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DIFFERENTIAL INHIBITION OF RAT TISSUE CATALASE BY CYANAMIDE

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Abstract—The relative sensitivity of rat tissue catalase to inhibition by intraperitoneally administered cyanamide was liver > kidney > heart > brain, whereas the activity of the erythrocyte enzyme was affected minimally The measured ED_{50} values for cyanamide in these tissues were 31, 44, 107 and 680 μmoles/kg body weight for liver, kidney, heart and brain respectively On a molar basis, cyanamide was approximately twenty times more potent than 3-amino-1,2,4-triazole (3-AT) in inhibiting hepatic catalase *in vivo* in the rat Like 3-AT, cyanamide inhibited erythrocyte catalase activity *in vitro* in the presence of hydrogen peroxide The apparent similarities between the inhibition of hepatic catalase by cyanamide and 3-AT *in vivo* suggest that cyanamide belongs to the family of 3-AT-like catalase inhibitors.

Cyanamide, an alcohol deterrent agent§, is metabolized via two distinctly different pathways. The primary route which represents a detoxication/elimination pathway is catalyzed by an acetyl-S-CoA dependent *N*-acetyltransferase yielding acetylcyanamide, the major urinary metabolite of cyanamide [1]. The second, but minor, pathway is an activation process catalyzed by catalase [2–4]; however, the products of this latter reaction remain to be identified. Nevertheless, it is clear that one of these reaction products is a potent inhibitor of aldehyde dehydrogenase (AlDH‖), and this activated metabolite of cyanamide accounts for the inhibition of AlDH by cyanamide *in vivo*

In the bioactivation of cyanamide by catalase, catalase itself is inhibited [2, 3]. This report describes the inhibition of catalase activity in rat liver, kidney, heart, brain and erythrocytes by cyanamide administered *in vivo* and the time course for the recovery of catalase activity in these tissues The similarity between the *in vivo* inhibition of catalase by cyanamide and by 3-amino-1,2,4-triazole (3-AT), the classic *in vivo* inhibitor of catalase, is discussed.

MATERIALS AND METHODS

Materials. Glucose oxidase and cyanamide were purchased from the Sigma Chemical Co. (St. Louis, MO) The latter was prepared using isotonic saline as vehicle in dosage forms such that 1 0 ml of the injection solution was administered per 100 g rat body weight Male rats of Sprague–Dawley descent were obtained from the BioLab Corp. (St. Paul, MN) and maintained on a standard rat chow diet (Purina Ralston Co , St. Louis, MO) and water *ad lib.* until used. Reagents for protein determinations were purchased from the Pierce Chemical Co. (Rockford, IL).

In vivo *studies* The animals (weighing 269 ± 6 g) were fasted overnight before use. Cyanamide was administered intraperitoneally, and the rats were killed by exsanguination under ether anesthesia 1 hr later. Approximately 1.0 ml of whole blood was taken by open chest cardiac puncture for catalase measurement, after which the organs were perfused *in situ* by whole body perfusion using 300–500 ml of heparinized (1000 units/l) isotonic saline [5]. The saline solution was infused into the left ventricle, and a small incision was made in the right ventricle for the effluent. Perfusion was continued until the kidneys were visibly cleared of erythrocytes The brain, liver, kidney and heart were excised, and 10% homogenates of brain, kidney and heart were prepared with 0.1% Triton X-100 in 10 mM potassium phosphate buffer, pH 7.0 The liver homogenate (25%) was prepared with 0 25 M sucrose–0.1 mM EDTA, pH 7 5. All homogenates were stored at 0° and were assayed for catalase activity the same day

Tissue catalase activity was measured using a Yellow Springs Oxygen Monitor equipped with a Clark style oxygen electrode essentially as previously described [2]. The reaction cell was temperature controlled and maintained at 25°. A 0 01 mM potassium phosphate buffer, pH 7 0, (1.7 ml) was deoxygenated in the reaction cell with a stream of N_2 Hydrogen peroxide (7 6 μmoles in 10 μl) was added to the deoxygenated buffer at zero time, and the base line O_2 formation rate was recorded Then at 1 min, a 25-μl aliquot of diluted (or of undiluted for brain) tissue homogenate was added The difference

