Biochemical Pharmacology, Vol. 26, pp. 2331–2336. Pergamon Press, 1977. Printed in Great Britain.

COMPARISON OF THE PROPERTIES OF METABOLITES OF CCNU*

GLYNN P. WHEELER, THOMAS P. JOHNSTON, BONNIE J. BOWDON, GEORGE S. MCCALEB, DONALD L. HILL and JOHN A. MONTGOMERY

Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, U.S.A.

(*Received* 25 *December* 1976; *accepted* 4 *March* 1977)

Abstract—Properties of the six isomeric *N*-(2-chloroethyl-*N'*-(hydroxycyclohexyl-*N*-nitrosoureas, which have been identified by other investigators as metabolites of *N*-(2-chloroethyl-*N'*-cyclohexyl-*N*-nitrosourea (CCNU), have been compared with those of CCNU and 2-[[[(2-chloroethyl)nitrosoamino]-carbonyl]amino]-2-deoxy-D-glucose (chlorozotocin). There are significant differences in the physicochemical, chemical, and biological properties of these metabolites, and the properties of some of them are significantly different from those of CCNU and chlorozotocin. The position of the hydroxy group and the steric configuration of the compound markedly affect the alkylating and carbamoylating activities of the compounds. The metabolites having the higher alkylating activities and the lower carbamoylating activities produce lethal toxicity to mice at lower molar doses but have somewhat better therapeutic indexes. The data are consistent with the hypothesis that the biological effects observed following the administration of CCNU are due to a large extent to the major metabolites with lesser effects contributed by the minor metabolites. Some of the metabolites have slightly better therapeutic indexes against murine leukemia L1210 than CCNU and chlorozotocin, and they are more soluble in water than CCNU but are active against both intraperitoneally implanted and intracerebrally implanted L1210 leukemia. There might be some therapeutic advantage to administering one of the minor metabolites instead of CCNU or chlorozotocin to cancer-bearing animals.

May, Boose and Reed [1] and Hill, Kirk and Struck [2] presented evidence that hydroxylation of the cyclohexyl ring occurs very rapidly when CCNU† is incubated with a liver microsomal preparation in the presence of oxygen and NADPH. Reed and May [3, 4] identified five metabolites (of the six possible metabolites) which they obtained in *in vivo* experiments with rats and in *in vitro* experiments with rat liver microsomes. They did not specify the configuration of the 2-hydroxy derivative, but in a personal communication Dr. Reed stated that it is the *cis* isomer. Hilton and Walker [5] independently identified the two pairs of 3- and 4-isomers and the *trans*-2-isomer as products of *in vitro* incubation and also found them in the plasma of rats. Only the *cis*-4-isomer and the *trans*-4-isomer were found in human plasma following the intravenous administration of CCNU; these isomers were present in approximately equal quantities [6]. The data of all of these groups of investigators indicate that the rate of metabolic hydroxylation exceeds the rate of chemical breakdown of CCNU, and therefore, it is likely that the hydroxylated metabolites are major contributors of the therapeutically active moieties. There is also evidence [1, 2] that hydroxylation of MeCCNU occurs. Because of these facts the various hydroxylated compounds have been synthesized [7,8 and this report] and compared with each other and with CCNU and chlorozotocin with respect to several physicochemical, chemical, biological, and chemotherapeutic properties.

MATERIALS AND METHODS

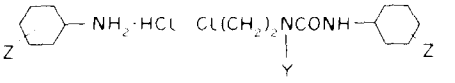
CCNU (NSC-79037) and chlorozotocin (NSC-178248) were obtained from the Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute. *N*-(2-Chloroethyl)-*N'*-(*cis*-4-hydroxycyclohexyl)-*N*-nitrosourea (NSC-239724) and its trans isomer (NSC-239717) were available from previous synthesis in this laboratory [7]; 3- and 2-hydroxy and 2-acetoxy derivatives of CCNU were prepared as described below with nitrosations that gave the hydroxy derivatives being performed essentially as described for the 4-hydroxy derivatives. The synthetic scale was such that 2–5 g samples were available for biological testing. Characterizing data for new compounds (and three others) in this series are given in Table 1.

