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# STUDIES ON THE CYANAMIDE–ETHANOL INTERACTION

## DIMETHYLCYANAMIDE AS AN INHIBITOR OF ALDEHYDE DEHYDROGENASE *IN VIVO*

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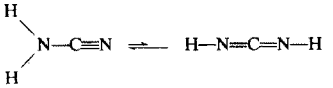
**Abstract**—Administration of dimethylcyanamide (DMC) to rats caused a marked elevation in ethanol-derived blood acetaldehyde (AcH) and depressed the specific activity of the low $K_m$ mitochondrial aldehyde dehydrogenase (AlDH) by 90% at 12–24 hr, coincident with depletion of hepatic glutathione levels. Comparison of the relative efficacy of DMC and cyanamide in elevating blood AcH measured at 2 hr and 1 hr post-drug treatment, respectively, indicated that DMC was at least one-fifth as active as cyanamide. However, since the comparison was not made at optimal times for DMC (12–24 hr), it is likely that its activity *in vivo* approaches that of cyanamide itself. DMC was essentially inactive *in vitro* as an inhibitor of the low $K_m$ AlDH isozyme in intact rat liver mitochondria. Although methylcyanamide, the product of *N*-demethylation of DMC, was too unstable to be prepared for this evaluation, the higher monoalkyl cyanamide, *n*-propylcyanamide, was synthesized chemically and was shown to be a good inhibitor of the mitochondrial enzyme *in vitro*. These results suggest that DMC must be *N*-demethylated before being converted to a reactive species that inhibits AlDH activity.

Cyanamide, the active component of the drug Temposil, is a potent inhibitor of aldehyde dehydrogenase (AlDH) and elicits a pharmacological response similar to disulfiram (Antabuse) [1–4]. Although it is well established that this cyanamide-mediated sensitization to ethanol is causally related to the inactivation of AlDH *in vivo*, the molecular basis of this inhibitory effect has not yet been elucidated [5]. The lack of activity of cyanamide *in vitro* with purified preparations of AlDH, despite its demonstrated inhibition of a wide spectrum of AlDH isozymes *in vivo*, suggests that cyanamide must be metabolically converted to an active form for inhibition of AlDH [6].

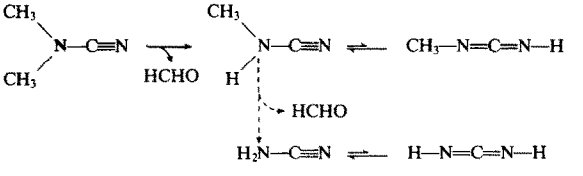
The possibility that addition products of cyanamide with amines or thiols might be the active inhibitors was examined *in vitro* by Kitson and Crow [5]. Dicyandiamide and aminoethylisothiouronium ion (AET), as well as two possible by-products of the reaction of cyanamide with thiols, viz. thiourea and formamidine disulfide, were found to be inactive when tested against a partially purified sheep liver AlDH *in vitro*. That cyanamide may act via its tautomeric carbodiimide form (HN=C=NH) [7] has, however, not been examined experimentally. This cyanamide → carbodiimide conversion may not



Cyanamide Carbodiimide

occur spontaneously, but rather may require *metabolic* activation since cyanamide, although inactive against a *purified* preparation of AlDH *in vitro*, is a good inhibitor of the low $K_m$ AlDH when incubated with *intact* rat liver mitochondria.

If an activated carbodiimide form were in fact involved, then a cyanamide derivative which cannot form a carbodiimide until it is metabolized would not be expected to exhibit AlDH inhibitory activity *in vitro*, but it might be active *in vivo*. Dimethylcyanamide (DMC), an *N,N*-dialkylated cyanamide with the amino nitrogen of cyanamide completely substituted, cannot form a carbodiimide until it is metabolically *N*-methylated *in vivo*. On the basis of this working hypothesis, we set out to compare the



inhibitory effects of DMC and of cyanamide itself on AlDH, both *in vivo* and *in vitro*. Following DMC or cyanamide administration to rats, the elevation of ethanol-derived blood acetaldehyde (AcH) and the specific activity of the low $K_m$ AlDH isozyme of liver mitochondria were determined. *N*-Demethylation of DMC by microsomal cytochrome P-450 enzymes and the enhancement of demethylation by phenobarbital (PB) pretreatment were evaluated by incubating liver microsomes (100,000 *g* fraction), isolated from untreated (control) and PB-pretreated rats, with DMC and assaying the formaldehyde produced. The activities of DMC and of a monoalkyl cyanamide, *n*-propylcyanamide, as AlDH inhibitors *in vitro* were also assayed by incubation with isolated liver mitochondria.

