The Enzymatic Basis of the Selective Action of Cyclophosphamide

Peter J. Cox, Barry J. Phillips, and Peter Thomas

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London SW3 6JB, England

SUMMARY

The initial metabolic products of cyclophosphamide (4-hydroxy-cyclophosphamide and aldophosphamide) were prepared biologically in unpurified form. Their toxicity to tumor cells were tested by bioassay techniques and in cell culture, and the deactivation abilities of various tissue-soluble fractions were quantitated. Liver and kidney cytosol effectively deactivated the primary metabolites, whereas cytosols from gastrointestinal tract mucosa, Walker ascites tumor, and spleen were less efficient. When [14C]cyclophosphamide was activated and incubated with liver cytosol, 34% of all radioactivity was identified as carboxyphosphamide, by mass spectrometry of the methyl ester.

Measurement of alcohol dehydrogenase (EC 1.1.1.1) and aldehyde dehydrogenase (EC 1.2.1.3) activities by reduced nicotinamide adenine dinucleotide production revealed a qualitative correspondence between aldehyde dehydrogenase activity and deactivation ability. Unpurified aldophosphamide and the analogs prepared from 6-methyl- and 5,5-dimethylcyclophosphamides were substrates for nicotinamide adenine dinucleotide-requiring enzymes, whereas incubation of 4-hydroxy-4-methylcyclophosphamide in an unfractionated incubation mixture with liver soluble enzymes did not cause reduced nicotinamide adenine dinucleotide production.

Activated 4-methylcyclophosphamide was deactivated by liver cytosol to the same extent as phosphoramid mustards (dose reduction factors, 2.2 and 2.7, respectively); that for liver cytosol and activated cyclophosphamide was 49.1.

It was concluded that the selective action of cyclophosphamide when compared to other nitrogen mustards is largely dependent on the balance between the enzymatic production of nontoxic metabolites (principally carboxyphosphamide) and chemical decomposition of aldophosphamide to the ultimate alkylating agent (phosphoramid mustards).

INTRODUCTION

The metabolism of the alkylating agent, cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,3,2-oxazine-2-oxide) has been the subject of extensive study (cf. Refs. 13 and 18) since its discovery (1, 2) in 1958. Various schemes relating known and presumed metabolites have been proposed during this time, leading to the current scheme linking the major metabolites proposed by a number of groups (6, 7, 23). Metabolism occurs by 4-hydroxylation of the ring by microsomal enzymes utilizing NADPH, and 4-ethoxy-cyclophosphamide has been isolated (7) as a stable derivative of this hydroxy metabolite. Aldophosphamide semicarbazone has been conclusively identified (23) as a stabilized product of aldophosphamide, the acyclic isomer of 4-hydroxycyclophosphamide, following the demonstration of an alkylating aldehyde (22) in vivo and in vitro. The principal urinary excretion product, carboxyphosphamide, and 4-keto-cyclophosphamide, both of which are relatively nontoxic (7, 24, 26), were identified (24, 26) before the intermediates, and conversion of aldophosphamide to carboxyphosphamide has been demonstrated using purified aldehyde oxidase (14), a commercial preparation of aldehyde dehydrogenase (14) and hepatic cytosol (14, 22).

Phosphoramid mustards, probably derived by a chemical β elimination of acrolein from aldophosphamide, has been reported both in in vitro systems (6, 7) and in vivo (12, 25). Either aldophosphamide or 4-hydroxy-cyclophosphamide (e.g., Ref. 11) have been presumed to exert the cytotoxic properties of cyclophosphamide in vivo, although it has been suggested that breakdown to phosphoramid mustard is essential for this activity (7, 15). 5,5-Dimethylcyclophosphamide has been shown to be metabolized to the hydroxylated intermediate (9), the acyclic isomer of which cannot liberate phosphoramid mustard by β elimination due to the gem-dimethyl grouping, and this metabolite is of very low toxicity to tumor or normal tissue (3, 9).

