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Differential distribution and covalent binding of two labeled forms of methyl-CCNU in the Fischer 344 rat

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Summary. *The present study compares the organ distribution and covalent binding of MeCCNU labeled either within the carbamylating ([cyclohexyl-1-^{14}C]MeCCNU; Chx-^{14}C-MeCCNU) or alkylating ([2-chloroethyl-1,2-^{14}C]MeCCNU; Cle-^{14}C-MeCCNU) region of the compound in an animal model shown to be suitable for studying the nephrotoxicity of the nitrosoureas. Extraction of tissue homogenates with organic solvents of increasing polarity, and subsequent analysis of these extracts by HPLC showed fat to accumulate the highest concentration of parent compound. Kidney accumulated the highest levels of the more polar ether- and methanol-extractable metabolites and/or degradation products of either cyclohexyl-derived or chloroethyl-derived ^{14}C-MeCCNU. Striking differences were apparent in the accumulation, degradation and/or metabolism, and tissue distribution of covalently bound radioactivity for the chloroethyl and cyclohexyl moieties. For example, approximately twice as much cyclohexyl-derived ^{14}C was bound covalently to protein of kidney than to protein of liver or lung. In contrast, approximately twice as much chloroethyl-derived ^{14}C was bound to lung protein than to liver of kidney protein. No radioactivity was bound covalently to tissue DNA following Chx-^{14}C-MeCCNU administration. On the other hand, at 4 h, chloroethyl-derived ^{14}C was irreversibly bound to DNA in the relative amounts of kidney (5.0 nmol/mg), liver (2.7 nmol/mg), and lung (1.25 nmol/mg). These results demonstrate that MeCCNU metabolites and/or degradation products are preferentially accumulated in kidney, a primary target organ for MeCCNU toxicity. Moreover, kidney protein and DNA were subject to extensive carbamylation and alkylation reactions as measured by irreversibly bound cyclohexyl-derived and chloroethyl-derived ^{14}C, respectively. These data suggest that the extent of irreversibly bound drug to tissue macromolecules may be a valid predictor of MeCCNU toxicity. However, the relative toxicological significance of either protein carbamylation or DNA alkylation in mediating MeCCNU-induced nephropathy is not yet understood.*

Abbreviations: MeCCNU, 1-(2-chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea; Chx-^{14}C-MeCCNU, [cyclohexyl-1-^{14}C]-MeCCNU; Cle-^{14}C-MeCCNU [2-chloroethyl-1,2-^{14}C]-MeCCNU]; HPCL, high performance liquid chromotography; PAH, p-aminohippuric acid

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Introduction

1-(2-Chloroethyl)-3-(trans-4-methylcyclohexyl)-1-nitrosourea (MeCCNU; semustine) is a widely employed investigational anticancer drug that has been used in the treatment of certain advanced cancers. Preclinical toxicity studies showed the kidney to be a target organ for MeCCNU toxicity [6, 20]. However, early clinical trials did not show MeCCNU to be nephrotoxic [16, 26, 27]. More recently, however, a relationship between MeCCNU and a delayed irreversible and often fatal nephrotoxicity in patients has become established [25]. A suitable animal model for studying this problem in the Fischer 344 rat has been developed [14].

MeCCNU is known to decompose spontaneously in aqueous solutions to form carbonium ion alkylating agents as well as isocyanates that are capable of carbamylation reactions (Fig. 1) [17, 18]. MeCCNU also is known to be rapidly metabolized in the liver by a cytochrome P-450-dependent reaction [15]. A number of previous studies have shown that the antitumor activity of MeCCNU is due to alkylation and subsequent cross-linking of nucleophilic sites in DNA by the 2-chloroethyl carbonium ion [4, 13], which along with the isocyanate comprises the major reactive intermediates of MeCCNU. It has previously been suggested by others that carbamylation is unnecessary for the antitumor activity of the nitrosoureas and that carbamylation reactions may be responsible for unwanted toxic side-effects to normal host tissue [11].

