Metabolism and Binding of Cyclophosphamide and Its Metabolite Acrolein to Rat Hepatic Microsomal Cytochrome P-450


ABSTRACT

The hepatic cytochrome P-450-mediated metabolism and metabolic activation of [chloroethyl-3H]cyclophosphamide ([chloroethyl-3H]CP) and [4-14C]cyclophosphamide ([4-14C]CP) were investigated in vitro in the reconstituted system containing cytochrome P-450 isolated from phenobarbital-treated rats. In addition, hepatic microsomal binding and the hepatic microsomal-mediated metabolism of [14C]acrolein, a metabolite of [4-14C]CP, were also investigated.

The metabolism of [chloroethyl-3H]CP and [4-14C]CP to polar metabolites was found to depend on the presence of NADPH and showed concentration dependence with respect to cytochrome P-450 and NADPH:cytochrome P-450 reductase. Km and Vmax values were essentially similar (Km, 0.44 and 0.42 mM; Vmax, 4.8 and 7.0 nmol of polar metabolites formed/min/nmol of cytochrome P-450 for [4-14C]CP and [chloroethyl-3H]CP, respectively). The patterns of inhibition by microsomal mixed-function oxidase inhibitors, anti-cytochrome P-450 antibody, and heat denaturation of the cytochrome P-450 were essentially similar, with subtle differences between [4-14C]CP and [chloroethyl-3H]CP metabolism. The order of inhibition by various mixed-function oxidase inhibitors was SKF > α- and β-naphthoflavones > metyrapone.

The in vitro metabolic activation of CP in the reconstituted system demonstrated predominant binding of [chloroethyl-3H]CP to nucleic acids and almost exclusive binding of [4-14C]CP to proteins. Gel electrophoresis-fluorography of the proteins in the reconstituted system treated with [4-14C]CP demonstrated localization of the 14C label in the cytochrome P-450 region. To examine this association further, hepatic microsomes were modified with [14C]acrolein in the presence and the absence of NADPH. The results confirmed covalent association between [14C]acrolein and cytochrome P-450 in the microsomes and also demonstrated further metabolism of [14C]acrolein, apparently to an epoxide, which is capable of binding covalently to proteins.

The results of these investigations not only confirm the significance of primary metabolism but also emphasize the potential role of the secondary metabolism of cyclophosphamide in some of its toxic manifestations.

INTRODUCTION

CP, an oxazaphosphorine derivative of the classical alkylating agent nitrogen mustard, is used commonly in the treatment of a variety of neoplastic diseases (8). In addition, because of its immunosuppressive properties, CP has also found use in the treatment of diseases thought to be of autoimmune etiology (7). CP, inactive per se, requires metabolic activation by the hepatic microsomal cytochrome P-450-dependent mixed-function oxidases (Chart 1). In addition to the microsomal-mediated biotransformation, cytosolic reductases/oxidases also participate in the detoxification of activated metabolites of CP (4, 17).

Since the biological effects of CP are believed to result from the binding of activated metabolites to tissue macromolecules, during the past several years, we have investigated the microsomal metabolism-mediated binding of CP to macromolecules, especially to proteins and nucleic acids. Such microsomal preparations are known to contain several different forms of cytochrome P-450 (10, 11, 23). These different cytochromes P-450, while possessing some overlapping substrate specificities, are known to have widely differing specificities in the metabolism of various chemicals (10, 11, 23). To define more clearly the role of CP metabolism in the toxicity of CP, we have now used a purified cytochrome P-450 reconstituted system to study the metabolism and metabolic activation of CP labeled in the chloroethyl side chain with 3H and in the ring with 14C. These investigations have helped to delineate the specificity of the macromolecular binding, especially to cytochrome P-450, of various metabolites of CP.

Our previous reports have suggested that the predominant binding of [4-14C]CP to microsomal proteins is most likely due to the binding of [14C]acrolein produced during the metabolism of CP (14, 16). Acrolein, a highly reactive olefinic aldehyde, may itself undergo microsome-mediated metabolic activation, possibly to acrolein oxide (27). Acrolein, a toxic chemical (6, 19, 20, 25), has been identified recently as the causative agent of CP-associated hemorrhagic cystitis (2, 3, 5). Moreover, earlier reports from this laboratory have implicated acrolein as the metabolite of CP responsible for the inhibition and denaturation of hepatic microsomal cytochrome P-450 (16, 24). In the present report, using [14C]acrolein and [4-14C]CP, we have also: (a) made comparative evaluation to define the role of acrolein in the binding of 14C label from [4-14C]CP to hepatic microsomal cytochrome P-450; and (b) investigated further metabolism of acrolein to protein-binding metabolites.

