Biochemical Pharmacology. Vol. 24, pp. 2153–2158. Pergamon Press. 1975. Printed in Great Britain.

# HYDROXYLATION OF 1-(2-CHLOROETHYL)-3-CYCLOHEXYL-1-NITROSOUREA

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(*Received* 2 *January* 1975; *accepted* 7 *March* 1975)

**Abstract**—Rat liver microsomal mixed function oxidase catalyzes the hydroxylation of the cyclohexyl moiety of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) to give at least five metabolites. When exposed to alkaline pH at 100° CCNU and its metabolites quantitatively release their cyclohexyl moiety as cyclohexylamine and aminocyclohexanol respectively. The *N*-(2,4-dinitrophenyl) derivatives of cyclohexylamine and aminocyclohexanols were separated by high pressure liquid chromatography. The metabolites *in vitro* and *in vivo* have been identified as *trans*-2-hydroxy CCNU, *cis*-3-hydroxy CCNU, *trans*-3-hydroxy CCNU, *cis*-4-hydroxy CCNU and *trans*-4-hydroxy CCNU. Ring hydroxylation axial to the 1-(2-chloroethyl)-1-nitrosourea group (*cis*-2-, *trans*-3-, *cis*-4-) is favored over equatorial attack (*trans*-2-, *cis*-3-, *trans*-4- ). Pretreatment of rats with phenobarbital leads to an increased rate of hydroxylation and a change in the relative amounts of the hydroxylated products. The significance of hydroxylation in relation to the antitumor activity of CCNU is discussed.

1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU, NSC-97037)* is a member of a group of halogenated alkyl-nitrosoureas [1] which have antineoplastic activity against a wide range of experimental and human tumors. Of particular significance is the activity of this class of compounds against intracerebral tumors [2]; a property which may be related to their lipid solubility. Although it has been shown that the haloalkyl-nitrosoureas possess both alkylating and carbamylating activity both *in vivo* [3] and *in vitro* [4], the active antineoplastic moiety has not been elucidated.

Two recent observations indicate a role of hepatic mixed function oxidase in the metabolism of CCNU. May *et al.* [5] have described enzymic hydroxylation of the cyclohexyl moiety and have identified a number of hydroxylated products,† and Bowie and Walker [6], from this laboratory, have observed a marked decrease in the toxicity of 1-(2-chloroethyl)- 3-(*trans*-4-methylcyclohexyl)-1-nitrosourea (methyl-CCNU) in mice with elevated hepatic mixed function oxidase activity after their pretreatment with phenobarbital.

Alkanes and cycloalkanes are known to be substrates for the hepatic microsomal mixed function oxidase. Heptane is hydroxylated to a mixture of isomeric heptanols [7], cyclohexane yields cyclohexanol [8], and cyclohexylamine is hydroxylated at the 2-, 3- and 4-positions [9]. The major urinary metabolite of *N*-cyclohexyl-*N*-methyl-(2-amino-3,5-dibromobenzyl) ammonium chloride (Bisolvon, Boehringer) is the *trans*-4-hydroxycyclohexyl compound [10].

It is the purpose of this report to describe the isolation and characterization of polar metabolites of CCNU *in vitro* and *in vivo*.

