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INHIBITION BY CYANAMIDE OF 4-HYDROXYCYCLOPHOSPHAMIDE/ALDOPHOSPHAMIDE OXIDATION TO CARBOXYPHOSPHAMIDE*

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Abstract—The relative rates at which normal and malignant cells oxidize 4-hydroxycyclophosphamide/aldophosphamide to carboxyphosphamide, a reaction catalyzed by NAD-linked aldehyde dehydrogenases and by aldehyde oxidase, may be potentially important in determining the relative sensitivities of these cells to cyclophosphamide. Potential inhibitors of the NAD-linked aldehyde dehydrogenase catalyzed reaction were sought in the present investigation. Pretreatment with cyanamide markedly depressed (70–98 percent) the catalytic activity of NAD-linked aldehyde dehydrogenases present in the 105,000 *g* soluble fraction and the solubilized 105,000 *g* particulate fraction of rat and mouse liver and kidney, and of W256 ascites carcinosarcoma cells, when butyraldehyde was used as the substrate. Disulfiram, diethyldithiocarbamic acid or allyl alcohol pretreatment was relatively ineffective. Acrolein or cyclophosphamide pretreatment was without effect. Oxidation of 4-hydroxycyclophosphamide/aldophosphamide to carboxyphosphamide by hepatic soluble and solubilized particulate fractions under incubation conditions optimal for the expression of NAD-linked aldehyde dehydrogenase activity was virtually abolished when the subcellular fractions were obtained from rats or mice treated with cyanamide. Conversion of 4-hydroxycyclophosphamide/aldophosphamide to carboxyphosphamide *in vivo* was markedly depressed in cyanamide-treated, functionally anephric, mice. Cyanamide should prove to be a useful tool in ascertaining whether the rate of 4-hydroxycyclophosphamide/aldophosphamide oxidation to carboxyphosphamide is an important determinant in the sensitivity of normal and malignant cells to cyclophosphamide.

Cyclophosphamide is a prodrug widely used as an antitumor and immunosuppressive agent. Its pharmacology and metabolism have been reviewed [1–4]. The current understanding of its metabolism is summarized in Fig. 1. It is first hydroxylated to 4-hydroxycyclophosphamide/aldophosphamide (HCP/AP), also a prodrug. The biochemical basis for its relatively good therapeutic index apparently resides with HCP/AP but it has not been elucidated [2–4]. The selective action of cyclophosphamide may depend, at least in part, on the relative rates at which HCP/AP is converted to cytotoxic and non-cytotoxic metabolites in sensitive and insensitive normal and neoplastic cells [5–10]. The principal urinary metabolite of cyclophosphamide is carboxyphosphamide [1–4]. Oxidation of HCP/AP gives rise to carboxyphosphamide, a metabolite with little cytotoxic activity [5, 10–16]. Aldehyde oxidase and NAD-linked aldehyde dehydrogenases are known to catalyze this reaction [9, 10, 13–16]. *In vitro* experiments suggest that the activity of NAD-linked aldehyde dehydrogenases may be biologically more important in this catalysis [9, 10, 14–16].

Cyanamide depresses NAD-linked aldehyde dehydrogenase activity [17–19] and thus may be potentially useful in investigations designed to determine the relative importance of NAD-linked aldehyde dehydrogenase activity with regard to the pharmacological response elicited by cyclophosphamide.

The present investigation was designed to assess the ability of cyanamide and other agents to inhibit the oxidation of HCP/AP to carboxyphosphamide.

MATERIALS AND METHODS

Materials. Sidechain (1.9 mCi/mmole)- and ring (C-4; 2.75 mCi/mmole)-labeled [^{14}C]cyclophosphamide were obtained from the New England Nuclear Corp., Boston, MA. Radiochemical purity, as determined by thin-layer chromatography in five different systems, was more than 98 percent.

Cyclophosphamide was supplied by Dr. W. A. Zygmunt, Mead Johnson & Co., Evansville, IN. Carboxyphosphamide and 4-ketocyclophosphamide were supplied by Dr. Robert F. Struck, Southern Research Institute, Birmingham, AL. Phosphoramide mustard·cyclohexylamine was supplied by Dr. H. B. Wood, Jr., Drug Development Branch, Division of Cancer Treatment, National Cancer