MATERIALS AND METHODS

Chemicals. CP (4-14C, ring-labeled) with a specific activity of 11.25 mCi/mmol was purchased from New England Nuclear (Boston, MA). [Chloroethyl-3H]CP with a specific activity of 440 mCi/mmol was obtained from Amersham/Searle Corp. (Arlington Heights, IL). Both [4-14C]CP and [chloroethyl-3H]CP were repurified prior to use as described previously.

[4-14C]CP, [4-14C]cyclophosphamide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; [1,3-3H]acrolein, [14C]acrolein.
Preparation of Purified Micromosomal Enzymes. Cytochrome P-450 and NADPH-cytochrome P-450 reductase were purified from the hepatic microsomes of phenobarbital-treated rats according to the method of Guengerich and Martin (13). Only “Peak B” (see Ref. 13) was found to have significant activity toward CP, either [chloroethyl-3H]-CP or [4-14C]-CP, and this peak, which appeared as a single band on SDS-PAGE, was, unless otherwise stated, utilized in the metabolism studies.

The purified cytochrome P-450 had a specific activity of 14 to 17.5 nmol/min per mg of protein. The protein migrated as a single protein species on SDS-PAGE and had an apparent Mr of 52,000. The purified NADPH-cytochrome P-450 reductase had a specific activity of 57 nmol/min per mg of protein and had an apparent Mr of 75,000.

Metabolism of CP by the Reconstituted Cytochrome P-450 System. The metabolism of [4-14C]-CP and [chloroethyl-3H]-CP to aqueous metabolites was studied in the reconstituted system consisting of purified cytochrome P-450, purified NADPH-cytochrome P-450 reductase, and synthetic lipid, dilauroylphosphatidylcholine (Serozyme Research Laboratory, Hamilton, Ontario, Canada). Unless otherwise stated in the chart legends, a typical incubation mixture in phosphate buffer (0.09 M, pH 7.4) contained the following: the NADPH-generating system (14, 16), 300 nmoL of labeled cyclophosphamide, 0.25 nmoL of cytochrome P-450, 1 unit NADPH:cytochrome P-450 reductase (1 unit = 1 µmol of cytochrome c reduced/min) and 10 µg of dilauroylphosphatidylcholine in a total volume of 1 ml. The reconstituted enzyme system (cytochrome P-450, the reductase, and dilauroylphosphatidylcholine) was preincubated for 5 min at 37° in the presence of the substrate. The reaction was initiated by the addition of the NADPH-generating system (incubated previously for 15 min at 37°), and the incubation was continued for 30 min at 37°. Control incubations contained all of the above except NADPH. The reaction was stopped by the addition of 6.0 ml of chlorofom:isoamylic alcohol (95:5), followed by 1 ml of water. The tubes were capped, the contents were mixed vigorously on a vortex mixer, and the mixtures were centrifuged for 5 min at 2000 rpm. One ml of the aqueous layer was counted in a Beckman scintillation counter to determine the formation of NADPH-mediated aqueous metabolites of [4-14C]CP or [chloroethyl-3H]-CP. After subtraction of the background, the enzymatic activity is expressed as nmol of [chloroethyl-3H]-CP or [4-14C]CP related aqueous metabolites formed per nmol of cytochrome P-450 per min.

While phosphoramidemustard and non-nitrogen mustard (degradation product of phosphoramidemustard) would be among the major metabolites in chloroform-extracted aqueous phase of the incubation mixture, other polar metabolites remaining in the aqueous phase could include some acrolein and dechlorinated metabolites of CP reported to be produced by sheep (1). However, as reported previously (17), the ease with which the polar metabolites of CP can be measured as radioactivity in the aqueous phase has provided a rapid assay for detailed investigations of the enzymology of CP metabolism catalyzed by the purified cytochrome P-450 in the reconstituted system.

Binding of [chloroethyl-3H]-CP, [4-14C]-CP, and [14C]Acrolein to Proteins. The binding of [14C]acrolein per se and/or the metabolism-mediated binding of [chloroethyl-3H]-CP, [4-14C]-CP, and [14C]acrolein to proteins were studied with the incubation mixtures containing either hepatic microsomes or the reconstituted system.