## MATERIALS AND METHODS

*Chemicals.* CCNU (NSC-97037) was obtained from the Cancer Chemotherapy National Service Center with the $^{14}C$-label in either the 2-chloroethyl moiety ([U-$^{14}C$-2-chloroethyl]-CCNU, 9·91 mCi/m-mole) or the cyclohexyl ring ([$^{14}C_1$-cyclohexyl]-CCNU, 11·33 mCi/m-mole). The specific activity of the labeled CCNU was adjusted by the addition of unlabeled compound. *Trans*-2-aminocyclohexanol–HCl was a gift from Smith, Kline & French Laboratories, Philadelphia, Pa. *Trans*-3-aminocyclohexanol and *cis*-3-aminocyclohexanol were obtained by the catalytic hydrogenation of 3-acetamindophenol in ethanol in the presence of 5% rhodium on Alumina at 3·5 atm pressure and 50° for 3 hr [11]. The *N*-acetyl group was removed with hot alkali, and the aminocyclohexanols were extracted into ether at alkaline pH. The isomers were separated as described by Burkhardt *et al.* [12]. *Trans*-3-aminocyclohexanol was obtained as the crystalline oxalate m.p. 194–195°, and the *cis*-3-aminocyclohexanol was obtained as the hydroperchlorate salt m.p. 135–140°. (Burkhardt *et al.* [12] gave m.p. 193–194° for *trans*-3-aminocyclohexanol oxalate and 144–145° for the *cis*-3-aminocyclohexanol hydroperchlorate.) Analysis of the *N*-(2,4-dinitrophenyl) derivative of the *trans*-isomer by high pressure liquid chromatography indicated a trace contamination by the *cis*-isomer, whereas the *cis*-isomer itself contained 11 per cent of the *trans*-form.

Commercial 4-aminocyclohexanol, a mixture of the *cis*- and *trans*-isomers, was obtained from Pfaltz & Bauer, Flushing, N.Y. *Cis*- and *trans*-4-aminocyclohexanols were separated by fractional crystallization of the benzamides in chloroform–hexane [13]. Crystalline *trans*-4-benzamidocyclohexanol m.p. 209–211° and *cis*-4-benzamidocyclohexanol m.p. 130–135° were

* Abbreviations used: *trans*-2-hydroxy CCNU, 1-(2-chloroethyl)-3-(*trans*-2-hydroxycyclohexyl)-1-nitrosourea; *cis*-3-hydroxy CCNU, 1-(2-chloroethyl)-3-(*cis*-3-hydroxycyclohexyl)-1-nitrosourea; *trans*-3-hydroxy CCNU, 1-(2-chloroethyl)-3-(*trans*-3-hydroxycyclohexyl)-1-nitrosourea; *cis*-4-hydroxy CCNU, 1-(2-chloroethyl)-3-(*cis*-4-hydroxycyclohexyl)-1-nitrosourea; *trans*-4-hydroxy CCNU, 1-(2-chloroethyl)-3-(*trans*-4-hydroxycyclohexyl)-1-nitrosourea.

† D. J. Reed, personal communication.



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obtained (Della and Jefferies [13] gave m.p. 211° for the *trans*-4-benzamidocyclohexanol and 136° for the *cis*-4-benzamidocyclohexanol). Analysis of the benzamides by high pressure liquid chromatography indicated that *cis*-4-benzamidocyclohexanol contained 6 per cent of the *trans*-isomer; the *trans*-4-benzamidocyclohexanol was pure.

*N*-[2,4-*dinitrophenyl*] (DNP-) *derivatives of aminocyclohexanols.* The crystalline *cis*- and *trans*-4-benzamidoaminocyclohexanols were hydrolyzed to the parent aminoalcohol by refluxing in 2·5 N NaOH for 2 hr, followed by extraction of the aminoalcohol into ether at alkaline pH. After drying the ether extract over anhydrous sodium sulfate, the solvent was evaporated to leave the aminocyclohexanol as a clear viscous liquid. The *cis*- and *trans*-4-aminocyclohexanol were heated at 100° for 40 min in 0·25 M sodium 2,4-dinitrophenylsulfonate dissolved in saturated sodium borate solution (pH 9·2), and the resulting DNP-aminoalcohols extracted into benzene [14].

The salts of *trans*-2-aminocyclohexanol, *cis*-3-aminocyclohexanol and *trans*-3-aminocyclohexanol were added directly to the solution of 2,4-dinitrophenylsulfonate in saturated sodium borate to yield the DNP-aminoalcohols which were extracted into benzene.

The DNP-derivatives of the five aminocyclohexanols and cyclohexylamine were separated by high pressure liquid chromatography.

*Alkaline degradation of CCNU and hydroxylated CCNU.* The addition of CCNU (amount expressed as nitroso equivalents) to a saturated solution of sodium borate containing 0·25 M sodium 2,4-dinitrophenylsulfonate at 100° leads, after 40 min, to the quantitative formation of DNP-cyclohexylamine. Similarly, addition of hydroxylated CCNU yields a mixture of DNP-aminocyclohexanols which were identified by comparison of their elution times with the elution times of the authentic DNP-aminocyclohexanols during high pressure liquid chromatography.