The selective action of cyclophosphamide in vivo, i.e., the low toxicity compared to antitumor activity, giving a large therapeutic index, compared to other active alkylating compounds, has been attributed (7, 8) to the balance between activation processes (releasing phosphoramid mustard) and deactivation by enzymatic oxidation (yielding 4-ketocyclophosphamide and carboxyphosphamide) of the hydroxylated intermediate. It has been suggested (11) that the differential sensitivity of various tumors may be due to varying levels of aldehyde dehydrogenase.

We now report on studies of the deactivation capability of various tissues and the nature of the deactivation process.

MATERIALS AND METHODS

Cyclophosphamide, 4-methylcyclophosphamide, and 6-methylcyclophosphamide were gifts of Ward Blenkinsop.
P. J. Cox et al.

L. Ltd., Wembley, London, England: 5,5-dimethylcyclophosphamide was a gift of N. Brock, Asta-Werke AG, Bielefeld, West Germany; and phosphoramidate mustard (cyclohexylammonium salt) was obtained through Dr. H. Wood, National Cancer Institute, Bethesda, Md. [6-14C]Cyclophosphamide (specific activity, 3.95 mCi/mmole) was purchased from New England Nuclear, Swindon, Wiltshire, England.

Preparation of Tissue Fractions. Washed rat liver microsomes were prepared as previously described (7) from animals treated for 10 days with sodium phenobarbital (1 g/liter drinking water). They were stored as pellets at —30°.

The soluble enzyme fractions of rat tissues were prepared freshly as follows. The required tissue was excised, cooled rapidly to 4°, and washed with cold 0.15 M KCl. For cells from the gastrointestinal mucosa, the complete ileum was removed and cooled, and the lumen was thoroughly washed out with 0.15 M KCl. The ileum was then slit open and the mucosal cells were scraped off with a square-ended spatula. The cells were dropped into 0.1 M Tris-HCl buffer, pH 7.4, and their wet weight was obtained by difference. Bone marrow cells were prepared from the femurs of 25 Wistar rats, expelling the cells from the marrow cavity with sterile 0.9% NaCl solution. Approximately 2 g, wet weight, of cells were obtained in this manner. Walker ascites tumor cells were removed from the abdominal cavity of a routine passage animal, separated from the ascitic fluid by centrifugation (500 x g), and suspended in 0.3% NaCl solution to chase from New England Nuclear, Swindon, Wiltshire.

Preparation of 4-Hydroxy-5,5-dimethylcyclophosphamide. 5,5-Dimethylcyclophosphamide was oxidized (9) with KMnO4 and the 4-hydroxy derivative was isolated by extraction (CH2C12), thin-layer chromatography on silicic acid, and elution of the appropriate band (RF previously reported. Prior to use, the ethanol solution was taken to dryness under reduced pressure and the residue was dissolved in an appropriate volume of buffer.

Bioassay. The procedure was essentially the same as that previously described (4), the principal difference being that 10° Walker ascites cells/ml were used. Portions (1 ml) of the incubation mixtures (see “Results”) were added to the cell suspension (9 ml) and incubated for 2 hr at 37° prior to the injection i.p. of 1 ml (10° cells) into female Wistar rats. Groups of 5 animals were used for each treatment, and the survival times of the animals were recorded.

Cell Culture Assay. Walker tumor cell lines were established and maintained as previously described (20). The cytotoxicity assay system was essentially the same as that reported earlier (20). Cells from log phase cultures were resuspended in fresh medium (Dulbecco’s modified Eagle medium plus 10% fetal calf serum, containing penicillin and streptomycin), at a density of 5 x 10^6/ml. The suspension was dispensed in 4.5-ml portions into centrifuge tubes, and 0.5 ml of test solution was added to each. The tubes were incubated at 37° for 1 hr and then centrifuged at 800 x g for 3 min. Each cell pellet was resuspended in 10 ml of fresh medium and dispensed in 2-ml amounts into 3 dishes of a Linbro Multi-dish tray comprising 24 dishes, each dish 16 mm in diameter. Thus, each dish contained about 5 x 10^4 cells. A 0 time count was made on the remaining suspension. Trays were incubated at 37° in an atmosphere of 10% CO2 in air and cell counts were made, using 1 dish of each series, after 2, 3, and 4 days. The rate of multiplication of treated cells was compared with that of controls, to give an estimate of percentage inhibition of cell growth.

