ABSTRACT

The presence of therapeutically significant amounts of 4-hydroxycyclophosphamide-aldophosphamide in the blood of cyclophosphamide treated female C 57BL x DBA/2F1 mice was established by cochromatography of the isolated semicarbazone derivative with authentic aldophosphamide semicarbazone. The kidneys of these mice had been functionally removed before the administration of cyclophosphamide by tying off the renal arteries and veins. Radiochromatography was utilized for quantification. The total amount of radioactivity present in the blood reached a maximum within 30 min after cyclophosphamide administration and remained at that level throughout the remainder of the 4-hr observation period. The blood concentration of 4-hydroxycyclophosphamide-aldophosphamide reached a maximum of approximately 50 nmol/ml (about 15% of the total radioactivity present in the blood) between 5 and 30 min after the i.p. administration of cyclophosphamide, 65 mg/kg. 4-Hydroxycyclophosphamide-aldophosphamide was rapidly converted to carboxyphosphamide; concomitant with the appearance of carboxyphosphamide was a fall in blood 4-hydroxycyclophosphamide-aldophosphamide concentration to less than 3% of the total radioactivity present 90 min after cyclophosphamide administration. Relative to 4-hydroxycyclophosphamide-aldophosphamide and carboxyphosphamide concentrations, the maximum amounts of all other metabolites found in the blood were small. These observations do not support the view that 4-hydroxycyclophosphamide-aldophosphamide is converted to other metabolites at the site of its formation so that it never enters the circulation in appreciable amounts. Rather, they support the contention that 4-hydroxycyclophosphamide-aldophosphamide serves as a transport form of the ultimate cytotoxic metabolite(s) of cyclophosphamide.

INTRODUCTION

Cyclophosphamide is a prodrug widely used as an antitumor agent. For this reason, its metabolism is of special significance and has been investigated intensively. The current understanding of its metabolism is summarized in Chart 1. Cyclophosphamide is first hydroxylated to 4-hydroxycyclophosphamide by a mixed-function oxygenase system present largely in the liver. 4-Hydroxycyclophosphamide is believed to exist in equilibrium with its ring-opened tautomer, aldophosphamide. It is generally agreed that, as such, 4-hydroxycyclophosphamide-aldophosphamide is without significant antitumor activity and that it can undergo 2 fates: conversion to relatively nontoxic metabolites, namely, 4-ketocyclophosphamide, carboxyphosphamide, and alcophasphamide; and conversion to cytotoxic metabolites, namely, acrolein, phosphoramid mustard, and bis(2-chloroethyl)amine.

Still subject to controversy is whether 4-hydroxycyclophosphamide-aldophosphamide serves as a relatively long-lived transport form of the ultimate cytotoxic metabolites or as a short-lived intermediate that gives rise to these and other metabolites almost as quickly as it is formed. Previous investigations in our laboratory (14), although not definitive, indicated the presence of detectable amounts of 4-hydroxycyclophosphamide-aldophosphamide in the blood of rats given a very high dose of cyclophosphamide, namely, 400 mg/kg. Struck et al. (21) were unable to detect 4-hydroxycyclophosphamide-aldophosphamide in the blood of cyclophosphamide-treated (300 mg/kg) mice. They concluded that, upon its formation in the liver, 4-hydroxycyclophosphamide-aldophosphamide is quickly converted to other metabolites so that it never enters the circulation in appreciable amounts and that, therapeutically, the significant circulating metabolite is phosphoramid mustard. Voelcker et al. (24) were unable to detect the presence of 4-hydroxycyclophosphamide-aldophosphamide in the serum or urine of humans given cyclophosphamide but did report its presence in the serum and urine of cyclophosphamide-treated rats and mice. Wagner et al. (25) treated blood and urine of humans given cyclophosphamide and blood of cyclophosphamide-treated rats and mice with benzylmercaptan and were able to detect the 4-(S-benzyl)mercapto derivative of 4-hydroxycyclophosphamide. Moreover, Fenselau et al. (8) treated the plasma of a human given cyclophosphamide with cyanide and were able to detect the cyanohydrin derivative of aldophosphamide. However, attempts by Struck et al. (22) to detect 4-hydroxycyclophosphamide-aldophosphamide as a stabilized cyanohydrin derivative in cyclophosphamide-treated mice were unsuccessful.

