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BCNU-induced quantitative and qualitative changes in hepatic cytochrome P-450 can be correlated with cholestasis*

James C. Stolzenbach and Robert E. Larson**

College of Pharmacy, Oregon State University, Corvallis, Oregon, USA

Summary. Male Sprague-Dawley rats were given single i.p. injections of 1,3-bis(2-chloroethyl)-1-Nitrosourea (BCNU) to investigate changes in hepatic microsomal cytochrome P-450 content and metabolic activity. On day 14 after treatment (20 mg/kg), cytochrome P-450 content had decreased by approximately 25% and ethylmorphine *N*-demethylase activity (nmol product/nmol P-450/min) had decreased by 36%. In contrast, ethylmorphine *O*-deethylase and 7-ethoxycoumarin *O*-deethylase activities were not significantly decreased by BCNU treatment. Hepatic delta-aminolevulinic acid synthetase activity was only 60% of control values, and microsomal heme oxygenase activity was slightly but not statistically elevated. Cytochrome P-450 content in control and BCNU-treated rats increased in a similar manner after phenobarbital or β-naphthoflavone induction. Electrophoretic analysis of cytochrome P-450 proteins isolated from hepatic endoplasmic reticular membranes of treated and control rats suggested that alterations in these proteins occurred in BCNU-treated rats. These changes in cytochrome P-450 content and activity are very similar to those reported in isolated systems exposed to bile acids or in rats with experimentally produced cholestasis. BCNU has been shown to produce cholestasis, which precedes its effects on microsomal mixed-function oxygenase activity. Thus, the delayed effects of BCNU on microsomal drug metabolism are probably secondary to its interference with bile formation.

Introduction

Early in the development of 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine, BCNU), decreases in hepatic mixed-function oxygenase (MFO) activity and microsomal cytochrome P-450 were observed in rats receiving the drug [28, 29, 45]. The appearance of the effects seemed to be characteristically delayed by 1 week or more and were progressive after single i.p. or oral doses. Wilson and Larson [49] and Wilson et al. [50] later reported similar observations in mice and described differential responsiveness of BCNU-treated mice to various MFO inducers and inhibitors. No mechanistic explanations of these observations have appeared to date. Hepatotoxicity accompanied by decreases in MFO activity have also been described after administration of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lonustine, CCNU) [2, 46].

Recently the cholestatic effect of BCNU in rats has been described [17]. Between 36 and 48 h after single i.p. doses of BCNU, rats develop an intrahepatic cholestasis that is characterized by a deficit in the so-called bile salt-independent bile flow and an inability to concentrate exogenous organic anions in the bile. Because BCNU elicits profound effects on both bile flow and microsomal drug metabolism, we reexamined the early time course during which cholestasis develops after BCNU treatment for indications of MFO impairment. We also compared the changes in MFO activity during BCNU-induced cholestasis to those reported in the literature after bile duct ligation and treatment with other cholestatic agents. From these studies it seems clear that in the case of BCNU, the effects on MFO follow the development of cholestasis and are probably the result of that action of the drug.

Materials and methods

Unformulated BCNU and 1,3-bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea (BHCNU) were provided by the Drug Synthesis and Chemistry Branch of the NCI. Umbelliferone, β-naphthoflavone sodium succinate, pyridoxyl-5-phosphate, *p*-dimethylaminobenzaldehyde, and bovine hemin were purchased from Sigma Chemical Co. (St. Louis, Mo). Cytochrome *c*, glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (E. C. 1.1.1.49), and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Calbiochem Behring (La Jolla, Calif). Horse-heart cytochrome *c* was purchased from Boehringer-Mannheim (Indianapolis, Ind). The 7-

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** *Present address:* Searle Research and Development, 4901 Searle Parkway, Skokie, IL 60077, USA

Abbreviations. ALA, delta aminolevulinic acid; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine); BHCNU, 1,3-bis(*trans*-4-hydroxycyclohexyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (lomustine); EM, ethylmorphine; EMND, ethylmorphine *N*-demethylase; EMOD, ethylmorphine *O*-deethylase; ECOD, ethoxycoumarin *O*-deethylase; BAPH, benzo[a]pyrene hydroxylase; MFO, hepatic microsomal mixed-function oxygenases

Offprint request to: J. C. Stolzenbach

ethoxycoumarin was a gift from Dr. J. D. Hendricks of the Oregon State University Department of Food Science and Toxicology. 1,3,6-[^{3}H]-Benzo[a]pyrene (66.3 Ci/mmol) was purchased from New England Nuclear (Boston, Mass). Scint A counting fluor was purchased from Packard Instrument Co. (Downers Grove, Ill), and HPLC-grade acetonitrile was obtained from J. T. Baker and Co. (Philipsburg, NJ). The 1-hexane sulfonic acid counterion was obtained from Waters Associates (Milford, Mass) Ethylmorphine, morphine, and normorphine were all generously donated by the Research Technology Branch, Preclinical Research Division of the National Institute of Drug Abuse (Washington, DC).

