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INHIBITION OF ALDEHYDE DEHYDROGENASE IN BRAIN AND LIVER BY CYANAMIDE

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Abstract—Cyanamide ($H_2N—C\equiv N$) is effective as an agent to treat alcoholism presumably because it inhibits aldehyde dehydrogenase. In this study it was found that, *in vivo*, cyanamide is a very potent inhibitor of liver aldehyde dehydrogenases, but less effective against the brain enzymes. The ED_{50} for liver was found to be 8 mg/kg when given intraperitoneally. The inhibition diminishes with time but is measurable for at least 24 hr, even though the bulk of the ^{14}C-labeled cyanamide is excreted within 6 hr. Cyanamide is not effective when added to the assay mixture *in vitro*, suggesting that a metabolite is the inhibitor *in vivo*. A urinary metabolite has been isolated and partially characterized. It is an acid with a p*Ka'* of 3.9 and an extinction coefficient of 1.72×10^3 in base at 219 nm; the compound apparently retains the cyano group. However, it does not inhibit the enzyme *in vitro*.

Cyanamide, as citrated calcium carbimide (Temposil), has been employed in man for treatment of alcoholism [1] because of its ability to increase blood acetaldehyde levels after ethanol intake [2] and also to attempt to block the formation of oxalic acid in hyperoxaluria [3, 4]. Previous studies have shown that inhibition of rabbit liver aldehyde oxidation could be obtained only after administration of cyanamide *in vivo* [5]. A preliminary report described attempts at determination of the structure of the urinary excretion product of cyanamide [6].

The recent reawakening of interest in acetaldehyde and its metabolism as a component of some of the actions of ethanol [7] and our desire to utilize an aldehyde dehydrogenase inhibitor for studies of alcohol withdrawal in mice [8] necessitated a more thorough investigation of the properties of cyanamide. This report describes the results of these studies.

MATERIALS AND METHODS

Cyanamide was obtained from Aldrich Laboratories and was recrystallized from dry ether and stored desiccated at −20°. The purity was ascertained by thin-layer chromatography on precoated cellulose or Silica gel plates (Distillation Products No. 6d5 or No. K301R). The solvent was butanol–ethanol–water, 90:20:10. Cyanamide, dicyandiamide, thiourea and urea were located by spraying the developed plates with an acidic solution of *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) as described by Milks and Janes [9]. Preparative plates of cellulose were prepared and developed in the butanol–ethanol–water solvent system.

Cyanamide, ^{14}C-labeled (47 mCi/m-mole), was purchased from International Chemical and Nuclear Corp. Ten μCi was dissolved in 1 ml of absolute ethanol and kept at −20°. A second lot of 100 μCi was packaged in 10-μCi lots in sealed glass ampules in ether with a trace of acetic acid. Only chromatographically homogenous [^{14}C]cyanamide, as indicated by radioautography, was used. Saline (1 ml) with or without carrier cyanamide was added to the labeled cyanamide and the material injected intraperitoneally into experimental animals. Small aliquots of the injected material were weighed from a syringe by difference and placed on planchets for thin window, gas flow counting. Other aliquots were spotted on thin-layer chromatograms along with aliquots of urine from the experimental animals which had received the labeled cyanamide. After development, the thin-layer chromatograms were air dried and overlaid with X-ray film and stored from 3 to 11 days depending upon the amount of radioactivity applied. The X-ray film was removed and developed and the chromatogram sprayed with Ehrlich's reagent.

Animal experiments. Sprague–Dawley rats (150–250 g) of both sexes were injected with 10 μCi/kg of ^{14}C-labeled cyanamide, as described above. In some experiments CO_2 was collected by drawing air through a metabolism chamber which contained the animal. The CO_2 was trapped in 1 N NaOH. Aliquots of the NaOH solution were added to 5 ml of saturated Ba $(OH)_2$ in centrifuge tubes. The $BaCO_3$ was washed three times with small amounts of H_2O, transferred to planchets, dried and counted. The metabolism chamber contained two screens to separate the urine from the feces. Aliquots of urine were applied directly to planchets, dispersed with ethanol, dried and counted.

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At the end of the experiment, the animals were anesthetized with ether and blood was drawn from the abdominal aorta into a heparinized syringe. Tissue and blood samples were weighed and frozen until analyzed for ^{14}C content. A combustion train was used to burn the tissue samples. The resultant CO_2 was collected in 1 N NaOH and converted to $BaCO_3$ and the radioactivity determined as described above.

