

Microsomal Metabolism of Nitrosoureas¹

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SUMMARY

N,N'-Bis(2-chloroethyl)-*N*-nitrosourea (BCNU) is a substrate for a microsomal enzyme of mouse liver. The reaction requires NADPH, and the product is 1,3-bis(2-chloroethyl)urea. This activity is also found in mouse lungs but not in several other tissues. With reaction conditions under which BCNU is not chemically degraded, the K_m for BCNU with liver microsomes is 1.7 mM; nicotine is a competitive inhibitor with a K_i of 0.6 mM. *N*-Methyl-*N*-nitrosourea is denitrosated in a similar reaction.

N-(2-Chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea and *N*-(2-chloroethyl)-*N'*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea are also substrates for microsomal enzymes, but the products of these reactions are ring-hydroxylated derivatives. The K_m value for *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea is 3.0 mM and that for *N*-(2-chloroethyl)-*N'*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea is 1.0 mM. The hydroxylase activity is also present in lungs, but not in the other mouse tissues.

The rates of microsomal metabolism of BCNU, *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea, and *N*-(2-chloroethyl)-*N'*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea are fast enough to allow metabolism of large portions of administered doses before chemical decomposition of the drugs occurs.

INTRODUCTION

Several nitrosoureas have remarkable activity against mouse leukemia L1210 (22); 3 of these, BCNU,² CCNU, and MeCCNU, have been used in the treatment of clinical cancer (4, 9, 30). The mechanism of action for these nitrosoureas is not known with certainty, but there is evidence that they decompose chemically to yield reactive intermediates (19). From the part of the molecules containing the nitroso group, vinyl carbonium ions (19) and perhaps chloroethyl carbonium ions (15) are formed. The other parts of the molecules give rise to isocyanates, which have carbamoylating activity (1, 2, 19). The 2-chloroethylisocya-

nate formed from BCNU can also give rise to 2-chloroethylamine, an alkylating agent (29). MNU has activity against experimental tumors (13) and also has carcinogenic (6) and mutagenic (7) effects. This reactive compound decomposes to give a methylcarbonium ion and isocyanic acid (19).

Although the pharmacological distribution and urinary excretion of nitrosoureas and their products have been studied (5, 16, 17, 20, 23, 24, 26, 28) and evidence for their biotransformation has been presented (20), only 1 report of their enzymatic metabolism has appeared. May *et al.* (18) have found that CCNU is enzymatically hydroxylated on the cyclohexyl ring by an enzyme of rat liver microsomes.

This report concerns the characterization from mouse liver of microsomal enzymes that metabolize BCNU, CCNU, MeCCNU, and MNU.

MATERIALS AND METHODS

[¹⁴C]BCNU, labeled in the carbonyl group and, separately, in the chloroethyl carbons and [¹⁴C]CCNU and [¹⁴C]MeCCNU labeled in the cyclohexyl ring were obtained from Dr. Robert Engle, Drug Development Branch, National Cancer Institute, Bethesda, Md. The preparations of [¹⁴C]BCNU were further purified by thin-layer chromatography, and the radioactivities of both samples were diluted to 5.2 μ Ci/mg. CCNU (52.3 μ Ci/mg) and MeCCNU (22.3 μ Ci/mg) contained no detectable radioactive impurities and were used without dilution. [*methyl*-¹⁴C]Nicotine was purchased from the Amersham/Searle Corporation, Arlington Heights, Ill. [³H]MNU labeled in the methyl group and [5-¹⁴C] cyclophosphamide were obtained from New England Nuclear, Boston, Mass. Neither contained detectable impurities.

Microsomal oxidations of [¹⁴C]cyclophosphamide and [¹⁴C]nicotine were accomplished as reported previously (12). The microsomal oxidation of [¹⁴C]BCNU was accomplished similarly with washed microsomes from livers of female DBA/2 mice, 2 months old and weighing 18 to 23 g. The livers of 3 or more mice were pooled for each experiment. The standard reaction mixture contained the following: [¹⁴C]BCNU, 110 nmoles; NADPH, 400 nmoles; microsomes equivalent to 40 mg of liver; Tris-chloride buffer (pH 7.5 at 37°), 15 μ moles; and water in a total volume of 175 μ l. Incubation of the preparation was for 6 min at 37°. The reactions were stopped by adding 50 μ l of ethanol and streaking 50- μ l portions onto paper strips. Substrate and metabolite were separated by paper chromatography with isopropyl alcohol:15 N ammonium hydrox-

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² The abbreviations used are: BCNU, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea (carmustine); CCNU, *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea; MeCCNU, *N*-(2-chloroethyl)-*N'*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea; MNU, *N*-methyl-*N*-nitrosourea; LD₁₀, lethal dose for 10%.

