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STUDIES *IN VITRO* ON THE INACTIVATION OF MITOCHONDRIAL RAT-LIVER ALDEHYDE DEHYDROGENASE BY THE ALCOHOL-SENSITIZING COMPOUNDS CYANAMIDE, 1-AMINOCYCLOPROPANOL AND DISULFIRAM*

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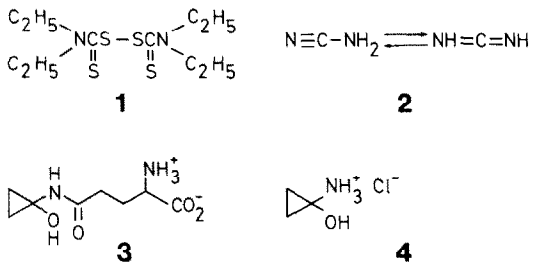
Abstract—The inhibition of the low-K_m, rat-liver mitochondrial aldehyde dehydrogenase (ALDH) by the alcohol-sensitizing agents cyanamide, 1-aminocyclopropanol (ACP) and disulfiram was studied *in vitro*. All three compounds caused a progressive decline in the enzyme activity. Restoration of activity could not be achieved by gel-filtration, dilution or by the addition of excess thiol. High concentrations of acetaldehyde partly restored the activity of the cyanamide-inactivated enzyme but had no effects on the disulfiram- or ACP-inactivated enzyme. In the presence of saturating concentrations of the coenzyme (NAD^+), the inactivation process followed first-order kinetics at fixed concentrations of the inhibitors. Plots of the apparent first-order rate constants against inhibitor concentration were curved, suggesting the formation of saturable, reversible holoenzyme–inhibitor complexes prior to the covalent reactions. In the absence of NAD^+, the rate of inactivation by disulfiram was biphasic and considerably higher than that in the presence of NAD^+. In contrast, no inactivation was obtained with cyanamide in the absence of NAD^+. Likewise, the presence of NAD^+ greatly promoted the inactivation by ACP. The esterase activity of the enzyme was also affected by the inhibitors, although to a lesser extent than was the dehydrogenase activity. The results obtained suggest that all three inhibitors inactivate the enzyme through covalent reactions with the thiol groups at the active site. It is proposed that binding of NAD^+ limits access of disulfiram to the thiols at the active site but provides a situation that favours an electrophilic attack of cyanamide and ACP on the thiol groups.

The alcohol-sensitizing effect of disulfiram (**1**) (Antabus®), cyanamide (**2**) (Temposil®, Dipsan®) and coprine (**3**) (a compound isolated from the inky-cap mushroom *Coprinus atramentarius*) (shown in Scheme 1) is mainly due to the inhibition of NAD^+-dependent aldehyde dehydrogenase (ALDH), which causes an elevated acetaldehyde level after ethanol ingestion [1–9]. The mechanisms of inactivation of ALDH by these agents *in vitro* have not been fully elucidated. It was previously believed that disulfiram was a reversible inhibitor of ALDH, being competitive with respect to NAD^+, and either non-competitive or uncompetitive with respect to the aldehyde substrate [10]. However, a number of studies have shown that disulfiram inhibits ALDH irreversibly, and it has been suggested that disulfiram forms mixed disulfides with the sulfhydryl groups of the enzyme [2–4].

Cyanamide has been reported to be an uncompetitive inhibitor of beef-liver ALDH, with respect to both acetaldehyde and NAD^+ [11]. However, Deitrich [12] and Deitrich *et al.* [13] could not detect any inhibitory action by cyanamide on ALDH from beef, rabbit or mouse liver *in vitro*. Likewise, no inhibition was found with cyanamide and ALDH from sheep-liver cytosol [14]. Also, DeMaster *et al.* [15] were unable to demonstrate inhibition of the mitochondrial rat-liver low-K_m ALDH *in vitro*. However, since they found cyanamide to be a potent inhibitor when incubated with intact rat-liver mitochondria, they suggested that metabolic activation was required. In conflict with these results, it has been demonstrated that cyanamide inactivates the low-K_m ALDH both in experiments with deoxycholate-disrupted mitochondria [16] and with the semi-purified enzyme [9].



Scheme 1

Coprine, which has been isolated and identified as a cyclopropanone derivative [N^5-(1-

* Parts of this investigation have been presented in a Ph.D. thesis at the University of Uppsala, 1979 [39].

hydroxycyclopropyl)-L-glutamine] [17], is not an inhibitor of ALDH *in vitro* [18–20]. It probably acts via the acidic hydrolytic product 1-aminocyclopropanol (**4**) (ACP), which is a potent inhibitor both *in vivo* and *in vitro* [18, 19].