Chromatographic materials for the isolation of cytochrome P-450 isozymes were purchased from the following sources: sodium deoxycholate, sodium cholate, 1,8-diaminooctane, flavin mononucleotide, phenylmethylsulfonyl fluoride, dithiothreitol, lubrol PX, 2′-adenosine monophosphate, sodium dodecyl sulfate, and adenosine 2′5′-diphosphate agarose were obtained from Sigma Chemicals, Inc. (St. Louis, Mo). Sepharose 4B was obtained from Pharmacia, Inc. (Piscataway, NJ) and cyanogen bromide, from J. T. Baker and Co. All other electrophoretic chemicals and standards came from Bio-Rad (Richmond, Calif), as did the cation (Ag 50w-X4, 100–200 mesh) and anion (Ag 1-XB, 100–200 mesh) exchange resins used for the isolation of urinary delta-aminolevulinic acid (ALA). All other chemicals were of the highest reagent quality available.

Animals and treatment. Male Sprague-Dawley rats weighing 225–300 g were obtained from the OSU Laboratory Animal Resources Center and housed in the College of Pharmacy animal facilities. They were kept 3–4/cage in standard stainless-steel cages suspended over processed and dried hardwood chips; the room was maintained at 21° ± 1° C, 60% relative humidity, with a 12-h light-dark cycle. BCNU was dissolved in corn oil (15 or 20 mg/ml) and injected i.p. (0.1 ml/100 g body weight) to deliver a dose of 15 or 20 mg/kg, respectively. Controls received the same volume of corn oil. BHCNU was dissolved in propylene glycol-EtOH [80:20, v/v] and given in a similar manner to deliver 27 mg/kg, a dose approximately equimolar to 20 mg/kg BCNU. Sodium phenobarbital was given in saline to control and BCNU-pretreated rats by a single i.p. injection (80 mg/kg) on 4 consecutive days, and the rats were sacrificed 24 h after the last dose. β-Naphthaflavone was given in corn oil by a single i.p. injection (100 mg/kg) 40 h prior to sacrifice.

Microsomal preparation. Rats were killed by cervical dislocation and the livers were immediately removed and placed in ice-cold 0.25 *M* sucrose. The livers were minced and washed three times with fresh ice-cold 0.25 *M* sucrose, and a 20% homogenate was prepared using an ice-chilled Potter-Elvenhjem homogenizer. Microsomal pellets were obtained by standard differential centrifugation techniques [44]. The microsomal pellets were resuspended in TRIS-KCl buffer [0.1 *M* TRIS(hydroxymethyl) aminomethane, 1.15% KCl (pH 7.4)] to approximately 5 mg/ml protein. Protein concentrations were determined by the method of Lowry et al. [27].

Spectral determinations for enzyme assays and cytochrome P-450 were done on an Aminco DW-2a UV-Vis spectrophotometer. Cytochrome P-450 was determined by the method of Omura and Sato [35]. A spectral binding constant (K_s) was measured for ethylmorphine as described by Estabrook et al. [13].

Enzyme assays. NADPH cytochrome *c* reductase activity was determined at 25° C in the TRIS-KCl buffer in a single cuvette containing 50 μ*M* cytochrome *c*, 0.8 m*M* KCN, and 100 ml microsomes in a 3-ml total volume. The reaction was started by the addition of NADPH (final concentration, 0.1 m*M*) and the absorbance was monitored at 540–550 nm in the dual-wavelength mode of the Aminco spectrophotometer for 2 min. An extinction coefficient of 18.5 cm^2/mol was used [51].

Total ethylmorphine (EM) metabolism was measured by simultaneous detection of products from the *N*-demethylase and O-deethylase reactions as described by Jarvi et al. [21]. Ethylmorphine *N*-demethylase activity was also assayed by measuring the formation of formaldehyde by the method of Nash [33]. Metabolism of 7-ethoxycoumarin was asseyed as described by Ullrich and Weber [47] and modified by Elcombe and Lech [11], using a Turner Modell 111 fluorometer. Umbelliferone (7-hydroxycoumarin) was used as a standard for product formation. The linear reaction rate between 1–4 min was used for specific activity determinations. Metabolism of tritiated 3,4-benzo[a]pyrene was measured by the method of DePierre et al. [8]. The substrate was purified and redissolved in acetone containing unlabeled benzo[a]pyrene to a final stock concentration of 3.17 m*M* and a specific activity of 12.6 mCi/mmol. The chemical and radiochemical purity was 97%. Activity of the incubates was measured in a Packard Instrument Model 3385 liquid scintillation spectrometer. Heme oxygenase activity was measured in rat microsomes isolated as previously described [41]. Liver homogenates were assayed for aminolevulinic acid synthetase activity as described by Lifen and Beattie [26]. Urinary delta-aminolevulinic acid (ALA) was measured in control and BCNU-treated rats kept in individual metabolic cages by the method of Davis and Andelman [7].

