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METABOLISM OF 4-HYDROXYCYCLOPHOSPHAMIDE/ALDOPHOSPHAMIDE *IN VITRO**

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Abstract—The metabolism of 4-hydroxycyclophosphamide/aldophosphamide (HCP/AP), the primary metabolite of cylophosphamide, was studied *in vitro*. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and bovine-liver catalase did not metabolize HCP/AP. Neither did a rat hepatic 1000 *g* soluble fraction when conditions were made optimal for phosphamidase-catalyzed metabolism. Purified horse liver alcohol dehydrogenase converted HCP/AP to a metabolite tentatively identified as alcophosphamide. This metabolite was also found when HCP/AP was incubated with murine hepatic 105,000 *g* soluble fractions under conditions optimal for the expression of alcohol dehydrogenase activity. Murine hepatic 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions catalyzed the oxidation of HCP/AP to carboxyphosphamide when incubation conditions were made optimal for the expression of NAD-linked aldehyde dehydrogenase activity. Acrolein, disulfiram and butyraldehyde inhibited carboxyphosphamide formation in this system whereas pyridoxal, menadione and allopurinol did not. Oxidation of HCP/AP to carboxyphosphamide by murine hepatic 105,000 *g* soluble or solubilized 105,000 *g* particulate fractions was not observed in the absence of NAD or when incubation conditions were made optimal for the expression of aldehyde oxidase activity. NAD-linked aldehyde dehydrogenase activity was found in all of the normal and neoplastic tissues examined when butyraldehyde or benzaldehyde was used as the substrate. Aldehyde oxidase activity was found in some of the normal tissues but not in the neoplasms. NAD-linked aldehyde dehydrogenase activity greatly exceeded aldehyde oxidase activity in all tissues. These findings suggest that carboxyphosphamide formation from HCP/AP is predominantly catalyzed by NAD-linked aldehyde dehydrogenases.

Cyclophosphamide is a prodrug widely used as an antitumor and immunosuppressive agent; its pharmacology and metabolic fate have been reviewed recently [1–4]. It is first hydroxylated to 4-hydroxycyclophosphamide by a mixed function oxygenase system present largely in hepatic endoplasmic reticulum. 4-Hydroxycyclophosphamide is believed to exist in equilibrium with its ring-opener tautomer, aldophosphamide. 4-Hydroxycyclophosphamide/aldophosphamide (HCP/AP) is also a prodrug and undergoes one of two fates: either hydrolysis to phosphoramide mustard, and acrolein, each of which is cytotoxic; or conversion to the relatively noncytotoxic metabolites, carboxyphosphamide, 4-ketocyclophosphamide and alcophosphamide. Phosphoeamide mustard can give rise, via hydrolysis, to bis(2-chloroethyl)amine, yet another cytotoxic metabolite. Carboxyphosphamide is the principal metabolite excreted in the urine and can also give rise, via hydrolysis, to bis(2-chloroethyl)amine.

The biochemical basis for the selective toxicity of cyclophosphamide apparently resides with HCP/AP but has not been elucidated [2–5].

The selective action of cyclophosphamide may depend, at least in part, on the relative rates at which HCP/AP is converted to cytotoxic and noncytotoxic metabolites in sensitive and insensitive normal and neoplastic cells [6–11]. Little is known about the catalysis of these conversions other than that aldehyde oxidase and NAD-linked aldehyde dehydrogenases are capable of catalyzing the oxidation of HCP/AP to carboxyphosphamide [8, 10–14]. The relative importance of the two enzyme activities in the catalysis of this reaction is unknown.

The present investigations are the first in a series intended to gain more knowledge about the metabolism of HCP/AP.

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MATERIALS AND METHODS

Materials. Side chain (1.9 mCi/mmole) labeled [^{14}C]cyclophosphamide was obtained from the New England Nuclear Corp. Boston, MA. Radiochemical purity, determined by chromatography in five different systems, was above 98 per cent.

Cyclophosphamide was supplied by Dr. W. A. Zygmunt, Mead Johnson & Co., Evansville, IN. Carboxyphosphamide and 4-ketocyclophosphamide were supplied by Dr. R. 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 Institute, Bethesda, MD. Propranolol hydrochloride was supplied by the Ayerst Research Laboratory, New York, NY. Authentic aldophosphamide semicarbazone was synthesized in our laboratories [5].

Phenobarbital sodium was purchased from Merck & Co., Rahway, NJ. Cyanamide, butyraldehyde, benzaldehyde, acrolein, bis(2-chloroethyl)amine hydrochloride and pyridoxal hydrochloride were purchased from the Aldrich Chemical Co., Milwaukee, WI. Disulfiram, menadione sodium bisulfite, diethyldithiocarbamic acid, allopurinol, NAD, NADH, NADPH, purified rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (one unit reduces 1 μmole of 3-phosphoglycerate to glyceraldehyde-3-phosphate per minute at pH 7.6 and 25°), purified bovine liver catalase (one unit decomposes 1 μmole of hydrogen peroxide per minute at pH 7 and 25°) and purified horse liver alcohol dehydrogenase (1 mg converts approximately 1.3 μmoles of ethanol to acetaldehyde per minute at pH 8.8 and 25°) were purchased from the Sigma Chemical Co., St. Louis, MO. Glucose-6-phosphate dehydrogenase (one enzyme unit reduces 1 μmole of NADP per minute at pH 7.4 and 20°) was purchased from the Boehringer Mannheim Corp., New York, NY. Chloral hydrate was obtained from the University of Minnesota Hospitals Pharmacy, Minneapolis, MN.