The aim of the present investigation was to study the mechanism of inhibition of ALDH *in vitro* by cyanamide and ACP. For comparison, disulfiram was also included. The experiments were performed on the low-K_m ALDH isolated from rat-liver mitochondria. This enzyme was chosen since it is responsible for the main part of the acetaldehyde elimination in rat liver during ethanol metabolism [21, 22].

MATERIALS AND METHODS

Chemicals. 1-Aminocyclopropanol hydrochloride (ACP) was synthesized as described by Lindberg *et al.* [17]. The compound was dissolved in water and stored at 4°. Under these conditions it is stable [23]. Cyanamide was obtained from Schuchardt (Munich, F.R.G.) and was extracted with ether and dried *in vacuo* before use. Disulfiram and acetaldehyde were supplied by Fluka AG (Buchs, Switzerland). Disulfiram was recrystallized twice in 99.5% ethanol. Acetaldehyde was freshly distilled before use. *p*-Nitrophenyl acetate was purchased from Aldrich (Beerse, Belgium). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Enzyme preparation. The primary aim of the enzyme purification was to obtain a preparation of the low-K_m aldehyde dehydrogenase free from other forms of the enzyme with higher K_m values for acetaldehyde. All steps were carried out at 4°. The liver from a Sprague–Dawley rat was cut into small pieces which were rinsed in a sucrose medium containing 0.25 M sucrose, 5 mM Tris–HCl and 0.5 mM EDTA (pH 7.2). Liver (10 g in 40 ml of sucrose medium) was homogenized in a Potter–Elvehjem homogenizer. The homogenate was made up to 10% (w/v) and centrifuged for 10 min at 800 *g*. The supernatant was carefully decanted into new tubs and was centrifuged for 10 min at 7000 *g*. The supernatant was discarded. The pellet (mitochondrial fraction) was gently suspended in 20 ml of sucrose medium and washed three times for 10 min at 7000 *g* to reduce the content of cytosolic enzymes. The final pellet was suspended in 10 ml (1 ml/g of liver) of 10 mM K^+-phosphate buffer (pH 7.5), and the suspension was sonicated for 3 × 15 sec at 21 kHz using a MSE 100 W Ultrasonic Disintegrator. The sonicated suspension was centrifuged for 60 min at 105,000 *g* to remove fragments of mitochondrial membranes. The supernatant was then passed through a Millipore filter of 0.45 μm pore size. Saturated, neutralized ammonium sulphate was then added to the clear supernatant (5–7 mg/ml of protein) to give 40% saturation, and the mixture was stirred for 10 min. After centrifugation at 15,000 *g* for 15 min, the supernatant was decanted and brought to 60% saturation with ammonium sulphate. The mixture was stirred for 10 min and then centrifuged again. The pellet was dissolved in 2 ml of 10 mM K^+-phosphate buffer (pH 7.5) to give a protein concentration of 4–5 mg/ml. The specific activity of the enzyme preparation was 37.5 nmole of NADH formed per min per mg of protein (measured with 20 μM acetaldehyde, 250 μM NAD^+ in 50 mM sodium pyrophosphate, pH 8.8, at 20°), which is a five-fold purification as compared to the specific activity of the liver homogenate at similar conditions (6.5 nmole of NADH formed per min per mg of protein). The preparation did not contain cytosolic dehydrogenases or high-K_m aldehyde dehydrogenases as judged by separate activity measurements. The purified enzyme was stored at −76°.

Enzyme assay and kinetic studies. The aldehyde dehydrogenase activity was assayed in 50 mM K^+-phosphate buffer containing 1.0 mM $MgCl_2$ (pH 7.5) or in 50 mM Na^+-pyrophosphate buffer (pH 8.0) either spectrophotometrically or fluorimetrically as previously described [24, 25]. The esterase activity of the enzyme was measured spectrophotometrically at 400 nm ($\varepsilon = 16\ mM^{-1}cm^{-1}$) by following the formation of *p*-nitrophenol from *p*-nitrophenyl acetate (0.2 mM) in 50 mM Na^+-pyrophosphate buffer, pH 8.0 [26]. The temperature was 20°.