*Thin-layer chromatography.* Diethyl ether extracts of enzymic incubation systems or plasma were dried overnight at 0–4° with anhydrous sodium sulfate, evaporated at room temperature under reduced pressure and finally dried over phosphorous pentoxide *in vacuo*. The residues containing nitrosoureas were dissolved in anhydrous acetone and applied to Silica gel plates containing fluorescent U.V. indicator (E. M. Laboratories, Elmsford, N.Y.). Plates, equilibrated for 30 min in the solvent atmosphere, were developed for 15 cm in 60% dichloromethane–20% tetrahydrofuran–20% toluene (v/v).

*High pressure liquid chromatography.* Separation of benzamides and DNP-derivatives of cyclohexylamine and aminocyclohexanols was achieved by chromatography on 100 cm × 2 mm Micropak Si-10 using a 4200 Series high pressure liquid chromatograph equipped with multiple wavelength u.v.-visible detector (Varian Instrument Division, Palo Alto, Calif.). Benzamides were eluted by 70% dichloroethane–30% tetrahydrofuran (v/v) and detected by their absorption at 230 nm. DNP-amines were eluted by 85% dichloroethane–15% tetrahydrofuran (v/v) and detected by their absorption at 350 nm. Peak elution times were recorded and peak areas integrated by an Autolab System IV integrator (Spectra–Physics, Mountain View, Calif.).

*Animals.* Male, Fischer strain rats (Charles River Breeding Labs., Wilmington, Mass.) were maintained under controlled lighting conditions of 12-hr light and 12-hr dark, housed over paper tray liners with food and water available at all times. Hepatic microsomal mixed function oxidase activity was elevated by i.p. administration of phenobarbital (80 mg/kg) on 3 successive days. Control animals received an equivalent volume of saline.

*CCNU metabolism* in vitro. Washed microsomes were prepared from minced liver which was homogenized in 5 vol. 1·15% (w/v) KCl solution. The homogenate was centrifuged at 9000 *g* for 10 min and the resulting supernatant solution centrifuged at 105,000 *g* for 60 min to yield a microsomal pellet. The microsomal pellet was washed once by homogenization in 1·15% KCl and recentrifugation at 105,000 *g* for 30 min. The final microsomal pellet was resuspended in a volume of 1·15% KCl such that the microsomes from 1 g liver were contained in 1 ml.

The rate of CCNU metabolism was determined by measurement of the amount of [$^{14}$C]-CCNU remaining after 10 min of incubation with rat liver microsomes and an NADPH-generating system containing NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase. The remaining [$^{14}$C]-CCNU was extracted with 5 vol hexane at 0° for 10 min. The radioactivity of a portion of the hexane extract was determined and the amount of [$^{14}$C]-CCNU remaining after incubation was calculated from a knowledge of its specific radioactivity. The rate of loss of hexane extractable parent compound represents the sum of the rates of enzymic hydroxylation and non-enzymic breakdown. The rate of non-enzymic breakdown was determined in reaction mixtures containing microsomes but lacking the NADPH-generating system and the true rate of enzymic hydroxylation obtained by subtraction.

For the determination of $K_m$ and $V_{max}$, reaction mixtures were incubated for 5 min with five CCNU concentrations ranging from 0·067 to 0·5 mM. Reaction mixtures contained either microsomes from phenobarbital-pretreated rats (0·2 mg microsomal protein/ml of reaction mixture) or microsomes from saline-pretreated rats (0·5 mg microsomal protein/ml of reaction mixture). Approximately 10 per cent of the substrate was metabolized during the incubation period.