More detailed and specific analyses of the formation, distribution, and covalent binding of metabolites and degradation products of MeCCNU in various tissues in a relevant in vivo model are needed. To address these goals, the following tissue distribution and binding studies were conducted in Fischer 344 rats administered nephrotoxic doses of MeCCNU labeled within either the carbamylating ([cyclohexyl-1-^{14}C]MeCCNU) or the alkylating ([2-chloroethyl-1,2,^{14}C]) MeCCNU) region of the compound.

Methods

Male Fischer 344 rats (125–150 g; Taconic Farms, Germantown, NY) received either cyclohexyl-1-^{14}C-MeCCNU (40 mg/kg, 0.5 mCi/m*M*) or 2-chloroethyl-1,2-^{14}C-MeCCNU (40 mg/kg, 0.1 mCi/m*M*) by SC injection, dissolved in sesame oil (Sigma Chemical Co., St. Louis, Mo). At each time interval (0.5, 1, 2, 4, 8, 12, and 24 h), the rats (5 per group) were sacrificed by cervical dislocation and the livers, kidneys, lungs, fat, and blood were removed. Each tissue sample was

then placed in 2 vol of H_2O and homogenized. An aliquot of the homogenate was digested with protosol (New England Nuclear, Boston, Mass) and counted by liquid scintillation spectrometry (Tracor Analytic, Elk Grove, Ill) for the determination of total tissue levels of MeCCNU. The remaining homogenate was extracted sequentially with hexane (3 × 2 vol), ether (3 × 2 vol), and finally methanol, until radioactivity in the supernatant reached background levels. An aliquot of each extract was counted and the nanomoles of extractable MeCCNU equivalents per gram of wet tissue weight were calculated. Another aliquot of extract was used for the determination of parent compound by reverse-phase HPLC (Radial-Pak, μ-Bondapak C_{18} column; Waters associates) using an isocratic solvent system of acetonitrile and H_2O (1 : 1) at the rate of 2 ml/min. The 60-s fractions containing the radiolabeled MeCCNU metabolites and/or degradation products and the unchanged MeCCNU were collected directly into scintillation vials and counted. The remaining pellet was resuspended in 0.6 *N* perchloric acid at 70° C for 30 min and any undissolved material was sedimented by centrifigation at 1,500 rpm for 10 min. An aliquot of the supernatant was counted, and a second aliquot was used to determine the DNA concentration [3]. The irreversible binding to this fraction was calculated as nanomoles of MeCCNU equivalents bound per milligram of DNA. The final pellet was solubilized overnight in 1 *N* NaOH at 37° C. An aliquot was counted and another was used for protein determination [1]. Covalent binding to protein was calculated as picomoles of MeCCNU equivalents bound per milligram of protein.

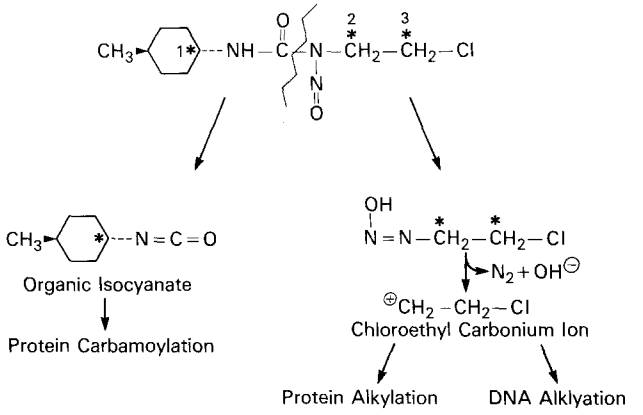

Fig. 1. Abbreviated scheme for the nonenzymatic degradation of MeCCNU. ★ Denotes position of ^{14}C label: position 1, [cyclohexyl-1-^{14}C]-; positions 2 and 3, [2-chloroethyl-1,2-^{14}C]MeCCNU [2]

