The presence of an aldehyde dehydrogenase inhibitor in animal diets and its effects on the experimental results in alcohol studies

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1. The effects of chronic ethanol administration on the metabolism of ethanol and acetaldehyde were studied in rats fed on a commercial diet containing an aldehyde dehydrogenase inhibitor, calcium cyanamide (calcium carbimide), as a contaminant in the calcinated bone-meal fraction of the diet.

2. Rats given an ethanol solution (150 ml/l) for 3 months and fed on a diet containing calcinated bone meal showed two times higher activity of the low-K_m acetaldehyde dehydrogenase in the liver, 26 % higher rate of ethanol elimination, and two to three times lower acetaldehyde levels in blood during ethanol elimination compared with control rats pair-fed on the same diet.

3. The results obtained from the ethanol-treated rats were similar to those obtained in experiments on control rats fed on diets not containing calcinated bone meal.

4. Experiments performed in vitro and in vivo on the inhibition of the acetaldehyde metabolism by cyanamide suggested that the apparent effects of chronic ethanol intake were really caused by calcium cyanamide in the diet.

An aldehyde dehydrogenase inhibitor has recently been found in a commercial standard diet for small animals (Tottmar & Marchner, 1975; Marchner & Tottmar, 1976*a*, *b*). The inhibitor was detected in the calcinated bone-meal fraction of the diet, and it was isolated and identified as a cyanamide derivative (probably calcium cyanamide). Rats fed on diets containing calcinated bone meal showed a markedly decreased ethanol and acetaldehyde-oxidizing capacity due to the inhibition of the acetaldehyde dehydrogenase activity in the liver.

Feeding animals on diets containing calcinated bone meal may cause serious misinterpretations of the results obtained in alcohol studies. In the present study, it will be shown that the increase in the acetaldehyde- and ethanol-oxidizing capacity found after long-term ethanol treatment of rats fed on such a diet was caused by the presence of calcium cyanamide in the diet.

MATERIALS AND METHODS

Animals and diets

Female Sprague–Dawley rats, obtained from Anticimex, Sollentuna, Sweden, were used. At the beginning of the ethanol feeding study, the body-weights of the ethanol-treated and the pair-fed control rats were 70–75 g. In all other experiments rats weighing 200–250 g were used.

Before arriving at this Institute, the rats had been fed on a standard diet containing calcinated bone meal (diet 1 A), obtained from Astra-Ewos Co., Södertälje, Sweden. The ethanol-treated rats and the pair-fed control rats (eight rats in each group) were given this diet throughout the study. Another group of fourteen control rats, not pair-fed with the ethanol-treated rats, were fed on a diet (diet 1 B) of exactly the same composition as diet 1 A, except for the replacement of calcinated bone meal by calcium phosphate. Rats used in experiments involving parenteral administration of calcium cyanamide and ethanol were

fed on a diet not containing calcinated bone meal (the control diet), obtained from Anticimex, Sollentuna, Sweden. The rats were fed on the control diet or diet 1B for at least 2 weeks before the experiments. Details of the diets have been given previously (Marchner & Tottmar, 1976*a*, *b*).

Ethanol treatment

Eight rats were given an ethanol solution (150 ml/l) as the only drinking fluid for 3 months and had free access to diet 1A. Eight pair-fed controls were given a daily amount of diet 1A corresponding to the daily total energy intake in the ethanol-treated rats. The ethanol-treated rats consumed approximately 25% of their total energy intake in the form of ethanol. At the end of the treatment, four rats in each group were used to determine the rate of ethanol elimination and the acetaldehyde level in blood (see below). Thereafter, all rats in each group were killed and the livers were removed for measurements of enzyme activities.