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## MATERIALS AND METHODS

*Materials.* Dimethylcyanamide (DMC) which was available commercially contained formaldehyde as impurity and was therefore chemically purified before use. DMC (Tridom-Fluka Chemical Co., Hauppage, NY) was added to an aqueous solution of semicarbazide hydrochloride (30 g, 0.27 mole). The reaction mixture was heated on the steam bath for 30 min, saturated with sodium chloride after cooling, and subjected to exhaustive extraction with ether. The ethereal solvent was replaced with methylene chloride, the solution was dried over anhydrous sodium sulfate, and the material was distilled. The fraction which boiled at 71.6–73.4° (90 mm Hg) was collected, $n_D^{21}$ 1.4070. Reaction of this chemically purified DMC with the Nash reagent used for the determination of formaldehyde gave values which were essentially indistinguishable from a water blank. Acetaldehyde (AcH), purchased from the Aldrich Chemical Co. Inc. (Milwaukee, WI), was distilled before use. *n*-Propylcyanamide was prepared according to Mukaiyama *et al.* [8]. The following chemicals were purchased from the source indicated: cyanamide, glucose-6-phosphate, glucose-6-phosphate dehydrogenase (yeast), reduced glutathione, and NADP (Sigma Chemical Co., St. Louis, MO); sodium phenobarbital (USP) (Ganes Chemical Inc., Pennsville, NJ); nicotinamide (Nutritional Biochemical Corp., Cleveland, OH); and semicarbazide hydrochloride (Fisher Chemical Corp., Chicago, IL). SKF-525A (β-diethylaminoethyl 2,2-diphenylvalerate·HCl) was gift from Smith Kline & French Laboratories, Philadelphia, PA.

Male Sprague–Dawley rats weighing 151–175 g, purchased from Biolab Corp. (St. Paul, MN), were allowed free access to Purina Lab Chow and water. When indicated, PB was administered in the drinking water (1 mg/ml) for 1 week with a fresh solution provided every other day. The animals were provided fresh water and fasted the night before they were killed.

*Blood AcH determination.* Blood AcH levels were measured 1 hr after ethanol administration (2 g/kg, i.p.) in treated and control animals essentially as described previously [9]. The animal was stunned by a quick blow to the head, and blood was immediately withdrawn by open chest cardiac puncture. Aliquots (0.2 ml) were placed in ice-cold 20 ml serum vials containing 1.0 ml of 5 mM sodium azide. The vials were immediately capped, frozen on dry ice, and kept frozen at −20° until assayed. AcH was determined in duplicate samples by a headspace gas chromatographic technique as previously described [10] and quantitated using a standard curve based on known concentrations of AcH. This procedure is a modification of the hemolysis method [11, 12] and, with the addition of sodium azide, the artifactual generation of AcH is negligible [13].

*Mitochondria preparation.* Male Sprague–Dawley rats that had been fasted overnight were decapitated. Care was taken throughout the isolation procedure to maintain the mitochondrial preparation at 0°. The livers were perfused via the portal vein with 20 ml of saline, immediately removed, and minced in 0.25 M sucrose solution containing 0.1 mM EDTA (pH 7.5). The liquid was decanted, and the minced livers were homogenized in 30 ml of fresh solution using a Potter–Elvehjem homogenizer (three up and down strokes of the Teflon pestle). The homogenate was centrifuged at 755 *g* for 12 min, and the supernatant fluid was carefully decanted and recentrifuged at 12,000 *g* for 15 min. The pellet was gently suspended in 15–25 ml of sucrose–EDTA solution and centrifuged (12,100 *g*) for 10 min. This washing process was repeated twice with 0.25 M sucrose. The final pellet was suspended in about 1.5 ml of 0.25 M sucrose. Protein concentration was determined by the method of Lowry *et al.* [14].

