. The kinetics of inhibition are presented in Figure 3A. As is the case with aldehyde dehydrogenase from mouse liver, 100% inhibition is not achieved. Such a result is consistent with the possibility that the inhibition is reversible. The line in Figure 3A was calculated assuming that the approach to equilibrium is (pseudo) first order with a half-life of 3.1 min. Since this half-life represents a rate of approach to equilibrium, it represents the sum of the forward and reverse rates. The half-lives for the forward and reverse reactions are calculated from this half-life and the equilibrium constant to be 3.2 (forward, inhibition reaction) and 64 min (reverse reaction). The equilibrium constant for formation of inhibited enzyme is calculated to be 2000 M^{-1} from the amount of active enzyme remaining at equilibrium (4.8%).

The reversibility of the inhibition was tested directly. Aldehyde dehydrogenase was incubated with cyclopropanone hydrate until equilibrium was established and diluted 2000-fold. Aliquots were then periodically assayed for recovery of activity. The results are presented in Figure 3B. The data show that upon dilution, the enzyme regains activity in a first-order process. Nearly 100% of the original activity is recovered. From these data, $t_{1/2}$ for the dissociation of the enzyme–inhibitor complex was found to be 230 min. This is four times greater than $t_{1/2}$ determined from the data of Figure 3A. We believe that this discrepancy is due to the difference in protein concentration in the two experiments. The experiment of Figure 3B is done at much lower protein concentrations than that of Figure 3A. Possibly, dissociation of the enzyme–inhibitor complex occurs more slowly at lower protein concentrations. An experiment will be cited later in this paper which supports this interpretation.

The data in Figure 3B show a burst of recovery of activity on dilution of the inhibited enzyme. The amount of inactive enzyme changes from 95%, the equilibrium value before dilution, to 85% within 10 min after dilution. This burst is not related to the inactivation by cyclopropanone hydrate but is due to the presence of a small amount of air-oxidized enzyme in this enzyme preparation which regained activity on dilution into buffer containing a high concentration of 2-mercaptoethanol.

Inhibition with [^3H]Cyclopropanone Hydrate. Although it is clear from the data in the preceding section that the inhibition reaction is reversible, these data do not require that the inhibition be an equilibrium reaction, i.e., that dihydroxycyclopropane both binds to and is released unchanged from the enzyme. An alternate explanation of the data would be that the inhibitor is just a very poor substrate for the enzyme, i.e., that cyclopropanone hydrate binds to the enzyme but is released in some modified form and that the "equilibrium" concentration of active enzyme is in reality a steady-state concentration. This problem was addressed by inhibiting the enzyme with [^{3}H]cycopropanone hydrate (see experimental details in Figure 4). The inhibited enzyme was passed through a Sephadex G-25 column to remove unreacted inactivator. The enzyme was then maintained at pH 8.0 for