N-(2-Chloroethyl)-N'-(cis-3-hydroxycyclohexyl)-N-nitrosourea (NSC-253945) and its trans *isomer (NSC-260599).* Low-pressure catalytic hydrogenation (5% Rh–Al_2O_3, 50 psi) of *N*-(3-hydroxyphenyl)acetamide in ethanol (cf. Ref. [8]) and alkaline hydrolysis of the *N*-(3-hydroxycyclohexyl)acetamide resulting in theoretical yield gave 3-aminocyclohexanol as a *cis-trans* mixture, which was resolved as the hydrogen oxalates [9]. The *trans* salt, m.p. 200–201° (lit. [8, 9] m.p. 193–194°), crystallized from methanol in 36 per cent yield and the methanol-soluble *cis* salt, m.p. 142–143° (lit. [8] 141–143°), from ethanol in 18 per cent yield. The *cis* hydrochloride [m.p. 175–177°, IR (KBr) 2045 cm^{-1} (NH_3^+)] derived from the oxalate was

* This work was supported by contracts NO1-CM-43762 and NO1-CM-43784, Division of Cancer Treatment, NCI, NIH, DHEW.

† CCNU, *N*-(2-chloroethyl-*N'*-cyclohexyl-*N*-nitrosourea (NSC-79037); MeCCNU, *N*-(2-chloroethyl)-*N'*-(*trans*-4-methylcyclohexyl)-*N*-nitrosourea (NSC-95441).

Table 1. Characterization of some hydroxy and acetoxy derivatives of CCNU and some of the corresponding intermediates



A. Amine hydrochlorides B. Y = H: Ureas
C. Y = NO: Nitrosoureas

NSC No.	Compound type	Substituent Y	Substituent Z	Configuration	M.p. †	Infrared absorption*, cm^{-1} OH	NH	NH_3^+	Ester C=O	Urea C=O	Urea CNH	Urea N=O
	A		3-OH	*trans*	196–198			2060				
	A		2-OAc	*cis*	215–217‡			2030	1745			
	A		2-OAc	*trans*	241–242			2040	1730			
	B	H	3-OH	*cis*	99–100					1615	1560	
	B	H	3-OH	*trans*	84–86					1620§	1575	
	B	H	2-OH	*trans*	91–92					1625	1570	
	B	H	2-OAc	*cis*	165–166		3330		1735	1610	1560	
	B	H	2-OAc	*trans*	112–113		3335‖		1730	1620	1575	
	B	H	2,6-diOH	*trans, trans*	119–120¶					1630	1580	
253945	C	NO	3-OH	*cis*	(yellow oil)					1715	1520	1480
260599	C	NO	3-OH	*trans*	(yellow oil)					1710	1520	1480
253946	C	NO	2-OH	*cis*	55–57	3500	3395			1695	1520	1470
253947	C	NO	2-OH	*trans*	94–95	3445	3330			1685	1535	1485
204817	C	NO	2-OAc	*cis*	51–52		3365		1730	1695	1525	1470
204818	C	NO	2-OAc	*trans*	65–66		3345		1730	1710	1525	1480
264395	C	NO	2,6-diOH	*trans, trans*	127–128 dec**	3490, 3410	3285			1700, 1685††	1540	1490

* Spectra of solids measured in KBr disks and spectra of liquids, as capillary films between KBr windows.

† Melting points determined in capillary tubes with a Mel-Temp. apparatus and are uncorrected.

‡ Cf. product, m.p. 213–213.5°, of attempted recrystallization of *cis*-3a,3.4,5,6,7,7a-hexahydro-2-methylbenzoxazole hydrochloride [25].

