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Title: Impaired Organic Anion Excretion and Cholestasis Caused by 1,3-bis-(2-chloroethyl)-1-nitrosourea (carmustine or BCNU) in Rats

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Previous investigation showed that 20 mg/kg BCNU caused inhibition of sulfobromophthalein (BSP) excretion and inhibited bile salt independent bile flow (BSIF) in rats by 48 h after treatment (Hoyt, 1984).

The present investigation demonstrated that BCNU inhibited the canalicular step in the excretion of BSP. This was indicated by the inhibition of maximal excretion of BSP, inhibition of the biliary excretion of the non-metabolized dye, indocyanine green and the lack of inhibition of the conjugation of BSP with glutathione. Canalicular effects were also suggested because BCNU-pretreatment inhibited the excretion of endogenous reduced glutathione and oxidized glutathione.

The inhibition of BSP excretion by BCNU did not

appear to result from increased hepatic content of reduced or oxidized glutathione because these materials fell to normal levels at 72 h after 20 mg/kg BCNU. BSP excretion was inhibited by this dosage for at least 96 h (Hoyt, 1984). Inhibition of BSP excretion was not due to accumulation of bile salts because their depletion had no effect on BSP excretion in BCNU-treated rats.

Pretreatment of rats with pentobarbital sodium prevented BCNU-induced cholestasis and inhibition of BSP excretion. Pentobarbital may have stimulated BSIF, altered the response of the liver to BCNU, changed the amount and/or types of interactions of BCNU with liver or reduced the amount of BCNU reaching sites of action.

Theophylline and glucagon infusions stimulated bile flow in BCNU-treated rats. These agents may have increased intracellular cyclic adenosine monophosphate (cAMP) content. The research of others indicates that cAMP may stimulate sodium-coupled chloride transport in epithelia. Therefore, this transport or hepatocellular cAMP may be depressed in BCNU-treated rats. Theophylline and glucagon may have stimulated bile flow so that the effect of BCNU on other systems was masked.

Analysis of biliary bile salt composition demonstrated that there was no increase of the more hydrophobic, toxic bile salts. No action of BCNU is related to the hypothesized accumulation. The opposite

trend, a shift to hydrophilic bile salts, was observed. This shift is observed in cholestasis caused by alpha-napthyl isothiocyanate, ethinyl estradiol and bile duct ligation, suggesting that it is a result of cholestasis.

Impaired Organic Anion Excretion and Cholestasis Caused by 1,3-bis-(2-chloroethyl)-1-nitrosourea (carmustine or BCNU) in Rats

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Impaired Organic Anion Excretion and Cholestasis Caused by 1,3-bis-(2-chloroethyl)-1-nitrosourea (carmustine or BCNU) in Rats

GENERAL INTRODUCTION

I. History and Toxicity of BCNU

1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) is an antineoplastic drug. Development of nitrosoureas as clinically useful antineoplastic agents was initiated by the discovery that methyl-N-nitrosonitroguanidine reduced the tumorigenic response to intraperitoneal (i.p.) inoculation with L1210 leukemia cells (DeVita et al., 1965). The Cancer Chemotherapy National Service Center screened related compounds and found that methylnitrosourea was active on i.p. and intracerebrally (i.c.) injected L1210 cells. Southern Research Institute then began screening nitrosoureas and found BCNU to be the most active and capable of prolonging the survival of mice that received L1210 cells i.c. (DeVita et al., 1965). In clinical trials the drug was also active against a wide variety of carcinomas and sarcomas in about 20% of patients tested.

Toxicity was apparent in the clinic as well. Acutely, intravenous (i.v.) administration caused flushing of the skin and nausea. Topical application

caused erythrema but no blistering often associated with other alkylating agents. DeVita et al. (1965) found that leukopenia and thrombocytopenia were the major dose-limiting toxicities. Maximal cytopenia occurred about one month after a single i.v. injection of 300 to 400 mg/sq.m and was dose related. Hepatic and renal toxicity were evidenced by the release of marker enzymes into blood and urine, but these effects were not as serious as the action on bone marrow (DeVita et al., 1965).

Many toxic effects were delayed in onset. This is a characteristic of many of the actions of BCNU. Delayed pneumotoxicity in the form of fibrosis is a clinically limiting effect. Pulmonary fibrosis occurs in as many as 50% of individuals who have received a cumulative dosage of 1500 mg/m^2 regardless of the schedule of administration (Aronin et al., 1980). Biochemical changes in the lungs of rats that may predict fibrosis, such as release of angiotensin converting enzyme from endothelium and increased collagen content of the tissue, occured several weeks after a single dose of BCNU (20 mg/kg, or about 120 mg/sq.m; Jarvi and Larson, 1985) and several weeks into multiple dosing schedules (Smith and Boyd, 1983).

The cardiovascular toxicity of BCNU has been examined only recently. A single i.p. injection of 20

mg/kg in rats caused supersensitivity of the caudal artery to norepinephrine *in vitro* after 2 weeks. It occurred 1 week after 25 mg/kg. The effect was abolished by denervation of arteries with 6-hydroxydopamine suggesting a prejunctional site of action. BCNU caused toxicity to the endothelial lining of the vasculature as evidenced by release of angiotensin converting enzyme and its acute effect on cultured endothelial cells (Jarvi and Larson, 1985; Nicolson and Custead, 1985; Rawson and Larson, 1985). The role of the endothelium in acetylcholine-mediated relaxation of the rat caudal artery *in vitro* was inhibited 1 day after BCNU treatment *in vivo*. As in the lung, arterial collagen content was increased (Rawson and Larson, 1985). This action was delayed, occurring 14 d after 20 mg/kg BCNU.

BCNU affects the function of platelets. *in vitro* exposure of normal platelets to BCNU inhibited the release of serotonin stimulated by collagen. Platelets failed to aggregate in the presence of epinephrine, adenosine diphosphate, collagen and arachidonic acid (McKenna *et al.*, 1983)

In the rat liver, BCNU causes effects that develop as early as 24 h and progress through 80 to 120 days after a single administration of 20 mg/kg. Thompson and Larson (1969) first characterized the hepatotoxicity. One of the earliest effects noted was

retention of sulfobromophthalein (BSP) at 36 h. This finding indicated that hepatic excretory function was impaired. Hyperbilirubinemia was not observed until 2 weeks after 20 mg/kg BCNU however.

Prolongation of pentobarbital-induced sleep occurred 7 days after treatment with BCNU. This observation led to a series of investigations on the effect of BCNU on the hepatic monooxygenase activity in rats and mice and their responses to enzyme inducers (Lu and Larson, 1970; Wilson and Larson, 1981, 1982). Recent work indicated that BCNU changed the nature of the microsomal cytochromes since metabolic activity was reduced in reconstituted systems and because patterns of electrophoretic banding were altered (Stolzenbach, 1984). These effects occurred by 14 days after 20 mg/kg BCNU. Both ethylmorphine-N-demethylase and O-deethylase activities of microsomes were inhibited primarily by an increased apparent Km (Stolzenbach et al., 1984). It was noted by Stolzenbach that extrahepatic cholestasis can cause changes in heme metabolism and monooxygenase activity that are similar to his observations (Stolzenbach et al., 1984; Stolzenbach, 1984). This is a provocative mechanistic idea given the cholestatic actions of the drug (Thompson and Larson, 1969; Hoyt, 1984).

The effects of BCNU on the liver progress for

months after a single administration. Eighty-four days after 20 mg/kg Thompson and Larson noted the appearance of "hob nail" livers in rats. Histologically, there was proliferation of connective tissue and biliary epithelium (Thompson and Larson, 1969). Now it seems that the delayed connective tissue proliferation, first characterized in the liver, may be a more general action occurring in the lung and cardiovascular system as well.

The observations of BSP retention, hyperbilirubinemia, and actual cholestasis by Thompson and Larson led to the previous and present investigations into the mechanisms of action of BCNU on hepatic excretory function (Hoyt, 1984).

II. Pharmacokinetics

BCNU is commonly administered to humans i.v. in solution with 10% ethanol (Wasserman, 1976). The drug is usually given i.p. to laboratory animals. BCNU is a lipophilic substance as evidenced by its ability to affect intracerebrally inoculated L1210 cells, its solubility in corn oil, and its octanol:water partition coefficient of 37 (Weinkam et al., 1980). Nitrosoureas are unstable in aqueous solution and much has been concluded regarding the biological activity of various products of spontaneous breakdown (Montgomery, 1976). The half-life of BCNU in aqueous solutions buffered at

pH=7.4 is about 50 min. The half-life in serum is much shorter (11 to 14 min in man, 26 min in rats) and is increased by addition of triglycerides (Levin et al., 1979; Weinkam et al., 1980).

The pharmacokinetic behavior of BCNU was adequately described by a 2 compartment open system (Levin et al., 1979). The bioavailability of i.p. injections of BCNU in 10% ethanol in rats was 0.57 (Levin et al., 1979). This value is affected by hepatic metabolism of the drug. Levin et al. (1979) showed that phenobarbital pretreatment had a profound effect on the plasma concentrations of BCNU in rats. The bioavailability i.p. injections was reduced to 0.087 in phenobarbital pretreated animals. The area under the curve was reduced by 64% after i.v. administration of BCNU and by 90% after i.p. administration of BCNU. The maximal rate of disappearance of BCNU in 9000 x g rat liver supernatants was increased 2.5 fold by phenobarbital pretreatment (control supernatant metabolized BCNU in a saturable fashion at a maximal rate of 1.7 nmol/min/mg protein). Calculations were made by Levin et al (1979) indicating that the life of the drug in the body is closely related to hepatic metabolism. The hepatic metabolism of the nitrosoureas is primarily denitrosation to give bis(2-chloroethyl) urea which may react with glutathione (Hill, 1976).

Denitrosation was not completely inhibited by anaerobic conditions and weakly inhibited by carbon monoxide (Hill et al., 1975). Subsequent research showed that NADPH-cytochrome P-450 reductase catalyzed the denitrosation of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in the absence of phenobarbital-inducible cytochrome in reconstituted systems. This reaction was stimulated in the presence of flavin mononucleotide and not flavin adenine dinucleotide (Potter and Reed, 1983). Urinary excretion accounts for 80% of an administered dose of BCNU in the form of degradation products (Calabresi and Parks, 1980).

BCNU decomposes in aqueous solution by deprotonation at N-3 generatiing chloroethyl isocyanate containing that nitrogen. The isocyanate can hydrolyze to give carbon dioxide and chloroethylamine. (Montgomery, 1976). The isocyanate is probably responsible for carbamylation reactions which have been related to some toxic effects of BCNU (Sariban, et al., 1984; Scudiero et al., 1984). Lymphoblasts take up intact molecules of BCNU by passive diffusion in vitro. The addition of chloroethyl isocyanate reduced the uptake of radioactivity from [U-^{14}C]-BCNU by 72%, implying that the isocyanate may be the major species taken up after decomposition of BCNU (Begleiter et al., 1977). Chloroethyl diazohydroxide is also produced and

upon loss of the hydroxyl at N-2, denitrogenation, which is strongly favored, yields chloroethyl carbonium. This is a probable alkylating species. The carbon attached to N-1 can lose a proton resulting in the formation of vinyl carbonium after denitrogenation also (Montgomery, 1976).

III. Molecular Actions

It is implied that the actions of BCNU are a result of the effects of the spontaneously formed products. The genotoxicity of BCNU may be related to the ability of carbonium ions to alkylate DNA and/or to cross-link DNA at guanine and cytosine (Tong et al., 1982). In aqueous solution BCNU reacts primarily with polyguanosine monophosphate and polycytidine monophosphate (Ludlum et,al., 1975). Other effects of BCNU are related to actions of chloroethyl isocyanate. Wheeler et al. (1974) correlated physical characteristics, biologic activity and alkylation and carbamylation activity in a series of nitrosoureas. They concluded that alkylation was the main factor contributing to antitumor action while carbamylation contributed to the lethality to the animal. High lipid solubility was important for therapeutic action also.

The cytotoxic action of BCNU toward neoplastic

cells is probably a result of this bifunctional activity. DNA is damaged by alkylation and cross-linking as described and the chloroethyl isocyanate appears to inhibit DNA repair by O^6-alkylguanine alkyltransferase (Kann et al., 1974a; Sariban et al., 1984; Scudiero et al., 1984). Chloroethylamine, formed by decarboxylation of isocyanate, is not inhibitory (Kann et al., 1974a). DNA ligase activity is inhibited by chloroethyl isocyanate in vitro, implying that excision-incision repair may be depressed by BCNU also (Fornace et al., 1978). Thus, BCNU causes DNA damage and inhibits its repair. BCNU inhibits basal thymidine incorporation into DNA, but this is not required for cytotoxic action since nitrosoureas lacking effect on thymidine can be cytotoxic (Kann, 1978). BCNU and chloroethyl isocyanate inhibit RNA processing and transport from the nucleus, but this is not directly related to cytotoxic action (Kann et al., 1974b).

BCNU inhibits a number of other enzymes in vitro and in vivo. Most investigations implicate carbamylation by chloroethyl isocyanate in mechanisms. Although all possible enzymes have not been tested for inhibition, a salient point is that not all enzymes tested are inhibited by therapeutic levels of the drug in vitro or after isolation from pre-treated subjects (Frischer and Ahmad, 1977; McKenna et al., 1983). BCNU

inhibited glutathione reductase at a lower concentration than other enzymes tested (McKenna et al., 1983). BCNU did not alter the activity of glycolytic enzymes, enzymes of the hexose monophosphate pathway, glutathione peroxidase, adenylate kinase or adenosine triphosphatase in human erythrocytes taken within 1.5 hours after i.v. administration of the drug (McKenna et al., 1983). Ten minutes after i.p. administration to mice, glutathione reductase was inhibited in kidney, liver, lymph nodes, brain, spleen, lung, skeletal muscle and heart (Frischer and Ahmad, 1977). Reductase was inhibited in platelets after in vivo exposure (McKenna et al., 1983). Chloroethyl isocyanate was shown to be responsible for this action on the reduced form of the enzyme in vitro (Babson and Reed, 1978). BCNU also inhibited the reduced form of lipoamide dehydrogenase in vitro (Ahmad and Frischer, 1985). The active sites of these 2 enzymes possess extensive amino acid sequence homology and contain 2 cysteine residues important for electron transfer (Ahmad and Frischer, 1985). Reductase was inhibited 50% at about 10 μM BCNU while the dehydrogenase was inhibited 50% at about 100 μM BCNU. Other flavoenzymes such as glucose and xanthine oxidase were not inhibited (Ahmad and Frischer, 1985). Lipoamide dehydrogenase of platelets was not reduced after in vivo exposure, probably because it exists mainly in the

oxidized form (McKenna et al., 1983). Brain creatine kinase and brain, heart and liver malate and lactate dehydrogenases were inhibited in mice 96 h after i.p. injection of 50 mg/kg BCNU (Maker et al., 1983). BCNU inhibited serum angiotensin converting enzyme in vitro in the millimolar concentration range and the enzyme was inhibited after 80 mg/kg BCNU i.p. in rats (Smith and Boyd, 1983).

Butyl isocyanate inhibited serine proteases such as chymotrypsin and elastase by covalent binding to active site serine (Brown and Wold 1973). Butyl isocyanate also inhibited yeast alcohol dehydrogenase and pig liver transglutaminase by reaction with active site cysteine (Twu and Wold, 1973; Gross et al., 1975). Chloroethyl isocyanate and BCNU inhibited also tubulin polymerization in vitro (Brodie et al., 1980).

The main point is that BCNU generates an isocyanate that may interact with nucleophilic sites of a number of macromolecules. Some enzymes are inhibited by the action of BCNU and others are unaffected. BCNU also produces chloroethyl and vinyl carbonium species that are thought to cause DNA alkylation and cross-linking.

It is interesting that many of these actions are delayed in onset, because it appears that the actions of BCNU, both therapeutic and toxic, are a consequence of

the early formation of reactive species in the body It could be that some effects are secondary to DNA damage causing cellular accumulation of inactive or inappropriate proteins. Stolzenbach (1984) found that although specific cytochrome P-450 content of hepatic microsomes was decreased, microsomal protein content was not reduced by BCNU pretreatment. Rats also responded to inducing agents such that P-450 contents were equal in induced control and treated rats (Stolzenbach, 1984). These data imply that the ability to synthesize protein is not generally compromised. However, as mentioned previously, the isozymes of microsomal cytochromes were altered as evidenced by reduced enzyme activity in a reconstituted system and by electrophoretic analysis (Stolzenbach, 1984). This is an example of an alteration in gene products due to BCNU-pretreatment. A delay in onset of effects could then be explained by the time required to turnover cell protein. BCNU might act on cell proteins directly as observed for the isocyanates. A consequence could be enzyme inhibition or stimulation. In this case, delayed onset of effects would be related to the time required to accumulate or lose an endogenous substance.

The ultimate impact of BCNU is most probably due to multiple interactions with cells however. Indeed, the antineoplastic, cytotoxic action of BCNU appears to

be a consequence interactions with both DNA and protein. Over the long time courses it is difficult to relate drug action to a discrete event, and actions in some organs may be secondary to a toxic effect in others. As an example, cholestasis might cause the accumulation of material in plasma, such as bile salts, which are known to be toxic to other organs such as the heart (Better and Harari, 1983). Delineation of the time-course of action is very important in determining the mechanisms associated with the toxicity of BCNU.