Unless otherwise stated, the incubation mixtures for studies on the binding of [14C]acrolein and [4-14C]-CP to hepatic microsomes contained: 0.09 M potassium phosphate buffer, pH 7.4; 5 mM MgCl2; 1.4 mM MnCl2; 0.54 mg of hepatic microsomal protein, NADPH generating system (14, 16); 65 nmoL of acrolein or 150 nmoL of CP (final volume of incubation mixture, 0.5 ml). The metabolism of [chloroethyl-3H]-CP and [4-14C]-CP to protein binding metabolites was also studied in the reconstituted cytochrome P-450 system. The incubation mixture was similar to that described for studies on the metabolism of CP to polar metabolites. The reaction mixture for either type of incubation was incubated for 30 min at 37°. At the end of the incubation, proteins were precipitated from the incubation mixture by the addition of 4 ml of cold 5% trichloroacetic acid. The resulting protein pellet was washed with cold 5% perchloric acid followed by ethanol and air dried, digested, and counted as described before (16, 17).
Quantities of aqueous (polar) metabolites derived from [chloroethyl-3H]CP and [4-14C]CP, and the radioactivity remaining in the aqueous phase was used to calculate the amount of protein-bound polar metabolites. Each value is a mean of 2 or 3 determinations. Over 85-fold greater binding of the 14C label to cytochrome P-450 as described by Thomas et al. (31). The IgG fraction from the immunized sera was isolated as described by Guengerich (9).

Preparation of Antibody against the Cytochrome P-450. Rabbit antibody was prepared against the purified phenobarbital-induced cytochrome P-450 as described by Thomas et al. (31). The IgG fraction from the immunized sera was isolated as described by Guengerich (9).

RESULTS

Metabolism and Binding of CP. As shown in Table 1, the metabolism of [4-14C]CP and [chloroethyl-3H]CP by the reconstituted cytochrome P-450 system (consisting of purified cytochrome P-450, purified NADPH-cytochrome P-450 reductase, dilauroyl phosphatidycholine, and NADPH) resulted in the formation of protein binding, nucleic acid binding, and polar metabolites. Over 85-fold greater binding of the 14C label to cytochrome P-450 protein was observed compared to the binding of the 3H label. However, the pattern of binding of [chloroethyl-3H]CP and [4-14C]CP to nucleic acids was nearly completely reversed. The metabolism of CP resulted in greater binding (10-fold) to E. coli tRNA of the 3H label than that of the 14C label. Similar results were obtained with calf thymus DNA (data not shown).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pmol of metabolite bound</th>
<th>To protein (per mg of cytochrome P-450)</th>
<th>To nucleic acid (per mg of E. coli tRNA)</th>
<th>Polar metabolites (pmol of labeled metabolites produced/min/mg of cytochrome P-450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[4-14C]CP</td>
<td>17,200</td>
<td>170</td>
<td>3,260</td>
<td></td>
</tr>
<tr>
<td>[chloroethyl-3H]CP</td>
<td>203</td>
<td>1,840</td>
<td>8,730</td>
<td></td>
</tr>
</tbody>
</table>

The incubation mixture was extracted with chloroform/isooamyl alcohol (95:5), and the radioactivity remaining in the aqueous phase was used to calculate the quantities of aqueous (polar) metabolites derived from [chloroethyl-3H]CP and [4-14C]CP.

Measurement of radioactivity remaining in the chloroform-extracted aqueous phase following the metabolism of [chloroethyl-3H]CP or [4-14C]CP indicated that more 3H label than 14C label was recovered in the aqueous phase. Similar results were obtained previously for the hepatic microsomes (14, 16). The formation of the activated and polar metabolites depended upon the presence of NADPH and cytochrome P-450 since, in the absence of either, very little metabolite formation occurred.

In order to confirm that protein binding 14C label from [4-14C]CP was associated with cytochrome P-450, we separated the proteins in the reconstituted system by SDS-PAGE following the metabolism of [4-14C]CP and visualized the radio-labeled proteins by fluorography. As shown in Fig. 1, the greatest amount of protein-bound radioactivity migrated in one region of the autoradiogram. This is the area of migration of cytochrome P-450, since comparable experiments in which the proteins were stained have shown that cytochrome P-450 migrates in this region of the gel. Essentially no binding was observed when

