Role of Glutathione in the Metabolism-dependent Toxicity and Chemotherapy of Cyclophosphamide

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ABSTRACT

The role of glutathione in the biological effects of cyclophosphamide (CP) was evaluated by investigating the following: effect of CP on hepatic glutathione levels; relationship between hepatic glutathione depletion (repletion) and the binding of [chloroethyl-3H]CP and [4-14C]CP to hepatic macromolecules; effects of interaction between CP (or acrolein) and diethyl maleate (a classical glutathione depletor), and/or between CP and cysteine on the binding of labeled CP to hepatic macromolecules, on the induction of hematuria, on the content of hepatic cytochrome P-450, on weight gain in rats, on survival in mice, and on the chemotherapeutic efficacy of CP against Walker 256 carcinoma in rats.

CP and acrolein produced dose-dependent depletion of hepatic glutathione in mice, whereas phosphoramide mustard was at least one order of magnitude less effective. Acrolein caused death in mice; CP became covalently bound to hepatic macromolecules, prevented weight gain in rats, and produced hematuria and depression of hepatic cytochrome P-450 in vivo. These effects of CP (or acrolein) were enhanced by diethyl maleate but partially blocked by cysteine. On the other hand, reduction in the volume of Walker 256 carcinoma in rats by CP was not antagonized by cysteine.

All these investigations point to the following conclusions: (a) acrolein produced during the metabolism of CP binds to proteins and, by doing so may denature these proteins; and (b) acrolein in vivo preferentially reacts with glutathione, and sulfhydryl-containing compounds may protect against acrolein toxicity and at the same time not interfere with the chemotherapeutic activity of CP. And is not cytotoxic. In vivo, however, activation of CP by the microsomal mixed-function oxidase takes place predominantly in the liver. As shown in Ref. 20, Chart 1, initial steps of the metabolic activation of CP involve the formation of 4-hydroxy-CP, which is believed to rearrange to its isomer aldoxophosphamide (34). If not consumed in the reaction catalyzed by “soluble enzymes,” aldoxophosphamide can undergo β-elimination to release acrolein and PM.

Since most of the recent research proposes PM as the biological alkylator and effective chemotherapeutic moiety (10, 17, 34), it is important to realize that, for each molecule of PM released from the parent compound, a molecule of acrolein must be eliminated as well. Acrolein is a highly reactive aldehyde, and its formation from CP was first demonstrated by Alarcon and Meienhofer (2), with the suggestion that acrolein might play a significant role in the antitumor activity of CP. However, Sladek (33) showed that the cytotoxicity of acrolein to Walker 256 ascites cells in vitro was considerably less than the cytotoxicity of activated CP metabolites. Although acrolein may not be a specific antitumor agent, it is free to bind to cellular macromolecules. Acrolein:cysteine conjugates have been found in the urine of patients receiving CP (1). Mannello et al. (26) have reported denaturation by acrolein of the hepatic microsomal cytochrome P-450 in vitro, and this denaturation was blocked by cysteine. Furthermore, during the metabolism of [14C]CP in vitro, 14C label (recovered in acrolein) became bound almost exclusively to the microsomal proteins, and this binding was also blocked by cysteine (18). Both these observations suggest a protective role for free sulfhydryl groups, and since glutathione is a readily available source of endogenous sulfhydryl groups, its role in the metabolism-related toxicity of CP was investigated and is the subject of this report.

INTRODUCTION

CP is a valuable chemotherapeutic agent used in the treatment of many tumors, including breast carcinoma (9), lymphoma (23, 31), multiple myeloma (21), carcinoma of the lung (24), and some sarcomas (15, 19). It has also been used for immunosuppression prior to organ transplantation (3, 36) as well as for the treatment of many tumors, including breast carcinoma (9), lymphoma (23, 31), multiple myeloma (21), carcinoma of the lung (24), and some sarcomas (15, 19). It has also been used for immunosuppression prior to organ transplantation (3, 36), and since glutathione is a readily available source of endogenous sulfhydryl groups, its role in the metabolism-related toxicity of CP was investigated and is the subject of this report.