IV. Review of Cholestatic Actions of BCNU

Cholestasis (reduced bile production) in rats was produced 48 h after a single i.p. injection of BCNU at a dose of 20 mg/kg (Hoyt, 1984). This effect was associated with a selective reduction of bile salt independent bile production. Plasma sodium was reduced and potassium was increased. The possible inhibition of sodium/potassium adenosine triphosphatase was not investigated. Bile salt excretion rate was reduced only because the flow of bile was inhibited. The rats actually maintained an elevated biliary concentration of bile salts. Therefore, the mechanism of bile salt transport was not inhibited. Elevated biliary bile salt concentrations and increased bile:plasma osmolality

ratios implied that permeability between bile and plasma was not increased.

In contrast, rats were unable to concentrate sulfobromophthalein (BSP) in bile, even prior to the onset of cholestasis. Since BSP excretion is facilitated by conjugation with glutathione (Whelan et al., 1970), the effect of BCNU on the reduced glutathione content of liver was investigated. Glutathione was elevated before the inhibition of sulfobromophthalein excretion (Hoyt, 1984). There was an adequate supply of glutathione in the liver to act as substrate for conjugation of sulfobromophthalein. This was a paradox when one only considered the action of BCNU as an inhibitor of oxidized glutathione reductase, a system that resynthesizes reduced glutathione from the oxidized form (Meister, 1983). In addition, the pattern of metabolites of sulfobromophthalein appearing in bile was altered by BCNU (Hoyt, 1984). A number of additional metabolites not associated with amino acid were found in bile from BCNU-treated rats. This might have been a consequence of an action on the conjugating enzymes (glutathione-S-transferases) or simply due to the delay in excretion which could allow time for additional metabolism of BSP conjugated with glutathione.

V. Purpose

There were 2 purposes that motivated the present investigation. One was to describe how BCNU inhibits organic anion excretion. The other was to investigate the cause of cholestasis further.

In addition, pharmacological prevention or reversal of the actions of BCNU on bile production and organic anion excretion was attempted as a possible means to gain further mechanistic insight into the actions of BCNU.

The hypotheses were that BCNU inhibits xenobiotic organic anion excretion by an action at the canalicular membrane and that this action and cholestasis do not involve an accumulation of toxic bile salts.

GENERAL METHODS

All of the experiments were carried out using male, Sprague-Dawley rats weighing 250 to 350 grams. The rats were purchased from Simonsen Laboratories, Gilroy, CA. Rats were housed in groups of 5 in standard suspended steel cages (41 x 24 x 18 cm). They were maintained on a 12 h light/dark cycle at 21 $\pm$ 1 ^{o}C, and at 60 $\pm$ 5% relative humidity with 15 complete air changes per day. Rats were provided with free access to water and food unless otherwise indicated.

When surgery was required, rats were anaesthetized with pentobarbital sodium (60 mg/kg intraperitoneally) dissolved in ethanol:propylene glycol:water, 1:2:7. To determine bile flow, a short midventral incision was made and the common bile duct was isolated. A PE 10 cannula was inserted into the bile duct to the hilum of the liver and bile was collected into plugged, graduated, glass syringes marked in increments of 10 µl unless otherwise noted. The liver was removed and weighed at the end of the collection period. Bile flow was determined in µl/min/g liver. Whenever the concentration of some material in bile was determined, its excretion rate could be calculated as the product of bile flow and concentration in appropriate units.

Data were compared using analysis of variance and the Student's t- test unless otherwise indicated in a subsection entitled "Data Analysis" under the individual experiments. When the variance for the experimental groups in question were unequal in an F-test at alpha = .05, a modification of the t-test, the t'-test, was used. When $P < .05$, a significant effect was assumed (Snedecor and Cochran, 1980).

I. The Effect of BCNU on the Maximal Rate of BSP Excretion

INTRODUCTION

Materials that are excreted in bile fall into 3 classes based on the ratio of concentrations in bile and plasma during steady-state. Sucrose, inulin and albumin are examples of molecules that are excluded from bile to a large extent (Klaassen and Watkins, 1984). Urea, erythritol, mannitol, sodium and potassium are examples of molecules that equilibrate to a 1:1 ratio (Klaassen and Watkins, 1984). A third set of compounds, the cholephils, are concentrated in bile relative to plasma. The cholephils are classified into 3 groups based on electric charge. Ouabain is a neutral compound that is concentrated in bile (bile:plasma ratio approaches 70, Klaassen and Watkins, 1984). N-ethylprocainamide ethobromide is an example of a cation that is concentrated in bile to a bile:plasma ratio of about 10 (Klaassen and Watkins, 1984). Bile salts, such as taurocholate, and organic anions, such as bilirubin glucuronides, sulfobromophthalein (BSP) and indocyanine green (ICG), are examples of negatively charged compounds that are concentrated in bile (Klaassen and Watkins, 1984). The bile:plasma ratio in the isolated,

perfused rat liver approaches 40,000 for taurocholate and BSP (Krell et al., 1982). Compounds that are concentrated in bile are characteristically of high molecular weight. In fact there is a "molecular weight threshold" for predominant biliary excretion around 300-600 g/mol and it varies from species to species (Smith, 1973).

In previous investigations, BCNU inhibited the ability of rat liver to concentrate BSP, but not bile salts, in bile (Hoyt, 1984). The excretion of BSP, or any other substance concentrated in bile, occurs in steps (Wheeler et al., 1960). In the present studies, BSP was administered i.v. It distributes and is taken up by the liver across the sinusoidal or basolateral membrane of the hepatocyte. Albumin has the effect of preventing the development of a concentration gradient within the sinusoids from the portal to venous outlet during anterograde perfusion and vice versa during retrograde perfusion of the isolated liver (Gumucio et al., 1984). Albumin decreases the first pass extraction of BSP relative to a perfusion without albumin (Gumucio et al., 1984). This is a reflection of the ability of albumin to bind BSP (Gumucio et al., 1984). Based on mathematical modeling of transport, Forker and Luxon (1983) have concluded that binding of albumin-ligand complexes to the sinusoidal membrane facilitates uptake

of the ligand. This apparently accounts for high extraction ratios that seem to be consonant with low rates of dissociation of ligands from albumin (Forker and Luxon, 1983). Because BSP is subject to efficient extraction by the liver, its excretion could also be sensitive to changes in hepatic blood flow (Pang, 1980).

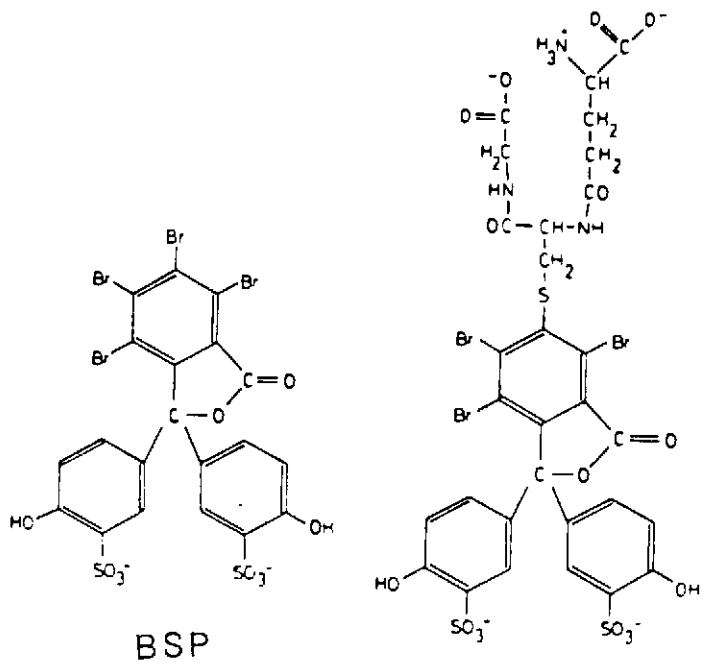
The uptake of BSP by isolated hepatocytes follows Michaelis-Menten kinetics at concentrations below 10 µM but deviates from this at higher concentrations (Schwenk et al., 1976). Uptake was found to be independent of a sodium gradient, membrane potential or metabolic energy supply implying that it is diffusional in nature (Schwenk et al., 1976). Some investigators have isolated proteins from the plasma membrane that bind organic anions with high affinity (Klaassen and Watkins, 1984). One or more of these may be a transport protein involved in uptake, but it is important to remember that uptake is not zero-order with respect to BSP concentration at high BSP concentrations. The amounts of BSP administered in the present investigations yield such high plasma concentrations for such a prolonged time that passive diffusion probably represents the dominant mechanism of uptake (after a single i.v. injection of 50 mg/kg BSP the plasma concentration in control rats peaked at 1.2 mM and approached 10 µM 45 min later, Hoyt, 1984). The uptake

of organic anions may be facilitated by binding to glutathione(GSH)-S-transferases intracellularly (Levi et al., 1969; Kaplowitz, 1980). Uptake is the most rapid step in the excretion of BSP and is not rate-limiting in normal animals (Forker, 1977).

Bilirubin is an endogenous compound that shares the uptake system with BSP (Klaassen and Watkins, 1984). The uptake system for bilirubin and BSP is relatively separate from that for bile salts, but there is some overlap. Bile salts are taken up mainly by a sodium-dependent mechanism but they also enter the cell by a sodium-independent system that is competitively inhibited by BSP and bilirubin and by passive diffusion (Anwer and Hegner, 1978; VanDyke et al., 1982). Laperche et al. (1981) demonstrated that BSP uptake is competitively inhibited by taurocholate and that the Ki is about the same as its Km for sodium-independent bile salt uptake suggesting that these are the same systems. BSP and bilirubin non-competitively inhibited sodium-dependent bile salt uptake and it was suggested that BSP and bilirubin are not subject to the sodium-dependent system (Anwer and Hegner, 1978). This is consistent with the results of Schwenk et al. (1976) demonstrating that BSP uptake is not sodium-dependent.

Organic anions, including BSP,ICG and bilirubin, associate with GSH-S-transferases and membrane lipids in

the cell (Kaplowitz, 1980; Boyer et al., 1983). It has been hypothesized that GSH-S-transferases may facilitate movement of bound material to the bile canalicular side of hepatocytes and that membrane associated material might reach the canalicular membrane by lateral diffusion (Kaplowitz, 1980; Boyer et al., 1983). The hepatic content of transferase does correlate with the development of excretory function after birth, but it does not correlate with the difference in the ability of mice and rats to excrete BSP (Klaassen, 1975; Gregus and Klaassen, 1982). Once in the cell, organic anions may or may not be metabolized. ICG is not metabolized before excretion whereas BSP is conjugated with GSH by the action of GSH-S-transferases (Gregus and Klaassen, 1982). The structures of BSP, BSP conjugated with glutathione (BSP-SG) and indocyanine green are shown in figure 1. Bilirubin is conjugated with glucuronic acid by the action of UDP-glucuronyl transferases found in microsomes and glucuronidation is almost absolutely required for bilirubin excretion into bile (Hochman and Zakim, 1983; Gordon et al., 1984; Klaassen and Watkins, 1984). Conjugation is necessary for maximal excretion of BSP although some unconjugated BSP appears in bile also (Whelan et al., 1970; Gregus and Klaassen, 1982). It is apparent that the cytosolic GSH-S-transferases do not have access to membrane bound BSP (Boyer et al., 1983).



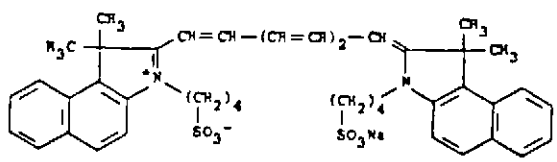


Figure 1. The structures of sulfobromophthalein (BSP), its glutathione conjugate (BSP-SG) and indocyanine green (ICG).

The liver possesses a microsomal form of the enzyme but its relationship to this consideration has not been investigated (DePierre and Morgenstern, 1983).

The last step in excretion is passage across the canalicular membrane into bile. This is considered as a potential rate-limiting step in excretion because the maximum transport rates of BSP, ICG and taurocholate are less than the uptake rates (Forker, 1977). The maximum excretion rate for BSP (BSP Tm) is 60% of the maximum uptake rate (Klaassen and Watkins, 1984). The excretion across this membrane may be dependent on the membrane potential which is about -30 mV inside (Boyer, 1980). This has been shown to be the case for the anion, taurocholate, in canalicular membrane vesicles (Inoue et al., 1984b; Meier et al., 1984b). The transport in these preparations was saturable implying that it is carrier mediated (Inoue et al., 1984; Meier et al., 1984). The possibility exists that BSP and conjugated BSP (BSP-SG) are subject to this system since taurocholate and BSP compete for excretion under conditions of maximal transport (O'Maille et al., 1966). The transport of some GSH conjugates, such as S-dinitrophenyl glutathione, in canalicular membrane vesicles was stimulated by an appropriate membrane potential (Inoue et al., 1984a). Transport was saturable and GSH, GSSG and another glutathione conjugate,

S-benzyl glutathione inhibited transport suggesting that these 4 compounds share transport systems (Inoue et al., 1984a). BSP-SG did not inhibit transport of S-dinitrophenyl glutathione, raising the possibility that GSH conjugates may be excreted into bile by multiple mechanisms (Inoue et al., 1984a). This is reminiscent of the situation at the sinusoidal membrane with respect to uptake. The possibility that GSH, GSSG or bile salts might be involved in the inhibition of BSP excretion are considered in chapters IV and V. It was once thought that transport into bile might be driven by association with bile salt-cholesterol-phospholipid mixed micelles in bile (Scharschmidt and Schmid, 1978), but this effect has been ruled out (Ritt and Combes, 1967; Delage et al., 1976).

The determination of the maximal rate of BSP excretion (BSP Tm) represented a step toward defining the anatomical site of action of BCNU in blocking the excretion of BSP. The maximal rate of BSP excretion was determined under the influence of BCNU. Under the conditions of maximal transport, the rate-limiting step in excretion, presumably the canalicular step, should be saturated. This experiment also eliminated a complication associated with the collection of a single bile sample upon the appearance of BSP in bile (Hoyt,

1984). In those experiments, a reduced rate of transport of BSP in bile could have been responsible for lower concentrations of BSP in the samples. The determination of biliary BSP concentration during maximal transport indicates the maximum ability to concentrate BSP in bile.

MATERIALS and METHODS

Polyethylene tubing (PE 10, PE 50) was puchased from Clay Adams, Parsippany, NJ. Pentobarbital sodium was obtained from City Chemical Corp., NY. Sulfobromophthalein (BSP) was purchased from Sigma Chemical Co., St. Louis, MO. Sodium phosphate was from J.T. Baker Chemical Co., Phillipsburg, NJ. Sodium toluene-p-sulfonate was obtained from Eastman Kodak Co., Rochester NY.

Surgery was performed 48 h after treatment with 20 mg/kg BCNU or corn oil. In addition to cannulation of the bile duct, the right femoral artery and vein were cannulated with PE 50 tubing. The venous cannula was connected to an infusion pump (Gilson Minipuls 2) for constant infusion of BSP in 0.9% NaCl at a rate of 2.5 mg/min/kg and 38 µl/min. Bile was collected in 15 min intervals from the time that BSP first appeared in bile. A blood sample was taken from the arterial cannula at the end of each interval.

The concentration of BSP in bile was determined by the difference in absorbance at 580 nm after alkalinization and acidification of samples (Whelan *et al.*, 1970). Twenty µl of bile was added to 3.0 ml of 0.9% NaCl and a drop of 0.1 N NaOH or 0.1 N HCl was

added. The extinction coefficients of BSP and its glutathione conjugate (BSP-SG) are nearly equal (Whelan et al., 1970), so the total concentration of BSP was measured on a Coleman Junior II model 6120 spectrophotometer.

Plasma BSP was measured by the procedure of Richterich (1969). Arterial blood samples (150 µl) were taken in heparinized capillary tubes and were centrifuged 2000 rpm x 20 min using an Intrenational Centrifuges hematocrit rotor (#927). Plasma was added to 500 µl of alkaline buffer (229 mM dibasic sodium phosphate, 10.2 mM tribasic sodium phosphate, 10.2 mM sodium toluene-p-sulfonate, pH=10.6) and the absorbance at 578 nm was measured with a Bausch and Lomb Spectronic 600 spectrophotometer. One hundred µl of 2.0 M monobasic sodium phosphate was added to acidify the mixture and give the blank value. The concentration of BSP in bile and plasma was calculated as the following:

$$[BSP] = A_{(sample)}/A_{(standard)} \times \text{dilution factors} \times \text{concentration of standard}$$

where A is the difference in absorbance between alkalinized and acidified samples.

The establishment of maximal transport was

assumed to occur when a plateau in the excretion rate of BSP was observed despite increasing plasma concentrations (Klaassen et al., 1969). This occurred in every individual rat during the final 3 intervals. The values of BSP excretion rate and biliary [BSP] were averaged for the last 3 intervals to estimate maximal transport.

RESULTS

In this experiment cholestasis was not evident 48 h after 20 mg/kg BCNU administration (fig. 2). It was observed that the concentration of BSP in plasma increased in both experimental groups throughout the infusion (fig. 3), and this was the case in each individual rat tested (data not shown). There was a tendency toward higher plasma concentrations in the BCNU-treated group but the differences were not significant. The data in fig. 4 indicate that the BSP excretion rate reached a maximal value despite the rising concentration in plasma in both groups of rats. This implies that the excretory system was saturated (Klassen et al., 1969). The BSP excretion rate in the treated animals was depressed in all 4 intervals. This effect was due to an inability of the treated rats to concentrate BSP in bile (fig. 5). Values for maximal BSP excretion rate (BSP Tm) and biliary concentration of BSP during maximal excretion, which were estimated by the average of those values during the last 3 intervals, are presented in table 1. The BSP Tm was decreased 45% ($P < 0.01$) and the maximal biliary concentration of BSP was decreased 47% ($P < 0.005$). The inability to concentrate BSP accounts completely for the excretory deficit.

