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CYTOCHROME P-450-DEPENDENT FORMATION OF ALKYLATING METABOLITES OF THE 2-CHLOROETHYLNITROSOUREAS MeCCNU AND CCNU

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Abstract—Rat liver microsomes catalyzed the biotransformation of the clinically important nitrosourea anticancer agents 1-(2-chloroethyl)-3-(*trans*-4-methyl-cyclohexyl)-1-nitrosourea (MeCCNU) and 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) to alkylating metabolites that bound covalently to microsomal protein and to DNA. The enzyme-mediated microsomal alkylation required NADPH and oxygen and was inhibited by carbon monoxide, indicating the participation of a cytochrome P-450-dependent monooxygenase. Additional studies with inhibitors such as piperonyl butoxide and with the inducers 3-methylcholanthrene and phenobarbital were consistent with this view. In contrast to these observations on the formation of alkylating metabolites, carbamylation reactions were not affected significantly by microsomal metabolism. Reduced glutathione, cysteine or *N*-acetylcysteine decreased the microsomal alkylation by MeCCNU and produced a corresponding increase in the formation of polar metabolites that was resolved by HPLC as three distinct *N*-acetylcysteine–MeCCNU adducts. The addition of semicarbazide to the reaction decreased microsomal alkylation by 30%, indicating that the formation of the alkylating species may proceed via an aldehyde intermediate. Renal microsomes were not found to catalyze the alkylation reaction. Moreover, MeCCNU inhibited the renal slice accumulation of *p*-aminohippuric acid only in the presence of liver microsomes and NADPH, suggesting that a liver metabolite may be responsible for the renal toxicity of the parent nitrosourea.

The nitrosoureas (e.g. BCNU†, CCNU, MeCCNU and chlorozotocin) represent a large and important class of anticancer agents that have widespread application in the treatment of human malignancies. These agents are inherently unstable and can degrade to form reactive isocyanate intermediates that can participate in carbamylation reactions, and alkylating species such as the 2-chloroethyl carbonium ion [1, 2]. The antitumor activity of the chloroethyl nitrosoureas is believed to be due to the alkylation and subsequent cross-linking of nucleophilic sites in DNA by the 2-chloroethyl carbonium ion [3, 4]. In contrast, carbamylation reactions inhibit a variety of metabolic functions [5–7] and may be responsible for some of the host toxicity of the parent nitrosourea. However, several of the more lipophilic nitrosoureas (e.g. MeCCNU and CCNU) are metabolized to a variety of hydroxylated metabolites by cytochrome P-450-dependent enzymes [8, 9]. Formation of the hydroxylated metabolites occurs more rapidly than degradation *in vivo* [10], and it is generally believed that the biological activity of the nitrosoureas is expressed through the subsequent degradation of the hydroxylated metabolites [11].

The nitrosoureas display a range of dose-limiting target tissue toxicities to specific organs including the lungs, kidney, liver or pancreas [12]. These toxic effects seriously limit the utility of this class of anticancer drugs. For example, BCNU treatment is associated with the development of a fatal lung injury [13, 14], whereas renal toxicity has been observed in patients receiving MeCCNU [15]. The mechanism(s) for the different tissue specific toxicities of the nitrosoureas is not understood. However, recent studies have provided evidence which suggests that the renal toxicity of MeCCNU in rats may be related to the formation of a metabolite in the liver which accumulates in the kidney en route to urinary excretion [16]. In the present investigation, we report on the activation of MeCCNU by hepatic microsomes *in vitro*, to form reactive alkylating intermediates that bound irreversibly to microsomal protein and DNA. These studies demonstrate that an alkylating metabolite of the parent nitrosourea may contribute to the toxicity and perhaps antitumor activity of MeCCNU and structurally related nitrosoureas.