*Measurement of mitochondrial AlDH activity.* The activity of the low $K_m$ AlDH isozyme was assayed in *intact* mitochondria by measuring the rate of AcH disappearance from a closed incubation system as described by DeMaster and Nagasawa [15]. This assay system is dependent on intramitochondrial oxidation of NADH and is specific for the low $K_m$ isozyme located in the mitochondrial matrix [15]. The incubation mixture (1.0 ml) contained 0.25 M sucrose, 5 mM $MgSO_4$, 1.0 mM EDTA, 10 mM KCl, 10 mM arsenate (pH 7.5) and intact rat liver mitochondria. The selection of arsenate buffer was to completely uncouple oxidative phosphorylation in this system [16]. The reaction was initiated by addition of AcH (200 nmoles) and, after incubation for 5 min at 38°, quenched with $HClO_4$ (final concentration, 0.5 N). AcH was quantitated by essentially the same procedure used for determination of blood AcH. Specific activity is expressed as nmoles AcH oxidized per min per mg protein.

*Microsomal N-demethylation of DMC.* Microsomes (100,000 *g* fraction) were prepared from livers of untreated (control) and PB-treated rats as previously described [9]. The washed microsomal pellet was suspended in 0.05 M phosphate buffer (pH 7.4) to a final concentration of 1.0 g wet weight liver/ml. The *N*-demethylation of DMC by microsomal enzymes was followed by assaying the formaldehyde formed after incubation at 37° in a shaking water bath for the time specified. The incubation mixture (12.0 ml) contained: DMC (20–40 mM), sodium phosphate buffer (pH 7.4, 83 mM), $NADP^+$ (2.0 mM), glucose-6-phosphate (2.5 mM), KCl (16.5 mM), $MgCl_2$ (4.0 mM), nicotinamide (8.3 mM), semicarbazide hydrochloride (pH 7.4, 4.0 mM), glucose-6-phosphate dehydrogenase (38 units), and liver microsomes from PB-treated or untreated rats corresponding to 500 mg wet weight liver. The reaction was initiated by addition of enzyme and quenched after 30 min with zinc sulfate (15% w/v, 2.0 ml) and saturated barium hydroxide (2.0 ml). After centrifugation, the supernatant fraction was assayed for formaldehyde using the Nash reagent [17]. Tissue blank controls containing the complete incubation mixture except for DMC were routinely subtracted in this standard procedure.

*Measurement of mitochondrial respiration.* Rates of oxygen utilization in the presence of test compounds (0.1 to 1 mM, or equivalent volume of water) were measured at 38° using a Clark oxygen electrode [18] (Yellow Springs Instrument Co., Yellow Springs, OH) in a reaction medium (1.8 ml) identical to that used for the measurement of mitochondrial

Table 1. Effects of DMC and related compounds on blood AcH levels and AlDH activity after ethanol treatment*

| Treatment | Blood AcH ($\mu$M) | AlDH activity [nmoles AcH oxidized·min$^{-1}$·(mg protein)$^{-1}$] |
|---|---|---|
| Experiment 1 | | |
| Saline (control) | 10.6 ± 0.8 (4) | 10.09 ± 0.54 |
| DMC (1.0 mmole/kg) | 588 ± 22† (3) | 6.04 ± 0.32 |
| Cyanamide (1.0 mmole/kg) | 1516 ± 55† (3) | 1.92 ± 0.30 |
| Experiment 2 | | |
| Saline (control) | 10.3 ± 2.7 (4) | |
| Dicyandiamide (0.5 mmole/kg) | 11.0 ± 1.4‡ (5) | |
| Cyanamide (0.5 mmole/kg) | 1213 ± 169† (5) | |

* Each animal was given saline or drug (i.p.) followed by ethanol (2 g/kg, i.p.) 1 hr later, and killed 2 hr after the drug. Blood AcH and mitochondrial AlDH were measured as described in Materials and Methods. All values are means ± S.E. Values in parentheses denote the number of animals in each treatment group. Statistical comparisons are based on Student's *t*-test.

† $P < 0.001$ compared to respective saline control.

‡ P = NS compared to saline control.

AlDH activity *in vitro*. The reaction was initiated by the addition of mitochondria (1.0 to 2.0 mg protein/ml of reaction mix). The mitochondria were preincubated with test compound for 5 min before $\beta$-hydroxybutyrate (40 mM, final concentration) was added. Oxygen uptake (n-atoms oxygen consumed per mg protein) was compared to controls without test compounds.