These discrepancies raise questions concerning the validity and/or sensitivity of the various assays thus far used. An independent procedure was utilized in the experiments reported herein in an attempt to resolve this controversy.

MATERIALS AND METHODS

Side-chain (1.9 mCi/mmole)- and ring (C-4; 2.75 mCi/mmole)-labeled [14C]cyclophosphamide were obtained from New England Nuclear, Boston, Mass. Radiochemical purity, as determined by chromatography in 5 different systems, was greater than 98%. Carboxyphosphamide and 4-ketocyclophosphamide were supplied by Dr. Robert F. Struck, Southern Research Institute, Birmingham, Ala. Cyclophosphamide (NSC 26271)
was supplied by Dr. W. A. Zygmunt, Mead Johnson and Company, Evansville, Ind.

Female C57BL × DBA/2F1, (hereafter called BD2F) mice (ARS/Sprague-Dawley, Madison, Wis.), maintained on a standard chow diet provided ad libitum and weighing 18 to 20 g, were utilized to investigate the metabolism of cyclophosphamide in vivo. They 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 cyclophosphamide was injected. [14C]Cyclophosphamide was injected i.p., 65 mg/kg (5 μCi of side-chain-labeled or 10 μCi of ring-labeled), in a 0.2-ml solution of 0.9% NaCl solution. Ninety-μl blood samples were collected from the tail vein by using a heparinized pipet at various times following cyclophosphamide administration. The samples were immediately placed in glass centrifuge tubes containing 0.6 mg of semicarbazide hydrochloride dissolved in 90 μl of a 10 mM dibasic sodium phosphate-monobasic potassium 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 an aqueous 5.5% zinc sulfate solution followed by 90 μl of an aqueous 4.5% barium hydroxide solution were added to precipitate proteins. The mixture was centrifuged at 9000 × g for 20 min at 2 ± 2°, and the resultant supernatant was saved for radiochromatographic analysis. Separation was effected in Systems 1 and 2 (Table 1). Radioscans were obtained with a Packard Model 7201 radiochromatogram scanner; a minimum of approximately 0.001 μCi could be detected. Metabolites detected as peaks on the radiochromatograms were quantitated by determining the area under the recorded curves.

In some experiments, peaks were not completely resolved. Areas of these peaks were estimated by observing the degree of tailing and shape of other peaks where complete separation was achieved. The contribution made by each of the overlapping peaks could then be estimated. When there was no separation of metabolites, e.g., cyclophosphamide and 4-ketocyclophosphamide (Chart 2, silica gel) and cyclophosphamide and an unidentified metabolite (Chart 2, paper), the contribution made by cyclophosphamide was estimated by determining the amount of 4-ketocyclophosphamide and unidentified metabolite present on chromatograms where these metabolites were separated from cyclophosphamide. 4-Ketocyclophosphamide was separated from cyclophosphamide on paper (Chart 2), and the unidentified metabolite, believed to be aldo- phosphamide (6), was separated from cyclophosphamide on silica gel (Chart 2). The contribution made by cyclophosphamide to the overlapping peak detected on the silica gel and paper chromatogram was then estimated by subtracting the contribution made by 4-ketocyclophosphamide and the unidentified metabolite, respectively.

The identity of the metabolite in the blood suspected of being 4-hydroxycyclophosphamide-aldophosphamide was established by cochromatography of the semicarbazone derivative with authentic aldophosphamide semicarbazone.