*CCNU metabolism* in vivo. CCNU was administered to 150–200 g rats in ethanol–fat emulsion* (1 : 9) at a dose of 30 mg/kg by tail vein (5 ml/kg) as a single 15-sec push. Blood samples obtained by cardiac puncture were drawn into heparinized syringes and kept at 0–4° during the preparation of plasma. The plasma was subjected to high speed centrifugation to separate lipid material which migrated to form a pellicle at the surface of the plasma. CCNU and its hydroxylated metabolites were obtained by three 10-min extractions of the plasma with 3 vol. diethyl ether at 0–4°.

* Fat emulsion containing in water, soybean oil (10%, w/v), glycerol (2·25%, w/v) and egg yolk phospholipid (1·2%, w/v); supplied as Intralipid, Cutter, Berkeley, Calif.

*Other methods.* Nitroso groups were determined by the method of Loo and Dion [15]. The method was successfully applied directly to scrapings from thin-layer chromatograms by incubating the scrapings at 50° for 40 min in 0·5 ml of 0·5% sulfanilamide (w/v) in 2 N HCl plus 1·0 ml water. After cooling, 0·1 ml of 0·3% *N*-naphthylethylenediamine diHCl (w/v) was added and the solution clarified by centrifugation. The absorbance at 540 nm was determined after 10 min. Suitable areas of the chromatogram which did not contain nitrosated products were treated identically to give a background value for the method.

Protein was determined by the biuret method. Radioactivity was measured in a Packard Tri-Carb model 3375 liquid scintillation spectrometer. CCNU and its metabolites dissolved in small volumes of acetone were added to 10 ml Aquasol (New England Nuclear, Boston, Mass.). Counting efficiency was 86 per cent. Radioactivity from thin-layer chromatograms of [$^{14}$C]-CCNU and its metabolites was determined by the direct addition of scraped sections of the chromatograph to 10 ml Aquasol. Counting efficiency was 78 per cent.

## RESULTS

CCNU is rapidly metabolized during incubation with rat liver microsomes and a source of NADPH. The disappearance of the parent compound is dependent on the presence of microsomes and NADPH and is inhibited by carbon monoxide (Table 1). Non-enzymic breakdown of CCNU with loss of nitroso function occurs during the incubation at 37°. The chemical half-life of CCNU and related nitrosated metabolites under the conditions of the mixed function oxidase assay was 51 min. The half-life was determined by assay of nitroso content of the incubation system at intervals during a 60-min incubation at 37°. The value was not affected by the presence or absence of the NADPH-generating system. This implies that hydroxylation of the cyclohexyl moiety of CCNU does not alter the chemical lability of the nitrosourea.

The initial rates of hydroxylation of CCNU in the mixed function oxidase system were analyzed by the method of Lineweaver and Burk [16]. With hepatic microsomes from saline-pretreated animals, values of $V_{max}$, 42·5 nmoles CCNU hydroxylated/min/mg of microsomal protein, and $K_m$, 0·42 mM, were obtained. With hepatic microsomes from phenobarbital-pretreated animals, the values were $V_{max}$, 67·5 nmoles CCNU hydroxylated/min/mg of microsomal protein, and $K_m$, 0·24 mM.

Table 1. Requirements for CCNU metabolism*

| Conditions | Rate CCNU metabolism† |
|---|---|
| Complete | 23·3 |
| Minus NADPH | 0·5 |
| Minus microsomes | 0·0 |
| Complete plus CO | 12·7 |

* The complete incubation system contained: 0·05 M Tris-HCl buffer, pH 7·4, 0·4 mM NADP, 5 mM glucose 6-phosphate, 5 mM $MgCl_2$, 0·3 units/ml of yeast glucose 6-phosphate dehydrogenase, 0·5 mg microsomal protein/ml and 0·5 mM [$^{14}$C-cyclohexyl]-CCNU (25 $\mu$Ci/m-mole). Incubation was for 10 min. Complete minus NADPH conditions were achieved through the omission of NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase. In complete plus CO, 50% carbon monoxide–50% air replaced air as the gas phase.

† Expressed as nmoles/mg of microsomal protein/min.