For the inactivation experiments, the enzyme (0.5 mg of protein) in buffer with or without coenzyme and inhibitors present was incubated in a glass vessel at 20°. At different times, aliquots were withdrawn and transferred to the fluorimeter cuvette. The reaction was started by the addition of acetaldehyde and NAD^+ (final concentrations 20 and 250 μM, respectively). The reaction rates were constant for 4–5 min in experiments with cyanamide and ACP. In experiments with disulfiram, a slow but progressive inactivation occurred during the assay, and the initial rates were calculated from the first minute of the reaction. The reactions of ACP and cyanamide with cysteine were measured at 20° by following the rate of disappearance of the cysteine–SH through titrations with *N*-ethylmaleimide (NEM) [27]. The reaction with cysteine and the respective inhibitor was initiated by mixing 0.2 ml of 1 M inhibitor solution with 2.0 ml 0.01 M cysteine solution. The solutions were freshly prepared in Na^+-pyrophosphate buffer and adjusted to the same pH before mixing. At appropriate time intervals, 0.2 ml aliquots were removed and added to 2.8 ml of a 1 mM solution of NEM in 0.2 M K^+-phosphate buffer (pH 7.0). The absorbance at 300 nm was determined within 30 min for each sample. Control values for cysteine solutions without inhibitor were determined over the time course of the reaction. Pseudo-first order rate constants were determined from semi-logarithmic plots.

Lines were fitted to the experimental points by the method of least-squares.

RESULTS

Inactivation of ALDH in the presence of NAD^+

In the presence of a saturating concentration of NAD^+ (250 μM) (K_m NAD^+ = 7 μM at pH 7.5; unpublished results), disulfiram, cyanamide and 1-aminocyclopropanol (ACP) caused a time-dependent inactivation of the mitochondrial low-K_m aldehyde dehydrogenase (ALDH). Semi-logarithmic plots of the remaining activity against time were linear in all experiments with disulfiram and ACP,

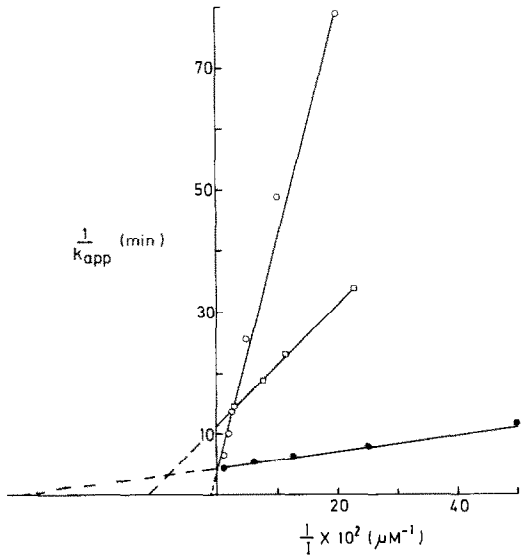


Fig. 1. Kinetics of the inactivation of rat-liver low-K_m aldehyde dehydrogenase by cyanamide (●), ACP (○) and disulfiram (□), showing the double reciprocal plot of the apparent first-order rate constants of inactivation as a function of inhibitor concentration. Incubation medium contained 50 mM K^+-phosphate, 1 mM $MgCl_2$, 0.25 mM NAD^+; pH 7.5.

whereas an initial delay followed by a strict pseudo-first order reaction was observed in experiments with cyanamide. When the apparent first-order rate constants (k_{app}) were plotted against inhibitor concentration, non-linear plots were obtained indicating an approach to a maximal value of the rate of inactivation as the inhibitor concentration was increased. This saturation effect suggests that reversible complexes are formed between the inhibitors and the enzyme prior to covalent binding. Thus, the inactivation processes may be written:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E \cdot I \xrightarrow{k_{inac}} E\text{–}I \qquad (1)$$

where E and E·I are the holoenzyme and the reversible holoenzyme–inhibitor complex, respectively. k_{inac} is the inactivation rate constant for the conversion of E·I to the covalent complex E–I. A steady-state treatment of this mechanism [28, 29]

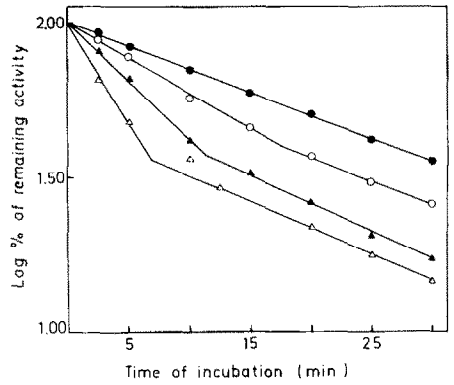


Fig. 2. Protection by NAD^+ against disulfiram inactivation of the low-K_m ALDH. Inactivation by 4 μM disulfiram in the absence (△), and in the presence of NAD^+: 10 μM (▲); 50 μM (○); 100 μM (●). pH 7.5.

leads to the following expression for the rate of enzyme disappearance:

$$-\frac{d(E)}{dt} = \frac{k_{inac}(E)(I)}{K_I + I} \qquad (2)$$

where $K_I = \frac{k_2 + k_{inac}}{k_1}$.