Authentic aldophosphamide semicarbazone was generated in vitro by microsomal activation of cyclophosphamide in the presence of semicarbazide. Microsomes were prepared from the livers of 180- to 220-g male Holtzman rats (The Holtzman

Table 1

<table>
<thead>
<tr>
<th>System</th>
<th>Support</th>
<th>Solvent</th>
<th>Aldophosphamide semicarbazone</th>
<th>Carboxyphosphamide</th>
<th>Cyclophosphamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Analytical silicagel</td>
<td>Chloroform:methanol (9:1)</td>
<td>0.45</td>
<td>0.09</td>
<td>0.90</td>
</tr>
<tr>
<td>2</td>
<td>Whatman no. 1 paper</td>
<td>Isopropyl alcohol:ammonium hydroxide (4:1)</td>
<td>0.72</td>
<td>0.46</td>
<td>0.85</td>
</tr>
<tr>
<td>3</td>
<td>Analytical silicagel</td>
<td>Butanol:glacial acetic acid:water (3:1:1)</td>
<td>0.72</td>
<td>0.46</td>
<td>0.85</td>
</tr>
<tr>
<td>4</td>
<td>Preparative silicagel</td>
<td>Chloroform:methanol (3:1)</td>
<td>0.58</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Preparative silicagel</td>
<td>Chloroform:methanol (9:1)</td>
<td>0.20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Preparative silicagel</td>
<td>Chloroform:methanol (9:1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Chart 1. Proposed route of cyclophosphamide metabolism.
Co., Madison, Wis.) as described previously (13) and were used immediately after preparation. The rats had been maintained on a standard chow diet ad libitum and had been given i.p. injections of phenobarbital sodium, 40 mg/kg once daily, for 5 days prior to sacrifice to induce hepatic microsomal mixed-function oxygenase activity. They were sacrificed 24 h after the last dose of phenobarbital. Conversion of cyclophosphamide to aldophosphamide semicarbazone was accomplished in a reaction mixture containing the following constituents adjusted to pH 7.4 in 25-ml Erlenmeyer flasks: NADP, 2 μmol; dibasic sodium phosphate—monobasic potassium phosphate buffer, 200 μmol; semicarbazide hydrochloride, 37.5 μmol; magnesium chloride, 10 μmol; glucose 6-phosphate, 20 μmol; glucose-6-phosphate dehydrogenase, 2 enzyme units (1 enzyme unit will reduce 1 μmol of NADP per min at pH 7.4 and 20°C; Boehringer Mannheim Corp., New York, N. Y.); cyclophosphamide, 13.8 μmol; microsomes obtained from 500 mg of wet liver; and 1.15% potassium chloride solution in a final volume of 5 ml. A control preparation was prepared by omitting cyclophosphamide from the incubation mixture. The reaction was started by addition of the microsomal preparation. Incubation was at 37°C in open flasks for 15 min in a Dubnoff metabolic shaker (120 oscillations/min). The reaction was stopped by the addition of 2 ml of an aqueous 5.5% zinc sulfate solution followed by 2 ml of an aqueous 4.5% barium hydroxide solution. Precipitated protein was removed by centrifugation at 9000 × g for 20 min at 2 ± 2°C. The supernatant fraction was then chromatographed on preparative silica gel plates (Analtech, Inc., Wilmington, Del.) in chloroform:methanol (9:1). The approximate location (RF 0.2) of the metabolite suspected of being aldophosphamide semicarbazone was determined by the 4-((p-nitrobenzyl)pyridine assay on a separate plate (14). This
the solvent and tetramethylsilane as the internal reference (chemical shift, 0.00). After the methanol extract was dissolved in dimethyl sulfoxide-d6 (0.3 ml), the sample was placed in 5-mm (outside diameter) tubes, sealed, and analyzed at 100 MHz. Spectral peaks obtained with the control preparation were subtracted from the spectral peaks obtained with the sample preparation. The net spectrum (6) was compared to that reported by Struck (18) for synthetic aldophosphamide semicarbazone. The only substantial difference between the spectrum obtained in the present experiments and that reported by Struck (18) for chemically synthesized aldophosphamide semicarbazone was an absence of a triplet peak with a chemical shift of 2.36 to 2.56. This peak was probably obscured by the solvent, dimethyl sulfoxide-d6, which is known to produce the type of multiplet observed at this chemical shift. A probable explanation for the lack of solvent obfuscation in Struck’s analysis is that his sample concentration was approximately 10-fold greater than that used in the present experiment. Hence, the signals of the derivative may have been sufficiently intense relative to those of the solvent to prevent obfuscation of the former by the latter. Additional evidence to support the peak assignments was obtained by the addition of D2O to the sample used in the PMR analysis. This caused the disappearance of bands 4.30 to 4.44, 6.13, and 9.90, indicating the presence of groups with exchangeable protons (12).