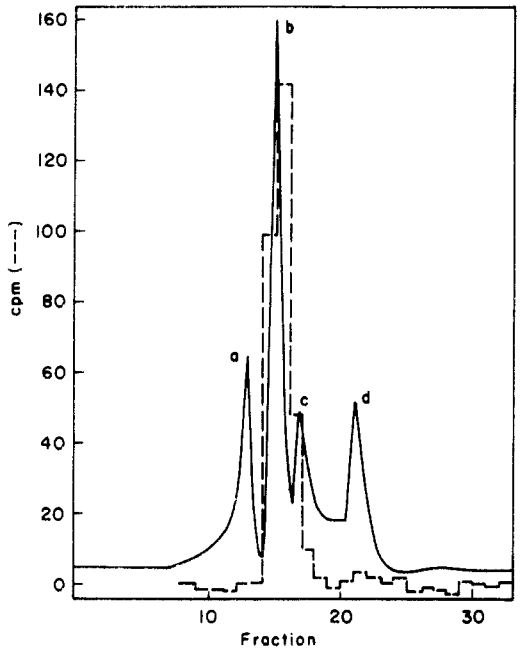


FIGURE 4: Identification of species released from yeast aldehyde dehydrogenase in the reverse of the inhibition reaction. Enzyme, 2.2 IU, was incubated in 0.2 mL of buffer containing 30% glycerol, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 3.6 mM [^{3}H]cyclopropanone hydrate (1.8 Ci/mol). After 15 h at 25 °C, the enzyme–inhibitor solution was chromatographed on Sephadex G-25 (medium, 0.5 × 29 cm) in 10 mM potassium phosphate, pH 8.0, 0.1 mM EDTA. The protein-containing fractions were pooled (0.4 mL), made 10 mM in cyclopropanone hydrate, kept at 25 °C for 26 h, and then adjusted to pH 7 by addition of 4 μL of 1 M KH_2PO_4. Radioactive species which were released from the enzyme during this time were separated from protein by chromatography on Sephadex G-25 (medium, 0.5 × 24 cm) in 10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA. Recovery of total radioactivity was 78%; 18% of the total radioactivity remained bound to enzyme. The small molecule fractions containing the radioactivity which was released from the enzyme were pooled (1.5 mL), and 80 μL (920 dpm) was mixed with 0.5 μL of 1 M HCl and 10 μL each of 1 M cyclopropanone hydrate (which contains an equal concentration of ethanol) and 1 M propionic acid. This solution was analyzed by high-pressure liquid chromatography on a μC_{18}-Porasil column (Waters Associates Inc., Milford, MA, 0.4 × 30 cm) in water. (—) Peaks detected by refractive index: (a) buffer salts, (b) cyclopropanone hydrate, (c) ethanol, and (d) propionic acid. (···) Radioactivity (cpm − background, background = 18 cpm); 745 dpm were recovered in the cyclopropanone hydrate peak (81% of the radioactivity applied).

15 h to allow the enzyme–inhibitor complex to dissociate. The enzyme was then again passed through a Sephadex G-25 column, and the small molecule fraction was pooled. The pooled fraction was examined by high-pressure liquid chromatography (Figure 4). One major peak of radioactivity was detected and this peak comigrated with cyclopropanone hydrate. A possible small peak of radioactivity eluting as propionic acid could be accounted for entirely by the slow decomposition of cyclopropanone hydrate to propionic acid under these conditions (half-life = 70 h at 25 °C, pH 8).

The base-catalyzed ring-opening reaction of cyclopropanone hydrate (Wasserman et al., 1974) was used as the second criterion for identification of the species released from the enzyme. An aliquot (100 μL, 1150 dpm) of the Sephadex small molecule fractions from the above experiment was adjusted to pH 8–8.5 with 0.7 μL of 1 M NaOH and applied to Dowex 1-X8 (1 mL, 200–400 mesh, Cl^- form); 918 dpm (80%) were eluted with water and 55 dpm (5%) were eluted subsequently with 1 M HCl. Another 100-μL aliquot was adjusted to pH 12–12.5 with 2 μL of 1 M NaOH, heated 1 min at 100 °C, adjusted back to pH 8–8.5 with 1 μL of 1 M HCl, and then chromatographed as above on Dowex 1-X8. In this case, no radioactivity was eluted with water but 1250 dpm (109%) were eluted with 1 M HCl, consistent with the known decomposition of cyclopropanone hydrate to propionic acid at pH 12.

These results establish that the inhibition by cyclopropanone hydrate is truly reversible and that cyclopropanone hydrate is not chemically modified by aldehyde dehydrogenase.

The stoichiometry of binding of cyclopropanone hydrate to the enzyme was also determined in this experiment; 0.82 mol of cyclopropanone hydrate was calculated to be bound per mole of active sites after correcting for the equilibrium concentration of active enzyme and assuming a subunit molecular weight of 56 000 (Clark & Jakoby, 1970) (the enzyme is normally a tetramer of four identical subunits).

Effects of Substrate Concentrations on Inhibition by Cyclopropanone Hydrate. The effects of NAD and benzaldehyde on the rate of inhibition of yeast aldehyde dehydrogenase by cyclopropanone hydrate are presented in Tables I and II, where they are compared to the effects on inhibition by iodoacetamide and on the enzyme-catalyzed oxidation of benzaldehyde.