§ Presence of the cyclized urea, 2-[(*trans*-3-hydroxycyclohexyl)amino]-2-oxazoline hydrochloride, indicated by medium absorption (shoulder) at 1690 cm^{-1} (C = N).

‖ Also a shoulder at 3300 cm^{-1} (NH).

¶ Lit. m.p. 125–127° [20].

** Lit. m.p. 125–126° dec [20].

†† Double carbonyl absorption due to solid state effect.

Note: Elemental analyses (C, H, and N) were performed on each compound, and the experimental values agreed with the theoretical values within commonly accepted limits.

identical with the hydrochloride derived in five steps from 3-hydroxybenzoic acid via 2-oxa-4-azabicyclo-[3.3.1]nonan-3-one [10], a cyclic carbamate of fixed *cis* configuration (the aminocyclohexanol was freed from the oxalate with strong aqueous sodium hydroxide solution and extracted into chloroform, and the chloroform solution was treated with ethanolic hydrogen chloride and evaporated to dryness under reduced pressure). *cis*-3-Aminocyclohexanol, m.p. 75° (lit. m.p. 68–70° [9], 70° [10]), was freed from the oxalate and converted to the urea in 52 per cent yield by treatment with 2-chloroethyl isocyanate in acetonitrile with subsequent dilution with ether. Nitrosation of 3.3 g (15 m-moles) of the urea gave 3.5 g (94 per cent yield) of the nitrosourea, a yellow oil, after treatment with methanol (for destruction of nitrous acid ester); analysis by reversed-phase high-pressure liquid chromatography (HPLC) [11] indicated ~1.2 per cent of a UV-absorbing impurity, presumably the *N'*-nitroso isomer, which was also detected by NMR spectroscopy (see Ref. [12]).

trans-3-Aminocyclohexanol, m.p. 95° (lit. [9, 10] m.p. 94–95°), freed from the oxalate and further purified as the hydrochloride, was converted to the urea in chloroform containing triethylamine (~4 ml/g of aminocyclohexanol); removal of the solvent left a semisolid residue from which the nitrosourea, a yellow oil whose purification involved recovery from a filtered hot carbon tetrachloride solution and treatment with methanol, was derived in 43 per cent yield (from the aminocyclohexanol). The product was somewhat contaminated with the *N'*-nitroso isomer as indicated by NMR spectroscopy [12] and as quantitated (~7.8%) by HPLC [11]. Easy cyclization to the corresponding oxazoline hydrochloride appeared to complicate the isolation of a characterizable sample of the urea; a sample for analysis (but not entirely free of the oxazoline) was prepared in ether and triturated several times, with much loss, in acetonitrile. *trans*-3-Aminocyclohexanol hydrochloride was recrystallized for analysis by dilution of a solution of 350 mg in 4.0 ml of ethanol with 20 ml of acetonitrile.

N-(2-Chloroethyl)-N'-(cis-2-hydroxycyclohexyl)-N-nitrosourea (NSC-253946) and its trans *isomer (NSC-253947).* Reported procedures were used for the preparation of *trans*-2-aminocyclohexanol hydrochloride [13] and its three-step inversion to *cis*-2-aminocyclohexanol hydrochloride [14]. The trans hydrochloride was converted to the corresponding (2-chloroethyl)-

urea in cold chloroform by first adding triethylamine (1.6 ml/g) and then the isocyanate; the residue after evaporation of the solvent was washed with ether, triturated in cold water, and recrystallized from acetonitrile, giving a 42 per cent yield. The *cis*-2-hydroxy urea was described previously [12].

The *cis*-2-hydroxy nitrosourea was obtained in 83 per cent yield after treatment with methanol and trituration in petroleum ether and was essentially free of UV-absorbing impurities as indicated by HPLC [11]. The *trans*-2-hydroxy nitrosourea was recrystallized twice from acetonitrile (yield 25 per cent), and the product that was recovered by evaporation of the filtrates was recrystallized from acetonitrile by the addition of water (2 vol.); the total yield was 61 per cent. This product contained ~1.5% of the *N'*-nitroso isomer as shown by HPLC [11].