MATERIALS AND METHODS

Chemicals and Radiolabeled Compounds. [14C]CP (11.5 mCi/mmol) was obtained from New England Nuclear (Boston, Mass.). [3H]CP (440 mCi/mmol) was custom ordered from Amersham/Searle (Arlington Heights, Ill.). All radioisotopes were stored at -70° and were repurified immediately before use by thin-layer chromatography, and the purity (99% radiochemical purity) was established by chromatography, using plastic thin-layer chromatography plates developed in 2 separate solvent systems [chloroform:isopropyl alcohol:hexane (80:10:10); and chloroform:ethanol (90:10)]. PM and CP were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, Md. Reduced glutathione was obtained from Boehringer Mannheim (Mannheim, West Germany), Ellman’s reagent [5,5'-dithiobis(2-nitrobenzoic acid)] was from Calbiochem-Behring (La Jolla, Calif.), and diethyl maleate and acrolein were obtained from Eastman Kodak (Rochester, N. Y.). All other chemicals used were of the highest available quality.

Animals. Female mice, 6 to 10 weeks old, weighing 20 to 25 g, were obtained from the animal facilities of this Institute. Mice were caged in
groups of 6 and maintained identically. Mice received commercially available mouse diet and water ad libitum until the day of sacrifice.

Male Sprague-Dawley rats, 100 to 125 g or larger, were obtained from A. R. Schmidt Co. (Madison, Wis.). Rats were caged in groups of 4 and received commercially available rat diet and water ad libitum until the day of sacrifice.

**Chemical Treatments.** For radiolabeled CP binding experiments, animals received either [*H]CP or [*C]CP diluted with unlabeled CP at various doses. For toxicity studies, animals received various doses of CP or acrolein which was diluted with distilled water. Pretreatment with diethyl maleate was included in glutathione depletion experiments. Cysteine and alanine were given at 200 mg/kg by i.p. bolus or 200 mg/kg/24 hr by continuous tail vein infusion. Details are described in the legends to charts and to tables.

**CP Binding in Vivo.** [*H]CP and [*C]CP were used to study the binding characteristics of activated CP in vivo. Following a given dose of radiolabeled CP (either *C* or *H*), mice were killed by cervical dislocation, the livers were removed, and rinsed in cold 0.9% NaCl solution. Each organ was homogenized in 15 ml ice-cold 6% p-amino-salicylic acid (sodium salt), and duplicate 300-μl samples were removed to determine the amount of radiolabeled CP metabolite covalently bound to proteins. These samples were first treated to remove nucleic acids by digestion in 5 ml of trichloroacetic acid (5%) for 15 min at 90°C. The samples were cooled, spun at 2500 rpm for 10 min, and decanted, leaving the precipitated residue in the tube. This procedure was repeated. The protein precipitate was washed successively with cold 5% perchloric acid (repeated twice), ethanol, and finally diethyl ether. The air-dried protein residue was digested with 0.5 N NaOH and neutralized, and the radioactivity was counted by scintillation spectrometry. Protein was determined by the method of Lowry et al. (25). The remaining homogenate was extracted to isolate nucleic acids, using phenol:creosol:8-hydroxyquinoline (555 ml water-saturated phenol, 50 ml m-cresol, and 50 mg 8-hydroxyquinoline). Ten ml of this extraction solution were added to the 15-ml homogenate in p-amino-salicylic acid. The tubes were shaken continuously for 1 hr, and spun at 13,000 rpm for 25 min. The aqueous phase, which contained the nucleic acids, was transferred to 35-ml conical tubes and extracted again with an additional 10 ml of phenol:creosol:8-hydroxyquinoline solution. Nucleic acids present in the aqueous layer were precipitated by an equal volume of ethanol:creosol (9:1), and the tubes were cooled at −20°C and left overnight. The tubes were spun at 2000 rpm for 20 min, and the nucleic acid pellet was dissolved in 3 ml of 0.015 M sodium chloride:0.0015 M sodium citrate buffer, pH 7.0. The nucleic acids were precipitated by the addition of 4.5 ml ethanol, and the tubes were cooled to −20°C and left overnight. The nucleic acid pellet, redissolved in 4 ml sodium chloride:sodium citrate buffer (as above), was extracted twice with chloroform:isoamyl alcohol (95:5). The nucleic acids in the aqueous phase were reprecipitated with 0.5 ml 1 M NaCl and 5 ml ethanol (95%) and placed in the freezer (−20°C) overnight. The tubes were spun at 2000 rpm for 20 min, and the resulting pellet was redissolved in 3.0 ml of sodium chloride:sodium citrate buffer (as above). Absorbances of the resulting solutions were determined at 260 nm in a Gilford spectrophotometer; an absorbance of 1 was taken to represent approximately 50 μg of nucleic acid per ml of solution (20). Nucleic acid-bound radioactivity was measured by counting 1.5 ml of this solution by scintillation spectrometry. The radioactivity data were corrected for quenching by using an external standard and a computer program set up by the late Dr. C. Dave of this Institute: dpm were converted to pmol CP bound per mg nucleic acid.

