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Title:

Hepatic Mixed Function Oxygenase Activity in Rats During Cholestasis Following 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) Treatment

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Robert E. Larson

Rats were given single i.p. injections of BCNU to investigate changes in hepatic cytochrome P-450 content and specific activity in male rats. Microsomal P-450 levels were unchanged from 6 hours to 7 days after BCNU treatment but by day 21 they had decreased 45%. Ethylmorphine N-demethylase activity (nm product x nm $P\text{-}450^{-1}$ x $minute^{-1}$) was only 67% of controls 14 days after BCNU treatment while ethylmorphine O-deethylase activity only fell 12%. Metabolism of benzo(a)pyrene and 7-ethoxycoumarin was not decreased to the extent of ethylmorphine N-demethylation. β-naphthoflavone increased 7-ethoxycoumarin metabolism more in BCNU pretreated rats than in controls.

Microsomal heme oxygenase activity was slightly elevated in BCNU treated rats 14 days after dosing. Delta-amino-levulinic acid synthetase activity was only 60% of controls in the liver homogenates of BCNU treated rats. No difference in microsomal total heme was detected.

Partial purification of P-450 from control and BCNU treated rat hepatic microsomes was achieved. Reduced, CO exposed preparations from treated rats had a bathochromic shift of 1.3 nm as compared to controls. Reconstituted systems with P-450 from controls had approximately 3 times higher ethylmorphine N-demethylase activity than similar systems using P-450 from BCNU treated rats. Differences between electrophoresis banding patterns were also observed. These results support the hypothesis that different P-450 isozymes exist in the treated animals.

Many of the changes seen in drug metabolism and hepatic heme metabolism after BCNU treatment mimic those reported from other laboratories in rats with cholestasis. BCNU produces cholestasis in rats and this may explain how the drug causes prolonged differences in P-450 content and specific activity.

Hepatic Mixed Function Oxygenase Activity in Rats During Cholestasis Following 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) Treatment

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It is impossible for me to indicate the influences responsible for my love of biology, but my parents and family supported me despite the fact they often wondered just how I got involved in this demanding discipline. For all of their trust, many, many thanks and much love.

...I found the star thrower...

Silently I sought and picked up a still-living star, spinning it far out into the waves. "I understand," I said. "Call me another thrower." Only then I allowed myself to think, he is not alone any longer. After us there will be others.

The Star Thrower

By Loren Eiseley

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Hepatic Mixed Function Oxygenase Activity in Rats During Cholestasis Following 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) Treatment

I. General Introduction

Attempts to decrease the number of cancer related deaths in this country have lead to a tremendous amount of research for new, more selective and potent chemotherapeutic agents. Development of antimetabolites, such as methotrexate, new antibiotics, such as various modifications of bleomycin, and numerous other cytotoxic agents has been continuous in the past 20 years. Along with these compounds are the nitrosoureas which were first introduced in 1963 when 1-methyl-1-nitrosourea (MNU) was found to kill intracerebrally innoculated L1210 leukemia in mice (1). This was the first report of an agent capable of passing through the blood brain barrier and slowing the spread of malignant cells in the central nervous system. One of the nitrosoureas, 1,3 bis-(2-chloroethyl)-1-nitrosourea (BCNU) was found to possess a potent killing effect on primary brain tumors (2,3). Since BCNU's discovery it has been used clinically as the main treatment for these types of tumors or as an adjunct to other chemical agents. Closely related compounds such as 1-(2-choroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) or 1-(2-chloroethyl)-3-(4 methylcyclohexyl)-1-nitrosourea (MeCCNU) have also been widely used. Better regimes of

therapy using these and other compounds have lead to greatly increased survival times for the cancer patient. This trend has lead to increasing concern about the long term toxicities associated with the nitrosoureas and how they might interact with other therapeutic agents being taken concurrently.

BCNU is a highly lipophillic compound which degrades rapidly in aqueous solutions under physiologic conditions. Pharmacokinetic studies have demonstrated the drug has a half life of only 15-30 minutes in the plasma with little or no parent drug being detectable in the serum after 5 minutes (4). Degradation of BCNU is largely non-enzymatic and elimination occurs mostly through the kidneys and biliary tract. Despite these properties the clinically limiting toxicities of the drug often do not appear for up to 6 weeks post dosing and may be of prolonged duration. The most life threatening of these side effects are severe myelosuppression and lung fibrosis (5,6).

Hepatic toxicity, while not immediately life threatening to the cancer patient, has been reported during clinical use (5). Serum levels of bilirubin, glutamic oxalacetic transaminase (SGOT) and alkaline phosphatase were monitored and 25% of the patients had increases in at least one of these markers that could not be attributed to the primary disease (5). These symptoms appeared from 6 to 66 days post dosing.

Thompson (7) described delayed lethality and bimodal hepatotoxicity in rats after a single i.p. injection of the drug. Even at the highest dosage (75 mg/kg) death was delayed for at least 5 days. As the dosage decreased the time to lethality was increased. This pattern was also seen when liver function was measured by serum enzymes, bromosulfophthalein (BSP) retention and pentobarbital sleep times. Though the cause for the delay in lethality and toxicity is not known, Thompson (7) speculated it may be due to several factors. These included recirculation of the drug in the enterohepatic system, a long lived metabolite, or a self-propagating biochemical and physiological effect.

Thompson and Larson (8) found biliary excretion of BSP was decreased for as long as 84 days post treatment with BCNU. Serum bilirubin was also increased throughout this period and these facts lead to direct measurement of bile flow in treated and control animals. Rats given 30 mg/kg BCNU 3 days previously had only 46% of the bile flow recorded in control rats. Flow began to return to normal by day 7 (76% of control) but decreased again by day 28 to 34% of the control value. A 20 mg/kg dosage of BCNU did not decrease bile flow after 3 days but by day 28 flow was only 12% of the control value (7). After 84 days the rats given 20 mg/kg BCNU had near normal bile flow. Histopathology of livers taken from rats given 30 mg/kg BCNU revealed inflammation and necrosis of the intraparenchymal biliary epithe-

lium by day 7 and necrosis of the bile duct epithelium by day 28 (7).

Subsequent studies done in this laboratory by Hoyt (9) have demonstrated that BSP excretion in rats is decreased 25% within 24 hours of a 20 mg/kg dosage of BCNU. The excretion is decreased to only 5% of controls 48 hours after treatment. Bile flow is decreased to less than 50% of controls after 48 hours. Some preliminary work on longer time periods after 20 mg/kg BCNU has shown flow begins to return to normal by day 7 but is decreasing again at day 14 (unpublished data).

More detailed analysis of the reduction in bile flow by Hoyt (9) has found that bile salt excretion is not decreased to the same extent as BSP excretion. During his experiments the excretion of bile salts in treated rats (20 mg/kg) was 70% of control rats after 48 hours though bile flow was 50% or less of the controls. Thus, the bile salt dependent flow seems rather refractory to the effects of BCNU while the bile salt independent flow is more sensitive to inhibition. Despite the relative lack of an inhibitory effect on the bile salt excretion mechanism there is little doubt bile acids must build up into the liver due to the very low bile flow in treated animals. Direct measurement of bile acids in hepatic tissues of control and treated animals remains to be done. Rats given 15 mg/kg BCNU had no significant decrease in bile flow up to 15 days post dosing but BSP

retention was evident at 10, 12 and 15 days (9).

The increases in pentobarbital sleep time also reported by Thompson and Larson (8) suggested a possible interaction between BCNU and the hepatic mixed function oxidase (MFO) system. Later experiments by Lu and Larson (10,11) concentrated on the in vitro metabolism of various substrates using a post mitochondrial supernatant and found that ethylmorphine N-demethylation, hexobarbital oxidation, analine hydroxylation and p-nitrobenzoic acid reduction activities (nm product x mg protein^{-1} x min^{-1}) were all decreased in rats 13 days after a single i.p. injection of BCNU (30 mg/kg). Also, total P-450 content of the liver microsomes was decreased when compared to controls. These data indicated that rats were largely compromised in their ability to metabolize xenobiotics after treatment with BCNU. The effects were maximal approximately 21 days after dosing and were dose dependent. One intriguing aspect of this toxicity was the increasing time delay before symptoms appeared as the dose of BCNU was lowered.

Despite the effects on cytochrome P-450 in Lu's experiments (10,11), BCNU did not appear to decrease cytochrome b_5 content or the total microsomal protein concentrations in rat liver microsomes. Due to the known anorexia and nausea BCNU causes in human patients, Lu & Larson (10,11) studied these effects in relation to P-450 activity and found that the BCNU-related decreases in meta-

bolism could not be explained by nutritional deficiencies. The delay between dosing and the effects on mixed function oxidase activity was fairly unique among chemotherapeutic agents (12). Despite the significant effect on cytochrome P-450 the treated rats appeared to respond to phenobarbital injections in Lu and Larson's experiments though levels of activity for most substrates did not reach those of induced control animals (10,11). General mechanisms to explain these effects were suggested but the cause of decreased metabolism remained speculative.

Attempts to isolate possible mechanisms led to studies using mice as a model (13). The compound had many of the same effects in mice as in rats but important differences were observed. Once again BCNU decreased the P-450 content and altered the activity (nm product x nm $P\text{-}450^{-1}$ x min^{-1}) of hepatic microsomes. Total ethylmorphine metabolism was decreased while benzo(α)pyrene activity was increased in the experiments with mice and 7-ethoxycoumarin activity was unchanged. Experiments adding BCNU directly to the in vitro assay did not alter metabolism and the inhibitory effects of BCNU on P-450 mediated metabolism were also seen in vivo in mice by measuring antipyrine half life.

Wilson's (13) experiments indicated mice were not as sensitive to the decreased metabolism of xenobiotics as were rats. The mice generally were more likely to recover from effects of the drug after approximately a month as compared

to rats given equal dosages in mg/kg. Additionally, in mice phenobarbital injections completely reversed the effects of BCNU with induced treated animals having the same P-450 content and activity as induced controls to all the substrates tested. Indeed, for most substrates induced BCNU treated animals had higher specific activities than did induced controls. In mice, as in the rat, many microsomal proteins such as cytochrome b_5 did not seem altered by BCNU. Wilson also measured cytochrome c reductase and found no decrease in activity (13).

Attempts to explain the effect of BCNU on mixed function oxidase activity have been unsuccessful despite a great deal of information known about the chemical characteristics of the compound. The major breakdown products are the 2-chloroethyl isocyanate carbamylating species and a 2-chloroethyl carbonium ion which causes alkylation (14-18). Many investigators feel therapeutic effects of the drug are derived from its alkylating ability while carbamylation causes more of the toxic side effects (15,19,20).

BCNU is known to halt cell division and has its maximal lethality to cells in the G_1 or early S phase of the cell cycle (15,21). This effect may be due to the ability of the drug to enter the nucleus and alkylate DNA as well as hampering the activity of DNA repair enzymes (22,24). Other experiments suggest a decrease in purine synthesis or the carbamylation of tubulin may kill the cells (25,26).

Whether or not these effects can be used to explain the hepatic toxicities involved with the drug is not known.

Recent studies have shown BCNU specifically inhibits many enzymes by alkylating their active site. In a clinical study Frischer and Ahmad (27) found that BCNU decreases erythrocyte glutathione reductase activity minutes after dosing and Babson and Reed (28) showed the enyzme was stoichiometrically inhibited by the 2-chloroethyl isocyanate moiety. Other enzymes such as chymotrypsin, alcohol dehydrogenase, creatine kinase and malate dehydrogenase have been shown to be directly inhibited by BCNU (28-30).

It is known that BCNU does reach the endoplasmic reticulum in liver cells and is actively metabolized at that site. The reaction is NADPH reductase dependent and the product, of this reaction 1,3 bis (2-chloroethyl) urea is formed fast enough to precede chemical breakdown in rats (31,32). Levin et al (33) reported that phenobarbital pretreatment of rats decreased the antitumor effect of the drug and increased its rate of elimination. Experiments done by Potter & Reed (34) found that BCNU undergoes microsomal denitrosation to inactive forms in rats. The related drug, CCNU, was reported by them to be degraded by the P-450 reductase and not the phenobarbital induced P-450 isozyme(s). Recent studies have shown BCNU metabolism to be a glutathione dependent reaction (35). It seems possible from these results BCNU may be able to reach and damage many

components of the endoplasmic reticulum.

By entering the cell membranes BCNU may damage the lipid bilayer itself. The drug is known to alter the endoplasmic reticulum in rat glioma cell monocultures (36). Changes in urinary bladder epithelial plasma membrane ultrastructure and cytoplasmic vesicles have been described for a related compound, N-methyl-N-nitrosourea (37,38). Cytochrome P-450 is known to be highly dependent upon a proper lipid environment and alterations in membrane structure will effect its activity (39,40).

The experiments described herein were designed to determine if the alterations in xenobiotic metabolism caused by BCNU are the result of direct effects of the drug on P-450 or secondary to some other process. Direct effects may include alkylation of DNA, damage to the ability of the cell to produce protein or attack on lipid components of the membranes. Alkylation of P-450 proteins or the lipid surrounding them may destroy critical protein-lipid interactions. A secondary effect may include damage to the enzymes caused by the prolonged cholestasis found in treated animals. Results from the experiments reported herein will be analyzed with an attempt to indicate that BCNU treated animals often have similar changes in P-450 metabolism as seen in animals after bile duct ligation (BDL) or after treatment with other agents known to cause cholestasis.

The first chapter of this study describes experiments

designed to better correlate differences between the rat and mouse models in order to better understand the underlying mechanism of the alterations to MFO activity caused by BCNU. This data also provides a comparison to data reported by other laboratories on animals after BDL or treatment with cholestatic agents. This is necessary largely due to a relatively small amount of work done on cholestasis in mice.

In the second study, experiments are described which measure two of the enzymes in heme metabolism in control and treated rats. This work addresses the problem of BCNU causing damage not to the P-450 system directly but to other enzymatic pathways necessary for its proper function.

The last study involved the isolation of P-450 isozymes from the livers of BCNU treated and control animals. These experiments also include reconstituted systems used to measure ethylmorphine N-demethylase activity. These may indicate whether or not BCNU directly damages the protein or the lipid components of the endoplasmic reticulum.

These experiments are more important than ever in view of the continued use of the nitrosoureas in cancer therapy. Additionally, as combination protocols become more common it is critical to understand the effects of BCNU on MFO activity.

II. The Effects of BCNU on Rat Liver Microsomal Cytochrome P-450 Content and Activity

INTRODUCTION

Numerous studies conducted previously have documented dose dependent changes in drug metabolism following BCNU pretreatment (8,10,11,13). These changes have been found in both mice and rats and can be measured by either *in vivo* or *in vitro* techniques. Despite a drug plasma half life of only 30 minutes the onset of altered drug metabolism may be delayed for weeks and last for a month or more (5,6). Two events mark the observed effects, both relating to the cytochrome P-450 enzyme system. First, the level of cytochrome P-450 in microsomes obtained from BCNU pretreated mice or rats is lower than control groups (10,11,13). Secondly, depending on the substrate, the specific activity of the system (nm product x nm $P\text{-}450^{-1}$ x min^{-1}) is decreased, increased, or unchanged after a single i.p. injection of BCNU. Despite the often drastic effects BCNU has on cytochrome P-450 itself, many other microsomal enzymes seem unaffected. Indeed, in mice, the microsomal protein content is often higher in the treated animals than controls (13). Theories on the mechanism causing these effects have been proposed but, while many possibilities have been eliminated the underlying causes have remained elusive.

Because BCNU carbamylates and alkylates numerous types of proteins and nucleic acids it is difficult to explain its rather selective effects on microsomal enzymes. Even more difficult to rationalize are the selective changes in the specific activity of P-450 toward certain substrates. The delayed effects would seem to exclude the possibility that BCNU acts directly on the P-450 system itself and the lack of effect when BCNU is added directly to in vitro systems supports this idea. The possibility exists that BCNU works through some secondary mechanism(s) and if this mechanism can be isolated the toxic side effects of BCNU may be better understood.

The experiments in this section are designed to more fully compare and contrast the effect of BCNU on the mouse and rat models. Despite the large amount of data described in the general introduction most of the studies done between the species do not overlap and indeed many of the techniques used today were not readily available when the original rat work was done. Many similarities exist in the response of the 2 species to BCNU but they also show many different reactions and a better description of these differences may aid in our understanding of the drug's mechanism of action. Thus, these experiments were done in rats and designed for comparison to the data from mice obtained by Wilson and Larson (13).

Another comparison will be to more completely describe

the effect of BCNU immediately following administration of the drug. Because the drug has a short half life and is known to enter into the endoplasmic reticulum (4,33) it is interesting to speculate whether or not the mixed function oxidase system is changed within 48 hours after injection of BCNU.

These experiments will also describe more completely the effect of BCNU on ethylmorphine metabolism. Wilson & Larson (13) studied total ethylmorphine metabolism as measured by the disappearance of substrate and did some work on N-demethylase activity as measured by the Nash reaction (41). Ethylmorphine O-deethylation also occurs and does not seem greatly affected by phenobarbital induction (42,43). Duquette and Holtzman (43) have developed a radioassay to measure both of these activities simultaneously but described herein is a high performance liquid chromatographic (HPLC) technique which eliminates the need for labeled substrates. Because of the selective effects of BCNU both of these activities will be studied in more detail.

Wilson & Larson (13) described for the first time the enhancing effect BCNU has on benzo(α)pyrene metabolism in the mouse. They tried to induce this activity with a P-448 inducer, β-naphthoflavone, but found their mice to be nonresponsive. A similar set of experiments were done in the rat to explain more clearly the effect of BCNU on this

MFO activity.

Recently a new nitrosourea compound, 1,3-bis(hydroxycyclohexyl)-1-nitrosourea (BHCNU), has become available which only carbamylates but does not alkylate biologic materials (44). By use of this compound I hope to isolate carbamylation from alkylation to see which is more important in altering MFO activity and also give some indication on the relative toxicity of the two compounds. Ultimately, this combination of comparative studies and new methodology may help to explain BCNU's mechanism of action. These experiments may also suggest further areas of investigation that will allow more understanding on how all the nitrosoureas react in the body.