MATERIALS AND METHODS

Chemicals and reagents. MeCCNU [1-(2-chloroethyl)-3-(*trans*-4-methyl-cyclohexyl)-1-nitrosourea]; [*cyclohexyl*-1-^{14}C]MeCCNU; [2-*chloroethyl*-1,2-^{14}C]MeCCNU; CCNU [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea]; and [2-*chloroethyl*-1,2-^{14}C]CCNU were obtained from the Developmental Therapeutics Program (NCI, Bethesda, MD) and were purified by preparative thin-layer chromatography. Glutathione (GSH), cysteine, *N*-acetyl-

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† Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(*trans*-4-methyl-cyclohexyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosurea; 3-MC, 3-methylcholanthrene; PB, phenobarbital; GSH, glutathione; Cys, cysteine; NAC, *N*-acetylcysteine; and PAH, *p*-aminohippuric acid.

cysteine (NAC), NADP, NADPH, *p*-aminohippuric acid (PAH), 3-methylcholanthrene (3-MC), semicarbazide and calf thymus DNA were purchased from the Sigma Chemical Co. (St Louis, MO). Phenobarbital (PB) and chromatography solvents were obtained from the Baker Chemical Co. (Phillipsburg, NJ). Piperonyl butoxide was purchased from the Fluka Chemical Corp. (Hauppauge, NY).

Animals and pretreatments. Male Fischer 344 rats (Taconic Farms, Germantown, NY), weighing 150–175 g, were given a standard laboratory diet and water *ad lib.* for a period of 1 week after arrival from the supplier. PB was injected i.p. (80 mg/kg) once daily for 4 days and microsomes were prepared 24 hr after the last dose of PB. 3-MC was dissolved in corn oil and was adminstered i.p. (50 mg/kg) once daily for 2 days. Microsomes were prepared 48 hr after the last dose of 3-MC.

Incubations. Liver and kidney microsomes were prepared by differential centrifugation of tissue homogenates by the method of Borton *et al.* [17]. Protein in the microsome-enriched fraction was estimated by the method of Bradford [18], and the suspension was diluted to a final protein concentration of 25 mg/ml (100 mM KPO_4/5 mM EDTA/0.25 M sucrose; pH 7.4) and stored at −70° until use. Incubations were essentially as described by May *et al.* [9] and contained in a final 1-ml volume: 0.1 M K_2HPO_4 (pH 7.4), 1 mM NADP, 1 mM NADH, 3.5 mM glucose-6-phosphate, 3 units glucose-6-phosphate dehydrogenase, 3.5 mM $MgCl_2$, and 2 mg microsomal protein. When indicated, additions to the reaction (i.e. GSH, cysteine, NAC, semicarbizide) were at 2.5 mM. After a 3-min preincubation at 37°, the reaction was initiated by adding MeCCNU in 10 μl MeOH at a final concentration of 0.25 mM MeCCNU. MeCCNU, labeled either in the chloroethyl [2-*chloro*-1,2-^{14}C]MeCCNU) or cyclohexyl ([*cyclohexyl*-1-^{14}C]MeCCNU) moiety, was at a final specific activity of 1 μCi/μMol. Where indicated, [*chloroethyl*-^{14}C]CCNU was used at the same concentration and specific activity as MeCCNU. Incubations were at 37° for 60 min with shaking. Reactions were terminated by placing on ice and were extracted immediately with 3 × 2 ml hexane, which quantitatively removed parent MeCCNU, followed by 3 × 2 ml extractions with MeOH which removed metabolites and degradation products. The resulting protein precipitate was washed exhaustively with hot MeOH until 1 ml of the MeOH contained less than 100 dpm. An aliquot of the combined hexane or MeOH extracts was counted by liquid scintillation spectrometry (Packard Instrument Co., Downers Grove, IL) to estimate the parent MeCCNU remaining and total product formed respectively. The washed protein pellet was redissolved in 1 ml of 1 N NaOH, an aliquot of the protein digest was assayed for protein, and the remaining digest was assayed for ^{14}C.

DNA binding. The amount of [*chloroethyl*-^{14}C]MeCCNU that bound to DNA was determined essentially as described for protein binding with the following modifications. Calf thymus DNA (1 mg) was added to the reaction mixture and the reaction was terminated by extraction with 2 vol. of CIP (chloroform:isoamyl alcohol:phenol, 24:1:25). The resulting emulsion was centrifuged at 2000 *g* for 10 min. The aqueous phase was reextracted with CIP and made 0.1 M with respect to NaCl. DNA was precipitated with 2 vol. of EtOH at −70°. The DNA was redissolved in 1 ml of 15 mM citrate/150 mM NaCl, pH 7.0, and was reprecipitated with EtOH. This process of redissolving and reprecipitating the DNA was repeated until 1 ml of the EtOH supernatant fraction contained less than 100 dpm. The DNA was then redissolved in 1 ml of 0.5 N perchloric acid, and aliquots were assayed for DNA content [19] and radioactivity.