Male DBA mice ($\approx$ 25 g) were obtained from Jackson Laboratories in Bar Harbor, Me., or from the Institute for Behavioral Genetics in Boulder, Colo. Animals were injected intraperitoneally with recrystallized cyanamide dissolved in normal saline. Brains and liver were taken at the times indicated in each table or figure and homogenized in 0.25 M sucrose containing 1% Triton X-100 to make 20% homogenates for liver and 30% homogenates for brain. When subcellular-fractionation studies were to be carried out, the Triton was omitted. The liver homogenates were centrifuged for 60 min and the brain homogenates for 90 min at 100,000 *g* and used immediately.

Subcellular fractionation of liver was carried out in 0.25 M sucrose by standard techniques. The mitochondrial and nuclear pellets were washed once and then resuspended in a volume of 0.25 M sucrose–1% Triton equal to the original wet weight of the tissue; the microsomal pellet was resuspended without washing in the same fluid in one half this volume. All these suspensions were then centrifuged at 50,000 *g* for 1 hr. Aliquots of the homogenate and the post-microsomal supernatant were made to 1% Triton and also centrifuged in the same manner. Aldehyde dehydrogenase was assayed spectrophotometrically utilizing 3.3 mM acetaldehyde, 1 mM NAD as substrate in 0.03 M pyrophosphate, pH 9.6. For liver 0.1 ml of the enzyme source in a 3-ml assay volume was used, while for brain 0.1 ml of the enzyme source in a 1-ml assay volume was employed. Protein was determined by the biuret method using bovine serum albumin as a standard.

Isolation and characterization of the cyanamide metabolite. Preparative plates of cellulose were streaked with urine containing the [^{14}C]cyanamide metabolite, and developed using the butanol–ethanol–water solvent. Standards of urea and cyanamide were included. Radioautograms were made of the plates. Ehrlich's reagent was used on a small portion of the plate to locate the urea and cyanamide standards. Exposed spots on the developed film were used to locate the radioactive metabolite on the plate. This band of material was scraped from the plate and eluted successively with water and ethanol. A band of similar size not containing ^{14}C label or Ehrlich-reacting material was used as a blank. The ultra-violet spectrum of the water-eluted material was determined in acid and base and revealed a strong absorption at 219 nm in base which disappeared in acid. The pK_a^1 and molar absorbency of the metabolite were determined by titration with standard acid and back titration with standard base by following the 219 nm absorption.

Paper electrophoresis of the metabolite was carried out in 0.1 M phosphate buffer, pH 7.0, for 20 min at 100 V. The ultraviolet spectrum of material isolated in this manner was determined.

To obtain larger quantities of the metabolite, urine containing the ^{14}C-labeled metabolite was applied to a 40 cm × 20 mm column of Amberlite IRA 401, H^+ form, and the column was washed with water. A linear gradient consisting of equal volumes of 0.15 N HCl in the reservoir and H_2O in the mixing flask was then started. Fractions were collected and aliquots taken for radioactivity determination and ultraviolet spectroscopic analysis. Tubes containing the radioactive metabolite were combined and the pH was adjusted to 10 with KOH. The solution was evaporated to near dryness *in vacuo*. This solution was then made acidic in the cold and extracted with ether several times until most of the 219 nm absorbing material was extracted into the organic phase. The ether was evaporated to a small volume and chilled. The frozen water layer was removed and the amount of metabolite present in the ether was approximated from the 219 nm absorption after extraction into base. The ether was extracted with a calculated amount of NaOH solution to remove the metabolite present. This material (pH 5.6) was then placed on a DEAE cellulose column (pH 7.0) and the column washed with water. The metabolite was eluted with 0.01 N NaCl, the pH of the eluate was adjusted to 10.0 and the solution concentrated, acidified and extracted with ether as above, then re-extracted into base. Large amounts of the metabolite were isolated in this manner by injecting 75 mg/kg of cyanamide IV into rabbits and collecting the urine for 24 hr. Metabolite from mice was obtained by injecting ten animals with 75 mg/kg and collecting urine for 24 hr. The isolation procedure was the same as described above. Dogs could not be used for this portion of the study, since they are very susceptible to the toxic effects of cyanamide.