Infrared analysis was performed by the thin-film method (12) using chloroform to transfer the metabolite suspected of being aldophosphamide semicarbazone to sodium chloride plates. The spectrum obtained (6) was essentially identical to that reported by Struck (18) for synthetic aldophosphamide semicarbazone.

Attempts to establish the identity of the putative aldophosphamide semicarbazone by mass spectral analysis using the chemical ionization technique were unsuccessful. No quasi-molecular ion and only 2 chlorine-containing fragments (m/e 185, 1 Cl; m/e 106, 1 Cl) were observed. The identity of the metabolite suspected of being carboxyphosphamide was established by cochromatography with authentic aldophosphamide semicarbazone (Table 1, Systems 1 and 2) with authentic carboxyphosphamide and by mass spectral analysis of the methylated derivative. The electron impact technique and a Finnigan Model 3200 mass spectrometer were used for the latter. Female BD2F1 mice, whose renal arteries and veins were ligated, were given i.p. injections of cyclophosphamide, 140 mg/kg. Three hr later, blood was removed from the tail vein and deproteinized as described above. The supernatant was chromatographed in System 2 (Table 1), and the approximate location (Rf 0.46) of the metabolite suspected of being carboxyphosphamide was determined by the 4-(p-nitrobenzyl)pyridine assay (14) on a separate strip of paper. The metabolite was then removed from the paper by extraction with methanol and methylated with diazomethane (1, 6, 20). A control preparation was prepared by using blood obtained from untreated mice. Samples were introduced into the mass spectrometer through the direct probe inlet system. The mass spectrum obtained with the control preparation was subtracted from the mass spectrum obtained with the sample preparation. The net mass spectrum (6) compared favorably to that reported by other investigators for methylated carboxyphosphamide (1, 20). All fragments reported by Bakke et al. (1) were observed with the exception of a chlorine-containing fragment at m/e 239. This fragment was not observed by Struck et al. (20) either, perhaps because of its weak intensity. Two fragments (m/e 155 and m/e 105) were detected that were not reported by the other investigators. Because of the relatively low signal:noise ratio at these mass ranges, it was difficult to determine if the peaks were caused by fragments from the isolated metabolite or by contaminants.

RESULTS

The objective of the present investigation was to determine, by an independent procedure, whether 4-hydroxycyclophosphamide-aldophosphamide was present in therapeutically significant amounts in the blood of mice given cyclophosphamide. A specific, sensitive, and economical assay was desired. Detection and quantitation were expected to be difficult because the metabolite was likely to be unstable to most laboratory manipulations (19, 24).

Previous investigations in this and another laboratory indicated that 4-hydroxycyclophosphamide-aldophosphamide could be trapped and stabilized as the semicarbazone derivative (14, 18). Moreover, the semicarbazone derivative could be separated from cyclophosphamide and the other metabolites of cyclophosphamide in a variety of chromatographic systems (14, 18).

Based on these observations, the following method was judged most probably to prove fruitful. The putative 4-hydroxycyclophosphamide-aldophosphamide was to be trapped as the semicarbazone, its identity was to be established by cochromatography with authentic aldophosphamide semicarbazone, and quantification was to be achieved by radiochromatographic analysis.

The metabolism of cyclophosphamide in vivo was monitored in female BD2F1 mice given injections of [14C]cyclophosphamide. Preliminary experiments dictated that a minimum of 5 to 10 μCi of radiolabeled cyclophosphamide had to be administered to mice in order to quantitate metabolite appearance and disappearance. Blood samples were collected at various times after cyclophosphamide injection. Representative radiochromatographic scans of the 30-, 90-, and 180-min samples are shown in Chart 2. Blood levels of cyclophosphamide, carboxyphosphamide, and 4-hydroxycyclophosphamide-aldophosphamide over the 4-hr collection period are presented in Chart 3.

The total amount of radioactivity present in the blood reached

It cannot be stated which tautomer, namely, 4-hydroxycyclophosphamide or aldophosphamide, is the predominant form present in the blood because the addition of semicarbazide induces the conversion of 4-hydroxycyclophosphamide to aldophosphamide by forming the semicarbazone of the latter (18).