MATERIALS & METHODS

Chemicals

Glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD E.C. #11149), nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Calbiochem Behring, ^{3}H benzo(a)pyrene was from New England Nuclear, 7-ethoxycoumarin was a gift from Dr. J.D. Hendricks, Scint-A was from Pakcard Instrument Co., Umbelliferone and β-naphthoflavone were obtained from Sigma Chemical Co., horse heart cytochrome c was from Boehringer Mannheim. HPLC grade acetonitrile was obtained from J.T. Baker Chemical Co., 1-hexane sulfonic acid counter ion (Pic-B6®) was from Waters Associates and the C_{18} reverse phase column was also from Waters Associates. Ethylmorphine, morphine and normorphine were all generously donated by the Research Technology Branch, Pre-clinical Research Division of the National Institute of Drug Abuse. All other materials used were reagent grade.

Animals and Treatment

Male Sprague-Dawley rats weighing 200-250 g were housed in the OSU College of Pharmacy animal facilities. Five animals per cage (17.5" x 10" x 7") were allowed food and water ad libitum and maintained in a 12 hour light-dark cycle.

BCNU was dissolved in corn oil (15 or 20 mg/ml) and

injected intraperitoneally, (i.p.), the volume of injection was 0.1 ml/100 g body weight. The 30 day LD_{50} of BCNU by this method of dosing was determined to be 30 mg/kg (7). Controls received injections of only corn oil. BHCNU was dissolved in a propylene glycol/EtOH (80/20, v/v) mixture (20 or 27 mg/ml, equivalent molar dosage to 15 or 20 mg/kg BCNU respectively) and controls received only the vehicle. Phenobarbital was dissolved in saline and given i.p. (80 mg/kg) 4 days consecutively. The last dose was given 24 hours prior to sacrifice. β-naphthoflavone was suspended in corn oil and given i.p. (100 mg/kg) 40 hours prior to sacrifice. The following steps were all conducted at 4°C. The homogenate was centrifuged (β-fuge, Model A-2, Lourdes Instruments Co.) at 600 xg for 12 minutes, the supernatant was then decanted from the pellet and spun again at 12,000 xg for 12 minutes. Microsomes were pelleted from the resulting supernatant in a Beckman Model L3-50 ultra centrifuge using a type 42.1 rotor at 105,000 xg for 60 minutes. The pellet was resuspended in tris/KCl buffer (0.1 M tris, 1.15% KCl, pH 7.4) and resedimented in the ultracentrifuge. This pellet was resuspended in the tris/KCl buffer to approximately 5 mg protein/ml. Protein was determined by the method of Lowry et al (45). All microsomal suspensions were kept ice cold and used within 12 hours of isolation.

Spectral determinations were performed on an Aminco DW-2a UV-vis spectrophotometer (46). Cytochrome P-450 was

determined using the method of Omura and Sato (47). An extinction coefficent of $E_{450-490}$ 91 x mM^{-1} x cm^{-1} was used. Cytochrome P-420 was measured as described by Omura and Sato (48). A spectral binding constant (Ks) was measured for ethylmorphine as described by Schenkman et al (49).

Enzyme Assays

NADPH cytochrome c reductase was determined at 25°C in the tris/KCl buffer (pH 7.4) in a single cuvette containing 50 uM cytochrome c, 0.8mM KCN and 100 ul of microsomes in 3 ml total volume. The reaction was started by the addition of NADPH (0.1 mM final concentration) and monitored at 550 nm - 540 nm in the dual wavelength mode of the Aminco spectrophotometer for 2 minutes. An extinction coefficient of 18.5 x mM^{-1} x cm^{-1} was used (50).

Total ethylmorphine metabolism was measured by simultaneous detection of products formed from N-demethylation and O-deethylation of the substrate. The assay mixture was 2.5 ml of total volume (tris/KCl buffer) containing 10 mM $MgCl_2$, 0.2mM nicotinamide, 0.8 units/ml glucose-6-phosphate dehydrogenase, 4 mM glucose 6 phosphate and 0.9 mM ethylmorphine. The protein concentration was 1 mg protein/ml for most determinations. Duplicate samples were incubated for 5 min. at 37°C in a Dubnoff metabolic shaker water bath and the reaction was then started by the addition of $NADP^+$ (final concentration 0.4 mM). Blanks received only the buffer. Incubation was carried out for 7 minutes in air

and stopped by the addition of 1.5 ml of 0.5 M potassium phosphate (pH 8.7) to the flasks which were then put on ice. The assay mixture was then poured into 15 ml capped tubes and unmetabolized ethylmorphine and the products of metabolism were extracted by the addition of 15 ml of 20% isopropanol in dichloromethane containing 1.8 µg/ml codeine sulphate used as an internal standard. The tubes were shaken by hand for 4 minutes, centrifuged to enhance separation of the layers for 5 minutes and the upper aqueous layer was aspirated and discarded. The organic phase was evaporated under nitrogen at 60°C and brought up to a volume of 150 µl in the HPLC mobile phase consisting of 85% Waters reagent Pic-B6® (1-hexane sulfonic acid) in 1% acetic acid and 15% CH_3CN.

Samples were then injected (0.01 ml using a Waters Intelligent Sample Processor (WISP) Model 710) onto a µBondapak C_{18} (3.9 mm x 30 cm) reverse phase column with a flow rate of 2 ml per minute at 2100 psi. The compounds of interest were measured with a U.V. spectrophotometric detector set at 254 nm and data were recorded on a linear strip chart recorder. Total run time was 12 min. Activity was expressed as nm morphine/nm P-450/min or nm norethylmorphine/nm P-450/min. The reactions were linear up to 10 minutes at 1 mg protein/ml. Ethylmorphine N-demethylase activity was also measured in separate experiments by the Nash reaction using the same assay conditions.

[^{3}H]-benzo(α)pyrene metabolism was measured by the method of DePierre et al (51). Benzo(α)pyrene [1,3,6-^{3}H] (in hexane) was washed with 0.25 m NaOH/40% EtOH. The Hexane layer was removed, evaporated to dryness and the benzo(α) pyrene was redissolved in acetone containing cold 3,4 benzo(α)pyrene to a final stock concentration of 3.17 mM and a specific activity of 12.6 mCi/mM. The metabolism was carried out in 1.0 ml total volume tris/KCl buffer containing the NADPH generating system described previously with 0.5 mg microsomal protein and 80 mM benzo(α)pyrene (1.0 mCi [^{3}H]-BP). Assays were started by the addition of $NADP^+$ after a 5 minute preincubation at 37°C and terminated after a 20 minute reaction time with 1.0 ml of 0.5 M NaOH in 80% ethanol/H_2O. The assays were extracted with 3.0 ml of hexane. The activity was analyzed by removing 25 µl of the hexane extract (containing unreacted substrate) and putting it into 5 ml of Scint-A used as fluor. A 0.3 ml aliquot of the lower phase (containing the product) was put into 10 mls of the fluor. These were then placed in a Packard Instrument Model 3385 liquid scintillation spectrometer, counted, and activity was expressed as nm product x nm $P\text{-}450^{-1}$ x $minute^{-1}$. The reaction was linear for 25 minutes and from 0.1 mg protein to 0.5 mg protein per ml.

Metabolism of 7-ethoxycoumarin (mp 87-87.5°C) was assayed as described by Ullrich and Weber (52) and modified by Elcombe and Lech (53). The reaction took place in 3.0 ml

of 66 mM tris/HCl buffer (pH 7.4) containing 0.10 mM 7-ethoxycoumarin (added in DMSO stock), 0.5 mg microsomal protein per ml and was started by the addition of NADPH (50 mM final concentration) at 25°C. The increase in fluorescence at 450 nm was monitored in a Turner Model III Fluorometer filtered with a narrow pass filter #7-60 for 360 nm excitation and a narrow pass filter #48 plus sharp cut filter #2A for emission at 450 nm. Umbelliferone (7-hydroxycoumarin) was used as a standard for product formation. The first minute of the reaction was ignored because it was not linear with microsomal protein content. The next 4 minutes of the reaction were linear and were used for specific activity determination which was expressed as nm umbelliferone x nm $P\text{-}450^{-1}$ x $minute^{-1}$.

All data was compared using Student's t-test with the level of significance set at $p \leq 0.05$ unless otherwise stated. All data are reported as the mean $\pm$ standard error.

RESULTS

Rats pretreated with BCNU (15 or 20 mg/kg) experienced an initial period of anorexia lasting 1 or 2 days. Despite resumption of eating, treated rats continued to have lower body weights than controls when sacrificed at 14, 21 or 28 days. Rats treated at the 20 mg/kg dose lost approximately 20% of their original weight by day 14. The lower dose caused less weight loss, on the order of 10%, at this time point. Studies done on rats 24 hours after they were given 20 mg/kg BCNU did not show any weight loss.

Liver weights did not decrease proportionately to body weights. (Table I) Treated animals showed a statistically significant increase ($p \leq 0.05$) in liver to body weight ratios after induction with phenobarbital or β-naphthoflavone but the effect was not as pronounced with the P-448 type inducer (Table I).

Some animals treated with BCNU (20 mg/kg) demonstrated clinical signs of jaundice by day 21. These signs included yellowish adipose tissue and in some cases a cloudy yellow urine. In severe cases the liver itself appeared yellowish though this usually did not occur until day 28 or later. These animals were noted and compared to other treated groups. As expected, they had lower body and liver weights than treated animals not showing jaundice and often had very low microsomal P-450 levels and activity. The supernatant obtained from the first 105,000 xg centrifugation was

TABLE I: Liver to body weight ratios in control and BCNU treated (20 mg/kg) male rats.

	Noninduced	Phenobarbital Induced[b]	-Naphthoflavone Induced[c]
Control	4.02 ± 0.12 (19)[d]	5.04 ± 0.17 (19)	4.51 ± 0.19 (11)
BCNU[a]			
Day 14	4.85 ± 0.15[e] (11)	6.38 ± 0.24[e] (11)	5.15 ± 0.10[e] (4)
Day 21	4.94 ± 0.17[e] (11)	5.92 ± 0.22[e] (11)	4.97 ± 0.25[e] (4)
Day 28	5.33 ± 0.47[e] (7)	5.68 ± 0.15[e] (6)	4.57 ± 0.41 (4)

All data presented as weight percent liver to body in grams ± standard error of the mean

a. animals injected i.p. with 20 mg/kg BCNU in corn oil

b. animals injected i.p. with 80 mg/kg for 4 consecutive days before sacrifice

c. animals injected i.p. 100 mg/kg in corn oil 40 hours before sacrifice

d. number of animals per group

e. significantly different from controls of that group ($p \leq 0.05$)

yellowish in these animals though it was clear after the 2nd ultracentrifugation step. These animals responded poorly to induction with phenobarbital or β-naphthoflavone. Animals given dosages of 15 mg/kg rarely showed signs of jaundice. The experiments were designed to minimize the occurrence of these signs and most animals in the study looked and acted much like controls except for some weight loss and lack of preening behavior.

BCNU caused a dose dependent decrease in the cytochrome P-450 content of liver microsomes which first became significant 14 days after the 20 mg/kg dosage (78% of control). Animals treated with 15 mg/kg also showed significant decreases by day 14, but they were not as severe (88% of control). It should be noted that the higher dose caused measurable decreases in P-450 levels before the decrease was seen at lower doses. These data are presented in Tables II, III, IV, and Figure 1. In this study the high dose animals were maximally effected by day 21 (P-450 content 57% of controls) (Fig. 1). Some animals treated with the higher dose began to recover by day 28 but others were extremely sick. This may explain the high variability of the data at this time point. Decreases in P-450 levels are not related to increases in P-420. The percentage of P-420 to P-450 was never more than 10% in control or treated groups.

Treatment with phenobarbital (80 mg/kg, 4 days) caused the expected rise in P-450 content as compared to noninduced

TABLE II. The effects of BCNU (20 mg/kg) on cytochrome P-450 content and specific activity in male rat hepatic microsomes

	nm P-450/mg Protein	Ethylmorphine[a]	7-Ethoxycoumarin[a]	Benzo(α)pyrene[a]
(A) NONINDUCED ANIMALS				
Control	1.1 ± 0.04 (19)[e]	2.96 ± 0.27 (12)	1.41 ± 0.12 (15)	0.58 ± 0.08 (6)
BCNU[b]				
14 days	0.85 ± 0.03*(11)	1.80 ± 0.29*(7)	1.49 ± 0.65 (7)	---
21 days	0.63 ± 0.04*(11)	1.87 ± 0.35*(6)	0.37 ± 0.15 (3)	
28 days	0.74 ± 0.10*(7)	1.08 (2)	---	
(B) PHENOBARBITAL INDUCED ANIMALS[c]				
Control	2.6 ± 0.11 (19)	3.6 ± 0.34 (15)	4.28 ± 0.22 (17)	0.35 ± 0.023 (8)
BCNU[b]				
14 days	2.19 ± 0.19*(11)	1.92 ± 0.15*(7)	5.12 ± 0.49 (11)	0.66 ± 0.03* (4)
21 days	1.62 ± 0.16*(11)	2.44 ± 0.28*(7)	4.14 ± 0.7 (9)	0.35 ± 0.04 (4)
28 days	1.18 ± 0.20*(6)	2.45 ± 0.08*(4)	8.50 ± 0.90 (5)	---
(C) β-NAPHTHOFLAVONE INDUCED ANIMALS[d]				
Control	1.58 ± 0.48 (11)	---	5.54 ± 0.64 (6)	1.93 ± 0.24 (4)
BCNU[b]				
14 days	0.97 ± 0.07*(4)	---	---	2.23 ± 0.15 (4)
21 days	1.26 ± 0.13 (4)	---	3.85 ± 1.13*(4)	1.19 ± 0.25 (4)
28 days	1.30 ± 0.14 (4)	---	12.40 ± 0.08 (4)	0.93 ± 0.21*(4)

a. nm Product/nm P-450/minute, mean ± standard error of the mean
b. 20 mg/kg i.p.
c. 80 mg/kg: 4 days consecutively i.p. The last dose was given 24 hours prior to sacrifice
d. 100 mg/kg: 1 dose i.p. 40 hours prior to sacrifice
e. number of samples
* significantly different from controls of that group ($p \leq 0.05$)

TABLE III. Liver microsomal metabolism (nm Product/mg Protein/minute) in BCNU treated (20 mg/kg) male rats expressed as percent of control
All metabolism expressed as mean ± S.E.

	nm P-450/mg Protein	Ethylmorphine[a]	7-Ethoxycoumarin	Benzo(α)pyrene
(A) NONINDUCED ANIMALS[a]				
Day 14	77	53	107	—
Day 21	57	38	46	36
Day 28	67	34	--	—
(B) PHENOBARBITAL INDUCED ANIMALS[c]				
Day 14	84	68	89	106
Day 21	62	67	104	90
Day 28	45	46	104	—
(C) β-NAPHTHOFLAVONE INDUCED ANIMALS[d]				
Day 14	61	—	--	69
Day 21	80	--	168	60
Day 28	82	—	158	37

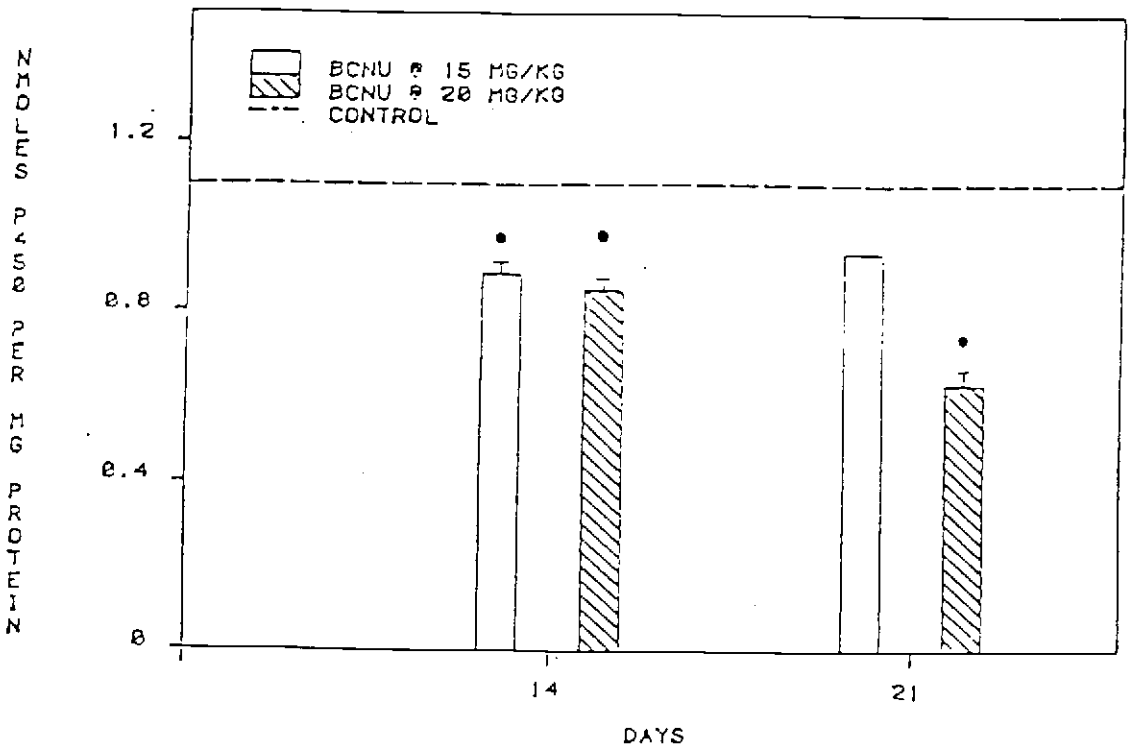
a. Control values: ethylmorphine 3.16 ± 0.25 nm; 7-ethoxycoumarin 1.42 ± 0.1; benzo(α)pyrene 0.59 ± 0.08
b. Control values: ethylmorphine 7.15 ± 0.40 nm; 7-ethoxycoumarin 11.1 ± 0.8; benzo(α)pyrene 0.99 ± 0.16
c. 7-ethoxycoumarin 9.1 ± 0.81 benzo(α)pyrene 3.6 ± 0.51