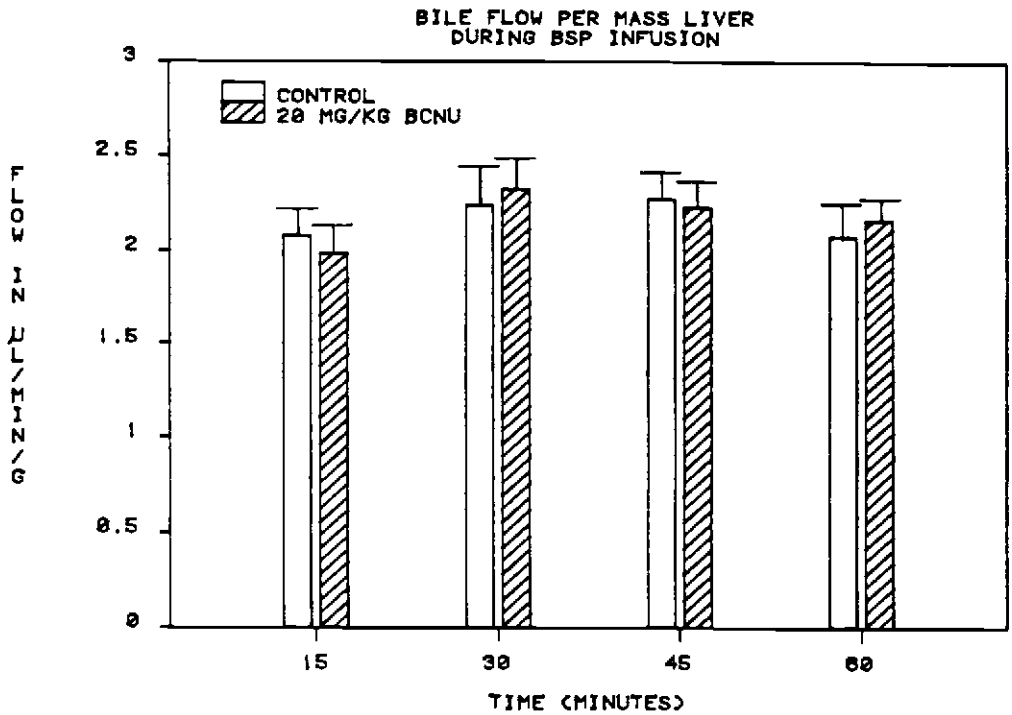


Figure 2. Bile flow in control and BCNU-treated rats during constant i.v. infusion of BSP (2.5 mg/min/kg). Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) 48 h earlier. The mean and standard error for 6 rats/group are indicated.

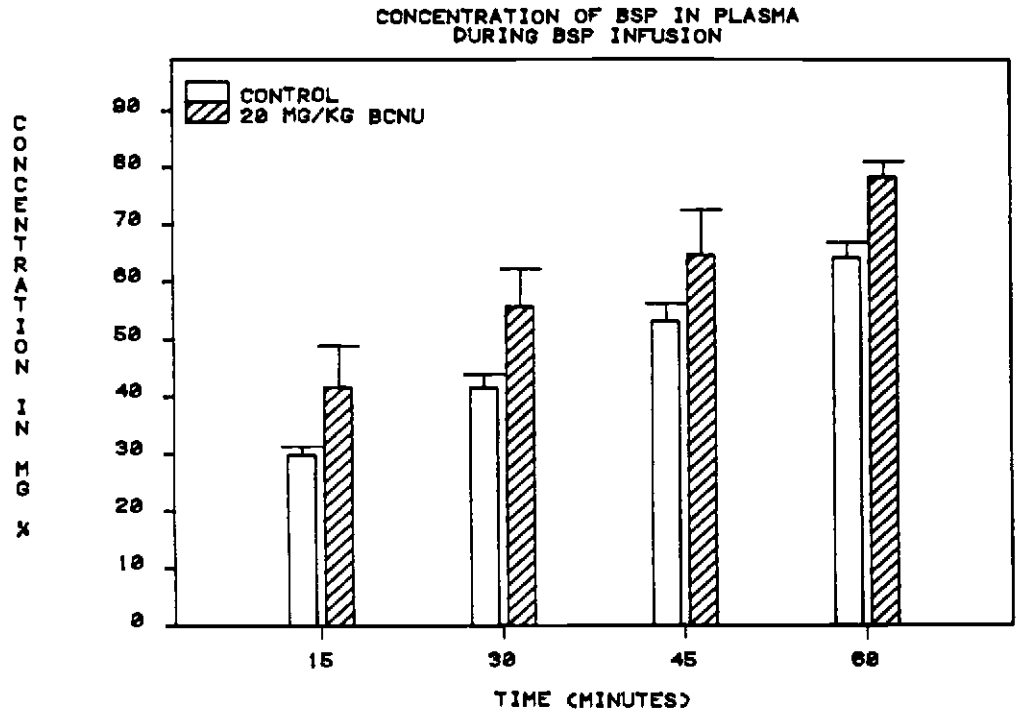


Figure 3. Concentration of BSP in the plasma of control and BCNU-treated rats during constant i.v. infusion of BSP (2.5 mg/min/kg). Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) 48 h earlier. The mean and standard error for 6 rats/group are indicated.

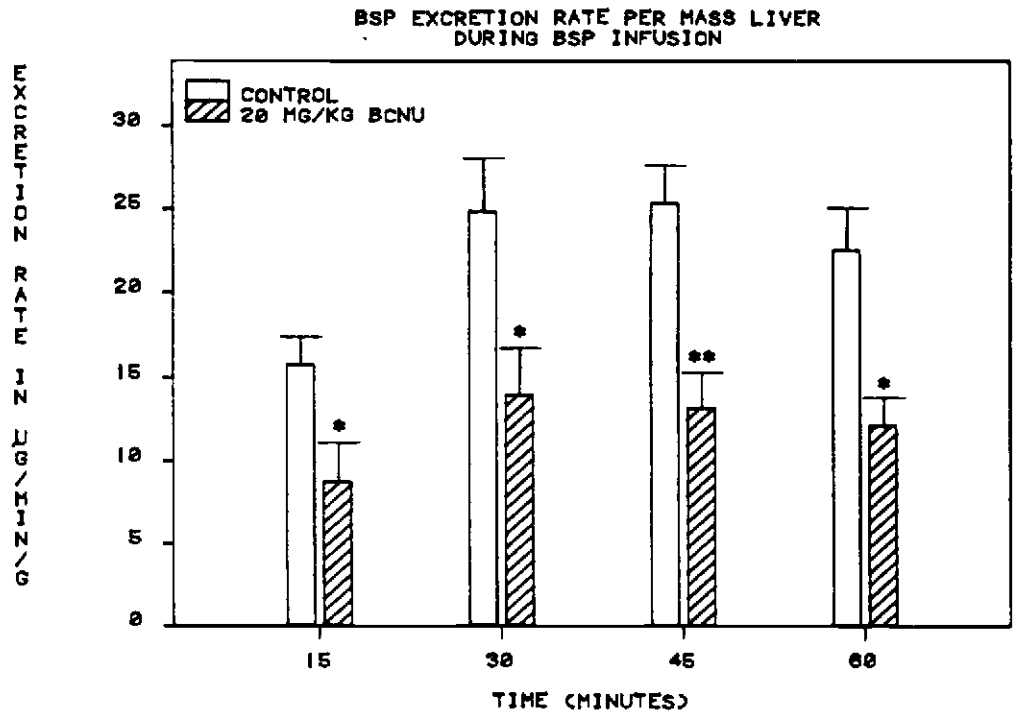


Figure 4. BSP excretion rate in control and BCNU-treated rats during constant i.v. infusion of BSP (2.5 mg/min/kg). Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) 48 h earlier. The mean and standard error for 6 rats/group are indicated (*, $P < 0.05$; **, $P < 0.005$).

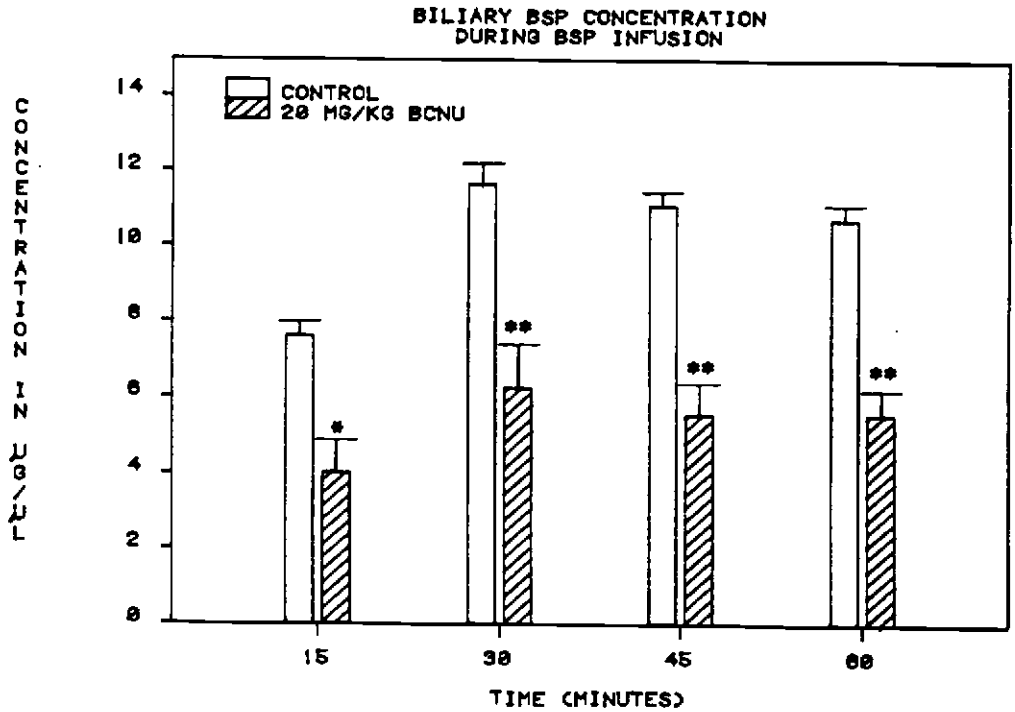


Figure 5. Biliary BSP concentration in control and BCNU-treated rats during constant i.v. infusion of BSP (2.5 mg/min/kg). Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) 48 h earlier. The mean and standard error for 6 rats/group are indicated (*, $P < 0.01$; ** $P < 0.005$).

Table 1. The Effect of BCNU (20 mg/kg) on the Maximal Excretion of BSP During Constant I.V. Infusion[a]

Group	N	BSP Tm (µg/min/g)	Maximal [BSP] in Bile (µg/µl)
Control	6	24.70 ± 2.20	11.15 ± 0.344
BCNU	6	13.47 ± 2.24[b]	5.86 ± 0.844[c]

a) values are mean ± standard error
b) $P < 0.01$
c) $P < 0.005$

DISCUSSION

BCNU compromised the ability of rats to maximally concentrate BSP in bile. In this experimental setting the possible effect of an altered transit time was eliminated. For example, if it took more time for BSP to enter bile, the concentration of BSP would be reduced in an initial sample, and with time, rats might produce a sample with a normal concentration of BSP. The results of this experiment indicated that the ability to concentrate BSP was inhibited.

One explanation of the inhibition of BSP Tm is that canalicular excretion of BSP and BSP-SG is the site of action of BCNU. This is reasonable because uptake from plasma is not rate-limiting in normal rats. If the rate-limiting step in excretion in BCNU-treated rats is the canalicular step, then the effect of BCNU was to induce canalicular selectivity for bile salts because bile salt excretion was not inhibited (Hoyt, 1984; see also chapter V). If canalicular transport occurs by a carrier-mediated process (O'Maille et al., 1966; Inoue et al., 1984b; Meier et al., 1984a) that is shared by BSP and taurocholate, BCNU may alter the ability of such a structure to transport BSP and/or BSP-SG, leaving bile salt transport unaffected. For example, the affinity of the carrier for BSP relative to bile salts may have been

decreased.

The reduction of BSP Tm means that the maximum ability to concentrate BSP was reduced. It is known that BSP and its non-metabolized analog, dibromosulfophthalein (DBSP) undergo countertransport. That is, BSP and DBSP can enter the liver from bile (Peterson and Fujimoto, 1973; Vonk et al., 1979). The reduced Tm could be due to increased countertransport. One possibility is that the canalicular membrane was altered so that permeability to BSP was increased. BSP then might distribute from bile to liver in response to the same driving force(s) that cause uptake from plasma. The change would have to be selective for BSP relative to bile salts, however. The decrease in excretion cannot be attributed to increased liver size which would dilute BSP intracellularly because liver weights were not altered by 20 mg/kg BCNU (see table 13 in the appendix).

It is unlikely that BSP leaked back into plasma by the paracellular route because bile salt excretion should be susceptible to such a shunt induced in treated animals. The tight junctions possess a barrier to anion flux (Bradley and Herz, 1978). BSP, a disulfonic acid, would be more ionized than bile salts, which are monocarboxylic acids. BSP is of higher molecular weight than bile salts and permeability varies inversely with

molecular weight (Smith and Boyer, 1982). Finally, agents that increase permeability between bile and plasma inhibit both bile salt and BSP excretion (Krell et al., 1982).

A possibility that was not investigated is that BCNU selectively inhibits organic anion uptake from plasma. This seems relatively unlikely because it is not a rate-limiting step in normal rats. The possibility that the uptake step was inhibited was unlikely considering also the effects of BCNU on bilirubin in plasma. Thompson and Larson (1969) showed that BCNU caused conjugated bilirubin to increase at the expense of free bilirubin 3 d after a 30 mg/kg dosage. This implied that uptake was normal, allowing bilirubin to enter hepatocytes and be conjugated. In fact, a block of canalicular excretion would be a good explanation for this result. It should be noted that hyperbilirubinemia did not occur until 2 weeks after 20 mg/kg BCNU implying that BSP excretion was not reduced by competition with high levels of bilirubin in the present study (Thompson and Larson, 1969).

At the high plasma concentrations of BSP in these experiments, diffusion is probably the major component of uptake (Schwenk et al., 1976). It is still possible that BCNU decreased this uptake, perhaps by a change in membrane permeability, thickness or surface

area. It could explain the selective action of BCNU on organic anion excretion vs. bile salt excretion because bile salts are taken up by a different sodium-dependent mechanism (Anwer and Hegner, 1978; Laperche et al., 1981; VanDyke et al., 1982; Meier et al., 1984a).

It is possible that BCNU induced an increase in endogenous substances that might compete for excretion such as glutathione species (see chapter IV). Both oxidized and reduced glutathione were elevated but returned to normal values at a time when BSP excretion was inhibited in previous experiments (Hoyt, 1984; chapter IV). There was no evidence for accumulation of toxic, hydrophobic bile salts that might inhibit BSP transport (chapter VIII).

Although BCNU causes anorexia, there was no evidence that fasting affected BSP excretion. Data on the effect of fasting are presented in table 12 in the appendix.

Because BSP is efficiently extracted from blood by the liver, its excretion might be affected by changes in hepatic blood flow (Pang, 1980). Data presented in the appendix suggest that hepatic blood flow was normal. The technique applied to measure blood flow, LASER Doppler Velocimetry, only assays to a small depth into tissue, so that given the acinar heterogeneity in enzyme systems important in BSP excretion (Redick et al.,

1982), redistirbution of flow might still account for decreased excretion.

Under the conditions of maximum transport, the sites in the liver that make excretion a rate-limited process should be saturated (Wheeler et al., 1960; Klaassen et al., 1969). Since uptake from plasma is not a rate-limiting step, saturation is an intracellular phenomenon composed of binding to protein and organelles, metabolism and passage across the canalicular membrane (O'Maille et al., 1966; Whelan et al., 1970; Kaplowitz, 1980; Boyer et al., 1983). BCNU might have altered the metabolism of BSP which might decrease excretion. However, experiments presented in chapters II and III demonstrated that the excretion of non-metabolized ICG was inhibited and that cytosolic GSH-S-transferase activity was not reduced by BCNU-pretreatment.

BCNU could decrease excretion by inducing an increase in intracellular binding capacity and/or affinity. This possibility could not be evaluated by a determination of hepatic BSP content because a block of canalicular excretion would also explain an increase in intracellular dye. One would have to determine binding parameters in vitro after cell fractionation. BCNU does not decrease microsomal or cytosolic protein content (Stolzenbach, 1984; chapter III), and GSH-S-transferases

already represent about 10% of cytosolic protein (Kaplowitz, 1980). The extreme plasma levels of BSP in the later phases of the constant infusion (fig.3) would seem to imply that the intracellular sites are filled.

In summary, the inability to concentrate BSP accounts for decreased excretion. It appeared that BSP excretion was inhibited by an action of BCNU at the canalicular side of the cell. An effect on uptake from plasma, though unlikely, was not ruled out.

II. The Effect of BCNU on the Excretion of Indocyanine Green (ICG)

INTRODUCTION

ICG is another xenobiotic organic anion that is excreted in bile (Gregus and Klaassen, 1982). Its structure is shown in figure 1. ICG and BSP compete for various steps in excretion but ICG is not metabolized before excretion (Forker, 1977; Gregus and Klaassen, 1982). In fact, this was the reason for analyzing ICG excretion under the influence of BCNU. It was hypothesized that BCNU might inhibit BSP excretion by preventing the GSH-S-transferase reaction. If this was the only site of action of BCNU in inhibiting organic anion excretion, then the excretion of ICG should be unimpaired. If cholestasis was evident, the rats should still concentrate ICG in bile normally. In contrast, if rats were unable to concentrate ICG after BCNU pretreatment, then the action of BCNU could not have been confined to GSH-S-transferase.