Isolation of NAC derivatives. Incubations were for 60 min and were identical to those previously described with the exception that reactions were in a 5-ml volume and contained 2.5 mM NAC. The combined MeOH extracts were centrifuged at 10,000 *g* for 30 min and were filtered with a 0.5 μm filter (Millex-SR; Millipore Corp., Bedford, MA). Carboxymethyl esters of the isolated NAC adducts were formed from methanol using thionyl chloride as a catalyst [20]. The resulting carboxymethyl–NAC adducts were separated by reverse phase HPLC using a 5 μm ODS-3 Partisil column. The metabolites were eluted at 1.5 ml/min with a solvent system containing acetonitrile:water:glacial acetic acid (30:70:0.1) for 20 min, followed by a 15-min linear gradient to 100% acetonitrile containing 0.1% glacial acetic acid. Fractions were collected directly into scintillation vials at 1-min intervals.

Effect of MeCCNU metabolites on renal slice p-aminohippuric acid uptake. Renal cortical slices (100 mg) were prepared from F344 rats and were added to 5 ml of a microsome incubation containing MeCCNU (0.5 mM) and an NADPH-generating system as previously described. After a 1-hr incubation, renal slices were removed, blotted dry, and transferred to fresh vials for measuring organic anion accumulation (*p*-aminohippuric acid; PAH) as described previously [21]. Renal slice uptake was expressed as the slice-to-medium ratio (S/M).

Statistics. Statistical significance was calculated by the multiple *t* statistics of Dunnett [22].

RESULTS

Effects of inducers of drug metabolism on the covalent binding of ^{14}C-labeled MeCCNU. The covalent binding of [*chloroethyl*-^{14}C]- and [*cyclohexyl*-^{14}C]MeCCNU was used, respectively, to estimate the formation of alkylating and carbamylating intermediates of MeCCNU (Fig. 1). Labeled MeCCNU was incubated with rat liver microsomes obtained from control rats or from rats pretreated with either 3-MC or PB to induce the cytochrome P-450 monooxygenase system (Fig. 1). In the absence of an NADPH-generating system, carbamylating species accounted for >98% of the total reactive intermediates that bound to microsomal protein. Neither the addition of the NADPH-generating system or 3-MC pretreatment had an effect on the carbamylation reaction. However, in the presence of NADPH, PB-induced microsomes catalyzed a 25% decrease in protein carbamylation. In contrast to these studies on the formation of carbamylating intermediate(s), the alkylation of microsomal protein by MeCCNU