The effects of benzaldehyde were straightforward (Table I). Benzaldehyde alone did not affect the rate of inhibition by cyclopropanone hydrate, even at concentrations as high as 25 mM. In the presence of NAD, however, benzaldehyde protected against inhibition, and the K_m for benzaldehyde determined for the inhibition reaction was the same as that for the normal oxidation reaction.

The effects of NAD on the rate of inhibition by cyclopropanone hydrate were different than the effects on inhibition by iodoacetamide (Table II). NAD depresses the rate of inhibition by iodoacetamide, and the K_m for this reaction is the same as the K_m for NAD in the oxidation of benzaldehyde. NAD, however, increases by a factor of 30 the rate of inhibition by cyclopropanone hydrate, and the K_m determined for this reaction is 10-fold higher than the K_m for the oxidation reaction.

Effect of Cyclopropanone Hydrate Concentration on the Rate of Inactivation. The data in Figure 5 demonstrate that inhibition of yeast aldehyde dehydrogenase by cyclopropanone hydrate shows saturation kinetics with respect to inhibitor concentration. The K_m for the inhibitor is 10 mM in the absence of NAD. Although in the presence of NAD inhibition was too fast to measure above 10 mM inhibitor, the data at this concentration and below indicate that the K_m in the presence of NAD is also 10 mM.

By comparison, the rate of inhibition of yeast aldehyde dehydrogenase by iodoacetamide varies linearly with inhibitor concentration up to at least 20 mM in the absence of NAD and up to at least 4 mM in the presence of 1 mM NAD. The rate of inhibition was too fast to measure at higher concentrations of iodoacetamide.

Effect of Iodoacetamide on the Inhibition by Cyclopropanone Hydrate. Yeast aldehyde dehydrogenase has three cysteine residues per subunit (Clark & Jakoby, 1970). The enzyme is inactivated by iodoacetamide, which presumably reacts with one of the sulfhydryl groups. We examined the effect of iodoacetamide on the inactivation by cyclopropanone hydrate as well as the effect of cyclopropanone hydrate on iodoacetamide inhibition to determine whether these two inactivators interact at or near the same site. When aldehyde dehydrogenase is exposed to [^{14}C]iodoacetamide, radioactivity is incorporated into the enzyme with first-order kinetics. Enzyme activity is lost at the same rate at which radioactivity is incorporated into the enzyme. Iodoacetamide binds to the

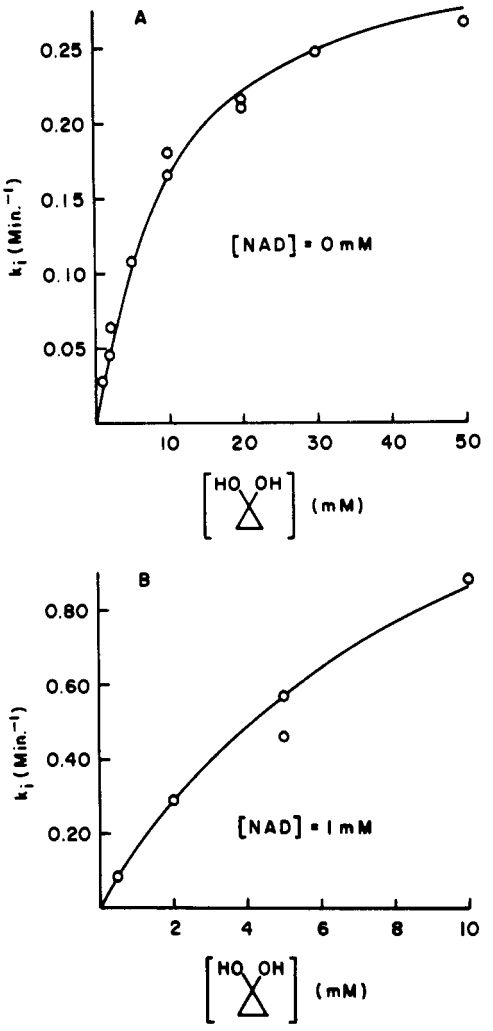
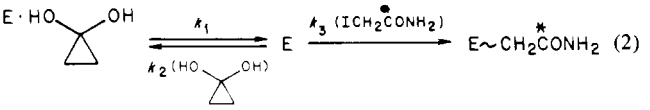