N-(2-*Chloroethyl*)-N-[cis-2-(*acetyloxy*)*cyclohexyl*]-N-*nitrosourea NSC-204817 and its* trans *isomer NSC-204818.* The acetic esters of *cis*- and *trans*-2-aminocyclohexanol hydrochloride were prepared as previously described [15] for a 4-isomer, which has since been shown to be identical with the acetate derived from *trans*-4-amino-cyclohexanol hydrochloride [16]. The *cis* acetate hydrochloride precipitated from the chilled reaction mixture and was washed with a little acetonitrile; yield 33 per cent. The less soluble trans acetate hydrochloride precipitated from the hot reaction mixture, but was collected cold and washed with a little acetonitrile; yield 81 per cent. (2-Chloroethyl)ureas were prepared from the above acetoxy amine hydrochlorides in chloroform after liberation of the free amine with triethylamine (2 ml/g); after 1 hr the solvent was removed *in vacuo* and the residue washed well with hexane and with cold water; yields 96 (*cis*) and 85% (*trans*). The acetoxy ureas were nitrosated in cold concentrated hydrochloric acid with dinitrogen trioxide (*cf.* the preparation of chlorozotocin [17]) during ~60 min; the products were isolated by extraction of the reaction mixtures with chloroform and purified by precipitation from ethanol with cold water; yields ~75 per cent. The extracted *cis* nitrosourea was a yellow oil before precipitation from ethanol.

N-(2-*Chloroethyl*)-N'-[(1α,2β,6β)-2,6-*dihydroxycyclohexyl*]-N-*nitrosourea* (*NSC-264395*). Preparation of the intermediate (1α,2β,3α)-2-amino-1,3-cyclohexanediol involved the condensation of glutaraldehyde with nitromethane [18] and reduction of the resulting (1α,2β,3α)-2-nitro-1,3-cyclohexanediol [19]. The (2-chloroethyl)urea was formed from a suspension of the aminocyclohexanediol in *N,N*-dimethylformamide (addition of the isocyanate at 5°, subsequent stirring at ~25°) and precipitated by addition of ether (23 vol.); recrystallization from acetonitrile gave a 68 per cent yield. A suspension of the urea in 0.5 N hydrochloric acid was nitrosated at 0° with dinitrogen trioxide (1.5 hr); the resulting nitrosourea was purified by dissolving in hot ethanol, filtering, and diluting with hexane (5 vol.), giving a 73 per cent yield in two crops. The preparation of this nitrosourea was recently reported by Machinami *et al.* [20].

Determination of chemical and physicochemical properties. The methods for determining half-lives, alkylating activities, and carbamoylating activities were those that have been described previously [21], except that Tris buffer, pH 7.4, was used in the test for alkylating activity. The half-lives were determined by measuring the decrease in optical density accompanying the decomposition of the nitrosourea, and the determination of alkylating activity was based upon the alkylation of 4-(*p*-nitrobenzyl)pyridine at approximately physiological conditions. The carbamoylating activity was measured by determining the extent of carbamoylation of ^{14}C-lysine upon incubating mixtures of the nitrosoureas and ^{14}C-lysine. The distribution coefficients (P) for the 1-octanol–water system [22] were determined experimentally for the *cis*-4- and *trans*-4-isomers by W. J. Haggerty, Jr., Midwest Research Institute, Kansas City, Missouri, and were estimated from elution data from reversed-phase HPLC for the other isomers [11].

Determination of biological effects. The single-dose LD_{10}'s and the single-dose ED_{50}'s (doses causing at least 45-day survival of 50 per cent of the mice after i.p. implantation of 10^5 L1210 cells or after i.c. implantation of 10^4 L1210 cells) were determined by standard procedures by the Chemotherapy Department of this Institute. In the chemotherapy experiments the agents were injected i.p. 1 day after the implantation of the L1210 cells.