Equation (2) may be applied in the form

$$\frac{1}{k_{app}} = \frac{1}{k_{inac}} + \frac{K_I}{k_{inac}} \cdot \frac{1}{I}. \qquad (3)$$

The plots shown in Fig. 1 indicate good agreement with equation (3) and with the mechanism described. The values obtained are summarized in Table 1.

Inactivation of ALDH in the absence of NAD^+

The low-K_m ALDH from rat liver was much more sensitive to disulfiram in the absence, than in the presence, of NAD^+. In the absence of NAD^+, the inactivation by disulfiram was biphasic, starting with a rapid reaction which was followed by a slower process (Fig. 2). The rate of the initial reaction was not linear with respect to disulfiram concentration, suggesting that a reversible complex was formed between the apoenzyme and this inhibitor. The values of K_I and k_{inac} obtained for the initial reaction were 12×10^{-6} M and 7.8×10^{-3} sec^{-1}, respectively.

In contrast to the results obtained with disulfiram, the enzyme was completely insensitive to cyanamide

Table 1. Comparison of the inactivation of the low-K_m ALDH from rat-liver mitochondria

	Presence of NAD^+			Without NAD^+		
	K_I (10^6 M)	k_{inac} (10^3 sec^{-1})	SI (M^{-1} sec^{-1})	K_I (10^6 M)	k_{inac} (10^3 sec^{-1})	SI (M^{-1} sec^{-1})
Cyanamide	3.0	3.6	1200	0*	0*	0*
ACP	63	2.8	44	–	–	7.7
Disulfiram	9.0	1.5	167	12	7.8	650

The second-order constant k_{inac}/K_I is used as an index of specificity (SI). In the absence of NAD^+, inactivation by ACP did not show saturation kinetics hence the straightforward second-order constant is given as SI.

* Incubation with cyanamide up to 10 mM.

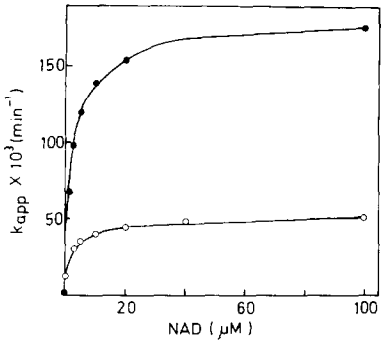


Fig. 3. Promotion effect of NAD^+ on the rate of inactivation by cyanamide (●) and ACP (○). The apparent first-order rate constant of inactivation is plotted against NAD^+ concentration at a fixed concentration (25 μM) of cyanamide and ACP. pH 7.5.

in the absence of NAD^+, even at very high inhibitor concentrations (10 mM). Likewise, the rate of inactivation of the apoenzyme was considerably lower than that of the holoenzyme when ACP was used as the inhibitor. However, in this case, there was no evidence for the formation of a saturable complex prior to the covalent reaction. Thus, equation (2) may be simplified to $-d(E)/dt = k_1(E)(I)$, and the apparent second order rate constant for the inactivation by ACP can be estimated to be 7.7 M^{-1} sec^{-1}.

Effect of NAD^+ concentration on the inactivation of ALDH

Since the rate of inactivation by both cyanamide and ACP was enhanced by the presence of NAD^+, incubations were carried out at different concentrations of the coenzyme at a fixed concentration of inhibitor. The k_{app} obtained from these experiments was plotted against the concentration of NAD^+, as shown in Fig. 3. Both curves reached a limiting value at high concentrations of NAD^+, corresponding to saturation of the enzyme with NAD^+, i.e. total conversion of the apoenzyme to holoenzyme.

The protection by NAD^+ against disulfiram inactivation was much more pronounced in the initial rapid reaction than in the subsequent slower reaction (Fig. 2). Accordingly, at a high concentration of NAD^+ (100 μM) the biphasic inactivation was abolished, and the loss of enzyme activity followed first-order kinetics.

Table 2. The effect of thiol reagents on the activity of the low-K_m ALDH *in vitro*

Incubations*			Remaining† activity
Compound	mM	NAD^+ (250 μM)	% of control
Iodoacetamide	1.0	+	<1
		−	31
Iodoacetate	1.0	+	92
		−	90
O-Iodosobensoate	0.005	+	100
		−	45
O-Iodosobensoate	0.010	−	34

* Incubations for 10 min in 50 mM phosphate buffer containing 1 mM $MgCl_2$, pH 7.5.