Enzyme assays

The low- and high-Michaelis constant (K_m) acetaldehyde dehydrogenases in liver homogenates were assayed spectrophotometrically at 25° by measuring the reduction of NAD^+ at 340 nm as detailed previously (Tottmar & Marchner, 1976). The alcohol dehydrogenase (*EC* 1.1.1.1) activity in liver homogenates was assayed at 25° using acetaldehyde as the substrate, as described previously (Tottmar, Pettersson & Kiessling, 1973).

The inhibition experiments in vitro were performed on the low-K_m acetaldehyde dehydrogenase partially purified by ammonium sulphate fractionation of a matrix + intermembrane fraction isolated from rat-liver mitochondria (Tottmar & Marchner, 1975).

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin as a standard.

Parenteral administration of calcium cyanamide and ethanol

Calcium cyanamide (Cyanamide Ltd, Montreal, Canada) was prepared as a suspension in gum arabicum (Acacia; 50 g/kg) by sonic irradiation and was given by stomach tube at doses of 10 and 30 mg/kg body-weight. Ethanol was given intraperitoneally as a solution in saline (9 g sodium chloride/l) (78 g/l) at a dose of 1·50 g/kg body-weight.

Determination of ethanol and acetaldehyde in blood

Experiments on rats given ethanol chronically were, in general, started 5–6 h after withdrawal of the drinking bottles, and in all cases, at approximately 3 h after the ethanol concentration in blood had decreased to zero. Blood samples were taken from the tip of the tail. Acetaldehyde was determined fluorimetrically with the use of rat-liver aldehyde dehydrogenase, and ethanol was determined spectrophotometrically with yeast alcohol dehydrogenase, as described previously (Tottmar & Marchner, 1976). The rate of ethanol elimination was calculated as described by Wallgren & Barry (1970).

A statistical treatment of the results was made using Student's *t* test.

RESULTS

Effects of long-term ethanol feeding on the metabolism of ethanol and acetaldehyde in rats fed on a diet containing calcinated bone meal

The rate of ethanol elimination was 26% higher ($P < 0.001$) and the acetaldehyde level in peripheral blood was two to three times lower ($P < 0.05$) in rats given an ethanol solution (150 ml/l) for 3 months as compared to pair-fed control rats (Fig. 1).

The activities of the low- and high-K_m acetaldehyde dehydrogenases in the liver were 138

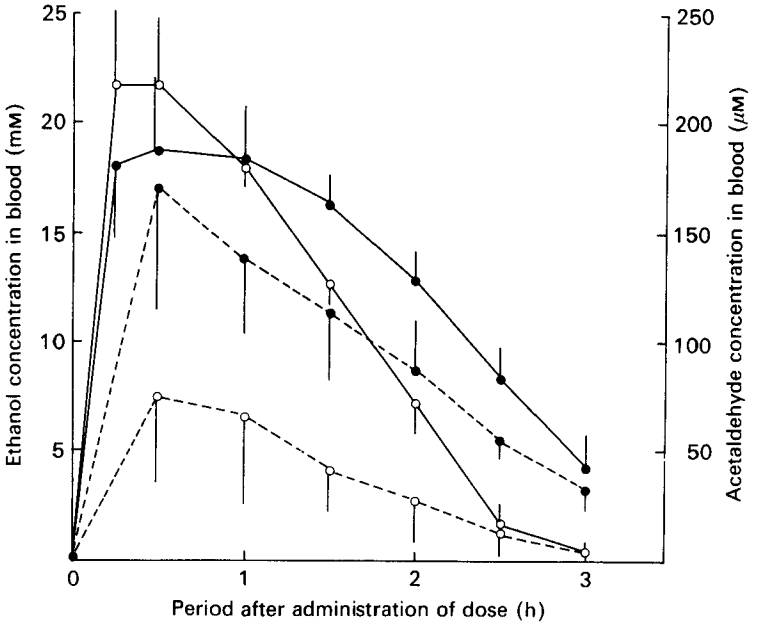

Fig. 1. Ethanol (——) and acetaldehyde (– – –) levels in peripheral blood from ethanol-treated rats (○) and control rats (●) fed on a diet contaminated with calcium cyanamide. Ethanol was given intraperitoneally at a dose of 1·50 g/kg body-weight 3 h after the blood ethanol concentration in the ethanol-treated rats had decreased to zero. Each point represents the mean value for four rats, and the vertical bars represent the standard deviations.

and 40% higher respectively ($P < 0{\cdot}001$) in the ethanol-treated rats as compared to the control rats (Table 1). The alcohol dehydrogenase activity in liver homogenates was similar in the two groups (Table 1).