Enzyme Kinetics. The activities of alcohol dehydrogenase and aldehyde dehydrogenase in the soluble enzyme fractions of the various tissues were determined essentially by the method of von Wartburg et al. (27) for alcohol dehydrogenase (EC 1.1.1.1) and the method of Dietrich et al. (10) for aldehyde dehydrogenase (EC 1.2.1.3). Initial rates of NADH formation were followed using a Pye Unicam SP 500 monochromator (Pye Unicam Ltd., Cambridge, England) with a Gilford Model 220 absorbance indicator (Gilford Instruments Laboratories, Inc., Oberlin, Ohio), the output of which was connected to a Honeywell strip chart recorder (Honeywell Ltd., Bracknell, Berkshire, England). The fullscale deflection of the recorder was set to 0.1, and the initial rates were measured at 340 nm in silica cells of 10-mm path length housed in a cell compartment thermostatically maintained at 30°. Control determinations in the absence of substrate were carried out, and the ΔE340 of the controls was subtracted from the test values. Assays were carried out in 0.1 M sodium phosphate buffer, pH 7.2 throughout, to ensure a more correct relationship with physiological pH values.

Preparation of 4-Hydroxy-5,5-dimethylcyclophosphamide. 5,5-Dimethylcyclophosphamide was oxidized (9) with KMnO4 and the 4-hydroxy derivative was isolated by extraction (CH2C12), thin-layer chromatography on silicic acid, and elution of the appropriate band (RF 0.39; CHCl3:C2H5OH, 9:1) with ethanol. Yields ranged from 1.7 to 3.2%, the higher yields being obtained from reactions of 18 hr duration compared to the 2 hr previously reported. Prior to use, the ethanol solution was taken to dryness under reduced pressure and the residue was dissolved in an appropriate volume of buffer.
Enzymatic Preparation of Activated Cyclophosphamides.

In order to study the reaction of liver cell supernatant with aldophosphamide and its 4-methyl, 6-methyl, and 5,5-dimethyl analogs, the appropriate substrates (1 mM) were incubated with washed microsomes as described above for cyclophosphamide. (The analogs of aldophosphamide formed from 4-methyl-, 6-methyl-, and 5,5-dimethylcyclophosphamides have been described as such for convenience, taking the numbering from the parent compounds.) These conditions are known to cause substantial metabolism of the substrates (7–9). After the 45-min incubation period, the incubations were cooled to 0° and centrifuged at 65,000 × gav for 30 min. The clear supernatant was used as the substrate solution in enzyme assays. Control incubations were also prepared, omitting substrates.

Isolation of Carboxyphosphamide. Following metabolism of [6-14C]cyclophosphamide by microsomes and tissue cytosols, proteins were precipitated from the incubation medium by the addition of ethanol (4 volumes). After centrifugation at 1200 × gav, the supernatant was concentrated to an aqueous residue at 30°/10 mm and extracted (3 times) with chloroform, prior to adjustment to pH 2 with 2 M HCl and further extraction (3 times) with CHCl3. The extracts at each pH were combined and dried with anhydrous sodium sulfate. The pH 2 extracts were conventionally methylated with diazomethane (10 min) and chromatographed in chloroform:ethanol (9:1).