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§ Temposil, Dipsan, Abstem

‖ Abbreviations AlDH, aldehyde dehydrogenase, 3-AT, 3-amino-1,2,4-triazole, ED_{50}, dose of drug which produces 50% of maximal effective response

between the rate of O_2 formation before and after the addition of tissue homogenate was taken as the actual reaction rate. For analysis, the heart, kidney and liver homogenates were diluted 5-, 100- and 500-fold, respectively, with 0.1% Triton X-100 in 10 mM potassium phosphate buffer, pH 7 0, while the blood was diluted 500-fold with 10 mM potassium phosphate buffer, pH 7 0 The dilutions were made immediately before analysis Tissue catalase activity is expressed in units of (a) nmoles O_2 formed per min per μg protein and (b) mmoles O_2 formed per min per g wet weight tissue. Erythrocyte catalase activity is expressed in units of nmoles O_2 formed per min per μg hemoglobin Hemoglobin was measured spectrophotometrically using Drapkin's reagent. Protein was determined using the bicinchoninic acid reagent with bovine serum albumin as the standard [6]

In vitro study The inhibition of erythrocyte catalase by cyanamide was determined by a sequential procedure as follows The primary reaction cell contained 20 μl heparinized whole rat blood (3 3 mg hemoglobin), 2.0 units glucose oxidase, 10 mM glucose, 1.0 mM cyanamide, and 10 mM potassium phosphate buffer, pH 7.0, in a final volume of 1 0 ml. The primary reaction was initiated by the addition of whole blood and the mixture was incubated for 20 min at 25°. A 5-μl aliquot of the primary mix was removed and immediately added to the secondary reaction mix contained in a temperature-controlled reaction cell, and the rate of O_2 formation was recorded The details for measuring catalase activity were as described above There was no loss of erythrocyte catalase activity during the 20-min incubation of the control primary mix (see Table 3)

Dose–response curves The equations for the hyperbolic plots of measured tissue catalase activity versus cyanamide dose were solved by an iterative least squares method [7]. The actual curves given for response vs *log* dose (Fig 1) represent these solutions

Statistical analysis. The results are expressed as mean ± S E M The analyses of variance were determined using Student's *t*-test. P values of <0 05 were accepted as significant

RESULTS

The catalase activities in homogenates of whole liver, kidney, heart and brain and in erythrocyte lysates from control animals are shown in Table 1 The relative order of activities for these tissues were liver > kidney > erythrocyte > heart > brain, based on catalase activity per g wet weight tissue, or for erythrocytes, activity per ml whole blood.

The potency of cyanamide as an *in vivo* inhibitor of catalase was evaluated using dose–response curves (Fig 1), and the ED_{50} values for cyanamide were calculated (Table 2) Of the tissues examined, hepatic catalase was the most sensitive to inhibition by cyanamide, whereas erythrocyte catalase was the least inhibited, viz 14% inhibition at the highest cyanamide dose

The time course for the recovery of catalase activity in these same tissues was determined using a cyanamide dose of 310 μmoles/kg (Fig. 2) Maximum inhibition occurred within 1 hr of cyanamide administration and catalase activity returned to within 60% of control values by 24 hr post cyanamide treatment. The degree of inhibition achieved for each respective tissue reflected its ED_{50} for cyanamide. Again, erythrocyte catalase activity was minimally affected by cyanamide

It has been reported that erythrocyte catalase is also insensitive to inhibition by 3-AT *in vivo* [8] but is inhibited by 3-AT *in vitro* [9, 10] Since the failure of 3-AT to inhibit erythrocyte catalase activity *in vivo* has been attributed to a lack of hydrogen peroxide in the circulation [9], the ability of cyanamide to inhibit rat erythrocyte catalase *in vitro* was also studied with and without added hydrogen peroxide (Table 3) Cyanamide, in the presence of a hydrogen peroxide source, significantly inhibited erythrocyte catalase, however, when the hydrogen peroxide source was omitted from the incubation, inhibition was not observed