TABLE IV. The effects of BCNU (15 mg/kg) on P-450 content and activity in male rat liver microsomes

	nm P-450/mg Protein	Ethylmorphine[a]	7-Ethoxycoumarin[a]	Benzo(α)pyrene[a]
(A) NONINDUCED				
Control	1.1 ± 0.04 (19)[c]	6.63 ± 0.08 (4)	1.41 ± 0.53 (15)	0.58 ± 0.08 (6)
BCNU[b]				
14 days	0.89 ± 0.04 (9)	4.09 ± 0.49 (6)*	1.54 ± 0.27 (9)	0.50 ± 0.10 (5)
21 days	0.94 (2)	—	1.25 (2)	—
(B) PHENOBARBITAL INDUCED				
Control	2.6 ± 0.11 (19)	—	4.28 ± 0.22 (17)	0.35 ± 0.023 (8)
BCNU[b]				
14 days	1.97 ± 0.28 (6)	—	3.23 (2)	0.38 ± 0.07 (4)
21 days	1.70 ± 0.08 (3)	—	4.21 ± 0.24 (3)	—
(C) β-NAPHTHOFLAVONE INDUCED				
Control	1.42 ± 0.10 (6)	3.06 ± 0.64	—	—
BCNU[b]				
14 days	1.12 ± 0.064(6)	2.43 ± 0.91	—	—

* significantly different from controls ($p \leq 0.05$)
All data expressed as mean ± standard error
a. nm Product/nm P-450/minute
b. 15 mg/kg, i.p.
c. number of samples

Figure 1. Cytochrome P-450 Content



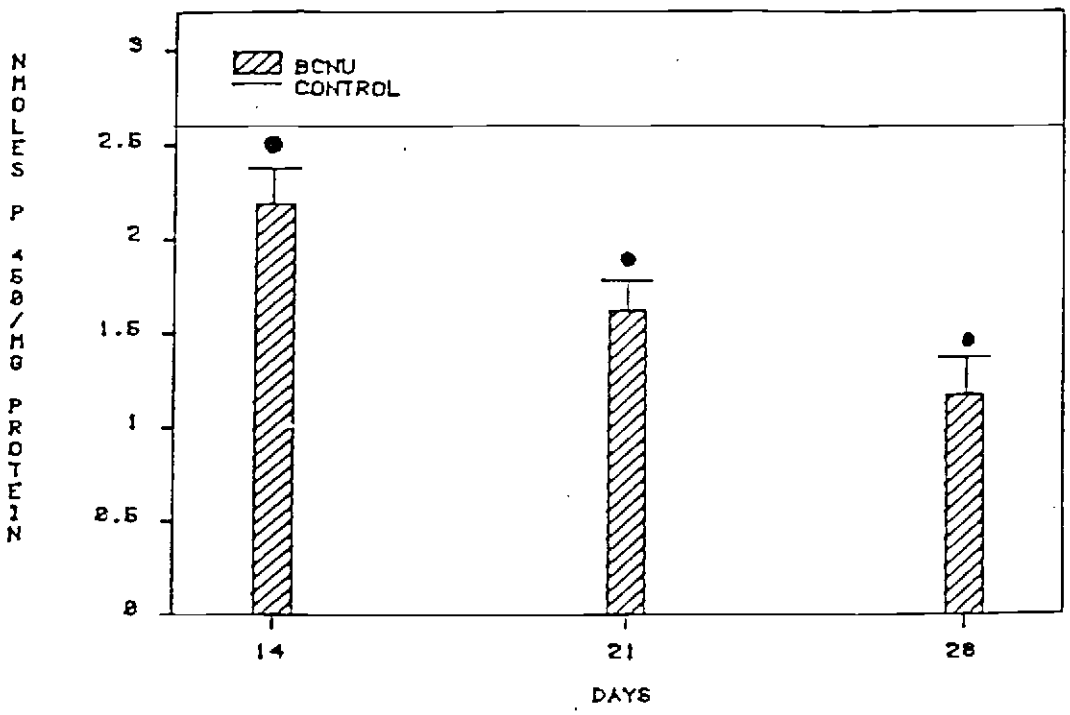
BCNU, dissolved in corn oil, was given i.p. to male rats.

Control rats received only the vehicle. Hepatic microsomal protein and P-450 content was measured as described in the text. Treated groups significantly different from controls ($p \leq 0.05$) are indicated by black dots.

rats for both control and BCNU treated animals (Tables II, III, IV). Phenobarbital given for 6 days at this dosage failed to raise the amount of induction over that seen in animals given the drug for only 4 days. Pretreatment with BCNU (20 mg/kg) decreased the amount of induction obtained by 38% at day 21 (Figure 2). BCNU pretreated animals did, however, always have higher P-450 levels than noninduced controls at days 14 and 21 despite the large decreases that occurred after BCNU alone at these times.

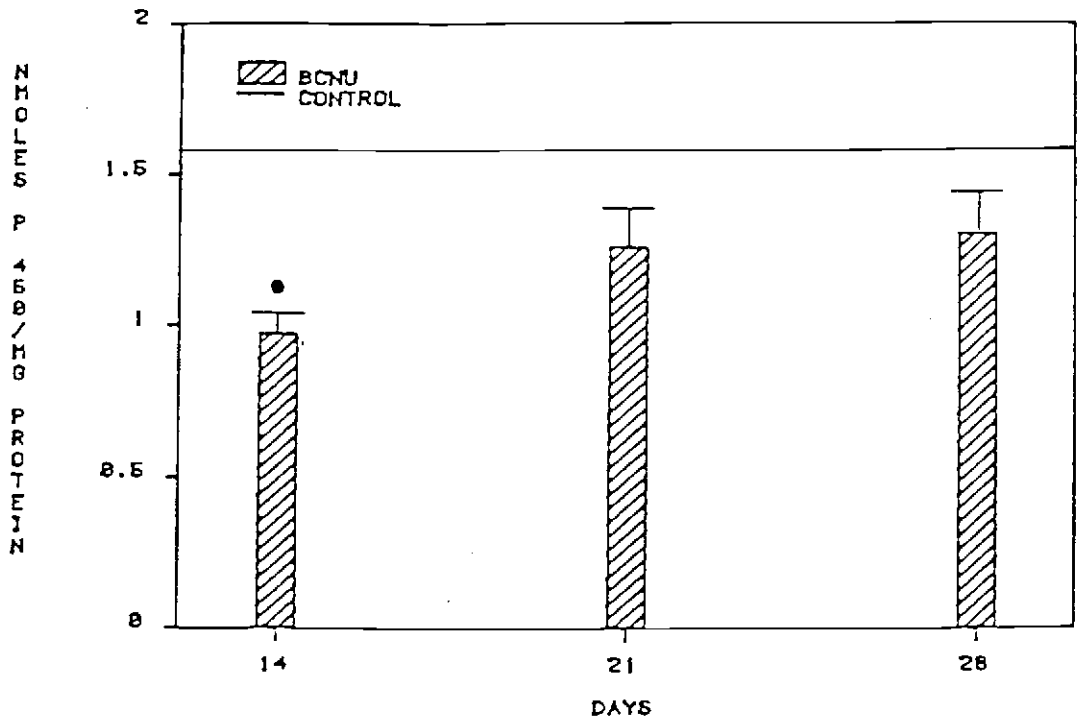
β-Naphthoflavone induction was more variable than phenobarbital induction in both control and treated animals. (Tables II, III, IV). Both groups did, however, show the spectral shift from 450 nm to 448 nm that is common with 3-methylcholanthrene type inducers and demonstrated increased levels of mixed function oxidase content over noninduced controls. Animals pretreated with BCNU did not, however, induce to the same levels as the control groups at day 14 which is in agreement with the experiments using phenobarbital (Figure 3). Animals given 15 mg/kg BCNU were only induced to 83% of induced control groups at day 14 while the 20 mg/kg groups only responded 58% as much as controls. By days 21 and 28, however, there was no difference between induced controls and induced BCNU treated rats. This differs from the results obtained after phenobarbital induction.

Figure 2. Cytochrome P-450 Content after Phenobarbital Induction in Control and BCNU Treated Animals



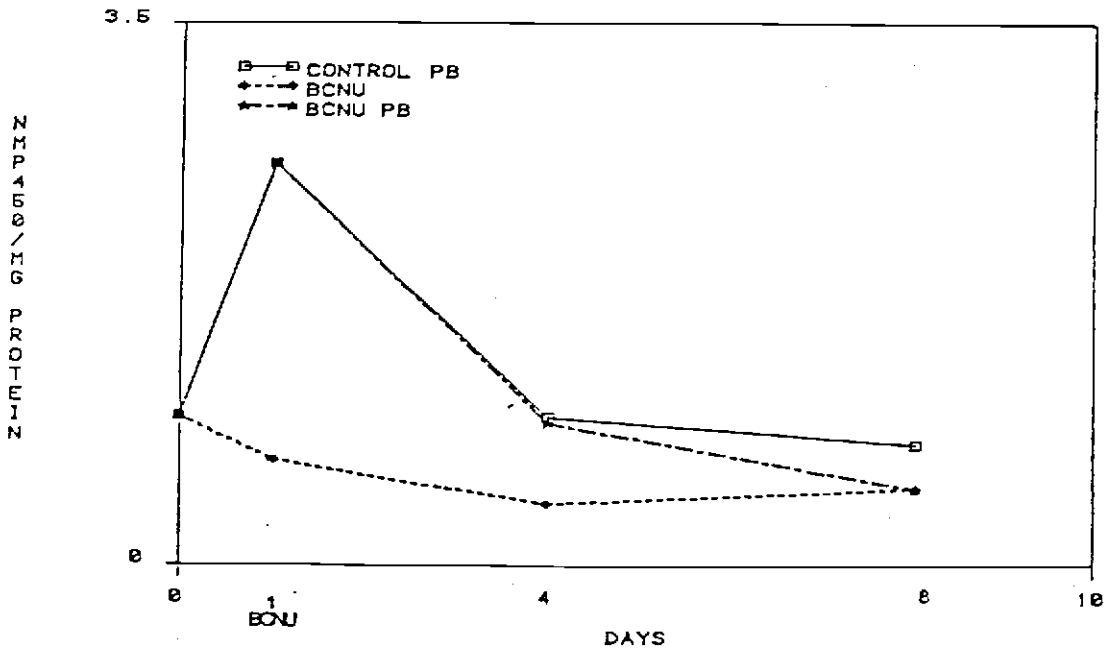
Male rats were given BCNU (20 mg/kg) dissolved in corn oil and injected i.p. (80 mg/kg) for 4 consecutive days. Animals were sacrificed 24 hours after the last phenobarbital injection. Hepatic microsomal protein and P-450 was measured as described in the text. Treated groups significantly different from controls ($p \leq 0.05$) are indicated by black dots.

Figure 3. Cytochrome P-450 Content after β-Naphthoflavone Induction in Control and BCNU Treated Animals



Male rats were given BCNU (20 mg/kg) dissolved in corn oil and injected i.p. β-naphthoflavone was dissolved in corn oil and rats were injected i.p. (100 mg/kg) 40 hours before they were sacrificed. Hepatic microsomal protein and P-450 content were measured as described in the text. Treated groups significantly different from controls ($p \leq 0.05$) are indicated by black dots.

Figure 4. P-450 Content VS Time (BCNU 15mg/kg) Following Phenobarbital



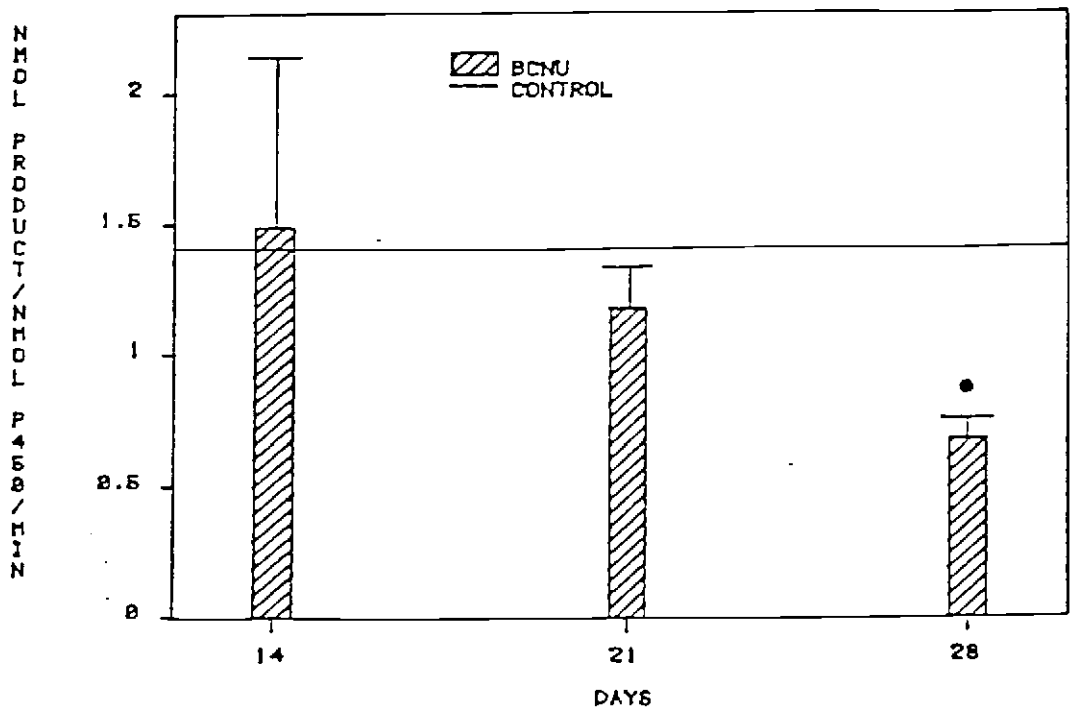
Male rats were induced with (80 mg/kg) given for 4 consecutive days i.p. BCNU was dissolved in corn oil and given to some animals 24 hours after the last phenobarbital injection. Some noninduced rats were also given BCNU. Hepatic microsomal protein and cytochrome P-450 content were measured as described in the text. Each point is the mean of 2 animals.

Several groups of rats were pretreated with phenobarbital (4 days, 80 mg/kg) and then given BCNU (15 mg/kg) 24 hours later to measure any increased rate of P-450 degradation in the BCNU animals as compared to induced controls not given the drug. Some rats were just given BCNU for comparison. As shown in Figure IV no difference in the rate of return to noninduced levels of P-450 was seen.

Experiments were conducted in control and BCNU pretreated animals on the in vitro metabolism of several P-450 substrates. No effect on ethylmorphine N-demethylation was seen as measured by the Nash reaction when BCNU was added directly to assays containing control microsomes. These experiments were done at concentrations of BCNU up to 5 times what could be expected in vivo in the liver at the dosages given the animals. Measurements of P-450 conversion to P-420 during the incubation period revealed no differences between control and treated groups. Conversion was never greater than 5% over a 10 minute incubation at 37°C.

Animals receiving 20 mg/kg BCNU did not show any decreased 7-ethoxycoumarin activity when product formation was normalized to either nm P-450 or mg protein. After 14 days, however, the metabolism of 7-ethoxycoumarin was decreased if calculated on a mg protein basis but showed no change if corrected for nm P-450. This, is of course, due

Figure 5. 7-Ethoxycoumarin Metabolism in Control and BCNU Treated Animals



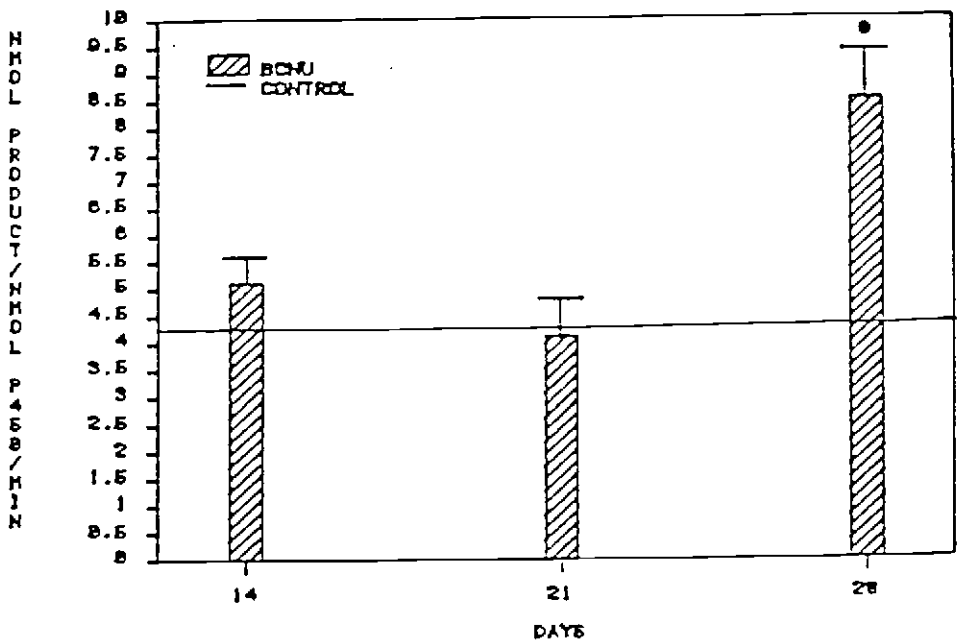
Male rats were given BCNU (20 mg/kg) dissolved in corn oil and injected i.p. Controls received only the vehicle. Hepatic microsomal metabolism of 7-ethoxycoumarin was measured by the increase in fluorescence at 450 nm after excitation with 360 nm light. Umbelliferone was used as a standard for product formation. Treated groups significantly different from controls ($p \leq 0.05$) are indicated by black dots.

to the large decrease in the content of P-450 in the microsomes by this time (Table II, Figure 5). By day 21 the activity was lower even if based on product formation per nm P-450. Thus, there was a steady decrease in the animal's ability to metabolize this substrate up to 21 days after dosing.

Phenobarbital completely reversed the effects of BCNU on 7-ethoxycoumarin metabolism at day 14 and there was no difference between the activity of induced controls and induced treated animals. By day 21, however, the activity of induced treated animals was only 66% of induced controls when corrected to mg microsomal protein (Table III). The specific activity of the P-450 enzyme(s) was not affected and was even somewhat higher in treated induced animals. By day 28 the specific activity of P-450 had increased to 2 times that of the induced controls. It should be stressed the amount of cytochrome P-450 per mg microsomal protein was still lower for the induced treated animals when compared to induced controls at this time. Thus, though the BCNU animals 28 days after treatment had less P-450 content, the enzymes were more effective in metabolizing 7-ethoxycoumarin (Figure 6).