![Fig. 1. Gel electrophoresis-fluorography of purified cytochrome P-450 treated with [4-14C]CP in the reconstituted system. Purified cytochrome P-450 was incubated in the reconstituted system with [4-14C]CP in the presence and absence of NADPH. The proteins from the incubation were precipitated and subjected to gel electrophoresis. Gels with separated radio-labeled proteins were subjected to fluorography. Details are described in the text.](image-url)
NADPH was omitted from the reaction mixture. In separate experiments, essentially identical results, i.e., binding of the 14C label to cytochrome P-450, were obtained when purified cytochrome P-450 was treated with [14C]acrolein in the absence of NADPH. In these experiments, gel electrophoresis-fluorography of the modified proteins demonstrated that major band of radioactivity is associated with cytochrome P-450. These results, reported previously (15), are in agreement with our earlier suggestion (14, 16) that the CP metabolite binding to cytochrome P-450 is acrolein.

Kinetic Analysis of [4-14C]CP and [chloroethyl-3H]CP Metabolism. Optimum conditions for cytochrome P-450-catalyzed formation of the polar metabolites of [4-14C]CP and [chloroethyl-3H]CP were determined with respect to incubation time, amount of purified cytochrome P-450, and the amount of NADPH-cytochrome P-450 reductase. Optimization experiments conducted with [4-14C]CP and [chloroethyl-3H]CP as the substrate yielded nearly identical results. Therefore, optimal conditions (e.g., 30 min of incubation at 37°C, 0.25 nmol of cytochrome P-450, 4 units of reductase/nmol of cytochrome P-450) defining a linear rate of reaction were used in subsequent studies carried out to measure kinetic parameter of [chloroethyl-3H]CP and [4-14C]CP metabolism.

In order to determine whether there are qualitative differences for the catalysis of [chloroethyl-3H]CP and [4-14C]CP in the reconstituted system, K_m and V_max values were measured. The metabolism data derived for both [4-14C]CP and [chloroethyl-3H]CP could be fitted to linear Lineweaver-Burke plots (Chart 2); [4-14C]CP exhibited a K_m of 0.44 mM and maximum velocity of 4.8 nmol of aqueous metabolites formed/nmol of cytochrome P-450/min, while [chloroethyl-3H]CP exhibited similar K_m and V_max values (0.42 mM and 7.0 nmol of aqueous metabolites formed per nmol of cytochrome P-450 per min, respectively). In addition, there was an excellent correlation (r^2 = 0.99) between the reciprocals of product formation and substrate concentration in the Lineweaver-Burk plots for both [4-14C]CP and [chloroethyl-3H]CP metabolism.

Effects of Inhibitors on [4-14C]CP and [chloroethyl-3H]CP Metabolism by the Reconstituted Cytochrome P-450 System. SKF-525A and metyrapone are known inhibitors of mixed-function oxidase enzyme activities induced by phenobarbital and similarly acting chemicals, whereas naphthoflavones (α and β) are potent inhibitors of the enzyme activities induced by 3-methylcholanthrene and similarly acting chemicals (26, 30). Studies were conducted to determine the effects of these inhibitors on the metabolism of both [4-14C]CP and [chloroethyl-3H]CP to aqueous metabolites. The data are presented in Chart 3. All the inhibitors tested, SKF-525A, metyrapone, α-naphthoflavone and β-naphthoflavone, produced a concentration-dependent inhibition of the metabolism of both [chloroethyl-3H]CP and [4-14C]CP, with minor differences between the 2 labels. The order of potency was SKF-525A > α- and β-naphthoflavones > metyrapone.

Effect of Anti-Phenobarbital-induced Cytochrome P-450 Antibody on the Metabolism of [4-14C]CP and [chloroethyl-3H]CP by the Reconstituted Cytochrome P-450 System. The IgG antibody raised against the cytochrome P-450 was determined to be specific by Ouchterlony double-diffusion analysis against the cytochrome P-450 (Peak B2) isolated from phenobarbital-treated rats. This antibody was tested for its ability to inhibit [chloroethyl-3H]CP and [4-14C]CP metabolism. The anti-

![Chart 2. Lineweaver-Burk plot of [4-14C]CP metabolism by the reconstituted cytochrome P-450 system. A similar plot was obtained for [chloroethyl-3H]CP metabolism. Other details are described in the text.](chart2.png)