Results

Organ distribution of total drug

Radiolabel derived from Chx-^{14}C-MeCCNU reached peak levels in kidney and liver within 1 h and exhibited a biphasic biological half-live in kidney of 9 and 20 h, respectively (Fig. 2A). The area under the curve for kidney was 191% and 346% greater than that for liver and lung, respectively (Table 1). Cle-^{14}C-MeCCNU reached peak levels in all tissues at 12 h, and was cleared slowly from kidney with a terminal-phase, half-life of 23 h. The area under the curve for Cle-^{14}-MeCCNU was 251% and 625% greater in kidney than in liver or lung, respectively. In kidney, the area under the curve for Cle-^{14}C-MeCCNU was 130% greater than that for Chx-^{14}C-MeCCNU. In liver the ratio of Cle-^{14}C-MeCCNU to Chx-^{14}C-MeCCNU was 0.99, and that in lung was 0.73.

Distribution of parent compound

Unchanged ^{14}C-MeCCNU was entirely removed by hexane extraction of the organ homogenates as shown by HPLC. Further extraction by ether and methanol failed to remove any more parent compound. Hexane extracted not only parent drug, but an unidentified metabolite and/or degradation product that was less lipophlic than MeCCNU. This unidentified metabolite accounted for 10% of the hexane-extractable radioactivity in fat and up to 75% of the Chx-^{14}C-MeCCNU-derived hexane-extractable radioactivity in kidney. Only trace amounts of this derivative were found in kidney following administration of Cle-^{14}C-MeCCNU. The greatest concentrations of parent drug were found in fat (Fig. 2B and Table 1) which accumulated 10 times more parent drug (per gram wet weight/24 h) than either lung or kidney, and 37 times more than liver.

Table 1. Accumulation at 24 h of total and ether-, hexane-, and methanol-extractable radioactivity derived from either Chx-^{14}C-MeCCNU (40 mg/kg SC) or Cle-^{14}C-MeCCNU (40 mg/kg SC) in the F344 rat

Tissue	Position of radiolabel	$AUC_0^{24\,hr}$ (nmol/g)[a]							
		ΣAUC	% Kidney	AUC_{hexane}	% Kidney	AUC_{ether}	% Kidney	AUC_{MeOH}	% Kidney
Kidney	Chx	1380[b]	–	145 (11)[c]	–	444 (32)	–	791 (57)	–
	Cle	1790	–	44 (2)	–	37 (2)	–	1706 (96)	–
Liver	Chx	632	46[d]	52 (8)	36	127 (20)	29	453 (72)	57
	Cle	636	36	12 (2)	27	16 (3)	43	608 (96)	36
Lung	Chx	423	31	121 (29)	83	105 (25)	24	197 (47)	25
	Cle	308	17	37 (12)	84	13 (4)	35	169 (55)	10
Fat	Chx	485	35	372 (77)	257	85 (18)	19	28 (6)	4
	Cle	546	31	443 (81)	1007	57 (13)	154	46 (8)	3

[a] Areas under the curves were calculated using the trapezoidal rule and were expressed as nmol/g wet tissue weight
[b] Mean of five animals; standard errors were less than 10% of the respective means
[c] Numbers in parenthesis represent the percentage of total radioactivity/g wet weight
[d] Expressed as percentage of radioactivity obtained from the equivalent kidney extract