Table 1 Normal levels of catalase activity present *in vivo* in liver, kidney, heart, brain and whole blood of the rat

	Catalase activity		
	mmoles O_2 formed/min	nmoles O_2 formed/min	
Tissue	g wet weight tissue	μg protein	Relative activity*
Liver	14 5 ± 1 1	85 8 ± 5 6	1 0
Kidney	3 82 ± 0 41	31 9 ± 2 9	0 26
Heart†	0 24 ± 0 02	8 1 ± 0 7	0 016
Brain	0 047 ± 0 004	0 48 ± 0 06	0 0032
Blood‡	2 82 ± 0 12	17 6 ± 0 5	0 19

Data (N = 6) represent tissue catalase activities for control animals from the experiment shown in Fig 1

* Tissue catalase activities from the first data column were normalized against the liver value which was set at 1

† The protein fractions were centrifuged to remove fibrous matter before assaying for protein

‡ Catalase units for the first and second columns are mmoles O_2 formed/min/ml whole blood and nmoles O_2 formed/min/μg hemoglobin respectively

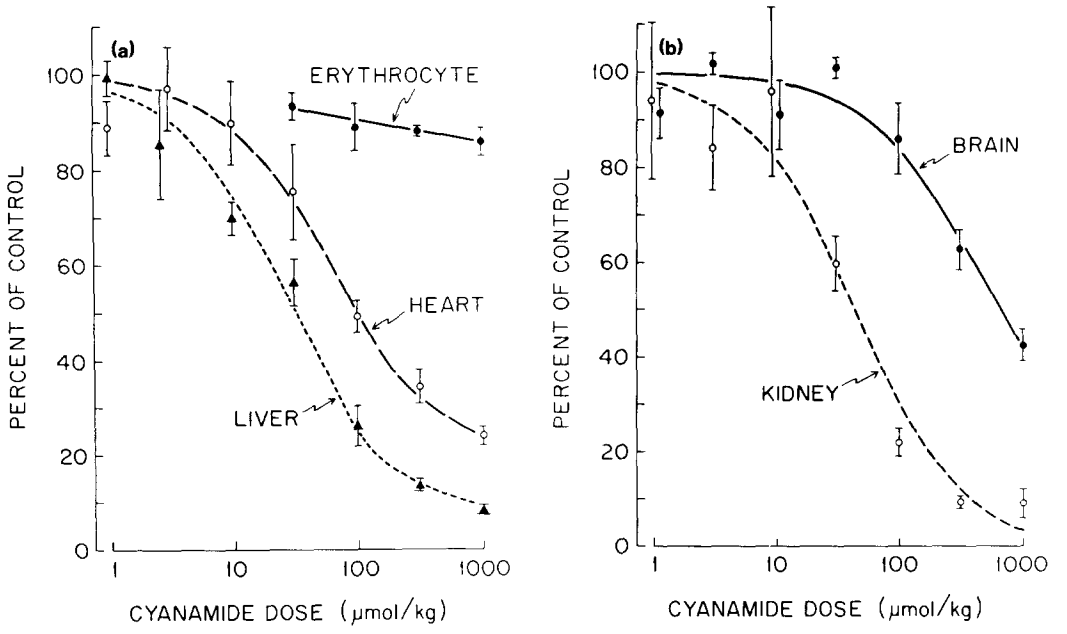

Fig 1 Log dose vs response curves for the *in vivo* inhibition by cyanamide of catalase in rat liver, heart, erythrocyte (left panel), kidney and brain (right panel) Each animal received cyanamide (i p) or isotonic saline (control) 1 hr before being killed Each data point represents the mean ± S E for a minimum of four animals. The tissue catalase activity plotted as percent of control was calculated from the data in units of nmoles O_2 formed/min per μg protein or nmoles O_2 formed/min per μg hemoglobin for the erythrocyte enzyme Normal values for the saline-treated controls are presented in Table 1

DISCUSSION

The effect of cyanamide on catalase activity based on the ED_{50} values for the different rat tissues (Table 2) indicates a differential pattern of inhibition. With the exception of the erythrocyte enzyme, the relative potency of cyanamide among the tissues examined followed the same order as the relative tissue catalase activity, i.e. liver > kidney > heart > brain. A possible explanation for this variable pattern of inhibition is the formation of a cyanamide-derived inhibitory product. Thus, the liver with high catalase activity could produce higher steady-state intracellular concentrations of active inhibitor than, for example, the brain. Alternatively, the relative sensitivity of the respective tissue catalases to cyanamide may simply reflect tissue hydrogen peroxide levels with no mechanistic implications.