The results obtained from the ethanol-treated rats fed on diet 1 A were similar to those obtained in experiments on control rats fed on diet 1 B (calcinated bone meal replaced by calcium phosphate) (Table 1), or another commercial standard diet (the control diet) used in previous studies (Marchner & Tottmar, 1976*a*, *b*).

Effects of calcium cyanamide on the acetaldehyde metabolism in intoxicated rats

In this experiment, the rats had been fed for at least 2 weeks on a control diet not containing calcinated bone meal, and they had not been subjected previously to ethanol treatment. Administration of a single oral dose of calcium cyanamide (30 mg/kg body-weight) caused a marked decrease in the activity of the low- and high-K_m acetaldehyde dehydrogenases in the liver (89 and 70% inhibition, respectively, compared with saline-treated controls) (Table 2). Much lower inhibition was found in rats given an intraperitoneal injection of ethanol (1·5 g/kg body-weight) 15 min before the administration of calcium cyanamide (35 and 11% inhibition, respectively, compared to ethanol-treated controls). The differences in inhibition between these two groups were statistically significant ($P < 0{\cdot}01$ and $P < 0{\cdot}001$ respectively). The acetaldehyde dehydrogenase activity in ethanol-treated control rats was similar to that found in untreated or saline-treated control rats.

The acetaldehyde level in peripheral blood in rats given ethanol 15 min before calcium cyanamide (10 mg/kg body-weight) was similar to that found in rats given ethanol only (20–50 μM), and no increase in the level was found during the period of ethanol elimination (4 h), whereas in rats given calcium cyanamide 15–60 min before ethanol, the acetaldehyde

Table 1. *Effects of long-term ethanol feeding on the metabolism of acetaldehyde and ethanol in rats fed on a diet containing calcinated bone meal*

(Mean values and standard deviations; numbers of animals used are given in parentheses)

Experimental group*	Acetaldehyde dehydrogenase activity (nmol NADH formed/min per mg protein)		Alcohol dehydrogenase (*EC* 1.1.1.1) activity (nmol NADH oxidized/min per mg protein)	Acetaldehyde level in blood (μM) Time after ethanol administration		Rate of ethanol elimination (mg ethanol/h per kg body-wt)
	Low-K_m enzyme	High-K_m enzyme		30 min	90 min	
Diet containing calcinated bone meal						
Pair-fed control rats	2·6±0·8 (8)	10·6±1·0 (8)	85·3±5·6 (8)	170±54 (4)	114±32 (4)	335±14† (4)
Ethanol rats	6·2±0·3§ (8)	14·8±2·2§ (8)	85·4±7·8 (8)	74±40‡ (4)	40±19‡ (4)	453±23†§ (4)
Diet containing no calcinated bone meal						
Control rats	6·8±0·7 (14)	13·4±1·3 (14)	—	52±16 (7)	45±13 (7)	478±41 (7)

K_m, Michaelis constant.

* For details of treatments and diets, see p. 317.

† Calculated from the ethanol-elimination curves shown in Fig. 1.

‡ $P < 0.05$, § $P < 0.001$ for differences between ethanol rats and the pair-fed control rats.