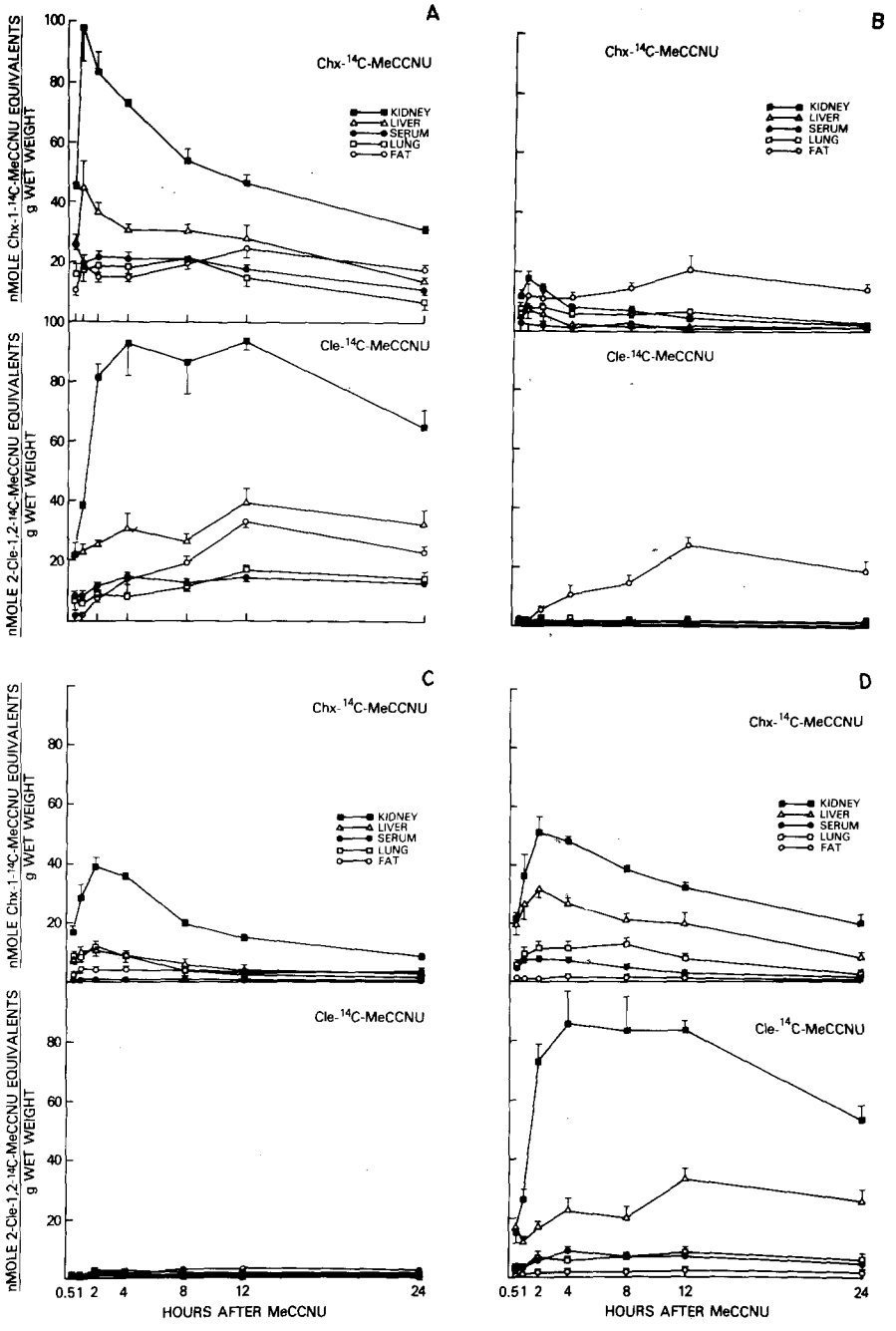

Fig. 2A–D. Tissue distribution of radiolabeled compounds at various times after the administration of Chx-^{14}C-MeCCNU and Cle-^{14}C-MeCCNU. Rats were given single SC injections of either Chx-^{14}C 40 mg/kg or Cle-^{14}C-MeCCNU 40 mg/kg and sacrificed at the times indicated. Each value is the mean ± SEM of 5 rats. **A** Total radiolabel; **B** hexane-extractable radiolabel; **C** ether-extractable radiolabel; **D** methanol-extractable radiolabel