Table 2. Thin-layer chromatography of ether-soluble products of CCNU metabolism *in vitro**

| Time (min) | $R_f$ | Per cent total cpm applied | |
|---|---|---|---|
| | | [$^{14}$C-chloroethyl]-CCNU | [$^{14}$C-cyclohexyl]-CCNU |
| 0 | 0·60 | 91·5 | 91·6 |
| | 0·53 | 1·7 | 0·2 |
| | 0·37 | 3·8 | 4·2 |
| | 0·07 | 1·5 | 2·4 |
| 2 | 0·60 | 57·3 | 54·9 |
| | 0·53 | 4·9 | 5·3 |
| | 0·37 | 32·4 | 35·6 |
| | 0·07 | 2·3 | 3·1 |
| 5 | 0·60 | 2·4 | 1·2 |
| | 0·53 | 8·9 | 10·1 |
| | 0·37 | 77·3 | 77·7 |
| | 0·07 | 5·3 | 7·6 |
| 10 | 0·60 | 1·0 | 0·6 |
| | 0·53 | 11·6 | 14·0 |
| | 0·37 | 71·7 | 72·1 |
| | 0·07 | 7·8 | 8·3 |
| 20 | 0·60 | 0·3 | 0·6 |
| | 0·53 | 17·6 | 16·3 |
| | 0·37 | 70·5 | 70·5 |
| | 0·07 | 8·2 | 8·2 |

* CCNU, [$^{14}$C]-labeled in either the chloroethyl or cyclohexyl moieties (0·1 mM, 100 $\mu$Ci/m-mole), was incubated for the times indicated under conditions described as 'complete' in Table 1. Ether extracts of the incubation mixtures were chromatographed on Silica gel with 60% dichloromethane–20% tetrahydrofuran–20% toluene (v/v) and the developed plates analyzed for radioactivity in sequential 0·5-cm sections. Recovery of radioactivity from the chromatograms averaged 96 per cent (range 92–99 per cent). The major components separated by thin-layer chromatography were subsequently identified by the formation of the DNP-derivatives of their cyclohexyl moieties: $R_f$ 0·60, CCNU; $R_f$ 0·53, *trans*-2-hydroxy CCNU; $R_f$ 0·37, *trans*-3-hydroxy CCNU, *cis*-4-hydroxy CCNU and *trans*-4-hydroxy CCNU; $R_f$ 0·07 was not identified.

Analysis by thin-layer chromatography of the CCNU metabolites produced during incubation of [$^{14}$C]-CCNU with microsomes reveals the presence of one major and two minor polar metabolites. The data in Table 2, which expresses the distribution of radioactivity derived from the parent compound as a percentage of the total radioactivity applied to the chromatogram, indicate the near complete loss of parent compound within the first 5 min of incubation. This rapid loss is accompanied by an equally rapid appearance of a metabolite with an $R_f$ of 0·37 together with the slower appearance of metabolites with $R_f$ values of 0·53 and 0·07. Colorimetric determination of nitroso function demonstrated that the parent compound and the three metabolites are nitrosated. Analysis of assays containing either [$^{14}$C-chloroethyl]-CCNU or [$^{14}$C-cyclohexyl]-CCNU (Table 2) indicates a similar distribution of radioactivity throughout the metabolites at all times during the

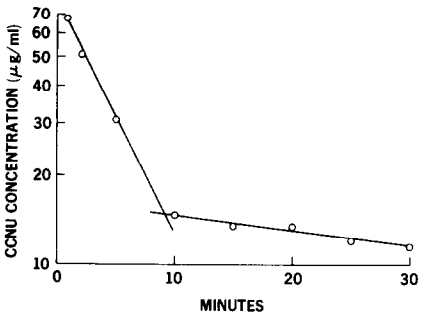


Fig. 1. Plasma disappearance of CCNU and nitrosated metabolites during 30 min, after i.v. injection of 30 mg/kg of CCNU. Each point represents the average plasma concentration from two or three animals.

incubation, which strongly suggests that the nitrosourea remains intact but is modified in polarity by the addition of polar groups.

After i.v. injection of CCNU in rats, the plasma level of CCNU and related nitrosated metabolites falls exponentially with a half-life of 6·5 min during the initial distributive phase (Fig. 1). Thereafter, the plasma level falls exponentially with a half-life of 47 min—a value similar to that found during incubation *in vitro* at pH 7·4 and 37°. Wheeler *et al.* [4] have obtained a chemical half-life of 53 min for CCNU in 0·1 M phosphate buffer, pH 7·4, at 37°.