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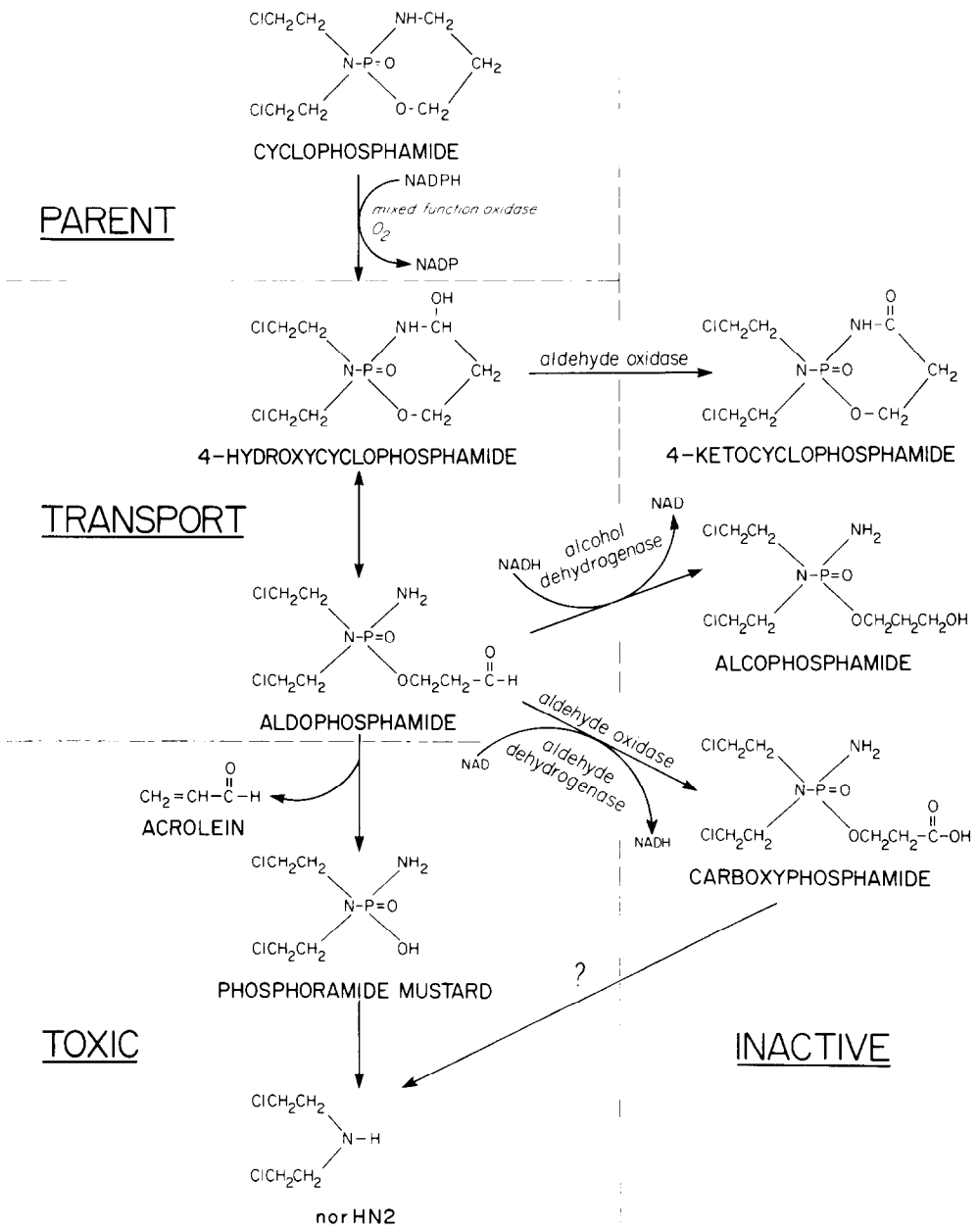

Fig. 1. Proposed route of cyclophosphamide metabolism.

Institute, Bethesda, MD. Authentic aldophosphamide semicarbazone was synthesized in our laboratory [20]. Cyanamide, butyraldehyde, benzaldehyde, allyl alcohol, acrolein, and bis(2-chloroethyl)amine hydrochloride were purchased from the Aldrich Chemical Co., Milwaukee, WI. Disulfiram, diethyldithiocarbamic acid, NAD and NADH were purchased from the Sigma Chemical Co., St. Louis, MO.

Male Holtzman rats, weighing 180–220 g, were purchased from the Holtzman Co., Madison, WI. Female BDF_1 mice, weighing 18–20 g, were purchased from ARS/Sprague–Dawley, Madison, WI. Rats were housed in hanging wire cages and mice in plastic cages filled with ground corn cob bedding. All animals were fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, MO) *ad lib.* Walker 256 rat mammary carcinosarcoma ascites cells were maintained by weekly transfer; their growth characteristics have been described previously [5, 21].

Drug administration. Cyanamide was dissolved in normal saline and was injected i.p. or s.c. in a volume of approximately 0.2 ml. Disulfiram was suspended in a 4% aqueous solution of carboxymethylcellulose sodium and given p.o. in a volume of approximately 1 ml. Diethyldithiocarbamic acid was dissolved in water and given p.o. in a volume of approximately 1 ml. Allyl alcohol, acrolein and cyclophosphamide

were placed in normal saline and were injected i.p. in a volume of approximately 0.5 ml. Dosages and administration schedules are noted with each experiment. All of the drug solutions and suspensions were administered immediately after preparation.

Tissue preparation. The 105,000 *g* soluble fractions and the solubilized 105,000 *g* particulate fractions were prepared from the livers and kidneys of rats and mice as described previously [16].

A 105,000 *g* soluble fraction and a solubilized 105,000 *g* particulate fraction were also prepared from Walker 256 carcinosarcoma cells. Ascites tumor cell-bearing rats were stunned by a blow to the head and decapitated, and the peritoneal cavity was opened. The tumor cells were withdrawn from the peritoneal cavity and diluted with 2 vol. of 1.15% potassium chloride solution. Saponin, pH 7.4, was added to the fluid to a final concentration of 0.17%. The mixture was allowed to stand at room temperature for 5 min to lyse any red blood cells present [22]. The suspension was then centrifuged at 100 *g* for 5 min at room temperature. The resultant supernatant fraction was aspirated, and the pellet, containing the tumor cells, was washed three times with ice-cold 1.15% potassium chloride solution. After resuspension in 1.15% potassium chloride solution, the tumor cells were counted with the aid of a hemocytometer and lysed with a Savant Insonator, model 1000, set at 90. Light microscopic examination revealed that complete cell lysis was achieved within 5 sec. The 105,000 *g* soluble fraction and the solubilized 105,000 *g* particulate fraction were then prepared as described previously [16]. The final preparations were diluted with 1.15% potassium chloride solution so that each milliliter contained subcellular fractions obtained from 1×10^9 cells.

