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ACUTE EFFECTS OF THE ALDEHYDE DEHYDROGENASE INHIBITORS, DISULFIRAM, PARGYLINE AND CYANAMIDE, ON CIRCULATING KETONE BODY LEVELS IN THE RAT

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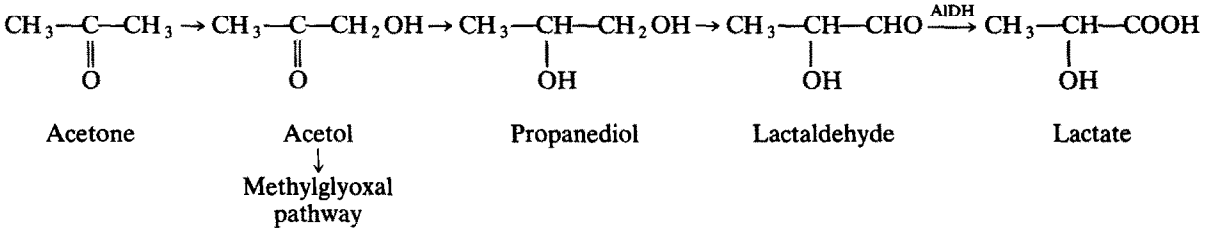
Abstract—Acetonemia is generally associated with the ketogenic states of fasting and diabetes. Disulfiram (DS), an inhibitor of aldehyde dehydrogenase (AlDH) that is used as an alcohol deterrent drug, is also known to elevate blood acetone in humans, but in the absence of a commensurate increase in its metabolic precursor, acetoacetate. We reexamined the effects of DS and other AlDH inhibitors on circulating ketone body levels in male rats of Sprague–Dawley descent and again demonstrated a 6- and 16-fold increase in blood acetone along with normal levels of acetoacetate at 6 and 24 hr after DS. Pargyline, another inhibitor of AlDH, maintained normal blood acetone levels in the presence of *reduced* acetoacetate levels. A third inhibitor of AlDH, cyanamide, administered to fasted and nonfasted rats, elevated blood acetone levels 10-fold over controls, with, however, a commensurate 5- and 7-fold increase in blood acetoacetate levels. The threshold values for the cyanamide-induced elevation of blood acetone and acetoacetate were equivalent, i.e. approximately 0.25 mmol/kg body weight (i.p.). The elevation of acetoacetate and the inhibition of hepatic catalase activity by cyanamide are not mechanistically linked, since 3-amino-1,2,4-triazole, another inhibitor of catalase, elevated blood acetone but not acetoacetate levels. These findings suggest that DS-induced acetonemia is due to inhibition of acetone metabolism, whereas enhanced acetone formation through acetoacetate contributes significantly to cyanamide-induced acetonemia.

Disulfiram (DS†), an alcohol deterrent drug‡, is known to elevate blood acetone levels in rats and humans [1, 2]. However, at least in humans, the other ketone bodies, i.e. β-hydroxybutyrate and acetoacetate, are within the normal range [2]. With the exceptions of DS therapy and isopropyl alcohol poisoning [3, 4], acetone accumulation in the circulation is the result of increased fatty acid oxidation, the acetone arising from the decarboxylation of acetoacetate. In the metabolic ketogenic states of uncontrolled diabetes [5] and fasting [6], blood levels of all three ketone bodies are elevated.

Acetone is metabolized in the rat to lactate via the propanediol pathway [7]. The initial reaction which is catalyzed by acetone monooxygenase is shared with a second route of acetone metabolism, viz. the methylglyoxal pathway [7]. Since aldehyde dehydrogenase (AlDH) is involved in the metabolic disposition of acetone, and since DS represents one of a number of known *in vivo* inhibitors of AlDH, we reexamined the effects of DS and other AlDH inhibitors on the levels of ketone bodies in the circulation.

MATERIALS AND METHODS

Disulfiram, cyanamide, pargyline hydrochloride, 3-amino-1,2,4-triazole, acetoacetate and β-hydroxybutyrate were obtained from the Sigma Chemical Co. (St. Louis, MO).



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† Abbreviations: DS, disulfiram; AlDH, aldehyde dehydrogenase; and 3-AT, 3-amino-1,2,4-triazole.

‡ Antabuse.

Groups of male rats (283 ± 5 g) of Sprague–Dawley descent (BioLab, Inc., St. Paul, MN) maintained on a standard Purina rat chow diet were adminstered drug regimens as described in the tables and figures. These studies were performed in adherence with the guidelines established in the *Guide for the Care and*

Use of Laboratory Animals published by the U.S. Department of Health and Human Resources (NIH Publication No. 85-23, revised 1985). Animals were housed in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC), and the research protocol was approved by the Animal Study Subcommittee of the Minneapolis VA Medical Center.