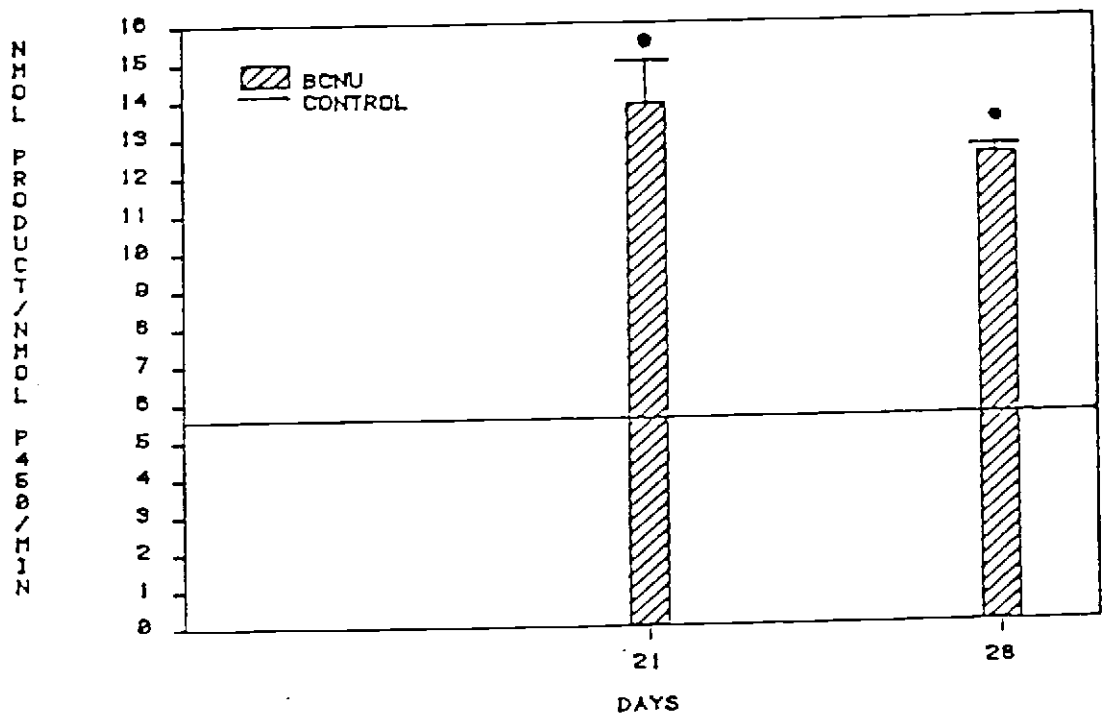
Induction with β-napthoflavone elevated the activity of BCNU treated animals to levels well above control induced groups towards 7-ethoxycoumarin on days 21 and 28 (Figure 7). This was true whether the data was calculated on a per

Figure 6. 7-Ethoxycoumarin Metabolism After Phenobarbital Induction in Control and BCNU Treated Animals



Male rats were given BCNU (20 mg/kg) dissolved in corn oil and injected i.p. Controls received only the vehicle. Phenobarbital was dissolved in saline solution and injected i.p. (80 mg/kg) for 4 consecutive days. Animals were sacrificed 24 hours after the last phenobarbital injection. Hepatic microsomal metabolism of 7-ethoxycoumarin was measured by the increase in fluorescence at 450 nm after excitation with 360 nm. Treated groups significantly different from controls ($p \leq 0.05$) are indicated by black dots.

Figure 7. 7-Ethoxycoumarin Metabolism After β-Naphthoflavone Induction in Control and Treated Animals



Male rats were given BCNU (20 mg/kg) dissolved in corn oil and injected i.p. Controls received only the vehicle. β-naphthoflavone was dissolved in corn oil and injected (100 mg/kg) i.p. 48 hours after the animals were sacrificed. Hepatic microsomal metabolism of 7-ethoxycoumarin was measured by the increase in fluorescence at 450 nm after excitation with 360 nm. Treated groups significantly different from controls ($p \leq 0.05$) are indicated by black dots.

mg protein basis or per nm P-450. There was no difference between the treated animals on days 21 or 28 after β-naphthoflavone induction.

Benzo(α)pyrene metabolism was decreased in animals given 20 mg/kg BCNU after 21 days (Tables II, III, IV). Phenobarbital induction reversed this decrease and once again at day 14 the specific activity of the enzyme was higher for induced treated animals than for induced controls. By day 21, however, phenobarbital failed to cause such a dramatic increase in activity. β-naphthoflavone was unable to raise benzo(α)pyrene metabolism in BCNU pretreated animals to levels above that seen with induced controls at any of the time points.

Ethylmorphine N-demethylase activity was altered to a greater extent than any other type of metabolism after BCNU treatment (Tables II, III, IV). Experiments done at early time points (Table V) show a temporary decrease in N-demethylation after 24 hours though P-450 content was not decreased. This effect was transitory and by day three activity seemed to recover to normal values. Soon after this time point the decrease in P-450 content and long term changes in activity began to appear. This short term effect did not always occur at 24 hours, however, and some animals sacrificed at this time point were no different than controls.

TABLE V. The effects of BCNU (20 mg/kg) on P-450 content and specific activity in male rat liver microsomes immediately after treatment

	nm P-450/mg Protein	Ethylmorphine[a]	7-Ethoxycoumarin[a]
Control	1.1 ± 0.04 (14)[c]	4.8 ± 0.42 (14)	1.93 ± 0.13 (3)
BCNU			
6 hrs	1.03 ± 0.09 (5)	3.58 ± 0.39 (5)	
12 hrs	1.1 ± 0.23 (10)	5.05 ± 0.7 (4)	
24 hrs	1.05 ± 0.15 (16)	2.45 ± 0.35 (16)[b]	2.24 ± 0.4 (5)
36 hrs	0.95 ± 0.08 (3)	5.60 ± 0.33 (4)	
48 hrs	1.0 ± 0.14 (7)	4.36 ± 0.45 (6)	
72 hrs	1.1 ± 0.07 (5)	5.87 ± 0.27 (5)	
336 hrs (14 days)	0.62 ± 0.062 (4)*		

a. nm product x nm $P\text{-}450^{-1}$ x $minute^{-1}$, mean ± S.E.

b. Significantly different from controls ($p \leq 0.05$)

c. Number of samples

By day 14, however, almost every animal tested showed decreases not only in the P-450 content of the microsomes but in the ability of the animal to metabolize ethylmorphine as measured by the Nash reaction (41). At day 21 the activity was only 36% of controls when corrected to mg protein and 57% when standardized to nm P-450. Unlike 7-ethoxycoumarin metabolism, phenobarbital did not increase activity to induced control levels at any time point. β-naphthoflavone decreased the specific activity of the MFO system towards ethylmorphine N-demethylase activity in control and treated animals (Table IV).

The high performance liquid chromatographic (HPLC) method developed in this laboratory allowed measurement of the activity of both types of P-450 isozymes which metabolize ethylmorphine (Table VI, VII). As reported by other groups (42,43), N-demethylase activity was greater than O-deethylase activity in control animals. At no time did we detect any production of normorphine which would be the product of sequential metabolism of ethylmorphine by these 2 isozymes (Figure 8). Our results also demonstrate the O-deethylase activity is not greatly increased by phenobarbital treatment which agrees with results from other laboratories (42,43). It should be noted that control values for ethylmorphine N-demethylase activity were higher in these experiments than for those previously reported (Tables II, VI). This seems to be due to a change in our

TABLE VI. HPLC determination of ethylmorphine N-demethylase and O-deethylase activity.

	nm P-450 x mg P^{-1}	O-deethylase	N-demethylase
Control	0.88 ± 0.024	2.44 ± 0.45[a]	5.89 ± 0.91[a]
	---	2.77 ± 0.50[b]	6.69 ± 0.80[b]
Treated[c]	0.62 ± 0.062[d]	1.51 ± 0.18[a]	2.68 ± 0.53[a,d]
	---	2.43 ± 0.23[b]	4.32 ± 0.70[b,d]
Phenobarbital Induction[e]			
Control	1.91 ± 0.35	2.13 ± 0.48[a]	14.04 ± 1.2[a]
Treated[f]	2.09 ± 0.19	2.68 ± 0.77[a]	16.51 ± 1.6[a]

For all groups n=4, Data presented as the mean ± S.E.

a. nm product x mg $protein^{-1}$ x min^{-1}

b. nm product x nm $P\text{-}450^{-1}$ x min^{-1}

c. 20 mg/kg BCNU 14 days post treatment

d. Significantly different from control ($p \leq 0.05$)

e. 4 days 80 mg/kg

f. 15 mg/kg BCNU

TABLE VII. Ethylmorphine N-demethylase and O-deethylase kinetics in control and BCNU treated male rats

	O-deethylase		N-demethylase	
	Vm[b]	Km[c]	Vm[b]	Km[c]
Control	3.03	23.8	7.7	108
Treated[a]	3.50	1.70	5.2	467
Measured as described by Nash[d]				
Control	---	---	9.6	105
Treated[a]	---	---	2.7	435
Control[e]	1.8	52	5.0	250

a. 20 mg/kg BCNU 14 days post dosing

b. nm product x mg protein^{-1} x minute^{-1}

c. μMolar

d. Nash (41)

e. Duquett, P.H. and Holtzman, J.C. (43)

Diagram of Possible Routes of Metabolism for Ethylmorphine

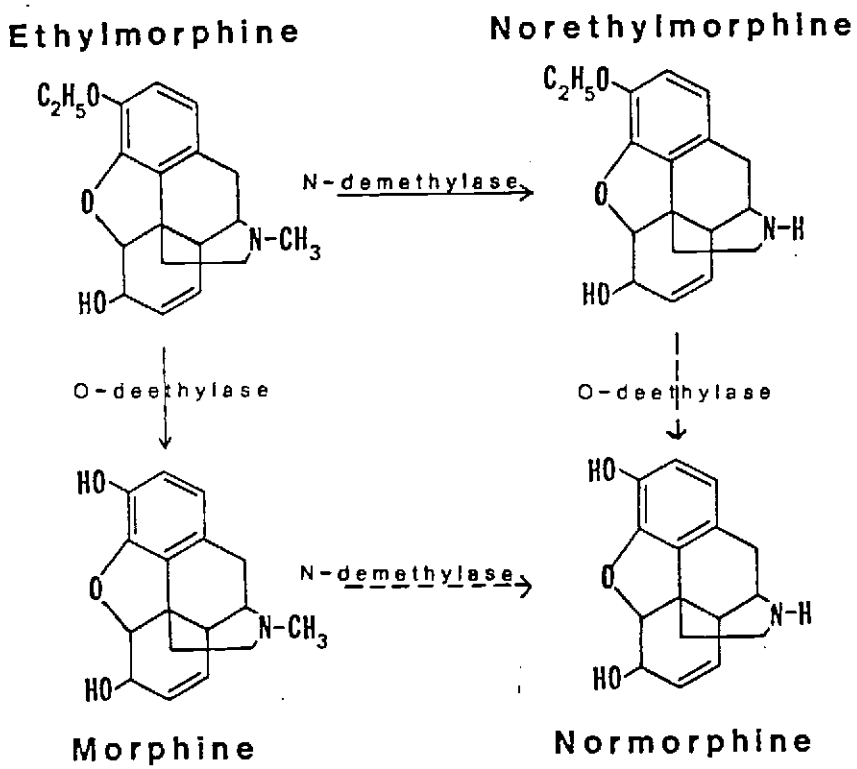


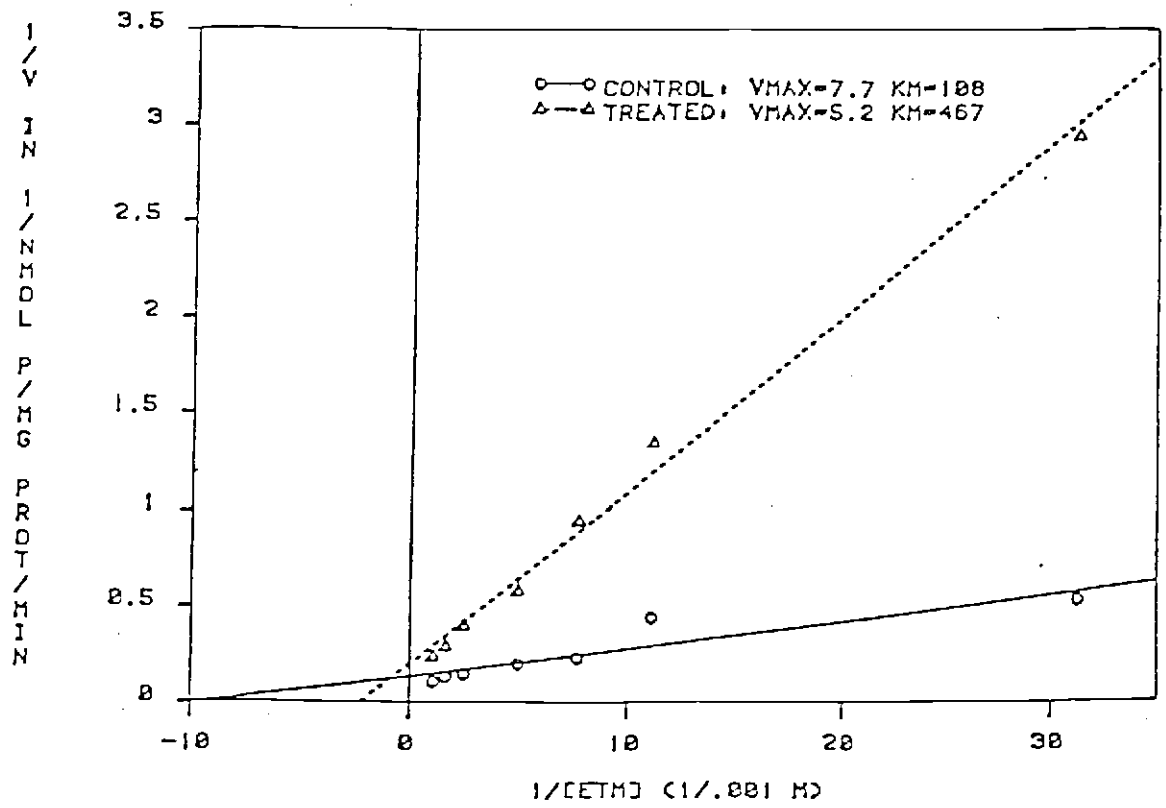
Figure 8. Possible routes of ethylmorphine metabolism in rat hepatic microsomes. HPLC analysis detected morphine and norethylmorphine as metabolic products but normorphine was not formed.

source of Sprague-Dawley rats because all of the HPLC results were confirmed using the Nash reaction which had been used previously to measure N-demethylation. Results from the experiments show N-demethylase activity was greatly depressed in BCNU animals after 14 days but O-deethylase activity was not significantly altered. Because of this differential effect the percentage of morphine produced out of total ethylmorphine metabolism was higher for treated than control animals (35% vs 30% respectively).

Results from kinetic experiments were plotted in typical Lineweaver-Burk fashion and are seen in Figures 9 and 10. After 14 days animals pretreated with BCNU had a 15% increase in the Vmax of O-deethylase activity. The Vmax for the N-demethylase enzyme was decreased. The km for both activities was increased at least four fold (Table VII). These results indicate a seemingly tremendous competitive inhibition of both isozymes and an indication of noncompetitive inhibition on the N-demethylase isozyme. It seems unlikely that BCNU itself could be causing this competitive effect and this may be due to other factors such as the presence of bile acids caused by cholestasis produced in BCNU treated animals.

