HYDROXYLATION OF THE CARCINOSTATIC 1-(2-CHLOROETHYL)-3-CYCLOHEXYL-1-NITROSOUREA (CCNU) BY RAT LIVER MICROSOMES

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Summary

Ring hydroxylation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea was shown to occur in the presence of liver microsomes prepared from both normal and phenobarbital induced rats. The metabolite was identified by mass spectrometry after selective extraction and purification by liquid chromatography. The microsomal catalyzed reaction was oxygen and NADPH dependent, inhibited by carbon monoxide and induced 4-5 fold by in vivo phenobarbital pre-treatment. Phenobarbital induced microsomes hydroxylated the substrate at a rate of 17.6 nmoles/min/mg protein at 37°. A Type I difference spectrum was observed with phenobarbital induced microsomes that also displayed a substrate binding constant (K_s) of 4 x 10^{-5} M.

The nitrosoureas which include CCNU[1] are currently being intensively evaluated clinically as highly promising drugs in the chemotheraphy of brain tumors (1), Hodgkins disease (2) and lung cancer (3). According to Carter et al. (4) the nitrosoureas are one of the most exciting groups of compounds to arise from the Chemotheraphy Program of the National Cancer Institute. They represent a rather unique class of antineoplastic agents that are lipid soluble, are distributed widely to tissues in vivo and have an extremely short plasma half life (5). While these drugs are known to undergo chemical degradation at room temperature (6) little is known about their possible metabolism. Metabolic transformation of CCNU to a more hydrophilic nitrosourea should yield a more potent less toxic drug according to the prediction of Hansch et al. (7).

Liver endoplasmic reticulum catalyzes a cytochrome P-450 dependent hydroxylation of carbon of many different compounds including cyclohexane (8,9),

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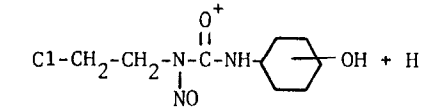
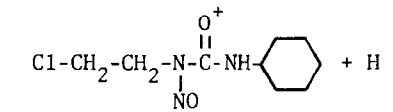
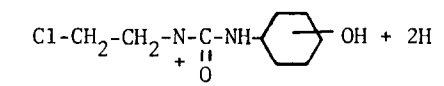
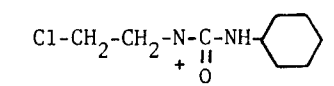
[1]Abbreviations: CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; Methyl-CCNU, 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea; cis-4-Hydroxyl-CCNU, 1-(2-chloroethyl)-3-(cis-4-hydroxycyclohexyl)-1-nitrosourea; Cyclohexyl [^{14}C]-CCNU, [^{14}C]-CCNU labeled uniformly in its cyclohexyl moiety; EI, electron impact mass spectrometry; CI, chemi ionization mass spectrometry.

TABLE 1

Mass Spectral Identification of Microsomal Metabolite of CCNU.

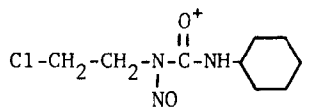
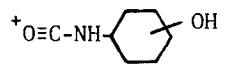
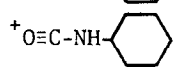
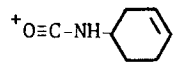
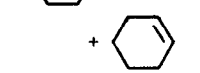
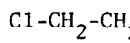
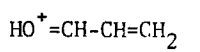
Incubation system; see Table 2. The product was isolated from incubates by ether extraction after prior removal of CCNU by hexane extraction. Product was purified by liquid chromatography using a Vydac column and isooctane-chloroform (65:35). Only a partial listing of mass spectra fragments is given below (base peak intensity = 1,000).

A. Mass Spectra by Chemi Ionization Mass Spectrometry*

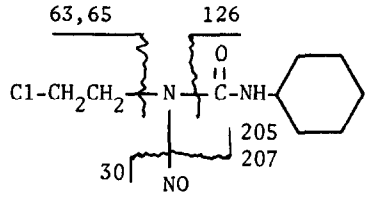
m/e	Structure Assignment	Relative Intensity CCNU	Relative Intensity CCNU Metabolite
252		4	20
250		16	70
236		262	10
234		420	25
223		0	190
221		0	400
207		84	20
205		300	50