* The abbreviation used is: PMR, proton magnetic resonance.
* R. F. Struck, personal communication.

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of the experiments are described in “Materials and Methods.” Each point
the kidneys were functionally ineffective. Blood levels of 4-
dioactivlty contributed by minor metabolites is not shown.
were prepared for chromatographic separation. After separation on paper (Table
1, System 2) or silica gel (Table 1, System 1), radioactivity was determined by a
radiochromatogram scanner. Metabolites were quantitated by weighing each
peak of the resultant tracing. Representative scans are shown in Chart 2. Details
of the experiments are described in “Materials and Methods.” Each point
represents the mean value of measurements made from 2 to 4 chromatograms
prepared from blood samples obtained from 2 mice. 1, cyclophosphamide; 0, 4-
hydroxycyclophosphamide-aldophosphamide; and \( \Delta \), carboxyphosphamide. Ra-
dioactivity contributed by minor metabolites is not shown.
a maximum within 30 min after cyclophosphamide administra-
tion and remained at that level throughout the remainder of the 4-
hr observation period (data not presented). Most of the cyclophosphamide was metabolized within 2 hr (Charts 2 and
3). 4-Hydroxycyclophosphamide-aldophosphamide trapped as
the semicarbazone derivative migrated with \( R_f \) values of 0.45 and
0.72 on silica gel and paper, respectively. Identification of this
metabolite as aldophosphamide semicarbazone was based in
part on the results of experiments where blood samples ob-
tained from cyclophosphamide-treated mice were incubated in
the presence or absence of semicarbazide. When semicar-azide was omitted, no peaks were detected at \( R_f \) 0.45 or 0.72
on silica gel and paper, respectively (data not presented).
Additional evidence was provided by cochromatography ex-
periments. When the metabolite migrating at \( R_f \) 0.45 in System
1 (Table 1) was isolated and cochromatographed with authentic aldophosphamide semicarbazone, \( R_f \) values were identical in
the 6 different systems listed in Table 1.
The half-life of 4-hydroxycyclophosphamide-aldophosph-
amide in the blood was considerably less than 1 hr even though
the kidneys were functionally ineffective. Blood levels of 4-
hydroxycyclophosphamide-aldophosphamide reached a maxi-
mum of approximately 50 \( \mu \)M (about 15% of the total radioac-
tivity present in the blood) between 5 and 30 min after cyclo-
phosphamide injection (Chart 3). Ninety min after cyclophos-
phamide injection, 4-hydroxycyclophosphamide-aldophosph-
amide accounted for only 2 to 3% of the total radioactivity
present in the blood.
The rapid metabolism of 4-hydroxycyclophosphamide-aldoph-
phamide to carboxyphosphamide apparently accounts for the
short half-life of the former (Charts 2 and 3). Carboxyphos-
phamide migrated with a \( R_f \) of 0.09 on silica gel and 0.46 on
paper (Chart 2). Detectable levels of carboxyphosphamide
were observed 5 min after cyclophosphamide injection. A
maximum of 65% of the total radioactivity present in the blood
was reached at approximately 90 min after cyclophosphamide
injection and persisted for the rest of the observation period.
Exhaustive efforts to establish the presence of other known
cyclophosphamide metabolites, e.g., phosphoramide mustard,
4-ketocyclophosphamide, alcosphamide, bis(2-chloro-
ethyl)amine, and acrolein, were not made. However, the radi-
ochromatograms presented in Chart 2 fail to show the presence
of large amounts of any radilabeled material other than that
which can be ascribed to cyclophosphamide, 4-hydroxycyclo-
phosphamide-aldophosphamide or carboxyphosphamide.
The \( R_f \) of authentic 4-ketocyclophosphamide is 0.62 in Sys-
tem 2. As judged by the amount of radioactivity at \( R_f \) 0.62
(System 2), less than 5% of the total radioactivity could be
attributed to this metabolite at any time point (Chart 2). The
peak at \( R_f \) 0.75 (Chart 2, System 1) is believed to represent
alcophosphamide (8); this metabolite appears to have migrated
with cyclophosphamide (\( R_f \) 0.88) in System 2. Less than 5%
of the total radioactivity could be attributed to this metabolite.
Of particular interest were the 2 small peaks observed at \( R_f \)
0.28 and 0.40 in System 2 (Chart 2). The peak at \( R_f \) 0.28 was
observed only when side-chain-labeled cyclophosphamide was
administered, and the peak at \( R_f \) 0.40 was observed only when
ring-labeled cyclophosphamide was administered. Of the
known cyclophosphamide metabolites that contain only the
side-chain or ring moieties, phosphoramide mustard or bis(2-
chloroethyl)amine and an acrolein derivative, respectively,
would appear to be the most probable candidates. The \( R_f \)’s
of authentic phosphoramide mustard cyclohexylamine in Systems
1 and 2 were 0.10 and 0.28, respectively. In any case, the
maximum levels of each of the metabolites represented by
these peaks was less than 5% of the total radioactivity present
in the blood.