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ide:water (80:5:15, by volume). The strips were scanned with a radiochromatogram scanner, and the amount of product was calculated. Assays for CCNU and MeCCNU oxidation were similar, except that the microsomes added were equivalent to 15 mg of liver; only 50 nmoles of substrate were added; and the time of incubation was 10 min. For MNU oxidation the reaction conditions were also similar, except that the buffer was phosphate at pH 6.6; microsomes equivalent to 20 mg of liver were present; 80 nmoles of substrate were added; and the time of incubation was 10 min. A solvent of 1-butanol:ethanol:water (4:1:1, by volume) was used to separate substrate and product in this reaction. To determine the tissue distribution of enzymes standard assay conditions were used, except that 15 mg of homogenized tissue replaced microsomes in each assay.

Nitrosoureas were dissolved in ethanol and added to the reaction mixtures so that the final concentration of ethanol was 1.2%, an amount that had no detectable effect on the reactions. All reactions were performed in duplicate and were linear with time over the period of incubation. The pH, amounts of enzyme, and amounts of NADPH were optimal (data not presented). Under the conditions used, neither BCNU, CCNU, nor MeCCNU were chemically degraded to a detectable degree; some loss of radioactivity from MNU was observed. This loss, probably due to formation of methanol from MNU, prevented precise quantitation of the reaction rate.

RESULTS

An indication that BCNU was a substrate for a microsomal enzyme was obtained from experiments in which it competitively inhibited the microsomal oxidation of nicotine to 5'-hydroxynicotine and of cyclophosphamide to aldophosphamide (Chart 1). The K_i for BCNU in the nicotine reaction was 0.15 mM, compared to a K_m of 1.7 mM. For the oxidation of cyclophosphamide, the K_i was 0.10 mM, compared to a K_m of 0.5 mM. Another micro-

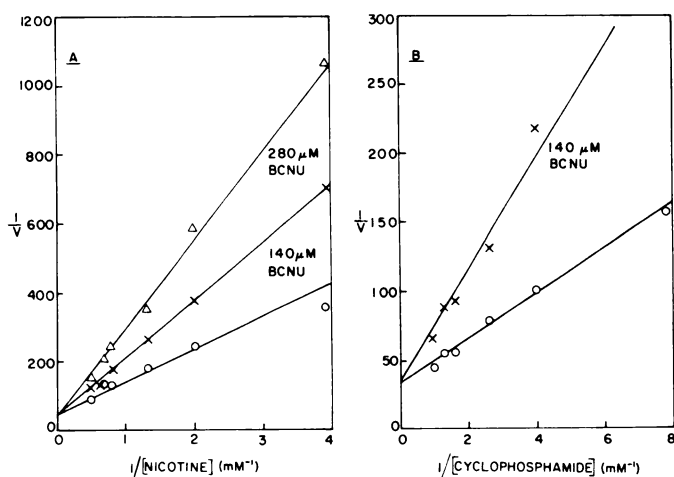


Chart 1. Inhibition by BCNU of nicotine oxidation to 5'-hydroxynicotine (A) and of cyclophosphamide to aldophosphamide (B). Values for the ordinate are reciprocals of μ moles of product formed.

somal, oxidative reaction for nicotine, the production of nicotine 1'-oxide (12), was not inhibited by BCNU.

In the same type of assay system, [14 C]BCNU labeled either in the carbonyl or chloroethyl groups was converted to a product with an R_F of 0.90, compared to an R_F for BCNU of 0.80. The formation of the product was dependent on the presence of microsomes and NADPH; neither NAD, NADH, nor NADP could be substituted (Table 1). Magnesium ions were not required and did not stimulate the reaction. When the reaction system was performed at 0.5 mm of pressure or in an atmosphere of nitrogen, product formation was reduced by 55%, indicating a possible requirement for oxygen. The reaction was inhibited moderately by carbon monoxide. In the presence of a mixture of 85% N_2 :5% O_2 :10% CO, the rate was 76% of that in 95% N_2 :5% O_2 . The value is an average of separate determinations of 77 and 74%. The K_m for BCNU in this reaction was 1.7 mM, an average of separate determinations of 1.4 and 2.0 mM. Nicotine competitively inhibited the reaction with a K_i of 0.6 mM (Chart 2), but cyclophosphamide did not inhibit more than 25% at a concentration of 25 mM. The reaction was not inhibited more than 20% by saturating amounts of 2-diethylaminoethyl 2,2-diphenylvalerate.