Analysis by thin-layer chromatography of ether-soluble products from the plasma of rats receiving CCNU i.v. (Fig. 2) indicates a rapid loss of parent compound ($R_f$ 0·60) with the equally rapid appearance of one major metabolite ($R_f$ 0·37) and the slow appearance of radioactivity at $R_f$ 0·07. The pattern and rate of appearance of the products *in vivo* are very similar to those observed *in vitro* with the exception that the $R_f$ 0·53 product *in vitro* is not clearly separated in plasma extracts, probably being included in the broad region ($R_f$ 0·37) containing the bulk of the hydroxylated metabolites. Within 5 min after a rapid intravenous injection of [$^{14}$C-cyclohexyl]-CCNU (30 mg/kg, approximately $LD_{10}$) in control animals, the ether-soluble radioactivity in plasma is about equally distributed between the parent compound and its major metabolite, with a small fraction present at $R_f$ 0·07. A similar distribution of radioactivity (Fig. 2) exists within approximately 1 min in animals which have been pretreated with phenobarbital.

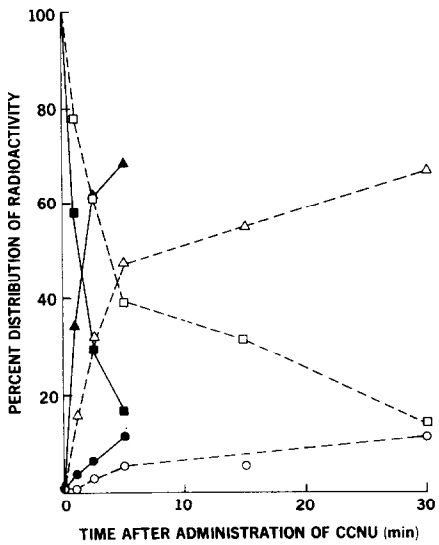


Fig. 2. Distribution of radioactivity from [$^{14}$C-cyclohexyl]-CCNU in rat plasma at various times after i.v. injection. The nitrosoureas contained in ether extracts of plasma were subjected to thin-layer chromatography, and the distribution of radioactivity between the parent compound ($R_f$ 0·60) and two polar metabolites ($R_f$ 0·37 and 0·07) was determined. Open symbols (○, △, □) refer to saline-treated animals; closed symbols (●, ▲, ■) to phenobarbital-pretreated animals. $R_f$ 0·60, □■; $R_f$ 0·37, △ ▲; $R_f$ 0·07, ○ ●. Each point represents the average per cent distribution of radioactivity between CCNU and its metabolites obtained from plasma of two to four animals.

The identity of the major metabolites of CCNU has been determined by the generation of DNP-derivatives of the cyclohexylamine moiety which is quantitatively released during exposure of the nitrosourea to alkaline conditions at elevated temperature. Comparison of these products with authentic DNP-derivatives of cyclohexylamine and aminocyclohexanols was made by high pressure liquid chromatography, each