MATERIALS and METHODS

Polyethylene tubing (PE 10, PE 50) was puchased from Clay Adams, Parsippany, NJ. Pentobarbital sodium was obtained from City Chemical Corp., NY. Indocyanine green (ICG) was purchased from Hynson, Westcott and Dunning Inc., Baltimore, MD. Bovine serum albumin was purchased from Calbiochem, San Diego, CA.

Rats were treated with 20 mg/kg BCNU in corn oil or corn oil alone (i.p., 1.0 ml/kg). At 0(controls), 24, 36, 48 h after treatment, rats were anaesthetized with pentobarbital sodium, i.p. The common bile duct was cannulated. After approximately 30 min ICG in 0.9% NaCl was injected into the inferior vena cava (31 µmol/kg, 1.0 ml/kg). This dosage produces maximal biliary excretion (Gregus and Klaassen, 1982). Upon appearance of ICG in bile, collection was started in a new container. At the end of the experiment the liver was removed and weighed.

The concentration of ICG in bile was determined spectrophotometrically at 805 nm on an Aminco DW 2A spectrophotometer (Gregus and Klaassen, 1982). Standard ICG and bile samples were diluted in 5.0% bovine serum albumin. The dilution of bile was generally 20 µl plus 5.0 ml solvent. The excretion rate was calculated as

the product of bile flow and concentration.

RESULTS

Rats pretreated with 20 mg/kg BCNU were cholestatic by 36 h after treatment (fig. 6) and were compromised in their ability to excrete indocyanine green (ICG, fig. 7). The ICG excretion rate was depressed significantly from 51.99 $\pm$ 4.83 nmol/min/kg in control to 14.23 $\pm$ 2.08 nmol/min/kg at 36 h and to 7.93 $\pm$ 6.18 nmol/min/kg at 48 h (mean $\pm$ standard error), $P < 0.001$ in each case (fig. 7). The large standard error at 48 h indicates that some rats were excreting essentially no ICG at this time. Furthermore, the inhibition of ICG excretion was not merely secondary to cholestasis, but was characterized by an inability to concentrate the material in bile (fig. 8). The biliary concentration of ICG after a single i.v. injection was reduced from 1.57 $\pm$ 0.079 µM in control to 0.876 $\pm$ 0.212 µM at 36 h and to 0.310 $\pm$ 0.138 µM at 48 h, $P < 0.005$ and 0.001 respectively. These results should be compared with with the effect of 20 mg/kg BCNU on the excretion of a single i.v. injection of 50 mg/kg BSP (Hoyt, 1984). Those results were calculated only on the basis of body weight and this is the reason that the excretion of ICG is presented on the basis of body weight. The results were the same when calculated on the basis of liver weight (data not shown). The action of BCNU on the excretion

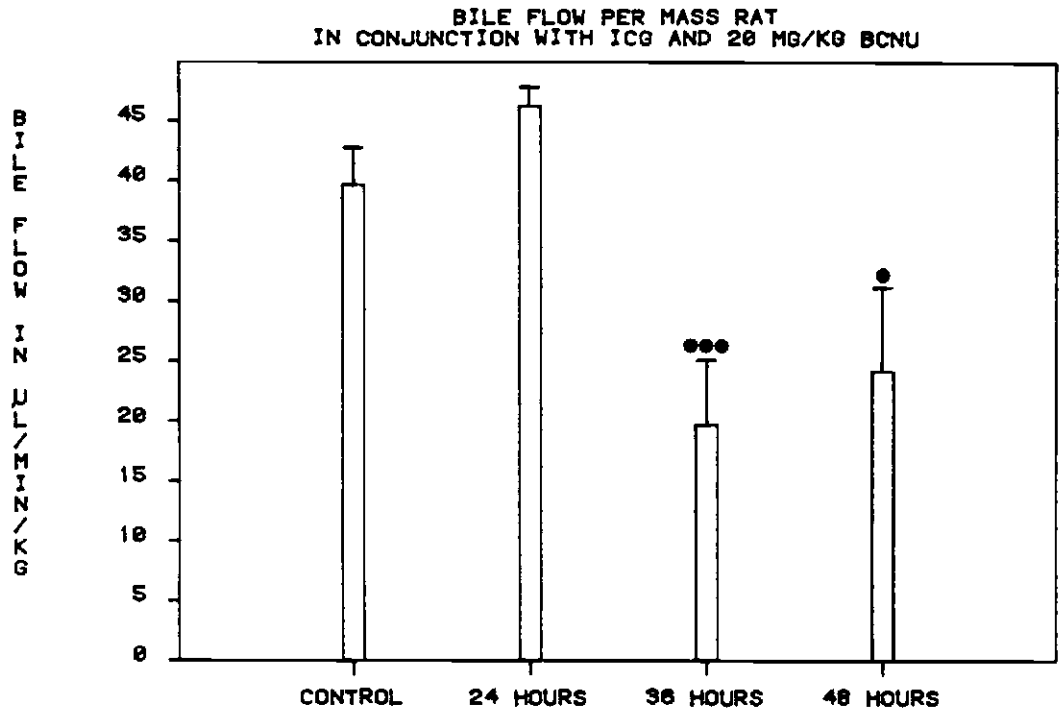


Figure 6. Bile flow in control and BCNU-treated rats. Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) and bile was collected under pentobarbital anaesthesia at the indicated time after treatment. After 30 min of bile collection, a single injection of ICG (31 µmol/kg) was given i.v. The mean and standard error are indicated (●, $P < 0.05$; ●●●, $P < 0.01$).

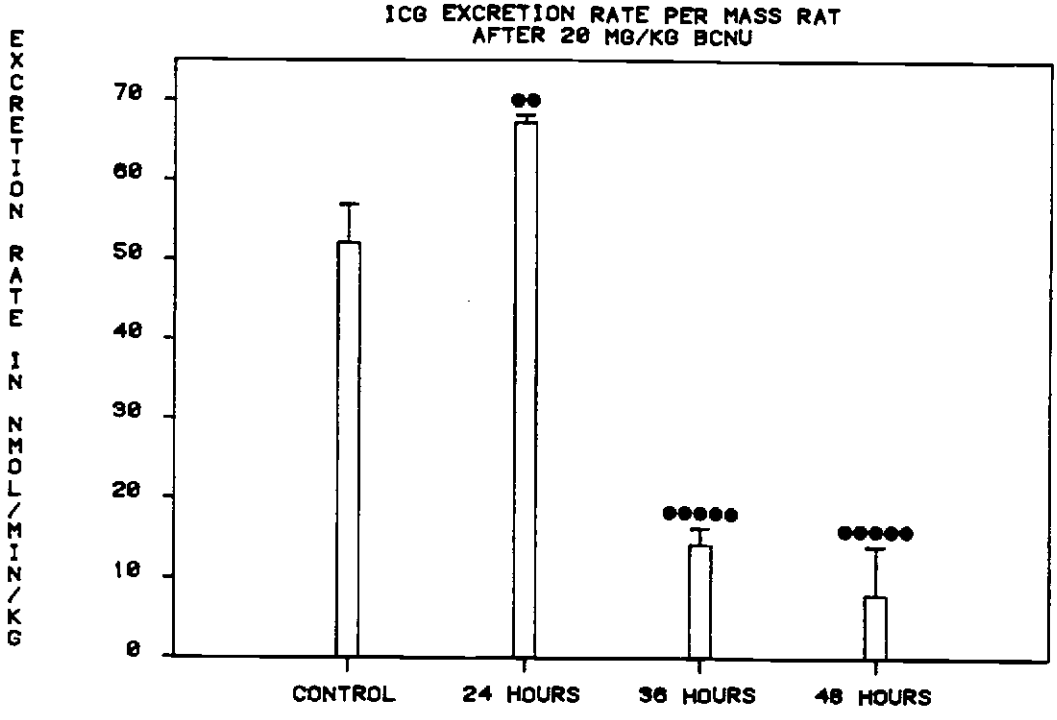


Figure 7. ICG excretion rate in control and BCNU-treated rats. Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) and bile was collected under pentobarbital anaesthesia at the indicated time after treatment. After 30 min of bile collection, a single injection of ICG (31 µmol/kg) was given i.v. The mean and standard error are indicated (●●, $P < 0.25$; ●●●●●, $P < 0.001$).

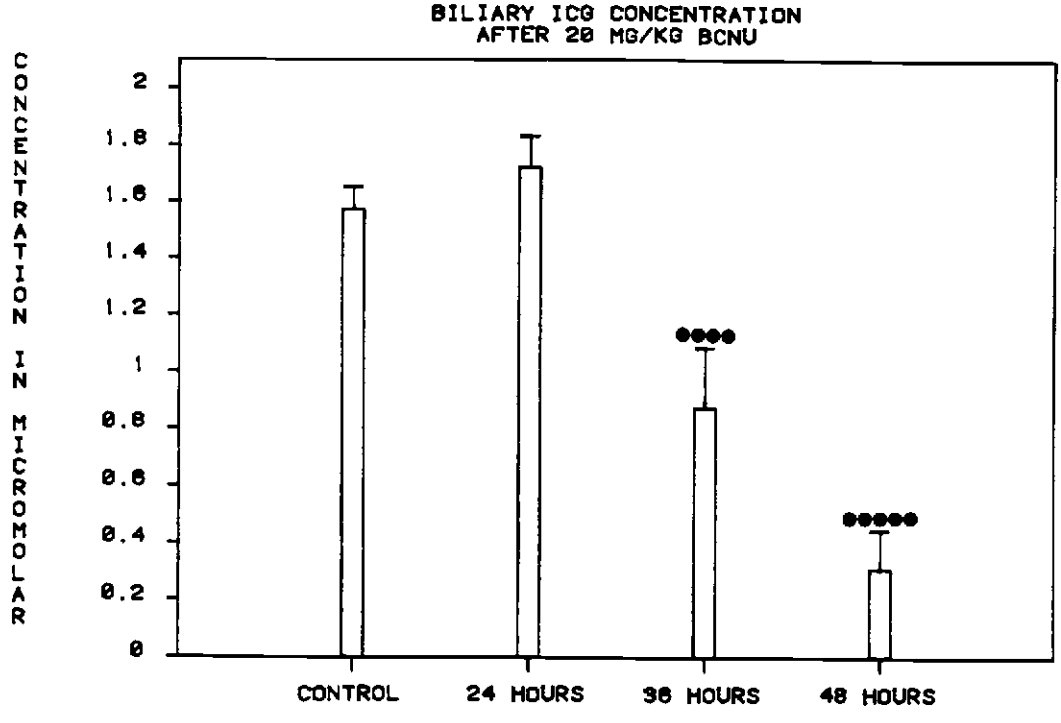


Figure 8. Biliary ICG concentration in control and BCNU-treated rats. Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) and bile was collected under pentobarbital anaesthesia at the indicated time after treatment. After 30 min of bile collection, a single injection of ICG (31 µmol/kg) was given i.v. The mean and standard error are indicated (●●●●, $P < 0.005$; ●●●●●, $P < 0.001$).

of ICG was similar to the action of BCNU on the biliary excretion of a single i.v. injection of BSP which was seen in previous studies (Hoyt, 1984).

DISCUSSION

The effect of 20 mg/kg BCNU on the excretion of a single i.v. injection of ICG closely resembled its action on the excretion of a single i.v. injection of BSP (Hoyt, 1984). Cholestasis was produced over about the same time course as in that previous study. Most importantly, the rats were unable to concentrate ICG to a normal extent.

The excretion of ICG is similar to the excretion of BSP in several respects. Both substances are taken up from plasma and they compete for uptake with each other (Forker, 1977). Both bind to GSH-S-transferases intracellularly with similar affinity (Takikawa *et al.*, 1985). However, ICG is not metabolized prior to excretion (Gregus and Klaassen, 1982).

These results indicate that the action of BCNU cannot be confined to inhibition of BSP metabolism if this were to occur at all. In fact, the results of *in vitro* experiments on GSH-S-transferase presented in chapter III indicated that the enzyme activity was not impaired.

In summary, the action of BCNU to inhibit xenobiotic organic anion excretion was not confined to BSP. Rats were also unable to concentrate the non-metabolized dye, ICG, in bile. The action does not

appear to involve GSH-S-transferase activity, suggesting that canalicular processes were affected.

III. The Effect of BCNU on Glutathione-S-Transferase Activity *In Vitro*

INTRODUCTION

The experiments of chapter I indicated that BSP excretion was probably blocked at a point following uptake from plasma. The results of the ICG experiment in chapter II demonstrated that the action of BCNU was not confined to GSH-S-transferase. To determine the actual relevance of this enzyme activity as a site of action, it was important to determine the effect of BCNU-pretreatment on conjugation activity *in vitro*.

GSH-S-transferases are a group of enzymes that catalyze the reaction of gamma-glutamylcysteinylglycine, reduced glutathione or GSH, with electrophilic substrates (Kaplowitz, 1980). There are about 8 isozymes. Each is a dimer of subunits of about 25 to 30 kilodaltons. The different forms result from different combinations of 6 monomers of slightly different molecular mass (Jakoby *et al.*, 1984). A molecule of GSH-S-transferase possesses a binding site for GSH, one for substrate to be conjugated, and a non-substrate binding site (i.e. bilirubin and ICG bind here, Kaplowitz *et al.*, 1980). The environment of the active site is such that the pKa of the cysteine sulfhydryl is

lowered allowing increased dissociation to produce the nucleophilic conjugate base (Kaplowitz, 1980). The sulfur then attacks electrophilic sites causing substitution (for example when a good leaving group like halogen is present as in the case of BSP, see fig. 1) or addition to unsaturated bonds or epoxides (Kaplowitz, 1980).

The isozymic forms differ in their ability to act on different substrates (Habig et al., 1974). Enzyme activity is inducible with agents such as phenobarbital, 3-methylcholanthrene, pregnenalone-16 alpha-carbonitrile and trans-stilbene oxide (Klaassen, 1975; Kaplowitz, 1980; DePierre et al., 1984). The soluble forms of GSH-S-transferase cannot metabolize substrates bound to phospholipids but act only on substrate as it is released into the aqueous phase (Boyer et al., 1983). As mentioned, the transferases may have a storage function provided by the non-substrate binding site and they may facilitate uptake of material from plasma by decreasing free intracellular concentration (Levi et al., 1969; Kaplowitz, 1980), but the exact relation of transferases to hepatic excretion (other than metabolism) is not clear.

The enzymes are primarily cytosolic in various organs such as lung, liver and kidney (DePierre and Morgenstern, 1983; Dawson et al., 1984). These enzymes

constitute about 10% of cytosolic protein in the liver (Kaplowitz et al., 1980). A form activated by N-ethyl maleimide was isolated only from liver microsomes Morgenstern et al., 1980). The distribution of transferases within the liver is not homogenous, being concentrated in centrilobular areas (Redick et al., 1982).

In contrast to the cytosolic activity, microsomal transferase was not induced by phenobarbital, 3-methylcholanthrene and trans-stilbene oxide (Frieberg et al., 1979; Morgenstern et al., 1980). The microsomal form is mainly responsible for the conjugation of hexachloro-1,3-butadiene, a lipophilic molecule (Wolf et al., 1983). Although the activity of this form is low compared with the cytosolic forms (about 10%), this might be an important consideration for BSP metabolism and for the detoxification and metabolic activation of lipophilic substances (Morgenstern et al., 1980; Boyer et al., 1983; Wolf et al., 1983).

In the following experiments, the GSH-S-transferase activity of a hepatocytosolic preparation towards BSP and 1-chloro-2,4-dinitrobenzene (CDNB), isolated from BCNU-pretreated rats, is described. Cytosol was isolated 24 and 48 h after pretreatment with 20 mg/kg BCNU. These represent times

before and after the inhibition of BSP and ICG excretion (Hoyt, 1984; see chapter II). There were 2 purposes for determining GSH-S-transferase activity. One was to determine whether BCNU affected the ability of the liver to metabolize BSP, since metabolites not associated with amino acids (i.e. not conjugated with GSH) were found in the bile of treated rats (Hoyt, 1984). Secondly, although the inhibition of ICG excretion implied that metabolism by itself was not sufficient to explain the decreased BSP excretion, it was of interest to see whether such an action might have contributed to the excretory deficit in BCNU-treated rats.

MATERIALS and METHODS

Sulfobromophthalein (BSP), and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma Chemical Co., St. Louis, MO. Potassium phosphate was from J.T. Baker Chemical Co., Phillipsburg, NJ. Reduced glutathione was from Boehringer Mannheim Gmbh, West Germany.

The procedure for the assay of glutathione (GSH)-S-transferase activity was that of Vessey and Zakim (1981). Rats were treated with 20 mg/kg BCNU or corn oil and the following procedures were carried out 24 and 48 h later. Rats were killed by cervical dislocation followed by thoracotomy and the liver was removed and weighed. The liver was homogenized in a glass/teflon homogenizer in 4 volumes of 1.15% KCl. The homogenate was centrifuged 20 min x 12000 x g. The supernatant was centrifuged 20 min x 20000 x g. This supernatant was the centrifuged 1.0 h x 105000 x g. The final supernatant (cytosolic fraction) was filtered under vacuum through 70 ml of DEAE cellulose equilibrated with 0.1 M potassium phosphate, pH=7.5. The protein content of the filtrate was determined at this point (Lowry et al., 1951), and GSH -S- transferase activity toward CDNB and BSP was measured.