Microsomes were prepared from the livers of phenobarbital-treated rats as described previously [16].

All tissue fractions were used immediately after preparation.

Enzyme assays. Aldehyde oxidase and NAD-linked aldehyde dehydrogenase activities in various tissue fractions were monitored spectrophotometrically as described previously [16]. Benzaldehyde and butyraldehyde, respectively, were used as substrates.

Cyclophosphamide metabolism in vitro. Metabolism of cyclophosphamide *in vitro* was monitored as described previously [16]. The general procedure was first to incubate sidechain-labeled [^{14}C]-cyclophosphamide with microsomes obtained from livers of phenobarbital-treated rats for 15 min; this served to generate HCP/AP. Incubation conditions were then adjusted to those optimal for the expression of NAD-linked aldehyde dehydrogenase or aldehyde oxidase activity: a hepatic 105,000 *g* soluble fraction or a solubilized 105,000 *g* particulate fraction obtained from control or cyanamide-treated rats or mice was added and the incubation was continued for an additional 15 min. Semicarbazide was then added and incubation was continued for yet another 15 min to allow aldophosphamide semicarbazone formation. Incubation with semicarbazide serves to trap both aldophosphamide and 4-hydroxycyclophosphamide because semicarbazide induces the conversion of 4-hydroxycyclophosphamide to aldophosphamide by forming the semicarbazone of the latter [23]. The incubation mixture was then deproteinized and the resultant deproteinized supernatant fraction was saved for radiochromatographic analysis.

Cyclophosphamide metabolism in vivo. Metabolism of cyclophosphamide *in vivo* was monitored as described previously [20]. Mice were anesthetized with ether, a dorsal incision was made, and the renal arteries and veins were ligated. The animals were allowed to recover from the anesthesia for 1 hr before any drugs were administered. Cyanamide (160 mg/kg, s.c.) was injected 45 min before the administration of [^{14}C]cyclophosphamide (65 mg/kg, i.p.; 5 μCi of sidechain- or 10 μCi of ring-labeled). Blood samples (90 μl) were obtained from the tail

Table 1. NAD-linked aldehyde dehydrogenase activity in hepatic subcellular fractions obtained from rats treated with various agents*

Treatment				NAD-linked aldehyde dehydrogenase activity (% of control)	
Agent	Dose (mg/kg)	Route	Time of administration (hr before killing)	Soluble	Particulate
Cyanamide	60	i.p.	1	2	3
Disulfiram	600	p.o.	48,24	78	67
Disulfiram	500	p.o.	96,72,48,24	70	71
Diethyldithocarbamic acid	700	p.o.	48,24	86	76
Diethyldithiocarbamic acid	500	p.o.	96,72,48,24	80	76
Acrolein	20	i.p.	3	100	100
Allyl alcohol	50	i.p.	3	77	98
Cyclophosphamide	200	i.p.	12	100	100

* Rats were treated according to the schedules indicated. Hepatic subcellular fractions were prepared, and NAD-linked aldehyde dehydrogenase activity was determined as described in Materials and Methods. The substrate was butyraldehyde. Control rates were 2.1 (soluble fraction) and 4.2 (particulate fraction) μmoles NADH formed · min^{-1} · (g liver)$^{-1}$. Each value is the mean of duplicate measurements performed on the designated liver fractions of each of two or three rats.

vein using a heparinized pipette at various times following cyclophosphamide administration. The samples were immediately placed in glass tubes containing 0.6 mg of semicarbazide hydrochloride dissolved in 90 μl of 10 mM phosphate buffer, pH 7.4. The mixture was then incubated at 37° for 15 min in a Dubnoff Metabolic Shaker (120 oscillations/min) to allow maximum aldophosphamide semicarbazone formation. At the end of this time, 90 μl of a 5.5% zinc sulfate solution was added, followed by 90 μl of a 4.5% barium hydroxide solution to precipitate proteins. The mixture was centrifuged at 9000 *g* for 20 min at 2 ± 2° and the resultant supernatant fraction was saved for radiochromatographic analysis.

Radiochromatographic analysis. Separation was effected on paper and silica gel. The solvent systems were isopropanol–ammonium hydroxide (4:1) and chloroform–methanol (9:1) respectively. Radioscans were obtained with a Packard Radiochromatogram Scanner, model 7201; a minimum of approximately 0.001 μCi could be detected. Metabolites detected as peaks on the radiochromatograms were identified and quantified as described previously [16, 20].

RESULTS

Treatment of rats with cyanamide, known to be a potent inhibitor of NAD-linked aldehyde dehydrogenase activity *in vivo* [17–19], almost totally eliminated *in vitro* hepatic soluble and particulate NAD-linked aldehyde dehydrogenase activity (Table 1). Cyanamide was virtually without inhibitory effect when added directly to incubation mixtures containing soluble or particulate fractions obtained from control animals [16]. These observations support the contention that a metabolite of cyanamide is responsible for the inhibition [19, 24].

