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Microsomal Monooxygenation of the Carcinostatic 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea. Synthesis and Identification of Cis and Trans Monohydroxylated Products[†]

Hubert E. May,[‡] Richard Boose, and Donald J. Reed

ABSTRACT: Liver microsomal hydroxylation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea was shown to occur on the cyclohexyl ring at positions 3 and 4. Four metabolites were isolated by selective solvent extraction and purified by high-pressure liquid chromatography. *cis*-4-, *trans*-4-, *cis*-3-, and *trans*-3-OH derivatives of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea were synthesized and their chromatographic, mass spectral, and nuclear magnetic resonance characteristics matched those of the metabolites. The position of ring hydroxylation and the identity of each geometric isomer were established by nuclear magnetic resonance using a shift reagent in conjunction with spin decoupling techniques. Microsomes from rats pretreated with pheno-

barbital showed a sixfold increase in hydroxylation rate (19.8 vs. 3.3 nmol per mg per min). The induction was quite selective for *cis*-4 hydroxylation (19-fold); however, induction of *trans*-4 (threefold), *cis*-3 (threefold), and *trans*-3 (twofold) hydroxylation did occur. Quantitatively the *cis*-4-hydroxy metabolite was 67% of the total product by phenobarbital-induced microsomes and 21% for normal microsomes. Microsomes from animals pretreated with 3-methylcholanthrene gave about the same rate and product distribution that normal microsomes gave. A mixture of 80% carbon monoxide–20% oxygen inhibited formation of all four hydroxy metabolites with the inhibition ranging from 55 to 78%.

Nitrosoureas including CCNU¹ are undergoing intensive clinical evaluation and are highly promising for use as chemotherapeutic agents for brain tumors (Walker, 1973), Hodgkins disease (Selawry and Hansen, 1972), and lung cancer (Takita and Brugarolas, 1973). Carter et al. (1972) have stated that these lipid soluble compounds are among the most promising antineoplastic agents to emerge from the Chemotherapy Program of the National Cancer Institute. They distribute widely to tissues and have a short plasma half-life (Oliverio et al., 1970). Montgomery et al.

(1967) and Reed et al. (1975) have shown that these compounds also have relatively short half-lives in buffers. We have begun an extensive study of the metabolism of CCNU in an effort to understand its mode of action as an antitumor agent and to understand its toxic action. Until recently, little was known about the metabolism of CCNU when it was found to be hydroxylated in the liver as an initial metabolic step (May et al., 1974). According to the prediction of Hansch et al. (1972), the introduction of a hydrophilic group into CCNU should make it a more potent less toxic drug.

Liver microsomes catalyze an NADPH and cytochrome P-450 dependent hydroxylation of the cyclohexyl group of a number of compounds (Ullrich, 1969; Diehl et al., 1970; McMahon et al., 1965; McMahon and Sullivan, 1966). We reported earlier that CCNU is hydroxylated by a microsomal system that requires NADPH and oxygen, that is inhibited by carbon monoxide and is inducible by pretreatment of animals with phenobarbital. Further evidence for the involvement of cytochrome P-450 was obtained when CCNU was found to bind to microsomes and produce a type I difference spectrum. The spectrally determined dissociation constant for the P-450–CCNU complex was $4 \times 10^{-5} M$ (May et al., 1974).

The present paper deals with the isolation and identifica-

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¹ Abbreviations used are: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; *cis*-4-OH-CCNU, 1-(2-chloroethyl)-3-(*cis*-4-hydroxycyclohexyl)-1-nitrosourea; *trans*-4-OH-CCNU, 1-(2-chloroethyl)-3-(*trans*-4-hydroxycyclohexyl)-1-nitrosourea; *cis*-3-OH-CCNU, 1-(2-chloroethyl)-3-(*cis*-3-hydroxycyclohexyl)-1-nitrosourea; *trans*-3-OH-CCNU, 1-(2-chloroethyl)-3-(*trans*-3-hydroxycyclohexyl)-1-nitrosourea; [*cyclohexyl*-1-¹⁴C]CCNU, [¹⁴C]CCNU labeled in its cyclohexyl moiety; i-CCNU, 1-(2-chloroethyl)-3-cyclohexyl-3-nitrosourea; Eu(dpm)₃, tris(dipivaloylmethanato)europium(III).

Table I: Liquid Chromatographic Conditions for Separation of OH-CCNU's.

Column ^c	Conditions ^b	Retention Times ^a			
		<i>trans</i> -3-	<i>cis</i> -3-	<i>cis</i> -4-	<i>trans</i> -4-
Bio-Sil A (10 × 500)	IDP (930:63:105) (4 ml/min)	24	24	32	56
LiChrosorb (5 μ) (3.2 × 250)	IDP (930:63:70) (1 ml/min)			9.1	13.7
	IDP (930:63:35) (1 ml/min)	10.0	10.8	13.1	20.5
	IDP (930:63:20) (1 ml/min)	16.9	18.9		
	10 μg/100 μl to 10 mg/0.5 ml				

^a Retention times are expressed in minutes. We have observed that retentions are somewhat variable especially with repeated analyses of biological extracts. Thus, retention times are given to indicate whether conditions will resolve mixtures. Internal standards have been used for identification purposes. ^b I, isooctane; D, dichloromethane; P, 2-propanol; Numbers in parentheses indicate volume ratios; flow rates approximate; sample injection, approximate quantity per injected volume in column solvent (dilute solutions) or in chloroform (concentrated solutions). ^c Trade names of packing, packing size, and column dimensions in mm. Bio-Sil A, Bio-Rad Laboratories, Richmond, Calif.; LiChrosorb, Altex, Berkeley, Calif.

tion of four monohydroxylated CCNU isomers, synthesis of these compounds for confirmation of identity, and quantitation of rates of formation of these compounds using microsomes from normal and drug-induced rats.