Male, Holtzman rats, weighing 170–220 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.*

Rat Walker 256 mammary carcinosarcoma cells were grown subcutaneously as described previously [15]. Mouse MC-79 and MBE sarcomas were grown subcutaneously and supplied by Dr. C. F. McKhann, University of Minnesota, Minneapolis, MN. These tumors originated in the leg muscles of mice administered 3-methylcholanthrene subcutaneously.

Subcellular fraction preparation. Washed microsomes, prepared from the livers of phenobarbital-treated rats or mice, were used to activate cyclophosphamide [16]. Phenobarbital sodium dissolved in 0.9% sodium chloride was injected i.p. to induce hepatic microsomal mixed function oxygenase activity. Rats and mice were injected once and twice daily, respectively, for 5 days (40 mg/kg per injection), and were killed 24 and 12 hr, respectively, after the last dose. Animals were stunned by a blow to the head and decapitated. Livers were quickly excised, placed into an ice-cold 1.15% potassium chloride solution, trimmed of all foreign tissues, blotted dry, and weighed. All subsequent procedures for the preparation of the tissue fractions were carried out at 0–4°. A Dounce homogenizer was used to prepare 25–50% homogenates in a 1.15% potassium chloride solution. The homogenate was centrifuged at 9,000 *g* for 20 min and the resultant supernatant fraction was then removed and centrifuged at 105,000 *g* for 60 min. The supernatant fraction was discarded and the microsomal pellet was resuspended, with the aid of a Dounce homogenizer, in 1.15% potassium chloride solution so that each ml contained the equivalent of 500 mg of liver. The suspension was centrifuged again at 105,000 *g* for 30 min, the supernatant fraction was discarded, and the pellet was resuspended as before. All microsomal fractions were used immediately after preparation.

Soluble (105,000 *g*), and solubilized particulate (105,000 *g*), fractions were prepared from various tissues of untreated rats and mice. The desired tissues were excised and whole homogenates were prepared as described above except that a Sorval Omni-mixer was used in addition to assist in the homogenization of skeletal muscle, stomach, and small intestine. The whole homogenates were then centrifuged at 105,000 *g* for 60 min at 2 ± 2°. The resultant soluble fractions were removed and diluted with 1.15% potassium chloride solution so that each ml contained the equivalent of 500 mg of tissue. The pellets were resuspended in 1.15% potassium chloride solution so that, following the addition of deoxycholate to a final concentration of 0.3% and thorough mixing to solubilize particulate proteins, each ml contained the equivalent of 500 mg of tissue. The suspension was then centrifuged at 105,000 *g* for 60 min at 2 ± 2°. The resultant supernatant (solubilized particulate) fractions were removed and diluted with 1.15% potassium chloride solution so that each ml contained the equivalent of 500 mg of tissue. All fractions were used immediately after preparation.

Nucleic acids were freed from whole homogenates by the method of Schneider [17], and the DNA content was determined by the method of Dische [18] as modified by Davidson and Waymouth [19]. Calf thymus DNA was used as the standard.

Soluble fractions (1000 *g*) were prepared from the livers of untreated rats by centrifugation of the whole liver homogenate, prepared as described above, at 1000 *g* for 10 min at 2 ± 2°. The resultant soluble fraction was removed and diluted with 1.15% potassium chloride solution so that each ml contained the equivalent of 100 mg of liver. These fractions were also used immediately after preparation.

Spectrophotometric assay for NAD-linked aldehyde dehydrogenase activity. The complete reaction mixture contained NAD (12 μmoles), pyrazole (600 μmoles), butyraldehyde or benzaldehyde (12 μmoles), potential inhibitor, the 105,000 *g* soluble fraction or the solubilized 105,000 *g* particulate fraction obtained from 10 to 20 mg of tissue, and sodium pyrophosphate buffer (96 μmoles) adjusted to pH 7.8 when monitoring activity present in the

soluble fraction and to pH 8.8 when monitoring activity in the solubilized particulate fraction, in a final volume of 3 ml. Preliminary experiments established these conditions as optimal for hepatic NAD-linked aldehyde dehydrogenase activity. All potential inhibitors of NAD-linked aldehyde dehydrogenase activity were added to the incubation media in aqueous solution except disulfiram, which was added in 10 μl of ethanol. The reaction was started by adding butyraldehyde or benzaldehyde and was followed by monitoring NADH formation at 340 nm in a Gilford 2400 automatic recording spectrophotometer equipped with a Haake Constant Temperature Circulator to maintain a constant reaction temperature of 30°. The buffer and pyrazole solutions were warmed to 30° before addition to the cuvettes. Subcellular fractions, butyraldehyde and benzaldehyde were kept on ice prior to their introduction into the incubation media. Initial velocities and a molar extinction coefficient of 6100 for NADH were used to quantify enzyme activity. Butyraldehyde and benzaldehyde were omitted from the blank mixture.