Disulfiram, a good inhibitor of NAD-linked aldehyde dehydrogenase activity *in vitro* [16], depressed activity in the present experiments by only 22–33 percent even at near lethal doses. Diethyldithiocarbamic acid, a metabolite of disulfiram [25], depressed activity by only 14–24 percent. The present results are in agreement with those reported by Lamboeuf *et al.* [26] but differ from those obtained by Deitrich and Erwin [27], who observed a 70–80 percent depression of NAD-linked aldehyde dehydrogenase activity with either agent in rats.

Acrolein did not depress NAD-linked aldehyde dehydrogenase activity even at near lethal doses. Acrolein reportedly reacts with all available sulfhydryl groups [28] and is a potent inhibitor of NAD-linked aldehyde dehydrogenase activity *in vitro* [16]. It is possible that most, if not all, of the administered dose was inactivated before it reached the target site. The effect of an acrolein precursor, viz. allyl alcohol, was examined in an attempt to circumvent this possibility. Allyl alcohol itself is not reactive and is readily metabolized to acrolein by alcohol dehydrogenase [29, 30]. Alcohol dehydrogenase activity is abundantly present in hepatic cytosol. Hence, it seemed possible to generate acrolein in the general proximity of the target enzyme, NAD-linked aldehyde dehydrogenase, and thus minimize the reaction of acrolein with non-specific sites. Treatment with allyl alcohol, however, only minimally depressed NAD-linked aldehyde dehydrogenase activity (Table 1). Incubation of cyclophosphamide with hepatic microsomes has been reported to yield acrolein as one of the metabolites [31]. Treatment with cyclophosphamide did not depress NAD-linked aldehyde dehydrogenase activity (Table 1).

The effect of cyanamide treatment on NAD-linked aldehyde dehydrogenase activity was investigated further (Table 2). Initial experiments were designed to determine the optimal dose and treatment schedule required for maximal depression of activity. Preliminary experiments revealed that doses as high as 160 mg/kg could be administered s.c. to either rats or mice without lethal effects. The s.c. route was selected for routine use because in future experiments, where tumor cells were to be grown i.p. and

Table 2. NAD-linked aldehyde dehydrogenase and aldehyde oxidase activities in subcellular fractions of tissues obtained from rats and mice treated with cyanamide*

Animal	Time after cyanamide injection (min)	NAD-linked aldehyde dehydrogenase activity (% of control)						Aldehyde oxidase activity (% of control)
		Liver		Kidney		W256		
		Soluble	Particulate	Soluble	Particulate	Soluble	Particulate	Liver
Mouse	45	2 ± 1	7 ± 1	3 ± 1	14 ± 4			55 ± 12
	240	8 ± 1	19 ± 2	8 ± 1	30 ± 5			65 ± 8
Rat	45	2 ± 0	4 ± 1	2 ± 1	10 ± 1	8 ± 2	17 ± 2	56 ± 8
	240	4 ± 0	9 ± 1	5 ± 1	13 ± 1	ND†	ND	64 ± 8

* Cyanamide was administered s.c. to rats (60 mg/kg) or mice (160 mg/kg) 45 or 240 min before killing the animals. Rats bearing W256 ascites carcinosarcoma cells were injected with cyanamide 6 days after the i.p. injection of 10^6 tumor cells. Butyraldehyde and benzaldehyde were used as the substrates to quantify NAD-linked aldehyde dehydrogenase and aldehyde oxidase activities respectively. Control rates for NAD-linked aldehyde dehydrogenase activity in liver and kidney subfractions were 1.6 (mouse liver soluble), 2.3 (mouse liver particulate), 2.1 (rat liver soluble), 4.1 (rat liver particulate), 0.2 (mouse kidney soluble), 0.5 (mouse kidney particulate), 1.1 (rat kidney soluble), and 0.7 (rat kidney particulate) μmoles NADH formed·min^{-1}·(g tissue)$^{-1}$. Control rates for NAD-linked aldehyde dehydrogenase activity in W256 tumor cell subfractions were 22 (soluble) and 200 (particulate) pmoles NADH formed·min^{-1}·$cell^{-1}$. Control rates for aldehyde oxidase activity in liver soluble fractions were 0.05 (rat) and 0.11 (mouse) μmole benzaldehyde disappeared·min^{-1}·(g tissue)$^{-1}$. Each value is the mean ±S.E. of duplicate measurements performed on the tissues of each of three animals.

† Not determined.

cyanamide was to be administered, direct exposure of the tumor cells to high concentrations of this agent would be avoided. Forty-five minutes after the administration of cyanamide to mice (160 mg/kg) or rats (60 mg/kg), less than 10 percent of the hepatic NAD-linked aldehyde dehydrogenase activity remained. Enzyme activity remained below 20 percent of control throughout the 4-hr observation period. These observations are in agreement with the findings of others [18, 19]. Smaller doses of cyanamide, viz. 20 mg/kg (rats) and 80 mg/kg (mice), were nearly as effective; hepatic NAD-linked aldehyde dehydrogenase activity in the two subcellular fractions ranged between 8 and 14 percent of control 45 min after administration of the drug (data not presented).