**Reduced Glutathione Determination.** Hepatic glutathione in the red blood cell was measured by a modification of the method of Ellman (14). A portion of liver (0.1 to 0.2 g) was homogenized in 5 volumes of 0.1 M phosphate buffer (pH 7.4), and an equal volume of 4% sulfosalicylic acid was added. The contents of the tubes were mixed on a vortex for 1 min and left on ice for 30 min. The tubes were centrifuged at 2000 × g (4200 rpm) for 30 min. Then, 0.5 ml of the clear supernatant was added to 4.5 ml of 1.0 M potassium phosphate buffer, pH 8.0. After the contents were mixed on a vortex, 3.0 ml were removed and placed in a test cuvette, and 0.2 ml Ellman’s reagent was added. The contents of the cuvette were mixed, and the absorbance was measured at 412 nm on a Gilford spectrophotometer. Control cuvette contained phosphate buffer in place of the tissue supernatant.

**Glutathione Depletion and Repletion.** As shown in Chart 1, the depletion of hepatic glutathione by acrolein, diethyl maleate, CP, or PM was dose dependent, with PM being at least 1 order of magnitude less effective than the other 3 chemicals. Time dependence of hepatic glutathione depletion was also studied (Chart 2). In the untreated animals, the level of hepatic glutathione was relatively constant during the 24-hr observation period, and cysteine per se had an insignificant effect on this level (data not shown). CP caused the glutathione to fall to its lowest level at 4 to 8 hr, after which it started to return toward the glutathione level of the untreated controls. Cysteine given in conjunction with CP, however, caused an earlier return of glutathione level toward normal (data not shown), and at 12

**RESULTS**

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**Chart 1.** Dose-dependent depletion of hepatic glutathione by diethyl maleate, CP, PM, and acrolein in mice. Six C3Hf/HeHa mice in each group were given i.p. injections of different doses of PM, acrolein, diethyl maleate, or CP. Two hr later, the mice were killed by cervical dislocation, and the livers were quickly removed, placed on ice, and immediately assayed individually for the levels of reduced glutathione. Each point is the mean of 6 animals. Individual values in each group varied less than 10% from the mean value.

**Chart 2.** Time-dependent depletion of hepatic glutathione by CP, diethyl maleate, or both. Six C3Hf/HeHa mice in each group were given i.p. injections of different doses of PM, acrolein, diethyl maleate, or CP. Two hr later, the mice were killed by cervical dislocation, and the livers were quickly removed, placed on ice, and immediately assayed individually for the levels of reduced glutathione. Each point is the mean of 6 animals. Individual values in each group varied less than 10% from the mean value.

The role of glutathione in in vivo toxicity of CP and acrolein. The effects of diethyl maleate and cysteine on the body weight gains in rats treated with CP and on the survival of mice treated with acrolein are shown in Chart 3 and Table 1, respectively. Diethyl maleate, cysteine, or 0.9% NaCl solution had no effect on the normal body weight gains determined by the final weight on Day 7, which was about 40 to 50 g more than that on Day 0. However, over the 7-day test period, CP treated-rats did not gain any weight and actually showed a slight weight loss. On the other hand, weight gain determined by the body weight on Day 7 in the CP-plus-cysteine group was significantly higher (although this trend was evident from Day 2 onward) than that in the CP group. Pretreatment with diethyl maleate enhanced the toxicity of CP, inasmuch as this group showed significantly (p < 0.05) greater weight loss than did the CP group.