Microsomal hydroxylation of CCNU. The microsomal oxidation was accomplished with washed microsomes from livers of female DBA/2 mice, as described previously [2]. The reaction mixture contained the following: CCNU, 440 nmoles; NADPH, 10 μmoles; microsomes equivalent to 300 mg of liver; Tris chloride buffer (pH 7.5 at 37°), 280 μmoles; and water in a total vol. of 3.4 ml. Incubation of the preparation was for 10 min at 37°. Controls lacked NADPH. The reactions were stopped by placing the preparations in an ice bath, and unchanged CCNU was removed by extraction with six 6-ml portions of hexane. Metabolites were extracted with three 6-ml portions of ether [8]. The combined ether extracts were dried over anhydrous sodium sulfate, and the ether solution was decanted. The metabolites were stored overnight in the ether solution at −80°. For analysis by reversed-phase HPLC [11], a methanolic solution of the metabolites was prepared as follows. The ether was evaporated in a gentle stream of nitrogen and the residue dissolved in an appropriately small vol. of methanol (<1 ml). Aliquots of up to 75 μl were chromatographed, and no interfering peak was observed with the control solution. The metabolites were identified by chromatographing with synthetic standards; estimation of peak areas gave an apparent isomeric ratio (Table 2).

RESULTS AND DISCUSSION

Table 2 shows the relative quantities of metabolites that were found in various *in vitro* and *in vivo* systems including the mouse liver microsomal system of the present study. The apparent lack of agreement of the data for the three microsomal systems is not surprising in view of the use of different buffers and different periods of incubation and in view of the fact that the quantity of each metabolite present at any time is dependent upon both the rate of formation and the rate of decomposition of that particular metabolite. Data for four of the five systems indicated that the *cis*-4-isomer was a major metabolite, and data

Table 2. Metabolites of CCNU

	Distribution (%)				
	Rat			Mouse	Human
	Microsomes			Microsomes	
Isomer	Phosphate* 5–10 min	Tris† 40 min	*in Vivo*† 20 min	Tris 10 min	Plasma‡ after i.v. CCNU
cis-2-OH	trace	—	—	—	—
trans-2-OH	—	14	8	1	—
cis-3-OH	30	trace	4	13	—
trans-3-OH	39	31	23	12	—
cis-4-OH	21	53	54	46	*ca* 50
trans-4-OH	9	3	12	28	*ca* 50

* Data of May, *et al.* [1].
† Data of Hilton and Walker [5].
‡ Data of Hilton and Walker [6].

for all of the systems show that only small quantities of the 2-isomers were formed.

Table 3 contains data that summarize the experimental results obtained to date with the metabolites and with the synthetic compounds, *trans*, *trans*-2,6-dihydroxy CCNU, *cis*-2-acetoxy CCNU, and *trans*-2-acetoxy CCNU.

As would be expected, the distribution coefficients of the monohydroxycyclohexyl compounds are between those for CCNU and for chlorozotocin. Although the log P values of the six isomers fall within a relatively narrow range, the lipoid solubilities of the isomers differ sufficiently to permit separation of them by reversed-phase HPLC [11]. All of the isomers are effective against intracerebrally implanted L1210 leukemia, in contrast to chlorozotocin, which does not cross the 'blood-brain barrier'.

The half-lives of all of the metabolites are between those of CCNU and chlorozotocin. The *cis*-4- and *trans*-4-isomers have about the same carbamoylating activity as CCNU, while the *cis*-2- and *trans*-2-isomers are like chlorozotocin in having very low carbamoylating activities; the carbamoylating activities of the *cis*-3- and *trans*-3- isomers are intermediate between those of CCNU and chlorozotocin.