*Spectra done by Dr. J. Daly at NIH

B. Mass Spectra by Electron Impact Mass Spectrometry at 70 volts.

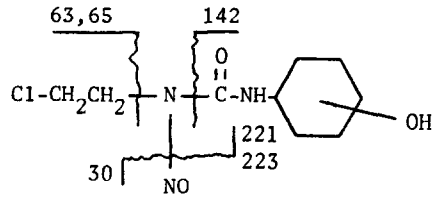
m/e	Structure Assignment	Relative Intensity CCNU	Relative Intensity CCNU Metabolite
235		4	1
233		8	1
142		1	326
126		272	5
124		8	202
83		1000	61
81		55	1000
65		16	56
63		43	135
57		19	292
30		75	258

Scheme I



M^+ = 233,235 (EI)
234,236 (CI)

CCNU



M^+ = 249,251 (EI)
250,252 (CI)

CCNU Metabolite

ethylbenzene (10) and 1-(4-acetylphenylsulfonyl)-3-cyclohexyl urea (acetohexamide) (11). In vivo formation of the glucuronides of various isomers of methylcyclohexanol after administration of methylcyclohexane is further evidence of cytochrome P-450 catalyzed hydroxylation of this alicyclic ring (12).

This report presents evidence, which includes mass spectral data, that CCNU hydroxylation in the cyclohexyl moiety is catalyzed by an O_2 and NADPH dependent, CO sensitive microsomal enzyme system whose activity is enhanced several fold when animals are treated with phenobarbital. Further, CCNU produces a Type I binding spectrum (K_s = 4 x 10^{-5} M).

Materials and Methods

Sprague Dawley adult male rats (250-350 g) were given three daily injections of phenobarbital (i.p. 100 mg/Kg). Microsomes were prepared according to the method of Borton et al., (13). Protein was determined by the method of Lowry et al., (14). Electron impact mass spectra were obtained by a Varian model M7 mass spectrometer using a direct probe at 110° and an ionizing potential of 70 volts. Chemi ionization mass spectra were done by Dr. J. Daly (NIH) using a modified AES MS-9 mass spectrometer, a probe temperature of 100° and isobutane as the chemical agent. Difference spectra were determined using an Aminco Chance DW-2 spectrophotometer from the laboratory of Dr. Norman Bishop, Department of Botany and Plant Pathology, Oregon State University. Liquid chromatography was done on a Chromatronix model 3100 liquid chromatograph equipped with an ultraviolet flow detector (254 and 280 nm) and a Vydac Adsorbent column.

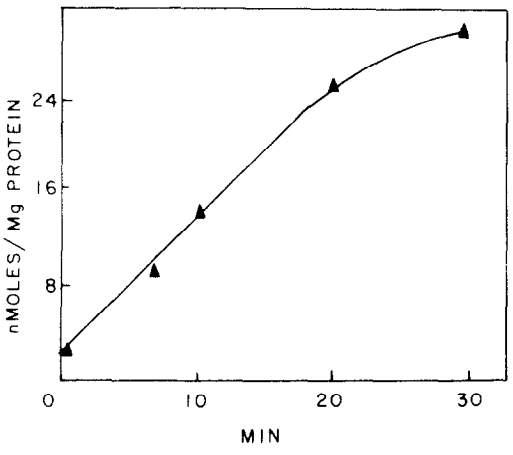


Figure 1. Time course of formation of hydroxylated CCNU.

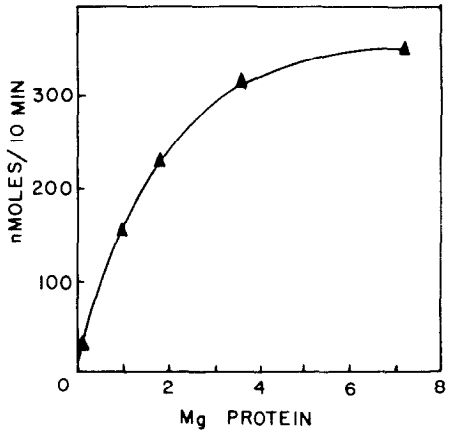


Figure 2. Effect of protein concentration on the rate of CCNU hydroxylation.

Results and Discussion

Incubation of liver microsomes from phenobarbital treated rats in the presence of NADPH-generating system caused a rapid disappearance of cyclohexyl [^{14}C]-CCNU and formation of a polar metabolite. The purified product was subjected to both electron impact and chemi ionization mass spectrometry and compared to the parent CCNU (Table 1).