![Chart 3. Effects of inhibitors of microsomal mixed-function oxidases on the metabolism of [4-14C]CP (A) and [chloroethyl-3H]CP (B) by the reconstituted cytochrome P-450 system. Reaction conditions are as described in the text. Control values for [4-14C]CP and [chloroethyl-3H]CP polar metabolites generated were 70 nmol of polar metabolites/nmol of cytochrome P-450 and 97 nmol of polar metabolites/nmol of cytochrome P-450, respectively.](chart3.png)
cytochrome P-450 antibody produced a concentration-dependent inhibition of [4-14C]CP metabolism, reaching a complete inhibition at an antibody concentration of 1 mg. The anti-cytochrome P-450 antibody also produced a concentration-dependent inhibition of [chloroethyl-3H]CP metabolism; however, at an antibody concentration of 1 mg, the metabolism of [chloroethyl-3H]CP was inhibited by only 70%. These results show subtle differences in the inhibition of the metabolism of [4-14C]CP and [chloroethyl-3H]CP by the anti-cytochrome P-450 antibody.

**Thermolability of the Purified Cytochrome P-450 Protein.** In this experiment, cytochrome P-450 was preincubated for various times (5, 10, or 15 min) at varying temperatures (37, 42, or 50°). After the incubation period, the remaining components of the complete reaction mixture were added to the cytochrome P-450, and the cytochrome P-450 was tested at 37° for its ability to support the metabolism of [chloroethyl-3H]CP and [4-14C]CP to aqueous metabolites. When cytochrome P-450 was preincubated at 37° for 5, 10, and 15 min, there was a 5 to 20% decrease in the metabolism of either [4-14C]CP or [chloroethyl-3H]CP. At 42°, the metabolism of [4-14C]CP or [chloroethyl-3H]CP was decreased by 10 to 20%. However, at 50°, there was a time-dependent loss of activity, reaching about 80% at 15 min for both [4-14C]CP and [chloroethyl-3H]CP metabolism.

**Effect of Thiols on the Binding of Acrolein.** We have reported previously that compounds containing free sulfhydryl group(s) block the binding of 14C label from [4-14C]CP to microsomes (14, 16). To further support our hypothesis that these chemicals blocked the binding by interacting with acrolein formed during [4-14C]CP metabolism, we studied the effects of compounds containing a free sulfhydryl and/or a free amino group on the binding of [14C]acrolein to the hepatic microsomes. The results of this study are given in Table 2. For comparison, we have included data on the binding of [4-14C]CP in this table. Of the chemicals tested, glutathione was the most potent in blocking the binding of [14C]acrolein and 14C label from [4-14C]CP; the binding of both [14C]acrolein and [4-14C]CP was inhibited by 90%. Cysteine, which was a very potent inhibitor of the binding of 14C label from [4-14C]CP, was equally effective in preventing the binding of [14C]acrolein. N-Acetylcycteine was somewhat less effective than was cysteine in preventing the binding of the radiolabel from both sources. Lysine was only marginally effective in blocking the binding either of [14C]acrolein or of 14C label from [4-14C]CP.

**Mixed Function Oxidase-mediated Activation of Acrolein.** The above studies on the binding of [14C]acrolein to microsomes were done in the absence of NADPH. Therefore, it was of interest to determine whether metabolism-mediated binding of [14C]acrolein to microsomal proteins occurred. Metabolism of [14C]-acrolein was assayed with microsomes isolated from phenobarbital-treated rats, and the results of this study are given in Table 3. While [14C]acrolein per se in the absence of metabolism showed significant binding to the microsomes, this binding was substantially enhanced in the presence of NADPH, suggesting that a metabolite of acrolein was also binding to the microsomes. The enhancement in the binding of [14C]acrolein is clearly metabolism-dependent, since the presence of 1 mm SKF-525A completely abolished the difference in the binding observed between plus and minus NADPH. In addition, compared to phenobarbital-treated microsomes, control microsomes did not generate a substantial amount of the NADPH-mediated metabolite (e.g., 2707 pmol of metabolite/mg of protein vs 23 pmol of metabolite/mg of protein).

**DISCUSSION**

**CP Metabolism by Cytochrome P-450: Kinetics and Inhibition.** Since it was of interest to study whether the different moieties of the CP molecule are metabolized similarly by cytochrome P-450, we studied the metabolism of [chloroethyl-3H]CP and [4-14C]CP in the reconstituted cytochrome P-450 system. Any observable differences between the metabolism of [4-14C]CP and [chloroethyl-3H]CP could possibly be detected by measuring the following: kinetic constants (Km and Vmax), sensitivity to known mixed function oxidase inhibitors, effects of heat denaturation of cytochrome P-450, and/or sensitivity to anti-cytochrome P-450 antibody.

