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STUDIES ON POSSIBLE MECHANISMS FOR THE INTERACTION BETWEEN CYANAMIDE AND ALDEHYDE DEHYDROGENASE

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Abstract—Cyanamide is known to interfere with the metabolism of alcohol by decreasing the activity of aldehyde dehydrogenase *in vivo*, thereby leading to an accumulation of acetaldehyde following the ingestion of ethanol. We have studied various mechanisms for the chemical interaction between aldehyde dehydrogenase and cyanamide (or some of its possible metabolites). Cyanamide was shown to react under physiological conditions with amino and thiol groups, forming guanidino and isothiouronium compounds respectively. However, it was found that the enzyme is not appreciably affected *in vitro* by high concentrations of cyanamide. Dicyandiamide and the aminoethylisothiouronium ion (AET) also have no effect *in vitro*. It was postulated that the pathway of *in vivo* enzyme modification by cyanamide may involve thiourea (formed by breakdown of isothiouronium compounds) and formamidine disulphide (an oxidation product of thiourea). However, although the disulphide is a moderately-effective inactivator of aldehyde dehydrogenase *in vitro*, administration of thiourea or AET to rats does not result in any significant loss of aldehyde dehydrogenase activity in either the cytoplasm or the mitochondria

It has long been known that a person who drinks after having ingested cyanamide will experience unpleasant symptoms (flushing, hypotension, palpitations, dizziness, etc.). For this reason the compound has been used as an alternative to the well-known disulfiram (Antabuse) [1] in attempts to dissuade alcoholics from drinking [2]. The compound may be administered as citrated calcium cyanamide (or carbimide), variously known as CCC, Temposil or Abstem, or as an aqueous solution of cyanamide itself. The main cause of the disulfiram–ethanol reaction is generally accepted to be an inactivation of hepatic aldehyde dehydrogenase and thus the occurrence of elevated blood acetaldehyde levels after the ingestion of ethanol; a similar explanation is thought to hold with cyanamide. In support of this, studies involving several different species (rats, mice, rabbits and humans) have shown that cyanamide administration results in decreased aldehyde dehydrogenase activity *in vivo* and consequent accumulation of acetaldehyde during ethanol metabolism [2–11].

Recently, further interest in the effects of cyanamide on alcohol metabolism has arisen from observations that rats maintained on certain standard laboratory diets show unusually high blood acetaldehyde levels after drinking ethanol. It appears that cyanamide is present in the calcined bonemeal fraction of such fodder [10].

The intention of the present study was to throw light upon the chemical basis of the action of cyanamide. To this end, cyanamide and various possible metabolites of this compound have been investigated as potential inhibitors of sheep liver cytoplasmic aldehyde dehydrogenase *in vitro*. The action of thiourea and 2-aminoethylisothiouronium bromide hydrobromide (AET) on rat liver aldehyde dehydrogenase *in vivo* was also examined.

MATERIALS AND METHODS

The following compounds were obtained from the sources indicated: cyanamide (as a 50% aqueous solution) and pyrazole, Aldrich Chemical Co. Inc. (Milwaukee, WI, U.S.A.); dicyandiamide, acetaldehyde, 2-mercaptoethanol and phenanthraquinone, Fluka A.G. (Buchs, Switzerland); AET, *N*-acetyl-L-cysteine and 5,5′-dithiobis-(2-nitrobenzoic acid), Sigma Chemical Co. (St. Louis, MO, U.S.A.); NAD^+, glycine and L-cysteine, BDH Chemicals (Poole, Dorset, U.K.); thiourea, Ajax Chemicals Ltd., Sydney, Australia; formamidine disulphide dihydrochloride, Pfaltz & Bauer Inc. (Stamford, CI, U.S.A.).