NAD-linked aldehyde dehydrogenase activity in extrahepatic tissues could also catalyze the oxidation of HCP/AP to carboxyphosphamide [16]. Experiments were thus designed to determine what effect cyanamide would have on the NAD-linked aldehyde dehydrogenase activity present in two of these tissues. The kidney was included in these studies because, next to the liver, it contains the highest level of NAD-linked aldehyde dehydrogenase activity [16]. W256 tumor cells contain relatively little NAD-linked aldehyde dehydrogenase activity [16] but were included as a representative of the ultimate target of cyclophosphamide therapy. NAD-linked aldehyde dehydrogenase activity was virtually eliminated in these tissues 45 min after cyanamide administration (Table 2). Recovery was minimal 4 hr following cyanamide treatment. These observations suggest that cyanamide is a potent inhibitor of NAD-linked aldehyde dehydrogenase activity in all body tissues.

Cyanamide also inhibited aldehyde oxidase activity (Table 2). However, the inhibitory effect of cyanamide was relatively specific for NAD-linked aldehyde dehydrogenase activity since the aldehyde oxidase activity present in rat and mouse hepatic 105,000 *g* soluble fractions was depressed by only about 40 percent, whereas NAD-linked aldehyde dehydrogenase activity in these fractions was virtually eliminated after identical treatment. Aldehyde oxidase activity remained depressed for at least 4 hr after cyanamide administration.

Soluble fractions (105,000 *g*) obtained from the livers of control rats oxidized HCP/AP to carboxyphosphamide when incubation conditions were made optimal for the expression of NAD-linked aldehyde dehydrogenase activity; soluble fractions (105,000 *g*) obtained from the livers of cyanamide-treated rats were essentially unable to do so (Fig. 2). Identical results were obtained when the 105,000 *g* soluble fractions were obtained from the livers of mice. Similarly, solubilized 105,000 *g* particulate fractions obtained from the livers of cyanamide-treated rats or mice were unable to oxidize HCP/AP to carboxyphosphamide when incubation conditions were made optimal for the expression of particulate NAD-linked aldehyde dehydrogenase activity, whereas solubilized 105,000 *g* particulate fractions obtained from the livers of control rats or mice were (data not presented).

No oxidation of HCP/AP to carboxyphosphamide

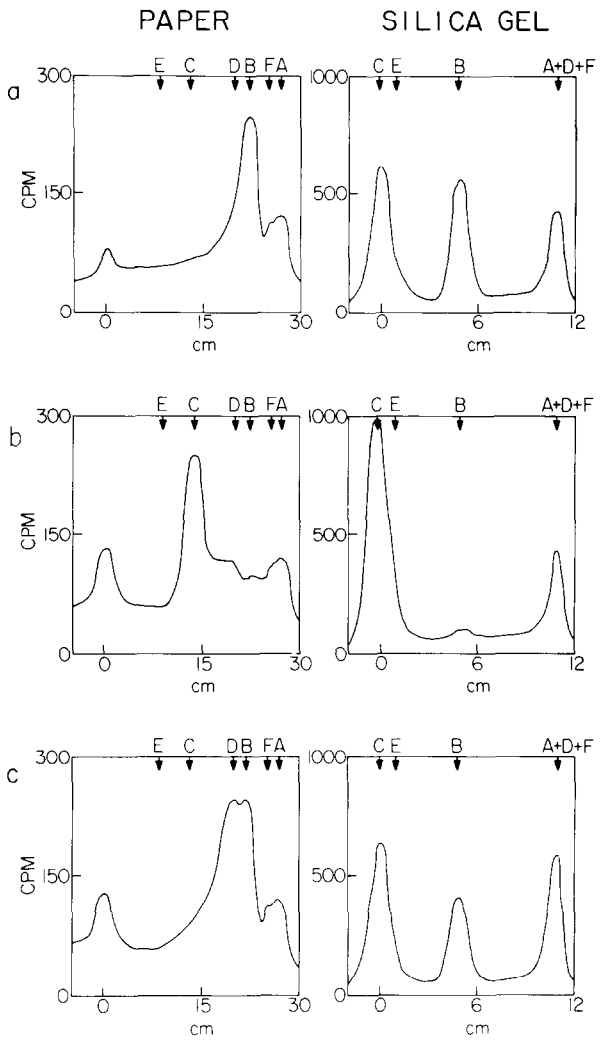

Fig. 2. Metabolism of cyclophosphamide *in vitro*. Except as noted below, sidechain-labeled [^{14}C]cyclophosphamide was incubated with rat hepatic microsomes for 15 min under conditions optimal for the expression of mixed-function oxygenase activity (first incubation); hepatic 105,000 *g* soluble fraction obtained from control or cyanamide-treated (60 mg/kg, s.c., 45 min before killing) rats was added after adjusting the incubation media to conditions optimal for the expression of soluble NAD-linked aldehyde dehydrogenase activity and incubation was continued for an additional 15 min (second incubation); semicarbazide was then added and incubation was continued for yet another 15 min (third incubation). Following incubation, cyclophosphamide and its metabolites were chromatographically separated on paper and silica gel. Representative radioscans of these chromatograms are shown. Details describing the preparation of subcellular fractions, incubation conditions, separation techniques, and radiochromatographic analysis are provided in Materials and Methods. The letters above each scan identify the areas of the chromatogram to which the authentic compounds migrate: A, cyclophosphamide; B, aldophosphamide semicarbazone; C, carboxyphosphamide; D, 4-ketocyclophosphamide; E, phosphoramide mustard·cyclohexylamine; and F, bis(2-chloroethyl)amine HCl. Panel a: The hepatic 105,000 *g* soluble fraction was omitted during the second incubation. Panel b: Hepatic 105,000 *g* soluble fraction from control rats was added prior to the second incubation. Panel c: Hepatic 105,000 *g* soluble fraction from cyanamide-treated rats was added prior to the second incubation.