FIGURE 5: Effect of cyclopropanone hydrate concentration on the rate of inhibition, k_i, of yeast aldehyde dehydrogenase. (A) Enzyme, 0.022 IU, was incubated in 0.5 mL of a solution containing 30% glycerol, 0.1 M Tris-HCl, pH 8.0, 0.2 M KCl, 1 mM EDTA, and varying concentrations of cyclopropanone hydrate. Aliquots, 80 μL, were removed periodically and added to 1 mL of assay buffer to determine enzyme activity (see Materials and Methods). At low inhibitor concentrations where the rate of loss of activity was not first order (as in Figure 3A), the initial rate of inhibition was taken as k_i. The line was calculated for K_m = 10 mM and k_i = 0.33 min^{-1} at saturating concentrations of cyclopropanone hydrate. (B) Same as 5A but with 1 mM NAD. The line is calculated for K_m = 10 mM and k_i = 1.73 min^{-1} at saturating inhibitor concentrations.

enzyme to the extent of 0.75 mol per mol of active site; this is the same stoichiometry obtained with cyclopropanone hydrate.

In order to determine whether enzyme inactivated with iodoacetamide would still bind cyclopropanone hydrate, yeast aldehyde dehydrogenase, 0.11 IU, was inactivated with iodoacetamide for 15 min at 25 °C in 10 μL of buffer containing 30% glycerol, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 20 mM iodoacetamide. [^{3}H]Cyclopropanone hydrate, 0.2 μL of a 150 mM solution, was then added and the incubation continued for 17 h. When this mixture was chromatographed on Sephadex G-25, only 20 cpm eluted with the protein fractions. This is 4% of the radioactivity expected on the basis of the known stoichiometry of labeling by cyclopropanone hydrate. In contrast, when control enzyme was treated in the same manner but with iodoacetamide omitted in the first incubation, 460 cpm eluted with the protein fractions (90% of theory). These results indicate that cyclopropanone hydrate does not bind to enzyme inactivated with iodoacetamide.

In the converse experiment, enzyme was inactivated by 10 mM cyclopropanone hydrate as described for Figure 3A. After 35 min (95% inactivation) 15 μL of 13.8 mM [^{14}C]iodoacetamide, 13 Ci/mol, was added to 150 μL of the enzyme–cyclopropanone hydrate solution, and aliquots were assayed subsequently for incorporation of iodoacetamide into the enzyme (see Materials and Methods). Under these conditions, radioactivity was incorporated into the enzyme with a half-life of 120 min. In a parallel experiment in which cyclopropanone hydrate was omitted, the half-life for incorporation of radioactivity was 2.9 min. The conditions of this experiment are represented by eq 2. Enzyme-bound



(2)

cyclopropanone hydrate is initially at equilibrium with a small amount of active enzyme (5% of the total enzyme) which is rapidly labeled on addition of [^{14}C]iodoacetamide. The further slow reaction with iodoacetamide is then determined by the microscopic rate constants k_1, k_2, k_3. The steady-state rate expression for formation of iodoacetamide-labeled enzyme is given in eq 3 where k_{obsd} is a (pseudo) first-order rate constant

$$k_{obsd} = \frac{k_1 k_3(\text{iodoacetamide})}{k_1 + k_2(\text{cyclopropanone hydrate}) + k_3(\text{iodoacetamide})} \quad (3)$$

when iodoacetamide and cyclopropanone hydrate are present in large excess over enzyme.