The conjugation of BSP with GSH was carried out in a final reaction volume of 1.0 ml containing 5.0 mM GSH in 0.1 M potassium phosphate at pH=7.5, 0.1 M potassium phosphate at pH=7.5, and varying amounts of BSP in the buffer and the cytosolic supernatant. The blanks contained buffer in place of supernatant. The reaction of BSP with GSH was monitored at 330 nm on an Aminco DW 2A spectrophotometer. The rate was taken from the linear increase in absorbance after initiation with supernatant. An extinction coefficient of 4.5 mM^{-1} cm^{-1} for the BSP conjugate was used (Habig et al., 1974).

CDNB conjugation with GSH was also carried out in a reaction volume of 1.0 ml. The reaction contained 0.1 M potassium phosphate at pH=6.5, 5.0 mM GSH in potassium phosphate at pH=6.5, varying amounts of CDNB in 95% ethanol and supernatant. The reaction was measured by the linear increase in the absorbance at 340 nm. An extinction coefficient of 9.6 mM^{-1} cm^{-1} was used in the calculations (Habig et al., 1974).

In some control reactions, BCNU in 95% ethanol was added directly to the reaction system immediately before initiation with supernatant. In no case was the concentration of ethanol greater than 4%.

Data Analysis. The values of maximum velocity (Vmax) and apparent Km were estimated by double reciprocal plotting. The double reciprocal relations

were established by least squares regression (Neter and Wasserman, 1974). Also, the averages of velocities at substrate concentrations near the Km and at the highest substrate concentrations used were compared by the Student's t-test or t'-test.

RESULTS

The average values of kinetic parameters for the *in vitro* conjugation of BSP and 1-chloro-2,4-dinitrobenzene (CDNB) are shown in table 2. These values were obtained by linear regression of reciprocal data. Comparisons of control and BCNU-treated rats were made at substrate concentrations approximating the apparent Km in the controls and at substrate concentrations that produced a reaction rate approaching the control Vmax (i.e. the highest concentration tested).

The results are summarized in tables 2 and 3. The double reciprocal relations of CDNB conjugation rate vs. CDNB concentration for the treated and control rats appear in figure 9. The estimated Vmax and Km are in table 2. BCNU-pretreatment caused a 40% reduction of Vmax at 24 h ($P<0.001$) but Vmax was normal at 48 h. The apparent Km's were not significantly altered by BCNU since the mean values for treated or control rats were contained in the confidence intervals of control or treated rats respectively.

At the highest CDNB concentration tested, 2.0 mM, conjugation was depressed by 47% 24 h after treatment with 20 mg/kg BCNU ($P < 0.05$, table 3). The reaction velocity normalized by 48 h. A similar pattern

Table 2. Estimated Maximal Velocity and Apparent Km for Conjugation of BSP and CDNB[a]

Group	Time	BSP Km	BSP Vmax	CDNB Km	CDNB Vmax
Control	----	0.01 (.006,.016)	5.96 (4.97,7.45)	0.27 (.161,.477)	765 (551,1155)
20 mg/kg BCNU	24 h	0.01 (.004,.022)	5.86 (4.18,9.83)	0.32 (.189,.755)	448 (318,755)
	48 h	0.02 (.009,.086)	9.17[b] (5.48,28.0)	0.19 (.130,.279)	713 (593,892)

a) rats received corn oil or BCNU (20 mg/kg, i.p.); cytosol was isolated at the indicated times after treatment, Km and Vmax were estimated by linear regression of double reciprocal plots; Km is in mM, Vmax is nmol conjugate formed/min/mg protein; for Km the 95% confidence interval obtained by calculation from maximum and minimum slopes and y-intercepts from double reciprocal plots are in parentheses; for Vmax the 95% confidence interval is in parentheses and differences were established by a t-test of the y-intercepts in the double reciprocal plots.

b) $P < 0.001$

Table 3. The Effect of BCNU-Pretreatment on the *In Vitro* Conjugation of BSP and CDNB

Group	N	Reaction Rate[a] 0.25 mM CDNB	2.0 mM CDNB	0.012 mM BSP	0.060 mM BSP
Control[b]	4	361 ± 48.1	699 ± 97.6	3.64 ± .4699	5.55 ± .523
BCNU[c]					
24 h	3	204 ± 25.0	373 ± 42.8	3.58 ± .2508	4.78 ± .4330
48 h	3	393 ± 45.4	698 ± 52.4	3.48 ± .3414	6.50 ± .6997

a) values are mean ± standard error; units are nmol conjugate formed/min/mg protein
b) 20 mg/kg BCNU in corn oil was given i.p. 24 or 48 h previously
c) $P < .05$

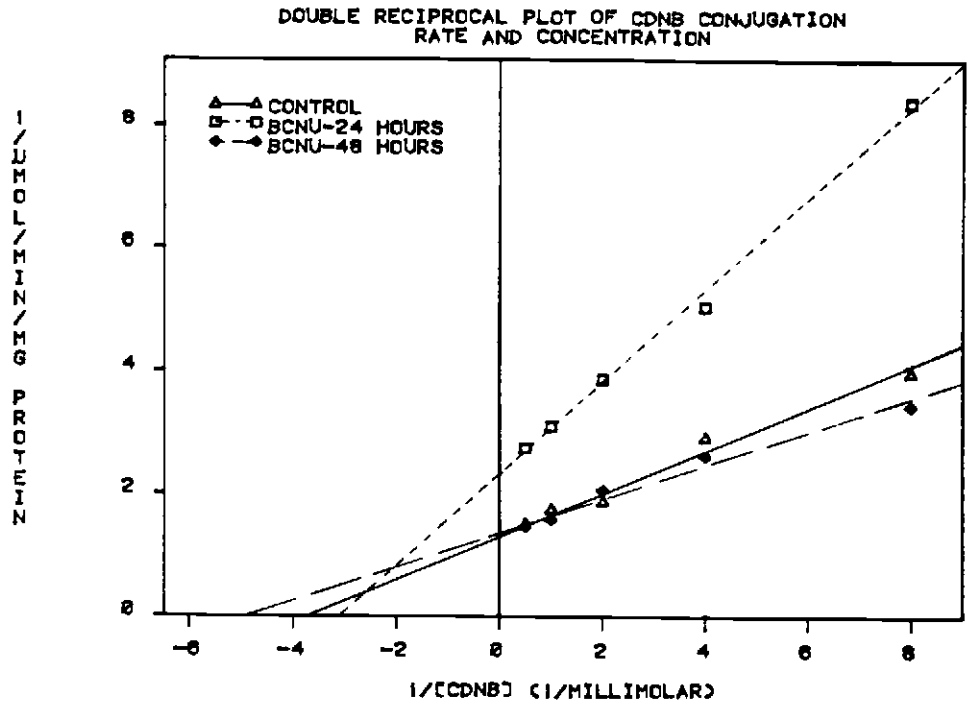


Figure 9. Double reciprocal relationship between the rate of formation of CDNB conjugate and [CDNB] mediated by the 105,000 x g supernatant from the livers of control and BCNU-treated rats. Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) and cytosol was isolated at the indicated time after treatment. The estimated kinetic parameters appear in table 2. Data points are the means of 3-4 rats, lines were established by least-squares regression of all data in the groups.

with time after treatment with BCNU was seen when the rate of reaction was measured at 0.25 mM, a concentration close to the estimated Km in controls (0.28 mM). The rate was 57% of control at 24 h ($P < 0.05$) and normalized 48 h after treatment. BCNU exerted a direct inhibitory effect on CDNB conjugation when added *in vitro*. The apparent Km was increased from 0.33 mM to 0.44 mM and the Vmax was reduced from 0.855 to 0.760 µmol/min/mg in the presence of 10 mM BCNU (fig. 10).

The double reciprocal relationship between BSP conjugation rate and BSP concentration for the cytosol of control and BCNU-pretreated rats curves upward at low substrate concentrations (fig. 11). This is in agreement with the results of Vessey and Zakim (1981) for the control reaction. Excluding the lowest substrate concentration allowed linear regression analysis of the remaining 3 to 5 points to be used (fig. 12). The estimated values of Vmax and Km appear in table 2. Vmax appeared to be increased at 48 h ($P<0.001$) and the apparent Km's were similar.

Because the Lineweaver-Burk plot was not linear, it may be more appropriate to compare the reaction rates at different BSP concentrations than to estimate kinetic parameters. The highest concentration of BSP tested was 0.060 mM. At this level there was no effect of

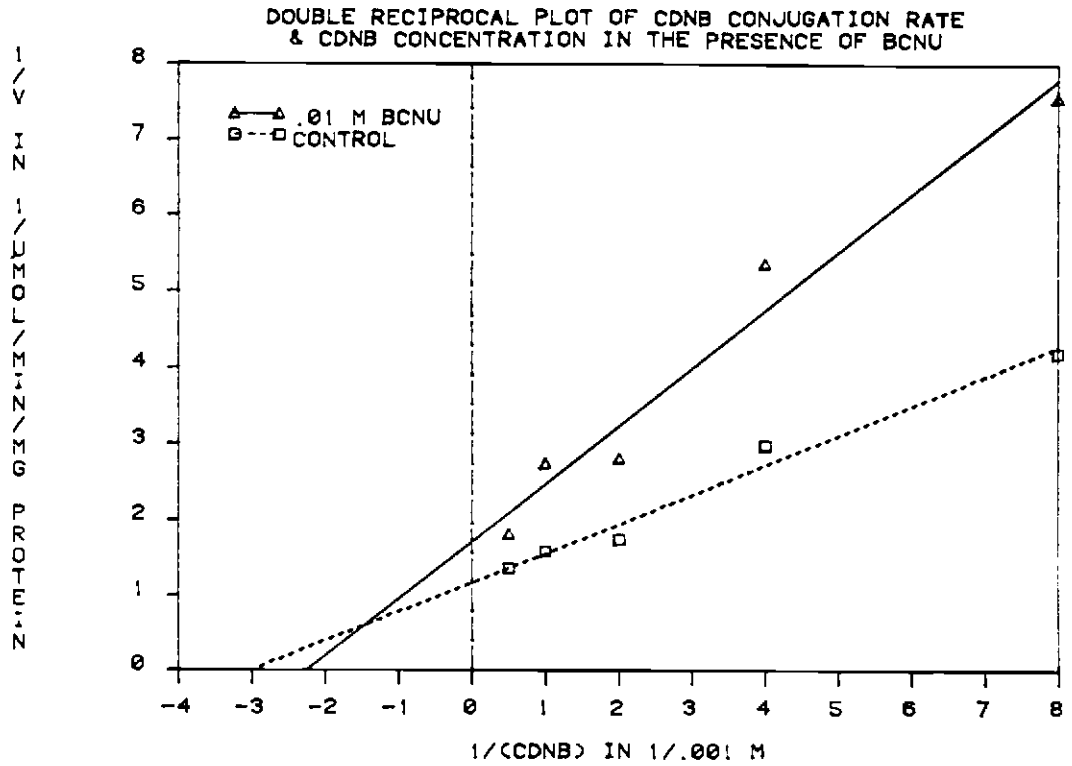


Figure 10. Double reciprocal relationship between the rate of formation of CDNB conjugate and [CDNB] mediated by the 105,000 x g supernatant from the liver of a control rat. 0.01 M BCNU was added to the reaction in vitro. Lines were established by least-squares regression.

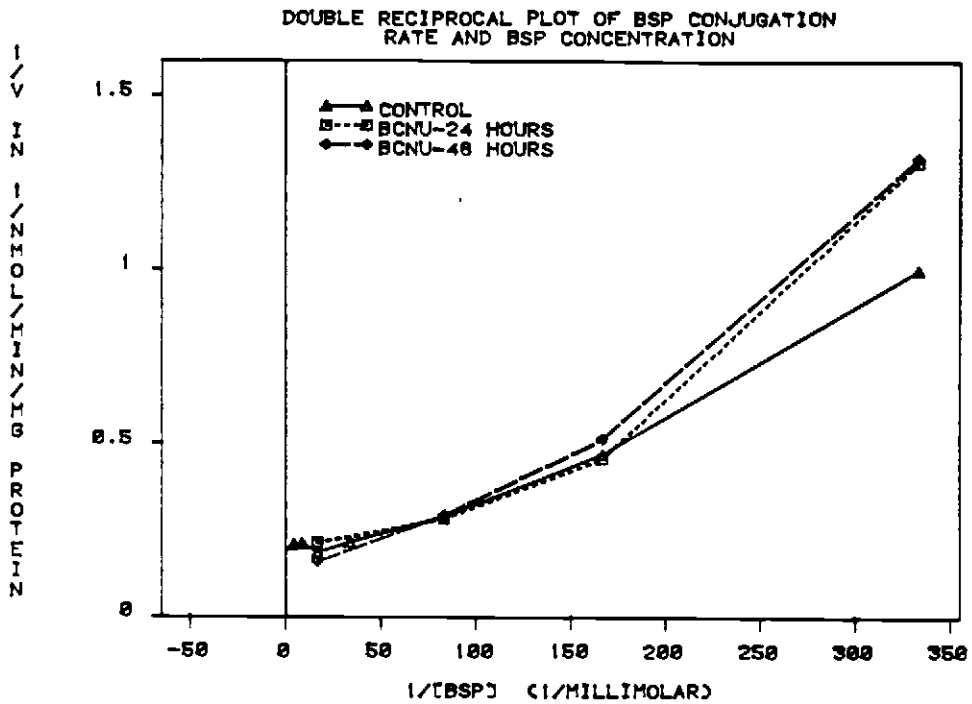


Figure 11. Double reciprocal relationship between the rate of formation of BSP conjugate and [BSP] mediated by the 105,000 x g supernatant from the livers of control and BCNU-treated rats. Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) and cytosol was isolated at the indicated time after treatment. Data points are the means of 3-4 rats.

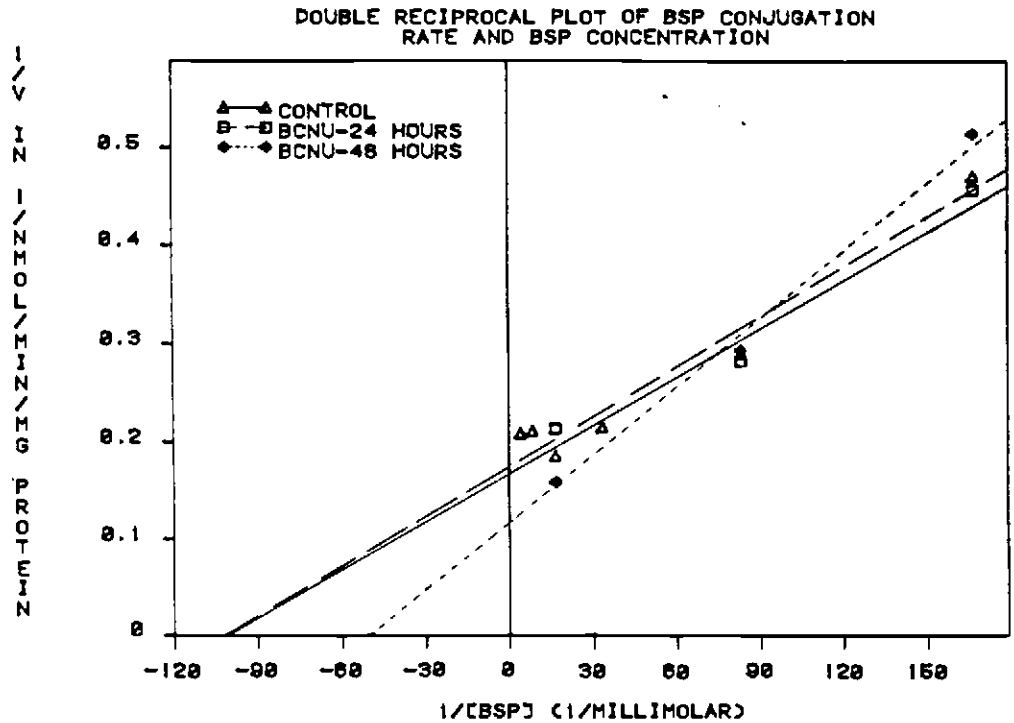


Figure 12. Double reciprocal relationship between the rate of formation of BSP conjugate and [BSP] mediated by the 105,000 x g supernatant from the livers of control and BCNU-treated rats, excluding the data at 3.0 mM BSP. Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg) and cytosol was isolated at the indicated time after treatment. Data points are the means of 3-4 rats. Lines were established by least-squares regression. The estimated kinetic parameters appear in table 2.

pretreatment with BCNU. Niether was there an effect when the reaction proceeded with 0.012 mM BSP (table 3). BCNU exerted a direct effect on the reaction when added *in vitro* (fig. 13). The double reciprocal relation without BCNU was linear for this control rat. The effect of BCNU was dose-related and there was a concave-up appearance not seen for CDNB conjugation. The reaction rate at 0.060 mM BSP was not reduced by 2.0 mM BCNU (5.5 $\pm$ .55 nmol/min/mg in control vs. 3.8 $\pm$ .41 nmol/min/mg, mean $\pm$ standard error) but was reduced to 2.39 $\pm$ .17 nmol/min/mg with 10 mM BCNU ($P<0.025$).