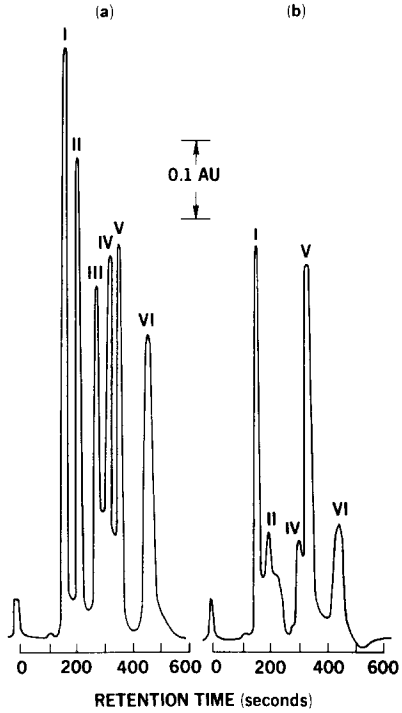


Fig. 3. (a) High pressure liquid chromatography of DNP-aminocyclohexanols on 100 cm × 2 mm Micropak Si–10 column eluted at 1 ml/min (4000 psi) with 85% dichloroethane–15% tetrahydrofuran (v/v) and effluent monitored at 350 nm. Sample in 1 μl benzene contained 1·5 nmoles each of DNP-cyclohexylamine (I), DNP-*trans*-2-aminocyclohexanol (II), DNP-*cis*-3-aminocyclohexanol (III), DNP-*trans*-3-aminocyclohexanol (IV), DNP-*cis*-4-aminocyclohexanol (V) and DNP-*trans*-4-aminocyclohexanol (VI). (b) High pressure liquid chromatography of DNP-derivatives of aminocyclohexanols generated during the alkaline degradation of hydroxylated CCNU extracted from the plasma of a phenobarbital-pretreated rat 2 min after i.v. injection of CCNU. Sample in 1 μl benzene equivalent to 200 μl extracted plasma. Conditions and designation of compounds I to VI as in Fig. 3a.

Table 3. Hydroxylated metabolites of CCNU, *in vitro* and *in vivo**

| Condition | % DNP-aminocyclohexanols† | | | | | |
|---|---|---|---|---|---|---|
| | *trans*-2-OH | *cis*-3-OH | *trans*-3-OH | *cis*-4-OH | *trans*-4-OH | % CCNU metabolized |
| *In vitro* | | | | | | |
| Control microsomes (40 min)‡ | 13·8 | trace | 30·8 | 52·8 | 2·6 | 77 |
| Phenobarb. microsomes (20 min) | 3·1 | 3·3 | 11·3 | 77·4 | 4·7 | 87 |
| *In vivo* | | | | | | |
| Control rats (20 min) | 8·0 | 3·9 | 22·6 | 54·0 | 11·5 | 96 |
| Phenobarb. rats (2 min) | 7·8 | trace | 9·3 | 61·5 | 21·4 | 78 |
| Phenobarb. rats (10 min) | 7·3 | trace | 11·7 | 50·9 | 30·1 | 85 |

* Metabolites *in vitro* were generated in the mixed function oxidase system described in the legend to Table 1, containing 1 mg/ml of microsomal protein and 0·4 mM CCNU. Metabolites *in vivo* were extracted from defatted plasma of animals which received i.v. CCNU (30 mg/kg). The nitrosoureas were extracted into ether at 0°, the ether was removed at room temperature and the residue heated at 100° for 40 min in saturated sodium borate solution containing 0·25 M 2,4-dinitrophenylsulfonate. The resultant DNP-aminocyclohexanols were separated by high pressure liquid chromatography and identified by comparison of their elution times with those of authentic DNP-aminocyclohexanols.

† DNP-derivatives of *trans*-2-aminocyclohexanol (*trans*-2-OH), *cis*-3-aminocyclohexanol (*cis*-3-OH), *trans*-3-aminocyclohexanol (*trans*-3-OH), *cis*-4-aminocyclohexanol (*cis*-4-OH) and *trans*-4-aminocyclohexanol (*trans*-4-OH).

‡ *In vitro*, time of incubation at 37°; *in vivo*, time after i.v. administration of CCNU at which blood samples were taken.

DNP-derivative having a reproducible, characteristic elution time (Fig. 3).

Hydroxylation of the cyclohexane ring of CCNU occurs at positions 2, 3 and 4 to produce, at least, the five hydroxylated CCNUs which yield the DNP-aminoalcohols analyzed (Table 3). *Cis*-4-hydroxy CCNU, yielding DNP-*cis*-4-aminocyclohexanol, is the major metabolite formed both *in vivo* and *in vitro*, making up more than half of the metabolites identified. The pattern of hydroxylation *in vitro* is dependent upon the source of microsomes. Microsomes from saline-treated animals produce significant amounts of *trans*-3-hydroxy CCNU (30 per cent) and *cis*-4-hydroxy CCNU (52·8 per cent) with a smaller amount of *trans*-2-hydroxy CCNU (13·8 per cent) and trace amount of *cis*-3-hydroxy and *trans*-4-hydroxy CCNU. Liver microsomes from phenobarbital-pretreated animals produce almost exclusively *cis*-4-hydroxy CCNU (77·4 per cent) with some *trans*-3-hydroxy CCNU (11·3 per cent) and small amounts of *trans*-2-hydroxy, *cis*-3-hydroxy and *trans*-4-hydroxy CCNU (3·1, 3·3 and 4·7 per cent respectively).