Rate constants k_1 and k_2 are known from the rate and equilibrium constants for inactivation by cyclopropanone hydrate in the absence of iodoacetamide (Figure 3A; 0.011 min^{-1} and 22 M^{-1} min^{-1}), and k_3 is known from the rate of inactivation by iodoacetamide in the absence of cyclopropanone hydrate (174 M^{-1} min^{-1}). The value of k_{obsd} calculated from these numbers is 0.0056 min^{-1} at the inhibitor concentrations used. This corresponds to a half-life of 124 min and compares with an experimentally determined half-life of 120 min.[3]

Inhibition of Other Enzymes by Cyclopropanone Hydrate. Cyclopropanone hydrate was found to inhibit a number of enzymes known to be sensitive to sulfhydryl reagents. The rates and equilibrium constants are presented in Table IV along with the rates for inactivation by iodoacetamide under the same conditions for comparison. It was not determined for how many of these enzymes inhibition by cyclopropane hydrate showed saturation kinetics. Within this limitation, the rate of inhibition by cyclopropanone hydrate relative to iodoacetamide varied by only two orders of magnitude.

Discussion

The mushroom toxin coprine inhibits ethanol metabolism and inactivates aldehyde dehydrogenase in vivo (Lindberg et al., 1977; Hatfield & Schaumberg, 1975; Tottmar & Lindberg, 1977). From the results presented here, it is clear that cyclopropanone hydrate, a logical metabolic product of coprine

[3] In practice, k_1 is often difficult to measure. As pointed out above, the value of k_1 is sensitive to enzyme concentration. Direct measurement of k_1 by dilution of inhibited enzyme, as in Figure 3B, is therefore not reliable. The values of k_{obsd}, k_2, and k_3 in eq 3, however, are readily determined at a given enzyme concentration. Using eq 3, the value of k_1 can be calculated for the same enzyme concentration. This calculation has proven useful in cases for which the equilibrium concentration of active enzyme in the presence of cyclopropanone hydrate cannot be accurately measured.

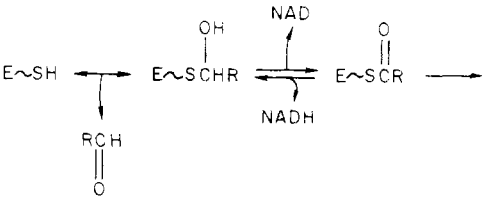
Table IV: Relative Inhibition Reactivities of a Series of Enzymes with Cyclopropanone Hydrate and Iodoacetamide

enzyme	pH	cyclopropanone hydrate k_{obsd}[a] (min^{-1})	cyclopropanone hydrate % active enzyme at equilibrium	iodoacetamide, k_{obsd}[b] (min^{-1})
yeast aldehyde dehydrogenase	8.0	0.22	5	0.17
mouse liver aldehyde dehydrogenase	9.3	0.046	20	
horse liver aldehyde dehydrogenase	9.3	0.036[c]	15	0.50[c]
yeast alcohol dehydrogenase	8.0	0.009		0.032
glyceraldehyde-3-phosphate dehydrogenase	7.6	0.06	<1	0.50
pyruvate decarboxylase	7.0	<0.002		
creatine kinase	9.3	5	6	0.46
pyruvate kinase	8.8	0.62[d]		0.058
		0.016[d]		<0.003
papain	5.5	0.46	28	0.33
chymotrypsin	7.8	<0.002		

[a] Rate of inhibition at 10 mM cyclopropanone hydrate. Where significant activity remains at equilibrium, k_{obsd} is the initial rate of inhibition (see Figure 3A). Yeast aldehyde dehydrogenase was inactivated as described in Figure 3A; mouse liver aldehyde dehydrogenase as described in Figure 2; all other enzymes as described in Materials and Methods. [b] Rate of inhibition by 1 mM iodoacetamide under the same conditions that the rate for cyclopropanone hydrate was determined. [c] In the presence of 0.5 mM NAD; the enzyme is not inactivated by cyclopropanone hydrate in the absence of NAD. [d] Pyruvate kinase has two reactive thiols; ca. 20% of the enzyme activity is lost on alkylation of the more reactive thiol by either of the inhibitors used here. The remaining activity is lost on alkylation of the second thiol.