On a molar basis chlorozotocin and the *trans*-2-isomer have more than a two-fold greater lethal toxicity than CCNU, while the *cis*-2-, *cis*-3-, and *cis*-4-isomers are about as toxic as CCNU, and the *trans*-3- and *trans*-4-isomers have intermediate toxicities. Also,

Table 3. Properties of metabolites of CCNU

			Per cent of value for CCNU							
							ED_{50}¶		ED_{50}/LD_{10}	
NSC No.	Compound	Log P*	$T_{0.5}$†	Alkylating‡ activity	Carbamoylating§ activity	LD_{10}‖	i.p.	i.c.	i.p.	i.c.
79037	CCNU	2.83	100	100	100	100	100	100	0.77	0.73
178248	Chlorozotocin	−1.02	46	489	4	38	30	—	0.62	—
253946	*cis*-2-OH CCNU	1.60	76	177	1	98	56	56	0.44	0.42
204817	*cis*-2-OAc CCNU	2.56	83	218	50	82	58	—	0.55	—
253947	*trans*-2-OH CCNU	1.30	66	329	6	41	16	29	0.29	0.52
204818	*trans*-2-OAc CCNU	2.56	92	213	100	60	30	—	0.39	—
264395	*trans,trans*-2,6-diOH	—	70	577	0.3	33	10	33	0.24	0.72
253945	*cis*-3-OH-CCNU	1.25	91	133	21	98	47	72	0.37	0.54
260599	*trans*-3-OH CCNU	1.28	84	112	61	74	41	53	0.42	0.53
239724	*cis*-4-OH CCNU	1.11	93	136	104	90	54	51	0.47	0.41
239717	*trans*-4-OH CCNU	1.00	87	140	100	82	36	48	0.33	0.43

* Logarithm of the distribution coefficient in the octanol–water system, as determined experimentally, calculated, or estimated with the aid of high-pressure liquid chromatography.
† Half-life in phosphate buffer, pH 7.4, 37°. The half-life of CCNU under these conditions is 53 min.
‡ Alkylating activity measured by the absorbance of the solution containing the product obtained upon reaction with NBP. Equimolar quantities were used in the tests.
§ Carbamoylating activity measured by the quantity of radioactivity present as reaction products after incubation of the compound with ^{14}C-L-lysine. Equimolar quantities were used in the tests.
‖ LD_{10}'s for BDF_1 mice were calculated on a molar basis, and those molar doses were compared with the molar LD_{10} for CCNU.
¶ Dosage (single dose i.p. day 1 only) giving 50 per cent long term (45 days or more) survivors when 10^5 leukemia L1210 cells were implanted i.p. or 10^4 leukemia L1210 cells were implanted i.c. Dosages were expressed on a molar basis for comparisons.

on a molar basis chlorozotocin and all of the metabolites have lower ED_{50}'s than CCNU, and they have better therapeutic indexes (ED_{50}/LD_{10}) than CCNU for both the intraperitoneal disease and the intracerebral disease.

Chlorozotocin and the *trans*-2-isomer, which have greater lethal toxicity than the other metabolites, have the highest alkylating activities and low carbamoylating activities. These results would seem to be in contradiction to the conclusion based upon previous studies [21] that the major effect of carbamoylation might be contributory to the whole-animal toxicity and to the observation that chlorozotocin causes less marrow toxicity than CCNU [23]. It is realized, however, that high alkylating activity can in itself be a major contributory factor toward whole-animal toxicity. For example, nitrogen mustard and other well-known alkylating agents are quite toxic. Among the isomers that have approximately similar alkylating activities (*cis*-2, *cis*-3, *cis*-4, and *trans*-4) those having the greater carbamoylating activities (*cis*-4 and *trans*-4) are somewhat more toxic.

For use in chemotherapy the absolute whole-animal toxicities are of less importance than the therapeutic indexes, such as ED_{50}/LD_{10}. The values for this ratio show that all of the metabolites have slightly better therapeutic indexes than CCNU and chlorozotocin.