The ketone bodies in blood were analyzed essentially as described by Eriksson [8] using head space gas chromatography. Blood was taken from each animal by open chest cardiac puncture [1]. Duplicate samples were prepared for analysis by the addition of 0.2-ml aliquots of blood to 1.8 ml of cold 0.6 N $HClO_4$ and mixed. The protein precipitate formed was removed by centrifugation at 4°. Aliquots (0.5 ml) of the protein free supernatant fraction were added to three glass septum vials, one for each of the ketone bodies. For acetone, the samples were neutralized with 0.115 ml of 3 N KOH, and the septum vials were sealed and maintained on Dry Ice until analyzed. The final pH of the acetone sample must be above pH 7.0 to prevent the decarboxylation of acetoacetate to acetone. For the determination of acetoacetate, 0.1 ml of 0.6 N $HClO_4$ was added, and the vials were sealed using either Teflon or aluminium faced septa. The acetoacetate was then converted to acetone by heating the samples at 100° for 90 min on a steam cone. These samples were cooled before analysis or stored on Dry Ice until analyzed. For β-hydroxybutyrate, the vials were sealed without further additions and heated to convert the acetoacetate to acetone as above. After the vials were cooled, 0.1 ml of 0.45% $K_2Cr_2O_7$ in 15.6 N H_2SO_4 was added through the septum, and the vials were again heated at 100° for 90 min to convert β-hydroxybutyrate to acetone. This two-step procedure for separately converting acetoacetate and β-hydroxybutyrate to acetone was used because the conversion of standard acetoacetate to acetone by acidified dichromate was found to be only about 80% complete compared to essentially 100% in perchloric acid alone.

Acetone, acetoacetate and β-hydroxybutyrate standards were used to construct standard curves which were linear over the observed concentration ranges. The calculations of the ketone body concentrations were all based on the gas chromatographic peak areas for acetone. The acetone in the acetoacetate samples after heating represents the sum of the acetone and acetoacetate concentrations (Fig. 1), and the acetone in the β-hydroxybutyrate samples after processing represents the sum of all three ketone bodies. The concentration of the individual ketone bodies could therefore be calculated from the differences in acetone concentrations of the three types of samples.

Hepatic catalase activity was measured using a Yellow Springs Oxygen Monitor equipped with a Clark style oxygen electrode as peviously described [9].

All results are expressed as mean ± SEM. Statistical analysis of the data was performed using Student's *t*-test (Table 1 and Fig. 1 only) or the Kruskal–Wallis test [10]. P values of <0.05 were accepted as significant.

RESULTS

Blood levels of the three ketone bodies were measured in separate groups of rats 6 and 24 hr following a single dose of DS (Table 1). The acetone levels in the DS-treated animals at these times were elevated 6 and 16-fold over their respective controls. Acetoacetate, the immediate metabolic precursor of acetone, was not elevated significantly by DS at either time. A modest increase in β-hydroxybutyrate was observed at 6 hr, but not at 24 hr. Overall, DS treatment caused a large increase in blood acetone levels in the absence of similar changes in acetoacetate or β-hydroxybutyrate concentrations, and the β-hydroxybutyrate/acetoacetate ratios were not different from controls.

Since AlDH has been implicated in the metabolism of acetone in the rat [7], the AlDH inhibitors, pargyline and cyanamide, were also evaluated for their

Table 1. Effect of DS on the levels of circulating ketone bodies in 24-hr fasted rats*

Group	Time after DS treatment (hr)	N	Acetone	Acetoacetate	β-Hydroxybutyrate†	β-Hydroxybutyrate† / Acetoacetate
			(mM)			
Control	6	4	0.059 ± 0.010	0.358 ± 0.071	1.05 ± 0.04	3.3 ± 0.7
DS	6	4	0.356 ± 0.032	0.377 ± 0.034	1.56 ± 0.15	4.2 ± 0.5
			(<0.001)‡	(NS)	(<0.05)	(NS)
Control	24	9	0.054 ± 0.008	0.405 ± 0.124	1.42 ± 0.28	2.8 ± 0.5
DS	24	8	0.895 ± 0.101	0.573 ± 0.068	1.29 ± 0.13	2.9 ± 0.3
			(<0.01)	(NS)	(NS)	(NS)

* A single dose of DS (0.59 g/kg in 5% gum acacia) was administered orally 6 or 24 hr prior to killing the animals. Controls received an equal volume of vehicle. Values are means ± SEM.