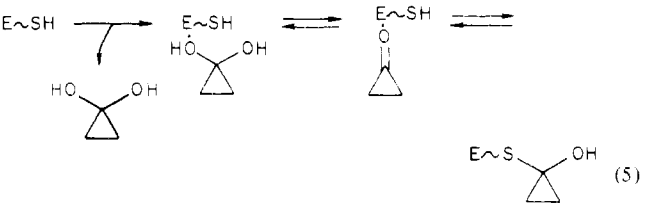
(eq 1), is capable of producing the same effects on ethanol metabolism that have previously been demonstrated for coprine. In addition, both coprine and cyclopropanone hydrate inhibit aldehyde dehydrogenase in vivo in mice. Only cyclopropanone hydrate, and not coprine, inhibited mouse liver aldehyde dehydrogenase in vitro, however. We conclude that coprine itself is inactive in vivo but is activated by hydrolysis to give initially cyclopropanone hemiaminal and ultimately cyclopropanone hydrate (eq 1). It has been previously reported (Tottmar & Lindberg, 1977) that cyclopropanone hemiaminal gives results equivalent to those reported here for cyclopropanone hydrate, although, as discussed in the introduction, we view these results with some caution. Given the mechanism which we will present below for the inhibition, we expect that cyclopropanone hemiaminal does in fact inhibit aldehyde dehydrogenase, but, due to the instability that we have observed for the hemiaminal, it may be difficult to prove this unambiguously. The rate constant that we have determined for hydrolysis of cyclopropanone hemiaminal to cyclopropanone hydrate ($t_{1/2}$ = 30 min, pH 7.5, 37 °C) predicts that on the time scale of the in vivo experiments, which is on the order of 1 h, both the hemiaminal and the hydrate would be present in comparable amounts. Which of these two species actually does the most damage to the aldehyde dehydrogenase in vivo seems a mute point. The mechanism to be presented for the inhibition reaction would predict the same form of the inhibited enzyme from either the hemiaminal or the hydrate.

The data that have been presented with respect to the mechanism of inhibition of yeast aldehyde dehydrogenase by cyclopropanone hydrate indicate that the inhibitor behaves as a substrate analogue. The mechanism that has been proposed (Feldman & Weiner, 1972; Jakoby, 1963) for the oxidation of aldehydes by aldehyde dehydrogenase is presented in eq 4.



$E\sim SH + RCO_2^-$ (4)

The key feature of this mechanism is the obligatory formation of a thiohemiacetal with an essential thiol at the active site. We propose a mechanism similar to that of eq 4 for the inhibition reaction (eq 5). Inhibited enzyme, in our view, is



(5)

the thiohemiketal between cyclopropanone and the essential thiol of the enzyme. This thiohemiketal would be inert to oxidation by NAD and would be kinetically stable in keeping with the known stability of adducts of cyclopropanone with various nucleophiles.[1]

As predicated by the mechanism of eq 5, the inhibition was found to be reversible. Cyclopropanone hydrate binds to and is released unchanged from the enzyme, and the stoichiometry of binding is 0.8 mol of cyclopropanone hydrate per mol of active site. The fact that cyclopropanone hydrate is released from inhibited enzyme also makes it clear that the inhibition is due to cyclopropanone hydrate itself and not to an impurity.

There is evidence which suggests that the inhibition involves a thiol at the active site. (a) Comparison of eq 3 and 4 indicates that substrates such as benzaldehyde should protect against inhibition by cyclopropanone hydrate by forming a thiohemiacetal with the active-site thiol. This was found to be the case. (b) Iodoacetamide reacts with aldehyde dehydrogenase with the same stoichiometry as observed for cyclopropanone hydrate and the one protects against reaction of the enzyme with the other. One would expect this result if both inhibitors reacted with the same residue. The usual reactivity of iodoacetamide implies that this residue is a thiol. (c) Cyclopropanone hydrate inhibits a number of enzymes known to be sensitive to thiol reagents.