In the mice (Table 1), diethyl maleate or cysteine alone had no effect on survival. Acrolein (4.5 mg/kg) was lethally toxic to the mice (mean survival time, 3.5 days). Pretreatment with diethyl maleate decreased the mean survival time approximately 50%, i.e., from 3.5 days to 1.75 days. On the other hand, pretreatment with cysteine produced a 100% increase in survival of the mice treated with a more toxic dose (8.7 mg/kg) of acrolein.

**Chart 3.** Effects of CP, cysteine, diethyl maleate, and combinations of these agents on body weight gain of Sprague-Dawley rats. Male rats (4 rats/group) received 0.9% NaCl solution i.p., cysteine, diethyl maleate, or CP, or combinations of CP and diethyl maleate or of CP and cysteine. Diethyl maleate and CP were given as single doses. One-half of the 200-mg/kg dose of cysteine was given i.p. 30 min before CP or 0.9% NaCl solution. Each point is the mean of 4 animals. Bars, S.E.
Effect of Glutathione Depletion on the Binding of CP in Vivo. Since our earlier data suggested that acrolein binds predominantly to proteins (18), it was thought that hepatic glutathione depletion might increase the binding of CP to proteins. This was tested in C3Hf/HeHa mice given a 60-mg/kg dose of CP (Table 2).

Diethyl maleate pretreatment significantly increased (p < 0.05; 30%) the binding to proteins of acrolein-associated 14C radioactivity. The data for [3H]CP showed that diethyl maleate pretreatment had no effect on the binding of [3H]CP to proteins.

Since the antitumor properties of CP may depend primarily upon the binding of CP metabolites to nucleic acids, the effect of glutathione depletion on the binding of CP to total nucleic acids was also investigated. The results of the nucleic acid binding study are also shown in Table 2. Diethyl maleate pretreatment had no effect on the binding of [3H]CP or [14C]CP to nucleic acids.

As demonstrated earlier (Chart 2), the depletion of hepatic glutathione by CP is time dependent. The relationship between the depletion of hepatic glutathione and the binding of radio-labeled CP to proteins over an 8-hr period is shown in Chart 4. Maximal depletion of hepatic glutathione occurred at 2 hr after CP administration. At this time, glutathione depletion was approximately 70% of the total, demonstrating the inability of this dose of CP to deplete the remaining 30% of glutathione. During the 8-hr period examined, the binding of [3H]CP to proteins in the liver steadily increased without any apparent relation to the depletion and repletion of hepatic glutathione. During the same 8-hr period, as the levels of hepatic glutathione fell (0 to 2 hr), the binding of [14C]CP to proteins steadily increased. On the other hand, as the level of hepatic glutathione returned to normal (2 to 8 hr), the binding of [14C]CP to proteins steadily decreased.

Toxicity and Antitumor Properties of CP under Conditions of Continuous i.v. Infusion with Cysteine. The effects of continuous i.v. infusion with cysteine or alanine on the toxicity and antitumor properties of CP in rats were investigated. In the control experiments, using the protocol described in Chart 5, it was observed that the infusion of cysteine or alanine alone had no effect on the body weight gain or on the cytochrome P-450 content of the liver, nor did it induce hematuria or affect the tumor growth of Walker 256 carcinoma implanted s.c. in the Sprague-Dawley rats.