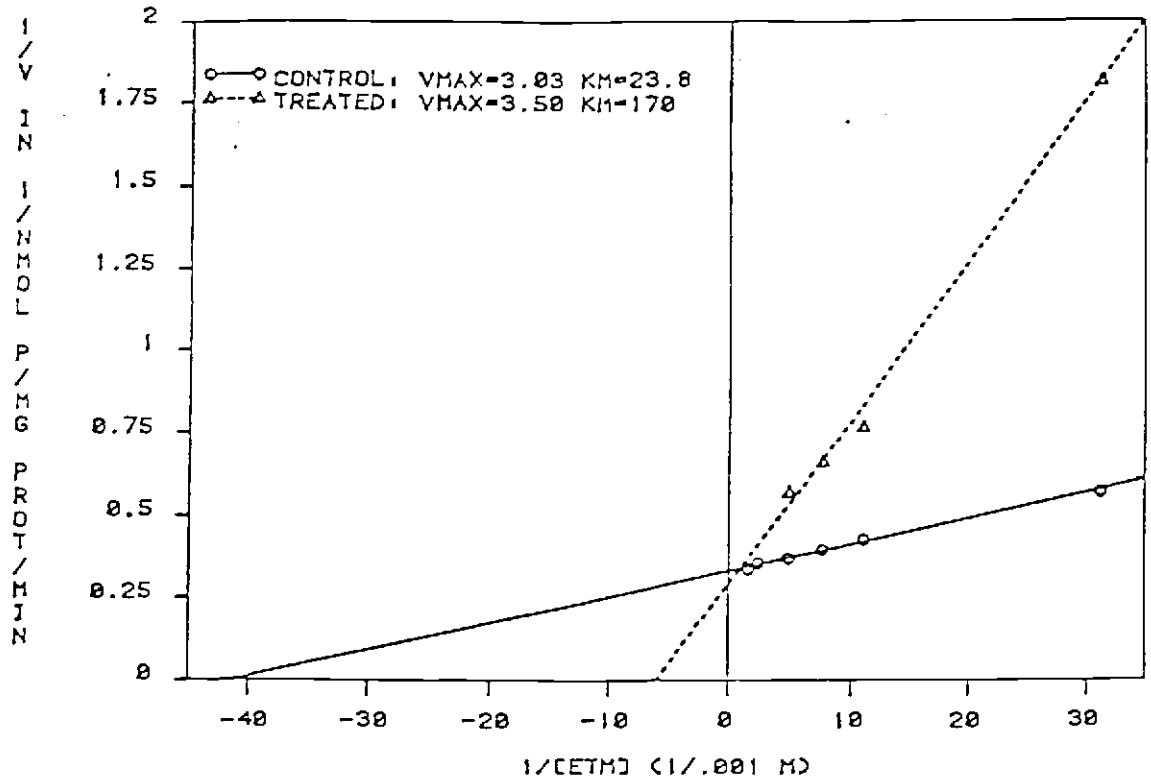
A spectral binding constant (Ks) was also determined for ethylmorphine and the results are shown in Figure (11). The Ks was increased 1.5 times in animals given 20 mg/kg BCNU 14 days before sacrifice. This increase in Ks demonstrates a

Figure 9. Ethylmorphine N-Demethylase
14 Days Post 20 mg/kg BCNU



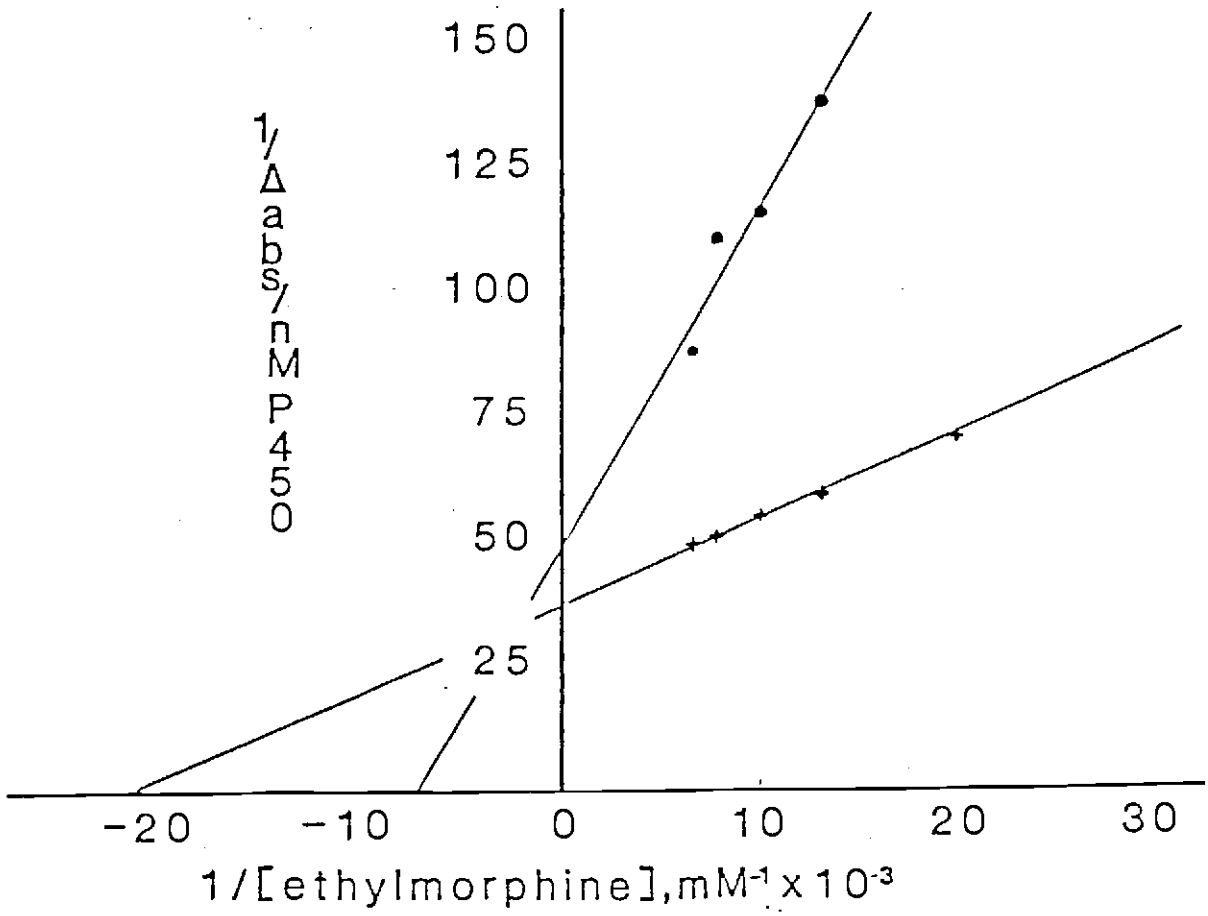
Ethylmorphine N-demethylase activity as measured by HPLC in control and BCNU treated male rat microsomes. Each point is the mean of triplicate samples. The Nash reaction (41) was used as a comparison to the HPLC method and comparable results were obtained. Vmax is expressed as nm product/mg protein/minute. The Km is in m.

Figure 10. Ethylmorphine O-Deethylase
14 Days Post 20 mg/kg BCNU



Ethylmorphine O-deethylase activity as measured by HPLC in control and BCNU treated male rat hepatic microsomes. Each point is the mean of triplicate samples. Vmax is expressed as nm product/mg protein/minute. The Km is in µm.

Figure 11. Binding Spectra for Ethylmorphine



A binding spectrum for ethylmorphine was obtained in control +---+ and BCNU treated (20 mg/kg, 14 days) animals *---* using an Aminco 2a UV-vis spectrophotometer. Each point is the mean of determinations at 2 different protein concentrations (1.0 mg Protein and 0.5 mg Protein per milliliter).
Control Amax = .024 Ks - 55 µm
Treated Amax = .016 Ks = 80 µm

decrease in binding affinity but does not seem able to completely explain the large increases seen in the Km after BCNU treatment. The Am was also decreased in treated animals demonstrating less binding of substrate to the enzyme.

Several experiments were performed to measure cytochrome c reductase activity in control and treated rats. The activity of this enzyme was not changed by BCNU at any time or dose of BCNU.

The studies using the experimental nitrosourea BHCNU are summarized in Table (VIII). Unlike BCNU, animals treated with this drug did not show any loss of weight or jaundice after 14 days. The dosages used (20 & 27 mg/kg) are equimolar with the 15 mg/kg and 20 mg/kg dosages respectively, used for BCNU in these experiments. Unlike BCNU we were unable to demonstrate any long term effects on ethylmorphine N-demethylase activity as measured by the Nash reaction. There was some evidence of decreased P-450 content after 24 hours with the higher dose but this effect was not seen after 7 or 14 days.

TABLE VIII. The effects of BHCNU on liver to body weight ratio and on hepatic microsomal P-450 content and specific activity in male rats.

	Liver/Body[a]	nm P-450/mg Protein	Ethylmorphine[b] N-demethylase
Controls	3.61 ± 0.10 (3)[c]	0.99 ± 0.05 (11)	---
BHCNU (20 mg/kg)			
Day 7	3.51 ± 0.17 (4)	0.53 ± 0.08 (4)	85
Day 14	3.47 ± 0.03 (4)	0.91 ± 0.04 (5)	70
BHCNU (27 mg/kg)			
24 hrs	3.7 ± 0.10 (5)	0.62 ± 0.04 (5)	70
6 days	3.19 ± 0.15 (6)	0.90 ± 0.09 (6)	125
14 days	3.75 ± 0.15 (5)	0.91 ± 0.05 (5)	80

a. % liver/body weight ± S.E.

b. Reported as percent of control. Control values 8.0 nm HCHO x nm $P\text{-}450^{-1}$ x min^{-1}

c. Number of Samples

DISCUSSION

Hepatic damage in rats treated with BCNU is not typical of many direct acting hepatotoxicants which display signs including central lobular necrosis or fatty change. Gross observation did not reveal fibrotic formation over the time course and dosages used in this experiment, though this has been reported in the liver several weeks after BCNU treatment (7,8). Animals which displayed gross effects appeared to suffer from cholestatic damage which is probably of intrahepatic origin (7,8).

Previous studies have eliminated weight loss and nutritional deficiency as possible mechanisms for the decrease in mixed function oxidase activity in mice and rats after BCNU treatment (10,11). Data from present experiments support this conclusion, as the livers from treated rats appeared to maintain weight despite decreased body size (Table I). The data does not indicate direct destruction of P-450 by BCNU though the drug is known to reach the endoplasmic reticulum and is partially metabolized in that organelle (31,32). It is doubtful that BCNU itself would keep P-450 levels depressed for weeks in view of its short residence in the body and the 1 to 3 day half life of the P-450 isozymes (4,54) unless the protein cannot be replaced; this possibility will be discussed below. Some type of direct action is probably responsible for the transient decline in N-demethylase activity at 24 hours but this is unlikely to be

the mechanism for the sustained progressive fall 14-21 days after dosing.

Cellular damage, as demonstrated by the tumor killing effects of BCNU, probably occurs due to alkylation and carbamylation but general protein metabolism in the endoplasmic reticulum of liver cells does not seem to be altered. This is evident by the lack of decline of protein content in the microsomes after BCNU and the response to inducing agents such as phenobarbital and β-naphthoflavone. Recent studies suggest phenobarbital causes an increase in the content of mRNA coded for P-450 and does not merely cause increased utilization of mRNA already present before induction (55-57). This implies DNA transcription is still possible and translation mechanisms are still intact after BCNU. Transport mechanisms must also still be functional to move newly synthesized P-450 into the smooth endoplasmic reticulum (SER). The possibility still exists that BCNU attacks only DNA sequences for cytochrome P-450 expressed in noninduced animals but leaves phenobarbital induced sequences intact. Further experiments are needed to eliminate this type of mechanism.

As previously discussed, BCNU is known to cause cholestasis in rats. A variety of experiments done by many investigators have shown that bile acid buildup is an important cause of changes in xenobiotic metabolism during cholestasis. Hutterer *et al* (58) described the *in vivo* effects

of bile duct ligation (BDL) and the *in vitro* changes caused by the addition of bile salts into the assay medium. Three stages of cholestasis occur after BDL which correspond to increasing levels of bile acids in the liver tissues. Stage I occurs soon after BDL when type I substrates for P-450 are metabolized less efficiently. Stage II is when type I substrate metabolism is severely depressed and some effects are seen on type II substrates. Stage III is reached when type II metabolism is altered noncompetitively and the content of P-450 is decreased. The effects on metabolism can be duplicated by *in vitro* addition of bile acids to incubations of control animal microsomes. It is believed the difference between type I and type II substrates is the way the substrate binds to the enzyme. Type I substrates bind to the lipoprotein portion of the enzyme and is very sensitive to membrane changes while type II substrates bind to the 6th ligand position of the iron in the heme group (40,58).

Shaffner (59) found BDL decreased aminopyrine demethylase activity 60% after 4 days but saw no change in aniline hydroxylase. The content of P-450 in the microsomes was decreased 30%. The Ks for hexobarbital was increased at this time indicating a lower binding affinity. No structural changes were observed with light microscopy but the electron microscope revealed serious alterations. Most noticable in his experiments was the decrease in rough endoplasmic reticulum. Dilation of the smooth endoplasmic

reticulum also occurs after BDL. Mitochondria had abnormal structures in hepatic cells and hepatocytes of ligated animals had increases in free cholesterol content. Serum transaminase levels were also increased.

Greim et al (60) reported that dihydroxy bile acids were better MFO inhibitors and more hepatotoxic than the trihydroxy acids and BDL caused an immediate increase in the dihydroxy type. These include chenodeoxycholic acid and ursodeoxycholic acid which are known to bind to cytochrome P-450 and decrease metabolism. Bile duct ligation did not, however, cause the concentration of these acids to increase enough to cause necrosis in the liver. One mechanism of this protection, according to Greim et al, is the P-450 mediated conversion of strong detergent acting acids like chenodeoxycholic to cholic or β-muricholic acids which are weaker acting. Because these conversions are mediated by P-450, metabolism of toxic bile acids to less harmful forms may be greatly decreased in long term cholestasis due to the decrease in P-450 content. This may cause the percentage of highly toxic bile acids out of total bile acid content in the hepatocyte to increase as cholestasis persists. Damage to cellular membranes would then be greater than predicted if only the increased concentration of bile acid in the cell was considered. This may be very important in BCNU treated animals where cholestasis may occur for weeks. Thus, even though BCNU does not stop bile flow as completely as BDL its

long term consequences on liver function may be quite severe.

Many of the changes caused by BDL are mimicked by BCNU and the time course of the drug's action on the MFO system corresponds well to its cholestatic properties. The 15 mg/kg dosage does not cause severe cholestasis as is evident at 20 mg/kg (9) and P-450 metabolism is also not as severely altered at the 15 mg/kg level. One of the major problems in explaining the effect of BCNU on liver metabolism was the differential alterations that occurred according to the activity measured. N-demethylation of ethylmorphine, (nm product x nm $P\text{-}450^{-1}$ x min^{-1}) for example, was greatly decreased even when the loss of P-450 was accounted for but the metabolism of 7-ethoxycoumarin was not decreased and even showed some increase in these animals. Benzo(α)pyrene metabolism also does not seem to be altered by BCNU as much as ethylmorphine N-demethylase activity. Bile duct ligation also causes differential effects on various substrates (61,62). Depierre and Dallner (63) have shown type I substrates are sensitive to destruction of phospholipids by a phospholipase. Membrane damage due to bile salts may mimic this type of inhibition and different type I substrates may be more or less sensitive to these effects depending on the P-450 isozymes which metabolize them.

The metabolism of ethylmorphine also demonstrates the differential effects that cholestasis may have on different

P-450 activities. Both the O-deethylase and N-demethylase activities showed large increases in their respective Km indicating competitive inhibition. This may be due to increasing bile acid concentrations in cholestatic animals which are known to competively inhibit metabolism by P-450 (58,59). Only the N-demethylase activity, however, had a lower Vmax when compared to controls which indicates a non-competitive inhibition.

Changes in bile flow caused by BCNU may explain some of the differences seen in the response of mice and rats to the drug. No direct measurements of BCNU's cholestatic effect on mice have been made but there has been comparative work done on the ability of the two species to excrete organic anions and on relative rates of bile flow. The mouse has a higher liver to body weight ratio (+48%) than the rat as well as increased basal bile flow (+48%) and bile acid excretion (+155%) (64). Though the mouse did display less BSP excretion and was more sensitive to the cholestatic effect of some organic anions, it is reasonable that the higher bile flow and bile acid excretion rates might aid the animal in reducing liver damage after BCNU. Mice generally recover from depressed MFO activity after BCNU better than rats and the activity in the species is generally not reduced to the extent seen in rats unless higher relative dosages are used (13). These results may indicate less initial hepatic damage due to cholestasis. It has been

demonstrated that ethinyl estradiol, known to decrease bile flow in the rat does not have this effect in mice (65). The mouse may be less sensitive than the rat to this cholestasis and therefore drug metabolism is not decreased by BCNU in mice to the same extent as in rats.

The changes in drug metabolism during cholestasis are not surprising because approximately 15% of the bile acids contained in the hepatocyte are located in the endoplasmic reticulum (66,67). As the concentration of bile acids increases due to BDL and possibly BCNU, changes in membrane fluidity, composition and lipid protein interactions occur. Most of these alterations are probably due to the detergent activity of the bile salts (61,62).

Previous experiments done in this laboratory (68) measured UDP-glucuronyl transferase (UDP-GT) activity on bilirubin or p-nitrophenol in control rats or rats given 20 mg/kg BCNU i.p. 13 days prior to sacrifice. The enzyme had higher activity towards both substrates in the treated animals. When Triton X-100 was added to the microsomes, as an activating agent, control preparations were activated approximately 20% more than the treated ones. After activation no difference existed between control rats and BCNU treated rats in microsomal UDP-Gt activity. These data are significant because it is believed UDP-GT operates in a latent or constrained form in the liver and microsomal preparations (69,70). Activation of the enzyme is possible by

altering membrane structure through such methods as addition of detergents and ultrasonication or by addition of compounds which damage membrane structure (71,72). Direct attack of the membrane by BCNU may be responsible for the results of the experiments described. Related compounds, such as methyl nitrosourea are known to cause lipid droplet formation in the endoplasmic reticulum and to damage membrane unit structure (37). It is also possible, however, that bile acids with detergent action disrupt the membrane and activate the enzyme due to the cholestatic action of BCNU.

The damage caused by the buildup of bile acids does not affect all microsomal proteins equally as demonstrated by the smaller decreases in P-450 reductase activity or cytochrome b_5 seen after cholestatic agents (61,62). These differences are not well understood but probably relate to the heterogeneity of the membrane lipid structure around the various proteins (73,74). A much neglected area of research is the effect other components of the bile, such as bilirubin, have in combination with the bile acids on the membrane during cholestasis.

Phenobarbital induction of the MFO system has been studied for many years and the effects of the drug on hepatic function are fairly well characterized. Liver cell size and number are both increased by the drug and both phase I and II enzymes involved in biotransformation have large

increases in activity after induction. In addition to these changes the content of smooth endoplasmic reticulum is increased in the cell after phenobarbital and membrane composition and fluidity are altered (75,76). Induction also increases bile flow in rats and this is mostly due to increases in bile salt independent flow though some data in primates suggests bile salt dependent flow is also increased (77).

Previous investigators have used phenobarbital concurrently with cholestatic agents, such as ethinyl estradiol, to reverse the decrease in bile flow which normally occurs (78-81). These experiments demonstrate, however, that the bile flow is not increased to levels obtained when phenobarbital is given alone.

Our experiments with induction after BCNU treatment are different from those described above mainly due to the long period of cholestasis experienced by the rat prior to phenobarbital administration. Induction in the BCNU pretreated rats was not as effective as it was in the control animals. The levels of cytochrome P-450 in the induced treated animals are lower than in induced controls. This is different from the previous experiments done in mice where induced BCNU treated animals had levels of cytochrome P-450 indistinguishable from induced controls. This again may be due to a smaller amount of membrane damage in the mouse than the rat. It is likely that phenobarbital is capable of

reversing many of the membrane alterations caused by cholestasis. If the drug does restore at least some of the bile flow to BCNU treated animals it may serve to remove some of the buildup of bile acids which has occurred in the animals. This is a strong possibility because bile salts associated with the endoplasmic reticulum seem only loosely affiliated with the membrane and would likely be removed as hepatic concentrations of bile acids decreased in the cytosol (66).