Expressed on the basis of the amount of protein present in the assay, homogenates of mouse lung tissue had 30% of the activity of liver homogenates, but there was no detectable activity (less than 5% of that in liver) in homogenates of mouse kidney, spleen, brain, muscle, or intestine, or in mouse serum.

For determination of the nature of the BCNU metabolite, a reaction mixture scaled up 100-fold was incubated for 10 min and extracted with chloroform (3×25 ml). The extract was dried over Na_2SO_4 , filtered, and evaporated to dryness. The residue was separated on a silica gel thin-layer plate with a solvent of chloroform. The plate was allowed to dry and was developed again with a solvent of chloroform:acetone (1:3, by volume). Two radioactive bands were observed, and BCNU was the faster migrating. The other band was collected and eluted with acetone. On thin-layer chromatography the product was identical to authentic N,N' -bis(2-chloroethyl)urea in solvents of chloroform (R_F 0.15) and acetone:chloroform (1:3, by volume) (R_F 0.65); on paper chromatography, it had an identical R_F of 0.90. Mass spectral analysis of the metabolite showed a molecular ion of 184 and the definitive fragments listed in Table 2.

Table 1
Effect of varying reaction components on the enzymatic denitrosation of BCNU

Components of reaction system	Product formed (nmoles)
Complete	22.3
- Microsomes	< 1.0
- NADPH	1.1
- NADPH, + NAD	2.0
- NADPH, + NADH	1.0
- NADPH, + NADP	< 1.0
Complete (stopped at 0 Time)	< 1.0
Complete + $MgCl_2$ (1 mM)	22.6

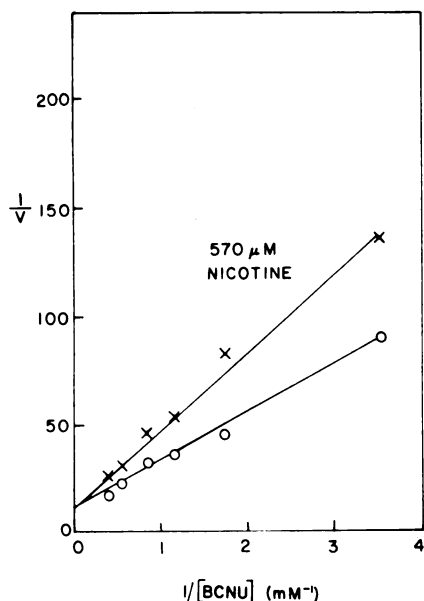


Chart 2. Inhibition of BCNU denitrosation by nicotine. Values for the ordinate are reciprocals of μ moles of product formed.

Identical fragmentation was observed in the mass spectrum of an authentic sample of *N,N'*-bis(2-chloroethyl)urea.

MNU was also a substrate for a NADPH-requiring enzyme of mouse liver. The product of this enzymatic reaction had R_F value of 0.55 in the paper chromatographic system using 1-butanol:ethanol:water as a solvent. MNU had a R_F of 0.93. In this solvent, *N*-methylurea also had a R_F value of 0.55; in an additional paper chromatographic system and in 3 thin-layer chromatographic systems, authentic *N*-methylurea had R_F values identical to those for the enzymatic product. Due to the chemical instability of the substrate, no kinetic values were obtained for this reaction.

In reaction systems similar to those used for denitrosation of BCNU, CCNU and MeCCNU were converted to products that migrated more slowly on paper chromatography than did the parent compounds and that were distinct from authentic denitrosated compounds. The metabolite of CCNU had a R_F of 0.74 compared to a R_F of 0.88 for CCNU; the MeCCNU metabolite had a R_F of 0.80, compared to a R_F of 0.94 for MeCCNU. In these reactions, CCNU had a K_m of 3.0 mM and MeCCNU had a K_m of 1.0 mM