Isolation of cytochrome P-450 proteins from the endoplasmic reticulum. *N*-Octylamino sepharose 4B was prepared as described by Cuatrecasus [6] and modified by Guengerich and Martin [15]. Commercial sodium cholate was recrystallized two times according to the method of Guengerich [14]. All steps were carried out at 4° C. Microsomes were obtained as previously described from male Sprague-Dawley rats (200–250 g) either left untreated or given 20 mg/kg BCNU 14 days prior to being killed. Livers were minced and pooled (8 rats/pool) prior to centrifugation. Solubilization was carried out as described by Imai [20]. Detailed descriptions of the isolation of the P-450 proteins have been published by Agosin et al. [1]. NADPH cytochrome *c* reductase (E.C. 1.6.2.4.) was isolated from liver microsomes of control rats that had been treated with phenobarbital (80 mg/kg, i.p., for 4 days) as described by Guengerich and Martin [14].

Sodium dodecyl sulphate polyacrylamide disc electrophoresis was carried out as described by Weber and Osborn [48]. Electrophoresis was carried out in a Hoefer Scientific disc electrophoresis unit. Molecular weights of the samples were determined by using a standard solution of proteins (obtained from Bio-Rad) for comparison.

Table 1. Liver:body weight ratios and hepatic microsomal protein and cytochrome P-450 content in control and BCNU-treated (20 mg/kg i. p.) male Sprague-Dawley rats

Time after treatment	Livers assayed (△)	Liver/body wt[a] (%)	Microsomal protein[a] (mg/g liver)	P-450[a] (nmol/mg protein)
Control	19	4.0 ± 0.1	6.5 ± 0.3	1.1 ± 0.04
6 h	5	4.3 ± 0.3	7.5 ± 0.3	1.0 ± 0.1
12 h	10	4.0 ± 0.3	8.1 ± 0.6	1.1 ± 0.2
24 h	15	4.0 ± 0.2	7.2 ± 0.3	1.1 ± 0.2
48 h	6	4.4 ± 0.5	7.5 ± 0.5	1.0 ± 0.1
72 h	5	4.7 ± 0.4	6.0 ± 0.2	1.1 ± 0.1
14 days	11	4.9 ± 0.2*	6.4 ± 0.4	0.9 ± 0.03**
21 days	11	4.9 ± 0.2*	5.9 ± 0.6	0.6 ± 0.04**
28 days	7	5.3 ± 0.5*	6.2 ± 0.2	0.7 ± 0.1**

[a] Mean ± SEM (for convenience of tabulation, control values were pooled since an F-test on the variance ratios revealed no statistical differences; $P < 0.01$)

* Significantly different from the control value of the group; $P < 0.05$

** Significantly different from the control value of the group; $P < 0.01$

Protein migration was monitored by staining the gels with 0.1% Coomassie blue dye (w/v) in 45% EtOH-10% acetic acid solution for 2-3 h and then destaining them overnight with EtOH-acetic acid-distilled water [10:7.5:82.5, by vol]. The gels were stored in distilled water until they were photographed.

Statistical analysis. Results shown in the text and tables are expressed as mean values ± SEM. Single statistical comparisons of a drug-treated group with its control were done using Student's two-tailed *t*-test. Differences were considered to be significantly different at $P < 0.05$. For convenience in presentation, the results of two or more replicated experiments were pooled when an F-test on the variance ratios showed no significant differences ($P < 0.01$) among the group variance.

Table 2. The metabolism of selected cytochrome P-450 substrates in male Sprague-Dawley rats 14 days after a single i. p. injection of 20 mg/kg BCNU

Substrate[a]	Control[b]	Treated[b]
	nmol product/mg protein per minute:	
ECOD	1.4 ± 0.1 (15)	1.5 ± 0.7 (9)
EMND	5.9 ± 0.9 (4)	2.7 ± 0.5 (4)*
EMOD	2.4 ± 0.5 (4)	1.5 ± 0.2 (4)*
BAPH	0.6 ± 0.1 (6)[c]	0.38 ± 0.45 (4)[c]
	nmol product/nmol P-450 per minute:	
ECOD	1.4 ± 0.1 (15)	1.5 ± 0.3 (9)
EMND	6.7 ± 0.8 (4)	4.3 ± 0.7 (4)*
EMOD	2.8 ± 0.5 (4)	2.4 ± 0.2 (4)
BAPH	0.6 ± 0.1 (6)[c]	0.2 ± 0.3 (4)[c]

[a] ECOD, ethoxycoumarin *O*-deethylase activity; EMND, ethylmorphine *N*-demethylase; EMOD, ethylmorphine *O*-deethylase; BAPH, benzo[a]pyrene hydroxylase