† Remaining activity was measured by the addition of 20 μM acetaldehyde and 250 μM NAD^+, when absent in the incubation.

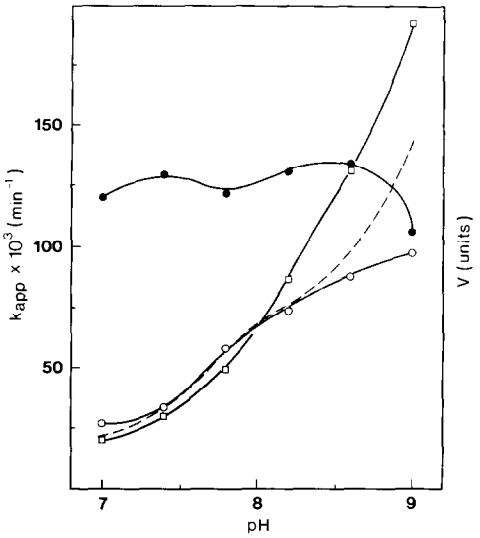


Fig. 4. pH-Inactivation rate profile showing the apparent first-order rate constant of inactivation as a function of pH in the presence of cyanamide (●; 5 μM), disulfiram (□; 10 μM) and ACP (○; 20 μM). The dotted line is the velocity of the non-inhibited enzyme-catalysed reaction in units at saturating concentrations of NAD^+ (250 μM) and acetaldehyde (20 μM). Buffers were made up with 0.2 M Na^+-pyrophosphate. Incubations were in the presence of 0.25 mM NAD^+.

Effect of NAD^+ on the inhibition of ALDH by sulfhydryl-reacting agents

As shown in Table 2, co-incubation with NAD^+ promoted the inhibition by iodoacetamide but protected against inhibition by iodosobensoate. Iodoacetate was not an inhibitor of the low-K_m ALDH when used at 1 mM.

Effect of pH on the inactivation of ALDH

In the presence of NAD^+ at a saturating concentration (250 μM), measurable spontaneous inactivation occurred only above pH 8.2. However, in the absence of NAD^+, the spontaneous inactivation in the higher pH range was too high to permit any accurate experiments with the inhibitors. The results given have been corrected for the spontaneous inactivation.

As shown in Fig. 4, the rate of inactivation by disulfiram increased rapidly with pH. The pH curve obtained, although not showing a distinct point of inflexion, supports the suggestion that disulfiram reacts with the thiol ion of the enzyme [3].

The rate of inactivation by ACP was also increased with increase in pH and followed the pH profile for the enzyme activity (the dotted line shown in Fig. 4) at pH values below 8.2 but deviated markedly at pH 8.2–9.0.

The rate of inactivation by cyanamide was not

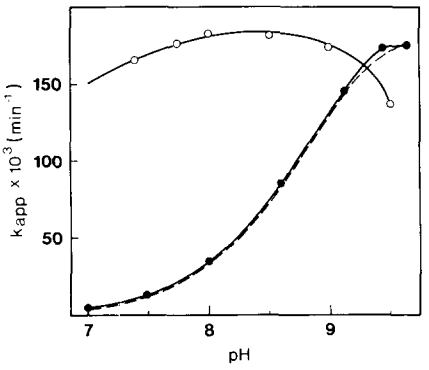


Fig. 5. pH-Dependence of the apparent first-order rate constant for the reaction of cysteine with cyanamide (●) and ACP (○). The dotted line is the theoretical curve for an ionizing group of pK_a 8.7. Buffers were made up with 50 mM Na^+-pyrophosphate.

influenced in the pH range 7.0–8.6 and was decreased at pH 9.0 (Fig. 4).

Reactivity of the inhibitors towards cysteine

Both ACP and cyanamide were found to react with cysteine. First-order kinetics were observed when the inhibitors, at a ten-fold molar excess (100 mM), were incubated with cysteine. At pH 7.5, the rate constants were 2.3×10^{-4} and 3.0×10^{-3} sec^{-1} for cyanamide and ACP, respectively. This large difference in reactivity, however, decreased with an increase in pH. As shown in Fig. 5, the reactivity of ACP towards cysteine did not vary much in the pH range 7.5–9.0 but appeared to decrease at higher pH. In contrast, the rate of reaction between cyanamide and cysteine increased markedly with pH and followed the theoretical titration curve for a thiol with a pK_a of 8.7.