In vitro *studies*. Cytoplasmic aldehyde dehydrogenase from sheep liver was purified as previously described [12, 13]. (The cytoplasmic enzyme was chosen for study as it is vastly more sensitive to disulfiram and similar compounds than the mitochondrial form [14].) Before use the enzyme solutions were thoroughly dialysed to remove 2-mercaptoethanol. Enzyme activity was assayed as previously described [14]. The compounds to be tested as possible inhibitors of aldehyde dehydrogenase were added as aqueous solutions to the enzyme and buffer mixture (at pH 7.3 unless otherwise stated) either before or after the reaction was initiated by the addition of substrates, as described in Tables 2 and 3. The final volume was 3 ml and in all cases suitable control assays were performed. Enzyme concentration in the assay mixture was approx. 0.4–0.6 μM (based on a molecular weight of 212,000 [15]).

The concentrations of thiols were determined spectrophotometrically using 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) by measuring the absorbance at 412 nm due to the 3-carboxy-4-nitrothiophenoxide ion.

Table 1. The reaction of cyanamide with amino and thiol groups *in vitro* at pH 7.3

NH_2CN (mM)	L-Cysteine (mM)	Ninhydrin test	Guanidino test	Thiol loss (%)
1		–	–	
	1	+	–	
1	1	+	+	*
2	1	+	+	*
20		–	–	
	20	+	–	
20	20	faint	+	4.3
40	20	–	+	84
250		–	–	
	250	+	–	
250	250	–	+	9.5
500	250	–	+	75
	N-Acetyl-L-cysteine (mM)			
	1		–	
1	1		–	8
	20		–	
20	20		+	28
	250		–	
250	250		+	71
	Glycine (mM)			
	1		–	
1	1		–	
	20		–	
20	20		+	
	250	+	–	
250	250	+	+	

* Not measurable, due to loss of thiol group from the control solution (cysteine, 1 mM) presumably by atmospheric oxidation.

Solutions of cyanamide and certain amino acids in 0.1 M phosphate buffer (pH 7.3) were allowed to stand overnight at room temperature. Aliquots of these solutions were then tested with ninhydrin and a guanidino stain as described under Materials and Methods. Where applicable, the percentage loss of free thiol groups was also determined.

Table 2. The effect of cyanamide, dicyandiamide and 2-aminoethylisothiouronium bromide hydrobromide on sheep liver cytoplasmic aldehyde dehydrogenase *in vitro* at pH 7.3

Cyanamide (mM)	Time of incubation of enzyme and inhibitor before addition of substrates	Enzyme activity (per cent of control rate); multiple figures refer to different enzyme preparations
1	30 min	81, 86
10	30 min	83, 43, 53
10*	30 min	88
10	overnight	76
50	30 min	32
50	overnight	38
Dicyandiamide (mM)		
1	0†	87
1	20 min	105
2-Aminoethylisothiouronium (mM)		
1	0†	92
1	20 min	104

* At pH 9.3.

† 'Inhibitor' added 1 min after substrates added to enzyme.

The presence of free amino and guanidino (or isothiouronium) groups in reaction mixtures initially containing cyanamide and amino acids was determined by spotting aliquots onto filter paper, drying, and treating with ninhydrin–cadmium reagent and phenanthraquinone, respectively. The latter process involves dipping the paper into a solution made from equal volumes of 0.02% (*w/v*) phenanthraquinone in ethanol and 10% (*w/v*) sodium hydroxide in 60% aqueous ethanol, drying, and then examining for fluorescence under a u.v. lamp at a wavelength of 366 nm [16].