Since there is at present no evidence indicative of polyhydroxylation of the cyclohexyl group of CCNU as a result of metabolism, one might speculate that if one administers a monohydroxy CCNU, further hydroxylation does not occur and the observed effects will be those of the administered compound. Upon this basis, or even if polyhydroxylation should occur, one might accomplish therapeutic benefit by administering the monohydroxy CCNU's that have the better biologic effects instead of CCNU. In view of the slightly better therapeutic index of the *trans*-2-isomer, the *trans,trans*-2,6-dihydroxy compound was synthesized and tested. This compound had properties and therapeutic efficacy similar to those of the *trans*-2-isomer, except the i.c. ED_{50}/LD_{10} was about the same as that for CCNU.

It was of interest to compare the properties of *cis*-2-acetoxy CCNU and *trans*-2-acetoxy CCNU to those of the corresponding hydroxy compounds to determine whether there might be some advantage to administering the acetoxy compounds. As might be expected, esterification resulted in greater lipid solubility of the compounds, and the log P values for the esters were similar to those for CCNU. The half lives in phosphate buffer were increased and were only slightly less than that of CCNU. The alkylating activities of the two acetoxy isomers were approximately the same, were intermediate between the values for the two hydroxy compounds, and were approximately two times as great as that of CCNU. The greater carbamoylating activities of the acetoxy compounds in comparison with the hydroxy compounds is consistent with the possibility that intramolecular carbamoylation might occur for the hydroxycyclohexyl isocyanates that would be generated from the hydroxy CCNU's, while such intramolecular carbamoylation would not occur for the acetoxycyclohexyl isocyanates generated from the acetoxy CCNU's. Intramolecular carbamoylation would decrease the amount of carbamoylation of lysine that could occur in the present tests and hence would give less carbamoylation in the present test. The two-fold difference in the carbamoylating activities of the *cis*-2-acetoxy CCNU and the *trans*-2-acetoxy CCNU is surprising, and the difference might be at least partially due to steric factors. The observed qualitative similarities of the *in vivo* biological effects of chlorozotocin [23] and of chlorozotocin tetracetate [24] suggest that deacetylation might occur in the animal, and similar deacetylation of the 2-acetoxy CCNU's might be anticipated. The data in Table 2 do not clearly indicate whether deacetylation did or did not occur, but it is evident that the values for the LD_{10}'s, the ED_{50}'s and the ED_{50}/LD_{10}'s are nearer those for the hydroxy compounds than those for CCNU. It appears that there would be no advantage in administering the acetoxy compounds instead of the corresponding hydroxy compounds.

The data presented here lead to the following conclusions:

(a) There are significant differences in the properties of the various metabolites, with some of them having much higher alkylating activities and much lower carbamoylating activities than the others and than CCNU. There are also differences in the biological effects.

(b) It appears likely that the biological effects observed following the administration of CCNU are due primarily to the major metabolites, but the minor metabolites probably contribute to some extent to those effects.

(c) The metabolites that have high alkylating activities and low carbamoylating activities are the most toxic on a molar basis, but they have therapeutic indexes against the intraperitoneal disease that are as good as, or slightly better than, those of the other metabolites. All of the metabolites are more toxic than CCNU on a molar basis but have better therapeutic indexes.

(d) The *trans*-2-isomer has alkylating activity, carbamoylating activity, and whole animal toxicity similar to those of chlorozotocin, but the *trans*-2-isomer has a better therapeutic index and is effective against the i.c. leukemia, whereas chlorozotocin is ineffective against the i.c. disease.

(e) The data indicate that administration of some of the metabolites, particularly the *trans*-2 isomer, would be advantageous over the administration of CCNU, since these metabolites have somewhat better therapeutic indexes and because their greater water-solubility might facilitate handling. These same metabolites have advantages over chlorozotocin in having better therapeutic indexes and in being effective against the i.c. leukemia.