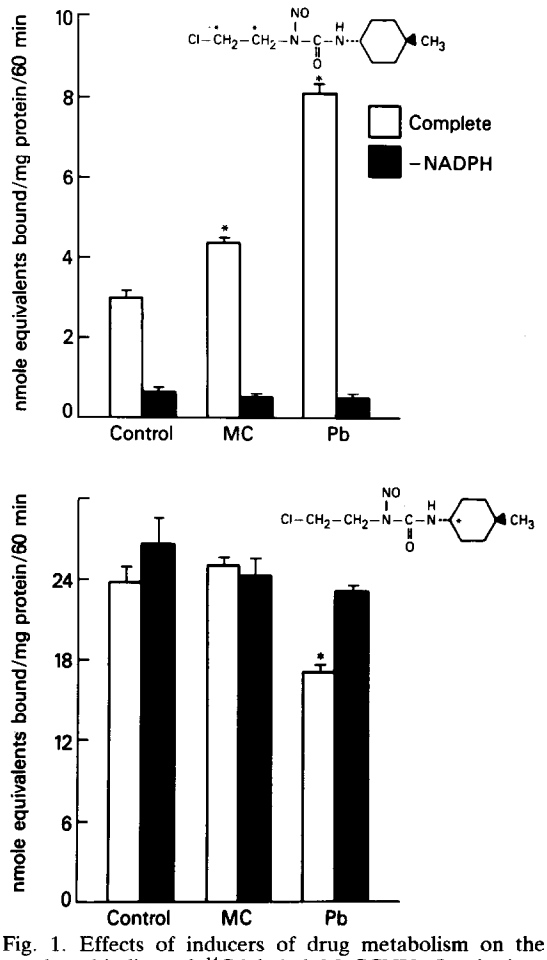

Fig. 1. Effects of inducers of drug metabolism on the covalent binding of ^{14}C-labeled MeCCNU. Incubations contained microsomes (2 mg/ml) from control, 3-MC or PB-pretreated rats; 250 μM MeCCNU; and, when indicated, an NADPH-generating system as described in Materials and Methods. Covalent binding by [*chloroethyl*-^{14}C]- or [*cyclohexyl*-^{14}C]MeCCNU (final specific activity, 1 μCi/μmole) was used, respectively, to assess the formation of the alkylating (top panel) and carbamylating (bottom panel) intermediates. Values are the means ± SE of triplicate determinations. Key: * significant difference, $P < 0.05$.

was found to be NADPH dependent. The addition of NADPH to the incubation resulted in a 6-fold increase in the formation of an alkylating intermediate that covalently bound to microsomal protein. The formation of this alkylating intermediate was increased an additional 1.5- and 3-fold by utilizing microsomes obtained from animals pretreated with 3-MC or PB respectively. In the presence of NADPH and PB-induced microsomes, the alkylating species accounted for 31% of the total reactive intermediates that bound to microsomal protein. This compares with the >2% that bound in the absence of NADPH.

CCNU, an analog of MeCCNU, also was metabolized to a reactive alkylating intermediate by liver microsomes (Table 1). Covalent binding of microsomal protein by [*chloroethyl*-^{14}C]CCNU was increased 8-fold by the addition of an NADPH-generating system to PB-induced microsomes.

Characterization of hepatic microsomal enzyme activities mediating the covalent binding of [chloroethyl-^{14}C]*MeCCNU.* Covalent binding by the reactive alkylating intermediate was dependent upon NADPH and was linear with respect to time (data not shown). The formation of this alkylating intermediate also required functional enzyme activity as boiled microsomes bound significantly less MeCCNU. Moreover, a carbon monoxide enriched, or a nitrogen atmosphere inhibited covalent binding as did the addition of the cytochrome P-450 inhibitor, piperonyl butoxide, to the reaction mixture (Fig. 2). Renal microsomes were not found to catalyze the formation of this alkylating MeCCNU metabolite. These findings, taken collectively, demonstrate a role for hepatic cytochrome P-450 in the formation of an alkylating metabolite of MeCCNU.

The kinetics of covalent binding of the alkylating metabolite of MeCCNU was examined in microsomes obtained from control and PB-pretreated animals (data not shown). Covalent binding of [*chloroethyl*-^{14}C]MeCCNU followed Michaelis–Menten kinetics and was linear over a range of MeCCNU concentrations (10–250 μM). The Michaelis constant (K_m) for binding of the alkylating metabolite was determined by the method of Lineweaver and Burk [23] and was 49 and 85 μM, respectively, for control and PB-induced liver microsomes. The V_{max} value for this reaction was 158 pmol bound/min/mg microsomal protein, and was increased 2-fold by PB treatment.

Effect of additions on the cytochrome P-450-dependent metabolism and covalent binding of [chloroethyl-^{14}C]*MeCCNU.* The addition of NADPH to the microsomal incubation not only increased the formation of bound alkylating metabolite(s) but also increased total MeCCNU and CCNU metabolism as indicated by a decrease in the amount of parent nitrosourea remaining after the 60-min incubation (data not shown), and to the increased formation of methanol-extractable radioactivity (Table 1). Methanol-extractable radioactivity, following the hexane extraction step to remove the parent nitrosourea, was used to estimate the formation of polar degradation products and metabolites of MeCCNU and CCNU. PB-induced microsomes did not affect the total metabolism of [*chloroethyl*-^{14}C]MeCCNU, but selectively increased the formation of the alkylating metabolite(s). The addition of the thiol compounds GSH, cysteine or *N*-acetylcysteine (NAC) markedly reduced protein alkylation in both control and PB-induced microsomes and produced a corresponding increase in the formation of polar methanol-extractable metabolites. Cysteine was less effective than either GSH or NAC at inhibiting the alkylation of PB-induced microsomal protein, presumably due to the rapid rate of auto-oxidation of this thiol. Semicarbazide inhibited protein alkylation by approximately 30%, demonstrating that a fraction of the alkylating metabolites may proceed via an aldehyde intermediate.