Table 2 ED_{50} values for the cyanamide inhibition of catalase activity for various rat tissues

Tissue	ED_{50} for Cyanamide* (mmole/kg, i p)	Relative potency†
Liver	0 031	1 0
Kidney	0 044	0 70
Heart	0 107	0 29
Brain	0 68	0 046
Blood	>1 0	<0 031

*ED_{50} Values were calculated from the data shown in the dose–response curves of Fig 1, as described in Materials and Methods

† Reciprocals of the ED_{50} values were normalized against the liver value, which was set at 1

Erythrocyte catalase was inhibited by cyanamide *in vitro* (Table 3), but not *in vivo* (Figs 1 and 2). This difference between the *in vitro* and *in vivo* results is similar to that reported for 3-AT [10, 11]. A low concentration of hydrogen peroxide in the erythrocytes is believed to be the basis for lack of inhibition by 3-AT *in vivo* [9], and this may also account for our findings with cyanamide.

Cyanamide is significantly more potent than 3-AT in inhibiting hepatic catalase *in vivo*. To our knowledge, the ED_{50} for the inhibition of hepatic catalase by 3-AT has not been reported for the rat, however, an ED_{50} of 4.0 mmoles/kg, i.p , has been reported for the mouse [8], and has been estimated to be 0 6 mmole/kg for the rat [12] These values can be compared to 0.031 mmole/kg for cyanamide that we found for the rat (Table 2) The standard dose of 3-AT generally used for *in vivo* studies is 11.9 mmoles/kg (or 1 g/kg) i.p , whereas a comparable degree of inhibition was achieved using a 0.31 mmole/kg dose of cyanamide similarly administered i.p. Using the above doses, maximal inhibition of tissue catalase by cyanamide occurred within 1 hr (Fig 2), whereas 2–3 hr were required for 3-AT [13] However, the recovery rates for hepatic catalase activity following maximal inhibition by cyanamide or 3-AT were similar (Fig. 2 and Ref. 14).

3-AT belongs to a family of catalase inhibitors that are believed to share a common mechanism of action [8, 15] It is possible that cyanamide also meets the minimum structural requirements for these 3-AT-like catalase inhibitors, as described by Margoliash *et al.* [15]. Evidence supporting this close similarity between cyanamide and 3-AT include: (a) blockade