Spectrophotometric assay for aldehyde oxidase activity. Aldehyde oxidase activity was measured by the method of Johns [20] under conditions determined in preliminary experiments to be optimal for this activity in liver fractions. The complete reaction mixture contained ammonium sulfate (390 μmoles), ethylenediaminetetraacetic acid tetrasodium (3 μmoles), sodium pyrophosphate buffer (9.6 μmoles) adjusted to pH 7.4, benzaldehyde (0.15 μmole), potential inhibitor, and the 105,000 *g* soluble fraction obtained from 40 mg of tissue, in a final volume of 3 ml. All potential inhibitors of aldehyde oxidase activity were added to the incubation media in aqueous solution except disulfiram which was added in 10 μl of ethanol. The reaction was started by adding benzaldehyde and was followed by monitoring the decrease in absorbance at 249 nm that resulted from the oxidation of benzaldehyde to benzoic acid, in a recording Aminco DW-2 UV/Vis spectrophotometer. A Haake Constant Temperature Circulator was used to maintain a constant reaction temperature of 30°. All constituents except benzaldehyde and the 105,000 *g* soluble fraction were warmed to 30° before addition to the cuvettes. Benzaldehyde and the subcellular fractions were kept on ice prior to their introduction into the incubation media. Initial velocities and a molar extinction coefficient of 17,540 for benzaldehyde were used to quantify enzyme activity. Benzaldehyde was omitted from the blank mixture.

Incubation conditions for the formation and metabolism of HCP/AP in vitro. The general procedure was to first incubate [^{14}C]cyclophosphamide with washed hepatic microsomes at 37° for 15 min; this served to generate HCP/AP. Radiolabeled HCP/AP had to be enzymatically generated in these investigations because it is relatively unstable [1–4] and not commercially available. Incubation conditions were then altered to those optimal for the specific enzyme activity to be investigated; various crude hepatic and purified enzyme fractions or the appropriate control solution were added and incubation was continued at 37° for an additional 15 min. Semicarbazide (1.5 μmoles in 5 μl, pH 7.4) was then added and incubation continued for yet another 15 min to allow aldophosphamide semicarbazone formation. Following the final incubation, protein was precipitated by the addition of 50 μl of 5.5% zinc sulfate solution and 50 μl of 4.5% barium hydroxide solution. The mixture was centrifuged at 9000 *g* for 20 min at 2 ± 2°, and the resultant supernatant fraction was placed on ice; samples were immediately submitted to thin-layer and paper chromatography. Following development, chromatograms were scanned for radioactivity.

The following mixture was incubated in 10-ml open glass tubes to generate radiolabeled HCP/AP: NADPH (0.5 μmole), sodium pyrophosphate buffer adjusted to pH 7.4 (1.4 μmoles), [^{14}C]cyclo- phosphamide (0.09 μmole, 0.1 μCi), and washed microsomes obtained from 20 mg of liver, in a total volume of 65 μl. A control reaction mixture was made by substituting 1.15% potassium chloride for the microsomal preparation. The reaction was started by the addition of microsomes. Following incubation at 37° for 15 min in a Dubnoff metabolic shaker, the reaction was stopped by placing the tubes on ice. This preparation was subsequently used to determine the ability of the various subcellular and enzyme fractions to metabolize HCP/AP. Before such determinations were made, the constituents of the incubation mixture were altered to those optimal for the specific enzyme activity to be investigated.

NAD (0.38 μmole) and 105,000 *g* soluble, or solubilized 105,000 *g* particulate fractions equivalent to 4 or 5 mg of liver, respectively, were added when assessing the ability of NAD-linked aldehyde dehydrogenases, present in the two subcellular fractions, to oxidize HCP/AP. The pH was adjusted to 7.8 when the 105,000 *g* soluble fraction was the source of enzyme activity and to pH 8.4 when the solubilized 105,000 *g* particulate fraction was used. The incubation medium used when investigating the ability of the solubilized 105,000 *g* particulate fraction to oxidize HCP/AP to carboxyphosphamide was adjusted to pH 8.4 rather than to the optimum (pH 8.8) determined in the spectrophotometric assay because HCP/AP was excessively degraded at the higher pH.

Ammonium sulfate (13 μmoles), ethylenediaminetetraacetic acid tetrasodium (0.1 μmole), and 105,000 *g* soluble fraction equivalent to 4 mg of liver were added when investigating the ability of aldehyde oxidase present in the 105,000 *g* soluble fraction to oxidize HCP/AP (pH 7.4).

The potential of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (6.8 units) or bovine liver catalase (0.5 units) for catalyzing the oxidation of HCP/AP at pH 7.8 and 8.4 was investigated under conditions otherwise optimal for these enzyme activities when glyceraldehyde-3-phosphate or H_2O_2, respectively, is used as the substrate [21]. NADH (0.38 μmole), horse liver alcohol dehydrogenase (0.1 mg) and sufficient sodium hydroxide to adjust the pH to 7.8 were added when the ability of alcohol dehydrogenase to catalyze the reduction of HCP/AP was determined.