RESULTS

Figure 1 presents the dose–response curves for inhibition of aldehyde dehydrogenase in mouse liver and brain. The marked difference in sensitivity of liver and brain enzyme is the most striking aspect of this study. In neither case, however, was complete inhibition obtained.

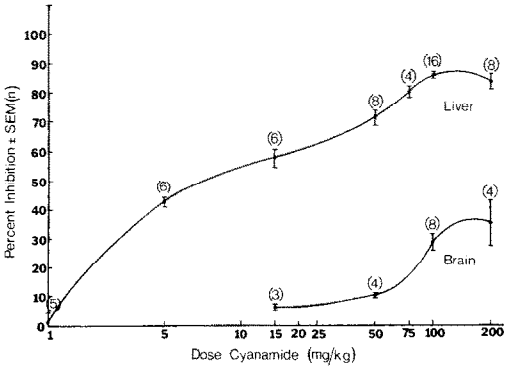

Fig. 1. Dose–response curve for inhibition of total mouse liver and brain aldehyde dehydrogenase by cyanamide given intraperitoneally 1 hr before death. Brackets are standard error and numbers in parentheses are the number of animals used.

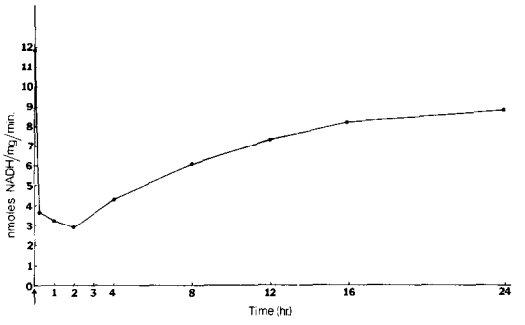

Fig. 2. Time course of inhibition and recovery of total mouse liver aldehyde dehydrogenase after intraperitoneal administration of cyanamide at 50 mg/kg given at 0 time (arrow). At least two animals were used at each time point. Activity at time zero was 11.8 nmoles NADH·mg protein^{-1}·min^{-1}.

The time course of inhibition in mice is presented in Fig. 2. The relatively short duration of the major part of the inhibition is contrasted by the very slow return toward control activity. Even after 48 hr the enzyme activity has not returned to control levels. This agrees with observations made by Koe and Tenen [10].

There are a number of aldehyde dehydrogenase enzymes in the mitochondrial, microsomal and supernatant fractions [11, 12]. The possibility of a differential inhibitory effect of cyanamide on one of these enzymes was next investigated. As shown in Table 1, there is a small but consistent difference in the inhibition of the supernatant enzyme activity as compared to inhibition of the mitochondrial and microsomal enzymes of mouse liver.

The relative immunity of the brain enzymes to injected cyanamide could be due to poor penetration of the active inhibitor into the brain. To circumvent this problem, cyanamide was injected directly into the lateral ventricle of the brain by the use of a Hamilton syringe. A small amount of blue dextran was added in order to verify placement of the injection. As shown in Table 2, however, when the amount of cyanamide which would be available to the brain, assuming complete distribution, was administered directly into the brain, no inhibition of the brain or liver enzyme could be detected. When the dose of cyanamide was comparable to that given peripherally, inhibition of only the liver enzyme was obtained and this inhibition was less than that observed when the same dose was given intraperitoneally.

Previously we had found that cyanamide was ineffective *in vitro* in inhibiting rabbit liver aldehyde dehydrogenase [5]. This was repeated for the mouse enzymes and again no significant inhibition was observed even at 10^{-3} M cyanamide. Mixtures of enzyme from an animal that had received cyanamide with similar enzyme from an animal that received no cyanamide, gave only additive results. Dialysis of liver homogenates from cyanamide-treated or control animals for 18 hr against large volumes of 0.25 M sucrose did not achieve reversal of inhibition.

Metabolite isolation. The ^{14}C label from injected cyanamide is rapidly excreted in the urine of rats (Table 3). By the end of 6 hr virtually all of the label appears in the urine. Negligible amounts of label are found in the expired CO_2. Only during hr 1 is there any significant amount of label in the liver and much less in the other tissues. [^{14}C]cyanamide that had been in aqueous solution for several days gave rise to large amounts of $^{14}CO_2$ in the expired air. Radioautograms made from thin-layer chromatography plates of the starting material after chromatography showed the presence of numerous radioactive spots but none that corresponded to the metabolite isolated from urine.