Distribution of ether- and methanol-extractable radioactivity

MeCCNU was rapidly degraded in vivo. Parent compound accounted for < 3% of the recoverable radioactivity in serum, liver, lung, or kidney within 30 min after SC drug administration (Fig. 2B and Table 1). Kidney accumulated the highest levels of the more polar ether- and methanol-extractable metabolites and/or degradation products derived from either Chx- or Cle-^{14}C-MeCCNU (Fig. 2C and D and Table 1). The chloroethyl moiety of MeCCNU was apparently cleaved rapidly from the intact molecule, as 96% of chloroethyl-derived radioactivity in liver, serum, or kidney was removed only by methanol extraction (Fig. 2D and Table 1). In contrast, considerable amounts of Chx-^{14}C-MeCCNU-derived radioactivity were removed by extraction with ether (Fig. 2C and Table 1). Ether-extractable radioactivity accounted for 32%, 25%, and 20% of the total radioactivity derived from Chx-^{14}C-MeCCNU in kidney, lung, and liver, respectively (Table 1). Ether-extractable radiolabel derived from Chx-^{14}C-MeCCNU was 3.5 and 4.2 times greater in kidney than in liver or lung, respectively. Methanol-extractable Chx-^{14}C-MeCCNU was 1.75 and 4 times higher in kidney than in liver and lung, respectively. The highest tissue levels of extractable radioactivity were obtained in the methanol extracts of kidney following Cle-^{14}C-MeCCNU administration. The kidney accumulated a concentration of MeOH-extractable Cle-^{14}C-MeCCNU that was 2.2 times that of methanol-extractable Chx-^{14}C-MeCCNU. Furthermore, kidney levels of the methanol-extractable Cle-^{14}C-MeCCNU were 2.8 and 10 times the concentrations found in liver and lung, respectively.

Tissue distribution of covalently bound MeCCNU

Both Chx- and Cle-derived ^{14}C-MeCCNU were bound extensively to serum protein (Fig. 3). With both derivatives, binding

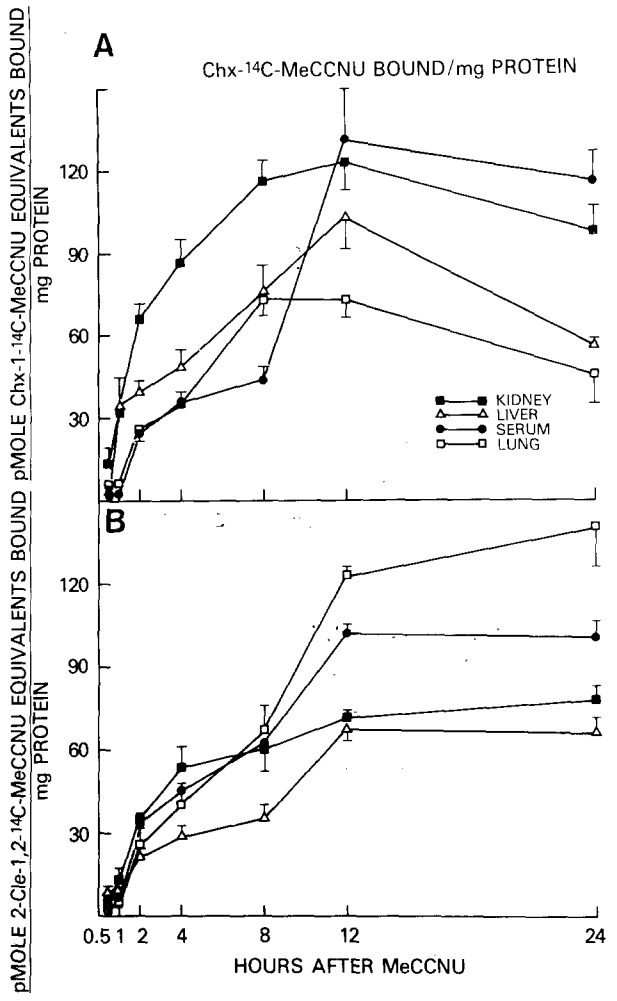

Fig. 3A, B. Tissue distribution of radioactivity bound covalently to protein. Rats were given single SC injections of either Chx-^{14}C-MeCCNU 40 mg/kg **(A)** or Cle-^{14}C-MeCCNU 40 mg/kg **(B)** and sacrificed at the times indicated. Each value is the mean ± SEM from five rats