Table 2. *Inhibition of the acetaldehyde dehydrogenase activity by cyanamide in livers of rats pretreated with ethanol*

(Mean values and standard deviations for six rats/group)

Experimental group*	Acetaldehyde dehydrogenase activity† (nmol NADH formed/min per mg protein)	
	Low-K_m enzyme	High-K_m enzyme
Saline	6·8±0·5	12·2±1·4
Saline+calcium cyanamide	0·8±0·3	3·6±0·5
Ethanol	7·1±0·7	13·2±1·0
Ethanol+calcium cyanamide	4·6±2·4‡	11·8±1·6§

K_m, Michaelis constant.

* Calcium cyanamide (30 mg/kg body-weight) or saline (9 g sodium chloride/l) were given per os 15 min after a single intraperitoneal injection of ethanol (1·50 g/kg body-weight) or saline.

† The acetaldehyde dehydrogenase activity was measured in liver homogenates 3 h after the start of the experiment.

‡ $P < 0·01$, § $P < 0·001$ for difference between the inhibitions due to cyanamide with saline and cyanamide with ethanol.

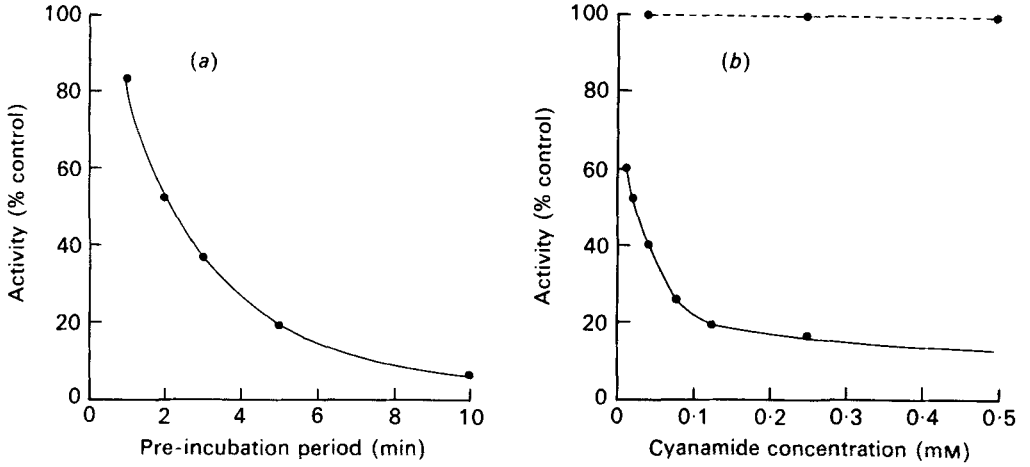

Fig. 2. Inhibition of the low-K_m acetaldehyde dehydrogenase from rat liver by cyanamide in vitro. The enzyme preparation was preincubated for various times at 23° in 50 mM-sodium pyrophosphate buffer (pH 8·8), containing 0·5 mM-NAD^+, in the presence or absence of cyanamide. (*a*) The activity after preincubation with inhibitor (0·1 mM) for 1–10 min before the addition of acetaldehyde (0·025 mM); (*b*) (——), the activity at different inhibitor concentrations after a preincubation period of 5 min; (– – –), the activity 5 min after the addition of cyanamide to the reaction mixture already containing acetaldehyde (0·025 mM). The reaction rates were linear for at least 10 min, and in the absence of the inhibitor, no decrease in the activity was observed during the course of the reaction.

level was several fold higher (200–300 μM). These results showed that the inhibition of the acetaldehyde dehydrogenases by cyanamide was prevented during the elimination of ethanol.