The pattern of hydroxylation *in vivo* is similarly dependent on the nature of pretreatment. Rats which have received saline pretreatment produce significant amounts of all five hydroxylated metabolites and in proportions similar to those found *in vitro*, with the exception of the larger amount of *trans*-4-hydroxy CCNU formed *in vivo*. In phenobarbital-pretreated animals, *cis*-4-hydroxy CCNU is the major product in a distribution which resembles the situation *in vitro*, but again with a much larger proportion of *trans*-4-hydroxy CCNU being formed *in vivo* than *in vitro*.

## DISCUSSION

Microsomal hydroxylation of the cyclohexyl moiety of CCNU is extremely rapid. More than 80 per cent of a carcinostatic dose of 30 mg/kg is metabolized in the rat within 30 min. The enzymic character of the hydroxylation indicates it is of a mixed function oxidase type, being dependent on a supply of NADPH and being inhibited by carbon monoxide. The enhanced rate of metabolism per mg of microsomal protein by liver microsomes derived from phenobarbital-pretreated animals is also consistent with hydroxylation by a mixed function oxidase. May *et al.* [5] have reported similar data on the microsomal hydroxylation of CCNU *in vitro* together with mass spectra data to indicate ring hydroxylation of CCNU and demonstration of a type I binding spectrum [17] when CCNU is added to microsomes.

The pattern of ring hydroxylation indicates that attack on the cyclohexyl ring is primarily axial to the 1-(2-chloroethyl)-1-nitrosourea group, with the predominant formation of *trans*-3-hydroxy CCNU and *cis*-4-hydroxy CCNU. Of the possible products of equatorial hydroxylation, only *trans*-2-hydroxy CCNU is formed in a significant amount *in vitro*, although equatorial attack at the 4-position to give *trans*-4-hydroxy CCNU occurs extensively *in vivo* and especially in animals pretreated with phenobarbital. The change in the position and direction of attack which occurs after phenobarbital pretreatment is evident both *in vitro* and *in vivo* and is probably related to the decrease in apparent $K_m$ for CCNU found *in vitro* with microsomes from phenobarbital-pretreated animals. While in both saline-treated and phenobarbital-pretreated animals *cis*-4-hydroxy CCNU is the major metabolite (54 and 61·5 per cent, respectively), the next most abundant metabolite is *trans*-3-hydroxy CCNU (22·6 per cent) in saline-treated animals but *trans*-4-hydroxy CCNU (21·4 per cent) after phenobarbital pretreatment. Similar changes in the pattern of hydroxylation after phenobarbital pretreatment have been reported for the microsomal hydroxylation of *N*-2-fluorenylacetamide [18], *n*-heptane [7] and the *n*-butyl side chain of *N*-*n*-butylbarbital [19].

The most striking implication of the rapid hydroxylation of CCNU relates to its bearing on the carcinostatic properties of the parent compound and the metabolites. The carcinostatic agent of the nitrosoureas is not known, although it is believed to be a reactive intermediate generated during the chemical breakdown of the molecule. Hydroxylation of the cyclohexyl moiety of CCNU probably does not influence the generation of the agent but will certainly influence such important parameters as protein binding, tissue distribution and mode and rate of excretion. Information on how these parameters influence the carcinostatic activity of the cyclohexyl-substituted nitrosoureas should lead to a better understanding of the mode of action of the nitrosourea antitumor agents.

*Acknowledgements*—We wish to acknowledge the technical assistance of Deborah M. Zito and helpful discussions on chemical aspects of this work with Dr. T. Karpetsky.

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