As shown in Chart 5A, animals treated with CP alone (non-infused) began to lose weight immediately and continued to lose weight over the 7-day period [17.25 ± 1.66 (S.E.) g after 7 days]. In the animals receiving infusions of 0.9% NaCl solution plus CP, weight loss was not significantly different (p >
of sterile 0.9% NaCl solution, s.c.). The tumor was allowed to grow until the or 0.9% NaCl solution alone had no effect on the weight gain profiles. Dawley rats received 0.5 g homogenized Walker 256 carcinoma (0.5 g in 0.5 ml profiles of rats bearing Walker 256 carcinoma. Each of the 11 male Sprague-Dawley rats treated with CP. Four male rats were infused with cysteine (200 mg per kg in 14 ml per 24 hr) or with 14 ml 0.9% NaCl solution per kg per 24 hr and 3 rats received no infusion (noninfused controls). Infusions were initially continued for 24 hr, then a single dose of CP (200 mg/kg i.p.) was administered, and infusions were continued for an additional 24 hr period. The infusions were then terminated, and the rats were weighed daily for the next 7 days. Each point represents the mean. Bars, S.E. In other studies, similar infusions with cysteine and the binding of [14C]CP and [3H]CP to hepatic macromolecules was investigated; and the effects of interaction between CP (or acrolein) and diethyl maleate, a classical glutathione dipeptidase (5, 6, 27), and/or between CP and cysteine on the toxic and chemotherapeutic properties of CP. The ability of CP to induce hepatic glutathione depletion was assessed; the relationship between hepatic glutathione depletion (repletion) and the binding of [14C]CP plus infusion of 0.9% NaCl solution, or CP plus infusion of 0.9% NaCl solution, or CP plus alanine infusion. Chart 5, B and C, shows the effects of cysteine infusion on the antitumor activity of CP against Walker 256 carcinoma. Weight gain profiles in Chart 5B demonstrate the toxicity of CP as evidenced by the loss of body weight in rats treated with CP. On the other hand, rats receiving cysteine infusion demonstrated less weight loss over the 9-day test period. Chart 5C shows the average tumor size in all groups of rats. As expected, tumor size in the untreated group continued to increase daily, but CP treatment suppressed the growth of the tumor to the same extent in both 0.9% NaCl solution- and cysteine-infused groups.

**DISCUSSION**

Extensive investigations were carried out to evaluate the effects of hepatic glutathione depletion and repletion on the toxic and chemotherapeutic properties of CP. The ability of CP to induce hepatic glutathione depletion was assessed; the relationship between hepatic glutathione depletion (repletion) and the binding of [14C]CP to hepatic macromolecules was investigated; and the effects of interaction between CP (or acrolein) and diethyl maleate, a classical glutathione depleter (5, 6, 27), and/or between CP and cysteine on the average diameter (length + width + 2) was approximately 15 cm. At this time, 4 rats were infused with cysteine (200 mg in 14 ml per kg per 24 hr, i.v.), and 4 were infused with 0.9% NaCl solution for 5 hr. Then, CP (100 mg/kg i.p.) was given to these 8 rats, and infusions were continued for an additional 24 hr. The infusions were then terminated, and total body weights were recorded each day for the next 8 days. The 3 untreated animals were also observed for weight gain. Each point represents the mean. Bars, S.E. In other studies, infusion of cysteine or 0.9% NaCl solution alone had no effect on the appearance of hematuria in the rats. The data suggest that infusion of 0.9% NaCl solution had no effect in preventing hematuria which appeared 1 day after CP administration. Alanine infusion prevented the appearance of hematuria in one animal on Day 1, but from Day 2 onward all animals had hematuria. Of the animals receiving cysteine, some of the animals were asymptomatic for up to 5 days, suggesting that some of the toxic side effects had been prevented by cysteine infusion.

As shown in Table 4, CP administration reduced the hepatic cytochrome P-450 from 0.83 ± 0.04 nmol/mg microsomal protein in untreated rats to 0.17 ± 0.01 in CP-treated rats. On the other hand, alanine, cysteine, or 0.9% NaCl solution (in the absence of CP) had no significant effect (p > 0.05) on the concentration of cytochrome P-450. However, infusion with cysteine, but not with alanine or 0.9% NaCl solution, decreased the toxicity of CP to a significant degree. The concentration of cytochrome P-450 in cysteine-infused animals was 50% higher (p < 0.05) than that in animals who received CP alone, or CP plus infusion of 0.9% NaCl solution, or CP plus alanine infusion.
Effects of cysteine or alanine infusion on the appearance of hematuria following CP treatment

Male Sprague-Dawley rats received either saline, alanine, or cysteine infusion in the presence or absence of CP (200 mg/kg). In the absence of CP, 0.9% NaCl solution, alanine, or cysteine infusion produced no blood in the urine of the rats; however, in the presence of CP, hematuria was observed in different degrees. Noninfused control rats received only CP. Details of the experiment protocol are described in the legend to Chart 5A.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclophosphamide</th>
<th>% of animals with hematuric cystitis at following times after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>Cysteine infusion  (200 mg/kg/24 hr)</td>
<td>200 mg/kg i.p.</td>
<td>25*</td>
</tr>
<tr>
<td>Alanine infusion   (200 mg/kg/24 hr)</td>
<td>200 mg/kg i.p.</td>
<td>75</td>
</tr>
<tr>
<td>Saline infusion    (0.9% NaCl/24 hr)</td>
<td>200 mg/kg i.p.</td>
<td>100</td>
</tr>
<tr>
<td>No infusion</td>
<td>200 mg/kg i.p.</td>
<td>100</td>
</tr>
</tbody>
</table>

*Percentage of rats with hematuric cystitis from a total of 4 rats in each group, 1 rat representing 25%, except in the non-infused group.