The activity of the P-450 system was increased by phenobarbital in both treated and control animals but the extent of the increase depended on the substrate. Ethylmorphine N-demethylase activity was decreased by BCNU treatment and induction with phenobarbital failed to increase BCNU treated animals to the levels of induced controls. This may be due to the existence of bile acids in the hepatocytes which would agree with the failure of phenobarbital to increase P-450 content in BCNU treated rats to induced control levels. It is known that some of the isozymes which cause ethylmorphine N-demethylation also metabolize bile salts (82). Thus, even after phenobarbital treatment, metabolism may still be inhibited.

Metabolism of 7-ethoxycoumarin, however, was not lower at days 14 or 21 in rats pretreated with BCNU and then induced as compared to induced controls. This again demonstrates the different effects BCNU has on different

substrates even after induction. By day 28 BCNU treated rats had higher 7-ethoxycoumarin metabolism after induction than induced controls. The effect of induction plus BCNU treatment seemed additive. Whether this is due to changes in membrane fluidity caused by long term cholestasis or a different response of the rats to induction at this time is not known.

β-naphthoflavone is an inducer in the same class as 3-methylcholanthrene and these compounds cause a more selective induction than phenobarbital (83). Thus, liver size, which is greatly increased after phenobarbital through increased cell number and cell size is not altered as greatly by β-naphthoflavone. These experiments agree with results from other laboratories where 3-MC given concurrently with a cholestatic agent still causes a shift in absorbance from 450 nm to 448 nm and the content of the cytochrome is greatly increased in the cell (81). In the rat, β-naphthoflavone induction increased P-450 content almost to control induced levels at days 21 and 28 after BCNU was given. This was not seen at day 14 where the BCNU treated rats did not respond to β-naphthoflavone as well as controls. This is the reverse of the pattern seen with phenobarbital where treated animals responded more poorly to induction as the time course after BCNU was lengthened. These patterns may be due to different sensitivities of the isozymes produced by phenobarbital and β-naphthoflavone to

the conditions caused by BCNU. There is some evidence all P-450 isozymes are not equally depressed by cholestasis (81) and this possibility will be considered again in section III.

Both benzo(α)pyrene and 7-ethoxycoumarin activity was increased by β-naphthoflavone in control and BCNU treated rats. Metabolism of 7-ethoxycoumarin was actually much higher in BCNU treated rats after induction than with induced controls. This response again suggests that 7-ethoxycoumarin metabolism is not inhibited greatly by BCNU and in fact after induction with phenobarbital, or more dramatically in the case of β-naphthoflavone, is greatly increased. The mechanism behind this difference is not clear and more research is needed to describe these responses in more detail.

Because phenobarbital has several effects on liver bile flow and hepatic cellular makeup which may not be seen with β-naphthoflavone, in vivo studies should be carried out to investigate more clearly which type of compound more completely reverses cholestasis and its effects on MFO activity. While β-naphthoflavone does increase MFO content during cholestasis, phenobarbital may demonstrate a more complete reversal of the various toxicities seen in vivo by partially restoring bile flow.

The hypothesis that many of the long term effects of BCNU on MFO activity are due to cholestasis is supported by

the results from injection of BHCNU into rats. This drug did not cause any obvious signs of jaundice or cholestasis at the dosages used and effects on cytochrome P-450 were minimal. While no measurements of anion or bile salt excretion have been performed on animals given this compound it is interesting to speculate that it may be the alkylating activity of BCNU that causes cholestasis because BHCNU is thought to act only as a carbamylating agent. This activity may be the critical mechanism that ultimately destroys much of the animal's ability to metabolize xenobiotics.

This section has compared the effects of BCNU to those known to occur after BDL or exposure to other cholestatic agents which have been fully described by various investigators (58-62, 80,81). The hypothesis that BCNU is not directly involved in long term decreases of MFO activity but acts through secondary mechanisms seems supported by the data. Though measurements of bile acid concentration in the liver have not been taken in BCNU animals, evidence of severe cholestasis in these animals has been presented by others (7,9). These experiments do not rule out, however, a direct action of BCNU on the SER which mimics the detergent action of increased bile acids in the liver. Experiments should be conducted, and are planned for future research in this laboratory, to finally prove or disprove this hypothesis.

III Heme Metabolism in Control and BCNU Treated Rats

INTRODUCTION

The previously described studies have detailed the profound and lasting effects BCNU has on levels of cytochrome P-450 in rat microsomes. The explanation for these long term effects is still questionable especially when the short half life of the drug is considered. The last section has given evidence that changes in cytochrome P450 levels and activity after BCNU treatment mimic changes seen after bile duct ligation. Experiments in this section are designed to study whether or not the effect of BCNU on P-450 metabolism is via a different, more direct mechanism. Due to the nonspecific alkylating and carbamylating effects of BCNU it is possible that other liver enzyme systems are altered which secondarily affect cytochrome P-450. Previous workers in this laboratory have suggested general hepatic heme metabolism may be altered by BCNU pretreatment (13). To test this idea fully it is necessary to analyze the effects BCNU might have on at least two regulatory enzymes in the heme metabolic pathway.

Heme synthesis is largely determined by the activity of the mitochondrial enzyme delta aminolevulinic acid (ALA) synthetase. This feedback-regulated enzyme catalyzes the condensation of glycine and succinyl CoA on the way to heme

production. The other enzyme of interest, microsomal heme oxygenase, is directed towards heme catabolism and forms the products biliverdin and carbon monoxide (84,85). Heme oxygenase appears to be substrate inducible so the activity is stimulated by increasing concentrations of unincorporated heme in the endoplasmic reticulum (84). Bissell and Hammaker (85) suggest that almost 50% of the heme available to the hepatic endoplasmic reticulum is taken up by cytochrome P-450 apoprotein. This suggests the content of P-450 in the endoplasmic reticulum may have a large affect on the activity of these enzymes. It is also possible that through the direct destruction of any of the enzymes in the synthetic pathway, BCNU may cause lowered levels of cytochrome P-450 while other less important heme containing microsomal enzymes would not be obviously effected.

These experiments may also serve to strengthen the possibility that BCNU mainly affects P-450 levels through its cholestatic action. Schacter et al (86) found bile duct ligation does alter the activity of the enzymes in the heme metabolic pathway. The decreases seen in P-450 content of rat liver microsomes after BDL have already been described in the drug metabolism section. Several investigators feel the decreased levels of P-450 after BDL are due to decreased synthesis and not increased degradation (81,86). Some of the data presented in this section would tend to indicate BCNU may work in the same manner. It is hoped that by a

careful comparison of our results with those reported after bile duct ligation, it will be possible to further speculate on whether or not cholestasis is the major cause of the effect BCNU has on P-450 content and activity.

MATERIALS AND METHODS

Chemicals

Sodium succinate, pyridoxal-5-phosphate, dimethylaminobenzaldehyde and bovine hemin were all obtained from Sigma Co. Cation (Ag® 50W-X4) and anion (Ag® 1-XB) resins were 100-200 mesh and were from Bio-Rad. All other reagents used were reagent grade.

Urinary ALA

Rats used for these experiments were treated and maintained as previously described. Delta-aminolevulinic acid (ALA) excretion in the urine was measured by the method of Davis (87). The animals were placed in individual metabolism cages overnight 12 hours before the time of interest. Rats were allowed water but no food over the collection period. Urine was collected in 25 ml graduated cylinders containing 0.2 ml of 50% glacial acetic acid. All samples were either analyzed immediately or stored for no more than 48 hours at 4°C.

Isolation of ALA was completed by passing the urine through two ion exchange columns. One ml of urine was first added to 3 g of anion exchange resin in the acetate form contained in a 10 ml disposable glass barrel syringe. This step removes porphobilinogen which interferes with the assay. The column was washed with 20 ml of distilled water which was then applied to a cation exchange column (3 g) in

the hydrogen form also made from a disposable 10 ml syringe. The cation column was then rinsed with distilled water until addition of Ehrlich's reagent, described below, failed to turn yellow indicating all urine had been removed from the column. Usually 40-45 ml was necessary for this rinse. After the distilled water, 5 ml of 1 M sodium acetate was passed through the column. No ALA was detected in this fraction. A second wash of 1 M sodium acetate of 7 ml removed all of the ALA from the column. This wash was poured into a 15 ml graduated test tube and 0.2 ml of purified grade pentanedione was added. The sample was then brought up to 10 ml with sodium acetate buffer (pH 4.6), the sample was then boiled for 10 minutes and cooled to room temperature. Two ml of the sample were removed and poured into a 10 ml test tube containing 2 ml of modified Ehrlich's reagent. The reagent includes 15 ml of glacial acetic acid, 5 ml of 70% perchloric acid 1.5% $HgCl_2$ and 500 mg of dimethylaminobenzaldehyde. The color was allowed to develop for 15 minutes and the optical density was read on a Bausch & Lomb spectronic 600 at 553 nm. Absorption was compared to a standard curve of ALA treated in a similar manner and sample recovery of ALA was typically 85%. After use the columns were cleaned by the method of Wilkinson (88). Anion columns were washed with 1 M sodium acetate until no chloride ion was detected. Cation columns were cleaned by soaking with 2 M sodium hydroxide overnight and then suc-

cessive rinses with distilled water, 4 M HCl, 2 M HCl, 1 M HCl and finally distilled water.

Liver Homogenate ALA Activity

Liver homogenates were tested for ALA synthetase activity as described by LiFen and Beatte (89). Treated rats (20 mg/kg), previously fasted for 36 hours, were sacrificed 14 days after BCNU was given. The livers were removed and pooled, 3 livers per sample, rinsed and washed 2 times, and then homogenized (10% liver) in ice cold TSEP buffer. The buffer was freshly made no longer than 24 hours prior to use and contained 50 mM tris/HCl (pH 7.4), 0.25 mM sucrose, 0.5 mM ethylenediaminetetraacetate (EDTA) and 0.2 mM pyridoxal 5 phosphate.

Assays were conducted in 16 x 100 mm test tubes to which 0.4 ml of stock assay mix containing 150 mM tris/HCl (pH 7.4), 250 mM glycine, 25 mM sodium succinate, 12.5 mM $MgCL_2$, 12.5 mM EDTA and 0.1 mM pyridoxal-5-phosphate had been added. Each assay mix also contained 500 ml of homogenate and 100 µl of cofactor mix containing 100 mM ATP and 1 mM succinyl CoA in distilled water (pH 7.4). Total final volume for the assay was 1 ml. All homogenate samples were tested in triplicate. The tubes were incubated for 40 minutes at 37°C in air.

The reaction was stopped by addition of 125 µl of 50% trichloroacetic acid. Blanks had TCA added at times 0. The denatured protein was sedimented in a Damon/IEC, HNS centri-

fuge at 5000 RPM for 5 minutes. A 0.6 milliliter aliquot of supernatant was removed and transferred to a 15 ml test tube. A fresh solution of 10% pentanedione in 1 M sodium acetate was prepared by heating the solution to no more than 45°C and vortexing. This mixture was allowed to cool and 0.3 ml was added to the test tube. The pH was checked to be at 4.6 with short range pH paper. The tubes were then capped and heated at 80°C for 10 minutes in a water bath to convert the aminoketones to pyrroles. The tubes were then placed in a water/ice bath for 30 minutes to cool.

To each test tube 30 ml of 10 N NaOH was added and the pH was tested with short range pH paper to be between 8 and 8.5.

Aminoacetone pyrroles were removed by ether extraction. Peroxide-free ether (168 ml) was equilibrated with 50 ml of assay mix, 50 ml of distilled water and 12 ml of 50% trichloroacetic acid, 56 ml of 1 M sodium acetate. The pH was adjusted to be between 8 and 8.5 with KOH.

Two ml of the equilibrated ether was added to each test tube and the mixture was vortexed for exactly 15 seconds and returned to the ice bath to enhance separation. The ether layer was aspirated to waste and 0.65 ml of the aqueous bottom layer was removed and transferred into a 2 ml test tube containing 50 µl of glacial acetic acid. After vortexing 0.7 ml of Erlich's reagent was added and color was allowed to develop for 15 minutes. The samples were scanned from

450 nm to 650 nm with the Aminco DW-2a UV-VIS specrophotometer in a 1 cm light-path cuvette. The zero time sample was used as a blank in the reference cell. Peak absorbance was measured at 552 nm with a shoulder at 525 nm. An extinction coefficient of $E_{552-650}$ equals 45 x mM^{-1} x cm^{-1} was used. Samples with a 525/552 ratio of less than 0.69 were discarded.

Heme Oxygenase

Heme oxygenase activity was measured in rat microsomes treated and isolated as previously described. The assay was performed as described by Schacter et al (90). A 6 ml total volume assay contained 18 mg of rat liver microsomes, 10 mg of the 105,000 x g supernatant (from control animals), 17 mM hemin (added as methem albumin), 2 mM $MgCl_2$ and a NADPH generating system as previously described in 0.1 M potassium phosphate buffer (pH 7.4). The 6 ml reaction mixture was evenly divided between 2 cuvettes and the reaction was started in the sample cuvette by the addition of NADP+ in 1% $NaHCO_3$ to a final concentration of 0.4 mM. The reference cuvette received an equal volume of 1% $NaHCO_3$ without NADPH. Bilirubin formation was followed at 468 nm with an extinction coefficient of 57 x mM^{-1} x cm^{-1}.

Total Heme

Total heme was measured as pyridine hemochrome (91). Pyridine was added to the microsomes to a final con-

centration of 3.2 M and an NaOH concentration of 83 mM. The sample cuvette was reduced with sodium dithionite and $K_3Fe(CN)_6$ was added to a final concentration of 25 mM in the reference curvette. An extinction coefficient of $E_{557-575}$ equals 32.4 $mM^{-1} \times cm^{-1}$ was used. All samples were corrected for hemoglobin content which was never more than 5% of the total heme (46).

RESULTS

The results of these experiments are presented in Tables IX and X. Animals in these experiments had a 26% decrease in microsomal P-450 content which agrees closely with the previous data reported in Chapter I. Also, in agreement with the earlier data was the lack of any significant change in total microsomal protein content in the treated animals though it was somewhat lower than the control values. The protein content of the liver homogenates in treated animals was, however, significantly lower than control values (Table IX).

Urinary excretion of ALA in BCNU pretreated rats was only 74% of control values but this was not significant at $p \leq 0.05$. Total microsomal heme, however, was not changed by BCNU pretreatment (Table X). Delta-aminolevulinic acid synthetase activity, the rate controlling enzyme in heme synthesis, was depressed to only 59% of control activity in the treated animals. Heme oxygenase, however, was increased by an amount almost equal to the decrease in ALA synthetase activity in BCNU treated rats. Though the activity of these enzymes may be closely linked, it is not believed at this time that there is a direct relationship between the decrease in ALA synthetase activity and the increase in heme oxygenase activity.

TABLE IX. Hepatic protein and microsomal P-450 content in control and BCNU treated rats.

	Control	Treated[c]
P-450[a]	1.0 ± 0.03 (5)[d]	0.74 ± 0.06*(6)
Microsomal Protein[b]	7.5 ± 0.15 (5)	6.3 ± 0.55 (4)
Homogenate Protein[b]	95.0 ± 2.24 (6)	86.0 ± 1.34*(5)

All data presented as the mean ± S.E.

a. nm P-450 x mg P^{-1}

b. mg Protein x gm $liver^{-1}$

c. 20 mg/kg BCNU, i.p. 14 days post treatment

d. Number of samples

* Significantly different from controls of that group ($p \leq 0.05$)

TABLE X. Hepatic microsomal heme metabolism in control and BCNU treated rats.

	Control	Treated
Total Heme[a]	0.74 ± 0.09 (4)[f]	0.67 ± 0.05 (4)
Heme Oxygenase[b]	0.03 ± 0.009 (4)	0.04 ± 0.005 (4)
Delta Amino-levulinic[c] Acid Synthetase	40.35 ± 5.01 (6)	23.73 ± 3.58[e] (5)
Urinary ALA[d]	0.54 ± 0.09 (5)	0.40 ± 0.02 (5)

All data presented as the mean ± S.E.

a. nm Heme x mg Protein^{-1}

b. nm Biliverdin x mg Protein^{-1} x min^{-1}

c. nm ALA g liver^{-1} x hr^{-1}

d. mg ALA x 100 mls urine^{-1}

e. Significantly different from controls of that group ($p \leq 0.05$)

f. Number of samples

g. 20 mg/kg BCNU 14 days post treatment

Both treated and control animals showed very low levels of hemoglobin contamination in the microsomes and the rats did not appear to be suffering an obvious hemolytic response to the BCNU which might tend to release large amounts of free heme to the liver and have an effect on the activity of the heme synthesis pathway. The treated animals were slightly jaundiced but as described in the last section this is believed to be due to the cholestatic effect of the BCNU.

DISCUSSION

These experiments were performed at a time when P-450 levels are known to be decreased by injection of 20 mg/kg BCNU. We have already discussed reasons for doubting that BCNU directly degrades P-450 during this period. Considerations of the data from this and the previous section make it unlikely the heme metabolic pathway is directly altered by BCNU. First of all, other heme containing enzymes, such as cytochrome b_5, do not seem as greatly affected (13). Secondly, and more persuasive are the results seen with phenobarbital administration to rats previously given BCNU where levels of P-450 are greatly increased over noninduced animals. Several investigators suggest phenobarbital causes an increase in cytochrome P-450 apoprotein which then draws on the general heme pool (84,85,92,93). This depresses the synthesis pathway and allows increased heme production. Experiments done by these groups argue against a direct induction of delta-aminolevulinic acid itself by phenobarbital. If these ideas are correct it would be impossible for phenobarbital to increase P-450 levels in BCNU treated animals if the heme synthesis enzymes had been destroyed. This hypothesis is supported by the fact that cobalt, which is known to block heme synthesis, does block P-450 induction by phenobarbital when spectral methods are used to measure enzyme content (93). At no time during the period after BCNU injection, however, have rats been inca-

pable of at least some induction by phenobarbital.