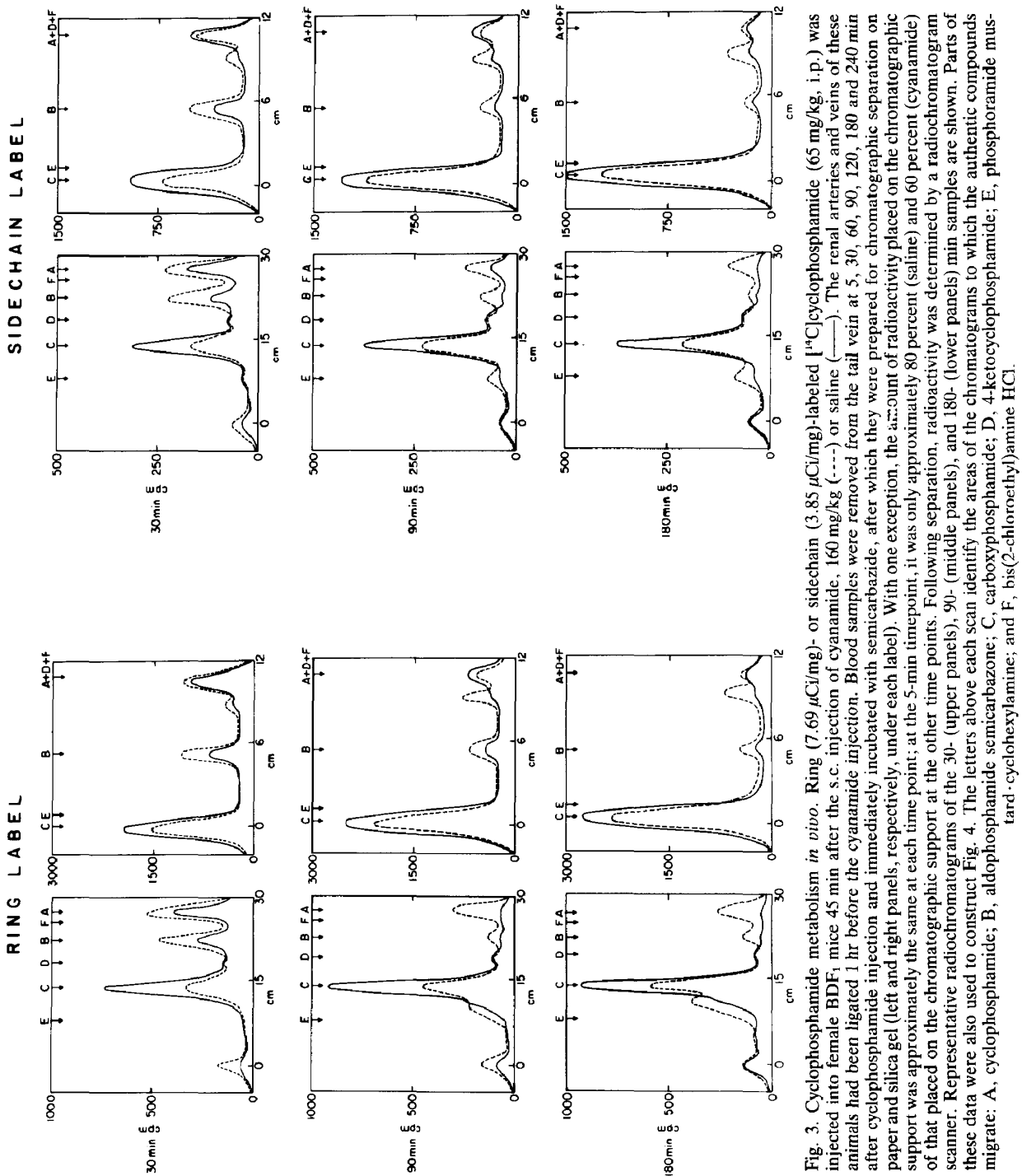

Fig. 3. Cyclophosphamide metabolism *in vivo*. Ring (7.69 μCi/mg)- or sidechain (3.85 μCi/mg)-labeled [^{14}C]cyclophosphamide (65 mg/kg, i.p.) was injected into female BDF_1 mice 45 min after the s.c. injection of cyanamide, 160 mg/kg (- - -) or saline (——). The renal arteries and veins of these animals had been ligated 1 hr before the cyanamide injection. Blood samples were removed from the tail vein at 5, 30, 60, 90, 120, 180 and 240 min after cyclophosphamide injection and immediately incubated with semicarbazide, after which they were prepared for chromatographic separation on paper and silica gel (left and right panels, respectively, under each label). With one exception, the amount of radioactivity placed on the chromatographic support was approximately the same at each time point; at the 5-min timepoint, it was only approximately 80 percent (saline) and 60 percent (cyanamide) of that placed on the chromatographic support at the other time points. Following separation, radioactivity was determined by a radiochromatogram scanner. Representative radiochromatograms of the 30- (upper panels), 90- (middle panels), and 180- (lower panels) min samples are shown. Parts of these data were also used to construct Fig. 4. The letters above each scan identify the areas of the chromatograms to which the authentic compounds migrate: A, cyclophosphamide; B, aldophosphamide semicarbazone; C, carboxyphosphamide; D, 4-ketocyclophosphamide; E, phosphoramide mustard·cyclohexylamine; and F, bis(2-chloroethyl)amine HCl.