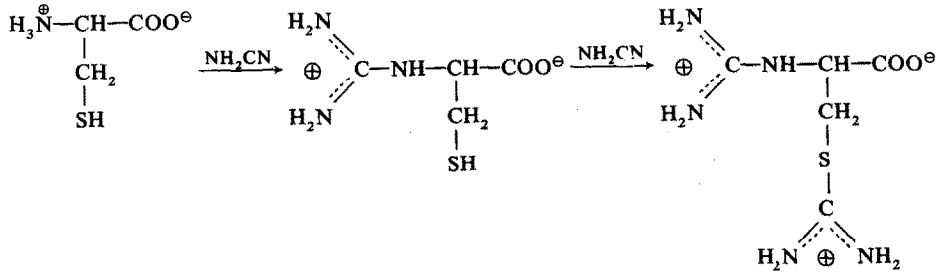
In vivo *studies*. Male albino Sprague–Dawley rats were injected i.p. with AET (200 mg/kg), thiourea (50 mg/kg) or saline for 5 consecutive days. During this time they had free access to a standard laboratory chow (Massey Mouse Diet, '77) and water *ad lib* and were maintained at 25° in a 12 h light/dark cycle. Approximately 24 hr after the last injection the rats were killed by cervical dislocation. Their livers were rapidly removed, rinsed with 0.01 M phosphate buffer (pH 7.3) containing 0.1 M KCl, blotted with absorbent paper and weighed. The livers were then homogenised in the same buffer (ice-cold) to yield 20% (*w/v*) homogenates which were subjected to 90 min centrifugation at 22,000 *g* and 0–4° to obtain the cytoplasmic fraction. Aliquots of the resulting supernatants were used for the measurement of aldehyde dehydrogenase activity at pH 7.3 as previously described [12, 14], except that the assay mixture contained pyrazole (33 mg/l) to inhibit interfering alcohol dehydrogenase activity. Alcohol dehydrogenase was assayed at 25° using 0.1 M potassium phosphate buffer (pH 7.3), 3.0 mM NAD^+ and 10 mM ethanol. Lactate dehydrogenase was assayed according to the method of Bergmeyer *et al.* [17].

Mitochondrial pellets were suspended in 5 ml ice-cold 0.01 M phosphate buffer (pH 7.3). The suspensions were sonicated for two 1 min periods and then centrifuged at 30,000 *g* for 90 min. The supernatants were assayed for aldehyde dehydrogenase as before, and glutamate dehydrogenase according to the method of Schmidt [18].

Protein concentrations were determined by the method of Bradford [19].

RESULTS

Reaction of cyanamide with amino and thiol groups. The reaction of cyanamide with amino and thiol groups at physiological pH was investigated and the results are presented in Table 1. The disappearance of these groups was followed by the use of ninhydrin and 5,5′-dithiobis-(2-nitrobenzoic acid) respectively, and the appearance of guanidino groups was detected by the formation of a fluorescent product with phenanthraquinone (as described above). It was found that the isothiouronium grouping also responded positively to the guanidino test. At the lowest concentrations studied (1 mM), little or no reaction was detected between cyanamide and glycine or *N*-acetyl-L-cysteine, but some phenanthraquinone-positive product was formed from cyanamide and L-cysteine. At the higher concentrations (20 mM and 0.25 M) the results were more clear cut. Under these conditions, reactions between cyanamide and the amino and thiol groups of glycine and *N*-acetyl-L-cysteine respectively were readily observed and both groups of cysteine were shown to react. In the latter case, with equimolar reagents, it was predominantly the amino group which reacted; extensive reaction of the thiol group only occurred when a 2-fold excess of cyanamide was used. The following scheme illustrates the reactions involved:



(1)

Effect of cyanamide on aldehyde dehydrogenase in vitro. It can be seen from Table 2 that cyanamide will cause a substantial drop in the activity of sheep liver cytoplasmic aldehyde dehydrogenase under the conditions used, but only when a high concentration of cyanamide is employed (10–50 mM). Maintaining the enzyme in the presence of cyanamide overnight before assay is carried out results in no further inhibition than that observed when only a 30 min incubation period was used.

Effect of dicyandiamide and 2-aminoethylisothiouronium bromide hydrobromide (AET) on aldehyde dehydrogenase in vitro. Table 2 also shows that dicyandiamide and AET have little or no effect on cytoplasmic aldehyde dehydrogenase *in vitro* at a concentration of 1 mM.