The rate of inhibition of aldehyde dehydrogenase by cyclopropanone hydrate shows saturation kinetics with respect to inhibitor concentration. We interpret this result (eq 5) to respresent fast, reversible binding of cyclopropanone hydrate to the enzyme followed by rate-limiting enzyme-catalyzed dehydration to give cyclopropanone. This model for the inhibition reaction raises the question of whether the enzyme can catalyze the dehydration of aldehydes in the normal oxidation reaction, although, so far as has been ascertained, there is no other evidence to support this possibility.

The interpretation of the above kinetics of inhibition demonstrates the desirability of knowing the equilibrium constant for the hydration of cyclopropanone. Our interpretation that dehydration of cyclopropanone hydrate is an obligatory step in the mechanism of inhibition rests on the assumption that the concentration of free cyclopropanone is

negligible. That the ketone is very much less stable than the hydrate has been amply demonstrated qualitatively, but, to our knowledge, the equilibrium constant has not been measured. In principle, an inhibition reaction such as the one studied here, might be the ideal method of determining this equilibrium constant. This is because the inhibition reaction can be measured at very high ratios of cyclopropanone hydrate to enzyme. At a high enough ratio of cyclopropanone hydrate to enzyme, the concentration of free cyclopropanone would be comparable to the enzyme concentration, and our model would predict that a burst of inactivation corresponding to the free cyclopropanone concentration would be observed. Our studies of this inhibition reaction have covered a range of ratios of inhibitor to enzyme from 50:1 to 10^5:1, and we have observed no such change in rate-determining step. From this observation, one can put a lower limit of 10^5 on the ratio of cyclopropanone hydrate to cyclopropanone in solution.

The data for the effects of NAD on inhibition of aldehyde dehydrogenase by iodoacetamide seem to be at variance with conclusions that have been published concerning the order of binding of NAD and aldehydes (benzaldehyde) in the normal catalytic reaction. Bradbury and Jakoby (1971) failed to detect binding of NAD to free enzyme and concluded that the order of binding was benzaldehyde first followed by NAD. Our data imply that NAD does bind to the free enzyme. NAD depresses the rate of inhibition by iodoacetamide. The K_m for NAD in this reaction is 10 mM. Saturation kinetics are not observed for inhibition by iodoacetamide, and it is expected that iodoacetamide reacts directly with the active-site thiol without any prior association step. If this is correct, then the only way that NAD could affect the rate of inhibition by iodoacetamide would be if NAD binds to the enzyme before iodoacetamide reacts. In fact, the K_m for NAD in the inhibition reaction would be an upper limit for the dissociation constant for NAD. In addition, we have evidence that benzaldehyde does not bind to aldehyde dehydrogenase in the absence of NAD. It was found that benzaldehyde protects against inhibition by cyclopropanone hydrate, i.e., forms a thiohemiacetal with the active-site thiol, only in the presence NAD, not in the absence. Our results are consistent then with an ordered mechanism in which NAD, and not benzaldehyde, binds first and with a dissociation constant for NAD of 10 mM. This discrepancy is being further investigated.

It appears that there may be some promise in the design of active-site-directed inhibitors derived from cyclopropanone. For example, cyclopropylamine has been found to inactivate plasma amine oxidase, and there is good evidence that inactivation requires oxidation of cyclopropylamine to cyclopropanone imine (C. Clapp, K. Berg, and R. H. Abeles, unpublished results). We expect that this enzyme is inhibited by formation of an adduct between cyclopropanone imine and a nucleophile on the enzyme, in analogy to the inhibition of aldehyde dehydrogenase by cyclopropanone hydrate. Inactivation of papain by aldehydes (Lewis & Wolfenden, 1977; Westerik & Wolfenden, 1972), furthermore, has obvious similarities to inactivation of aldehyde dehydrogenase by cyclopropanone hydrate. Wolfenden has found that analogues of normal substrates for papain that have an aldehyde moiety in the position of the normally hydrolyzed peptide bond form very stable thiohemiacetals with papain. If derivatives of cyclopropanone were used instead of aldehydes, the exceptional kinetic stability of cyclopropanone hemiketals might provide a useful improvement on this type of inhibition.

Acknowledgments

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