RESULTS

Initial experiments were carried out using the bioassay system. Cyclophosphamide was activated at 75 μg/ml prior to the addition of appropriate cell supernatants (0.5 volume). Incubation was continued for up to 24 hr before the further 10-fold dilution step with the cells yielded a final concentration of cyclophosphamide metabolites of 5 μg/ml. (In our hands and under the conditions described, the metabolism of cyclophosphamide by microsomes proceeded to completion, leaving no substrate.) At this concentration, in the absence of a tissue supernatant, cell kill approached 100% and the experimental animals survived longer than 30 days (control group, 7 days). Liver and kidney cell supernatants showed some effect, decreasing the survival time of the animals, but no effect was seen using gastrointestinal tract mucosa, tumor, or bone marrow cell supernatants. Table 1 related ISTa to percentage cell kill for 1 such experiment using liver cell supernatant. Cell kill was calculated from a bioassay, using Walker ascites tumor cells implanted (i.p.) into female Wistar rats. Exact details of the incubation conditions are given in “Materials and Methods” and “Results.” The length of incubation refers to the period of incubation with hepatic cytosol or buffer, subsequent to the 45-min microsomal activation and prior to treatment of the tumor cells. Percentage IST relates to a control value of 7 days, and each estimation was the mean of 5 animals.

<table>
<thead>
<tr>
<th>Length of incubation (hr)</th>
<th>Buffer</th>
<th>Liver cell supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% IST</td>
<td>% cell kill</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.83</td>
<td>&gt;310</td>
<td>99.99</td>
</tr>
<tr>
<td>2</td>
<td>&gt;270</td>
<td>99.95</td>
</tr>
<tr>
<td>4</td>
<td>220</td>
<td>99.82</td>
</tr>
<tr>
<td>24</td>
<td>83</td>
<td>99.00</td>
</tr>
</tbody>
</table>

The abbreviations used are: IST, increase in survival time; DRF, dose reduction factor; BSA, bovine serum albumin; ID50, 50% inhibitory dose; LD50, 50% lethal dose; ID90, 90% inhibitory dose.

1 The abbreviations used are: IST, increase in survival time; DRF, dose reduction factor; BSA, bovine serum albumin; ID50, 50% inhibitory dose; LD50, 50% lethal dose; ID90, 90% inhibitory dose.
Chart 1. Dose-response curves relating inhibition of Walker tumor cell growth in culture to the cyclophosphamide concentration. Cyclophosphamide was metabolized with microsomes and cofactors prior to the addition of 2 volumes of buffer (×), spleen cytosol (■), BSA (16 mg/ml) (□), Walker ascites tumor cytosol (●), gastrointestinal mucosa cytosol (▲), kidney cytosol (○), or liver cytosol (Δ). Following 2 hr incubation at 37°, cultured Walker cells were treated with these mixtures as described in "Materials and Methods," and inhibition of growth quantitated after 72 hr.

Table 2
Dose reduction factors for the deactivation of aldophosphamide by various tissue supernatants

The doses inhibiting growth of 50% of Walker cells in culture (ID50) have been derived from Chart 1 and, thus, each is dependent on at least 12 separate determinations.

<table>
<thead>
<tr>
<th>Concentration of added protein (mg/ml)</th>
<th>ID50 (µg/ml)</th>
<th>DRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (control)</td>
<td>0</td>
<td>0.56</td>
</tr>
<tr>
<td>BSA</td>
<td>16.0</td>
<td>0.76</td>
</tr>
<tr>
<td>Spleen</td>
<td>20.4</td>
<td>0.72</td>
</tr>
<tr>
<td>Walker tumor</td>
<td>15.4</td>
<td>1.62</td>
</tr>
<tr>
<td>Gastrointestinal mucosa</td>
<td>12.9</td>
<td>4.07</td>
</tr>
<tr>
<td>Kidney</td>
<td>16.8</td>
<td>9.55</td>
</tr>
<tr>
<td>Liver</td>
<td>24.0</td>
<td>27.5</td>
</tr>
</tbody>
</table>

Table 3
Dose reduction factors for phosphoramid mustard

Phosphoramid mustard (cyclohexyl ammonium salt) was incubated with microsomes and cofactors and subsequently with liver cytosol, spleen cytosol, or BSA solution as described in "Materials and Methods" and "Results." ID50 values were obtained from curves of at least 3 points, each point representing the mean of 3 experiments.