[b] All values are given as the mean ± SEM. The numbers in parentheses represent the numbers of livers assayed

[c] These data were taken 21 days after BCNU treatment

* Significantly different from the control values of the group; $P < 0.05$

Results

Initial, transient (1–2 days) anorexia and weight loss were typically observed among the BCNU-treated rats. Within the first 72 h of treatment a 15%–20% loss in body weight occurred and, in the manner described by Thompson and Larson [45], the treated animals continued to lag behind controls in weight gain after the resumption of feeding. These animals did not appear to be emaciated 14 days after BCNU administration, when most of these experiments were carried out. The only obvious symptom in BCNU-treated rats at this time was lower body weight as compared with controls of the same age. However, liver weights of BCNU-treated rats were not different from those of controls, and this resulted in higher liver: body weight ratios in those animals (Table 1). The rats became progressively jaundiced in a manner previously described as a result of the developing cholestasis [17, 45]. Jaundice became evident by day 21 after treatment and continued to increase in severity through day 28.

BCNU-induced decreases in cytochrome P-450 content

No change in P-450 content was seen before or during the time course over which cholestasis develops (36–48 h) in rats following BCNU administration. The earliest significant decrease in P-450 content was observed 14 days after the 20-mg/kg dose (Table 1). By days 14–21, when bile flow was maximally reduced at this dose to <50% of control [17], a 25%–45% decrease had occurred (Table 1). The decline in P-450 content was roughly equivalent 14 days after a dose of either 15 or 20 mg/kg (0.9±0.04 and 0.9±0.03 nmol P-450/mg protein, respectively). In rats receiving the higher dose the content continued to decline through day 21 (Table 1), whereas in animals receiving the 15-mg/kg dose it had apparently stabilized at the 14-day level (data not shown). We therefore focused most of our attention on the 14-day time point to take advantage of the observation that at this time and dose (20 mg/kg) the P-450 content was significantly diminished and still falling. This indicated that the toxic process was

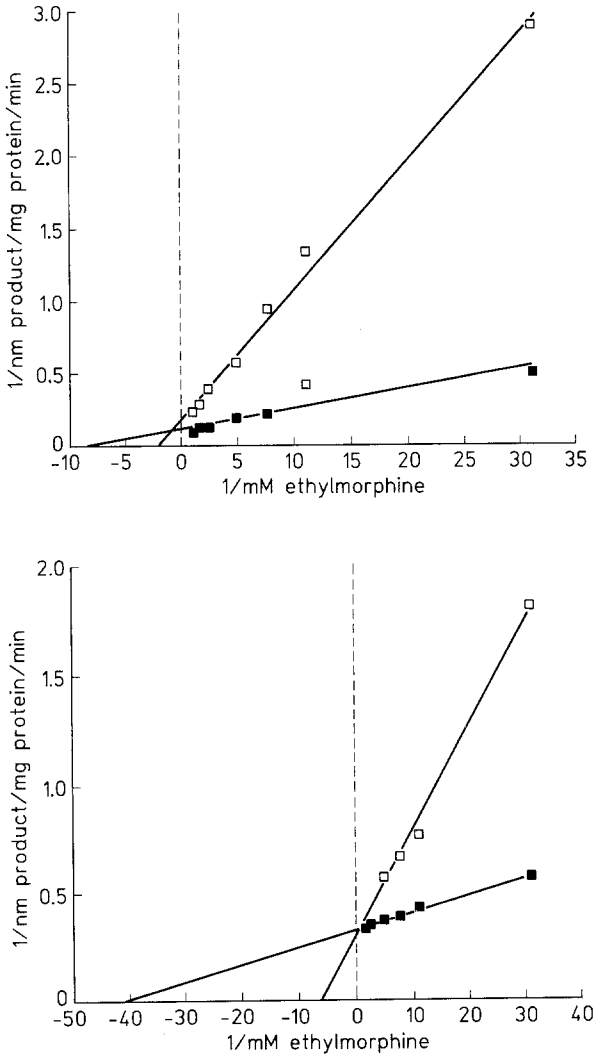

Fig. 1. Lineweaver-Burk representation of the kinetics of ethylmorphine *N*-demethylase (EMND, *top*) and ethylmorphine *O*-deethylation (EMOD, *bottom*) activities in liver microsomes isolated from control and BCNU-treated (20 mg/kg i.p.) male Sprague-Dawley rats at 14 days after treatment. Each point represents the average of three livers assayed. EMND: control K_m = 108 μM; V_{max} = 7.7 nmol product/mg protein per minute; treated K_m = 467 μM; V_{max} = 5.2 nmol product/mg protein per minute. EMOD: control, K_M = 23.8 μM; V_{max} = 3.03 nmol product/mg protein per minute; treated, K_m = 170 μM; V_{max} = 3.50 nmol product/mg protein per minute. ■ control, □ treated