The cytosolic protein contents per gram liver for the experiment were (mean $\pm$ standard error) 61.25 $\pm$ 3.14 mg/g in control (n=4), 56.49 $\pm$ 9.16 mg/g rats preated with BCNU 24 h earlier (n=3), and 56.23 $\pm$ 6.81 mg/g in rats pretreated with BCNU 48 h earlier. The differences were not significant compared to control. Liver weights in this experiment were (mean $\pm$ standard error) 13.35 $\pm$ 1.26 g in control, 11.67 $\pm$ .33 g in rats that received BCNU 24 h earlier, and 10.6 $\pm$ .5 g 48 h after treatment with BCNU. These were not significantly different in the t-test.

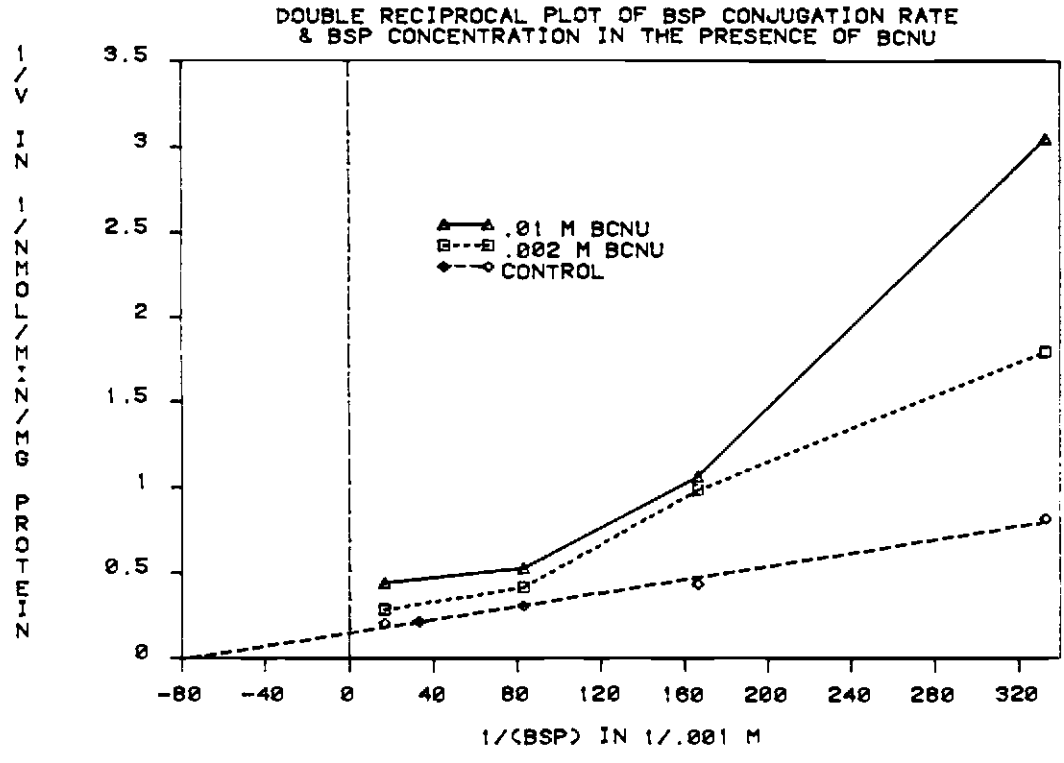


Figure 13. Double reciprocal relationship between the rate of formation of BSP conjugate and [BSP] mediated by the 105,000 x g supernatant from the liver of a control rat. 0.002 M and 0.01 M BCNU were added to the reaction in vitro.

DISCUSSION

The action of BCNU on GSH-S-transferase was investigated 24 and 48 h after pretreatment because the 20 mg/kg dosage was found to inhibit BSP excretion at 36 and 48 h but not 24 h (Hoyt, 1984). The object of this experiment was to see whether reduced metabolism contributed to the effect of BCNU on BSP excretion. It was shown before that BCNU increased the hepatic content of GSH at these times (Hoyt, 1984). Any effect on conjugation could not be due to depletion of this substrate.

The results indicated that pretreatment with BCNU had no significant effect on the activity of cytosolic GSH-S-transferase. BSP conjugation was not altered at any time except for a possible stimulation at 48 h (table 2). CDNB conjugation was reduced at 24 h but the activity was normal in rats given BCNU 48 h earlier. This does not correspond with the time course for the inhibition of BSP excretion. In addition, the hepatic content of cytosolic protein and liver weights were unaffected by the drug indicating that the overall reaction rate in the whole animal would be normal in treated rats.

Bile salts inhibit the conjugation of BSP *in vitro* (Vessey and Zakim, 1981). It is possible that bile

salts are accumulated *in vivo* in treated rats. During the isolation of cytosol, inhibitory substances would be diluted masking a relevant effect. The possibility that bile salts contributed to the decrease in BSP excretion is discussed in chapters I and V, and those results indicated that bile salts are not involved in the action of BCNU. Other unknown inhibitory substances may be present in BCNU-treated rats however.

The activity of the microsomal transferase was not investigated but this appears to be minimal compared with cytosolic activity (Wolf *et al.*, 1984). The microsomal form acts on lipophilic substrates (Wolf *et al.*, 1984). It may be important in BSP conjugation since the addition of phospholipid to soluble transferase systems inhibits the reaction although the microsomal metabolism of BSP does not appear to have been tested (Vessey and Zakim, 1981).

BCNU inhibited the conjugation of BSP and CDNB when added to a control system immediately before initiation. If a covalent interaction between the enzymes and a product of the breakdown of BCNU such as chloroethyl isocyanate occurred, inhibition might be pronounced with an increased incubation time in the presence of BCNU as was the case with GSSG reductase (Babson and Reed, 1978). It has been reported that BCNU is a substrate for hepatic GSH-S-transferase and this

could explain some of the *in vitro* inhibition (Hill *et al.*, 1976).

The maximum concentration of BCNU in plasma after an i.v. injection of 14 mg/kg in rats was about 65 μM (Levin *et al.*, 1979). Two and ten mM BCNU were used in the present *in vitro* studies. It may be that *in vitro* effects on transferase would not be observed at therapeutic concentrations of BCNU.

In summary, BCNU inhibited GSH-S-transferase *in vitro* at relatively high concentrations, but this was not relevant to the case involving *in vivo* exposure of rats to the drug. The *in vitro* metabolism of CDNB was depressed when incubated with cytosol isolated from rats treated with 20 mg/kg BCNU 24 h earlier but was normal when cytosol of rats treated 48 h before was used. BSP conjugation was not reduced by BCNU pretreatment at any time. Because BSP excretion was inhibited between 36 and 48 h after administration (Hoyt, 1984), inhibition of GSH-S-transferase cannot explain the depression of hepatic excretory function.

IV. The Effect of BCNU on Hepatic Glutathione Content

INTRODUCTION

In previous experiments, it was found that BCNU-pretreatment increased the level of hepatic reduced glutathione (GSH; Hoyt, 1984). This experiment was designed to investigate the effect of BCNU on oxidized glutathione (GSSG) and the distribution of GSH and GSSG between bile and liver. The concentration of GSH or GSSG observed at any time in the liver depends on the rate of synthesis and degradation of each and on the rate of efflux from the cells. Free GSH and GSSG are not taken up from extracellular fluid by hepatocytes (Hahn et al., 1978), although liposomally bound GSH can enter liver cells (Wendel and Jaeschke, 1982).

The first step in the synthesis of GSH (gamma-glutamylcysteinylglycine) is catalyzed by gamma-glutamylcysteine synthetase using glutamate and cysteine as substrates (Meister, 1983). The enzyme is subject to feedback inhibition by GSH (Meister, 1983). An important consideration in cases of drug overdosage where depletion of GSH occurs (for example with acetominophen) is the supply of cysteine (Kaplowitz et al., 1985). Cysteine can enter hepatocytes and it or methionine, which is taken up and metabolized to

cysteine, will increase GSH (Thor et al., 1979; Beatty and Reed, 1981). Methionine also decreases GSH efflux (Aw et al., 1984). Glycine is added to the dipeptide by GSH synthetase (Meister, 1983). GSH is the substrate for conjugation reactions mediated by GSH-S-transferases as described in chapter III (Meister, 1983). As mentioned, depletion of GSH occurs at high dosages of substrates for these enzymes (Meister, 1983). GSH and conjugates of GSH are degraded by gamma-glutamyl transpeptidase to cysteinylglycine and gamma-glutamyl amino acids (Meister, 1983). The enzyme is membrane bound and is in high concentration in the kidney (Tate and Meister, 1974). GSH is used in part to reduce free radicals and disulfides in cells and GSSG is formed in the process (Meister, 1983). Organic peroxides and hydrogen peroxide, which may be generated during normal metabolism and during drug metabolism by mitochondria and smooth endoplasmic reticulum, are detoxified by GSH peroxidase in the cytosol with production of GSSG from GSH (Sies et al., 1978; Wendel, 1981; Adams et al., 1983; Eklow et al., 1984; Guidi et al., 1984; Gunzler et al., 1984; Lauterberg et al., 1984). Glutathione reductase resynthesizes GSH from GSSG utilizing reduced nicotinamide adenine dinucleotide phosphate (NADPH, Meister et al., 1983). As discussed in the GENERAL INTRODUCTION, this enzyme is irreversibily inactivated

by chloroethyl isocyanate generated from BCNU (Babson and Reed, 1978; Ahmad and Frischer, 1985). This action has led to the use of BCNU in studies of GSH/GSSG homeostasis and responses *in vitro* (Bellomo *et al.*, 1982; Eklow *et al.*, 1984).

The levels of GSH and GSSG do not only depend on the action of enzymes. They are subject to efflux from the hepatocyte into blood and bile (Kaplowitz *et al.*, 1985).The concentration gradient for GSH from cell to plasma is about 5 µmol/g liver (or 10 mM based on 0.55 ml/g intracellular water; Akerboom *et al.*, 1982a) to 30-40 µM in hepatic vein plasma (Ookhtens *et al.*, 1985). Biliary efflux occurs down a lesser concentration gradient from about 5 µmol/g liver to about 1.5 mM in bile (see tables 4 and 5; Kaplowitz, 1985). There is a concentration gradient of about 25 nmol/g (45 µM) in the cell to 0.4 µM in systemic plasma for GSSG (Adams *et al.*, 1983). In contrast to GSH, GSSG is removed primarily by biliary excretion and is concentrated in bile relative to liver (tables 4 and 5, Adams *et al.*, 1983).

GSH efflux from the hepatocyte accounts for 85-90% of the turnover of hepatic GSH (Ookhtens *et al.*, 1985).Efflux across the sinusoidal membrane is greater than that across the canalicular membrane and accounts for 80-90% of the efflux of hepatic GSH (Ookhtens *et*

al., 1985). These investigators determined that the sinusoidal efflux of GSH from the isolated, perfused rat liver was saturated at high concentrations and found that BSP-SG inhibited this process (Ookhtens et al., 1985). This would suggest that movement across this membrane is by facilitated diffusion. However, others have demonstrated that biliary excretion of GSH across the canalicular membrane increases linearly with hepatic concentration and that saturation could not be obtained at 8 µmol GSH/g liver (Kaplowitz et al., 1983; Lauterberg et al., 1984). Biliary excretion by the isolated, perfused rat liver may have contributed to the plateau in sinusoidal efflux at the high hepatic GSH concentrations induced by cobalt chloride and 3-methylcholanthrene (Ookhtens et al., 1985). In agreement with these results, Inoue et al. (1984c) found that GSH was transported by saturable high and low affinity systems in sinusoidal membrane vesicles. Since the orientation of the vesicles was unknown, the 2 components may just represent transport by right side-out and inside-out vesicles (Inoue et al., 1984c). GSH transport was inhibited by GSH conjugates, as seen in the isolated liver, and by GSSG. The contribution of driving forces was not determined but the process appeared to be by facilitated diffusion in vivo as discussed above (Inoue et al., 1984c). The conjugate,

S-dinitrophenylglutathione was transported also and transport was inhibited by GSH and GSSG (Inoue et al., 1984c). GSSG was transported and GSH and S-benzylglutathione blocked the process (Inoue et al., 1984c). These data suggest that GSH, GSSG and GSH conjugates enter plasma from liver by facilitated diffusion mediated by the same system(s).

GSH may enter bile passively. Two groups have found that the excretion in bile is linearly related to the hepatic concentration in vivo (Kaplowitz et al., 1983; Lauterberg et al., 1984). Transport by canalicular membrane vesicles was by a a saturable mechanism affected by changes in membrane potential at low GSH concentration but nonsaturable above a concentration of 2.5 mM (Inoue et al., 1983). This would be a low GSH concentration in vivo. After a 48 h fast GSH only decreases 30% to around 4 µmol/g of liver or 7 mM and concentrations below 2.5 µmol/g (4.5 mM) were reached only after administration of a substrate for GSH-S-transferase (diethylmaleate) or an inhibitor of gamma-glutamylcysteine synthetase (buthionine sulfoximine) to decrease GSH synthesis (Ookhtens et al., 1985). However, the vesicles may simply be leaky compared to the canalicular membrane in vivo and transport in vivo might be seen to saturate if the intracellular concentration could be increased further.

The *in vitro* and *in vivo* results suggest that GSH excretion in bile is mainly passive in normal circumstances but this point is not certain.

GSSG transport into plasma appears to be along a concentration gradient as discussed above but is minor given that almost no efflux into the perfusate of the isolated, perfused rat liver occurs (Akerboom *et al.*, 1982a). Sinusoidal vesicles transport GSSG, GSH and GSH conjugates and they inhibit each other's transport (Inoue *et al.*, 1984c).

In contrast to GSH, GSSG is concentrated in bile relative to liver as mentioned above. The biliary excretion of GSSG in the isolated, perfused rat liver is identical to that *in vivo* and varies linearly with the cellular concentration (Akerboom *et al.*, 1982a). As for GSH, transport was not saturated at the highest cellular concentration attained leaving the question of carrier-mediated excretion open (Akerboom *et al.*, 1982a). However, transport in canalicular membrane vesicles was carrier mediated (Akerboom *et al.*, 1984b). The effect of membrane potential was not investigated, but others have found a GSSG-stimulated adenosine triphosphatase in liver plasma membranes and have proposed that it represents an adenosine triphosphate-dependent transporter of GSSG similar to the system in erythrocytes (Nicotera *et al.*, 1985).

The following experiment was designed to determine the effect of BCNU on the biliary excretion of GSH and GSSG and to examine the effect on hepatic GSH and GSSG. Recent studies have raised the possibility of competition between GSH, GSSG and GSH conjugates (for example, BSP-SG) for biliary excretion. This possibility was not apparent when previous investigations (Hoyt, 1984) were carried out (Akerboom et al., 1984a; Inoue et al., 1984a; Lauterberg et al., 1984).

MATERIALS and METHODS

Polyethylene tubing (PE 10, PE 50) was puchased from Clay Adams, Parsippany, NJ. Pentobarbital sodium was obtained from City Chemical Corp., NY. N-ethyl maleimide (NEM), 5,5'-dithiobisnitrobenzoic acid (DTNB) and NADPH: oxidized glutathione oxidoreductase, type III (EC 1.6.4.2., from baker's yeast) were purchased from Sigma Chemical Co., St. Louis, MO. Potassium phosphate, ethylene diamine tetraacetic acid (EDTA) and were from J.T. Baker Chemical Co., Phillipsburg, NJ. Oxidized glutathione was from Nutritional Biochemicals Corp., Cleveland OH. Reduced nicotinamide adenine dinucleotide phosphate was purchased from Calbiochem, San Diego, CA. Perchloric acid was obtained from Mallinckrodt Inc., Paris, KY.

Rats were treated with 20 mg/kg BCNU or corn oil i.p. Analyses were conducted 24, 48 and 72 h after treatment. For this experiment control and treated rats were pair-fed to control for the effects of reduced food consumption on the status of hepatic glutathione (Lauterberg and Mitchell, 1981). The food consumption of the BCNU-treated rats was was measured in 24 h intervals after injection with BCNU. The matched controls received corn oil 24 h after the BCNU-treated

rats were injected and received the amount of food consumed by their paired, treated rats in each corresponding 24 h period. At the time of administration of BCNU, rats were matched for weight as closely as possible also. Bile and liver were sampled between 9 and 10 a.m. to reduce the effect of diurnal variation (Jaeschke and Wendel, 1985)

For the 24 and 72 h groups, only hepatic GSH and GSSG were measured. Bile was collected from the 48 h group during pentobarbital anaesthesia directly into tared tubes containing 1.0 M perchloric acid and 2 mM EDTA. The tubes were kept on ice during and after collection since cold and acid prevent *in vitro* oxidation of biliary GSH (Eberle et al., 1981). Bile was collected in two 15 min intervals and volume was determined gravimetrically assuming a density of 1.0.

In the 24 and 72 h groups, the rats were killed by cervical dislocation and thoracotomy. Livers were removed, perfused with cold 0.9% NaCl and weighed. They were taken from rats in the 48 h group after 30 min of bile collection under pentobarbital anaesthesia and treated similarly.

GSH and GSSG were determined by the method of Smith and Boyd (1984) which is a modification of a method of Akerboom and Sies (1981). About 1.0 g of liver was homogenized in 4.0 ml of 1.0 M perchloric acid/2.0

mM EDTA using a Janke and Kunkel Ika-Werk Ultra-Turrax (trademark) homogenizer, model 1810. Bile samples were adjusted to a 1:5 dilution by addition of 1.0 M perchloric acid/2.0 mM EDTA.