† β-Hydroxybutyrate levels were measured in only four of the animals from each group.

‡ P values comparing the ketone bodies of DS-treated animals with their respective controls are given in parentheses. NS = not significant.

Table 2. Effect of pargyline on blood acetone and acetoacetate levels in fasted rats*

Group	Dose (mmol/kg)	N	Acetone (mM)	Acetoacetate (mM)	Acetone / Acetoacetate
Control	0.0	8	0.055 ± 0.007	0.363 ± 0.039	0.15 ± 0.02
Pargyline	0.625	5	0.082 ± 0.006 (NS)	0.237 ± 0.024 (<0.05)	0.37 ± 0.06 (<0.001)
Pargyline	1.0	7	0.071 ± 0.008 (NS)	0.138 ± 0.009 (<0.001)	0.53 ± 0.07 (<0.001)

* Pargyline (or isotonic saline) was administered intraperitoneally to fasted rats 4 hr before the animals were killed. Values are means ± SEM. P values (given in parentheses) compare each of the pargyline groups with controls. NS = not significant.

effects on circulating ketone bodies. Pargyline, when administered at a minimally toxic dose and at a dose equivalent to its LD_{50} [11], did not elevate significantly blood acetone levels, while it decreased acetoacetate levels compared to controls (Table 2). Thus, the acetone/acetoacetate ratio increased with increasing pargyline dose. This differential effect of pargyline on acetoacetate and acetone levels suggests that pargyline decreased both the rate of acetone formation and the rate of acetone metabolism.

The effect of cyanamide on circulating ketone bodies was initially evaluated using nonfasted rats by measuring the sum of acetoacetate and acetone at several time periods after treatment (Fig. 1). These ketone bodies were elevated 7-fold by cyanamide at 4 hr, but by 24 hr they returned to nearly control levels. This short-term effect of cyanamide on ketone body levels is consistent with the rapid rate of cyanamide clearance from the rat [12].

The response of the individual ketone bodies to cyanamide treatment was assessed in both fasted and nonfasted rats (Table 3). A minimum 10-fold increase in blood acetone was observed; however, this increase was also accompanied by a 5-fold (fed state) and 7-fold (fasted state) rise in the levels of acetoacetate. In the fed state, cyanamide also increased β-hydroxybutyrate levels, but to a lesser degree than acetoacetate; hence, a significant decrease in the β-hydroxybutyrate/acetoacetate ratio was observed in both sets of animals. These results indicate that the elevation of acetone levels in cyanamide-treated animals was due in major part to increased formation from its precursor, acetoacetate.

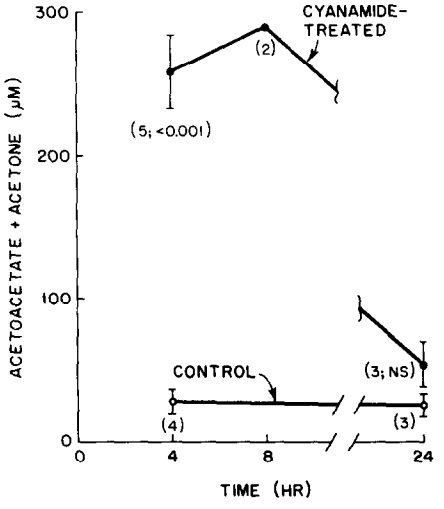

Fig. 1. Time course for the effect of cyanamide on the sum of acetoacetate and acetone blood levels in the rat. Rats (nonfasted) were administered cyanamide (0.50 mmol/kg in isotonic saline) or vehicle (control) intraperitoneally at zero time and were killed at the times indicated. The two ketone bodies were determined in combination as described in Materials and Methods. The N and P values (in parentheses) comparing cyanamide-treated animals against controls are given in the figure.

The potency of cyanamide as a modifier of circulating acetone and acetoacetate levels in the rat was indicated by their dose–response relationships (Fig. 2). The relative enhancement of these two metabolic intermediates by cyanamide increased in parallel and in a dose-dependent manner. The threshold values for the cyanamide doses required to elevate blood acetone and acetoacetate were estimated to be equivalent, viz. approximately 0.25 mmol/kg body weight. Cyanamide doses greater than 1.0 mmol/kg were not evaluated.

Other agents found to alter ketone body levels were ethanol and 3-AT. Both of these agents elevated blood acetone levels, but not acetoacetate, in the fasted rat (Table 3). In fact, ethanol decreased blood acetoacetate by 50% which therefore resulted in a corresponding increase in the β-hydroxybutyrate/acetoacetate ratio. Similar results were obtained using nonfasted animals (data not shown).