Table 2
Mass spectral analysis of CCNU and MeCCNU metabolites

Source	<i>m/e</i>	No. of Cl's	Relative abundance	Structure assignment
BCNU	184	2	8	$[\text{ClCH}_2\text{CH}_2\text{NHCONHCH}_2\text{CH}_2\text{Cl}]^+$
	149	1	11	$[\text{CH}_2\text{CH}_2\text{NHCONHCH}_2\text{CH}_2\text{Cl}]^+$
	135	1	21	$[\text{CH}_2\text{NHCONHCH}_2\text{CH}_2\text{Cl}]^+$
	106	1	10	$[\text{CONHCH}_2\text{CH}_2\text{Cl}]^+$
	78	1	22	$[\text{HNCH}_2\text{CH}_2\text{Cl}]^+$
	73	0	88	$[\text{H}_2\text{NCONHCH}_2]^+$
	63	1	100	$[\text{ClCH}_2\text{CH}_2]^+$
	49	1	90	$[\text{ClCH}_2]^+$
CCNU	142	0	15	$[\text{HO}-\text{C}_6\text{H}_{10}-\text{NHCO}]^+$
	124	0	12	$[\text{C}_6\text{H}_9-\text{NHCO}]^+$
	99	0	10	$[\text{HO}-\text{C}_6\text{H}_9]^+$
	81	0	100	$[\text{C}_6\text{H}_8]^+$
MeCCNU	156	0	9	$[\text{HO}-\text{C}_6\text{H}_9(\text{CH}_3)-\text{NHCO}]^+$
	138	0	19	$[\text{CH}_3-\text{C}_6\text{H}_8-\text{NHCO}]^+$
	113	0	11	$[\text{HO}-\text{C}_6\text{H}_8(\text{CH}_3)]^+$
	95	0	100	$[\text{CH}_3-\text{C}_6\text{H}_7]^+$

(Chart 3). Expressed on the basis of the amount of protein present in the assay, homogenates of mouse lung tissue had 50% of the CCNU and MeCCNU oxidase activity of liver homogenates; but, under the assay conditions used, there was no detectable activity (less than 5% of that in liver) in kidney, spleen, brain, muscle, intestine, serum, or leukemia L1210 cells.

A partial characterization of the microsomal metabolites of CCNU and MeCCNU was achieved by mass spectral analysis. Scaled-up reaction mixtures were incubated for 10 min and were extracted with heptane (3×10 ml) followed by chloroform (3×10 ml). The chloroform extracts were dried and evaporated as for the BCNU metabolite. Thin-layer chromatographic analysis of the 2 residues on silica gel in acetone:chloroform (1:9, by volume) revealed products with a R_f of 0.30 from CCNU and a R_f of 0.35 from MeCCNU. Mass spectral analysis of the CCNU and MeCCNU metabolites revealed no molecular ions, only the fragment peaks listed in Table 2. The mass spectrum of the enzymatic product of CCNU is in agreement with that reported by May *et al.* (18). Since these investigators compared their CCNU metabolite with authentic *N*-(2-chloroethyl)-*N'*-(4-hydroxycyclohexyl)-*N*-nitrosourea, the identity of this product, except for whether the hydroxyl group is *cis* or *trans*, is established. By analogy, the MeCCNU product may be *N*-(2-chloroethyl)-*N'*-(4-hydroxy-4-methylcyclohexyl)-*N*-nitrosourea.

Although CCNU and MeCCNU may also be substrates for the denitrosating enzyme, the rate of reaction is less than 5% of that for ring hydroxylation of the compounds.

DISCUSSION

Probably because nitrosoureas are very unstable chemically, little work has been done on the enzymatic metabolism of these compounds. It is known that rat liver microsomes catalyze the hydroxylation of the cyclohexyl ring of CCNU (18) and that rats excrete *N*-butylurea as a metabolite of the nitrosated compound (10). A guanidine derivative, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, is denitrosated by an enzyme in the soluble portion of rat liver, kidney, and stomach (25); but the microsomal enzyme that catalyzes denitrosation of BCNU and MNU is clearly not related to this enzyme, which does not require NADPH or any other cofactor. The mechanism for denitrosation of BCNU and MNU is not clear. Perhaps the nitroso group is oxidized to a nitro group and, after hydrolysis, is liberated as nitrate. The corresponding nitro analogs of BCNU and MNU have not been chemically synthesized, and their stability is unknown.

It is also not clear whether the enzyme that catalyzes denitrosation of BCNU and MNU is linked to cytochrome P-450. BCNU competitively inhibits the *C*-hydroxylation of nicotine, a P-450-mediated reaction (12), but not its *N*-oxidation; and nicotine in turn inhibits BCNU denitrosation. This could mean that cytochrome P-450 is involved. However, 2-diethylaminoethyl 2,2-diphenylvalerate, which inhibits the P-450-linked oxidations of many drugs but not the microsomal amine oxidase (31) or alkyldiazine oxidase (21), does not affect denitrosation. Further, the lack of

complete inhibition in the absence of oxygen and the weak inhibition of the denitrosation reaction by carbon monoxide are insufficient to allow the conclusion that cytochrome P-450 is required. The system catalyzing denitrosation apparently has properties different from those of cytochrome P-450-linked activities and those of microsomal amine oxidase.