The results from these experiments agree quite closely with Schacter et al (86) where rats were given bile duct ligations and the heme metabolic pathways were investigated. Within 48 hours they found decreases in ALA synthetase activity and heme oxygenase activity was increased to levels 2 times as high as control rats. Cytochrome P-450 levels were decreasing over this same time frame. Measurement of $^{3}H/^{14}C$ ratios in microsomal proteins indicated decreased heme synthesis and not increased degradation which led to reduced P-450 levels. A similar concluson was reached by other investigators looking at decreased P-450 levels after ethinyl estradiol treatment (81). To explain the increase in hepatic heme oxygenase activity in view of decreased synthesis Schacter suggests the primary event after BDL is the slowing of P-450 apoprotein production which causes an increase in the nonincorporated heme pool. This heme pool then regulates the activity of the synthetase and oxygenase in opposite manners to keep nonincorporated heme content at normal levels. This would explain why the values of heme oxygenase in BCNU treated animals were not as high as seen by animals in Schacter's group after bile duct ligation. The time after onset of cholestasis was long enough that excess heme had already been degraded and the oxygenase activity was closer to normal. Because BCNU animals continue to have decreasing levels of P-450 even after 14 days,

however, it is doubtful that experiments at later time points would show much lower oxygenase activity.

None of these results reflect on the central mechanism for regulating P-450 content of the endoplasmic reticulum. If Schacter (86) is correct, the mechanism causing lower apoprotein production is still unknown. The large concentrations of bile acids in cholestatic animals must produce changes which cause P-450 content to be less than control animals. The effect is fairly selective, however, in view of the fact total protein content is not greatly changed. How these membrane changes are interpreted by the cell to repress P-450 regulation is not understood. Decreased P-450 levels have not been explained even in BDL experiments and explanations are more difficult in the case of BCNU where many other factors may be important.

While these experiments were conducted to see what changes BCNU causes in heme metabolic pathways, they also illustrate another problem which Schacter et al. mention briefly. It is entirely possible that the products of cholestasis are most important in changing the activity of the heme metabolic pathway. The synthesis pathway is found in mitochondrial particles and catabolism occurs in the endoplasmic reticulum. Both organelles are known to store hepatic bile acids (66). These bile acids or other compounds may play a role in slowing down certain types of protein metabolism but these effects remain to be investigated.

IV. Isolation of P-450 from the Hepatic Microsomes of Control and BCNU Treated Rats

INTRODUCTION

Cholestasis, whether caused by bile duct ligation or by a variety of compounds, has long been known to alter the *in vivo* metabolism of a wide variety of P-450 substrates (58-61). These alterations have been confirmed *in vitro* where microsomal preparations from cholestatic animals are tested for P-450 content and specific activity. The previous chapters have described changes which occur in rat hepatic microsomes after a single i.p. injection of BCNU. Largely due to the time delay between the injection of BCNU and the appearance of the effects on P-450 content and activity, it has been argued herein that the drug causes the effect through a rather unique ability to cause prolonged and profound cholestasis in rats. While it is possible BCNU adducts damage endoplasmic reticulum proteins or lipids it seems more likely that normal turnover of these cell constituents would repair the damage before the observed effects of BCNU are evident. Results from other laboratories on cholestatic animals seem to agree fairly well with many of the effects reported herein with rats pretreated with BCNU (13,58,61).

Most investigators of cholestasis attribute the resulting alterations in P-450 mediated metabolism to the

increasing bile acid concentrations which occur in the liver (61). It is thought these bile acids act as detergents which disrupt the normal protein-protein or protein-lipid interactions of the endoplasmic reticulum. Despite these assumptions little is understood about these alterations and due to the lack of a good model, few investigations have been conducted in situations where cholestasis has existed for more than a week. The objective of the research outlined in this chapter is to more closely investigate the biochemical alterations that occur after BCNU and further investigate possible mechanisms by which the drug itself might change P-450 mediated metabolism over prolonged periods.

Investigators have long suspected and recent experiments have verified, that P-450 is not one enzyme but a mixture of different isozymes even in control animals not exposed to P-450 inducers. Estimates have been made that at least 11 different isozymes are present in noninduced animals (94-97). Induction with chemicals such as phenobarbital or 3-methylcholanthrene not only increase the total P-450 content of the microsomes but also change the makeup of isozymes in of the mixed function oxidase system (98). These changes in isozyme composition can be monitored in the microsomes by shifts in the Soret wavelength of maximum absorption, substrate activities, and changes in SDS polyacrylamide gel electrophoresis banding patterns. Due to

improved chromatographic techniques over the past several years these various P-450 proteins have been solubilized from the microsomal membrane and isolated from control and induced animals. The isozymes have then been characterized through the use of spectral determinations, electrophoresis, specific activities, immunoprecipitation and amino acid composition (98,99).

McKinnon et al. (81) suggested different P-450 isozymes are not altered by cholestasis in the same manner. Using gel electrophoresis they identified four bands which represented proteins in the molecular weight range of known P-450 isozymes. Bile duct ligation caused a decrease in 3 of the 4 bands while ethinyl estradiol, a known cholestatic agent, reduced only 2 band densities. The concurrent administration of phenobarbital or 3-methylcholanthrene with ethinyl estradiol was able to prevent decreases in some of the bands though each agent protected different isozymes. These changes in selected P-450 isozymes during cholestasis may explain to some degree the alterations seen in the specific activities of microsomes towards certain substrates when they are isolated from cholestatic animals and compared to controls. These experiments suggest that in addition to membrane changes due to bile acid build up in the cells, actual changes also occur in the type of proteins which constitute the membrane. While it has long been known that total P-450 content is decreased in cholestatic animals it

has not been previously possible to investigate which types of the enzyme are most effected.

One method of investigating changes in membranes caused by BCNU is to remove the P-450 protein(s) from the lipid bilayer, isolate them from other microsomal proteins and measure their physical characteristics. It should also be possible, through the use of reconstituted in vitro metabolism studies, to supply the protein an artificial lipid environment and measure substrate turnover. These types of investigations would effectively remove the influence bile acids have on the system in vivo and would eliminate membrane damage due to bile acid detergent action. Thus, if the P-450 proteins themselves are unchanged by the cholestasis much or all of the difference in activity between control and treated animals should be removed. This type of experiment would also tend to eliminate the possibility that BCNU has permanently damaged the membrane through alkylation or carbamylation. Because P-450 turnover is known to be fairly rapid it is unlikely that proteins damaged by the drug itself would still be present 14 days after BCNU was injected into the animal (4,54). Thus, any differences in activity would most likely be due to the cholestatic or some other secondary effect of the drug. In the following experiments P-450 was removed from the endoplasmic reticulum of control and 14 day BCNU pretreated animals and subjected to a series of biochemical measurements.

MATERIALS AND METHODS

Chromatographic gels were purchased from the following sources: Sepharose 4B, Pharmacia, Inc.; hydroxyapatite, Bio-Rad; and adenosine 2'5' diphosphate agarose, Sigma Chemical Co. Cyanogen Bromide (CNBr) was from J.T. Baker Co. Sodium deoxycholate, sodium cholate, 1,8 diaminooctane, flavin mononucleotide, phenylmethylsulfonyl flouride (PMSF), dithiolthriotal (DTT), Lubrol PX® and 2' adenosine monophosphate were obtained from Sigma Chemical Co. Cytochrome c and dilaurylphosphatidylcholine were purchased from Cal Biochem. Biobeads® and all electrophoresis chemicals and standards were bought from Bio-Rad except for sodium dodecyl sulphate (SDS) which was from Sigma Chemical Co.

N-octylamino Sepharose 4B was prepared as described by Cuatrecasus (100) and modified by Guengerich and Martin (101). All steps were carried out in a fume hood. Four hundred ml of Sepharose 4B was washed with 6 l of distilled water in a 3 l scintered glass funnel over a 4 l Buchner flask attached to an aspirator. The washed gel was poured into a 4 l beaker and stirred with a magnetic stirring bar in 1 l of distilled water. A pH electrode and thermometer were placed in the solution. CNBr (100 g) was dissolved in 300 ml of 1,4 dioxane and added dropwise to the stirring solution over a period of 10 minutes. As CNBr solution was added to the gel the pH was brought up to 11 and kept there

with 8M NaOH. Ice was added as necessary to keep the temperature between 20-25°C. After all the CNBr solution had been added, the reaction was continued for 10 min at 25°C and pH 11. The reaction was stopped by the addition of 600 ml of crushed ice. The suspension was poured into a 3 l scintered glass funnel fitted over a 4 l Buchner flask attached to an aspirator and washed with 4 l of distilled water at 4°C. A solution of 114 g of 1,8 diaminooctane in 400 ml of 4°C distilled water was immediately added to the gel and the pH was lowered to 10 with 6M HCl as measured by short range pH paper. This solution was stirred gently for 16 hours at 4°C. The resulting gel was successively washed with 10 l of distilled water, 2 l of 0.1M potassium phosphate buffer (pH 7.25) and 10 l of distilled water. The gel was stored in distilled water at 4°C until use.

Sodium cholate was recrystallized two times according to the method of Guengerich (102). The solid (60 g) was dissolved into 50% ethanol/distilled water (250 ml) and mixed with 0.1 g of powdered activated charcoal and stirred for 30 min. The solution was filtered, removing the charcoal, and the solvent was evaporated under a vacuum at 60°C leaving a precipitate which was dried to constant weight. A final 20% (w/v) solution was prepared in distilled water and filtered before use. Lubrol PX was also prepared as a 20% solution in distilled water.

Solubilizing buffer contained 0.1M potassium phosphate

(pH 7.25), 1.0 mM disodium ethylenediamine tetraacetate (EDTA), 0.4 mM PMSF, 20% glycerol, 0.6% (w/v) recrystallized sodium cholate and 1.0 mM DTT.

Buffer I contained 0.1M potassium phosphate (pH 7.25), 1.0 mM EDTA, 0.4 mM PMSF, 1 mM DTT and 20% glycerol.

Buffer II was 0.01M potassium phosphate (pH 7.25), 1 mM EDTA, 1 mM DTT, 0.05% lubrol PX and 20% glycerol.

Buffer III contained 0.3M potassium phosphate (pH 7.7) 0.1M EDTA, 0.1% lubrol PX and 20% glycerol.

Microsomes were obtained as described previously from male Sprague-Dawley rats (200-250 grams) either untreated or given 20 mg/kg BCNU 14 days prior to sacrifice. Livers were minced and pooled prior to centrifugation. Solubilization was carried out as described by Imai (103). This procedure involves resuspending the second 105,000 xg pellet to a concentration of 1.5 mg protein ml^{-1} in the solubilization buffer. The solution was gently stirred for 30 min at 4°C and centrifuged for 2 hours at 77,000 xg.

The 77,000 supernatant was poured at a rate of 30 ml x $hour^{-1}$ onto a 2.5 x 40 cm N-octylamino sepharose 4B column previously equilibrated with the solubilizing buffer. All the chromatographic steps were performed at 4°C. The column was loaded until approximately 1/3 was colored light red and then it was washed with 800 ml of buffer I containing 0.42% recrystallized sodium cholate. Cytochrome P-450 was eluted with 1600 ml of buffer I containing 0.33% recrystallized

sodium cholate and 0.06% (w/v) Lubrol PX. Collections were made in 5.0 ml fractions. Cytochrome P-450 containing fractions were identified by absorbance at 417 nm, pooled, and then diluted 10 fold with 20% glycerol/distilled water. This mixture was then loaded onto a hydroxyapatite column (10xl.5cm) previously equilibrated with buffer II. The column was washed with 3 volumes of buffer II and eluted with buffer II where the phosphate concentrations was increased to 0.1M (buffer IIB).

Cytochrome P-450 fractions were combined, diluted 16 fold with 20% glycerol/water and loaded onto a second N-octylamino sepharose 4B column (1.5x8cm) equilibrated with buffer IIB but the sodium cholate concentration was raised to 0.6%. The column was then washed with 60 ml of this solution and 1 band of cytochrome P-450 was eluted. Any cytochrome P-450 remaining on the column was removed with buffer IIB when the Lubrol PX concentration raised to 0.1%. Cytochrome P-450 containing fractions were pooled, added to biobeads® (1 g of beads x mg protein^{-1}) and stirred for 2 hours at 4°C to remove the detergent. Concentration of the protein was carried out in an Amicon PM-30 ultrafiltration cell. The resulting solution was dialyzed for 16 hours at 4°C with 50 volumes of 10 mM tris/HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. The protein was stored at -40°C in small batches.

Cytochrome P-450 content and reduced CO binding spectra

were determined by the method of Omura and Sato (47,48). Protein concentration of the fractions was measured by the Lowry reaction (45).

NADPH cytochrome c reductase (EC 1624) was isolated as described by Guengerich and Martin (101). Rats were treated with phenobarbital (80 mg/kg; 4 days) and sacrificed as previously described. The microsomes were isolated and solubilized and poured onto an octylamino Sepharose 4B column in the manner described above. The P-450 fraction was eluted as previously described and the reductase was removed from the column with 2 l of buffer I containing 0.35% sodium cholate, 0.15% sodium deoxycholate and 2 mM flavin mononucleotide. Fractions were tested for reductase activity (assay described below) and pooled. The reductase was then poured onto a 1.2x3cm column of 2'5'-ADP agarose, equilibrated with buffer III and then washed with 250 ml of the same buffer. The column was washed again with 250 ml of the same buffer but the phosphate concentration was lowered to 30 mM (buffer IIIB). The column was eluted with 50 ml of buffer IIIB containing 5 mM 2'AMP and 0.4 mM PMSF. These fractions were tested for reductase activity (described below), dialyzed 2 times for 16 hours each against 20 vol umes of buffer IIIB devoid of sodium deoxycholate, concentrated in an Amicon PM-30 ultrafiltration cell and stored at -40°C in small batches.

Purified NADPH cytochrome c reductase was assayed as

described by Yasukochi and Masters (50) in 0.3 M potassium phosphate (pH 7.7), 0.2 mM EDTA at 25°C. Cytochrome c was added to a final concentration of 0.05M and the reaction was initiated by addition of 0.1 mM NADPH. The reaction rate was monitored using the Aminco DW-2a Vis-UV spectrophotometer in the dual beam mode. Absorption at 550 nm was compared to the reference wavelength of 540 nm and an extinction coefficient of 18 x mM^{-1} x cm^{-1} was used.

In vitro metabolism of ethylmorphine was carried out in a reconstituted system described by Agosin et al (94). The final volume of the assay mix was of 1.0 ml and was prepared by the sequential addition of 0.45 nmoles P-450 (approximately 0.1 ml volume), 0.5 units NADPH cytochrome c reductase (approximately 0.1 ml volume; one unit of activity equals the reduction of 1 µmol cytochrome c at 25°C per minute), 50 g of dilaurylphosphatidylcholine (previously sonicated as a 0.1% suspension in 0.05 M tris buffer, pH 7.5), 50 g of sodium deoxycholate, 15 µmoles of $MgCl_2$ and substrate. The mixture was incubated at 35°C for 5 minutes and the reaction was initiated by the addition of 0.3 moles of NADPH. Blanks received only the tris buffer or were without the lipid and detergent. The reaction was stopped by the addition of 1.5 ml of 0.5M sodium phosphate after 10 min and activity was assayed by the HPLC method already described. Ethylmorphine N-demethylase activity was also measured by the Nash reaction (41). Due to very low acti-

vity, O-deelthylase product formation was not analyzed.

Sodium dodecyl sulphate (SDS) polyacrylamide disc electrophoresis (PAGE) was carried out as described by Weber and Osborn (104). Stock acrylamide solution was made by dissolving 22.2 g of acrylamide and 0.6 g of methylenebisacrylamide in enough distilled water to make 100 ml of solution. This mixture was filtered to remove particulates and stored at 4°C in a dark bottle. Gel buffer was made by mixing 7.8 g of $NaH_2PO_4 \cdot H_2O$, 20.45 of anhydrous Na_2HPO_2, and 2 g of SDS per liter of solution. To prepare a 10% acrylamide gel 15.0 ml of gel buffer, 13.5 ml of acrylamide mixture and 1.5 ml of ammonium persulphate (15 mg/ml) were mixed and degassed under a vacuum for 30 minutes. Polymerization was initiated by the addition of (0.015 ml) N,N,N',N' tetramethylethylenediamine (TEMED). The gel was then poured into clear glass tubes (5.0 ml I.D., 130 ml length) and before hardening a few drops of distilled water were placed over the mixture. The gels were allowed to set overnight at 4°C. The next morning a 3% stacking gel was prepared and added to the top of the separating gel (0.5 ml) and allowed to set for one hour.

Protein samples were diluted 1 to 2.5 in sample buffer containing 1.0 ml 0.5 M tris/HCl (pH 6.8), 1 ml of glycerol, 1 ml of 10% SDS (w/v), 0.1 ml of 2-mercaptoethanol and 0.9 ml of 0.05% (w/v) bromophenol blue. The samples were placed in boiling water for 5 minutes and then allowed to cool to

room temperature. Samples ranging from 25 to 100 µl (25-100 g protein) were added to each gel and the tubes were filled to the top with the cathode buffer described below.

The bottom chamber of a Hoefer Scientific disc electrophoresis unit was filled with 1 l of the anode buffer containing 0.025M tris/HCl (pH 8.3) with 0.19M glycine, and 0.1% SDS (w/v). The upper compartment was filled with the cathode buffer of 0.05M tris buffer (pH 8.3), 0.38M glycine and 0.1% SDS. Electrophoresis was carried out at 4°C using a constant current or 1.5 mamp/gel. Normal run time was approximately 6 hours. Gels were removed from the tubes using a syringe to insert water between the glass wall and the gel. A rubber bulb was used to gently exert air pressure which pushed the gel out in one unbroken piece. The gels were stained for 2 to 3 hours with 0.1% Coomassie Blue (w/v) in 45% EtOH/10% acetic acid. The gels were destained in a Hoefer Scientific Instrument chamber overnight with a 10% EtOH/7.5% acetic acid solution. Protein migration was measured and gels were stored in distilled water until they were photographed. Molecular weights were determined by using a standard solution containing lysozyme (M.W 14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000) bovine serum albumin (66,200) and phosphorylase B (92,500).