When measuring the ability of phosphamidase in the 1000 *g* soluble fraction (obtained from 10 mg liver) to cleave one of the P–N bonds of HCP/AP, HCP/AP

Table 1. NAD-linked aldehyde dehydrogenase and aldehyde oxidase activities in 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions of normal and neoplastic tissues*

Tissue or cell	DNA (mg/g tissue)	Aldehyde dehydrogenase activity [nmoles NADH formed per min·(mg DNA)$^{-1}$]				Aldehyde oxidase activity [nmoles benzaldehyde metabolized per min·(mg DNA)$^{-1}$]
		Butyraldehyde		Benzaldehyde		Benzaldehyde
		Soluble	Particulate	Soluble	Particulate	Soluble
Liver	2.8 ± 0.2	1072 ± 72	1798 ± 216	798 ± 124	1098 ± 117	20 ± 2
Kidney	3.4 ± 0.2	434 ± 85	195 ± 28	368 ± 24	154 ± 11	8 ± 2
Testicle	2.4 ± 0.3	67 ± 8	150 ± 3	16 ± 3	80 ± 3	9 ± 1
Lung	5.0 ± 0.4	23 ± 3	43 ±9	9 ± 1	40 ± 9	2 ± 1
Stomach	1.8 ± 0.1	250 ± 20	167 ± 15	ND†	ND	8 ± 3
Small intestine	3.0 ± 0.2	40 ± 7	219 ± 23	ND	ND	3 ± 1
Skeletal muscle	0.7 ± 0.0	40 ± 3	47 ± 3	ND	ND	0
Thymus	16.6 ± 1.7	2 ± 1	8 ± 2	ND	ND	0
Spleen	12.7 ± 0.9	5 ± 1	39 ± 3	ND	ND	0
Walker 256 tumor	5.3 ± 0.6	1 ± 1	16 ± 1	ND	ND	0
MC-70 tumor	6.0 ± 0.5	4 ± 1	8 ± 4	ND	ND	0
MBE tumor	5.8 ± 0.1	6 ± 2	7 ± 1	ND	ND	0

* Tissues were isolated, prepared, and assayed for enzyme activity as described in Materials and Methods. All normal tissues were obtained from rats. Butyraldehyde and benzaldehyde were used as substrates. Each value is the mean ± S.E. of duplicate measurements performed on tissues obtained from each of three animals. The same group of animals was not always used to obtain each of the values for any given tissue.

† ND, not determined.

was generated as before except that a sodium barbital (28.6 μmoles)–sodium acetate (28.6 μmoles) buffer (pH 7.4) was used rather than the sodium pyrophosphate buffer, because phosphamidase activity was inhibited by inorganic phosphate. Preliminary experiments established a V_{max} of 790 nmoles *p*-chloroaniline formed per min·(g liver)$^{-1}$ and a K_m of 1.3 mM when a 1000 *g* soluble fraction obtained from rat liver was used as the enzyme source and *p*-chloroanilidophosphonic acid was used as the substrate. The acetate–barbital buffer had no detectable effect on the microsomal activation of cyclophosphamide.

All reactions in which the further metabolism of HCP/AP was investigated were started by addition of the subcellular or enzyme fraction. Control reaction mixtures were prepared by substituting water for the tissue or enzyme fraction. Total volume during the second incubation period was always 95 μl.

Radiochromatography. Separation of cyclophosphamide and its metabolites was effected on paper and silica gel as described previously [5]. The solvent systems used were isopropanol–NH_4OH (4:1) and chloroform–methanol (9:1) respectively. 4-Ketocyclophosphamide and cyclophosphamide migrated with similar R_f values on silica gel. Separation of these two compounds was accomplished by extracting the material at this R_f from silica gel with chloroform and rechromatographing the extract on analytical alumina plates using chloroform–methanol (4:1) as the solvent. Extraction efficiency was approximately 80 per cent. In some experiments analytical silica gel plates (Eastman Kodak Co., Rochester, NY) were developed in two dimensions to separate bis(2-chloroethyl)amine from the other metabolites. Chloroform–methanol (9:1) was used for development in the first dimension and 1-butanol–glacial acetic acid–water (3:1:1) was used for development in the second dimension.

After chromatographic development, the metabolites were detected and quantified by radioactivity measurements using a Packard radiochromatogram scanner, model 7201, as described previously [5].

RESULTS

The relative amounts of NAD-linked aldehyde dehydrogenase and aldehyde oxidase activities in several normal and neoplastic tissues were determined (Table 1). Butyraldehyde and benzaldehyde were used as substrates in these initial investigations because of the relative ease of monitoring enzyme activity with these substrates.