Evidence accumulated thus far is all consistent with the CCNU metabolite being intact CCNU with monohydroxylation of the cyclohexyl ring. Comparison of the mass spectrum of CCNU with that of its metabolite (Table 1) shows that fragments 234-236, 205-207 (CI) and 126 (EI) (Scheme I) from the parent CCNU have corresponding fragments in the metabolite spectrum 16 mass units higher (250-252, 221-223 and 142). All of these fragments contain the cyclohexyl ring. The EI mass spectrum of the metabolite also gives evidence that dehydration of the cyclohexyl ring (142 → 124) is occurring during fragmentation in the mass spectrometer. The metabolite spectrum contains fragments which correspond to an unsaturated ring (124, protonated cyclohexenyl isocyanate and 81, cyclohexenyl ion) which are not present in the spectrum of parent CCNU. The corresponding structures with the saturated ring, 126 and 83

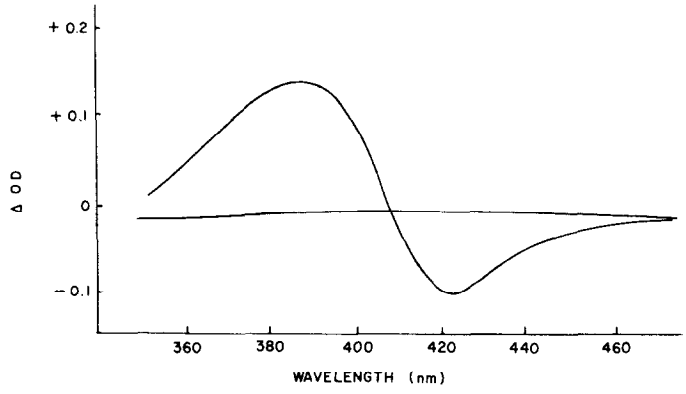


Figure 3. Difference spectrum of liver microsomes from phenobarbital treated rats after the addition of CCNU. Microsomes (8 mg protein) were added to 8 ml of 0.1 M Tris, pH 7.5. Equal aliquots (3 ml) were pipetted into cuvettes and base line determined. An acetone solution of CCNU (1mM final after 10 µl addition) was added to the sample cuvette and an equivalent volume of acetone was added to the reference cuvette.

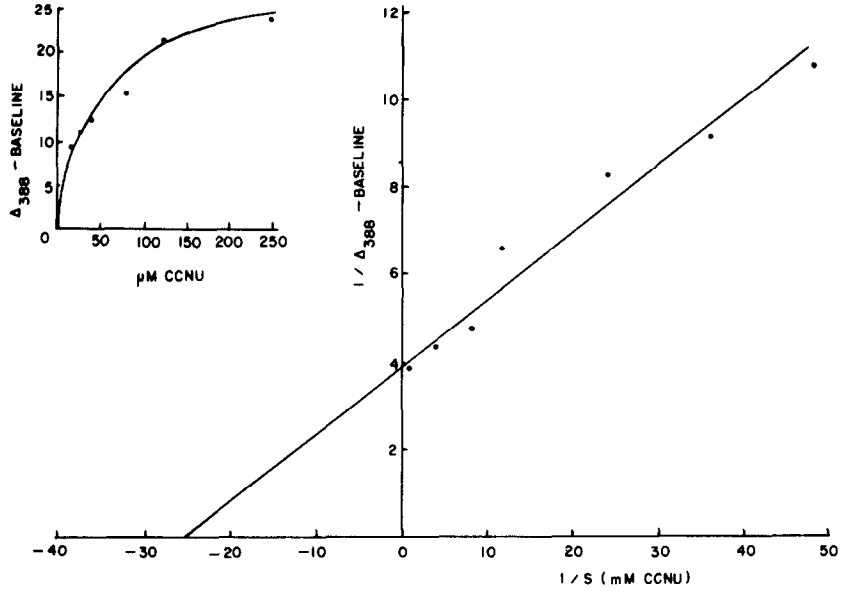


Figure 4. Ks of CCNU for phenobarbital-induced microsomes. Ks = -1/x-intercept or CCNU concentration at 1/2 maximal ΔOD (Inset).

respectively, are present in the CCNU spectrum but absent in that of the metabolite. The presence of large amounts of mass 57 in the metabolite spectrum which is absent in the CCNU spectrum is another indication of ring

TABLE 2

Substrate Requirements for Hydroxylation of CCNU by Rat Liver Microsomes.