Materials and Methods

Chemicals. CCNU, synthesized by Parke Davis, lot 7503 X 105, and [cyclohexyl-1-¹⁴C]CCNU were supplied by the National Cancer Institute. 4-Hydroxycyclohexylamine was obtained from Pfaltz and Bauer.

3-Acetamidophenol was supplied by Aldrich Chemical Co. Rhodium (5%) on charcoal was purchased from Englehard Industries.

Formic acid (88%) and all solvents including those used in liquid chromatography were J. T. Baker reagent grade. Tri-Sil BSA formula P and 2-chloroethyl isocyanate were obtained from Pierce Chemicals.

***N*-(*cis*,*trans*-3-Hydroxycyclohexyl)acetamide.** 3-Acetamidophenol was recrystallized three times from water (mp 147–148.5°). An ethanol solution of the phenol (23 g/120 ml; 152 mmol) was hydrogenated at 60° and 1700 psi H₂ for 22 hr in the presence of 2 g of 5% rhodium on charcoal as catalyst (Marshall²).

***cis*,*trans*-3-Hydroxycyclohexylamine.** *N*-(*cis*,*trans*-3-Hydroxycyclohexyl)acetamide (12 g, 76 mmol) was refluxed in 50 ml of 2.5 M NaOH for 18 hr. The hydroxycyclohexylamines were isolated by ether extraction. Yield of yellow oil, 7.6 g; 86%. The oil was analyzed for *cis* and *trans* content as the bis(trimethylsilyl) derivatives by gas chromatography as described below.

Separation of *cis*- and *trans*-3-hydroxycyclohexylamines. The *cis*- and *trans*-3-hydroxycyclohexylamines were isolated as the oxalate salts by fractional recrystallization from methanol. Melting point (193–194°) of *trans* agreed with Burckhardt et al. (1967). Melting point of the *cis* product was 141–143°.

1-(2-Chloroethyl)-3-(*cis*,*trans*-4-hydroxycyclohexyl)-1-nitrosourea (*cis*,*trans*-4-OH-CCNU). A modification of the method of Johnston et al. (1966) was used. 4-Hydroxycyclohexylamine (2 ml; 17 mmol), triethylamine (2 drops), and 2-chloroethyl isocyanate (1 ml; 11.4 mmol) were mixed without solvent. After 12 hr at room temperature the volatile materials were removed. The crude 1-(2-chloroethyl)-3-(*cis*,*trans*-4-hydroxycyclohexyl)urea (2.7 g) was nitrosat-

ed at 0° using 15 ml of formic acid (88%) and 43 mmol of sodium nitrite. The reaction mixture was neutralized to pH 6 with solid sodium bicarbonate, saturated with sodium chloride, extracted with ether (3 × 50 ml), and dried over anhydrous sodium sulfate. Solvent evaporation gave a light yellow oil (0.7 g) which was stored at –12°.

cis- and *trans*-4-OH-CCNU were isolated by preparative high-pressure liquid chromatography using a 10 × 500 mm column packed with Bio-Sil A. The *trans* isomer could be induced to crystallize from a solvent mixture (isooctane-dichloromethane-2-propanol; 930:63:70).

1-(2-Chloroethyl)-3-(*cis*,*trans*-3-hydroxycyclohexyl)-1-nitrosourea (*cis*,*trans*-3-OH-CCNU), 1-(2-Chloroethyl)-3-(*cis*-3-hydroxycyclohexyl)-1-nitrosourea (*cis*-3-OH-CCNU), and 1-(2-Chloroethyl)-3-(*trans*-3-hydroxycyclohexyl)-1-nitrosourea (*trans*-3-OH-CCNU). The method was the same as described for *cis*,*trans*-4-OH-CCNU except *cis*,*trans*-3-hydroxycyclohexylamine or *cis*-3-hydroxycyclohexylamine or *trans*-3-hydroxycyclohexylamine was used as the starting amine.

The ureas were not crystalline and were nitrosated without purification. Isolation of *cis*- and *trans*-3-OH-CCNU was achieved by preparative high-pressure liquid chromatography using a 3.2 × 250 mm column packed with LiChrosorb (5-μ particle size) as described below.

1-(2-Chloroethyl)-3-(*cis*-2-hydroxycyclohexyl)-1-nitrosourea (*cis*-2-OH-CCNU). 2-Acetamidophenol was hydrogenated using 5% Rh on charcoal as described above. Recrystallization of the crude product from acetone gave *N*-(*cis*-2-hydroxycyclohexyl)acetamide (mp 144–146°). *cis*-2-Hydroxycyclohexylamine was extracted (ether) from the alkaline hydrolysate of the amide and was used as described above to synthesize *cis*-2-OH-CCNU.

Methods

Mass spectra were obtained by a Varian Model M7 mass spectrometer using a GC port or a direct probe at 110° and an ionizing potential of 70 V.

Nuclear magnetic resonance (NMR) spectra were obtained using a 100-MHz Varian Model HA-100 nuclear magnetic resonance spectrometer equipped with a Varian Model C-1024 time averaging computer. Spectra were obtained at room temperature from solutions of sample in deuterated chloroform (Merck) (2–12 mg of sample/0.4 ml) using tetramethylsilane as an internal or external standard. In some instances of low sample concentration it was necessary to obtain computer accumulated spectra using a

² Method received from Dr. F. J. Marshall by private communication.