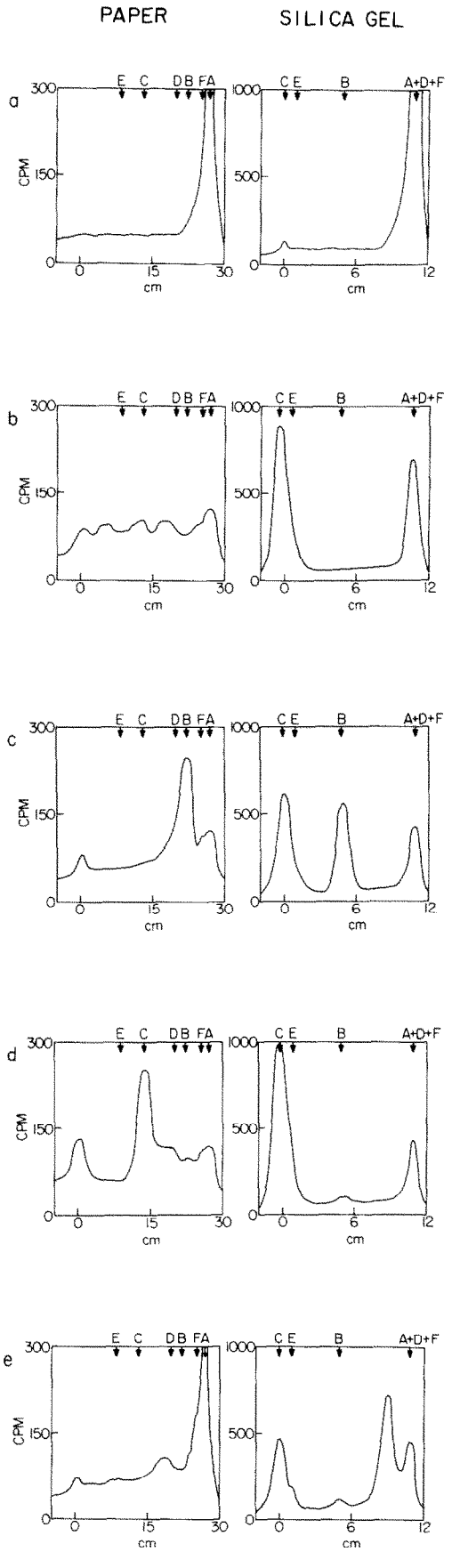
NAD-linked aldehyde dehydrogenase activity was found in both the 105,000 *g* soluble and the solubilized 105,000 *g* particulate fractions of each tissue examined, with the greatest activity being found in the liver (Deitrich [22] has reported similar finginds); the activity in subfractions of neoplastic tissues was relatively small. In other experiments, 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions obtained from cultured mouse P388 and L1210 lymphoma and from cultured Novikoff rat hepatoma cells contained only small amounts of NAD-linked aldehyde dehydrogenase activity, *viz.* 1–18 nmoles NADH formed per min·(mg DNA)$^{-1}$ when butyraldehyde was used as the substrate (data not presented). In agreement with the findings of others [23], aldehyde oxidase activity was found only in the

Table 2. Inhibition of rat hepatic NAD-linked aldehyde dehydrogenase and aldehyde oxidase activity *in vitro**

Inhibitor	Concn (mM)	Inhibition (%)		
		Aldehyde oxidase activity	NAD-linked aldehyde dehydrogenase activity	
			Soluble	Particulate
Cyclophosphamide	0.1	0	0	0
Acrolein	0.1	20	98	96
Disulfiram	0.1	ND†	91	34
Diethyldithiocarbamic Acid	0.1	ND	0	0
Cyanamide	1.0	ND	7	7
Cyanamide	0.1	ND	0	0
Pyridoxal HCl	1.0	100	0	0
Menadione	0.1	92	5	0
Allopurinol	0.2	80	0	0
Allopurinol	0.03	10	ND	ND
Chloral hydrate	0.1	ND	10	0
Propranolol	0.1	ND	0	0

* Hepatic 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions were prepared, and NAD-linked aldehyde dehydrogenase and aldehyde oxidase activities were determined spectrophotometrically, as described in Materials and Methods. The substrates for NAD-linked aldehyde dehydrogenase and aldehyde oxidase were butyraldehyde and benzaldehyde, respectively. Control rates for NAD-linked aldehyde dehydrogenase activity, expressed as μmoles NADH formed per min·(g liver)$^{-1}$, were 2.3 (soluble fraction) and 4.5 (particulate fraction). The control rate for aldehyde oxidase activity was 0.05 μmole benzaldehyde metabolized per min·(g liver)$^{-1}$. Each value is the mean of duplicate measurements performed on the designated liver fractions of each of one or two rats.

† ND, not determined.



105,000 *g* soluble fraction; it was not detected in any of the neoplastic tissues nor in some of the normal tissues. Moreover, NAD-linked aldehyde dehydrogenase activity greatly exceeded aldehyde oxidase activity in those tissues where the latter was found. A tumor burden, *viz.* Walker 256 carcinosarcoma or P388 lymphoma growing i.p., s.c. or i.m., did not alter the magnitude of hepatic NAD-linked aldehyde dehydrogenase or aldehyde oxidase activities (data not presented).

The effects of known and potential inhibitors of NAD-linked aldehyde dehydrogenase- and aldehyde oxidase-catalyzed reactions are presented in Table 2. Cyclophosphamide did not inhibit either enzyme activity. Acrolein was a potent inhibitor of both soluble and particulate NAD-linked aldehyde dehydrogenase activity; it was not a substrate for these enzymes. Inhibition was decreased when the concentration of the subcellular fraction was increased and was delayed when the concentration of NAD was increased (data not presented). In view of the evidence that NAD-linked aldehyde dehydrogenases contain thiol groups, possibly located at the catalytic site [24, 25], it seems likely that acrolein inhibited these enzymes by binding to the sulfhydryl groups located at these sites. Aldehyde oxidase also contains sulfhydryl groups [26]. However, 0.01 mM acrolein, which virtually eliminated NAD-linked aldehyde dehydrogenase activity, caused only a 20 per cent inhibition of aldehyde oxidase activity.