For the measurement of GSSG, 1.0 ml of liver homogenate was added to 0.25 ml of 0.21 M N-ethyl maleimide. For bile, a volume of the diluted sample was added to 1/4 of its volume of 0.21 M N-ethyl maleimide (NEM). Samples were neutralized to pH=6.2 with 2.0 M KOH (200 to 500 µl) while mixing, and then were centrifuged 5000 x g x 5 min. The supernatants were extracted 3 times with 20 ml ether to remove excess NEM (Smith and Boyd, 1984). The ether was removed by suction and bubbling of samples with nitrogen.

For the measurement of total glutathione (GSH+2GSSG), 1.0 ml of liver homogenate or about 1/2 of the dilute bile sample was neutralized to pH=6.2 with 2.0 M KOH (200 to 500 µl) while mixing. These samples were also centrifuged 5000 x g x 5 min. The liver supernatants were diluted 1:10 with 0.1 M potassium phosphate/5.0 mM EDTA, pH=6.2. Bile supernatants were assayed directly.

The method for determination of GSSG was essentially the same as for GSH+2GSSG. Treatment of samples with NEM removed GSH from them for measurement of GSSG alone (Akerboom and Sies, 1981). The recycling

assay of Akerboom and Sies (1981) was used to determine glutathione equivalents in the samples. This is a kinetic assay which allows observation of the linear increase in absorbance at 412 nm due to the formation of 5-thio-2-nitrobenzoate from 5,5'-dithiobisnitrobenzoate by reaction with endogenous GSH and GSH generated with NADPH and yeast glutathione reductase (EC 1.6.4.2). The cuvette contained 50 µl of NADPH (4 mg/ml in 0.5% sodium bicarbonate), 20 µl of 5,5'-dithiobisnitrobenzoate (1.5 mg/ml in 0.5% sodium bicarbonate), 20 to 100 µl of sample or 10 µM GSSG standard and enough 0.1 M potassium phosphate/1.0 mM EDTA, pH=7.0 to give a volume of 1090 µl. Reaction was initiated with addition of 20 µl of glutathione reductase, 6.0 units/ml. The total reaction volume was 1110 µl. The blanks contained buffer in place of sample.

A standard curve was constructed by least squares linear regression of the slopes of the absorbance vs. time curves on the concentration of GSSG ([GSSG]) in the cuvette. The regression relation was used to obtain the [GSSG] in the cuvette for the samples based on the slope of the absorbance vs. time curve. After conversion to biliary concentration and hepatic content by application of dilution factors, [GSH] was estimated by subtraction of 2 x [GSSG] from [GSH+2GSSG]. The ratio of GSH/GSSG in liver and bile was calculated

in addition to excretion rates (flow x concentration). The bile/liver ratio of GSH and GSSG were calculated for the 48 h group. Statistical comparisons were made with the paired t-test (Snedecor and Cochran, 1980).

RESULTS

BCNU altered the hepatic and biliary content of oxidized (GSSG) and reduced glutathione (GSH). In the liver, 20 mg/kg BCNU caused an increase in total glutathione (expressed as sulfhydryl equivalents or GSH + 2 x GSSG) and GSH at 24 and 48 h after its administration (table 4). GSH was increased about 150% at both times.BCNU caused an increase in the hepatic concentration of GSSG (200%), but not until 48 h after treatment. All values were not different from control by 72 h. Note that the values in table 4 include the average of the differences between pairs of rats that were matched for food consumption to control for dietary effects on glutathione concentration (Lauterberg and Mitchell, 1981). Examination of the independent means for controls in table 4 reveals the effect of dietary restriction on hepatic GSH. There was a 35% decrease in this value in 72 h. The means were slightly lower than reported control values around 5.5 µmol/g as expected (Akerboom et al., 1982a). In this table the paired differences are for controls subtracted from treated rats, so a positive value indicates an increase in the BCNU-treated group.

The bile was analyzed in the 48 h group. At this time 20 mg/kg BCNU had caused significant

Table 4. Hepatic Glutathione Concentration After 20 mg/kg BCNU[a]

Time	Group	N	Total(GSH+2GSSG) (µmol/g)	Oxidized(GSSG) (µmol/g)	Reduced(GSH) (µmol/g)
24 h	Control	5	4.80 ± 0.734	0.0424 ± 0.00698	4.72 ± 0.731
	BCNU	5	6.74 ± 0.925	0.0434 ± 0.00418	6.65 ± 0.922
			(1.94 ± 0.668)[b]	(0.001 ± 0.0110)	(1.93 ± 0.648)[b]
48 h	Control	5	4.21 ± 0.486	0.0186 ± 0.00266	4.17 ± 0.482
	BCNU	5	6.65 ± 0.284	0.0382 ± 0.00530	6.57 ± 0.282
			(2.44 ± 0.274)[d]	(0.020 ± 0.0050)[c]	(2.39 ± 0.277)[d]
72 h	Control	5	3.13 ± 0.592	0.0277 ± 0.00619	3.08 ± 0.582
	BCNU	5	3.94 ± 0.192	0.0319 ± 0.00544	3.88 ± 0.184
			(0.81 ± 0.712)	(0.004 ± 0.0008)	(0.80 ± 0.698)

a) values are the independently calculated means ± standard error, the mean paired difference (treated-control) and standard error of the difference are given in parentheses
b) $P < 0.05$
c) $P < 0.025$
d) $P < 0.001$

cholestasis. The average bile flow (mean ± standard error) in the control group was 2.33 ± 0.1392 µl/min/g of liver compared with 1.47 ± 0.2065 µl/min/g in the treated rats. The difference was significant at $P < 0.005$ using either the independent t-test or the paired t-test (average paired difference was 0.89 µl/min/g, standard error of the difference = 0.1865). The biliary concentrations of GSSG and GSH in controls are in good agreement with reported values in the pentobarbital-anaesthetized rat (Akerboom et al., 1982) The concentrations of total, oxidized and reduced glutathione in bile were reduced to about 32%, 50% and 26% of control respectively 48 h after 20 mg/kg BCNU (table 5). In table 5, the paired differences are for treated minus control so a negative value indicates a decrease due to treatment. The means for each experimental group are also given in table 5.

The independent group means of excretion rates and the paired differences in the excretion rates (treated - control) for GSH + 2GSSG, GSSG and GSH appear in table 6. The excretion rates depend on the bile flow rate and the biliary concentrations of the substances. In view of the effect on bile flow, it is not suprising that the paired differences were significantly negative, indicating that BCNU inhibited glutathione excretion.

Kaplowitz et al. (1983) and Lauterberg et al.

Table 5. Biliary Glutathione Concentration 48 h After 20 mg/kg BCNU[a]

Time	Group	N	Total(GSH+2GSSG) (mM)	Oxidized(GSSG) (mM)	Reduced(GSH) (mM)
48 h	Control	5	1.84 ± 0.159	0.220 ± 0.00780	1.38 ± 0.146
	BCNU	5	0.58 ± 0.148	0.111 ± 0.02320	0.36 ± 0.110
			(-1.24 ± 0.269)[b]	(-0.109 ± 0.02553)[c]	(-1.02 ± 0.228)[c]

a) values are the independently calculated means ± standard error, the paired difference (treated-control) and standard error of the difference are given in parentheses

b) $P < 0.01$

c) $P < 0.025$

Table 6. Biliary Excretion Rates of Total, Oxidized and Reduced Glutathione 48 h After 20 mg/kg BCNU[a]

Time	Group	N	Excretion Rate (nmol/min/g liver) Total(GSH+2GSSG)	Oxidized(GSSG)	Reduced(GSH)
48 h	Control	5	4.40 ± 0.577	0.514 ± 0.03688	3.37 ± 0.518
	BCNU	5	0.85 ± 0.355	0.149 ± 0.04099	0.55 ± 0.274
			(-3.55 ± 0.702)[b]	(-0.365 ± 0.05603)[c]	(-2.82 ± 0.607)[b]

a) values are the independently calculated means ± standard error, the mean paired difference (treated-control) and standard error of the difference are given in parentheses

b) $P < 0.01$

c) $P < 0.005$

(1984) demonstrated that the excretion rate for GSH was proportional to the hepatic concentration. The effect of BCNU on the ratio of biliary excretion rate to hepatic concentration is shown in table 7. These ratios were significantly depressed by BCNU-treatment, indicating again that excretion was depressed. In experiments conducted by Kaplowitz et al. (1983) and Lauterberg et al. (1984) in control animals, bile flow was not depressed. Cholestasis would contribute to the diferences in the observed excretion rates. For this reason the bile:liver concentration ratios were compared (table 8). It was obvious that the distribution of GSH and GSSG was changed. The ratios for total glutathione, GSSG and GSH were reduced by BCNU 48 h after administration of 20 mg/kg indicating that efflux into bile across the canilicular membrane was depressed.

The effect of BCNU on the relative amounts of GSH and GSSG in liver and bile is shown in table 9. At 24 h, the GSH/GSSG ratio was elevated in the treated livers ($P < 0.025$) but was normal at 48 and 72 h (table 9). This could also be deduced from table 4, where it is apparent that GSH was high at 24 and 48 h, that GSSG was high only at 48 h and that the hepatic values were not different from control at 72 h. In bile at 48 h, the concentrations of GSH and GSSG were decreased by BCNU (table 5). The relative changes were not the same

Table 7. Ratios of Biliary Excretion Rates of Total, Oxidized and Reduced Glutathione to Hepatic Concentration 48 h After 20 mg/kg BCNU[a]

Time	Group	N	Excretion Rate/Hepatic Concentration (nmol/min/μmol) Total(GSH+2GSSG)	Oxidized(GSSG)	Reduced(GSH)
48 h	Control	5	1.10 ± 0.1857	29.76 ± 4.707	0.846 ± 0.1485
	BCNU	5	0.13 ± 0.0569	5.17 ± 2.617	0.085 ± 0.0440
			(-0.96 ± 0.1810)[b]	(-24.60 ± 3.3728)[c]	(-0.761 ± 0.1465)[b]

a) values are the independently calculated means ± standard error, the mean paired difference (treated-control) and standard error of the difference are given in parentheses
b) $P < 0.01$
c) $P < 0.005$

Table 8. Ratios of Biliary to Hepatic Concentration of Total, Oxidized and Reduced Glutathione After 20 mg/kg BCNU[a]

Time	Group	N	Bile/Liver Concentration (µmol/ml/µmol/g) Total(GSH+2GSSG)	Oxidized(GSSG)	Reduced(GSH)
48 h	Control	5	0.456 ± 0.0645	12.8 ± 1.836	0.347 ± 0.0519
	BCNU	5	0.089 ± 0.0234	3.2 ± 0.930	0.056 ± 0.0175
			(-0.367 ± 0.0726)[b]	(-9.6 ± 1.329)[c]	(-0.292 ± 0.0577)[b]

a) values are the independently calculated means ± standard error, the mean paired difference (treated-control) and the standard error of the difference are given in parentheses
b) $P < 0.01$
c) $P < 0.005$

Table 9. Ratio of Reduced to Oxidized Glutathione in Liver and Bile[a]

After 20 mg/kg BCNU

Time	Group	N	Liver	Bile
24 h	Control	5	119.34 ± 22.11	n.d.
	BCNU	5	156.43 ± 19.36	
			(24.52 ± 6.71)[c]	
48 h	Control	5	229.44 ± 18.38	6.33 ± 0.76
	BCNU	5	190.16 ± 34.13	2.98 ± 0.58
			(-39.28 ± 31.97)	(-3.35 ± 1.07)[b]
72 h	Control	5	134.40 ± 28.84	n.d.
	BCNU	5	130.16 ± 12.91	
			(-4.24 ± 27.82)	

a) values are the independently calculated means ± standard error, the mean paired difference (treated-control) and standard error of the difference are given in parentheses, n.d. means not determined

b) $P < 0.05$

c) $P < 0.025$

since the GSH/GSSG ratio in bile was depressed relative to control (table 9).

DISCUSSION

The the previously reported increase in hepatic GSH due to 20 mg/kg BCNU (Hoyt, 1984) was confirmed in this study using a different assay. BCNU stimulated the synthesis of GSH. BCNU causes anorexia over at least 48 h (Hoyt, 1984). Fasting is known to deplete hepatic GSH, stimulate amino acid mobilization and to increase the rate of GSH synthesis (Mallette et al., 1969; Lauterberg and Mitchell, 1981). Dietary restriction of controls by pair-feeding should control for these responses. The elevation of hepatic GSH in BCNU-treated rats (table 4) may indicate that amino acid uptake important for GSH synthesis was not greatly inhibited (Thor et al., 1979).

Degradation of GSH might be inhibited by BCNU. The main route of degradation is via gamma-glutamyl transpeptidase (GGT) and the clearance of GSH from plasma is controlled mainly by this activity in the kidneys (Lauterberg and Mitchell, 1981; Adams et al., 1983). Because GSH is not taken up by the liver (Hahn et al., 1978),inhibition of this catabolic salvage of GSH component amino acids by BCNU might actually decrease the recirculation of cysteine into the hepatic pool thereby causing a decrease in GSH (Lauterberg and Mithcell, 1981). The activity GGT in the liver is low and primarily located in the canalicular membrane facing

the lumen (Hahn et al., 1978; Inoue et al., 1983). Inhibition of transpeptidase in the liver could increase GSH but would probably be insignificant if the enzyme activity is low in controls. BCNU-pretreatment did reduce the transferase activity toward chlorodinitrobenzene at 24 h (chapter III). Inhibition of some transferase reactions might directly increase GSH.

BCNU is a glutathione reductase inhibitor and is a substrate for GSH-S-transferase (Hill, 1976; Babson and Reed, 1978). Both actions would be expected to decrease the GSH content of liver. Depletion of GSH stimulates resynthesis which can overshoot the initial level (Valenzuela et al., 1983; Dalich and Larson, 1985). This is reasonable given the feedback inhibition exerted by GSH on gamma-glutamylcysteine synthetase (Meister, 1983). No decrease in hepatic GSH was observed at any time from 3 hours after treatment in a previous study however (Hoyt, 1984).

It seems that efflux across the sinusoidal membrane is the most important determinant of GSH content in the liver (Ookhtens et al., 1985). At 48 h, 20 mg/kg BCNU inhibited GSH excretion by causing cholestasis and preventing its distribution into bile as indicated by depressed biliary concentration and increased bile:liver ratio (tables 5 and 8). Efflux into

blood might also be decreased if BCNU exerted a general effect on the cell membrane or transporters. Sinusoidal efflux may occur by facilitated diffusion and BCNU might alter the function of a carrier. Although GSSG can inhibit sinusiodal transport of GSH (Inoue et al., 1984c), accumulation of GSH was observed at 24 h when GSSG was normal in BCNU-treated rats (table 4). Therefore, increased GSSG cannot completely explain the effect. The increased hepatic GSH at 24 h was not related to an effect of BCNU on GSSG since there was no change in GSSG.

Much of GSH excretion in bile may be due to passive diffusion (Inoue et al., 1983; Kaplowitz et al., 1983; Lauterberg et al., 1984). The inhibition of biliary excretion could be caused by an increase in membrane thickness, decreased surface area or decreased permeability of the canalicular membrane. Loss of canalicular microvilli is a common finding in cholestasis induced in many ways (Plaa and Priestly, 1977). The saturable portion of GSH transport in canalicular membrane vesicles is affected by membrane potential (GSH is transported as an anion; Inoue et al., 1983). BCNU did increase biliary potassium levels, but it did not inhibit bile salt excretion (Hoyt 1984) and, bile salt transport in vesicles is apparently driven by the membrane potential (Inoue et al., 1983; Meier et

al., 1984b).

Inhibition of GSSG reductase by BCNU could explain the increased hepatic content of GSSG depicted in table 4 (Babson and Reed, 1978). However, Frischer and Ahmad (1977) found that the enzyme was inhibited immediately after BCNU-treatment and hepatic GSSG was not elevated until 48 h after treatment in the present study (table 4).

Thiol transferase mediates the incorporation of GSSG into proteins as mixed disulfides (Kaplowitz et al., 1985). The hepatic content of mixed disulfides increases linearly with GSSG concentration (Brigelius et al., 1983). The basal level of mixed disulfides in rat liver is about the same as the GSSG content. BCNU may have shifted the distribution of glutathione by inhibiting the thiol transferase reaction but no evidence for this was gathered.

Biliary excretion of GSSG was also greatly inhibited (tables 5, 6 and 8). This could explain the increased hepatic content of GSSG at 48 h since GSSG is preferentially excreted in bile. Hepatic GSSG returned to control levels at 72 h (table 4). If biliary excretion was normal at 72 h, this hypothesis would be greatly strengthened but this was not determined. Because sinusoidal efflux of GSSG is not very extensive (Akerboom et al., 1982a), an effect of BCNU on that side

of the hepatocytes probably would be unimportant in blocking GSSG removal from hepatocytes. The accumulation of GSH and GSSG in the liver

The accumulation of GSH and GSSG in the liver may be relevant to the depression of BSP excretion caused by 20 mg/kg BCNU between 36 and 48 h after administration (Hoyt, 1984). In canalicular membrane vesicles both GSH and GSSG inhibit S-benzylglutathione and S-dinitrophenylglutathione transport (Inoue et al., 1983; Inoue et al., 1984a). However, BSP-SG did not inhibit the transport of S-dinitrophenylglutathione indicating that there may be more than one mechanism for excretion of GSH conjugates depending on the particular physicochemical properties of the material, such as molecular weight or lipophilicity (Inoue et al., 1984a; Kaplowitz et al., 1985). These inhibition data imply that increased hepatic GSH and GSSG content cannot explain the depression of BSP excretion.