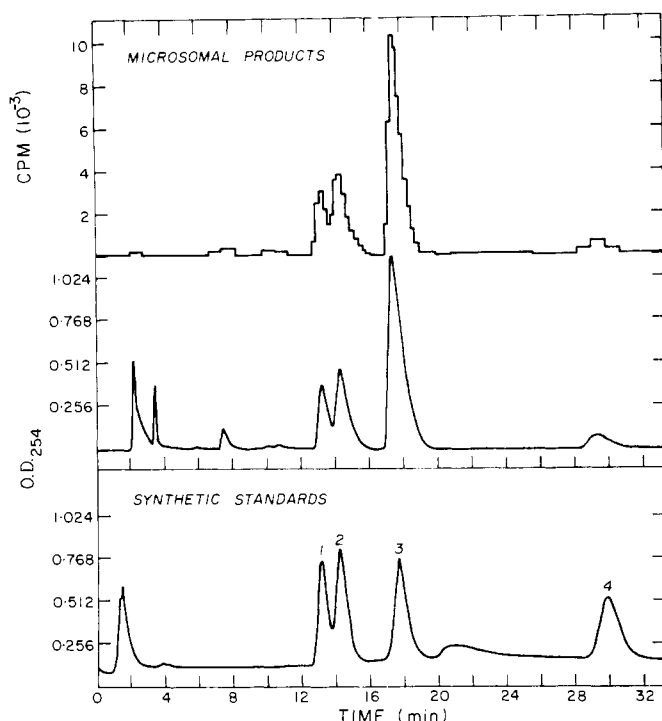


FIGURE 1: Liquid chromatography of OH-CCNU metabolites isolated from incubations of rat liver microsomes, NADP⁺, NADPH generating system, and [cyclohexyl-1-¹⁴C]CCNU. Microsomes were prepared from phenobarbital-induced rats. Details are described under Materials and Methods. Synthetic standards, which are compared to the OD₂₅₄ and ¹⁴C profiles of the metabolites, were chromatographed under identical conditions on a LiChrosorb column, 3.2 × 250 mm, with an eluting solvent system of isooctane-dichloromethane-2-propanol (930:63:35 v/v). Numbers denote (1) *trans*-3-OH-, (2) *cis*-3-OH-, (3) *cis*-4-OH-, and (4) *trans*-4-OH-CCNU. These correspond to metabolites 2, 3, 4, and 5, respectively. Metabolite 1 was eluted at 7–8 min at a flow rate of 1.0 ml/min.

time averaging computer in order to get acceptable spectra.

Studies concerned with the identification of geometric isomers utilized Eu(dpm)₃ (Ventron) as a shift reagent. After the unshifted spectrum was obtained, successive quantities (5 mg) of the shift reagent were added to the sample with the spectrum recorded after each addition. Addition of shift reagent was stopped when the spectrum became sufficiently simple for interpretation. At this point spin decoupling was done to facilitate assignment of protons.

Due to low solubility of Eu(dpm)₃ in deuterated chloroform some difficulty was experienced in obtaining stabilized shifted spectra required for spin decoupling.

GC-Mass Spectral Analysis and Identification of Hydroxycyclohexylamines. The bis(trimethylsilyl) derivatives of hydroxycyclohexylamines were separated by gas chromatography on a 1/8 in. × 6 ft stainless steel column packed with 3% OV-1 operating at 100 or 120°. The order of elution of these derivatives was *trans*-3-, *cis*-3-, *cis*-4-, and *trans*-4-. The commercially available *cis,trans*-4-hydroxycyclohexylamine gave *cis*- and *trans*-1,4-bis(trimethylsilyl) derivatives when treated with Tri-Sil BSA formula P for a few minutes at room temperature. Their identities were confirmed by GC-mass spectrometry and compared well with the results of Renwick and Williams (1972). 3-Hydroxycyclohexylamine gave *cis*- and *trans*-1,3-bis(trimethylsilyl) derivatives whose identities were also confirmed by mass spectrometry.

Table II: Comparison of Rate of Formation of OH-CCNU's from CCNU by Microsomes from Normal, Phenobarbital-Induced, and 3-Methylcholanthrene Induced Rat Liver.^a

OH-CCNU Metabolite	nmol per mg per min ^b		
	Normal	Phenobarbital	3-Methylcholanthrene
<i>trans</i> -3-	1.3	2.6	0.8
<i>cis</i> -3-	1.0	3.1	0.5
<i>cis</i> -4-	0.7	13.2	0.6
<i>trans</i> -4-	0.3	0.9	0.1
Total	3.3	19.8	2.0

^a For incubation system, method of analysis, and animal pretreatment, see Methods. ^b Quantities based on specific radioactivity of [cyclohexyl-1-¹⁴C]CCNU and confirmed by area under curve of liquid chromatographic trace at 254 nm. The data are averages of three experiments.

Table III: Carbon Monoxide Inhibition of CCNU Hydroxylation by Liver Microsomes from Phenobarbital-Induced Rats.^a

OH-CCNU Metabolite	nmol/10 min	
	O ₂ /N ₂ (20:80)	O ₂ /CO (20:80)
<i>trans</i> -3-	155	70 (45) ^b
<i>cis</i> -3-	170	38 (22)
<i>cis</i> -4-	860	213 (25)
<i>trans</i> -4-	156	56 (36)
Total	1341	377 (28)

^a Incubation and analysis as described in Materials and Methods. Each system was gassed with N₂ or CO then 20% of gaseous volume was replaced with O₂. ^b Numbers in parentheses are percent of O₂/N₂ (20:80).

Liquid Chromatographic Separation of Cis and Trans Isomers of 4-OH-CCNU and of 3-OH-CCNU. Geometric isomers of 4-OH-CCNU and 3-OH-CCNU were separated for quantitation and for preparative isolation by high-pressure liquid chromatography. Chromatographic conditions are summarized in Table I. A large Bio-Sil A column (10 × 500 mm) was used for preparative separations of *cis*- and *trans*-4-OH-CCNU (several hundred milligram quantities per sample injection) while a LiChrosorb column was used for preparative isolation of *cis*- and *trans*-3-OH-CCNU. The OH-CCNU's are stable a few days when stored without solvents and dry at -12° and may be stored weeks under the same conditions but at -80°.