DISCUSSION
It is generally agreed that cyclophosphamide is hydroxylated,
primarily in the liver, to 4-hydroxycyclophosphamide-aldophos-
phamide which in turn is converted to several metabolites, one
of which is phosphoramide mustard, and that the latter is the
active antitumor agent. The contention that phosphoramide
mustard, rather than 4-hydroxycyclophosphamide-aldophos-
phamide or bis(2-chloroethyl)amine, is the active antitumor
agent is supported by the observations that (a) at physiological
\( \text{pH} \), phosphoramide mustard is an alkylating species whereas
4-hydroxycyclophosphamide-aldophosphamide is not and
bis(2-chloroethyl)amine is a relatively weak alkylating agent (3, 10, 21),
(b) 4-hydroxycyclophosphamide-aldophosphamide can give rise to phosphoramide mustard (23), and (c) alklylation
by phosphoramide mustard of target molecules proceeds di-
rectly via an aziridinium intermediate rather than after cleavage
to bis(2-chloroethyl)amine or other metabolites (3).
Still in dispute is the length of the half-life of 4-hydroxy-
cyclophosphamide-aldophosphamide in vivo. At one extreme, 4-
hydroxycyclophosphamide-aldophosphamide could give rise
to phosphoramide mustard and other metabolites immediately
upon its formation in the liver. In that case, 4-hydroxycyclo-
phosphamide-aldophosphamide would not be present in cir-
culating blood; the cytotoxic action of cyclophosphamide would
be effected by phosphoramidé mustard generated outside the target cells, and the basis for the selective action of cyclophosphamide would reside solely with phosphoramidé mustard. At the other extreme, 4-hydroxycyclophosphamide-aldophosphamide would be generated in and leave the liver and give rise to phosphoramidé mustard only after entering tumor and other cells. In that case, it would be present in circulating blood for a significant length of time; the cytotoxic action of cyclophosphamidé would be effected by phosphoramidé mustard generated within target cells, and the basis for the selective toxicity of cyclophosphamide could reside with either 4-hydroxycyclophosphamide-aldophosphamide and/or phosphoramidé mustard.

The presence of 4-hydroxycyclophosphamide-aldophosphamide in the circulating blood of rodents and humans given cyclophosphamide has now been documented by 4 different assay systems in 3 independent laboratories. The failure in some investigations (21, 22) to find detectable amounts of 4-hydroxycyclophosphamide-aldophosphamide is probably due to the instability of this compound.

It could be argued that the detection of 4-hydroxycyclophosphamide-aldophosphamide in blood in our experiments is not indicative of its presence under more physiological conditions. Ligation of the renal arteries and veins would have the effect of preventing its excretion and metabolism by the kidney. The relative importance of the kidneys in determining the fate of this metabolite is not clear. Excretion may not play an important role since only relatively small amounts of 4-hydroxycyclophosphamide-aldophosphamide have been found in the urine (24, 25). This is as expected since significant amounts of the tautomeric pair may be bound to serum proteins rendering them unavailable for glomerular filtration (11, 24) and because active secretion of the tautomers into the urine is unlikely. Metabolism by renal enzymes, especially to carboxyphosphamide, could play a significant role in determining the fate of 4-hydroxycyclophosphamide-aldophosphamide. The kidney is known to be second only to the liver in its aldolase oxidase and NAD-linked aldehyde dehydrogenase content (6), and these enzymes are known to catalyze the formation of carboxyphosphamide, a relatively inactive antitumor agent, from 4-hydroxycyclophosphamide-aldophosphamide (4-6, 9, 14). The ability of kidney cytosol to inactivate 4-hydroxycyclophosphamide-aldophosphamide has been demonstrated (4, 5).