Disulfiram, a recognized inhibitor of NAD-linked aldehyde dehydrogenase activity [27], inhibited both

Fig. 1. Metabolism of cyclophosphamide *in vitro*. Except as noted below, [^{14}C]cyclophosphamide was incubated under conditions optimal for mixed-function oxygenase activity with rat hepatic microsomes for 15 min (first incubation); rat hepatic 105,000 *g* soluble fraction or alcohol dehydrogenase was added after adjusting the incubation media to conditions optimal for NAD-linked aldehyde dehydrogenase or alcohol 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). Panel a: Microsomes were omitted during the first incubation and neither the 105,000 *g* soluble fraction nor alcohol dehydrogenase was added prior to the second incubation. Panel b: Neither the 105,000 *g* soluble fraction nor alcohol dehydrogenase was added prior to the second incubation and semicarbazide was omitted from the third incubation. Panel c: Neither the 105,000 *g* soluble fraction nor alcohol dehydrogenase was added prior to the second incubation. Panel d: The 105,000 *g* soluble fraction was added prior to the second incubation. Panel e: Alcohol dehydrogenase was added prior to the second incubation. Appropriate control experiments were conducted as described in Materials and Methods. Following incubation, cyclophosphamide and its metabolites were chromatographically separated on paper and silica gel. Representative radioscans of these chromatograms are shown in this figure. 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 area of the chromatogram where 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.

the soluble and particulate enzymes; enzyme activity in the soluble fraction drop was more sensitive to disulfiram than was that in the particulate fraction. Disulfiram was not a substrate for these enzymes. Diethyldithiocarbamic acid, a metabolite of disulfiram, did not inhibit NAD-linked aldehyde dehydrogenase activity; cyanamide, believed to be an inhibitor of NAD-linked aldehyde dehydrogenase activity *in vivo* [28], had little effect on its activity *in vitro*.

Pyridoxal hydrochloride completely prevented benzaldehyde oxidation by aldehyde oxidase but had no effect on NAD-linked aldehyde dehydrogenase activity. Menadione, a known inhibitor of aldehyde oxidase [20], markedly inhibited it but was ineffective against NAD-linked aldehyde dehydrogenase activity. Allopurinol inhibited aldehyde oxidase activity but had no effect on NAD-linked aldehyde dehydrogenase activity. It may be a substrate for aldehyde oxidase [29] and, therefore, a competitive inhibitor of reactions catalyzed by this enzyme. Propranolol and chloral hydrate, each reportedly an inhibitor of NAD-linked aldehyde dehydrogenase [30, 31], were without inhibitory effect.

The ability of hepatic 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions to catalyze the oxidation of HCP/AP to carboxyphosphamide under conditions optimal for the expression of NAD-linked aldehyde or dehydrogenase or aldehyde oxidase activities was assessed next (Fig. 1). Authentic cyclophosphamide migrated near the solvent front in the chromatographic systems. Incubation of cyclophosphamide in the absence of microsomes did not result in the appearance of any new radioactive peaks in either system (Fig. 1a), indicating that cyclophosphamide did not degrade during the course of the incubation or chromatographic separation. Incubation of cyclophosphamide with microsomes resulted in the appearance of several new peaks on the paper chromatogram and the appearance of a single new peak at approximately the origin on the silica gel chromatogram (Fig. 1b). The new peaks are believed to represent HCP/AP and its breakdown products. The potential problems associated with what appeared to be spontaneous degradation of HCP/AP were minimized by incubating the product of microsomal activation with semicarbazide to yield a stable semicarbazone derivative [6, 13, 32]. A comparison of the radiochromatographic scans shown in Fig. 1b and 1c reveals the appearance of a new peak at R_f 0.72 (paper) and at R_f 0.45 (silica gel) following the addition of semicarbazide to the incubation mixture; the appearance of the new peak in the presence of semicarbazide was accompanied by the diminution of the peaks seen in its absence. Concentrations of semicarbazide above 15 mM did not further increase the size of the peaks. The relative sizes of the new peaks were consistently reproducible. Qualitatively identical results were obtained when cyclophosphamide was incubated with microsomes obtained from mice (data not presented). The metabolite represented by the new peaks was identified as aldophosphamide semicarbazone by co-chromatography with authentic aldophosphamide semicarbazone in six different systems [5]. Which tautomer, 4-hydroxycyclophosphamide or aldophosphamide, was the predominant form present in the incubation mixture is unknown, because addition of semicarbazide induces conversion of 4-hydroxycyclophosphamide to aldophosphamide by forming the semicarbazone of the latter [32]. After incubation of HCP/AP with rat hepatic 105,000 *g* soluble fraction, under conditions optimal for the expression of NAD-linked aldehyde dehydrogenase activity, a new peak at R_f 0.46 on the paper chromatogram and a peak of greater magnitude at R_f 0.09 on the silica gel chromatogram occurred (Fig. 1d) The metabolite represented by the new peak was identified as carboxyphosphamide by mass spectral analysis and cochromatography as described previously [5]. Appearance of the peaks representing carboxyphosphamide was accompanied by the virtual disappearance of the peaks representing aldophosphamide semicarbazone. Incubation of HCP/AP with rat or mouse hepatic solubilized 105,000 *g* particulate fractions or mouse hepatic 105,000 *g* soluble fractions under conditions optimal for NAD-linked aldehyde dehydrogenase activity gave essentially identical results (data not presented).