Effect of formamidine disulphide on aldehyde dehydrogenase in vitro. The inactivatory effect of formamidine disulphide on sheep liver cytoplasmic aldehyde dehydrogenase is recorded in Table 3. Relatively low concentrations of formamidine disulphide (10–20 μM) have a marked effect on the enzyme activity; this effect is greater when enzyme and inactivator are premixed before the substrates are added (even for only 1 min) than when the addition of inactivator occurs after the enzymic reaction has been initiated. The magnitude of

Table 3. The effect of formamidine disulphide on sheep liver cytoplasmic aldehyde dehydrogenase *in vitro* at pH 7.3

Formamide disulphide (μM)	Time of addition of inactivator to enzyme, before or after substrates	Enzyme activity (per cent of control rate) Expt. 1*	Expt. 2*
1	1 min before	94	
1	10 min before	74	
2	1 min before		84, 85
5	1 min before		73, 73
5	10 min before		74
5	20 min before		75
5	1 min after		84, 81
10	1 min before	11, 13, 12	
10	10 min before	16, 20	
10	1 min after	56, 56	
10	1 min before		34, 39
10	20 min before		43
10	1 min after		62, 67
20	1 min before		11
20	1 min after		45

* These two series of experiments were performed on different occasions with the same enzyme preparation.

the effect is not very reproducible (see the data obtained on different occasions with 10 μM formamidine disulphide in Table 3); this may be due to the instability of the disulphide in aqueous solution. Stock solutions of this compound were found to deposit a precipitate of sulphur when left standing. Neims *et al.* [20] commented that formamidine disulphide is a potent inactivator of D-amino acid oxidase, but gave no quantitative details because of the reagent's spontaneous decomposition.

A moderate excess of 2-mercaptoethanol (100 μM) was found to abolish the inactivatory effect of 20 μM formamidine disulphide if these reagents were mixed before the addition of enzyme and substrates, but not to reverse the inactivation when the 2-mercaptoethanol was added after the enzyme and formamidine disulphide were mixed. However, a much higher concentration of 2-mercaptoethanol (0.4 M) does largely reverse the inactivation caused by 20 μM formamidine disulphide. These results are very similar to those found with disulfiram [13].

Effect of thiourea and 2-aminoethylisothiouronium bromide hydrobromide (AET) on aldehyde dehydrogenase in vivo. In the first attempt to carry out this experiment, half of the rats (average weight 290 g) died after the first injection of thiourea (50 mg/kg), apparently from pulmonary congestion. The remaining half survived five consecutive daily injections with no adverse symptoms except reduced weight gain. In view of a report that thiourea is less toxic to rats under 2 months of age [21], the experiment was repeated with younger animals (average weight 125–215 g); in this instance all the animals survived and the following results were obtained.

Table 4 shows that thiourea (50 mg/kg) and AET (200 mg/kg) have no effect on rat liver cytoplasmic aldehyde dehydrogenase *in vivo*. Alcohol dehydrogenase is also unaffected but there is a slight decrease in lactate dehydrogenase activity in both cases. Both drugs cause a small but significant decrease in mitochondrial aldehyde dehydrogenase activity; however, this is not specific for aldehyde dehydrogenase—the results for glutamate dehydrogenase are virtually identical.

DISCUSSION

Many drugs and chemicals have been found to cause unpleasant reactions when ingested prior to the consumption of alcohol [1, 22]. Some such compounds (e.g. disulfiram or Antabuse, and cyanamide) are used deliberately in alcoholism therapy to curtail drinking; others, which are administered for unrelated medical reasons, may cause quite accidental reactions to alcohol. In general, although several other factors may be involved, the main mode of action of such compounds is thought to be through a blockage in the normal metabolism of acetaldehyde resulting in unusually high concentrations of this toxic compound. An extensive literature concerning the inhibitory effect of disulfiram on hepatic aldehyde dehydrogenase has recently been reviewed [1]. However, cyanamide has not received such detailed attention, although, as mentioned in the Introduction, its effect on aldehyde dehydrogenase and acetaldehyde levels *in vivo* is well established. It was the aim of the present study to investigate various possibilities for the chemical basis of the action of cyanamide.