Inhibition of the acetaldehyde dehydrogenase activity by cyanamide in vitro

The inhibition experiments were performed on a semi-purified preparation of the low-K_m acetaldehyde dehydrogenase isolated from rat-liver mitochondria. Cyanamide was found to be a potent inhibitor of the enzyme. A progressive decrease in the activity was observed when the enzyme and the inhibitor (0·10 mM) were preincubated for various periods before

the addition of acetaldehyde (0·025 mM) to the reaction mixture (Fig. 2*a*). At an inhibitor concentration of 0·02 mM the inhibition was 50% after a preincubation time of 5 min (Fig. 2*b*). No further decrease in the activity was observed after the addition of acetaldehyde. Similarly, no inhibition was found when cyanamide at a concentration of 0·02–1 mM was added to the reaction mixture already containing acetaldehyde (0·025 mM), and the activity did not decrease during the course of the reaction (5–10 min) (Fig. 2*b*). When all acetaldehyde had been used up in the reaction, the activity decreased during the incubation period before a new addition of acetaldehyde. These results showed that acetaldehyde protected the enzyme from inhibition by cyanamide in vitro. However, the inhibition obtained could not be reversed by acetaldehyde. Ethanol did not protect the enzyme from inhibition by cyanamide.

DISCUSSION

Moench (1950) reported that animal charcoal contained an alcohol-sensitizing compound. Clark & Hulpieu (1958) compared the effects of animal charcoal with those of disulfiram (Antabus®) and cyanamide (the active component of Temposil®), which are known to evoke a hypersensitivity to alcohol (Truitt & Walsh, 1971). Although they found that the onset and duration of action of animal charcoal and cyanamide were similar, they could not detect any cyanamide in their preparations. In our laboratory, however, it has recently been shown that calcinated bone meal, which has a composition very similar to the animal charcoal used by Clark & Hulpieu (1958), contains a cyanamide derivative (probably calcium cyanamide), and that this compound is formed when animal bone is heated at high temperatures (Marchner & Tottmar, 1976*b*). It has been known for many years that cyanamide evokes a hypersensitivity to alcohol (Koelsch, 1914; Ferguson, 1956). Calcium cyanamide (Temposil®) is used in the treatment of chronic alcoholics (Ferguson, 1956). Cyanamide is a potent aldehyde dehydrogenase inhibitor (Deitrich & Worth 1968; Deitrich, Troxell, Worth & Erwin, 1976; Tottmar, Marchner & Lindberg, 1977) and causes an accumulation of acetaldehyde during ethanol oxidation (Warson & Ferguson, 1955).

It was shown in a previous study (Marchner & Tottmar, 1976*b*) that a diet containing 20 mg of calcinated bone meal/g caused a marked decrease in the acetaldehyde dehydrogenase activity in rat liver. The same decrease was found in rats given a diet containing 0·2 mg calcium cyanamide/g, indicating that calcinated bone meal contained approximately 10 mg calcium cyanamide/g.

Calcinated bone meal is widely used in animal diets as a source of calcium and phosphorus. Rats fed on a diet containing calcinated bone meal showed a decreased capacity to oxidize acetaldehyde and ethanol, low acetaldehyde dehydrogenase activity in the liver, and a low sensitivity to disulfiram treatment (Marchner & Tottmar, 1976*a*, *b*; Tottmar *et al.* 1977). The lowered rate of ethanol oxidation was not caused by inhibition of alcohol dehydrogenase activity in the liver, but could probably be ascribed to the high level of acetaldehyde during ethanol oxidation and the kinetic characteristics of this enzyme, which favour the backward reaction, i.e. the reduction of acetaldehyde to ethanol (Tottmar & Marchner, 1975).

The present study shows the effects of long-term ethanol treatment in rats fed on a diet containing calcinated bone meal. It was found that long-term ethanol feeding caused a marked increase in the acetaldehyde dehydrogenase activity, accompanied by an increase in the rate of ethanol elimination and a decreased level of acetaldehyde during ethanol metabolism (Fig. 1, Table 1). However, the results obtained from the ethanol-treated rats were similar to those observed in control rats given a diet not containing calcinated bone meal, and furthermore, it had previously been found in this laboratory, that daily administration of ethanol to rats for 3 weeks actually caused a slight decrease in the acetaldehyde dehydrogenase activity (Tottmar, Kiessling & Forsling, 1974), and similar results have been reported

from other laboratories (Majchrowicz, Lipton, Meek & Hall, 1968; Koivula & Lindros, 1975). These facts prompted us to study whether the results obtained could be explained by the presence of calcium cyanamide in the diet.