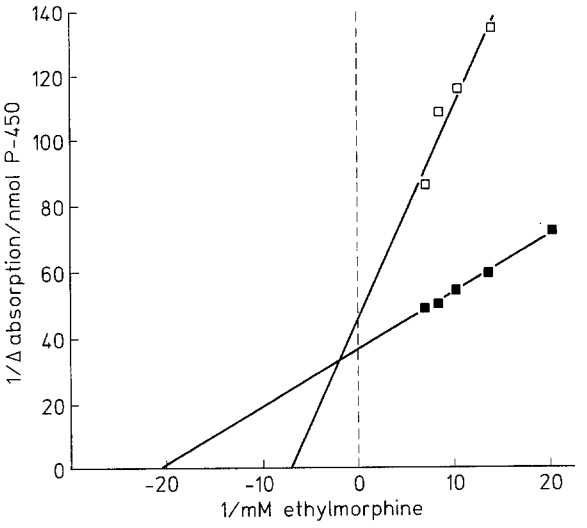

Fig. 2. A reciprocal binding plot of changes in absorbance $A_{390-420}$ per nanomole of cytochrome P-450 after the addition of ethylmorphine from liver microsomes isolated from control and BCNU-treated (20 mg/kg i.p.) Sprague-Dawley rats at 14 days after treatment. Each point represents the average of three livers assayed at two different protein concentrations (1.0 and 0.5 mg protein/ml) each. Control, K_s = 55 μM; A_{max} = 0.024. Treated, K_s = 80 mM; A_{max} = 0.016. ■ control; □ treated

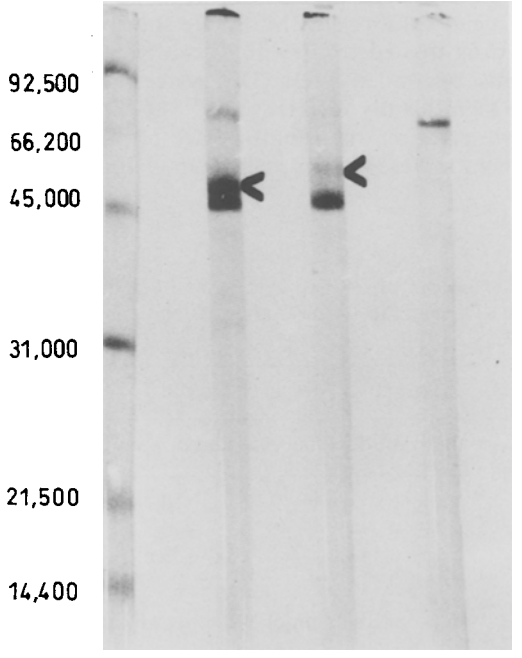

Fig. 3. SDS gel electrophoresis of partially purified cytochrome P-450 proteins from control and BCNU-treated (20 mg/kg i.p.) rat livers 14 days after treatment. Gel *A*, protein standards, approximate molecular weight (in daltons) indicated at *left*. Standards, from bottom to top: lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B. Gel *B*, from control rat livers, with major staining bands at apparent molecular weights of 53,700 and 56,200 daltons. Gel *C*, from livers of BCNU-treated rats, major staining bands at apparent molecular weights of 53,700 and 60,900 daltons. Gel *D*, purified cytochrome *c* reductase isolated from control rat livers; apparent molecular weight, 71,600 daltons. All gels contain 10% acrylamide

still in a dynamic stage and relatively uncomplicated by any general impairment to the health of the animals.

Effects of BCNU on P-450-mediated metabolism

When adjusted for either protein or P-450 content in the microsomes, the 7-ethoxycoumarin *O*-deethylase (ECOD) activity was not significantly altered from control values on day 14 after treatment with BCNU (Table 2). At 21 days after BCNU treatment this activity was not different relative to P-450 content, although it was significantly lower ($P < 0.05$) in terms of protein content due to the drop in the P-450 content of the microsomes at that time (data not shown). Similarly, BAPH activity was not affected at 14 days (not shown) and was variably but not significantly

Table 3. Liver: body weight ratios and hepatic microsomal protein and cytochrome P-450 content in control and BCNU-treated rats after phenobarbital or β-naphthoflavone induction

Days after[c] treatment	Liver/body wt. (%)	Microsomal protein (mg/g liver)	Cytochrome P-450 content[d]
	Phenobarbital[a, b]:		
Control[e]	5.0 ± 0.2 (19)	7.6 ± 0.4 (19)	2.6 ± 0.1 (19)
14	6.4 ± 0.2 (11)*	6.6 ± 0.3 (11)	2.2 ± 0.2 (11)
21	5.9 ± 0.2 (11)*	6.6 ± 0.3 (11)	1.6 ± 0.2 (11)*
28	5.6 ± 0.2 (6)*	6.6 ± 0.5 (6)	1.2 ± 0.2 (6)*
	β-naphthoflavone[a, f]:		
Control[e]	4.5 ± 0.2 (11)	7.3 ± 0.5 (7)	1.6 ± 0.5 (11)
14	5.2 ± 0.1 (4)*	7.8 ± 0.4 (4)	1.0 ± 0.1 (4)*
21	5.0 ± 0.3 (4)	6.2 ± 0.3 (4)	1.3 ± 0.1 (4)
28	4.6 ± 0.4 (4)	6.6 ± 0.4 (4)	1.3 ± 0.1 (4)