Hepatic microsomes also were found to catalyze a 3.5-fold increase in the alkylation of MeCCNU to calf thymus DNA (Table 1). The addition of DNA

Table 1. Effect of additions on the microsome-mediated metabolism and covalent binding of [*chloroethyl*-^{14}C]MeCCNU and [*chloroethyl*-^{14}C]CCNU*

Additions	Source of microsomes	(nmol [*chloroethyl*-^{14}C]Metabolites formed/60 min) MeOH extractable	nmol bound per mg protein	nmol bound per mg DNA
		[*chloroethyl*-^{14}C]MeCCNU		
None	Control	75 ± 8	0.6 ± 0.01	0.2 ± 0.04
NADPH	Control	178 ± 12	3.2 ± 0.03†	0.7 ± 0.02†
NADPH + GSH†	Control	198 ± 12†	1.7 ± 0.10†§	0.5 ± 0.10†§
NADPH + Cys	Control	202 ± 3†§	1.9 ± 0.03†§	0.4 ± 0.01†§
NADPH + NAC	Control	205 ± 4†§	1.9 ± 0.10†§	ND
NADPH + SCZ	Control	ND	2.4 ± 0.05†§	ND
None	Pb	75 ± 3	0.6 ± 0.03	ND
NADPH	Pb	161 ± 7†	11.0 ± 0.40†‖	ND
NADPH + GSH	Pb	177 ± 6†§	2.2 ± 0.20†§‖	ND
NADPH + Cys	Pb	182 ± 18†	6.0 ± 0.20†§‖	ND
NADPH + NAC	Pb	200 ± 6†§	3.4 ± 0.20†§‖	ND
		[*chloroethyl*-^{14}C]CCNU		
None	Pb	52 ± 1	0.8 ± 0.10	ND
NADPH	Pb	240 ± 10†	6.5 ± 0.50†	ND

* Methods for the preparation of microsomes, incubation systems and NADPH-generating system are described in Materials and Methods. All values are the means ± SE of three determinations. ND = not determined.

† Significant difference compared to no NADPH, $P < 0.05$.

‡ Additions of the thiols (GSH, glutathione; Cys, cysteine; NAC, *N*-acetylcysteine) and semicarbazide (SCZ) were at a final concentration of 2.5 mM.

§ Significant difference compared to the reaction run with an identical source of microsomes and an NADPH-generating system, $P < 0.05$.

‖ Significant difference compared to reaction run with NADPH-generating system and with microsomes obtained from non-phenobarbital-treated animals, $P < 0.05$.

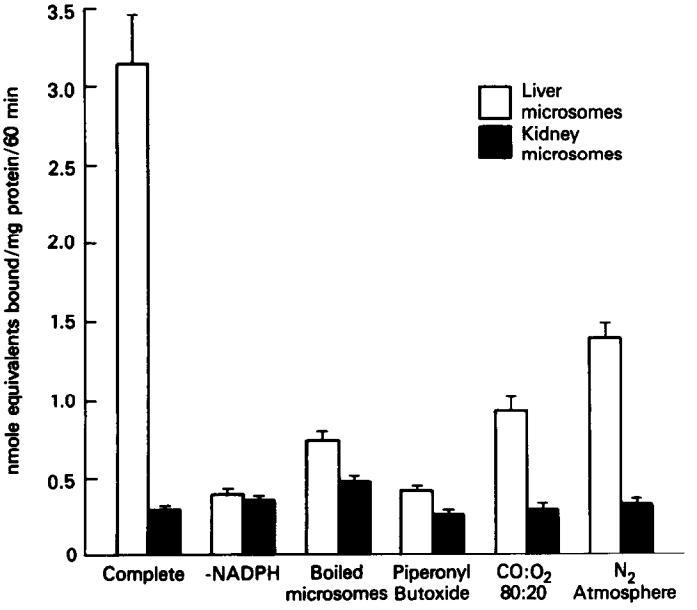

Fig. 2. Characterization of the liver and kidney microsomal enzymes which catalyze the formation of alkylating MeCCNU metabolites. All incubations were conducted with control microsomes and [*chloroethyl*-^{14}C]MeCCNU as described in the legend to Fig. 1 and in Materials and Methods. Values are the means ± SE of triplicate determinations.