Animals and Pretreatments. Sprague-Dawley adult male rats (250–350 g) were used. Phenobarbital was administered orally by using 0.2% solution for drinking water for 5 days. 3-Methylcholanthrene (30 mg/kg in corn oil) was administered i.p. daily for 2 days.

Incubation Procedure. Microsomes were prepared according to the method of Borton et al. (1974) and protein was determined by the method of Lowry et al. (1951). Incubations contained in a final 3-ml volume: 0.1 M phosphate buffer (pH 7.4), 1.0 μmol of NADP, 25 μmol of glucose-6-P, 1.2 units of glucose-6-P dehydrogenase, 1.8 μmol of [cyclohexyl-1-¹⁴C]CCNU (12,600,000 dpm), and 2 mg of microsomal protein. Incubations were carried out at 37° for 5 and 10 min and followed by 3 × 6-ml extractions with hexane to quantitatively remove unreacted CCNU and finally 3 × 6-ml extractions with ether to remove products. The combined ether extracts were dried over anhydrous sodium sulfate and the ether solution was decanted. The metabo-

Table IV: Comparison of Mass Spectral Fragments of Synthetic OH-CCNU's with Microsomal Metabolites.^a

<i>m/e</i>	CCNU	<i>i</i> -CCNU	<i>cis</i> -2-	M - 1	<i>trans</i> -3-	M - 2	<i>cis</i> -3-	M - 3	<i>cis</i> -4-	M - 4	<i>trans</i> -4-	M - 5
30	16	14	27	68	13	13	12	12	7	13	7	25
43	42	22	35	124	35	44	30	54	10	41	13	75
63	12	21	17	16	9	9	7	7	5	9	5	13
65	5	7	6	6	3	3	3	3	2	3	2	5
81	20	12	100	100	100	100	100	100	100	100	100	100
83	100	100	9	16	5	10	3	16	2	5	2	19
124	3	<1	52	24	5	5	10	10	20	17	4	7
126	27	<1	<1	1	<1	1	<1	2	<1	1	<1	2
128	<1	38	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
142	1	<1	96	29	7	5	19	18	19	16	5	5
144	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
232 ^b	1				2	14	10	4	1	1	20	12
234 ^b	46				<1	4	3	1	<1	<1	6	4
236 ^b	16				0	0	0	0	0	0	0	0
250 ^b	8				48	45	63	27	83	35	70	37
252 ^b	4				16	14	20	8	26	10	22	12

^a For method of incubation and isolation see Materials and Methods. Sample introduction via direct probe at 100° using ionizing potential of 70 V. ^b From chemi-ionization mass spectra. Base peak was *m/e* 81 for OH-CCNU's and 83 for CCNU.

Table V: Proton Assignments for 4-OH-CCNU's and *trans*-4-Hydroxycyclohexylacetamide in the Absence and Presence of Eu(dpm)₃.

Proton	I ^a		II ^b		III ^c	
	Normal	Shifted ^c	Normal	Shifted ^c	Normal	Shifted ^c
1-Axial	4.0	5.7	3.6	7.0	3.8-4.1	13.8
2,6-Axial	1.4-1.9	8.5	1.3	2.8	1.4	7.7
2,6-Equatorial	1.4-1.9	5.7	2.1	3.8	2.2	8.2
3,5-Axial	1.4-1.9	6.8	1.3	3.4	1.4	14.4
3,5-Equatorial	1.4-1.9	9.4	2.1	3.8	2.2	13.8
4-Axial			3.6	6.7	3.8-4.1	>14
4-Equatorial	4.0	>10				
Hydroxyl	1.6	>10	1.6	11.3	1.6	>14
Amide	6.9		5.4	7.4	6.7	12.8
Methylene	3.6, 4.3	4.7, 6.3			3.6, 4.3	5.6, 8.1
Methyl			2.1	4.3		

^a R = ClCH₂CH₂N(NO)C=O. ^b R' = CH₃C=O. ^c Molar ratio Eu(dpm)₃/sample: I = 0.96; II = 0.33; III = 0.90.

lites could be stored as an ether solution at -80° or preferably without solvent at -80°. Quantitative estimations of the amounts of the various OH-CCNU's were determined by liquid chromatography on a LiChrosorb column as described above. Fractions (70 or 20 drops) were collected and ¹⁴C counted in a Packard Model 2425 liquid scintillation counter utilizing a toluene fluor. Calculations were based on the specific radioactivity of the substrate and confirmed by the chromatographic trace at 254 nm.

Results

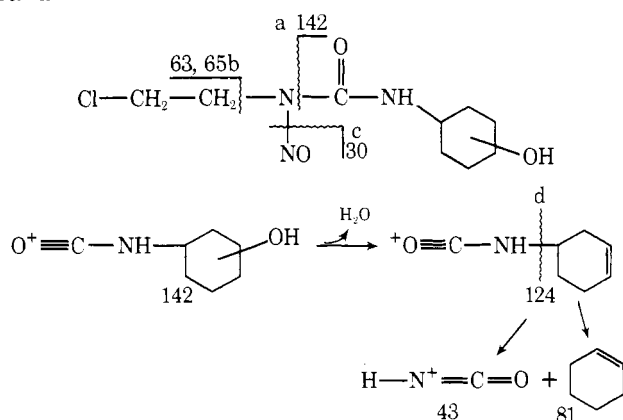
When liver microsomes from phenobarbital pretreated rats were incubated with [*cyclohexyl*-1-¹⁴C]CCNU and a NADPH-generating system at pH 7.4, five polar metabolites were separable by high-pressure liquid chromatography using a LiChrosorb column (Figure 1). The ¹⁴C profile matched the detector absorbance at 254 nm. Metabolites 2, 3, 4, and 5, numbered in the order of appearance from the column, had the same elution time as synthetic *trans*-3-, *cis*-3-, *cis*-4-, and *trans*-4-OH-CCNU, respectively (Figure 1).