RESULTS

The elution profiles during chromatography of the cytochrome P-450 bands obtained from the control and BCNU pretreated animals were similar though the bands were fainter in preparations obtained from the treated rats. Recovery of P-450 from the hydroxyapatite column was approximately 15-20% of the total P-450 protein originally present in the microsomes. Both treated and control P-450 preparations eluted from the octylamino-sepharose 4B column in a rather broad band in which all fractions with an absorbance greater than 0.1 at 417 nm were pooled. Chromatography on the hydroxyapatite column resulted once again in a broad band which was measured for absorbance at 417 nm and fractions containing the cytochrome were combined. Attempts to further isolate the P-450 isozymes and possibly separate them into pure fractions on the second octylamino column were unsuccessful, especially in the treated preparations, due to low recovery. Because of this, fractions from the hydroxypatite column were treated to remove detergent as described in the method section, concentrated, and frozen for further analysis. The specific content of the preparations was 5.5 nm P-450 x mg protein^{-1} for the control samples and 4.7 nm P-450 x ml protein^{-1} for the treated. This is approximately a 5 fold increase over the microsomal preparations. Neither preparation showed any P-450 reductase activity or measurable amounts of cytochrome

b_5. The cytochrome P-450 reductase was isolated to a highly pure form showing only one band on 10% or 13% acrylamide SDS gels and had an activity of 40.8 units per mg protein which was a 40 fold concentration over microsomes.

Results summarizing the characteristics of the P-450 preparations are presented in Table XI. During spectral analysis a bathochromic shift of approximately 1.3 nm was measured in the treated preparations as compared to the controls after solid soidum dithionite was added and CO monoxide was bubbled through the solutions. The oxidized form of the enzymes did not appear to differ in absorption characteristics. Ethylmorphine N-demethylase activity in the reconstituted systems is also shown in Table XI. Approximately 3 times higher specific activity was found in control preparations as compared to the treated (Table XI).

Photographs of the results from the electrophoresis analysis are shown in Figure 12. Only one band was detected in the reductase preparation with an apparent molecular weight of 71,600. As expected from the specific content of the preparations minor bands were detected in both control and treated P-450 samples which were outside of the molecular weight range normally considered to contain P-450 isozymes (45,000 to 65,000 daltons, 98,98). A major band was in both preparations with an apparent molecular weight of 53,700. Another band corresponding to a protein with an apparent molecular weight of 56,200 was found in the controls which

TABLE XI. Characteristics of partially purified P-450 samples from control and BCNU treated male rats

	Specific[a] Content	λMaximum Oxidized	λ Maximum Reduced - CO	Molecular Weight	Ethylmorphine N-demethylase
Control	5.5	417	450.9	Band I[b] 53,700 Band II[b] 56,200	22.6[c]
Treated[d]	4.7	417	449.6	Band I[b] 53.700 Band II[b] 60,900	7.26[c]

a. nm P-450 x mg Protein^{-1}

b. order from the anode

c. nm HCHO x nm P-450^{-1} x min^{-1}

d. 20 mg/kg BCNU, i.p., 14 days post treatment

SDS gel electrophoresis of partially purified cytochrome P-450 from control and BCNU treated male rats.

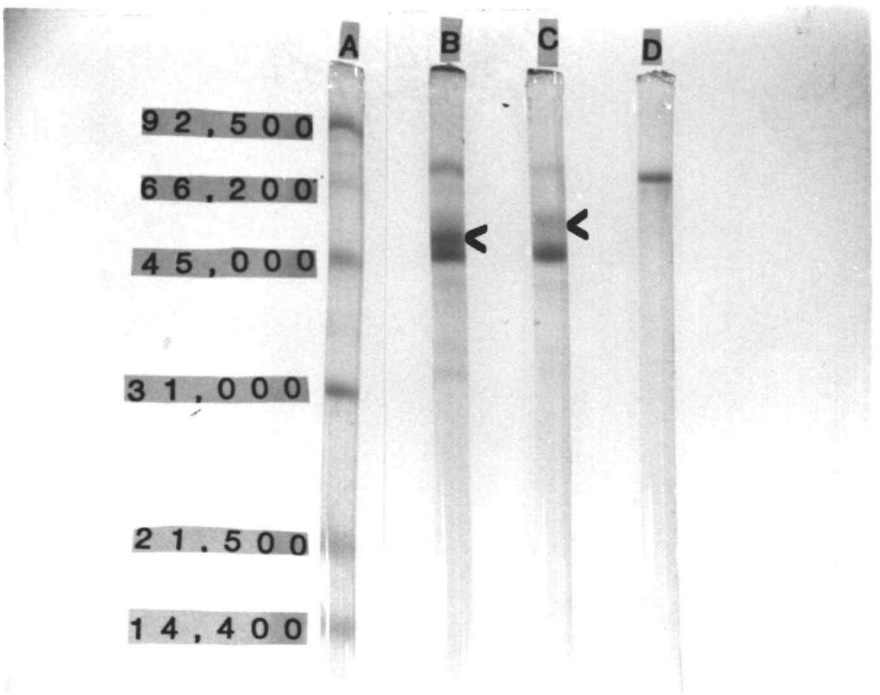


Figure 12. (A) Standard Proteins. Listed bottom to top; lysozyme (m.w. 14,400), soybean trypsin inhibitor (m.w. 21,500), carbonic anhydrase (m.w. 31,000), ovalbumin (m.w. 45,000), BSA (m.w. 66,200) and phosphorylase B (92,500). (B) Control Sample. Major bands at an apparent m.w. of 53,700 and 56,200. (C) Treated sample. Major bands at an apparent m.w. of 53,700 and 60,900. (D) Purified cytochrome creductase from control animals at an apparent m.w. of 71,600. ALl gels are 10% acrylamide.

did not appear in the treated sample. A light band with an apparent molecular weight of 60,900 was seen only in the treated sample.

DISCUSSION

The preparations from control and treated rats in this experiment were not pure P-450 proteins as indicated by specific content and electrophoresis. Instead the samples are mixtures of possibly several P-450 isozymes and other microsomal proteins. Only 15-20% of the original P-450 in the microsomes was recovered after the chromotography and it is possible the differences seen in the spectral characteristics and N-demethylase activity between the preparations may have been due to an artificial selection of different isozymes which were orginally present in both control and treated animals. Both of the preparations, however, acted similarly during the chromatographic steps and care was taken to pool all the fractions containing significant amounts of P-450 as described in the methods section. Recovery of 15 to 20% of the original P-450 is common at this stage of the purification (94,96). Agosin et al. (94) used a similar procedure for isolation of constitutive isozymes of P-450 and did not achieve separation of the different types of P-450 protein until they were applied to a second octylamino column. Because of large losses of P-450 caused by the second octylamino column step all of these preparations were tested after recovery from the hydroxyapatite column and subsequent removal of the detergents. Thus, it seems unlikely the accidental isolation of different isozymes from the preparations occurred.

All of the properties of these preparations are probably due to the sum characteristics of several P-450 isozymes because of the impure nature of the samples. These experiments do indicate, however, the major P-450 isozyme(s) found in control and treated animals differ from each other or at least that the sum total of the isozymes is not the same.

One of the properties which demonstrates the differences between the preparations is the difference in the wavelength of maximum absorption measured after the protein(s) are reduced with sodium dithionite and exposed to carbon monoxide. This has long been one method of determining different P-450 isozymes (95,98). This difference was not evident in the microsomal preparations. This may be due to the rather broad peaks obtained from the microsomes of treated animals which make it difficult to detect minor shifts in the Soret maximum. Absorption of light from other microsomal proteins which were removed from these partially purified preparations may also explain the lack of a measurable spectral shift in microsomal samples.

The other major factor indicating the two preparations are different from each other is the lower ethylmorphine N-demethylase activity of the treated animal preparations. Specific activities of P-450 isozymes towards different substrates are a primary method of differentiating them (98). Unfortunately, due to the small amounts of protein

isolated from the treated animals it was not possible to look at more than the single substrate. Because the P-450 reductase used for the reconstituted systems was isolated from control rats induced with phenobarbital there is no possibility this enzyme caused the differences seen in these *in vitro* experiments. Also, these *in vitro* assays remove the effects of membrane damage caused by BCNU directly or due to cholestasis, because the lipid environment was artificially supplied and was equivalent for both preparations. These results do not suggest membrane damage is unimportant *in vivo* but only that in addition to probable membrane disruption actual changes in the P-450 composition of the membrane have also occurred. These results would agree with the results by Mackinnon et al (81). As reported in the introduction they determined by SDS gel electrophoresis that different P-450 isozymes responded to cholestasis in varying degrees. Some forms were more depressed than others and this may help to explain some of the large decreases seen in ethylmorphine N-demethylase activity where the loss of total P-450 in the cholestatic animals does not explain all of the decreased activity.

Parkinson *et al* (105,106) found that the major form of P-450 in control animals showed an apparent molecular weight of 51,000 and that this isozyme, or mixture of isozymes was increased as the animals aged from day 28 to day 52. Both of the preparations in my experiment revealed bands during

SDS gel electrophoresis with apparent molecular weights of approximately 53,000. He also found that the band at 51,000 daltons could be decreased by the use of 3MC or β-naphthoflavone which demonstrates the content of this protein in the microsomes is subject to change. The two types of isozymes seen in my experiments also resemble 2 constitutive types isolated by Cheng and Schenkman when spectral properties and N-demethylase activities are compared (96). Caution must be taken when comparing preparations from these experiments to theirs, however, due to the purity of their isozymes. Commonly, noninduced rats seem to have at least 2 major forms of P-450 which vary in spectral properties and activity for ethylmorphine N-demethylase as well as other substrates (96). The control preparations in these experiments seem to show a majority of the P-451 type which has high ethylmorphine N-demethylase activity. In contrast, the treated animals have the P-449 type which has lower ethylmorphine N-demethylase activity. Neither isozyme seems similar to P-450 isozymes isolated from animals exposed to common inducing chemicals (98).

These results raise interesting questions concerning the regulation of P-450 isozymes in noninduced rats. How does regulation of the isozymes change during long term cholestasis? One possibility, of course, is that the regulation is not altered but one or more forms of P-450 are preferentially solubilized from the membrane which leaves a

predominance of an isozyme which is normally only a small percentage of the total P-450 in the cell. This seems unlikely because even during bile duct ligation the concentrations of bile acid reached in the liver are never high enough to solubilize integral proteins from the membrane (60). Also, analysis of the protein structures from the isozymes isolated to this date show large hydrophobic regions presumably intended for anchoring the protein into the membrane. Another possibility, aside from changes in P-450 regulation, is that the heme prosthetic group is more loosely attached to certain isozymes and it becomes separated from the apoprotein during cholestasis. This apoprotein may then be more subject to degradation. Some of the phenobarbital induced isozymes do seem to be more loosely associated with the heme group but this has not yet been shown for constitutive forms (93).

An interesting hypothesis is that there is a change in the isozymes which helps protect the animal from detergent bile acids during cholestasis. Greim et al (60) describe the mechanism by which rats are able to convert chenodeoxycholate, which has strong detergent properties, to less damaging forms such as ursodeoxycholate or β-muricholic acid. These transformations are made through P-450 dependent 6β and 7α hydroxylations. The 6β and 7α hydroxylation activity of bile duct ligated animals has been shown to increase when compared to controls (108). Additionally

Cheng and Schenkman (96) report their RLM_3 isozyme, which resembles the form isolated from our treated animals, is higher in 6β and 7α hydroxylation of testosterone than their RLM_5 isozyme which resembles the type predominating in our control animals. Therefore, even though the total content of P-450 decreases, the rat may be still able to form less toxic bile acids due to a relative increase in P-450 isozymes which have a high activity for those types of reactions.

The possibility that different P-450 isozymes may predominate in control and cholestatic animals suggest a highly coordinated control mechanism if the difference is not simply due to destruction of selected isozymes by bile acid buildup. Evidence from several laboratories suggests individual P-450's are highly regulated and the content of a particular isozyme of P-450 may be dependent to some extent by the presence of other forms of P-450 (105). Parkinson et al (105,106) report different half lives for several forms of P-450 which are present in low concentrations in control animals but increase dramatically after the animal is exposed to Arachlor 1254. These changes in P-450 levels and isozymal relationships are independent of the majority of other proteins found in the endoplasmic reticulum. This supports the finding that BCNU induced cholestasis decreases cytochrome P-450 levels but does not seem to greatly alter other microsomal proteins.

The loss of selected P-450 isozymes during cholestasis and the effects this might have on the regulation of other isozymes might help to explain some of the results seen in the drug metabolism of β-naphthoflavone induced animals pretreated with BCNU. Some of these animals showed very high levels of activity towards ethoxycoumarin despite somewhat lower levels of cytochrome P-450 when compared to β-naphthoflavone induced controls. It is possible the isozyme profile of the induced treated animals differed from the control induced animals and favored this type of metabolism. These arguments strongly support the contention that the isozymal makeup of control and treated animals does differ but there is a need for more definitive experiments.

SDS gel electrophoresis demonstrated some differences between the preparations in the molecular weight range usually considered to contain the major P-450 isozymes. While both control and treated animals seemed to have the same major band with an apparent molecular weight of 53,000 there were differences in minor bands between the two samples. The treated animals seemed to have a protein with an apparent molecular weight of 60,900 which was not as predominant in the control samples, but lacked the protein with an apparent molecular weight of 56,000 which was seen in control preparations. This would seem to support the hypothesis that there is a difference in the protein makeup of the microsomes between control and treated animals.

Future analysis of these proteins by isoelectric focusing or fragmentation digestion of more highly purified samples may prove these bands do indeed represent separate P-450 isozymes and may even find these bands contain more than a single P-450 protein.

Clearly the results from these experiments are not conclusive evidence that different isozymes dominate the control and treated microsomes. These results do strongly indicate, however, there are differences between the BCNU and control animals which cannot be entirely explained by the changes in membrane-protein interactions because the reconstituted systems have similar membrane structures. Many more experiments must be conducted to better characterize the changes which do occur and much more will have to be learned about the control of P-450 isozymes in the liver before it will be possible to explain how these changes take place. The experiments indicate the need for more studies on the long term consequences of cholestasis and suggest BCNU may be an excellent model compound for the production of prolonged cholestasis in rats.

V. Summary and Conclusions

BCNU has been shown to be hepatotoxic to both mice and rats through a series of experiments performed in this laboratory by several investigators using a wide variety of methods (8,10,13). The hepatotoxicity of BCNU which occurs in laboratory animals seems to be more severe than that seen in clinical situations (5). The possible importance of liver toxicity is often overlooked in clinical situations because of the more serious primary disease.

The experiments reported in this study have been designed to investigate the long term effects on MFO activity associated with BCNU after a single i.p. dose of the drug in rats. Results from the experiments have been interpreted to show that BCNU causes cholestasis in rats which then, via several mechanisms, alters hepatic microsomal function. These experiments do not, however, prove this hypothesis, and indeed the possibility exists that BCNU acts more directly on the MFO system, or a closely related enzyme system, to alter P-450 activity.

Several lines of evidence argue against the idea that cholestasis is the cause of decreased MFO activity after BCNU. Many investigators have shown BCNU does reach the SER and in fact is partially metabolized by microsomal systems (31,34). The drug is known to be cytotoxic and is a potent alkylator and carbamylator of cellular constituents (15). The relative lipophillic nature of the drug may allow some

of it, or the metabolites and breakdown products, to remain in lipid compartments for periods long exceeding the serum half-life and causing continuous damage to the SER. Indeed, the fact that BCNU does cause severe cholestasis is an indication it has long term effects which may be the result of membrane damage. Similar mechanisms of direct action could alter MFO activity. Certainly cholestasis does not account for the transient change in MFO activity seen approximately 24 hours after BCNU treatment because this is prior to detectable changes in bile flow. One other problem is that no good comparison between BCNU and other causes of cholestasis yet exists. These experiments need to be done.

Despite the arguments stated above against the hypothesis that long term changes in MFO activity after BCNU treatment are due to the cholestatic affects of the drug, I feel the evidence supports this idea. If BCNU acted directly and the lingering affects were due to long sequestering of the parent drug or its products, I would expect to find an initially large change in MFO activity with some incomplete recovery. This is not the pattern seen either in my experiments (Section I) or by others (10,13). Major changes in activity do not occur for one to two weeks after the drug has been given and this correlates well with the time course for cholestasis (7,9). Despite the lack of detailed comparisons between BCNU and other known cholestatic agents, results from section I correlate well with results from

other laboratories which have measured MFO activity during cholestasis (58,62). Recent results have shown that mice and rats do not react to the cholestatic effects of some drugs in the same manner (65). Wilson and Larson (13) produced evidence which indicates mice recover more quickly from hepatic damage produced by BCNU than rats. This difference may be due to the differences in cholestasis produced by the drug in mice and rats though no measurements of bile flow have been made in mice given BCNU.

Section II was a description of measurements made on delta aminolevulinic acid synthetase and heme oxygenase activity in rats after BCNU treatment. Many of the changes seen in heme metabolism mimic changes observed in other laboratories after bile duct ligation (84,85,86). BCNU does not appear to act like other known blockers of heme synthesis, like cobalt, which stops induction of MFO activity by phenobarbital (93). Evidence from other laboratories does not support the theory that increases in heme oxygenase activity are due to or the cause of increased P-450 degradation (84,86,93).

The last section was a report on experiments which were conducted to isolate P-450 proteins from control and treated animals. The physical properties of these proteins were then compared and ethylmorphine N-demethylase activities in reconstituted systems was determined. If only protein lipid interactions were changed by BCNU or the resulting cholesta-

sis then I would have expected proteins isolated from the animals would be identical and in reconsitution experiments equal activity between the two samples would be seen because identical lipid environments were supplied. This did not occur. Samples from treated and control animals had different patterns after SDS gel electrophoresis, different spectral properties and different ethylmorphine N-demathylase activity. Because the experiments took place two weeks after BCNU treatment it seemed likely some long term influence is causing these differences and cholestasis is one possibility. Changes in P-450 isozymal patterns have been reported during cholestasis and may explain the results seen here (81). It may be that these changes aid the rat in metabolizing bile acids and protect the animal from the more highly toxic forms. My results must be considered preliminary, however, because complete purification was not achieved and it was not possible to repeat the experiment at this time.

While these experiments have not proven the mechanism of action for the effect of BCNU on the MFO system they do establish a firm basis for further study. Certainly more details on the cholestasis produced by BCNU must be made. Purification of P-450 from control and treated animals should be achieved which might be made easier by new HPLC technology for protein purification. The pharmacokinetics of BCNU should be investigated more completely. An

interesting study would be a study of P-450 enzymes in other organs such as the lung or adrenal gland after BCNU. Clearly, despite gains made recently much more work is needed to study the mechanism of action of BCNU. The drug, however, is an excellent example of an agent causing multiple toxic effects and represents an interesting model for the better understanding of how organ systems respond to toxic injury.

VI. Biblography

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