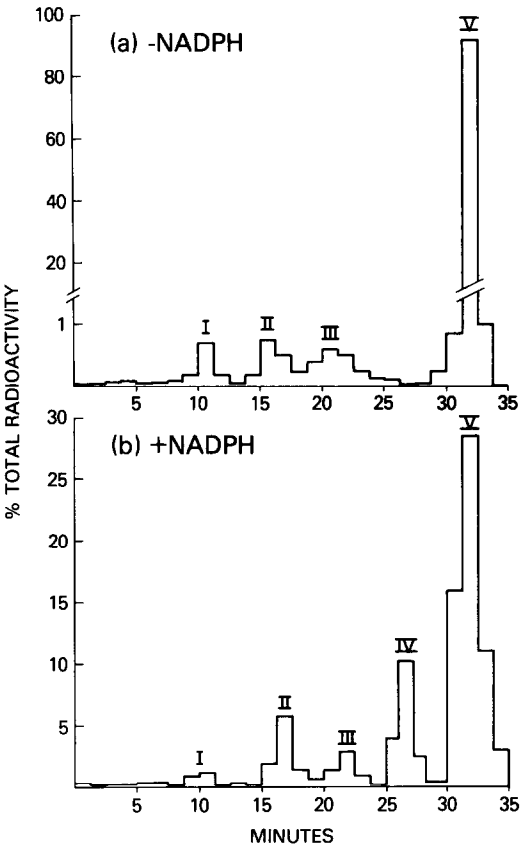

Fig. 3. Liquid chromatography of *N*-acetylcysteine-trapped [*chloroethyl*-^{14}C]MeCCNU metabolites isolated from phenobarbital-induced rat liver microsomes in the absence (a) or presence (b) of an NADPH-generating system. See Materials and Methods for details.

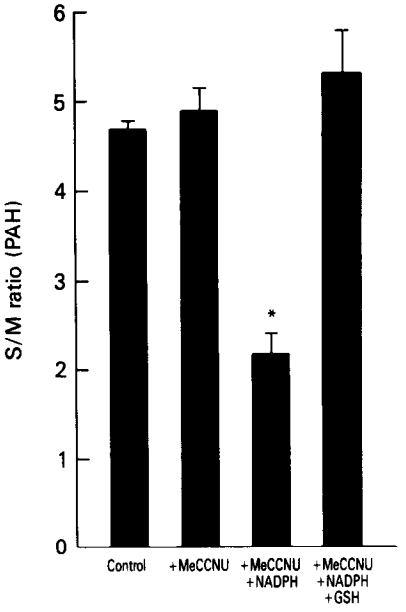

Fig. 4. Effect of liver microsomal metabolism and GSH on the toxicity of MeCCNU to rat kidney slices. *p*-Aminohippuric acid (PAH) accumulation was determined in renal slices (S/M; slice-to-medium ratio) following a 60-min preincubation with phenobarbital-induced liver microsomes, MeCCNU (0.5 mM) and when indicated, an NADPH-generating system and GSH. Values are the means ± SE of quadruplicate determinations. Key: * significant difference, $P < 0.05$.

to the reaction mixture resulted in a decrease in the amount of MeCCNU that bound to microsomal protein (data not shown). The decrease in protein binding resulting from the addition of the DNA was equivalent to the amount that bound to the DNA, suggesting that an identical alkylating species was responsible for both protein and DNA binding. The alkylation of DNA also was inhibited by thiol compounds (i.e. GSH and cysteine); however, GSH was more effective at inhibiting protein alkylation.