No experiments were performed on the reaction between disulfiram and cysteine, since, for every cysteine molecule reacting in the disulfide–thiol exchange reaction, a new thiol would be produced [30].

Attempts to restore the activity of the inhibited enzyme

No recovery from inhibition was obtained on dilution or by treating the inhibited enzyme with excess cysteine, not even when the inactivated enzyme had been passed through Sephadex G-25 (Table 3). However, in experiments with the cyanamide-inhibited enzyme, a large part of the activity could be restored if the enzyme was incubated in the presence of high acetaldehyde concentrations (Table 4). This recovery appeared to be instantaneous, since no further reactivation was observed when the activity was measured 10 min after the addition of acetaldehyde. No reactivation by acetaldehyde was observed when the enzyme was inhibited by ACP or disulfiram (Table 4).

Effect of the inhibitors on the esterase activity of ALDH

The esterase activity was also affected by the three inhibitors, although not as markedly as the dehydrogenase activity (Table 5). The inhibitory action of the compounds on the esterase function was affected by NAD^+ in a similar way as the dehydrogenase function. Thus, the inhibition of the esterase and the dehydrogenase activities by cyanamide required in both cases co-incubation with NAD^+, whereas the inactivation of both activities by

Table 3. Effect of gel filtration and treatment with cysteine on the inactivated enzyme

Inhibitor (mM)	% of initial activity after 10 min of preincubation			
	Before gel filtration	+ Cysteine*	After gel filtration	+ Cysteine*
None	100	115	69	75
Disulfiram (0.03)	38	42	30	34
Cyanamide (0.10)	12	13	6	5
ACP (0.10)	14	13	10	10

* The activity was measured 20 min after the addition of cysteine (10 mM). Incubation medium: pH 7.5, 0.25 mM NAD^+. Gel filtration on Sephadex G-25 coarse.

Table 4. Effect of acetaldehyde on the inactivated enzyme

Acetaldehyde (mM)	Activity (units)			
	Control	Cyanamide (10 μM)	ACP (20 μM)	Disulfiram (20 μM)
0.02	59	12	30	23
0.05	57	11	28	25
0.10	52	12	26	23
0.50	50	18	29	23
1.0	53	26	32	24

The residual activity was measured by the addition of different concentrations of acetaldehyde after 15 min of incubation with the respective inhibitor in the presence of 0.25 mM NAD^+ at pH 7.5.

Table 5. Comparison of the inhibition of the dehydrogenase and the esterase activity of ALDH

Incubations*		Activity after incubation	
Inhibitor (μM)	NAD^+ (250 μM)	Dehydrogenase†	Esterase‡
–	–	25.7 ± 2.8	66.3 ± 10.3
–	+	26.3 ± 1.6	66.0 ± 10.5
Cyanamide (185 μM)	–	25.1 ± 3.7	65.0 ± 11.5
Cyanamide (185 μM)	+	0	45.2 ± 8.7
ACP (185 μM)	–	8.7 ± 1.2	50.2 ± 6.2
ACP (185 μM)	+	0	42.8 ± 7.6
Disulfiram (20 μM)	–	0	23.5 ± 4.6
Disulfiram (20 μM)	+	13.6 ± 1.2	32.5 ± 5.6

* Incubations for 10 min in 50 mM Na^+-pyrophosphate, pH 8.0.

† Dehydrogenase activity is expressed as nmole of NADH formed per min per mg of protein. The activity was measured at 20 μM acetaldehyde and 0.25 mM NAD^+.

‡ Esterase activity is expressed as nmole of *p*-nitrophenol formed per min per mg of protein. The activity was measured at 0.2 mM *p*-nitrophenyl acetate and 0.25 mM NAD^+.

disulfiram was more pronounced in the absence, than in the presence, of NAD^+.

DISCUSSION

The results obtained in the present report confirm previous findings [9] that the alcohol-sensitizing compounds cyanamide, 1-aminocyclopropanol (ACP) and disulfiram inactivate the low-K_m aldehyde dehydrogenase (ALDH) from rat-liver mitochondria. With respect to disulfiram, the results obtained are in accordance with previous reports, suggesting the formation of covalent, irreversible bonds between the inhibitor and the SH-groups of the enzyme [3, 9, 31, 32]. It is noteworthy, however, that whereas disulfiram caused a total inhibition of the enzyme from rat-liver mitochondria (Table 5), only a 50% loss of activity was reported for the mitochondrial enzymes from sheep [33] and horse liver [34]. In this respect, the enzyme used in the present study resembles the cytoplasmic more than the mitochondrial enzymes obtained from these sources [3, 34].