Substrate requirements and various enzymic parameters for the formation of total hydroxylated products were re-

ported previously (May et al., 1974). When microsomes from phenobarbital-induced animals were used the major product was *cis*-4-OH-CCNU (67%) and other OH-CCNU's ranged from 5 to 16% of the total (Table II). The major product due to normal microsomes and those from 3-methylcholanthrene treated animals was *trans*-3-OH-CCNU. Phenobarbital-induced microsomes showed a six-fold increase in overall CCNU hydroxylation compared to normal microsomal activity and 19-fold increase in the formation of *cis*-4-OH-CCNU. 3-Methylcholanthrene, on the other hand, had no inducing effect for formation of any of the isomers (Table II).

It was previously reported that the enzyme system requires NADPH and oxygen and that CCNU binds rather tightly ($K_s = 4 \times 10^{-5}$ M) to cytochrome P-450 to produce a type I binding spectrum. Involvement of cytochrome P-450 in the hydroxylation mechanism was confirmed by showing hydroxylation was inhibited by carbon monoxide (May et al., 1974). In the present data, carbon monoxide inhibited total hydroxylation of CCNU by 72% when phenobarbital-induced microsomes were used. Inhibition was somewhat greater for formation of *cis*-4- and *cis*-3-OH-CCNU (Table III). It should be noted that microsomes

Scheme 1



from phenobarbital-induced animals also produce more of these compounds compared to normal animals (Table II).

Mass Spectral Characterization of OH-CCNU's and Microsomal Metabolites of CCNU. The purity of the synthetic standards and of the metabolites as isolated by liquid chromatography was established by a combination of mass spectral information and NMR. In each case the number of protons from integration of NMR spectra corresponded to 16 which is correct for a ring-hydroxylated CCNU. Thus the isolated compounds could not contain appreciable contaminating quantities of compounds containing two 2-chloroethyl groups, two nitroso groups, etc. The primary kind of contaminant isomer that one would expect to find would be the 3-nitroso compound and perhaps small amount of the parent CCNU in the case of the metabolite studies.

A comparison of the mass spectra of the synthetic standards and the microsomal metabolites is given in Table IV. The conventional electron impact mass spectrum did not give the molecular ion when normal quantities of sample were used, but the chemi-ionization mass spectrum did give the characteristic $M + 1$ peaks (m/e 250 and 252) for these monochlorinated compounds. This showed that the parent CCNU was hydroxylated by the microsomal system since the molecular weight of the parent CCNU was increased by 16. Scheme I accounts for the major fragments formed from compounds studied with the predominant bond cleavage occurring at a. CCNU did not contaminate the metabolites because m/e 126 corresponding to a protonated cyclohexyl isocyanate was absent. Further, m/e 236 corresponding to $M + 1$ for CCNU molecules containing ^{37}Cl was absent from all hydroxylated compounds. Masses m/e 232 and 234 found in spectra of OH-CCNU's and metabolites correspond to dehydration products ($M + 1 - 18$) formed in the mass spectrometer. The nitroso group (m/e 30) was present in all compounds. A comparison of CCNU (1-nitroso) with *i*-CCNU (3-nitroso) shows that fragments m/e 126 and m/e 128 occur, respectively. This indicates the cleavage of the nitrosamide bond predominates in both compounds. It is noted that these compounds form either m/e 126 or m/e 128 but not both. By analogy the 1-nitrosohydroxy-CCNU would give m/e 142 and 3-nitrosohydroxy-CCNU would give m/e 144. Since m/e 142 was a predominant peak and m/e 144 was absent in both the metabolites and synthetic compounds all OH-CCNU's were 1-nitroso compounds. Data by Johnston et al. (1966) confirm this conclusion since they observe that analogous 3-nitroso compounds show a much more complex NMR spectrum in the region of absorption of methylene protons of the chloroethyl group (δ 3.6). We observe well-defined triplets (see Figure 2) for the