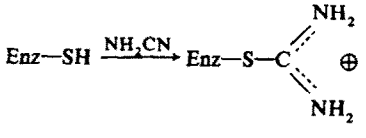
Cyanamide may be administered as such (NH_2CN) or as a combination of the calcium salt and citric acid. It is known to react with amines and thiols to give guanidines and isothiouronium compounds respectively [23]. The results in Table 1 show that such reactions occur readily at physiological pH and, bearing in mind the susceptibility of aldehyde dehydrogenase to thiol

Table 4. The effect of thiourea and 2-aminoethylisothiouronium bromide hydrobromide (AET) on rat liver aldehyde dehydrogenase and some other enzymes *in vivo*

	Cytoplasmic enzymes		
	Aldehyde dehydrogenase (nmoles/min/mg)	Alcohol dehydrogenase (nmoles/min/mg)	Lactate dehydrogenase (μmoles/min/mg)
Control (9)	2.24 ±0.23	6.83 ± 0.68	2.69 ± 0.17
Thiourea (15)	2.14 ± 0.11	6.74 ± 0.31	2.27 ± 0.09
AET (6)	2.15 ± 0.23	6.82 ± 0.25	2.30 ± 0.07
	Mitochondrial enzymes		
	Aldehyde dehydrogenase (nmoles/min/mg)	Glutamate dehydrogenase (μmoles/min/mg)	
Control (8)	24.2 ± 1.6	0.353 ± 0.035	
Thiourea (9)	17.9 ± 1.0	0.264 ± 0.028	
AET (9)	20.4 ± 1.2	0.302 ± 0.017	

Thiourea (50 mg/kg) and AET (200 mg/kg) were administered i.p. for 5 consecutive days. Enzymes were assayed as described under Materials and Methods. Measures of variability are S.E.M. Figures in brackets are the numbers of animals used in each experiment.

reagents such as disulfiram, it might be proposed that the enzyme would be inactivated by cyanamide according to scheme 2:



(2)

Accordingly, the effect of cyanamide on cytoplasmic aldehyde dehydrogenase *in vitro* was studied (see Table 2). It was found that only unrealistically high concentrations of cyanamide (10–50 mM) have an appreciable inhibitory effect. (The usual dose of citrated calcium cyanamide is 50–100 mg, but about 20 g of cyanamide would be necessary to give a concentration of 10 mM in the body-water compartment of the average person.) Thus direct action according to scheme 2 cannot explain the *in vivo* effects of cyanamide; presumably they must be mediated through some metabolite. This conclusion was also reached by Deitrich *et al.* [8] who could demonstrate no inhibition of purified beef liver aldehyde dehydrogenase by cyanamide. With 1.6 mM cyanamide, Ando and Fuwa [24] found uncompetitive inhibition of the partially purified beef liver enzyme, but the magnitude of the effect was small (less than 20%).

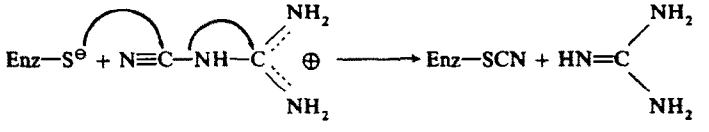
Cyanamide is known to dimerise slowly when left standing to form 'dicyandiamide' (cyanoguanidine). This process is rapid at alkaline pH. It might be proposed that the dimer is the active species *in vivo*, perhaps according to the hypothetical reaction in scheme 3:

(The bond broken in this reaction may be considered as 'energy-rich' by analogy with creatine phosphate.) However, dicyandiamide (1 mM) was shown to have a negligible effect on aldehyde dehydrogenase *in vitro* (see Table 2) and Ando and Fuwa [24] state that cyanamide loses its pharmacological action on dimerisation.