Like disulfiram, both cyanamide and ACP appear to inactivate the mitochondrial low-K_m ALDH through covalent reactions with the enzyme's active-site thiols. In favour of this view, cyanamide and ACP react covalently with the SH-group of cysteine. Further, acetaldehyde, which is known to bind to the cysteine residue of the active site through the formation of a thiohemiacetal [26], protects the enzyme from inhibition [9] and partly reactivates the cyanamide-inhibited enzyme at high concentrations (Table 3). Finally, all three inhibitors affect the esterase function of the enzyme. This is also in accordance with interactions taking place between the inhibitors and the enzymic thiols, since the esterase and the dehydrogenase activities probably occur at the same active site [26].

With respect to ACP, it has also been shown that cyclopropanone hydrate inactivates aldehyde dehydrogenase through interaction with the active-site thiol [35]. As pointed out by Wiseman and Abeles [35], cyclopropanone hydrate is not the first metabolite of coprine. According to Lindberg [23] the first degradation product of coprine is ACP which, however, is unstable as a free base and undergoes a series of rapid exchange reactions (Fig. 6). Since the hydroxyl moiety of aminocyclopropanol (**I**) is more easily displaced than the amine moiety, a highly reactive imminium salt is formed (**II**) which reacts readily with weak nucleophiles like ethanol [23]. However, in water solutions, aminocyclopropanol would probably also liberate ammonia to form a reactive cyclopropanone (**III**) which would be in equilibrium with cyclopropanone hydrate (**IV**). In a study on structure–activity relationships of cyclopropanone derivatives it was thus suggested that the aldehyde dehydrogenase inhibiting activity was inherited in the cyclopropanone hydrate or 1-aminocyclopropanols, i.e. the cyclopropanone function or its imminiumsalt moiety [23].

The observations made by Wiseman and Abeles [35] on yeast ALDH with cyclopropanone hydrate are somewhat similar to the present findings on the rat-liver low-K_m ALDH and ACP. Thus, the inactivation by the two compounds was considerably more rapid in the presence, than in the absence, of NAD^+ and showed in both cases saturation kinetics. However, whereas the dissociation constant for the ACP–low-K_m ALDH complex was within the

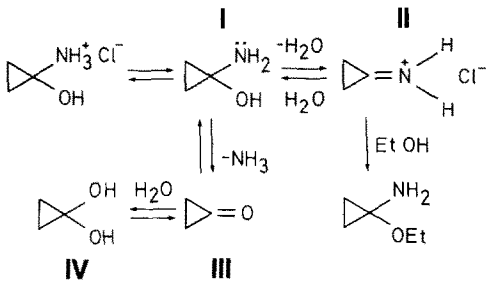


Fig. 6. Scheme showing exchange reactions of 1-aminocyclopropanone in ethanol and water according to Lindberg [23].

micromolar range (K_I = 60 μM, Table 1) the dissociation constant for the complex between yeast ALDH and cyclopropanone hydrate was 10 mM [35]. Also, the cyclopropanone hydrate inactivation of ALDH from yeast was reversible with $t_{1/2}$ = 230 min [35], whereas reversibility was not demonstrated for the ACP-inhibited low-K_m ALDH. The formation of a highly stable complex between ACP and the low-K_m ALDH *in vitro* is in accordance with earlier observations *in vivo* that the time course of recovery of the low-K_m ALDH is slow in rats given ACP either orally or by intraperitoneal injections [9].

It is not known whether the discrepancies between the inactivation by ACP and cyclopropanone hydrate are due to the different compounds being used, or to different sensitivity towards inhibition of the yeast ALDH and the low-K_m ALDH used in the present study. Nevertheless, yeast ALDH and the low-K_m ALDH differ in one important respect, namely their behaviour towards inhibition by iodacetamide. Thus, whereas NAD^+ depresses the rate of inhibition of ALDH from yeast [35], the presence of NAD^+ promotes the inactivation of the low-K_m ALDH by iodacetamide (Table 2).

Although there are many important indications of a reaction between cyanamide and the thiol group of the active site, as has already been discussed, the pH profile for the rate of inactivation (Fig. 4) does not support this. On the other hand, the pH-dependent rate of reaction of cyanamide with cysteine (Fig. 5) is consistent with the theoretical titration curve for a thiol with a pK_a of 8.7. The reason for these discrepancies is not known. However, a tentative explanation for the lack of a pH-effect on the rate of inactivation by cyanamide may be given on the assumption that the saturable binding involves a weak interaction of an acidic group of the enzyme and cyanamide, with cyanamide acting as a Lewis base through its free electron pair on the amino group. An increase in pH, which increases the ionization of the enzymic SH-group and thereby the covalent reaction with cyanamide, may at the same time affect the acidic site and reduce the necessary weak interaction or orientation. The net effect could be an unchanged overall rate of inactivation with change of pH.