Complete system contained in a final 3 ml volume: 0.1 M phosphate buffer pH 7.4, 1.0 μmole NADP, 25 μmoles glucose-6-PO_4, 1.2 units glucose-6-PO_4 dehydrogenase, 1.8 μmoles CH [^{14}C] -CCNU (12,600,000 dpm) and 2 mg microsomal protein from phenobarbital-induced animals except as indicated in experiment 2. Incubations were 10 min at 37°.

Modification of Complete System	*Ether Fraction dpm x 10^{-3}/mg protein/ml	**nmole product/ mg protein/min
Experiment 1		
None	114.4	-
Heated Microsomes	29.1	-
Minus Air, plus N_2	37.5	-
Minus NADP	20.5	-
Buffer only	21.0†	-
Experiment 2		
None	144.5	17.6
Minus NADPH gen. system	29.0	1.3
Normal Microsomes	54.8	4.0
Minus NADPH gen. system	30.1	1.3

* Extraction procedure given in Table 1.
** Based on purified metabolite radioactivity isolated by liquid chromatography on a Vydac column. See Table 1.
† Buffer dpm expressed as mg equivalent.

hydroxylation since 57 is a major fragment in the mass spectra of cyclohexanols (15). The chloroethyl group (63-65) and nitroso group (30) are present in both compounds. Other confirming lines of evidence for the intactness of the basic CCNU structure in the metabolite are: 1) the presence of the nitroso group by the method of Loo (16) and 2) cochromatography by thin layer and liquid chromatography of products derived from cyclohexyl [^{14}C]-labeled and 2-chloroethyl-[^{14}C]-labeled CCNU. Comparison of liquid chromatographic, mass spectral, and NMR data of the metabolite with that obtained from trans-4-hydroxy CCNU and cis-4-hydroxy CCNU strongly suggests that the metabolite is

TABLE 3

Effect of Carbon Monoxide on Microsomal Hydroxylation of CCNU

Conditions same as Table 2.

Gas Phase	Incubation time	Ether Fraction** dpm x 10^{-3}/mg protein/min	
Air	0	33.9	
Air	10	106.6	(72.7)
*20%O_2 + 80%CO	10	52.9	(19.0)
*20%)$_2$ + 80%N_2	10	85.1	(51.2)

* Gassing procedures were identical.
** Numbers in parentheses are corrected for zero time dpm.

cis-4-hydroxy CCNU. Preliminary experiments indicate that methyl-CCNU is also hydroxylated by liver microsomes to form several products.

The hydroxylating activity required NADPH and O_2, was inactivated by heating microsomes and was inducible (4-5 fold) by phenobarbital (Table 2). The activity was inhibited by 63% when the gas phase was 20% O_2 and 80% CO (Table 3).

Product formation was linear for about 20 min (Fig. 1) and up to about 0.7 mg protein/ml incubation (Fig. 2).

Phenobarbital induced microsomes gave a Type I difference spectrum (17) in the presence of CCNU (Fig. 3) and a spectrally determined K_s of 4 x 10^{-5} M (Fig. 4).

The substrate requirements, inhibition of enzyme activity by CO and the binding spectrum strongly imply that the drug metabolizing enzyme system of microsomes is responsible for the conversion of CCNU to its hydroxylated metabolite. The characteristics of the system are very similar to those described by Ullrich (8) for the hydroxylation of cyclohexane. He found a Type I binding for cyclohexane and a K_s value of 7.4 x 10^{-4} M. The K_s value for CCNU (4 x 10^{-5} M) compared to other Type I binding materials was lower than aminopyrine (3.3 x 10^{-4} M) and hexobarbital (1 x 10^{-4} M) but not as low as

SKF 525A (10^{-6} M) (18). The specific activity for CCNU hydroxylation was relatively high (17.6 nmole/min/mg) and somewhat less than that reported by Ullrich (8) for cyclohexane.

Work is currently in progress to determine the possible significance of these observations regarding the carcinostatic properties of CCNU and methyl CCNU.

Acknowledgement

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