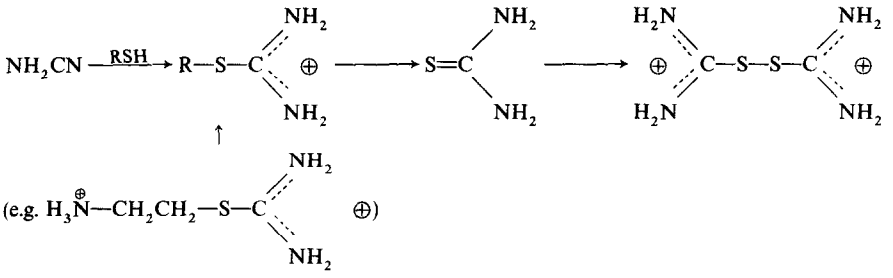
Recently it was reported that repeated administration of AET to rats causes a reduction in the voluntary intake of ethanol and a significant inhibition of aldehyde dehydrogenase *in vivo* [25]. On this basis, a possible mode of action of cyanamide *in vivo* might be through its metabolism to isothiouronium compounds. (As discussed above, such compounds are formed *in vitro* at physiological pH from cyanamide and thiols.) However, once again, Table 2 shows that purified aldehyde dehydrogenase is not significantly affected by AET (1 mM).

At this point, we hypothesised that the following sequence of reactions (scheme 4) may explain the effects of both cyanamide and AET *in vivo*.

In this sequence the breakdown of isothiouronium compounds to thiourea and the subsequent oxidation of thiourea to formamidine disulphide is envisaged. By analogy with other disulphides such as disulfiram (tetraethylthiuram disulphide), it was thought that formamidine disulphide might prove to be a potent inactivator of aldehyde dehydrogenase. Table 3 records the effect of this compound on cytoplasmic aldehyde dehydrogenase *in vitro*; an appreciable reduction in activity is caused by moderately low concentrations of the disulphide (10–20 μM). However, it must be pointed out that the concentration of disulfiram necessary to achieve a similar effect is smaller by at least an order of magnitude, and that the usual daily dosage of citrated



(3)



(4)

calcium cyanamide is only 50–100 mg, compared to 200 mg or more for disulfiram.

In order to gain more evidence on the possible involvement of scheme 4 in the cyanamide–ethanol reaction, the effect of thiourea on aldehyde dehydrogenase *in vivo* was examined. As stated above (and see Table 4) there was no significant inhibition of the cytoplasmic enzyme and only a small diminution in the mitochondrial activity (≃26%). The same extent of activity loss was also found with glutamate dehydrogenase. Thus it seems clear from this small, non-specific effect that thiourea cannot in fact be involved in the physiological effects of cyanamide.

A further point which is obvious from Table 4 is that, in our hands, AET does not reduce the activity of cytoplasmic aldehyde dehydrogenase *in vivo*. This result is in contrast to that of Messiha *et al.* [25], whose experimental protocol we followed, and who found a 46% inhibition. We have no explanation for this discrepancy. A small inhibition of the mitochondrial enzyme was found in the present work (~16%), but again this was parallelled by the effect on glutamate dehydrogenase.

In summary, although the present results have not established the mode of action of cyanamide *in vivo*, they do enable certain possibilities to be excluded. Thus it appears that aldehyde dehydrogenase is not directly affected by cyanamide itself, by dicyandiamide, or by AET; nor is the enzyme inhibited by a pathway involving thiourea and formamidine disulphide. Deitrich *et al.* [8], isolated, but did not identify, a urinary metabolite of cyanamide; however, this compound had no effect on aldehyde dehydrogenase *in vitro*. The biochemical basis of the cyanamide–ethanol reaction remains therefore an intriguing mystery. A further complication is that one of the characteristic symptoms (hypotension) of the *disulfiram*–ethanol reaction has been explained by an inhibition of dopamine-β-hydroxylase [26]. Cyanamide patients who drink also experience hypotension [27], but as yet the action of cyanamide or its metabolites on dopamine-β-hydroxylase has not been studied. Interestingly, it has recently been reported that ethanol and coprine (the pharmacologically active principle of *Coprinus atramentarius*) also produce hypotension, but this occurs without any inhibition of dopamine-β-hydroxylase [28].

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