<table>
<thead>
<tr>
<th>ID50 (µg/ml)</th>
<th>DRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>3.85</td>
</tr>
<tr>
<td>BSA</td>
<td>3.65</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.95</td>
</tr>
<tr>
<td>Liver</td>
<td>10.5</td>
</tr>
</tbody>
</table>

results obtained, giving DRF values of 2.2 for 4-methyl- and 6.9 for 6-methylcyclophosphamide. The ID50 concentration (in the absence of soluble enzymes) was approximately 300% greater than is required with cyclophosphamide.

The enzyme kinetics of the tissue supernatants was initially measured with model substrates, ethanol and acetaldehyde (Table 4). The tissue supernatants were dialyzed to remove endogenous substrates. Attempts were then made to make kinetic measurements using synthetic 4-hydroxy-5,5-dimethylcyclophosphamide. However, the rates of reaction were too slow to allow this, approximately 1.0% that observed with acetaldehyde. Comparative rates

were: liver, 100; gastrointestinal tract mucosa, 27; and Walker tumor, 8. For elimination of the possibility that the production of NADH was due to residual traces of ethanol from the preparation of the substrate (see "Materials and Methods"), an equal volume of ethanol was reduced to dryness and the residue was redissolved in buffer. When this solution was used in place of the substrate solution, no formation of NADH was observed.

However, studies with 4-hydroxy-5,5-dimethylcyclophosphamide showed it to be an inhibitor of the enzymes metabolizing ethanol and acetaldehyde.

Comparison of the reaction of liver cell soluble enzymes with various activated cyclophosphamides by measuring the formation of NADH showed that the relative rates of metabolism were: aldophosphamide, 100; 6-methyl analog, 34; and 5,5-dimethyl analog, 40. The 4-methyl analog of

4 In this paper, the terms aldophosphamide and 4-hydroxycyclophosphamide have been used to describe unfraccionated incubation mixtures containing the initial hydroxylated product of cyclophosphamide. Similarly, the analogs from methyl-substituted cyclophosphamides were prepared biologically and used as uncharacterized mixtures. These mixtures were always compared to controls that did not contain substrate or metabolites.
aldophosphamide was not metabolized, causing no production of NADH compared to a control incubation.

[14C]Cyclophosphamide was activated by microsomes at 75 μg/ml and subsequently diluted with buffer, gastrointestinal tract mucosa supernatant, or liver supernatant in a manner analogous to that used for the cell culture experiments (see above). After the centrifugation step, [14C]cyclophosphamide and its metabolites were extracted as described in “Materials and Methods.” Table 5 shows that the recovery of radioactivity in the pH 7.4 chloroform extract and in the aqueous residue after extraction were similar in the 3 samples. However, the radioactivity of the pH 2 extract was greatly increased in the case of the liver supernatant. The pH 2 extracts from the liver supernatant and gastrointestinal tract mucosa supernatant incubations, following methylation, yielded a single component (Rf 0.37), which gave mass spectral signals consistent with the structure of carboxyphosphamide, methyl ester (24). This compound was not identified in the methylated extract from the incubation with buffer.

DISCUSSION

The bioassay experiments reported here confirmed the previous observations (14, 22) that hepatic soluble enzymes could convert a cytotoxic metabolite of cyclophosphamide, aldophosphamide, to a nontoxic metabolite. However, as discussed in “Results,” the poor capability of the bioassay technique to discriminate accurately between samples of similar cytotoxicity rendered it unsuitable for quantitation except for gross effects. It can, however, be seen from Table 1 that the cytotoxic product produced by microsomal enzymes had considerable potency against the Walker tumor cell after 24 hr incubation. This finding shows that the activated material was not phosphoramid mustard, which is rapidly destroyed under identical incubation conditions (7), but was its precursor, aldophosphamide or the cyclic isomer 4-hydroxycyclophosphamide. Subsequently, the aldophosphamide could have broken down inside the tumor cells to release the cytotoxic phosphoramid mustard in situ.