As is apparent from Table 4, the ^{14}C label in the urine is neither urea, dicyandiamide, thiourea nor cyanamide. In order to further show that the compound is not urea, urine was incubated with urease before spotting. Urea was destroyed by this process but the radioactivity remained unchanged. Differentiation of the metabolite from thiourea was further accomplished by use of a spot test for R_2—N—C≡N groups consisting of cuprous acetate and benzidine. This test was strongly positive on the metabolite [13].

The ultraviolet spectrum of the metabolite eluted from the chromatographic plate was determined. This material has an absorption peak at 219 nm. With the addition of acid, the peak disappears. This is true for the metabolite from cyanamide-treated rats, mice and rabbits and for the metabolite isolated from the urine of rabbits treated with a combination of cyanamide and ethanol. The elution patterns of radioactivity with 219 nm absorbing material from an Amberlite anion exchange column is shown in Fig. 3. The curve for material absorbing at 208 nm is also in-

Table 1. Subcellular distribution of liver aldehyde dehydrogenase and inhibition by cyanamide*

Fraction	Subcellular distribution		Inhibition	
	Per cent activity ± S. E. M.	(N)	Per cent ± S. E. M.	(N)
Homogenate	100	(8)	80.7 ± 1.2	(8)
Nuclei	25.3 ± 2.9	(4)	66.4 ± 5.4	(4)
Mitochondria	28.3 ± 2.6	(8)	70.0 ± 2.4	(8)
Supernatant	20.5 ± 2.7	(8)	90.4 ± 1.6	(8)
Microsomes	5.6 ± 0.6	(4)	79.0 ± 3.8	(4)
Recovery	99.7			

* Mice were injected intraperitonally with 75 mg/kg of cyanamide 1 hr before taking the livers. Subcellular fractionation was carried out on livers from both control and injected mice by standard differential centrifugation techniques. Triton (1%) was added to all fractions after separation to clear the solutions for assay of aldehyde dehydrogenase as detailed in the text.

Table 2. Intracranial injection of cyanamide into mice*

Treatment	Tissue	
	Liver (nmoles NADH/mg protein/min)	Brain (nmoles NADH/mg protein/min)
Control	12.3 ± 0.70 (6)	1.17 ± 0.09 (6)
Cyanamide (55.6 mg/kg)	3.88 ± 0.24 (6)	1.01 ± 0.06 (4)
Cyanamide (0.6 mg/kg)	11.75 ± 1.29 (6)	1.11 ± 0.08 (4)

* Each animal received 15 μl cyanamide intracranially into the lateral ventricle. The average dosages were calculated based on the weight of the animals. The cyanamide concentrations were 83 mg/ml (55.6 mg/kg for an average weight of 22.4 g) and 1 mg/ml (0.6 mg/kg for an average weight of 25 g). Tissues were taken 1 hr after treatment.

Table 3. Fate of administered [^{14}C]cyanamide

Time (hr after [^{14}C]cyanamide)	Percentage of administered counts*		
	Expired CO_2	Urine	Liver
1	0.33	67.8	6.7
4	0.39	83.3	0
6	1.39	93.9	0

* Cyanamide, ^{14}C-labeled, was injected intraperitoneally into rats (10 μCi/kg) and the ^{14}C content of expired CO_2, urine and various tissues was determined at the intervals given as outlined in the text. The radioactivity found in tissues other than the liver was negligible.

cluded. Since the major radioactive material and 219 nm absorbing material coincided in several different isolation procedures, it was possible to administer unlabeled cyanamide and identify the metabolite by its absorption at 219 nm and the disappearance of the peak when the pH was lowered to 2.0. Urine collected from animals that had not received cyanamide, or urine to which cyanamide was added, did not contain the material absorbing at 219 nm.

The behavior of the compound on paper electrophoresis, on anion exchange columns, in solvent extractions and its u.v. spectra, suggested that it was an acid. The pK_a^1 was found to be 3.92 and molar absorbency of the metabolite in base has an average value of 1.7×10^3. The calculation of the molar absorbency assumes that only one titratable group is present in the pH range 2–10. If more than one ionizable group is present, it is not apparent from the titration curves, which appear to be smooth.

The compound is stable in dilute acid or base for prolonged periods. However, evaporation *in vacuo* under acidic conditions invariably led to destruction of the ultraviolet absorption at 219 nm.