The requirement of NAD^+ for the enzyme inactivation by cyanamide indicates that the inhibitor is bound to the enzyme in a fashion similar to the aldehyde substrate in the catalytic process. Thus, it has been shown that ALDH possesses half-of-the-sites reactivity to NAD^+ [36] and follows an obligatory sequential order of binding with NAD^+ prior to the aldehyde substrate [26, 37]. Since such properties are known to be connected with conformational changes within the enzyme [38], it is possible that the postulated acidic group is accessible only in the holoenzyme. Whether or not NAD^+ participates directly in the inactivation mechanism similar to the catalytic reaction is not known (i.e. the transfer of a hydride ion).

In order to summarize the inhibition of the low-K_m ALDH by cyanamide, the scheme may be outlined as shown in Fig. 7A. The inhibition by cyanamide is not readily reversible since simple dilution, passage through Sephadex or addition of excess of

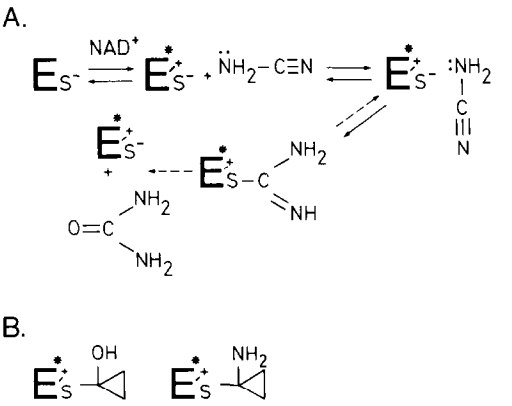


Fig. 7. (A) Scheme showing the proposed mechanism for the inactivation of the low-K_m ALDH by cyanamide in the presence of saturating concentrations of NAD^+. Holoenzyme is indicated by the asterisk. (B) The two suggested modified forms of the holoenzyme produced by ACP inactivation (see text).

thiols did not restore the enzyme activity. However, as the addition of high concentrations of acetaldehyde rapidly restored a large part of enzyme activity, the cyanamide inhibition might be termed pseudo-irreversible. This is also supported by the observation *in vivo* [9], which showed that more than 50% of the activity of the low-K_m ALDH was recovered within 12 hr. The inactivated enzyme may also be reactivated through the formation of urea.

The two most likely species of the covalently modified holoenzyme produced by the inactivation of ACP are shown in Fig. 7B. It is suggested that the inhibition by ACP starts with an interaction between the amino group of 1-aminocyclopropanol (**I** in Fig. 6) and the acidic group of the enzyme, similar to the interaction proposed for cyanamide (Fig. 7A). The subsequent reaction with 1-aminocyclopropanol and the enzyme may either proceed through the formation of the cyclopropanone intermediate (**III** in Fig. 6) or the imminium ion (**II** in Fig. 6), yielding the two modified enzyme forms respectively as shown (Fig. 7B).

In contrast to the unanimous reports on the potency of cynamide as an aldehyde dehydrogenase inhibitor *in vivo*, there are a number of conflicting observations concerning the activity of the drug *in vitro* (see Introduction). Several possible explanations to this may be given. (1) The incubation conditions are crucial since cyanamide and the enzyme require co-incubation with NAD^+ to yield an inactive enzyme. In this context it should also be mentioned that in the present study 1 mM of $MgCl_2$ was always added to the phosphate buffer in order to increase the enzyme-catalysed reaction [19]. However, this addition does not appear to affect the inactivation since a similar rate of inhibition was found also in pyrophosphate buffers lacking magnesium. (2) As previously discussed, susceptibility of the different aldehyde dehydrogenases obtained from different sources may vary. In this respect it has been shown that the microsomal high-K_m ALDH from rat liver is not inhibited by millimolar concentrations of

cyanamide *in vitro* [9]. (3) However, as the microsomal high-K_m enzyme is inhibited by low concentrations of cyanamide *in vivo* (60% inhibition by 5 mg cyanamide/kg rat, giving a concentration of approximately 170 μM in the body-water [9]), it is possible that a metabolite of cyanamide potentiates the effect *in vivo* and causes the inhibition of the microsomal high-K_m enzyme. Several such metabolic products have been suggested [13–15]; however, none has been found to be active *in vitro*.

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