The cell culture technique, measuring the inhibition of cell division, proved superior. The parallel sigmoid curves of Chart 1 amply demonstrate this. The dose reduction factors obtained from these measurements (Table 2) indicate clearly that tissues that do not show toxic effects after cyclophosphamide treatment in vivo, such as liver and kidney, deactivate activated cyclophosphamide very effectively under the experimental conditions described. Spleen soluble enzymes, on the other hand, are ineffective in the deactivation process. The deactivation capabilities of the tissue supernatants inversely correlate, at least qualitatively, with the proportion of 32P (derived from [32P]cyclophosphamide, 300 mg/kg) associated with the DNA compared to the radioactivity in the whole tissue 12 hr after treatment (P. Houghton, personal communication). It is possible that the proportion of the radioactivity bound to the DNA reflects the amount of available alkylating metabolites. Thus, the reduced level of alkylating metabolites expected in tissues with high deactivation activities would result in a lowered proportion of drug associated with the DNA.

Comparison of Tables 2 and 4 shows a qualitative similarity between increasing deactivation and increasing Vmax values for aldehyde dehydrogenase. No close correlation was seen for alcohol dehydrogenase activities. It was, however, possible that the deactivation capability measured by cell culture was due to binding of the active cyclophosphamide metabolites either nonspecifically to protein or by reaction of thiol groups with the aldehyde group of aldophosphamide.
phosphamide (15), or with the 2-chloroethyl side chains (21). When BSA was used in place of a tissue supernatant at 16 mg protein per ml, the percentage of deactivation that occurred was small (Chart 1; Table 2). Although BSA may not be a good model for tissue soluble proteins, it seems reasonable to conclude that nonspecific binding of cyclophosphamide metabolites to protein cannot account for the large DRF values shown by some tissue supernatants. Reaction of the mustard side chain with free thiols, e.g., glutathione, or with protein sulphydryl groups, could only be a contributory factor in the deactivation mechanism as shown by the results obtained with phosphoramide mustard (Table 3). Comparison of the DRF for phosphoramide mustard with liver supernatant (2.7) with the DRF values obtained for activated cyclophosphamide shows that phosphoramide mustard was deactivated considerably less effectively than was activated cyclophosphamide by liver supernatant (DRF 49.1). The deactivation was in fact less than that shown by Walker tumor cell supernatant and activated cyclophosphamide. The identical DRF for spleen with cyclophosphamide and phosphoramide mustard is of interest because it suggests that deactivation of activated cyclophosphamide by spleen supernatant is purely by binding or reaction with protein of the phosphoramide mustard released and that no specific mechanism operates on the intact molecule (aldophosphamide or 4-hydroxy-cyclophosphamide).

Hohorst's group have reported (15) that reaction of the aldehyde group of aldophosphamide with thiols resulted in deactivation. However, measurements of sulphydryl levels (5) (free and protein-bound) in rats have shown that, of the tissue types that we have studied, only the liver has a significantly different (and higher) level of acid-soluble sulphydryl groups. Although this could in part account for the superior protective action of liver soluble fraction against the toxicity of aldophosphamide, it remains inadequate to account for the range of supernatant deactivating abilities seen in our experiments. The types of compound reported by Hohorst, i.e., thiohemiacetals, should be easily hydrolyzed and may therefore perform a transport function in the blood.

We thus concluded that further enzymatic metabolism of the activated cyclophosphamide must account for the differences in tissue supernatant protection. Such metabolism could be further oxidation to 4-keto- and carboxyphosphamide. Further oxidation of the primary cyclophosphamide metabolites was proved by isolation of carboxyphosphamide from incubations of activated [14C]cyclophosphamide with liver and gastrointestinal tract mucosa soluble enzymes (see "Results"). In the case of liver cell supernatant, 99.7% of the radioactivity could be accounted for and 34.4% was identified as carboxyphosphamide, by mass spectrometry of its methyl ester. In the incubations with buffer alone and with gastrointestinal tract mucosa the radioactivity unaccounted for was presumably lost as [14C]acrolein, following β elimination from aldophosphamide. The identities of the products remaining in the aqueous residues after chloroform extraction are as yet unknown and may be derived from small molecule reactions with activated cyclophosphamide itself or with liberated acrolein.