Neither the purified metabolite nor that in crude urine significantly inhibits rat or mouse liver NAD-linked aldehyde dehydrogenase *in vitro*.

DISCUSSION

There are no potent specific inhibitors of aldehyde dehydrogenase available. Disulfiram is a relatively potent inhibitor *in vitro* but it is not potent *in vivo* and lacks specificity [14]. While the specificity of cyanamide inhibition awaits further testing, it is a potent inhibitor *in vivo* of liver aldehyde dehydrogenase with an ED_{50} of 8 mg/kg or about 2×10^{-4} M, assuming complete distribution. After intraperitoneal injection our results show that, while there is a high concentration of [^{14}C]cyanamide in the liver immediately after administration, it is rapidly removed. However, the inhibitory effect is evident at least 24 hr later. Apparently the inhibition by cyanamide is essentially irreversible, since dialysis did not achieve reversal of the inhibition. Additionally, in experiments where liver homogenates from cyanamide-treated animals were mixed with similar preparations from control animals, only additive rates were observed. This would indicate the absence of a dissociable inhibitor in the homogenate from treated animals.

Table 4. R_f values of cyanamide, urea and the cyanamide metabolite with Silica and cellulose thin-layer chromatography*

Compound	Silica	Cellulose
Urea	0.38	0.28
Cyanamide	0.76	0.65
Cyanamide metabolite	0.42	0.50
Thiourea		0.44
Dicyandiamide	0.65	

* The compounds were spotted on thin-layer plates and developed in butanol–ethanol–water, 9:2:1. The radioactivity was detected by radioautography and then the plates were sprayed with Ehrlich's reagent.

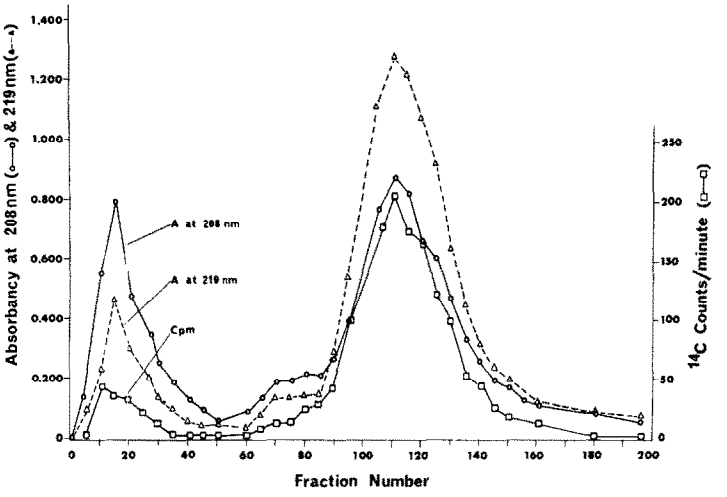

Fig. 3. Elution of [^{14}C]cyanamide metabolite from IRA 401 column.

The liver supernatant aldehyde dehydrogenase is more sensitive to inhibition than is the mitochondrial enzyme. Again the results of these experiments are consistent with a very tight binding of the inhibitor, since the mitochondria and nuclei were washed before solubilization. The inhibition remains high in these fractions, however. It is also of interest that we obtain a larger fraction of aldehyde dehydrogenase in the supernatant from the mice than is obtained in rats [12, 15]. The mitochrondria contain a low K_m aldehyde dehydrogenase which is most likely responsible for acetaldehyde oxidation during ethanol metabolism and also a smaller amount of a high K_m enzyme [12, 15]. The assay utilized in the study employed acetaldehyde concentrations that allow determination of the total activity in the mitochondria, although the majority of this activity is due to the low K_m enzyme.

It is apparent that a product of cyanamide and not cyanamide itself is responsible for the inhibition. This inhibitory molecule apparently cannot be formed in the brain nor gain easy access to it. Alternatively the brain enzyme may be less sensitive to inhibition. A urinary excretion product of cyanamide appears to be an acid with a low pK_a^1 and if the inhibitor also carries this group it would be expected to be excluded from the brain. To date, efforts of derivatization and analysis by gas chromatography–mass spectrometry have been unsuccessful.

Further studies are in progress to isolate and identify this inhibitor. Presumably it should be a very potent and effective inhibitor of the aldehyde dehydrogenase enzymes.

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