Acknowledgements—The authors are indebted to Ms. Anita T. Shortnacy for HPLC analyses, to Ms. Mary W. Trader for supervision of animal experiments, to Mr. Thomas C. Herren and Mr. Willie J. White for radioassays, and to Dr. William C. Coburn, Jr., Ms. Martha C. Thorpe, and Ms. Christine G. Richards for spectral data and elemental analyses.

REFERENCES

1. H. E. May, R. Boose and D. J. Reed, *Biochem. biophys. Res. Commun.* **57**, 426 (1974).
2. D. L. Hill, M. C. Kirk and R. F. Struck, *Cancer Res.* **35**, 296 (1975).
3. D. J. Reed and H. E. May, *Life Sci.* **16**, 1263 (1975).
4. D. J. Reed, *Proc. Am. Assoc. Cancer Res.* **16**, 92 (1975).
5. J. Hilton and M. D. Walker, *Biochem. Pharmac.* **24**, 2153 (1975).
6. J. Hilton and M. D. Walker, *Proc. Am. Assoc. Cancer Res.* **16**, 103 (1975).
7. T. P. Johnston, G. S. McCaleb and J. A. Montgomery, *J. med. Chem.* **18**, 634 (1975).
8. H. E. May, R. Boose and D. J. Reed, *Biochemistry* **14**, 4723 (1975).
9. U. Burckhardt, C. A. Grob and H. R. Kiefer, *Helv. chim. Acta* **50**, 231 (1967).
10. R. R. Burford, F. R. Hewgill and P. R. Jefferies, *J. chem. Soc.* 2937 (1957).
11. J. A. Montgomery, T. P. Johnston, H. J. Thomas, J. R. Piper and C. Temple, Jr., in *Advances in Chromatography*, (Eds J. C. Giddings, E. Grushka, J. Cazes and P. R. Brown), Vol. 15, p. 169. Marcel Dekker, New York (1977).
12. T. P. Johnston, G. S. McCaleb, P. S. Opliger and J. A. Montgomery, *J. med. Chem.* **9**, 892 (1966).
13. L. R. Hawkins and R. A. B. Bannard, *Can. J. Chem.* **36**, 220 (1958).
14. W. S. Johnson and E. N. Schubert, *J. Am. chem. Soc.* **72**, 2187 (1950).
15. T. P. Johnston, G. S. McCaleb, P. S. Opliger, W. R. Laster, Jr. and J. A. Montgomery, *J. med. Chem.* **14**, 600 (1971).
16. T. P. Johnston, G. S. McCaleb, S. D. Clayton, J. L. Frye, C. A. Krauth and J. A. Montgomery, *J. med. Chem.* **20**, 279 (1977).
17. T. P. Johnston, G. S. McCaleb and J. A. Montgomery, *J. med. Chem.* **18**, 104 (1975).
18. F. W. Lichtenthaler, *Chem. Ber.* **96**, 845 (1963).
19. T. Suami and S. Ogawa, *Bull. chem. Soc. Japan* **37**, 194 (1964).
20. T. Machinami, S. Nishiyama, K. Kikuchi and T. Suami, *Bull. chem. Soc. Japan* **48**, 3763 (1975).
21. G. P. Wheeler, B. J. Bowdon, J. A. Grimsley and H. H. Lloyd, *Cancer Res.* **34**, 194 (1974).
22. C. Hansch, N. Smith, R. Engle and H. Wood, *Cancer Chemother. Rep.* **56**, 443 (1972).
23. T. Anderson, M. G. McMenamin and P. S. Schein, *Cancer Res.* **35**, 761 (1975).
24. P. S. Schein, M. G. McMenamin and T. Anderson, *Cancer Res.* **33**, 2005 (1973).
25. R. A. B. Bannard, N. C. C. Gibson and J. H. Parkkari, *Can. J. Chem.* **49**, 2064 (1971).