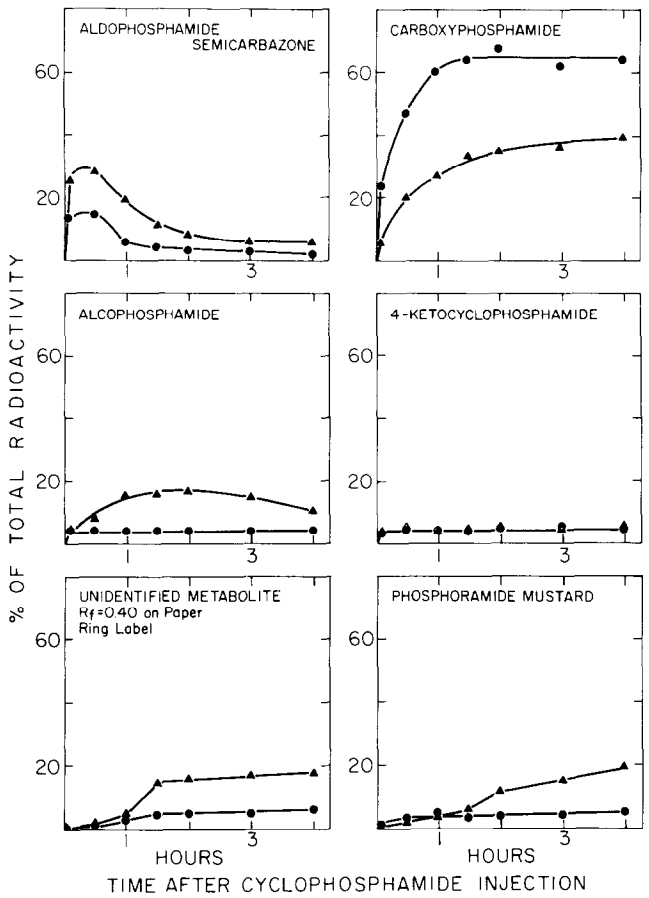

Fig. 4. Cyclophosphamide metabolism *in vivo*: Effect of cyanamide. Ring (7.69 μCi/mg)- or sidechain (3.85 μCi/mg)-labeled [^{14}C]cyclophosphamide (65 mg/kg, i.p.) was injected into female BDF_1 mice 45 min after the s.c. injection of cyanamide, 160 mg/kg (▲) or saline (●). The renal arteries and veins of these animals had been ligated 1 hr before the cyanamide injection. Blood samples were removed from the tail vein at the times indicated and immediately incubated with semicarbazide, after which they were prepared for chromatographic separation. Following separation, radioactivity was determined by a radiochromatogram scanner. Metabolites were quantified by weighing each peak of the resultant tracing. Representative scans and additional experimental details are presented in Fig. 3. Each point is the mean value of measurements made from two to four chromatograms prepared from blood samples obtained from two mice. As defined in Materials and Methods, the term aldophosphamide semicarbazone is synonymous with HCP/AP.

was observed when 105,000 *g* soluble fractions obtained from the livers of control or cyanamide-treated rats were used and media conditions were made optimal for the expression of aldehyde oxidase activity [16].

The effects of cyanamide treatment on the metabolism of cyclophosphamide *in vivo* are shown in Figs. 3 and 4. Maximum HCP/AP levels occurred between 5 and 30 min and were increased from the control value of 15 percent of the total radioactivity (about 50 μM) to 28 percent of the total radioactivity when mice were treated with cyanamide. Concurrently, the level of carboxyphosphamide decreased from 47 percent of the total radioactivity at 30 min in untreated animals to 20 percent of the total radioactivity at this time in cyanamide-treated mice. By 120 min after cyclophosphamide administration, the level of HCP/AP was less than 10 percent of the total radioactivity in both control and cyanamide-treated animals. Concurrent with the decline in HCP/AP concentrations, carboxyphosphamide concentrations increased to a maximum of 65 and 35 percent of the total radioactivity in control and cyanamide-treated mice respectively. These observations demonstrate that cyanamide treatment inhibits the oxidation of HCP/AP to carboxyphosphamide *in vivo*.

Cyanamide treatment had no effect on the *in vivo* level of 4-ketocyclophosphamide; however, it did cause an increased production of at least three other metabolites, apparently at the expense of HCP/AP (Figs. 3 and 4). Increased production of one of these metabolites ($R_f = 0.75$ on silica gel and 0.88 on paper), tentatively identified as alcophosphamide [16], was first detected 30 min after cyclophosphamide administration. Alcophosphamide levels in cyanamide-treated animals reached a maximum of 16 percent of the total radioactivity at 60 min. This level was maintained for approximately 1 hr, after which time a gradual decline was observed throughout the rest of the observation period.