*Measurement of glutathione.* Hepatic glutathione was assayed using the Ellman reagent (5,5'-dithiobis-2-nitrobenzoic acid) as described by Buttar *et al.* [19]. A 20% (w/v) liver homogenate was prepared in 5% trichloroacetic acid–5 mM $Na_2EDTA$ and centrifuged at 10,000 rpm for 20 min. Aliquots of the supernatant fraction (0.1 ml) were added to 4.8 ml of 0.1 M phosphate buffer (pH 8.0) followed by the addition of 0.050 ml of the Ellman reagent. The absorbance of the sample was read at 412 nm after 10 min, and glutathione was quantitated using a standard curve based on known concentrations of glutathione.

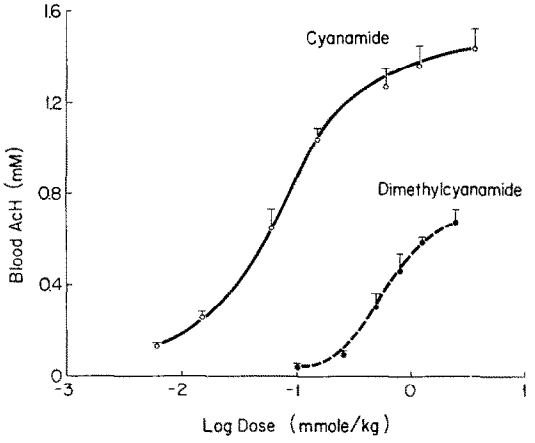


Fig. 1. Log dose versus response curves of the effect of DMC and cyanamide on blood AcH levels in the rat after ethanol treatment. Each animal received DMC (●) or cyanamide (○) (i.p.) followed by ethanol (2 g/kg, i.p.) 2 hr after DMC or 1 hr after cyanamide. All animals were killed 1 hr after ethanol. Blood AcH was measured as described under Materials and Methods. Each point is the mean ± S.E. from four or more animals.

## RESULTS

*DMC inhibition of AlDH* in vivo. DMC actively inhibited AlDH *in vivo* as indicated by the elevation of ethanol-derived blood AcH levels. As shown in Table 1, DMC administration caused a 49-fold elevation of blood AcH when measured 2 hr after DMC and 1 hr after the ethanol dose, compared to control values from rats receiving sham injection of saline and ethanol (2 g/kg, i.p.) The specific activity of the low $K_m$ mitochondrial AlDH was correspondingly decreased by 37%. Under the same conditions, an equimolar dose of cyanamide produced AcH blood levels that were 126-fold higher than control values and lowered the specific activity of the low $K_m$ mitochondrial AlDH by 80%. By contrast, dicyandiamide, a dimer of cyanamide, did not significantly elevate blood AcH at equimolar doses.

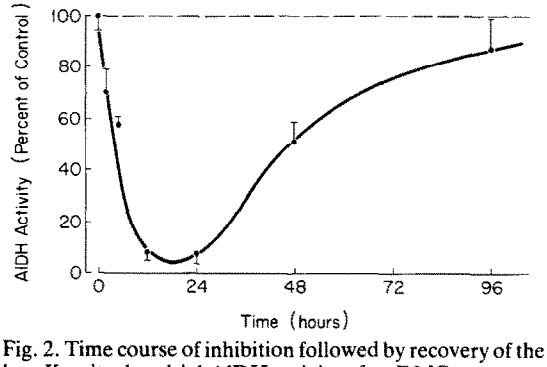


Fig. 2. Time course of inhibition followed by recovery of the low $K_m$ mitochondrial AlDH activity after DMC treatment in the rat. Animals were administered DMC (1.0 mmole/kg, i.p.) and killed at the times indicated. Liver mitochondria were isolated and the activity of the low $K_m$ AlDH isozyme was assayed as described in Materials and Methods. Individual points represent the mean ± S.E. from four or more animals. One hundred percent = 9.59 ± 0.55 nmoles AcH oxidized·min$^{-1}$ · (mg protein)$^{-1}$.