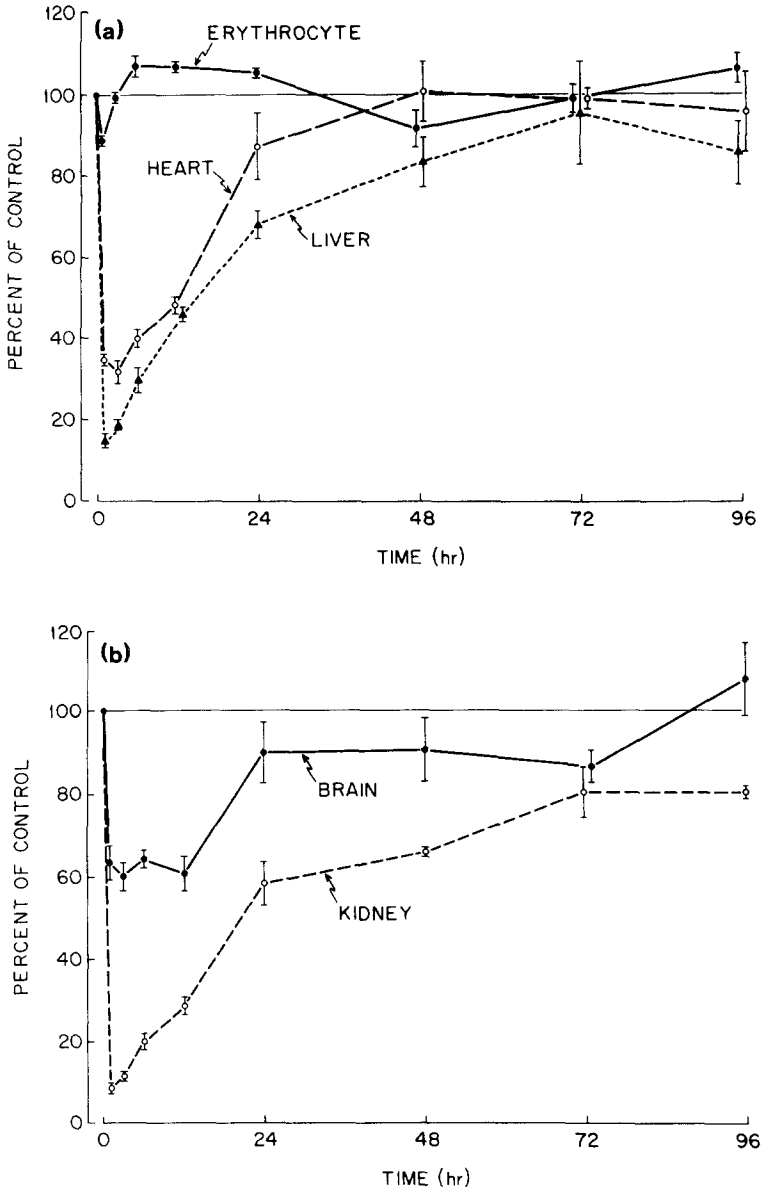

Fig 2 Time course for the recovery of catalase activity in rat liver, heart, erythrocyte (top panel), kidney and brain (bottom panel) following a single dose of cyanamide The animals were fasted 16 hr before use Each animal received an acute dose of cyanamide (0 31 mmole/kg body weight, i p) or saline and was killed at the times indicated At 8 hr following cyanamide treatment, the remaining animals were refed Catalase activities, expressed as percent of control, were calculated from the original data in units of mmoles O_2 formed/min per g wet weight tissue or nmoles O_2 formed/min per μg hemoglobin for the erythrocyte enzyme Control and test animals (N = 4) were killed at each time period, except for 3, 6 and 12 hr (N = 2) where control values were pooled

of their inhibitory effects on hepatic catalase by ethanol *in vivo* [9, 16, 17]; (b) inhibition of erythrocyte catalase *in vitro*, but not *in vivo* (Fig. 1 and Refs. 8 and 18); (c) a similar requirement for hydrogen peroxide in the inhibition of catalase *in vitro* (Table 3 and Refs 9 and 18); and (d) binding of both ^{14}C-labeled 3-AT and ^{14}C-labeled cyanamide to the apoprotein and not to the heme prosthetic group of catalase [15, 19]

The precise molecular mechanism for the inhibition of catalase by cyanamide is not known Although 3-AT is known to modify His-74 of the catalase apoprotein [20], a detailed mechanism for this inhibition has not been described There is, however, one significant difference between cyanamide and 3-AT, namely, the bioactivation of cyanamide by catalase results in the inhibition of hepatic AlDH *in vivo* [3], while the bioactivation of 3-AT does not [2] Studies are in progress to elucidate the relationship between cyanamide inhibition of catalase and the catalase-mediated inhibition of AlDH by cyanamide

Table 3 Inhibition of rat erythrocyte catalase by cyanamide *in vitro*

Cyanamide (1 0 mM)	H_2O_2*	Erythrocyte catalase activity† (% of control)	P value
−	+	100.0 ± 3 1	
+	+	19.2 ± 0 8	0 001
+	−	95 6 ± 1 3	NS‡

Experimental details were as described under Materials and Methods

* Glucose oxidase (2 0 units) and 10 mM glucose were used as the source of H_2O_2.

† Erythrocyte catalase activity corresponding to 100% of control was 16.6 ± 0 5 nmoles O_2/min/μg hemoglobin

‡ Not significant

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