Isolation of NAC–MeCCNU adducts. The ability of added thiol compounds to reduce the binding of alkylating MeCCNU metabolites to macromolecules, and to produce a corresponding increase in the formation of polar metabolites, was indicative of the formation of thiol–metabolite adducts. In the following study, the alkylating metabolite(s) was trapped by the addition of NAC to a reaction mixture containing PB-induced microsomes in the presence or absence of NADPH. The NAC–metabolite adducts thus formed were isolated by HPLC following hexane extraction and carboxymethyl esterification. Five radioactive peaks, with retention times of 11, 16.5, 22, 25.5 and 31 min were identified by this method (Fig. 3). Peak I accounted for only 2% of the total radioactivity and was formed in equal amounts either in the presence or the absence of NADPH. Peak II (2.3% of total radioactivity) also was formed in the absence of NADPH; however, microsomal metabolism resulted in a 5-fold elevation in the formation of this product. Peak III (2.1% of total radioactivity) was increased 2.5-fold by microsomal metabolism. Peak IV was found only when NADPH was added to the incubation and accounted for 17% of the total radioactivity. Peak V probably does not represent a single metabolite as this material eluted only at the end of the gradient (i.e. 100% acetonitrile). However, peak V did not include parent MeCCNU as this was removed by hexane extraction. Peak V accounted for 94% of the total radioactivity in the absence of NADPH, and microsomal metabolism decreased the radioactivity present in this peak by 42%. In the absence of NAC, radioactivity was only found in peak V.

Effect of glutathione and hepatic microsomal metabolism of MeCCNU on the uptake of p*-aminohippuric acid by kidney slices.* To assess the potential renal toxicity of the alkylating MeCCNU metabolite(s), kidney slices were coincubated with PB-induced hepatic microsomes and MeCCNU in the presence or absence of NADPH or GSH. After a 1-hr coincubation with microsomes, the kidney slices were removed and transferred to fresh medium for the measurement of renal anion accumulation utilizing PAH as a substrate (Fig. 4). MeCCNU had no effect on renal anion uptake when NADPH was not included in the microsomal coincubation

medium; however, in the presence of NADPH, MeCCNU inhibited PAH uptake by 50%. The addition of GSH to the preincubation medium completely protected against this decrease in PAH uptake. Preincubation of renal slices with microsomes, NADPH and GSH, but without MeCCNU, had no effect on PAH accumulation (data not shown).

DISCUSSION

The *in vitro* studies described here indicate that reactive alkylating metabolites of MeCCNU and CCNU were formed by rat liver microsomes (Fig. 1 and Table 1). Based on the requirement for NADPH and oxygen, inhibition by carbon monoxide or piperonyl butoxide, and induction by phenobarbital pretreatment (Fig. 2), the reaction was cytochrome P-450 dependent. The metabolism of MeCCNU yielded at least three alkylating species that formed adducts with NAC and were separated by HPLC (Fig. 3). Two of these adducts were formed independent of an active cytochrome P-450 system, but microsomal metabolism accelerated the formation of these conjugates by 3- to 5-fold. A third adduct was formed only in the presence of an intact cytochrome P-450 system and accounted for 17% of the total MeCCNU metabolites formed by PB-induced microsomes. These alkylating metabolites bound covalently to microsomal protein and to DNA (Table 1) and were toxic to rat kidney slices *in vitro* (Fig. 4). The renal toxicity of MeCCNU *in vitro* (Fig. 4) and the reaction with protein and DNA were reduced markedly by the addition of nucleophiles such as glutathione or NAC to the incubation (Table 1). In contrast to the formation of alkylating metabolites, carbamylation reactions were not shown to be affected significantly by microsomal metabolism (Fig. 1).

The present findings are entirely consistent with recent studies on the role of hepatic metabolism on the disposition and renal toxicity of MeCCNU in F344 rats [16]. In these studies, Kramer *et al.* demonstrated that PB pretreatment caused a marked increase in the urinary excretion and covalent binding of [*chloroethyl*-^{14}C]MeCCNU to renal and hepatic macromolecules and produced a corresponding increase in the renal injury caused by MECCNU. Conversely, pretreatment with piperonyl butoxide resulted in a marked decrease in the amount of reactive alkylating intermediates that bound irreversibly to renal macromolecules and produced a decrease in the MeCCNU-induced renal lesion. An early index of the MeCCNU-induced renal lesion was a decrease in the accumulation of PAH by kidney slices *in vitro* following the administration of MeCCNU *in vivo* [21]. In the present investigation, it was shown that MeCCNU produced a decrease in kidney slice uptake of PAH only in the presence of liver microsomes and an NADPH-generating system (Fig. 4). Moreover, kidney microsomes did not catalyze the formation of alkyklating MeCCNU metabolites (Fig. 2). These studies lend support to the conclusion that the MeCCNU-induced renal lesion was due to a reactive alkylating metabolite that was formed in the liver and accumulated in the kidney en route to urinary excretion [16].