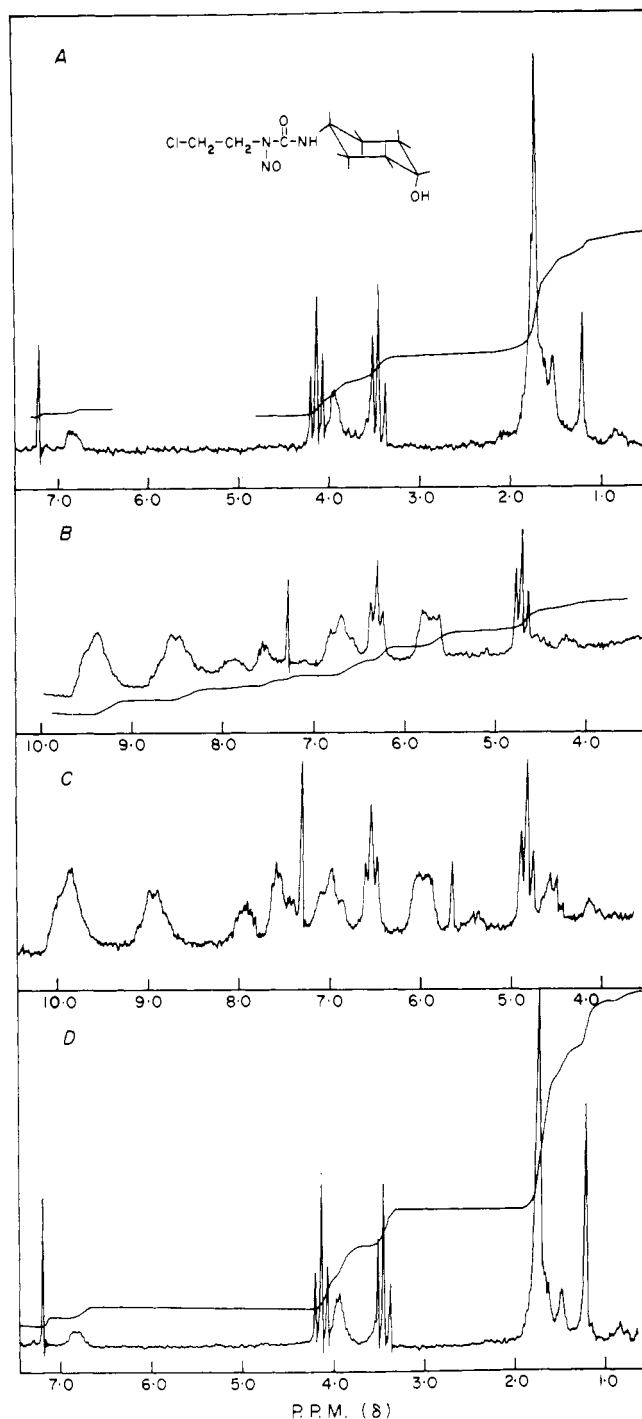


FIGURE 2: NMR spectra of (A) *cis*-4-OH-CCNU, (B) *cis*-4-OH-CCNU in the presence of $\text{Eu}(\text{dpm})_3$ at a molar ratio of $\text{Eu}(\text{dpm})_3/\text{sample}$ of 0.96, (C) same as B except with metabolite 4, and (D) metabolite 4.

synthetic OH-CCNU's and the metabolites. Comparison of fragments (Table IV) with Scheme I (bond b) shows that the chloroethyl group (m/e 63 and 65) was present in all cases. Cyclohexyl ring hydroxylation by the microsomal system was concluded because of m/e 142 analogous to m/e 126 in the parent CCNU and also because of the presence of ring dehydration fragments m/e 124 and 81.

Good agreement was observed between the mass spectra of microsomal metabolites and the synthetic standards. After NMR studies of synthetic standards permitted structure assignments it was concluded that metabolites 2, 3, 4, and 5 were respectively *trans*-3-, *cis*-3-, *cis*-4-, and *trans*-

Table VI: Spin Decoupling of Cyclohexyl Ring Protons.

Irradiation	Proton	Decoupled	Proton	Multiplicity Change
<i>cis</i> -4-OH-CCNU				
6.8	3,5-Axial	8.5	2,6-Axial	Quartet → triplet
6.8	3,5-Axial	9.4	3,5-Equatorial	Multiple → less complex
8.5	2,6-Axial	6.8	3,5-Axial	Triplet → doublet
8.5	2,6-Axial	5.7	2,6-Equatorial	Multiple → doublet
5.7	2,6-Equatorial	8.5	2,6-Axial	Quartet → triplet
6.3	Methylene	4.7	Methylene	Triplet → singlet
4.7	Methylene	6.3	Methylene	Triplet → singlet
<i>trans</i> -4-OH-CCNU				
14.4	3,5-Axial	7.7	2,6-Axial	Quartet → triplet
7.7	2,6-Axial	14.4	3,5-Axial	Quartet → triplet
13.8	1-Axial	7.7	2,6-Axial	Quartet → triplet
5.6	Methylene	8.1	Methylene	Triplet → singlet
<i>trans</i> -4-Hydroxycyclohexylacetamide				
7.0	1-Axial	2.8	2,6-Axial	Quartet → triplet
6.7	4-Axial	3.4	3,5-Axial	Quartet → triplet
3.8	3,5-Axial	3.8	3,5-Equatorial	Doublet → singlet
2.8	2,6-Axial	3.8	2,6-Equatorial	Doublet → singlet

4-OH-CCNU. Metabolite 1, which was obtained in quantities too small for NMR studies, had a mass spectrum much like that of the other OH-CCNU's. The relative retention time of metabolite 1 on LiChrosorb compared to *cis*-4-OH-CCNU was 0.44. Synthetic *cis*-2-OH-CCNU had the same relative retention time. It is noted that the *cis*-3 and the *cis*-4 isomers gave considerably more *m/e* 142 (20% of base) than the *trans* isomers (5% of base). This difference is strikingly enhanced with the synthetic *cis*-2-OH-CCNU (96% of base) and metabolite 1 (29%) of base. Thus, the evidence even without NMR data supports the conclusion that metabolite 1 is the *cis*-2-hydroxy isomer.

NMR Verification of Structure of *cis*-4- and *trans*-4-OH-CCNU. The proton assignments for *cis*-4- and *trans*-4-OH-CCNU as given in Table V are based on the use of the shift reagent Eu(dpm)₃ in conjunction with spin decoupling techniques (Table VI). Interpretations support the assumption that the bulky 2-chloroethylnitrosourea group is in the equatorial position and that these compounds exist primarily in a single conformation. Equatorial positioning of bulky groups have been observed for *cis*- and *trans*-4-*tert*-butylcyclohexanol (Demarco et al., 1970) and for *cis*- and *trans*-4-phenylcyclohexanol as well as *cis*-3-phenylcyclohexanol (Kanai et al., 1973).

The NMR spectra of *cis*-4- and *trans*-4-OH-CCNU are shown in Figures 2A and 3B, respectively. In both cases the integrated spectra gave 16 protons. The absorption of hydroxyl and amide protons shifted with temperature. The two methylene moieties of the 2-chloroethyl group are triplets at δ 3.6 and 4.3. Since the 1-axial proton and 4-axial or 4-equatorial protons are attached to carbons bearing electronegative atoms their absorptions were expected to be downfield compared to other ring protons and were observed at δ 3.6–4.0. Other ring protons of *cis*-4-OH-CCNU have high-field absorptions (δ 1.4–1.9) and could not be individually assigned. However, the 2,6,3,5-axial and 2,6,3,5-equatorial protons of *trans*-4-OH-CCNU could be assigned by comparison to *N*-(*trans*-4-hydroxycyclohexyl)acetamide (see Table V and compare Figure 3A and B).