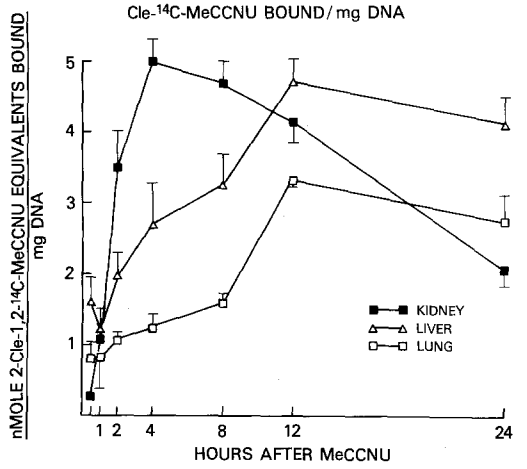

Fig. 4. Tissue distribution of Cle-^{14}C-MeCCNU bound irreversibly to DNA. Rats were given single SC injections of Cle-^{14}C-MeCCNU 40 mg/kg and sacrificed at the times indicated. Each value is the mean ± SEM from five rats

increased for up to 12 h, at which time maximum binding was achieved. Covalent binding of Chx-^{14}C-MeCCNU to serum protein was greater than in any tissue. Comparison of the distribution of covalently bound ^{14}C by either Cle- or Chx-^{14}C-MeCCNU indicates that kidney and liver underwent more extensive interaction with the cyclohexyl than the chloroethyl moiety. In contrast, lung tissue underwent more extensive interaction with the chloroethyl moiety. No discernible differences in the organ distribution of covalently bound Cle-^{14}C-MeCCNU were observed within 2 h after drug administration. At 4 h, kidney binding was marginally greater than that in the other tissues; however by 12 h, lung binding was nearly twice that of kidney or liver. Binding to lung, liver, kidney, and serum protein by Cle-^{14}C-MeCCNU was linear between 2 and 12 h, and was maximum at 24 h. Chx-^{14}C-MeCCNU was bound covalently to tissue protein more rapidly than Cle-^{14}C-MeCCNU. At 1 h, binding to liver or kidney protein by Chx-^{14}C-MeCCNU was 3 times that of Cle-^{14}C-MeCCNU. Moreover, the rank order for the tissue distribution of covalently bound Chx-^{14}C-MeCCNU was established within 2 h of drug administration, in contrast to 12 h for Cle-^{14}C-MeCCNU. Protein binding by Chx-^{14}C-MeCCNU was linear between 2 and 8 h in kidney and lung, and between 1 and 12 h in liver. Protein binding in all tissues was maximum at 12 h, and by 24 h nearly twice as much cyclohexyl-derived radioactivity was bound to kidney protein than to liver or lung protein.

Very high levels of Cle-^{14}C-MeCCNU were bound covalently to DNA (Fig. 4). The maximum binding of Cle-^{14}C-MeCCNU to kidney or liver DNA (nmol/mg DNA) was 70-fold greater than the maximal protein binding (pmol/mg protein) in the respective organs. Kidney DNA had the highest specific activity for covalent binding by Cle-^{14}C-MeCCNU. Binding to kidney DNA was linear for 4 h, at which time binding was maximum. However, by 24 h, Cle-^{14}C-MeCCNU-bound radioactivity had decreased by 60%. Peak DNA binding in liver and lung occurred at 12 h, and at 24 h had decreased by only 15%. The decrease in Cle-^{14}C-MeCCNU bound covalently to kidney DNA may partially be accounted for by a 100% increase in kidney DNA content (μg DNA/g wet weight) at 24 h (data not shown). In contrast, liver DNA had increased by only 20%, whereas no change was observed in lung DNA content. No Chx-^{14}C-MeCCNU was bound to DNA, as only background levels of radioactivity could be released by acid hydrolysis of the extracted tissue homogenates in Chx-^{14}C-MeCCNU-treated rats.