Even though the peak blood level of 4-hydroxycyclophosphamide-aldophosphamide, approximately 50 μM, that we obtained is undoubtedly higher than it would be under comparable conditions in the intact animal, our investigations demonstrate that 4-hydroxycyclophosphamide-aldophosphamide does not degrade, or is not enzymatically converted to other metabolites, immediately upon its formation, i.e., that significant amounts of it do leave the liver to enter the general circulation and that significant amounts of it remain intact in the body for at least the length of time that it takes to reach the kidney after its formation in the liver.

We found high levels of 4-hydroxycyclophosphamide-aldophosphamide, approximately 50 μM, present in the blood of mice for a relatively short period of time (less than 1 hr). Other investigators have reported somewhat different findings. Voelcker et al. (24) administered cyclophosphamide, 20 mg/kg i.v., to intact rats and observed that 4-hydroxycyclophosphamide-aldophosphamide serum levels remained fairly constant at 5 to 9 μM for at least 4 hr after introduction of the drug. Wagner et al. (25) reported that 4-hydroxycyclophosphamide-aldophosphamide was present at concentrations of 0.5 to 1.5 μM in the blood of humans 2 hr after the i.v. administration of cyclophosphamide, 10 mg/kg. The rate of 4-hydroxycyclophosphamide-aldophosphamide formation was relatively rapid in our experiments because we used mice. Mixed-function oxygenase activity is known to be higher in mice than in most species including rats and humans. Perhaps the low, persistent levels of 4-hydroxycyclophosphamide-aldophosphamide in humans and rats reflect a slower rate of formation accompanied by a "trapping" of 4-hydroxycyclophosphamide-aldophosphamide in target cells (7) so that it never accumulates in the blood.

On the basis of these observations and those reported herein, we take the view that the half-life of 4-hydroxycyclophosphamide-aldophosphamide in circulating blood is sufficient for this metabolite to make a major contribution towards cyclophosphamide antitumor activity, specifically, as a transport form of the ultimate cytotoxic metabolite(s).

Additional observations supporting this view include those of Brock (2) who reported that, in vivo, phosphoramidé mustard, acrolein, and bis(2-chloroethyl)amine are much less potent as antitumor agents than are cyclophosphamide and 4-hydroxycyclophosphamide. Moreover, Brock found 4-hydroxycyclophosphamide to have much greater cytotoxic activity, in vitro, than did phosphoramidé mustard, acrolein, or bis(2-chloroethyl)amine. In our laboratory, "activated" cyclophosphamide, 4-hydroperoxycyclophosphamide, and 4-hydroxyphosphoramidé phosphamide anhydro dimer were all found to be approximately 10 times more potent against cultured rat W256 carcinosarcoma and mouse P388 cells than was phosphoramidé mustard (15–17). Brock also observed that, in rats bearing the Yoshida ascites sarcoma, the therapeutic indices LD50/CD50, the dose lethal to 50% of the animals divided by the dose required to inhibit tumor growth 50% of cyclophosphamide and 4-hydroxycyclophosphamide were about 175 and 120, respectively, whereas the therapeutic indices for phosphoramidé mustard, acrolein, and bis(2-chloroethyl)amine were 3.5, <3.4, and 2.5, respectively (2). These observations not only support the contention that the cytotoxic action of cyclophosphamide is effected primarily by metabolites generated from 4-hydroxycyclophosphamide-aldophosphamide inside of target cells but also strongly suggest that the basis for the selective action of cyclophosphamide resides largely with 4-hydroxycyclophosphamide-aldophosphamide.

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Kinetics of Cyclophosphamide Biotransformation \textit{In vivo}

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