Final proof of structure of *cis*-4- and *trans*-4-OH-CCNU was obtained by use of Eu(dpm)₃ and spin decoupling. Both compounds possess four sets of equivalent ring protons, namely 2,6-axial, 2,6-equatorial, 3,5-axial, and 3,5-equato-

rial. For convenience of discussion 2,6-axial protons will be referred to as the 2-axial proton and 3,5-axial protons as the 3-axial proton, etc. For both compounds absorption by 2-axial proton in shifted spectra should be a broad quartet due to splitting by 1-axial, 3-axial, and 2-equatorial protons since J_{a-a} for vicinal protons and J_{a-e} for geminal protons are large compared to J_{a-e} and J_{e-e} for vicinal protons (Servis et al., 1975). The compounds should differ in multiplicity for absorption due to the 3-axial proton. *trans*-4-OH-CCNU should yield a quartet (splitting by 2-axial, 4-axial, and 3-equatorial protons) while *cis*-4-OH-CCNU should yield a triplet (splitting by 2-axial and 3-equatorial protons) since the 4-equatorial proton has a much smaller coupling constant than does the corresponding 4-axial proton of the *trans* isomer. These expected absorptions for 2-axial and 3-axial protons were observed for both compounds (Table V and Figures 2B and 3C). The downfield quartet of the *trans*-4 isomer was assigned to the 3-axial proton because of its closer proximity to the shift reagent (Demarco et al., 1970) and the upfield quartet was assigned to the 2-axial proton (see Figure 3C). Use of proximity to the shift reagent as criteria for proton assignment was confirmed in its use in the shifted spectrum of the *cis* isomer (Figure 2B). Europium complexes with oxygen of the hydroxyl group on the 4-axial side of the ring (Figure 2A) and is closer to the 2-axial than the 3-axial proton. Thus, the 2-axial proton (quartet) should experience a larger downfield shift than the 3-axial proton (triplet). The method was confirmed since it was expected that the 3-axial proton would be a triplet. Equatorial protons were assigned based on proximity to shift reagent and spin decoupling. In each case the 3-equatorial protons should experience the largest downfield shift. Irradiation at the resonance frequency of the geminal axial proton had the greatest effect on the equatorial proton (see Table VI). Irradiation of an axial proton reduced the multiplicity of the vicinal axial proton by one, for example, quartet to triplet (Table VI). It should be pointed out that decoupling of many geminal protons could not be observed because the differences in chemical shifts were too small, leading to instrumental noise in that region of the spectrum. This was particularly true of the *trans* isomer. Decoupling of both sets of methylene protons (triplets) produced the expected singlets.

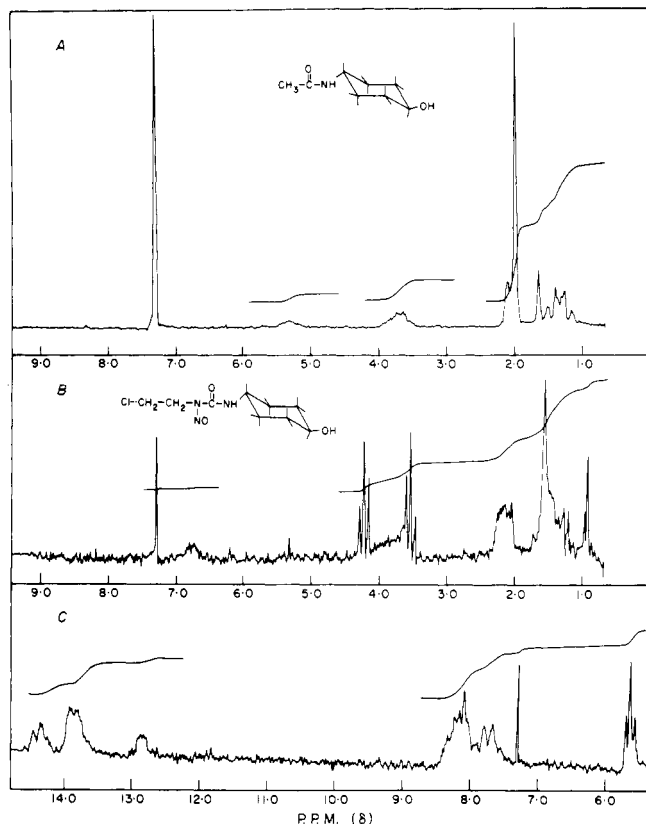


FIGURE 3: NMR spectra of (A) *N*-(*trans*-4-hydroxycyclohexyl)acetamide, (B) *trans*-4-OH-CCNU, and (C) *trans*-4-OH-CCNU in the presence of $\text{Eu}(\text{dpm})_3$ with a $\text{Eu}(\text{dpm})_3$ /sample ratio of 0.96.

The assignments made here are in agreement with those made by Demarco et al. (1970) for *cis*-4-*tert*-butylcyclohexanol and by Kanai et al. (1973) for *cis*- and *trans*-4-phenylcyclohexanol.

Comparison of *trans*-3-, *cis*-3-, and *cis*-4-OH-CCNU with Microsomal Metabolites 2, 3, and 4. Metabolite 4 was shown to be *cis*-4-OH-CCNU by its mass spectrum (Table IV) and by its normal (Figure 2A and D) and $\text{Eu}(\text{dpm})_3$ -shifted (Figures 2B and C) NMR spectra. Metabolite 4 also displayed the same spin decoupling as *cis*-4-OH-CCNU. *trans*-3- and *cis*-3-OH-CCNU were synthesized from the pure oxalate salts of *trans*-3- and *cis*-3-hydroxycyclohexylamine, respectively. Metabolites 2 and 3 chromatographed on liquid chromatography (Figure 1) and gave the same mass spectra (Table IV) as *trans*-3- and *cis*-3-OH-CCNU. Comparison of the NMR spectrum of metabolite 2 with *trans*-3-OH-CCNU (Figure 4) shows the compounds are the same. The NMR spectrum of metabolite 3 compares reasonably well with *cis*-3-OH-CCNU (not shown). Use of a shift reagent with the 3 isomers did not permit detailed structure analysis. However, decomposition of the isolated isomers in alkali and subsequent formation of 2,4-dinitrophenyl derivatives from the resulting *cis*- or *trans*-3-hydroxycyclohexylamines permitted structure verification by liquid chromatography.