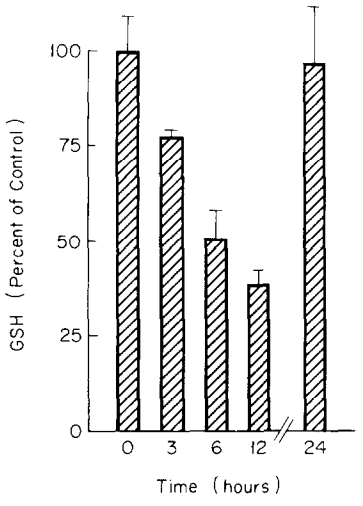


Fig. 3. Depletion of hepatic glutathione by DMC. DMC (1.0 mmole/kg, i.p.) was administered and the animals were sacrificed at the times indicated. A section of the lower median lobe was removed for determination of glutathione as described in Materials and Methods. Each value is the mean ± S.E. from four or more animals. One hundred percent = 7.94 ± 0.70 μmoles/g wet wt liver.

Table 2. Effects of SKF-525A and PB pretreatment on the DMC-mediated elevation of blood AcH in the rat following ethanol (2 g/kg)*

| Treatment | N | Blood AcH (μM) |
|---|---|---|
| Group I | | |
| Saline (control) | 4 | 16.2 ± 4.3 |
| SKF-525A (40 mg/kg) | 4 | 12.6 ± 6.7 |
| DMC (1.0 mmole/kg) | 6 | 597 ± 48† |
| SKF-525A + DMC | 6 | 591 ± 75† |
| Group II | | |
| PB control | 6 | 8.8 ± 2.3 |
| DMC (0.35 mmole/kg) | 6 | 86.6 ± 2.8‡ |
| PB + DMC (0.35 mmole/kg) | 6 | 99.2 ± 34.6‡ |

* Rats in Group I were administered saline or SKF-525A (40 mg/kg, i.p.) 4 hr before being killed, and DMC (1.0 mmole/kg, i.p.) or saline 3 hr before sacrifice. All animals received ethanol (2 g/kg, i.p.) 1 hr before they were killed. Rats in Group II were pretreated with PB as described under Materials and Methods. Both PB-treated and untreated rats were given DMC (0.35 mmole/kg, i.p.) 3 hr before sacrifice. PB control rats received saline. All animals received ethanol (2 g/kg, i.p.) 1 hr before being killed. Values are means ± S.E.

† P = NS compared to each other.

‡ P = NS compared to each other.

The relative efficacy of DMC and cyanamide in elevating blood AcH can be quantitatively compared by their dose–response curves in Fig. 1. The *in vivo* $ED_{50}$ calculated for DMC (0.54 mmole/kg) was five times larger than that for cyanamide (0.11 mmole/kg) when determined 2 hr post-DMC and 1 hr post-cyanamide. However, these results may not be optimal for DMC as examination of the time course for this inhibition of AlDH indicated that the loss of AlDH activity was somewhat delayed with maximum inhibition of AlDH occurring between 12 and 24 hr after DMC administration (Fig. 2). AlDH activity recovered slowly but was essentially completely restored by 96 hr. Corresponding to this reduction in AlDH activity, hepatic glutathione levels were markedly decreased with maximum depression of glutathione occurring 12 hr after DMC administration (Fig. 3). Under similar conditions, hepatic glutathione levels were not depressed when measured 3 hr after administration of cyanamide. In contrast to the slow recovery of AlDH activity after DMC, glutathione was rapidly restored to control levels after 24 hr.

Administration of SKF-525A (40 mg/kg, i.p.), a

Table 3. Inhibition of the low $K_m$ mitochondrial AlDH by DMC, cyanamide, and *n*-propylcyanamide *in vitro*

| Inhibitor* | AlDH activity [nmoles AcH oxidized·$min^{-1}$·(mg protein)$^{-1}$] | % Inhibition |
|---|---|---|
| Experiment 1 | | |
| Saline (control) | 14.24 ± 0.57 (7) | |
| DMC (200 μM) | 10.46 ± 0.05† (3) | 26.5 ± 0.4 |
| Cyanamide (200 μM) | 0.06 ± 0.05‡ (3) | 99.6 ± 0.4 |
| Dicyandiamide (200 μM) | 11.93 ± 0.18§ (3) | 16.2 ± 1.3 |
| Experiment 2 | | |
| Saline (control) | 10.75 ± 0.33 (3) | |
| *n*-Propylcyanamide (1.0 mM) | 1.84 ± 0.57‡ (3) | 82.9 ± 5.3 |
| *n*-Propylcyanamide (100 μM) | 3.01 ± 0.60‡ (3) | 72.0 ± 5.6 |
| *n*-Propylcyanamide (10 μM) | 7.84 ± 0.82† (3) | 27.1 ± 7.6 |