[a] All values are given as the mean ± SEM. The numbers in parentheses represent the numbers of livers assayed
[b] 80 mg/kg: 4 days of consecutive i. p. injections. The last dose was 24 h before the rats were killed
[c] BCNU, 20 mg/kg i. p. Controls received no BCNU but underwent the induction regimen with either phenobarbital or β-naphthoflavone. For comparisons with noninduced control values, refer to Table 1
[d] Units are expressed as nmol P-450/mg microsomal protein
[e] For convenience in tabulating, control values were pooled since an F-test on the variance ratios revealed no statistical differences; $P > 0.01$
[f] 100 mg/kg: one dose given i. p. 40 h before the rats were killed
* Significantly different from control values of the group; $P > 0.05$

($P < 0.05$) affected at 21 days, whether expressed in terms of P-450 content or microsomal protein (Table 2).

In contrast to the ECOD and BAPH activities, EM *N*-demethylase (EMND) was decreased by BCNU treatment. On day 14 after a 20-mg/kg dose of BCNU, EMND activity normalized to microsomal protein was depressed by 54%, whereas the P-450 content was only decreased by approximately 25%. Thus, the specific activity of the P-450 system (nmol product/nmol P-450 per minute) was also altered (Table 2). The 36% decrease in specific activity together with the lower P-450 content accounted for the overall reduction in EMND activity. Ethylmorphine *O*-dethylase (EMOD) activity was not decreased to the same extent as the *N*-demethylase activity. The decrease corresponded to the loss in P-450 content of the microsomes after BCNU treatment, but the specific activity of the EMOD system was not decreased (Table 2).

Cytochrome *c* reductase activity remained unchanged on day 14 after treatment with BCNU (20 mg/kg, data not shown). These data support previous observations that BCNU does not decrease cytochrome *c* reductase or cytochrome b_5 activity [29, 50].

Table 4. Alterations in hepatic cytochrome P-450 content and hepatic heme synthesis and degradation pathways 14 days after a single i. p. injection of 20 mg/kg BCNU

	Control[e]	Treated[e]
Cytochrome P-450 content[a]	1.0 ± 0.03 (5)	0.74 ± 0.06 (6)*
ALA synthetase[b]	40 ± 5.0 (6)	23.7 ± 3.60 (5)*
Heme oxygenase[c]	1.8 ± 0.1 (4)	2.4 ± 0.3 (4)
Urinary ALA[d]	0.54 ± 0.09 (5)	0.40 ± 0.02 (5)

[a] Units are expressed as nmol cytochrome P-450/mg microsomal protein
[b] Delta-aminolevulinic acid synthetase (ALA); units are expressed as nmol ALA/g liver per hour
[c] Units are expressed as nmol bilirubin/mg protein per hour
[d] Units are expressed as mg ALA/100 ml urine
[e] All values represent the mean ± SEM; the number of livers assayed is indicated in parentheses
* Significantly different from control values of the group; $P < 0.05$

Kinetic analysis of EMND and EMOD activities

In view of the apparent disparity between the losses in P-450 content and EMND activity, the kinetic parameters of the reactions were evaluated. These studies revealed sharp decreases in the apparent affinity, with little change in maximal velocity for the EMND and EMOD reactions in liver microsomes from BCNU-treated rats. Lineweaver-Burk plots of these data are presented in Fig. 1. There was a 4-fold increase in the K_m for EMND, from 108 μ*M* in controls to 467 m*M* in the treated groups. In the face of the unchanged specific activity of the EMOD reaction (Table 2), there was a 7-fold increase in its K_m (from 23.8 to 170 μ*M*). The decreased overall affinity of microsomes from BCNU-treated rats for EM was confirmed in spectral binding studies that demonstrated an increase of approximately 50% in the K_s and the A_{max} (Fig. 2).

Induction with phenobarbital and β-naphthoflavone

Induction with phenobarbital demonstrated that rats were responsive to this inducing agent even when the induction treatment was started up to 23 days after BCNU administration, when cytochrome P-450 content and activity

were greatly reduced in noninduced rats as compared with controls. Due to increased liver size after phenobarbital, BCNU-treated and control rats had higher liver: body weight ratios than the respective noninduced rats (Tables 1, 3). Hepatic microsomal P-450 content was also increased over values for noninduced rats in both groups. At 14 days after treatment the cytochrome P-450 content in induced, BCNU-treated rats was not statistically different from that in induced control rats (Table 3). Only after day 21 post-BCNU treatment did phenobarbital induction yield significantly lower P-450 content in treated rats than in controls; however, even at this time, the percentage of increase over the values for the respective noninduced rats was similar in both BCNU-treated rats (267%) and controls (236%).