Discussion

Results presented here and in a previous report (May et al., 1974) indicate that CCNU is rapidly hydroxylated by rat liver microsomes. Three separate ring positions of the cyclohexyl moiety are hydroxylated with both axial and equatorial carbon-hydrogen bonds being attacked. Based

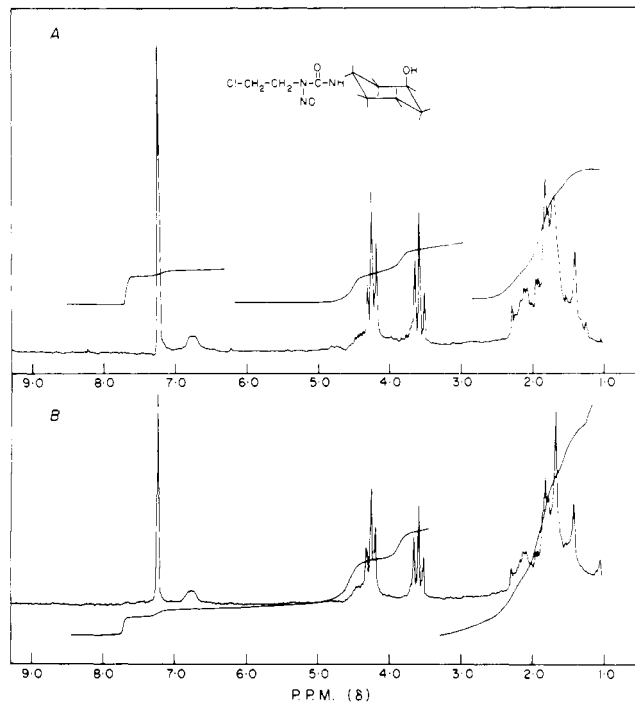


FIGURE 4: NMR spectra of (A) *trans*-3-OH-CCNU and (B) metabolite 2.

on substrate requirement (NADPH and oxygen), substrate binding spectrum (type I) (May et al., 1974), carbon monoxide inhibition (Table III), and enzyme induction by phenobarbital pretreatment (Table II), the reaction is cytochrome P-450 dependent.

The structures of four monohydroxylated products were determined by comparison of liquid chromatographic behavior, mass spectra, and NMR spectra of purified metabolites with synthetic compounds. Hydroxylation by normal microsomes occurred primarily (70%) at the 3 position of the cyclohexyl ring and the remaining 30% occurred at the 4 position. Interestingly, microsomes from phenobarbital-induced animals showed increased specific activity for production of all isomers but a much greater increase in rate of formation of *cis*-4-OH-CCNU which accounted for 67% of the total hydroxylated product. Microsomes from animals pretreated with 3-methylcholanthrene produced a distribution of hydroxylated products very similar to liver microsomes prepared from normal animals.

It is of interest that phenobarbital induction markedly changed the quantitative distribution of OH-CCNU metabolites from CCNU. A possible explanation is that the induced system contains at least two enzymes capable of cyclohexyl ring hydroxylation, the normal enzyme which produces primarily 3-OH-CCNUs and the induced enzyme which performs axial hydroxylation at the 4 position to produce *cis*-4-OH-CCNU. It has been reported that phenobarbital-induced liver microsomes compared to normal produce a different distribution of product alcohols when *n*-hexane (Kramer et al., 1974) and *n*-heptane (Frommer et al., 1972) are the substrates. Normal microsomes hydroxylate these aliphatic hydrocarbons at all possible positions and the quantitative distribution of the resulting alcohols make it possible to conclude that hydroxylation occurs more readily at a secondary carbon than a primary one and further in the case of *n*-hexane, hydroxylation at the secondary carbon is random. Such random hydroxylation did not occur with CCNU using either normal or induced microsomes.

The precise structures of the products of CCNU hydroxylation could be quite important. Biological activity, whether carcinostatic or toxicological, could very well depend not only on the ring position of the hydroxyl group but also on whether it is an axial or equatorial group. Evidence from NMR is consistent with the amide grouping of the OH-CCNU's being in the equatorial position at C-1. Thus these molecules seem to exist predominantly in one conformation. The resulting positioning of the hydroxyl group as either axial or equatorial should make the stereochemical aspects important in regards to chemical stability and metabolism of each individual isomer. A model of *cis*-3-OH-CCNU shows that the hydroxyl group is close enough to the urea nitrogens and oxygen for possible intramolecular hydrogen bonding. Burford et al. (1957) demonstrated intramolecular hydrogen bonds of the *cis*-3-aminocyclohexanols.

Preliminary evidence on the chemistry of OH-CCNU's suggests that stereochemistry of the ring hydroxyl group is important in regards to possible intramolecular carbamoylation. *cis*-3-OH-CCNU upon alkaline degradation undergoes virtually complete intramolecular carbamoylation to give the corresponding cyclic carbamate (urethane).³ *trans*-3-OH-CCNU does not appear to undergo such an intramolecular reaction. This difference in potential carbamoylation capability toward cellular constituents is currently under investigation.⁴

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³ Unpublished results; Reed, 1975.

⁴ After submission of this paper for publication, Johnston et al. (1975) reported the synthesis and biologic evaluation of 1-(2-chloroethyl)-3-(*cis*-4-hydroxycyclohexyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(*trans*-4-hydroxycyclohexyl)-1-nitrosourea.