Discussion

The purpose of the present study was to compare the organ distribution and covalent binding of two labeled forms of MeCCNU in an animal model previously shown to be suitable for studying the nephrotoxicity of the nitrosoureas [14]. Organ distribution and covalent binding studies, such as those described in the present investigation, have not previously been reported for MeCCNU. However, our data are in agreement with those recorded in studies of several structural analogs of MeCCNU, showing that within minutes of administration only trace amounts of intact drug could be found in blood, lung, kidney, or liver [7, 20, 23]. In the present investigation, the injection vehicle (sesame oil) did not contribute to the observed instability of MeCCNU in vivo, as HPLC showed that > 92% of MeCCNU was intact after 24 h in

this vehicle (data not shown). Extraction of tissue homogenates with organic solvents of increasing polarity, and subsequent analysis of these extracts, showed fat to accumulate the highest levels of parent compound, whereas kidney accumulated the highest levels of the more polar ether- and methanol-extractable metabolites and/or degradation products of MeCCNU. The accumulation of intact MeCCNU in fat could be expected from a drug as lipophilic as MeCCNU. Moreover, studies with CCNU [23] showed that brain also accumulated predominantly parent drug. The high levels of ether- and methanol-extractable derivatives of MeCCNU in kidney also could be anticipated from a compound such as MeCCNU, which is excreted primarily in the urine [12].

A comparison of the two labeled forms of MeCCNU reveals striking differences in the tissue accumulation, degradation and/or metabolism, and tissue distribution of covalently bound radioactivity of the chloroethyl and cyclohexyl moieties. For example, > 96% of radioactivity derived from chloroethyl-labeled MeCCNU was removed from tissue homogenates only by methanol extraction. In contrast, methanol extraction removed 47%, 57%, and 72% of the total extractable radioactivity derived from Chx-^{14}C-MeCCNU in lung, kidney, or liver, respectively (Table 1). The different extraction characteristics of the two labeled forms of MeCCNU probably reflects the overall metabolic fate of MeCCNU as described in detail for CCNU [15, 21, 22] and other nitrosoureas [7, 19]. In this scheme, rapid ring hydroxylation occurs, with concomitant degradation to alkylating species such as 2-chloroethyl carbonium ion and carbamylating species of cyclohexylisocyanate and monohydroxylated cyclohexylisocyanates. The major products derived from the ethyl-^{14}C groups of other nitrosoureas in vitro were water-soluble degradation products such as 2-chloroethanol and highly reactive volatile species such as vinyl chloride, chloroethyl cation, chloroethylene oxide, and chloroacetaldehyde [5, 17, 22]. These reactive chemical entities readily alkylate sulfhydryl-containing endogenous substances such as glutathione, and the presence of sulfur-containing metabolites in rat urine has been identified in the form of thiodiacetic acid and *s*-carboxymethylcysteine [22]. Cyclohexyl-derived radioactivity, on the other hand, could be found as ether-extractable hydroxycyclohexylamines or as methanol-extractable isocyanate-derived conjugates which would yield hydroxycyclohexylamines upon acid or base hydrolysis [12].

Differences were also apparent in the accumulation and biological half-life of the two labeled forms of MeCCNU, particularly in kidney. For example, the area under the curve for total drug showed kidney to accumulate 30% more chloroethyl than cyclohexyl-derived radioactivity (Table 1). Cyclohexyl-derived radioactivity reached peak levels at 1 h and was cleared rapidly from the kidney with an initial half-life of 9 h (Fig. 2a). In contrast, chloroethyl-derived radioactivity reached peak levels in kidney at 2 h and maintained these levels for 12 h. Such striking differences in the accumulation and distribution of the two labels were not apparent for the other organs studied.

The two labeled forms of MeCCNU also were found to differ in the organ distribution of covalently bound radioactivity (Fig. 3). In this regard, kidney was exposed to comparatively high levels of reactive MeCCNU intermediates. At 24 h, approximately twice as much cyclohexyl-derived radioactivity was bound covalently to kidney protein as to lung or liver protein (Fig. 3). In agreement with a study of CCNU, the cyclohexyl moiety was found to be extensively bound to blood protein [20]. Covalent binding of cyclohexyl-derived radioactivity is presumably due to carbamylation by the cyclohexylisocyanate of MeCCNU. The extensive carbamylation of kidney macromolecules is therefore consistent with the view that carbamylation may be responsible for unwanted toxic side-effects to normal host tissue [1]. However, kidney is also a target for alkylation by chloroethyl-derived radioactivity (Fig. 3). Moreover, the time-course for the increase and peak covalent binding of both forms of labeled MeCCNU correlated well with the time-course for the decrease in *p*-aminohippuric acid accumulation observed in kidney slices from MeCCNUtreated F344 rats [14]. Thus, the covalent binding of either labeled form of MeCCNU to tissue macromolecules appears to be a valid predictor of MeCCNU nephrotoxicity. However, these data do not provide sufficient evidence to implicate either carbamylation of a cyclohexyl-derived, or alkylation by a chloroethyl-derived, reactive intermediate, in causing the MeCCNU-induced nephropathy. Further studies in an appropriate animal model, using chloroethylnitrosoureas with low carbamylating activity (e.g., chlorozotocin), are needed to determine the reactive derivative responsible for the nephrotoxicity of MeCCNU.