* Each compound was preincubated with intact rat liver mitochondria (2.18 mg protein) for 5 min at 38° as described under Materials and Methods. AlDH activity was assayed as previously described. Values are means ± S.E.; the number of animals is given in parentheses.

† $P < 0.005$ compared to respective saline control.

‡ $P < 0.001$ compared to respective saline control.

§ $P < 0.05$ compared to respective saline control.

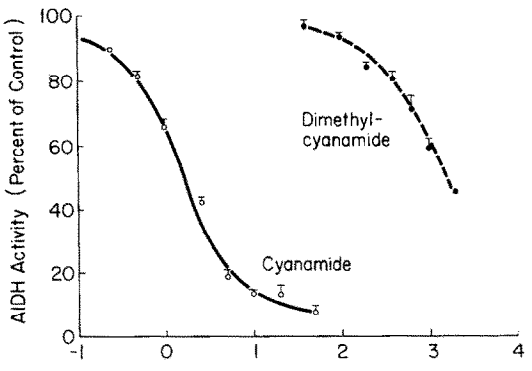


Fig. 4. Inhibition of the low $K_m$ mitochondrial AlDH isozyme by cyanamide (○) and DMC (●) *in vitro*. Intact rat liver mitochondria were preincubated for 5 min at 38° with cyanamide or DMC as described in Materials and Methods. All data points represent the mean ± S.E. of triplicate samples. One hundred percent = 14.38 ± 0.05 and 10.36 nmoles AcH oxidized·min$^{-1}$·(mg protein)$^{-1}$ for cyanamide and DMC respectively.

competitive inhibitor of the hepatic microsomal enzyme [20], did not prevent the accumulation of AcH in the blood when DMC was administered (Table 2). Stimulation of the synthesis of hepatic cytochrome P-450 enzymes by PB pretreatment did not enhance the inhibition of AlDH by DMC, as indicated by the absence of statistically significant differences between blood AcH levels in PB-pretreated and untreated (control) rats also treated with DMC.

*Effect of DMC and* n-*propylcyanamide on AlDH* in vitro. Preincubation of DMC or dicyandiamide (200 μM) with intact rat liver mitochondria for 5 min and assay of the remaining activity of the low $K_m$ AlDH isozyme indicated that they were poor inhibitors of this enzyme *in vitro*. Under these conditions, *n*-propylcyanamide, a monoalkyl cyanamide homologous to methylcyanamide, and cyanamide itself were potent inhibitors, reducing the specific activity of the enzyme by 83 and 99% respectively (Table 3). The comparative *in vitro* $ED_{50}$ values for cyanamide, *n*-propylcyanamide and DMC were estimated to be 2.0, 29 and 1600 μM (Table 3 and Fig. 4). As

Table 4. *N*-Demethylation of DMC by liver microsomes (100,000 *g*) derived from PB-pretreated and untreated rats

| DMC concn | Formaldehyde produced* [nmoles·(30 min)$^{-1}$·(mg protein)$^{-1}$] | |
|---|---|---|
| | PB-pretreated | Untreated |
| 20 mM | | |
| Complete system | 5.39 ± 0.02 | 2.24 ± 0.05 |
| Without cofactors | 0 | 0 |
| 40 mM | | |
| Complete system | 8.37 ± 0.2 | 2.97 ± 0.02 |
| Without cofactors | 0 | 0 |

* Assay conditions were as described in Materials and Methods. Each value is the mean ± S.E. of triplicate determinations.

the possibility existed that these cyanamides could alter mitochondrial oxidation of NADH, the effects of these compounds on mitochondrial respiration were determined under conditions used for the assay of AlDH in intact mitochondria. Using β-hydroxybutyrate as substrate, DMC, *n*-propylcyanamide and cyanamide at concentrations as high as 1.0 mM had no effect on mitochondrial respiration.