A K_a value of $40 \mu\text{M}$ for CCNU binding has been derived for microsomes from phenobarbital-induced rats (18). This value is considerably lower than the K_m reported here for microsomes from untreated mice (3 mM). Nevertheless, the mass spectral evidence indicates that the ring-hydroxylated metabolite of CCNU is apparently the same as that isolated from the rat liver microsomal system and tentatively identified as 4-*cis*-hydroxy-CCNU (18). The MeCCNU metabolite may have an analogous structure.

Although the nitrosoureas are metabolized by lung tissue, the relatively low rate of reaction and smaller weight of the lungs (~ 0.15 g) lead to the conclusion that lungs do not contribute greatly to the metabolism of these agents. A similar conclusion has been reached for other drugs (8).

The microsomal denitrosation of BCNU and MNU, which are the 1st enzymatic reactions reported for these compounds, results in products with very little antitumor activity (TRW-Hazelton Laboratories, Vienna, Va., personal communication). However, the hydroxylated derivatives of CCNU and MeCCNU have not yet been tested against experimental tumors.

In blood serum *in vitro* the half-life of BCNU is 45 min and in phosphate buffer at pH 7.2 the half-life of CCNU is 64 min (19). As judged by the killing of leukemia cells injected at various times after the drugs, the biological half-life of BCNU is between 15 and 30 min (3) and that for CCNU is 94 min (14). However, the half-life for unaltered CCNU is only 6 min in mice (20). The differences in these values are apparently due to *in vivo* metabolism of the agents, and the data are consistent with the conversion of BCNU to an inactive product and CCNU to one that retains antitumor activity.

From Charts 2 and 3, the V_{max} values for microsomal oxidation of BCNU, CCNU, and MeCCNU by mouse liver microsomes are 417, 2220, and 445 pmoles of product per min per mg of liver, respectively. Assuming that 1 g of liver tissue is present in a 20-g mouse and that half-maximal conditions for oxidation exist continuously in the liver, a mouse could metabolize 0.2, 1.1, and 0.2 μmoles of substrate per min, respectively. The LD_{10} values for BCNU, CCNU, and MeCCNU are 3.8, 3.4, and 3.0 μmoles per 20-g mouse (27). Accordingly, microsomal enzymes could metabolize a LD_{10} dose of BCNU in 19 min, a LD_{10} of CCNU in 3 min, and a LD_{10} of MeCCNU in 15 min.

In addition to the microsomal enzyme metabolizing BCNU, an enzyme is present in the supernatant of mouse liver that converts BCNU to an unidentified product at a half-maximal rate of $0.3 \mu\text{mol}/\text{min}/\text{mouse}$ (Ref. 11; D. L. Hill, unpublished results). With the same assumptions, the combined activities of these enzymes could metabolize a LD_{10} of BCNU in 7.6 min. CCNU and MeCCNU are not substrates for the enzyme in liver cytosol.

Due to differential tissue distribution of the drugs and

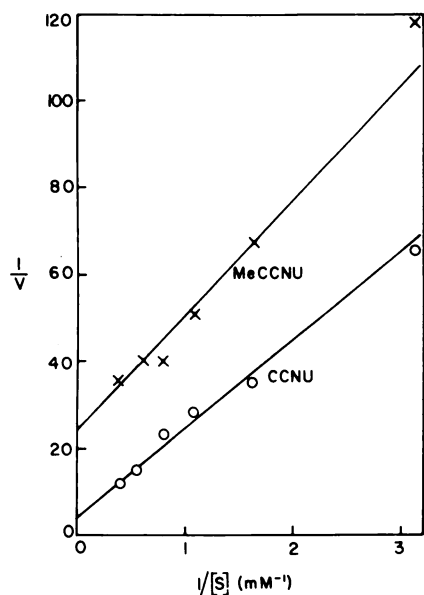


Chart 3. Lineweaver-Burk plot of CCNU and MeCCNU hydroxylation. Values for the ordinate are reciprocals of μ moles of product formed.

possibly other modes of biotransformation, there are hazards in extrapolating from assays of microsomal enzymes to metabolism in intact animals. However, the rates of reaction for microsomal enzymes involved in the metabolism of nitrosoureas are sufficiently high to allow biotransformation of large portions of administered doses before chemical decomposition of the drugs occurs.

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