The results presented here and in the aforementioned *in vivo* study by Kramer *et al.* [16] are in contrast to several reports that have demonstrated that PB pretreatment markedly reduces both the antitmour activity and acute lethality of several nitrosoureas, including MeCCNU [24–26]. Our results are not inconsistent with these studies if one regards the PB-dependent decrease in antitumour activity and toxicity as being due to the increased clearance of the alkylating species rather than a decrease in the formation of carbamylating intermediates, as was previously suggested. The renal lesion produced by MeCCNU treatment is delayed in onset and does not contribute to the acute lethality of MeCCNU [21]. Thus, the increased renal clearance of the alkylating species resulting from PB treatment correlated with increased renal injury and also may have had a sparing effect on other organs by decreasing exposure of these tissues to the reactive species.

The ability of GSH to reduce the covalent binding of MeCCNU *in vitro* (Table 1), and to protect against the *in vitro* renal toxicity produced by a metabolite of MeCCNU (Fig. 4), is consistent with *in vivo* studies demonstrating a role for GSH in the detoxification of MeCCNU. These previous studies demonstrated that MeCCNU preferentially decreased GSH concentrations in the liver and that pretreatment with an inhibitor of microsomal drug metabolism (i.e. piperonyl butoxide) protected against the MeCCNU-induced renal injury and decrease in hepatic GSH [16]. In contrast, pretreatment of animals with an inhibitor of GSH biosynthesis (i.e. buthionine sulfoximine) increased both the renal and hepatic toxicity of MeCCNU [27].

The capacity of hepatic microsomes to catalyze the formation of reactive alkylating metabolites of either MeCCNU or CCNU may explain the decrease in cytochrome P-450-dependent enzyme activities observed in liver microsomes obtained from rats treated with CCNU [28, 29]. The present studies suggest that a reactive metabolite of the nitrosoureas may selectively alkylate the cytochrome P-450 molecule that catalyzed its formation.

In this investigation we demonstrate that microsomal oxidation can yield reactive and possibly toxic alkylating species of the 2-chloroethylnitrosoureas. However, neither the metabolic route nor the chemical structure for these metabolites is known. Several pathways can be considered for bioactivation including the formation of unstable gem-chlorohydrin, carbonyl or chloroso intermediates. For example, a gem-chlorohydrin intermediate can be formed by oxygen insertion into the C—H bond of the beta-carbon of the chloroethyl side chain. This unstable gem-halohydrin intermediate can spontaneously decompose to form the reactive alkylating species 2-chloroacetaldehyde [30], and is similar to the bioactivation pathway described for 1,2-dichloroethane [31]. Data which support the involvement of this pathway in the bioactivation of the nitrosoureas were provided by the ability of the aldehyde trapping agent, semicarbazide, to reduce the microsome catalyzed covalent binding of MeCCNU (Table 1). However, it is unlikely that this pathway can fully account for the formation of reactive metabolites of the

nitrosoureas because semicarbazide only reduced covalent binding by 30%. The remainder of the reactive metabolites may be attributed to the formation of carbonyl or chloroso intermediates which are extremely reactive and would be difficult to detect. Moreover, further attempts to elucidate the metabolic route for activation are exacerbated by the fact that 2-chloroacetaldehyde, carbonyl or chloroso intermediates could combine with glutathione to ultimately give rise to similar urinary metabolites (i.e. thiodiacetic acid and *S*-carboxymethyl-NAC). These metabolites have been identified as major excretion products of CCNU [32] and provide further support for the biological relevance of the microsome-catalyzed bioactivation pathway described here.

Other possible bioactivation pathways for the nitrosoureas are less probable. For example, a relatively nonreactive degradation product of the nitrosoureas, 2-chloroethanol [8], can be oxidized to form 2-chloroacetaldehyde. However, this reaction was not shown to be catalyzed by the microsomal mixed-function oxidase system but, instead, by alcohol dehydrogenase [31]. Thus, microsome-catalyzed alpha-hydroxylation of the chloroethyl side chain [8] would most likely comprise a detoxification pathway by increasing the formation of 2-chloroethanol. Future studies will focus on identifying the alkylating species formed by the microsomal metabolism of the nitrosoureas, and in determining its possible role in contributing to the antitumor activity and toxicity of this important class of anticancer agent.

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