Since cyanamide and 3-AT are both inhibitors of catalase, their effects on hepatic catalase activity and blood acetoacetate levels were compared (Table 4). Only cyanamide elevated acetoacetate in the presence of reduced hepatic catalase activity, indicating that these two metabolic effects of cyanamide are not mechanistically linked.

DISCUSSION

Acetone is normally produced *in vivo* by the nonenzymatic decarboxylation of acetoacetate. Therefore, apart from isopropyl alcohol which is converted directly to acetone by alcohol dehydrogenase [3], acetonemia is usually associated with elevated levels of acetoacetate. However, acetonemia could also occur when one (or more) of

Table 3. Effect of cyanamide on the levels of circulating ketone bodies in fed and fasted rats*

Group	N	Acetone	Acetoacetate	β-Hydroxybutyrate	β-Hydroxybutyrate
		(mM)			Acetoacetate
Fed state					
Control	7	0.0090 ± 0.0028	0.048 ± 0.016	0.197 ± 0.035	4.9 ± 0.6
Cyanamide	6	0.097 ± 0.019 (<0.001)†	0.244 ± 0.077 (<0.001)	0.459 ± 0.120 (<0.01)	2.1 ± 0.2 (<0.001)
Fasted state					
Control	8	0.055 ± 0.007	0.36 ± 0.04	1.10 ± 0.11	3.7 ± 0.5
Cyanamide	7	0.67 ± 0.12 (<0.001)†	2.65 ± 0.45 (<0.001)	1.39 ± 0.33 (NS)	0.74 ± 0.20 (<0.001)
3-AT	4	0.31 ± 0.03 (<0.001)	0.58 ± 0.13 (NS)	1.07 ± 0.23 (NS)	2.0 ± 0.4 (<0.05)
Ethanol	3	0.10 ± 0.01 (<0.05)	0.16 ± 0.02 (<0.01)	1.07 ± 0.23 (NS)	6.4 ± 0.6 (<0.01)

* Fed and 18-hr fasted rats received cyanamide (0.50 mmol/kg) or isotonic saline (control) intraperitoneally and were killed 4 hr later, whereas ethanol (2 g/kg) and 3-AT (1 g/kg) were administered 5 and 7 hr before sacrifice respectively. Values are means ± SEM.

† P values comparing the drug-treated animals against controls are given in parentheses. NS = not significant.

the pathways of acetone metabolism is sufficiently inhibited. Our results show that acute treatment of rats with the AlDH inhibitors, DS, cyanamide and pargyline, did alter acetone metabolism. DS and cyanamide markedly elevated blood acetone levels, whereas pargyline maintained normal acetone levels in the presence of reduced acetoacetate levels. Of these, only cyanamide was found to also elevate the levels of acetoacetate and β-hydroxybutyrate.

The occurrence of DS-induced acetonemia in the fasted rat with normal fasting acetoacetate levels (Table 1) agrees with a similar observation in humans [2]. DS-induced acetonemia is best attributed to an inhibitory effect by DS on acetone metabolism. Two potential enzymatic sites for blockade in the metabolic pathway of acetone are: (a) the microsomal acetone monooxygenase, and (b) AlDH. DS is known to inhibit a number of microsomal mixed-function oxidases [13] as well as AlDH [14, 15]. The effects of pargyline on acetone metabolism (Table 2) can be similarly explained, since pargyline is a potent inhibitor of the low K_m AlDH isozyme *in vivo* [16, 17] and a substrate for microsomal cytochrome P-450 [17].

Cyanamide, another inhibitor of AlDH, caused a 10-fold rise in blood acetone in fed and fasted rats relative to controls (Table 3). Unlike DS-induced acetonemia, this rise in acetone lasted less than 24 hr (Fig. 1) and was accompanied by a significant rise in blood acetoacetate. This suggested that cyanamide effected a change in the rate of ketone body formation in addition to any effect it may have had on