An increased production of two other metabolites

was observed in the blood of cyanamide-treated mice. These metabolites were separated from the other metabolites on paper but not on silica gel (Fig. 3). One metabolite ($R_f = 0.40$) was detected only when ring-labeled cyclophosphamide was administered; the other metabolite ($R_f = 0.28$) was detected only when sidechain-labeled cyclophosphamide was given, and it migrated with authentic phosphoramide mustard. These metabolites appeared to remain at the origin in the silica gel chromatography system. Elevated levels of these metabolites first occurred at about 90 min after cyclophosphamide injection. Identification of the metabolite migrating at $R_f = 0.40$ was not made. However, of the known cyclophosphamide metabolites that contain only the ring moiety, an acrolein derivative is a probable candidate [28].

DISCUSSION

NAD-linked aldehyde dehydrogenase activity may be an important determinant in the metabolism of cyclophosphamide and thus the relative sensitivities of malignant and normal cells to cyclophosphamide [5, 6, 9, 10]. Several observations are consistent with this possibility. NAD-linked aldehyde dehydrogenases catalyze the oxidation of HCP/AP to carboxyphosphamide [9, 10, 13–16]. This enzyme activity is apparently present in all tissues but in varying amounts [16, 32]; neoplastic tissues contain relatively small amounts [16]. An inverse relationship between the sensitivity of tumor cells to the cytotoxic activity of aliphatic aldehydes and their content of NAD-linked aldehyde dehydrogenase activity has been reported [33]. Several phosphorylated acetals and aldehydes, all potential substrates for NAD-linked aldehyde dehydrogenases, potentiated the antitumor activity of cyclophosphamide [34]. Cox *et al.* [9, 10] observed a direct correlation between the amount of NAD-linked aldehyde dehydrogenase activity in various rat tissues, the amount of carboxyphosphamide formed by these tissues, and the ability of these tissues to detoxify the metabolite(s) generated by incubating hepatic microsomes and cyclophosphamide.

Some observations are inconsistent with the hypothesis. Gurtoo *et al.* [35] utilized several strains of mice differing genetically in sensitivity to phenobarbital induction of aldehyde dehydrogenase activity and concluded that hepatic aldehyde dehydrogenase activity is not a significant determinant in cyclophosphamide metabolism as it may relate to the chemotherapeutic activity of cyclophosphamide because they were unable to establish any correlation between these variables. The fact that tissues may contain several NAD-linked aldehyde dehydrogenases, each with its own range of substrate specificities [36], may complicate the interpretation of their observations. Administration of an inhibitor of NAD-linked aldehyde dehydrogenase activity such as disulfiram would be expected to potentiate the antitumor and lethal effects of cyclophosphamide if this enzyme activity is a significant determinant in the metabolism of, and pharmacological response to, cyclophosphamide. Administration of disulfiram did potentiate the antitumor effects of cyclophosphamide in one investigation [37], but in other investigations it did not [10, 38, 39]. Potentiation of the lethal effect of cyclophosphamide by disulfiram has been reported [10]. The inconsistency in these observations may be explained by the present and another investigation [26] which demonstrated that NAD-linked aldehyde dehydrogenase catalyzed oxidation of some aldehydes is not particularly sensitive to inhibition by disulfiram *in vivo*.

Cyanamide may prove to be a better agent to use for studies of this nature. The present experiments clearly demonstrate that the oxidation of HCP/AP to carboxyphosphamide *in vivo* is catalyzed by a cyanamide-sensitive enzyme, most probably one or more of the NAD-linked aldehyde dehydrogenases. Relative to those observed in control, functionally anephric animals, the plasma levels of HCP/AP, alcophosphamide, phosphoramide mustard, and of what may be a derivative of acrolein were elevated in cyanamide-treated, functionally anephric mice; in contrast, the plasma levels of 4-ketocyclophosphamide and carboxyphosphamide were unchanged and depressed, respectively, in these animals. These observations are consistent with the expectation that, when HCP/AP oxidation to carboxyphosphamide is inhibited, more of the HCP/AP is available for enzymatic and/or nonenzymatic conversion to other metabolites. Evidence for the contention that these changes in plasma levels of cyclophosphamide metabolites were due to an inhibition of NAD-linked aldehyde dehydrogenase activity effected by cyanamide was provided by experiments in which the oxidation of HCP/AP to carboxyphosphamide by hepatic 105,000 *g* soluble, or solubilized 105,000 *g* particulate, or fractions *in vitro* under incubation conditions optimal for the expression of NAD-linked aldehyde dehydrogenase activity was essentially abolished when the subcellular fractions were obtained from rodents treated with cyanamide.

The effect of cyanamide on the antitumor and toxic activities of cyclophosphamide is currently under investigation in our laboratory and will be the subject of a future communication.

Acrolein, a potential metabolite of cyclophosphamide, markedly inhibited the oxidation of HCP/AP to carboxyphosphamide by NAD-linked aldehyde dehydrogenases *in vitro* [16]. This observation is probably without significance *in vivo* since the administration of acrolein, allyl alcohol or cyclophosphamide to rats had little or no effect on NAD-linked aldehyde dehydrogenase activity.

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