Glutathione and CP Metabolism

Table 3

Effects of cysteine or alanine on the concentration of hepatic cytochrome P-450 in rats treated with CP

Details of the experimental protocol were similar to those described in Chart 5A and Table 3. Rats were killed on Day 6 after CP administration. Livers were removed, perfused with 0.9% NaCl solution, and processed to isolate microsomal fractions by differential centrifugation. Microsomes were analyzed by difference spectroscopy for the content of cytochrome P-450. Details are described in the text.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytochrome P-450 concentration (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment (3)</td>
<td>0.83 ± 0.04</td>
</tr>
<tr>
<td>0.9% NaCl solution infusion only (4)</td>
<td>0.76 ± 0.03</td>
</tr>
<tr>
<td>Cysteine infusion only (4)</td>
<td>0.80 ± 0.04</td>
</tr>
<tr>
<td>Alanine infusion only (4)</td>
<td>0.81 ± 0.04</td>
</tr>
<tr>
<td>CP only (3)</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>CP + 0.9% NaCl solution infusion (4)</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>CP + cysteine infusion (4)</td>
<td>0.25 ± 0.01</td>
</tr>
<tr>
<td>CP + alanine infusion (4)</td>
<td>0.18 ± 0.02</td>
</tr>
</tbody>
</table>

* Number in parentheses, number of rats per treatment group.

Glutathione Depletion and Repletion. While CP and acrolein both caused extensive depletion of hepatic glutathione, PM was at least one order of magnitude less effective. This differential effect of acrolein and PM on hepatic glutathione suggested that the chemotherapeutic activity of CP, which is believed to lie ultimately with PM (10, 34) and not with acrolein (7, 28, 33), may not be seriously compromised in the presence of sulphydryl compounds such as cysteine and N-acetylcysteine (3, 26). Similarly, CP-related in vivo depression of cytochrome P-450 content and aryl hydrocarbon [benzo(a)pyrene] hydroxylase activity is also blocked by N-acetylcysteine (3). Studies by Cox et al. (12) and Brock et al. (8) have also implicated acrolein as the causative agent in the production of hemorrhagic cystitis by CP; other investigations have also demonstrated the protection of the urotoxicity of CP by N-acetylcysteine (4, 30, 35).

Consistent with the suggestion that glutathione, via alkylation of acrolein, has an important role in the metabolism-related toxicity of CP is the observation that diethyl maleate enhanced the depletion of hepatic glutathione by CP in an additive manner, and cysteine afforded a certain degree of protection because it allowed an earlier return of glutathione levels to normal than when CP alone was administered.

Effects of Glutathione Depletion on the Binding of Labeled CP in Vivo to Liver Macromolecules. Our earlier studies (18) on the in vitro metabolism of [3H]CP and [14C]CP suggested that acrolein binds to proteins covalently. This binding is effectively blocked by glutathione and cysteine, which provide free sulphydryl groups for reaction with acrolein. The present investigations of the in vivo binding of [3H]CP and [14C]CP also clearly suggest that at least some of the metabolite (more than 50%) bound to liver proteins is acrolein. The data show that the binding of [14C]CP to proteins exceeds that of [3H]CP and that the binding of [3H]CP to nucleic acids exceeds the binding of [14C]CP, especially in extrahepatic tissues as reported elsewhere (20). These data can be interpreted to suggest that at least 2 types of metabolites bind to either proteins or nucleic acids. If the metabolite binding to a macromolecule has the intact structure of the parent compound, e.g., 4-hydroxy-CP, this would be reflected in the binding of equal quantities of both labels; an excess of either label could be interpreted to mean that one or the other portion of the metabolized (cleaved) CP is also binding. Therefore, the excess of [14C] over [3H] would suggest the binding of acrolein, and the reverse (i.e., excess of [3H] over [14C]), would suggest the binding of PM or norritrogen mustard.