Induction with phenobarbital failed to reverse the decrease in EMND specific activity, again demonstrating the sensitivity of this activity to BCNU pretreatment. After the induction, rats that had received BCNU 14 days earlier had only 53% of the EMND activity of induced controls when the activity was corrected for P-450 content. In contrast, phenobarbital increased ECOD activity in control and BCNU-treated rats to similar levels (data not shown).

Heme metabolism

To determine the extent to which alterations in heme anabolism and/or catabolism contributed to the decreased cytochrome P-450 content, ALA synthetase and heme oxygenase activities were assayed on day 14 after a dose of 20 mg/kg BCNU. These data are shown in Table 4. Heme oxygenase activity had increased by approximately 30% over control values by day 14 after BCNU treatment, but this increase was not statistically significant ($P > > 0.05$). However, there was a decrease of approximately 40% in ALA synthetase activity in the livers of the BCNU-treated rats at this time. In association with the decreased ALA synthetase activity, there was a tendency for reduced urinary excretion of ALA.

SDS gel electrophoresis

Cytochrome P-450 proteins were isolated from the endoplasmic reticulum and then analyzed by gel electrophoresis. The recovery of cytochrome P-450 proteins from the last stage in the process was approximately 20% of the total cytochrome P-450 protein originally present in the microsomes. Such recoveries are typical in these types of isolations [1]. The fractions containing cytochrome P-450 on the hydroxyapatite column that were obtained from control and treated rats were treated to remove the detergents, concentrated, and frozen at $-40°$ C. SDS gel electrophoresis of the products revealed different characteristics between the control and treated preparations (Fig. 3).

Two major staining bands with apparent molecular weights of 53,700 and 56,000 daltons were seen in the preparations from control rats. In the product from BCNU-treated rats, the band appearing at approximately 53,700 daltons was still evident, but the band at 56,200 daltons had disappeared and a faint band appeared in the 60,000-dalton region. Cytochrome *c* reductase isolated from control rats migrated on the gels with an apparent molecular weight of 71,600 daltons, comparing favorably with published values [51].

Results of BHCNU administration

Experiments using the compound BHCNU were conducted because it retains the carbamylating activity of BCNU but has a much lower alkylating activity [39]. This compound may be helpful in determining which of these activities is primarily responsible for the toxic effects seen with BCNU. The dose tested in these experiments (27 mg/kg) is approximately equimolar to that of 20 mg/kg BCNU. In contrast to animals that received BCNU, rats treated with BHCNU did not demonstrate weight loss, cholestasis, or jaundice (Hoyt and Larson, unpublished data). Liver: body weight ratios, hepatic microsomal protein, and P-450 levels were not significantly reduced after BHCNU treatment at this dose and in the time frame considered (data not shown).

Discussion

The time course of the decrease in P-450 content and EMND activity was consistent with the previous findings of Lu and Larson [28, 29] in rats and Wilson and Larson [50] in mice. It is doubtful that BCNU could directly produce these changes in vivo 14 days after dosing, in view of the drug's short residence time in the body [16, 24, 34] and the 1- to 3-day half-life of most P-450 isozymes [36, 37]. BCNU has been reported to interact with cytochrome P-450, producing a weak, type I substrate-binding spectrum [39], and these results have been duplicated in our laboratory (data not shown). The direct addition of BCNU at concentrations comparable with those expected in vivo into assay mixtures used to measure EM metabolism have never resulted in decreases in P-450 content or activity comparable with those observed in the present study. Thus, it would appear that the changes are due to biochemical or physiological effects that are not related to the direct attack of BCNU on the MFO system.

Nutritional inadequacy is a possible explanation for the delayed, progressive decreases in P-450 content and activity due to the anorexia experienced by the animals after BCNU administration. It is well established that the nutritional status of animals can alter their xenobiotic metabolizing capacity [5, 32]. Previous studies have demonstrated that the effects of BCNU are different from the changes in P-450 content and activity observed in pair-fed control rats and that force-feeding BCNU-treated rats with a nutritionally complete diet protects then from weight loss but not from changes in the P-450 enzyme system [29]. In the present studies, although the animals suffered from initial anorexia immediately after BCNU treatment, they soon resumed eating and did not appear emaciated on day 14, when most of these experiments were done. Liver weights were generally no lower in treated rats than in controls; however, due to smaller body weights that probably resulted from a lower percentage of body fat, the liver: body weight ratio was greater in treated rats. Total microsomal protein did not decrease in BCNU-treated animals. In previous experiments, the hepatic content of cytochrome *c* reductase and cytochrome b_5 was not altered by BCNU treatment on day 14 [29, 49]. In addition, the treated rats responded well to phenobarbital and β-naphthoflavone induction, suggesting that they could produce functional proteins and insert them into the endoplasmic reticulum. Although inadequate absorption of essential nutrients cannot be ruled out as a factor con-

tributing to our results, we feel that these contributions were minimal.