N-*Demethylation of DMC by rat liver microsomes*. DMC was *N*-demethylated by hepatic microsomal enzymes *in vitro* as indicated by the formaldehyde produced in the reaction (Table 4). Liver microsomes prepared from PB-pretreated rats were two to three times more effective in demethylating DMC than were microsomes from control animals. The concentration of the semicarbazide hydrochloride used to trap formaldehyde was 4 mM, in keeping with the report that semicarbazide may interfere with microsomal *N*-demethylation [21]. Concentrations of semicarbazide of 1–4 mM were found to have little effect on the amount of formaldehyde produced in these incubations.

## DISCUSSION

Among the compounds known to be potent inhibitors of ALDH *in vivo*, viz. disulfiram [22], pargyline [23, 24], coprine [25, 26], and cyanamide (*loc. cit.*), only the identity of the active inhibitory species from cyanamide remains unresolved. The present study was based on the hypothesis that a carbodiimide form of cyanamide might be responsible for inhibition of AlDH *in vivo*. Accordingly, we compared cyanamide with dimethylcyanamide, the latter being a cyanamide derivative which must be *N*-demethylated before a carbodiimide form can even be considered.

The elevation of ethanol-derived blood AcH and the inhibition of the low $K_m$ AlDH of liver mitochondria serve as indicators of significant AlDH inhibition [9]. That DMC satisfies these criteria *in vivo* is apparent from the data shown in Table 1 and Fig. 1. *In vivo* dose–response relationships (Fig. 1) indicate that DMC was at least one-fifth as effective as cyanamide on a molar basis. However, since the maximum inhibitory effect on AlDH by DMC was delayed, occurring between 12 and 24 hr after DMC administration (Fig. 2), and the above measurements were not made at optimal times, the $ED_{50}$ for DMC is very likely lower and might even approach that for cyanamide. The depletion of hepatic glutathione with time (Fig. 3) paralleled the time course of DMC-mediated AlDH inhibition (Fig. 2), with maximum depletion coinciding with the time (12 hr) at which the low $K_m$ AlDH was most inhibited by DMC. As glutathione plays an important detoxication role by inactivating chemically reactive intermediates formed in the metabolism of xenobiotic substances [27], these observations suggest that DMC is converted to a chemically reactive species *in vivo*—perhaps to a carbodiimide—which in turn can deplete hepatic glutathione. Surprisingly, cyanamide itself did not significantly lower glutathione when measured under similar conditions. This is a curious phenomenon which is currently under investigation.

The lack of effect of PB pretreatment on the

DMC-mediated elevation in blood AcH *in vivo* (Table 2)—where an enhancement might have been expected on the basis of the observed increase in demethylation of DMC by isolated microsomes (Table 4)—may have been due to the induction of the cytosolic $\phi$ (PB-inducible) enzyme under these conditions [28]. This was suggested somewhat by the lower blood AcH levels observed for the PB (control) compared to the saline (control) animals (Table 2). On the other hand, this may also have been due to the time of observation which was less than optimal as alluded to earlier. Similarly, the SKF-525A dose relative to that of DMC on a molar basis may have been too low to produce the expected inhibitory effect.

The $ED_{50}$ values for the inhibition of AlDH in intact mitochondria by cyanamide, *n*-propylcyanamide and DMC *in vitro* also lend support to the hypothesis that DMC must be converted to an active species *in vivo* before significant AlDH inhibition can be observed. Although methylcyanamide, the monodemethylated metabolite of DMC, could not be prepared in pure form to assess its activity *in vitro*—due to its high reactivity and facile trimerization to *s*-trimethylisomelamine [8, 29]—we were successful in synthesizing the more stable, two-carbon higher homolog of methylcyanamide, viz. *n*-propylcyanamide. This monoalkyl cyanamide proved to be a potent inhibitor of the low $K_m$ isozyme in the intact mitochondrial system, being nearly comparable to the activity of cyanamide itself (Table 3). Thus, the activity of DMC is very likely due to its monodemethylated product, methylcyanamide. However, the latter (as well as *n*-propylcyanamide) may still require metabolic activation, possibly by mitochondrial rather than by further microsomal action. Studies are in progress to determine the role of mitochondria in this activation process and to identify the active inhibitory species of cyanamide and of monoalkyl cyanamides.

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