Diethyl maleate enhanced the binding of acrolein (difference between [14C] and [3H] labels) to liver proteins by about 30%, while having essentially little effect on the binding of PM (difference between [3H] and [14C] labels) to nucleic acids. These
findings are consistent with the interpretation that CP-induced depletion of glutathione is attributable to its loss via reaction with acrolein. Furthermore, that acrolein is the major toxic metabolite of CP involved in glutathione depletion is also supported by a close relationship between hepatic glutathione depletion (repletion) versus \(^{14}C\)CP binding to liver proteins (Chart 4) and the lack of such relationship between glutathione depletion (repletion) versus \(^{3}H\)CP binding to the proteins.

**Role of Glutathione in Toxic and Antitumor Properties of CP.** Acrolein, being a very toxic chemical, has considerable potential for producing toxicity after it has been cleaved from aldophosphamide. Recent in *vitro* studies from this laboratory have demonstrated that of the various metabolites of CP tested, only acrolein and 4-hydroxy-CP (which releases acrolein in solution) denature cytochrome P-450 (3, 26). This *in vitro* denaturation of cytochrome P-450 was partially or completely blocked by cysteine and N-acetylcysteine (3), again suggesting that sulphydryl groups in these chemicals react with acrolein and prevent its binding to the microsomal protein. That a similar mechanism of acrolein toxicity (i.e., reaction with the sulphydryl groups in proteins) and the protection of this toxicity by glutathione are operating *in vivo* is evident from our data. The toxicity of CP to rats, demonstrated by the absence of any gain in body weight, was enhanced by diethyl maleate and partially decreased by cysteine (Chart 3). Direct administration to mice of acrolein in combination with diethyl maleate also resulted in additive toxicity (Table 1), suggesting a common mechanism of action, i.e., glutathione depletion. Here also, pretreatment with cysteine gave good protection (approximately 100% increased life span) against acrolein toxicity, reflecting rapid detoxification of acrolein by cysteine.

Toxicity of CP reflected in weight loss was also reversed somewhat by infusion of cysteine (Chart 5A) but not by alanine. Infusion of cysteine (24 hr prior to 24 hr post-treatment with CP (Chart 5A)) was not superior to daily treatment for 3 days prior to CP administration (Chart 3), indicating a biochemical effect of cysteine (stimulated glutathione repletion/biosynthesis?) instead of a direct acrolein-trapping effect. Cysteine but not alanine delayed the appearance of hematuria (Table 3), suggesting that cysteine, or an active derivative thereof, is present in bladders of rats in sufficient concentration to deactivate some acrolein, the causative agent of this CP toxicity (8, 12).

In a similar response, cysteine afforded some protection of cytochrome P-450 against CP toxicity (Table 4), revealing its ability to detoxify some of the acrolein generated *in vivo*. Thus, it appears that cysteine affords some protection against CP toxicity expressed as hepatic glutathione depletion in mice, weight loss in rats, hemorrhagic cystitis in rats, and cytochrome P-450 depletion in rats. Its rather dramatic protection against toxicity to mice of directly administered acrolein (Table 1) suggests a more efficient detoxification of acrolein present in high levels after direct administration, in comparison with presumably low levels generated from CP in *in vivo*. Cysteine protected modestly against weight loss in tumor-bearing rats (Chart 5B) and at the same time did not antagonize the antitumor effect of CP (Chart 5C).

The absence of antagonism of the antitumor properties of CP by cysteine, coupled with its reduction of various toxicities of CP, suggests a protective role for sulfhydryl-containing chemicals used in combination with CP. Superior protection (90 to 100%) against CP-induced depression of cytochrome P-450 in rats by N-acetylcysteine was recently observed in our laboratory (3), and its protective effect against CP-induced bladder toxicity was comparable to its protection of cytochrome P-450 depression *in vivo*. Other investigators have also reported protection by N-acetylcysteine of CP-related bladder toxicity (4, 30, 35) and lack of interference with the chemotherapeutic activity of CP (35).

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