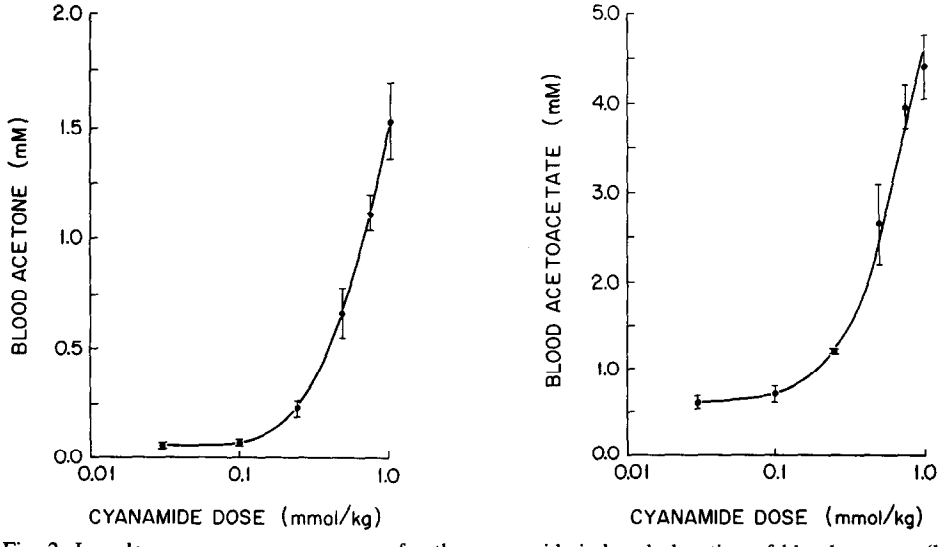

Fig. 2. Log dose versus response curves for the cyanamide-induced elevation of blood acetone (left panel) and acetoacetate (right panel) levels in 16-hr fasted rats. Each animal received cyanamide (i.p.) or isotonic saline (control) 4 hr before being killed. Each data point represents the mean ± SE for a minimum of three animals. Normal values for saline-treated controls are presented in Table 3.

Table 4. Comparison of the effects of cyanamide and 3-AT on hepatic catalase activity and blood acetoacetate levels*

Group	N	Blood acetoacetate (mM)	Hepatic catalase activity: mmoles O_2 formed/min g wet weight liver	Hepatic catalase activity: % of Control
Saline control	4	0.47 ± 0.05	14.9 ± 0.8	100
Cyanamide (0.5 mmol/kg)	3	1.24 ± 0.06 (<0.001)†	3.4 ± 0.3 (<0.05)	23
3-AT (1 g/kg)	4	0.38 ± 0.02 (NS)	1.0 ± 0.1 (<0.001)	7

* Overnight fasted rats were administered cyanamide or isotonic saline (i.p.) 1 hr before being killed, whereas 3-AT was given 4 hr before sacrifice. Values are means ± SEM.

† P values comparing the drug-treated animals against controls are given in parentheses. NS = not significant.

acetone catabolism. The absence of an observed cyanamide-induced rise in circulating ketone bodies reported earlier [1] can be attributed to the time of evaluation after drug administration.

The acetonemia induced by ethanol and 3-AT is probably due to the inhibition of acetone monooxygenase by these substances. Since the acetone monooxygenase described by Casazza *et al.* [7] appears to be identical to cytochrome P-450 LMeb or 3a [18, 19] i.e. the microsomal ethanol-oxidizing enzyme, ethanol may be a competitive substrate for acetone metabolism. Although 3-AT can decrease the level of microsomal cytochrome P-450 enzymes via inhibition of heme biosynthesis [20], the 3-AT-induced acetonemia can better be attributed to a direct inhibition of cytochrome P-450 by this agent [21, 22]. Ethanol may also interfere with acetone metabolism by way of its metabolite, acetaldehyde, through competition with L-lactaldehyde for AlDH.

The cyanamide-induced elevation of blood acetoacetate does not appear to be mechanistically linked with either the inhibition of hepatic AlDH or catalase in that other inhibitors of these enzymes did not cause a similar response. Using experimental conditions and dosages of pargyline and DS that are known to inhibit liver mitochrondrial AlDH activity 60–80% [14, 15, 17], these agents did not elevate blood levels of acetoacetate. Likewise, 3-AT inhibited hepatic catalase activity 93% without causing a rise in blood acetoacetate (Table 4). However, the overall time courses for the effect of cyanamide on AlDH activity [14], catalase activity [23] and ketone body levels (Fig. 1) were similar. Maximal inhibition of AlDH and catalase occurred within 1 hr [14, 23] whereas the ketone bodies peaked at about 8 hr after cyanamide treatment (Fig. 1). All three returned to within 40% of control values at 24 hr. The rise in blood acetoacetate is probably secondary to an early, but yet unknown, effect of cyanamide.

In conclusion, the results herein suggest that acetonemia, unrelated to the ketogenic states of fasting and diabetes, may be a relatively common phenomenon. The observation that cyanamide raised the concentration of all three ketone bodies may be of clinical importance because it suggests a condition which mimics the above ketogenic states. Further work is required to determine if cyanamide actually enhances fatty acid metabolism.

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