The apparent decrease in the specific activity of the EMND reaction (normalized to cytochrome P-450 content) was rather selective for that pathway, even when compared with *O*-deethylation of the same substrate. This may be due to the fact that the *N*-demethylation and *O*-demethylation of EM involve separate, multiple P-450 isozymes [10, 23, 25] that are not equally affected by BCNU treatment. Our kinetic studies suggest that both activities were altered by what appears to be competitive inhibition in the treated animals. The selective effect of BCNU was also demonstrated by the lack of a decrease in ECOD activity and only a late, marginal effect on BAPH.

Decreases in P-450 metabolic activities have been reported to occur mainly with type I substrates [13] after bile duct ligation or ethinyl estradiol treatment which is known to cause cholestasis in rats [30, 43]. For example, MacKinnon et al. [30] have also observed that EMND activity decreased dramatically after bile duct ligation. The decreases we observed in hepatic microsomal P-450 content were comparable with those that follow bile duct ligation [30, 43]. However, they were not as great as those associated with α-naphthylisothiocyanate (ANIT), which is believed to produce cholestasis and a loss of P-450 as separate effects [9, 12]. Thus, the changes observed in the present experiments may have been due to a decrease in bile flow resulting from BCNU treatment. This may provide an explanation for the apparent competitive inhibition seen in our experiments, because the decreased bile flow may have caused an increase in bile acids, bilirubin, or other endogenous products in the hepatocytes, which interfere with the metabolism of EM. The addition of bile acids to in vitro systems have produced changes in the kinetics of aminopyrine *N*-demethylation that closely resemble the changes reported here for EM [18, 19].

The decrease in ALA synthetase activity and the tendency toward increased heme oxygenase activity observed in the present study were also similar to the results of Schacter et al. [42] in bile-duct-ligated rats. In their experiments, within 48 h of bile duct ligation, heme oxygenase had doubled but ALA synthetase activity and P-450 content had decreased. The less prominent effect on heme oxygenase activity observed in the present study may be due to the more gradual development of cholestasis following BCNU as compared with the abrupt effect of bile duct ligation.

Although the relationship between P-450 content and control of the heme synthesis and degradation pathways is not well understood [31], it is unlikely that BCNU treatment directly damaged the heme regulatory pathways, in view of the long delay before the effects were noticeable. This hypothesis is supported by the fact that although cobalt, which is known to block heme synthesis, blocks P-450 induction by phenobarbital when spectral methods are used to measure enzyme content [38], rats treated with BCNU responded to phenobarbital induction in the present study. There is also no evidence from our data that a large increase in heme oxygenase activity is somehow responsible for the observed decrease in P-450 content, but a more complete time course evaluation is needed to rule out this possibility. Hutterer et al. [19] and others have suggested that bile salts accumulated after bile duct ligation can solubilize P-450 and associated enzymes from the endoplasmic reticulum, and this could also be a possible explanation for our observations.

Isolation of the microsomal cytochrome P-450 proteins from the endoplasmic reticulum and SDS gel electrophoresis of the proteins suggests that changes in the lipid environment alone cannot explain the alterations in MFO activity caused by BCNU treatment but that the proteins located in the membrane are actually different. MacKinnon et al. [30] have demonstrated differences in the electrophoretic patterns of microsomes after bile duct ligation that are similar to those reported here. This supports the theory that the changes observed in BCNU-treated rats are a response to the cholestasis produced in these animals and that cholestasis altered the type of cytochrome P-450 isozymes present in the endoplasmic reticulum. Selective changes in human cytochrome P-450 enzyme activities similar to those described in rats have also been seen in cholestatic human patients [22]. Therefore, differences in the cytochrome P-450 isozymes present in BCNU-treated rats as compared with controls probably contribute to the decreased EMND activity reported in the present study.

The studies with BHCNU, a congener of BCNU with low alkylation and high carbamylating activity, did not produce cholestasis (Hoyt and Larson, unpublished data). BHCNU also did not interfere with MFO activity at the dose and time course used in the present studies. Many of the toxic effects of BCNU and its cyclohexyl congener, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), have been ascribed to their carbamylating isocyanate degradation products, 2-chloroethylisocyanate and cyclohexylisocyanate, respectively [3, 4, 40]. We feel that studies with compounds such as BHCNU offer an excellent opportunity for investigating structure-related toxicities that occur with the nitrosoureas. Although the differences described between BCNU and BHCNU may ultimately be due to metabolic or pharmacokinetic considerations, the data support our hypothesis that the cholestatic effect of nitrosoureas such as BCNU and CCNU is primarily responsible for their effects on MFO activity.

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