Cyanamide was found to be a potent inhibitor of the low-K_m acetaldehyde dehydrogenase in the liver both in vitro and in vivo. This enzyme is responsible for the main part of the acetaldehyde oxidation in rat liver, and the acetaldehyde level in the liver and blood during ethanol metabolism seems to be directly related to its activity (Tottmar & Marchner, 1975, 1976). It was found that acetaldehyde protected the low-K_m enzyme from inhibition by cyanamide in vitro, and a similar protection by acetaldehyde seemed to occur also in vivo, where it was found that cyanamide caused much weaker inhibition in intoxicated rats than in control rats (Table 2). These results suggested that the observed increase in the acetaldehyde dehydrogenase activity in rats after long-term ethanol treatment was not caused by a true induction of the enzyme activity, but merely by a protection of the enzyme from the action of cyanamide, due to the presence of acetaldehyde in their bodies.

During the last 3 weeks of the experiment, the rats consumed ethanol at a rate close to their maximum ethanol-oxidizing capacity (8–9 g/kg body-weight per d), which means that they had ethanol and acetaldehyde in their bodies for most of the day. The ethanol concentrations in blood in the mornings varied between 10 and 20 mM. The acetaldehyde level in the liver during ethanol metabolism was 50–150 μM, and this level is quite sufficient to saturate the low-K_m enzyme, which has a K_m for acetaldehyde in the μM range (Grunnet, 1973; Tottmar *et al.* 1973; Tottmar & Marchner, 1975, 1976).

Although the present study does not exclude the possibility that chronic ethanol feeding affects the metabolism of ethanol and acetaldehyde in rats fed on a normal diet, it appears, however, quite conceivable that the dramatic effects observed in the present study were mainly caused by the presence of calcium cyanamide in the diet. It is also conceivable that the use of diets containing calcinated bone meal may cause serious misinterpretations of the experimental results in alcohol studies, and may also explain some of the controversial opinions about the metabolism and pharmacology of acetaldehyde (see Deitrich & Siew, 1974; Deitrich & Erwin, 1975; Tottmar & Marchner, 1975).

In the authors' laboratory, the diet containing calcinated bone meal had been used for 2 weeks when the inhibitor was detected. However, this diet and similar diets have been used in many laboratories for several years. It should also be mentioned that calcinated bone meal is the main component in several commercial mineral–vitamin preparations for dogs and humans.

The toxicology of cyanamide has been investigated by Benitz, Kramer & Dambach (1965), who found that feeding rats on a diet containing calcium cyanamide (calcium carbimide) at a concentration increasing from 0·5 to 2 mg/g during 14 weeks caused a general inhibition of growth, oedema, liver-cell necrosis and hypothyroidism accompanied by morphological changes in the pituitary gland, adrenal cortex and testes. These authors stated that the morphological signs caused by calcium cyanamide have to be regarded as potentially harmful side-effects.

As mentioned previously, the diet used in the present study was estimated to contain 0·2 mg calcium cyanamide/g, which, when considering the results reported by Benitz *et al.* (1965), should be regarded as too high to be neglected. To our knowledge, and according to the manufacturer of the diet, no abnormalities in rats fed on this diet have been reported, except for those observed in alcohol studies. However, it has been shown that calcium cyanamide interferes with the metabolism of neurotransmitters in the brain (Truitt & Walsh, 1971). It is possible, therefore, that the use of diets containing this inhibitor may affect the results in studies not only on the metabolism and pharmacology of ethanol and acetaldehyde, but also in studies on other drugs, biogenic amines and aldehydes.

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