The amount of conversion to carboxyphosphamide was shown to depend on the concentration of the subcellular fraction. The equivalent of 1 mg of liver had to be added to the incubation mixture before detectable amounts of carboxyphosphamide were formed with either the 105,000 *g* soluble or solubilized 105,000 *g* particulate fractions. Soluble (105,000 *g*) and solubilized particulate (105,000 *g*) fractions equivalent to 4 and 5 mg of liver, respectively, were selected for routine use in subsequent investigations. Approximately 90 per cent of the HCP/AP was converted to carboxyphosphamide when these amounts of subcellular fractions were used. Neither the 105,000 *g* soluble fraction nor the solubilized 105,000 *g* particulate fraction metabolized HCP/AP in the absence of NAD, indicating that the activity monitored was, in fact, catalyzed by NAD-dependent, aldehyde-oxidizing enzymes.

Metabolism of HCP/AP by rat and mouse hepatic 105,000 *g* soluble fractions under incubation conditions optimal for aldehyde oxidase activity was also investigated. No carboxyphosphamide was detected when a 105,000 *g* soluble fraction equivalent to 4 mg of rat liver was used.

The effect of disulfiram, acrolein, pyridoxal hydrochloride, menadione and allopurinol on the ability of hepatic subcellular fractions to catalyze the oxidation of HCP/AP to carboxyphosphamide under conditions optimal for the expression of NAD-linked aldehyde dehydrogenase activity is presented in Table 3. Acrolein and disulfiram inhibited the reaction by 90 per cent or more. A 50 per cent inhibition was observed when the disulfiram concentration was reduced to 0.1 mM. No inhibition was observed when the disulfiram concentration was reduced to 10 μM. Pyridoxal HCl, menadione and allopurinol did not inhibit carboxyphosphamide formation. Butyraldehyde (4.0 mM) inhibited carboxyphosphamide formation by 70 per cent.

In addition to NAD-linked aldehyde dehydrogenases and aldehyde oxidase, which have low substrate specificities, there are other enzymes that might metabolize HCP/AP. Glyceraldehyde-3-phosphate dehydrogenase is present in abundant quantities and

Table 3. Oxidation of HCP/AP to carboxyphosphamide by rat hepatic 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions: Inhibition by various agents*

Inhibitor	Concn (mM)	Inhibition of carboxyphosphamide formation (%)	
		Soluble	Particulate
Acrolein	1.0	100	100
Disulfiram	1.0	90	100
Pyridoxal HCl	1.0	0	0
Menadione	0.1	0	ND†
Allopurinol	1.0	0	ND
Butyraldehyde	4.0	70	ND

* [^{14}C]Cyclophosphamide was incubated with rat hepatic microsomes for 15 min; incubation conditions were made optimal for rat hepatic NAD-linked aldehyde dehydrogenase activity; the potential inhibitor or the appropriate vehicle and rat hepatic 105,000 *g* soluble fraction, solubilized 105,000 *g* particulate fraction, or the appropriate control solution, were added and incubation was continued for another 15 min; semicarbazide was added and incubation continued for yet another 15 min. The incubate was deproteinized and chromatographed on paper and silica gel; the developed chromatograms were then scanned for radioactivity. The sizes of the peaks migrating at the R_f of authentic carboxyphosphamide were determined and the percent inhibition of carboxyphosphamide formation calculated. Details describing the preparation of subcellular fractions, incubation conditions, separation techniques and radiochromatographic analysis are provided in Materials and Methods. All experiments were conducted twice and each value is the mean of the data obtained. Subcellular fractions were obtained from one or two rats.

† ND, not determined.

is reportedly capable of catalyzing the oxidation of various aldehydes to acids in the presence of NAD [33]. It did not catalyze the oxidation of HCP/AP to carboxyphosphamide. Another enzyme that might catalyze the metabolism of HCP/AP is catalase, a heme-containing enzyme capable of oxidizing primary alcohols to aldehydes and secondary alcohols to ketones that is present in nearly all animal cells and organs [34]. No new metabolites were detected and HCP/AP levels were not decreased when microsomally generated HCP/AP was incubated with catalase. Although it has been demonstrated conclusively that phosphamidase is not involved in the initial biotransformation of cyclophosphamide [35, 36], the possibility that it cleaves the P–N bonds of one or more of the metabolites of cyclophosphamide remains. Cleavage of one of the P–N bonds of HCP/AP would liberate bis(2-chlorethyl)amine. This metabolite was not detected when microsomally generated HCP/AP was incubated with a rat hepatic 1000 *g* soluble fraction under conditions optimal for the expression of phosphamidase activity.