Lineweaver-Burke analysis of the metabolism of [4-14C]CP and [chloroethyl-3H]CP revealed similar Km values for the formation of the labeled polar metabolites. The Michaelis constant (Km) was 0.42 mm and 0.44 mm for [chloroethyl-3H]CP and [4-14C]CP, respectively. These values obtained for the metabolism of the differentially labeled CP by the reconstituted system are very similar to the Km values reported for CP metabolism by microsomes from phenobarbital-treated rats (28). Siadek (28) reported that hepatic microsomes from phenobarbital-treated rats...

### Table 2

<table>
<thead>
<tr>
<th>Treatment (4 mm)</th>
<th>[14C]Acrolein bound as % of control</th>
<th>[4-14C]CP bound as % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glutathione</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Cysteine</td>
<td>18</td>
<td>12</td>
</tr>
<tr>
<td>N-Acetylcycteine</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Lysine</td>
<td>88</td>
<td>105</td>
</tr>
</tbody>
</table>

*Control value for acrolein alone averaged 37 nmol of [14C]acrolein bound/mg of protein.

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NADPH-mediated [14C]acrolein metabolite bound to microsomes (pmol of metabolite bound/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(PBMCs + NADPH) - (PBMCs - NADPH)</td>
<td>2707 (100)</td>
</tr>
<tr>
<td>(PBMCs + NADPH + 1 mm SKF-525A) - (PBMCs - NADPH + 1 mm SKF-525A)</td>
<td>6 (0.2) &lt;8 (&lt;0.1)</td>
</tr>
<tr>
<td>(MCS + NADPH) - (MCS - NADPH)</td>
<td>23 (4.5) &lt;10 (&lt;0.1)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses, percentage of control value.

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rats had a $K_m$ value for CP metabolism of 0.57 mw and that the $K_m$ value for untreated microsomes was 1.39 mw, suggesting, therefore, that phenobarbital pretreatment results in the formation of cytochrome(s) P-450 which exhibit greater affinity for CP substrate.

We studied the effects of inhibitors of mixed function oxidase-mediated reactions on the metabolism of [4-14C]CP and [chloroethyl-3H]CP. Inhibitors with preference toward certain types of induced forms of cytochrome P-450 have become important tools in differentiating between variants of the hemoprotein. SKF-525A and metyrapone preferentially inhibit mixed-function oxidase activities induced by phenobarbital and similar-acting chemicals, whereas 7,8-benzoflavone (α-naphthoflavone) and 5,6-benzoflavone (β-naphthoflavone) are potent inhibitors of 3-methylcholanthrene-inducible mixed function oxidase activities (26, 30).

While all 4 inhibitors tested produced inhibition of the metabolism of [chloroethyl-3H]CP and [4-14C]CP in a concentration-dependent manner, SKF-525A was by far the most potent inhibitor. This observation further strengthens the report that phenobarbital-inducible cytochrome P-450 preferentially metabolizes CP (14), and these results are in accord with the results of Sladek (29) on the microsomal metabolism of CP.

Activation of CP by the Cytochrome P450 Reconstituted System. The data presented in Table 1 clearly demonstrate that binding of the 14C label from [4-14C]CP to cytochrome P-450 protein exceeds that of the 3H label from [chloroethyl-3H]CP, and that the binding of the 3H label to E. coli tRNA exceeds the binding of the 14C label. A similar pattern of binding of the 14C label was observed when [14C]acrolein in the absence of metabolism was used to modify nucleic acids and cytochrome P-450 protein (data not shown). These results suggest that different moieties of the CP molecule bind to proteins and nucleic acids by different mechanisms. As shown in Chart 1, during the metabolism of [chloroethyl-3H]CP, the radioactivity could be retained in phosphoramide mustards which, being a very reactive alkylating agent, would avidly bind to nucleophiles located in nucleic acids (7). On the other hand, the metabolism of [4-14C]-CP would be expected to yield radiolabeled acrolein, a very reactive unsaturated aldehyde which denatures cytochrome P-450 by possibly alkylating critical sulfhydryl groups (16, 18). The treatment of hepatic microsomes with NADPH and mixed function oxidase is capable of further metabolism to a reactive metabolite, presumably an alkene oxide, which can alkylate proteins in microsomes; and (c) acrolein is the metabolite of CP responsible for some of the toxic effects associated with the parent compound.

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REFERENCES

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