Whereas more cyclohexyl-derived ^{14}C was bound in kidney, there was approximately twice as much cloroethyl-derived ^{14}C bound to lung protein than to liver of kidney (Fig. 3). It should be noted that the extensive binding of lung protein by chloroethyl-derived ^{14}C occurred although kidney accumulated 5 times more of these derivatives than did lung (Fig. 2A, D). A possible explanation for the extensive binding of chloroethyl-derived ^{14}C to lung protein may be the low levels of glutathione synthesis and reduced glutathione found in lung, relative to liver or kidney [9]. Moreover, MeCCNU has been shown to inhibit lung glutathione reductase in vitro (Smith and Boyd, unpublished observations). These effects may combine to deplete pulmonary glutathione levels enought to render the sulfhydryl groups of lung protein susceptible to extensive alkylation reactions. The reactive chloroethyl carbonium ion and/or other degradation products of the chloroethyl moiety (i.e., chloroacetaldehyde) are also formed by degradation of BCNU [5, 17], a nitrosourea which is toxic predominantly to the lungs rather than to the kidneys [24]. MeCCNU, on the other hand, is not known to be particularly toxic to the lungs. Further studies of the mechanism for the selective nephrotoxicity of MeCCNU relative to the pulmonary toxicity of BCNU are currently under investigation.

Previous studies have shown that the antitumor activity of MeCCNU, like that of most chloroethyl nitrosoureas, is due to alkylation and subsequent cross-linking of nulecophilic sites in DNA by 2-chloroethyl derived reactive intermediates [4]. Our data are in agreement with these studies. We did not find radioactivity to be associated with DNA when MeCCNU was administered with label in the cyclohexyl moiety. On the other hand, high levels of radioactivity were associated with DNA when MeCCNU was labeled within the chloroethyl moiety (Fig. 4). Binding to kidney DNA was linear for up to 4 h, at which time binding was maximum. At 4 h, binding to kidney DNA was 2-fold greater than in liver and 4-fold greater than in lung. However, at 24 h, DNA-bound MeCCNU had decreased by 60% of the peak values observed in kidney at 4 h, and was accompanied by a 100% increase in kidney DNA content (μg DNA/g wet weight; data not shown). In liver and lung, DNA binding at 24 h had decreased only by 15% of peak levels. It is well known that agents which cause injury to DNA frequently stimulate DNA synthesis. However, it is not known whether

the increase in kidney DNA content and corresponding decrease in the specific activity of covalently bound drug, observed 24 h after MeCCNU administration, was due to an increase in unscheduled DNA synthesis or phagocyte infiltration, or was part of a cellular proliferative response to MeCCNU-induced kidney damage. Organ weights were not higher at 24 h relative to controls, nor was there any histologic evidence of edema or lymphocyte infiltration. Nevertheless, the magnitude of the increase in DNA content reflected the initial levels of drug bound covalently to DNA. In this regard, liver DNA content had increased by only 20%, whereas lung DNA content remained a pretreatment levels.

Additional studies are necessary to understand the relative importance of target organ DNA alkylation as a possible mechanism for MeCCNU-induced nephropathy. The data presented in this paper are consistent with a hypothesis [14] according to which the extensive alkylation and carbamylation of kidney macromolecules observed after MeCCNU treatment may not only result in the initial nephrotoxic insult, but may also interfere with the normal cellular repair processes of the kidney.

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