Alcohol dehydrogenase is present in hepatic cytosol and catalyzes the oxidation of secondary alcohols to ketones in the presence of NAD, and the reduction of aldehydes to alcohols when NADH is supplied. In view of this activity, it seemed reasonable that it might catalyze the conversion of aldophosphamide to the corresponding alcohol in the presence of NADH and/or the conversion of 4-hydroxycyclophosphamide to 4-ketocyclophosphamide in the presence of NAD. A comparison of the radiochromatographic scans of Figs. 1c and 1e reveals the appearance of a peak of greater magnitude on the paper chromatogram at R_f 0.88 and a new peak on the silica gel chromatogram at R_f 0.75 when incubation was in the presence of horse liver alcohol dehydrogenase and NADH; the appearance of these peaks was accompanied by the virtual disappearance of the peaks representing aldophosphamide semicarbazone. The new metabolite was also formed when NADH and the 105,000 *g* soluble fraction of either mouse or rat liver, rather than horse liver alcohol dehydrogenase, was added to the incubation mixture. Conclusive identification of the new metabolite was not made. However, several of the major metabolites of HCP/AP, viz. carboxyphosphamide, phosphoramide mustard, bis(2-chloroethyl)amine and 4-ketocyclophosphamide, can be eliminated as possible candidates because their R_f values differ from those obtained for this metabolite in the two chromatography systems. The fact that this metabolite was formed from HCP/AP by alcohol dehydrogenase in the presence of NADH suggests that it is an alcohol. For these reasons it is tentatively identified as alcophosphamide. Addition of NAD, rather than of NADH, and alcohol dehydrogenase did not decrease the level of aldophosphamide semicarbazone or cause the formation of 4-ketocyclophosphamide or the putative alcophosphamide.

DISCUSSION

Hill *et al.* [12] reported that purified rabbit liver aldehyde oxidase and yeast NAD-linked aldehyde dehydrogenase are capable of oxidizing HCP/AP to carboxyphosphamide. It has also been observed that murine liver cytosol catalyzes this reaction in the presence or absence of added NAD [8, 10–14]. Additionally, there is evidence that 4-ketocyclophosphamide formation from HCP/AP may be catalyzed by aldehyde oxidase [8, 37, 38].

The present investigation extends these observations to include both murine liver cytosolic and particulate NAD-linked aldehyde dehydrogenases among the enzymes capable of catalyzing the oxi-

dation of HCP/AP to carboxyphosphamide and to include alcohol dehydrogenase among those capable of catalyzing the metabolism of HCP/AP, presumably by reducing it to alcophosphamide.

The relative importance of these enzyme activities in detoxifying HCP/AP *in vivo* is unknown. Alcohol dehydrogenase probably does not play an important role *in vivo* because alcophosphamide is a relatively minor urinary metabolite of cyclophosphamide [39, 40]. Alcophosphamide, however, could be a potential source of HCP/AP *in vivo* since reactions catalyzed by alcohol dehydrogenase are generally reversible. Oxidation to 4-ketocyclophosphamide by aldehyde oxidase is also probably relatively unimportant because 4-ketocyclophosphamide is also a relatively minor urinary metabolite of cyclophosphamide [38–42].

The major urinary metabolite is carboxyphosphamide [39, 41, 42]. Both aldehyde oxidase and NAD-linked aldehyde dehydrogenase could catalyze the formation of this metabolite. Moreover, there may be more than a single NAD-linked aldehyde dehydrogenase capable of this activity in each of the hepatic 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions [43]. Our investigations suggest that NAD-linked aldehyde dehydrogenases are more important. In support of this contention we have demonstrated that (1) hepatic 105,000 *g* soluble and solubilized 105,000 *g* particulate fractions can catalyze the oxidation of HCP/AP to carboxyphosphamide under conditions optimal for the expression of NAD-linked aldehyde dehydrogenase activity but not under conditions optimal for the expression of aldehyde oxidase activity; (2) aldehyde oxidase activity is limited to a few tissues, whereas NAD-linked aldehyde dehydrogenase activity may be ubiquitous; and (3) in tissues where both activities are found, the amount of NAD-linked aldehyde dehydrogenase activity is always substantially greater. Additional support has been provided by Cox *et al.* [10, 11], who 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.

The contention that, *in vivo*, NAD-linked aldehyde dehydrogenases play a greater role in the oxidation of HCP/AP to carboxyphosphamide than does aldehyde oxidase is based on *in vitro* experimentation. Only *in vivo* experimentation can confirm the validity of this hypothesis. Since HCP/AP is a much more potent cytotoxic agent than is carboxyphosphamide [6, 12, 42, 44], the administration of disulfiram, an inhibitor of NAD-linked aldehyde dehydrogenase activity, would be expected to potentiate the antitumor and lethal effects of cyclophosphamide if this enzyme activity is of major importance in the detoxification of HCP/AP *in vivo*. In one investigation [45], administration of disulfiram did potentiate the antitumor effects of cyclophosphamide, but in others [11, 37, 46] it did not. Potentiation of the lethal effects of cyclophosphamide by disulfiram has also been reported [11]. Pyridoxal, an inhibitor of aldehyde oxidase activity, has also been reported to potentiate the antitumor activity of cyclophosphamide [12]. However, we were unable to obtain potentiation with pyridoxal [37].

NAD-linked aldehyde dehydrogenase activity has been proposed as an important determinant in the relative sensitivities of malignant and normal cells to cyclophosphamide [6, 7, 10, 11]. The validity of this hypothesis remains to be determined. An inverse relationship between the sensitivity of tumor cells to aliphatic aldehydes and their content of NAD-linked aldehyde dehydrogenase activity has been demonstrated [47].

Acrolein, a potential metabolite of cyclophosphamide, markedly inhibited the oxidation of HCP/AP to carboxyphosphamide catalyzed by NAD-linked aldehyde dehydrogenases. The *in vivo* significance of this observation remains to be determined.

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