Studies by other workers (14) have shown that aldehyde oxidase is capable of forming carboxyphosphamide and that the reaction is prevented by classical inhibitors. However, aldehyde dehydrogenase (EC 1.2.1.3) has a much lower $K_m$ for model substrates (16) than does aldehyde oxidase (EC 1.2.3.1) (19).

Activated cyclophosphamide, 6-methylcyclophosphamide, and 5,5-dimethylcyclophosphamide were substrates for NAD$^+$-requiring enzymes in liver cell supernatant. Chemically prepared 4-hydroxy-5,5-dimethyl-cyclophosphamide was shown to bind to the enzymes, inhibiting both ethanol and acetaldehyde metabolism. The close correlation between the enzyme activity and the deactivating ability of each tissue supernatant lends some support to the importance of aldehyde dehydrogenase in the deactivation process. Activated 4-methylcyclophosphamide (the acyclic isomer of 4-hydroxy-4-methylcyclophosphamide) was not a substrate for these enzymes. The DRF for this compound with liver supernatant was 2.2, which was similar to the dose reduction factor for phosphoramide mustard (2.7), thus confirming the absence of specific enzymatic deactivation of activated 4-methylcyclophosphamide. The 6-methyl analog was deactivated, however, showing that insertion of a methyl group did not affect the deactivation process, unless placed in the C-4 position. The higher ID$_{50}$ (in the absence of soluble enzymes) noted for both 4- and 6-methylcyclophosphamides compared to that for cyclophosphamide reflects the effect of the methyl substituent either on the microsomal activation step or on the transport of metabolites into the cultured tumor cells.

Processes influencing the selectivity of cyclophosphamide should improve the therapeutic index in vivo. 4-Methylcyclophosphamide has an index of 54 against the ADJ/PC6 tumor (8) (LD$_{50}$, 430 mg/kg; ID$_{90}$, 8.0 mg/kg) which is only marginally different from the therapeutic index of phosphoramide mustard (index, 50: LD$_{50}$, 141 mg/kg; ID$_{90}$, 2.82 mg/kg) determined in the same way. As the latter compound does not possess an aldehyde grouping allowing reaction with thiols, it is likely that thiol reactions, which may occur with the hydroxyl group of 4-hydroxy-4-methylcyclophosphamide or with the carbonyl group of its acyclic isomer as well as with aldophosphamide, are unimportant for determining selectivity. (The possible reaction of 4-hydroxy-4-methylcyclophosphamide with thiols is currently under investigation.) Comparison of LD$_{50}$ and ID$_{90}$ values shows that higher dose levels of 4-methylcyclophosphamide than of phosphoramide mustard are required, which may be due to thiol deactivation of the activated compound.

From these studies, it is clear that enzymatic oxidation of activated cyclophosphamide by the soluble fractions of cells plays an important part in the protection of some tissues from toxic reactions. Thus, the hypothesis that the selective action of cyclophosphamide, compared to other nitrogen mustards, is the result of a balance between chemical release of the toxic phosphoramide mustard from aldophosphamide and enzymatic formation of the nontoxic 4-ketocyclophosphamide and carboxyphosphamide is supported by our observations. Further work to identify all the deactivation products in our systems and to study protection in intact cells is in hand.
ACKNOWLEDGMENTS

The authors wish to thank Dr. M. Jarman for determining the mass spectra, Dr. T. A. Connors and Dr. P. B. Farmer for many helpful discussions, and Professor A. B. Foster for his interest.

REFERENCES

The Enzymatic Basis of the Selective Action of Cyclophosphamide

Peter J. Cox, Barry J. Phillips and Peter Thomas


Updated version

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/35/12/3755

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.