Several reports indicate that induction of intracellular GSSG content by various methods inhibits the biliary excretion of taurocholate, bilirubin, BSP-SG (but not ICG) and S-dinitrophenylglutathione (Akerboom et al., 1982b; Akerboom et al., 1984a; Lauterberg et al., 1984). However, BSP excretion was depressed by 20 mg/kg BCNU from 36 through 96 h after treatment while hepatic GSSG was normal at 24 and 72 h and elevated only

at 48 h (table 4). Moreover, ICG excretion was depressed by BCNU-pretreatment (chapter II; Hoyt, 1984). In addition, BCNU did not inhibit bile salt transport into bile at times when BSP excretion was depressed implying that GSSG was not increased enough to cause such impairment of excretory function (chapter V; Hoyt, 1984). Taurocholate excretion was decreased 50% at 0.1 μmol/g intracellular GSSG, a concentration which is 5 to 20 times the amount seen in the present study (table 4; Akerboom et al., 1984a). For these reasons it appears that intracellular accumulation of GSSG was not the cause of impaired organic anion excretion.

Neither did the increased GSH in the liver appear to cause the inhibition of BSP excretion. Hepatic GSH was elevated at 24 and 36 h post-treatment, but normalized at 72 h (table 4). BSP excretion was inhibited after 36 h through 96 h (Hoyt, 1984). One would expect the two actions of BCNU to coincide if elevated GSH was directly responsible for reduced BSP excretion.

BCNU elicited a delayed change in the system that affected the excretion of GSH, GSSG, BSP and ICG but not bile salt excretion. The inhibition of GSH and GSSG excretion into bile tends to confirm the idea that, at the very least, net canalicular flux is inhibited. GSH and GSSG arise intracellularly so the complicating

consideration of the possibility of BCNU effects on sinusoidal uptake in the case of xenobiotic organic anion excretion does not apply. There could be a common cause for these actions such as a change in the canalicular membrane itself. The development of impaired excretion of organic anions and glutathiones might follow a similar time course but glutathione excretion was measured at one time point. Different time courses could be explained by differing sensitivities of the functions to the delayed action of BCNU. This might arise from differences in the transport mechanisms at the canalicular membrane as reviewed above. For example, GSH may be excreted by passive and/or facilitated diffusion while GSSG may be concentrated by an active system. Recall that BSP-SG does not block canalicular transport of all other GSH conjugates. The lack of effect of GSSG on ICG excretion while inhibiting BSP-SG excertion may indicate a difference in the mechanism of transport also (Lauterberg et al., 1984). Alternatively, BCNU may act by several mechanisms impacting on excretory function. Membrane structural alterations as well as direct and indirect actions on transporters may have occured.

One indirect mechanism might have involved an accumulation or loss of some unknown material that affects canalicular excretion. The possibility that

elevated bile salt concentrations after BCNU-treatment were responsible for reduced BSP excretion was ruled out because bile salt depletion had no impact on the excretory defect (chapter V; Hoyt, 1984). It remains to be determined whether bile salt depletion could alter the action of BCNU on GSH and GSSG excretion. This could be relevant since elevation of hepatic GSSG reduces the excretion of taurocholate (Akerboom et al., 1984a).

BCNU increased the ratio of GSH/GSSG in the liver at 24 h (table 9). This indicated that GSH was altered to a greater extent and sooner than GSSG. If BCNU inhibited efflux in general, this might be expected because of the much higher magnitude of GSH efflux although a small change in GSSG would also greatly affect the ratio. The increase might be indicative of the different mechnisms and directions of the major portions of efflux of GSH and GSSG. A high ratio of GSH/GSSG is an important determinate of hepatocellular calcium homeostasis and resistance of cells to oxidative stress and toxic agents (Gillette et al., 1974; Thor et al., 1979; Bellomo et al., 1982; Jones et al., 1983). The increased ratio in liver and elevated GSH may mean that rats would not be excessively sensitive to other agents early after exposure to BCNU unless their actions are terminated by biliary excretion. This awaits

confirmation.

The ratio of GSH/GSSG was decreased in bile by 20 mg/kg BCNU at 48 h (table 9). This could be due to cholestasis which might allow more time for degradation of GSH by gamma-glutamyltranspeptidase (Akerboom et al., 1984a).

In summary, GSH synthesis may be stimulated by BCNU-pretreatment, possibly as a result of an early depletion. Inhibition of degradation seems to be an unlikely cause of increased hepatic GSH. GSSG may increase as a result of inhibition of GSSG reductase but this should occur immediately after administration of BCNU. GSSG might have increased if thiol transferase activity was depressed. The most likely action of BCNU was to inhibit GSH and GSSG efflux. Because efflux into blood accounts for about 90% of GSH efflux, it seems likely that sinusoidal transport was depressed. Such an effect might indicate a contribution of the sinusoidal membrane as a site for reduced organic anion excretion after BCNU. This path is unimportant for GSSG, but the reduced excretion of GSSG in bile might explain its elevation intracellularly.

Increases in the glutathiones do not account for depression of excretory function by direct action. BCNU inhibited the excretion of GSH and GSSG. One simple

explanation is that BCNU acts on the canalicular side of the cells to inhibit excretion of BSP, ICG, GSH and GSSG but not bile salts. The data are consistent with a canalicular site of action with respect to BSP excretion as implied by the depression of BSP Tm (chapter I) and ICG excretion (chapter II).

V. The Effect of Cholestyramine on the Impairment of Excretory Function by BCNU

INTRODUCTION

Previous investigation indicated that although BCNU inhibited BSP excretion in rats, bile salt concentrations in bile were actually increased (Hoyt, 1984). This maintained bile salt excretion to some extent despite the presence of cholestasis although the rate was reduced with respect to controls (Hoyt, 1984). O'Maille et al. (1966) demonstrated that BSP and taurocholate could compete for excretion when one or the other was infused establishing a Tm. This suggested that BSP and bile salts might interact at the canalicular membrane. In addition, in vitro studies with canalicular membrane vesicles and studies in vivo and in isolated, perfused rat livers suggested that taurocholate, GSH, GSSG, glutathione conjugates of some compounds and BSP might be excreted by the same system or that they may share systems partially (Inoue et al., 1983; Akerboom et al., 1984a; Inoue et al., 1984a; Lauterberg et al., 1984). It was known that restriction of bile flow by bile duct ligation causes about a 100-fold increase of serum bile salts in 5 days, raising the question of whether BCNU-induced cholestasis could

cause impairment of BSP excretion (Kinugasa et al., 1981).

It was hypothesized that depletion of the bile salt pool would increase the ability of BCNU-treated rats to concentrate BSP in bile if bile salts were inhibiting organic anion excretion. Bile salts may be depleted by establishing a biliary fistula and allowing external drainage of bile for a time, such as overnight (Balabaud et al., 1977; Mackinnon and Kohler, 1980). Alternatively, one may administer a bile salt sequestrant such as cholestyramine (Gregus et al., 1980). This treatment was deemed advantageous because of the lack of trauma associated with surgery.

The effectiveness of the interruption of the enterohepatic circulation in reducing the body content of bile salts is based on the extensive reabsorbtion from the ileum and efficient hepatic extraction from plasma. Only about 5% of the bile salt content is lost by fecal excretion each day, for which de novo hepatic synthesis from cholesterol compensates (Packard and Shepard, 1982). Some bile salts, such as taurocholate, are taken up by the liver mainly by a sodium-dependent mechanism while others may traverse the membrane primarily by sodium-independent mechanisms such as diffusion (Van Dyke et al., 1982a). Reabsorption of of bile salts in the intestinal tract occurs by passive

diffusion in the duodenum and jejunum and by sodium-dependent cotransport at the brush-border of the ileum (Barnard et al., 1985).

Cholestyramine is a polymeric, anion exchange resin (quaternary ammonium) that is not absorbed from the gastrointestinal tract (Levy, 1980; Packard and Shepard, 1982). After oral administration, the anionic bile salts bind to the resin and fecal excretion is greatly facilitated (Packard and Shepard, 1982). Gregus et al. (1980) reported that a 1.0 g/kg oral dosage of cholestyramine in rats reduced the hepatic bile salt excretion rate by 80 to 85% 4 h after administration.

The purpose of the experiment was to determine whether bile salt depletion could increase the ability of BCNU-treated rats to concentrate BSP in bile (i.e. reverse the inhibition). Such an effect would indicate a role for bile salts in the impairment of organic anion excretion by BCNU.

MATERIALS and METHODS

Cholestyramine (Questran^{tm}) was obtained from Mead Johnson and Co., Evansville, Indianna. BSP, taurocholate, and 3-alpha-hydroxysteroid dehydrogenase (EC 1.1.1.50) were purchased from Sigma Chemical Co., St. Louis, MO. Oxidized nicotinamide adenine dinucleotide (NAD^+) was obtained from Calbiochem, San Diego, CA. Glycine, ethylene diamine tetraacetate (EDTA) and hydrazine sulfate were purchased from J.T. Baker Chemical Co., Phillipsburg NJ.

Ten rats were treated with 25 mg/kg BCNU and 10 rats received corn oil. After 48 h, rats were fasted overnight. On day 3, 5 rats in each group were given cholestyramine orally (1 g/kg, 10 ml/kg of .225 g/ml Questran^{tm}). Four h later rats were anaesthetized and bile flow and the excretion of a single i.v. injection of 50 mg/kg BSP were measured. After a basal period of bile collection, BSP was injected into the inferior vena cava and bile collection was initiated in 15 min intervals for 2 h. [BSP] was determined as before.

Bile salts in basal bile were measured by a modification of the method of Talalay (Talalay, 1960). Bile was diluted 1:10 with methanol and standards were made with cholate in methanol. Fifty µl of dilute

sample was added to 750 µl of buffer (1.3 M glycine, 7.1 mM EDTA, 0.51 M hydrazine sulfate, pH=9.4) and 100 µl of 5.32 mM nicotinamide adenine dinucleotide (NAD^+). Reduction of NAD^+ was initiated by addition of 100 µl of 3-alpha-hydroxysteroid dehydrogenase (EC 1.1.1.50, 0.7 units/ml). The reduction was monitored at 340 nm on an Aminco DW 2A spectrophotometer. The concentration of bile salts was determined by the standard relationship of the maximum change in the absorbance at 340 nm (after subtraction of the blank) to the concentration of the standard. Blanks contained methanol in place of bile salt. The maximum change in absorbance was taken when it failed to change by more than 0.002 in 15 min.

RESULTS

Bile salt concentrations in bile collected immediately after cannulation of the bile duct and prior to injection of BSP were 39.9 $\pm$ 3.6 mM in control and 6.17 $\pm$ 1.1 mM (mean $\pm$ standard error, n=5) in controls treated with cholestyramine ($P<0.005$ for comparison with untreated control). In the bile from BCNU-treated rats after cholestyramine administration the concentration was 25.5 $\pm$ 8.3 mM ($P<0.05$ vs. untreated control). The bile salt concentration was 32.8 $\pm$ 2.7 mM in rats treated with BCNU alone (not significantly different from control). The bile salt concentration was not affected by cholestyramine in 2 of 5 BCNU-treated rats (concentration was about 46 mM vs. about 12 mM in the other 3), which explains the high mean and standard error.

Bile flow in the rats treated with 25 mg/kg BCNU was depressed in the basal period ($P < 0.05$) but was normal after BSP injection (fig. 14). Cholestyramine alone caused a degree of cholestasis in all the intervals except at 60 and 75 min after the BSP injection (fig. 14). Bile flow was depressed in the BCNU-treated rats that also received cholestyramine throughout the experiment (fig. 14).

The effect of BCNU and cholestyramine treatment

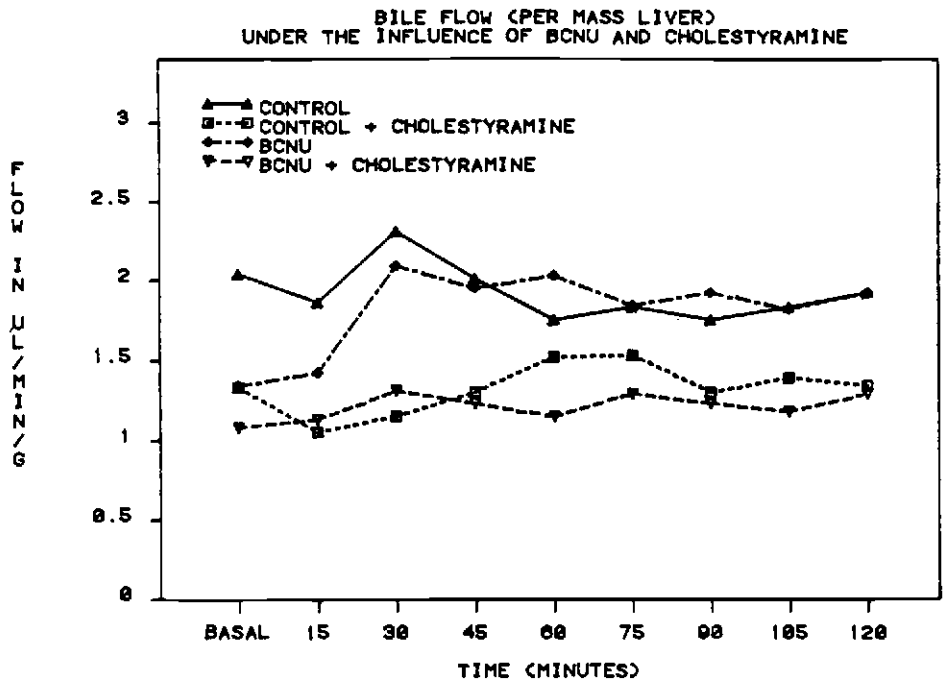


Figure 14. Bile flow in control rats, BCNU-treated rats, controls treated with cholestyramine and BCNU-treated rats treated with cholestyramine. Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Cholestyramine-treated rats received 1.0 g/kg orally 72 h later. Bile was collected under pentobarbital anaesthesia 4 h after treatment with cholestyramine. 50 mg/kg BSP was injected i.v. after a basal period (15 min) of bile collection.

on the BSP excretion rate is shown in figure 15. Cholestyramine treatment by itself reduced BSP excretion significantly only in the 15 and 75 min intervals. The peak mean rate (i.e. the mean in the interval with the highest mean) for the controls was 25.87 ± 5.467 µg/min/g of liver at 30 min. The peak mean in cholestyramine-treated controls was 14.81 ± 3.589 µg/min/g and occurred at 45 min. These values were not significantly different in an independent t-test. This mild reduction of the excretion rate was apparently due to reduced bile flow, not to inhibited transport of BSP into bile.

Cholestyramine had no effect on the ability of the rats to concentrate BSP in bile (fig. 16). Peak mean biliary BSP concentration in the controls was 10.99 ± 4.797 µg/µl at 30 min compared to the peak mean in the cholestyramine-treated controls of 11.01 ± 1.778 µg/µl at 45 min. The peak mean excretion rate in the latter group was 12.21 ± 3.275 µg/min/g at 30 min ($P < 0.05$ vs. control of 25.87 ± 5.47 µg/min/g). Consistent with earlier experiments, the depressed BSP excretion rates were due to a decreased ability of BCNU-treated rats to concentrate BSP (fig. 16, Hoyt, 1984). The peak mean concentration (5.75 ± 1.355 µg/µl, which is the mean value for the second interval) was not significantly different from control. It was noticed, however, that

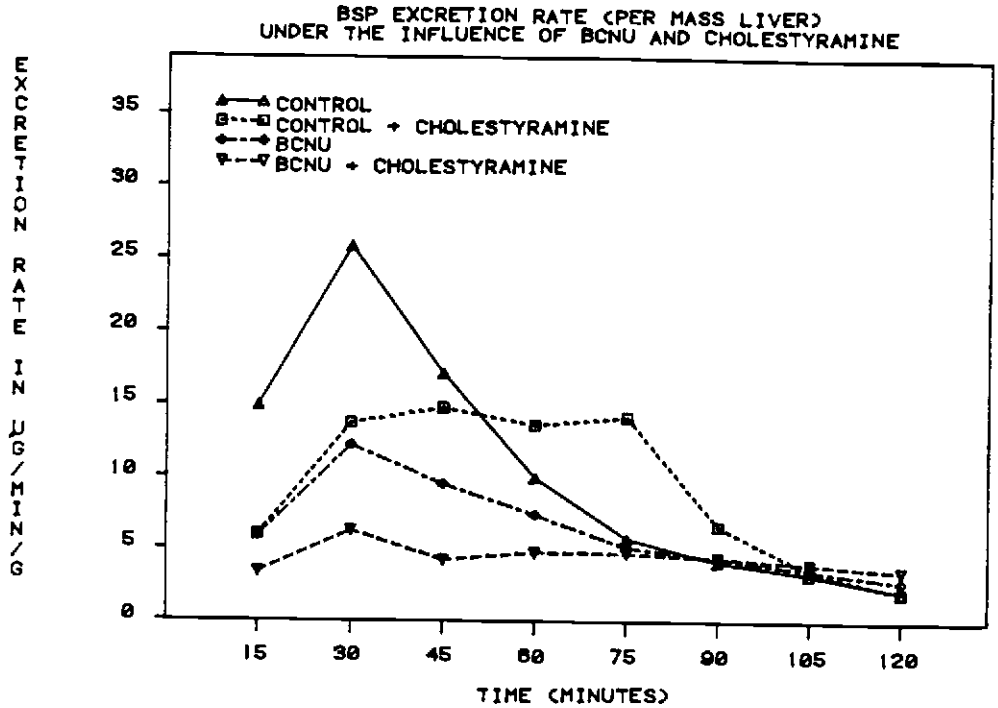


Figure 15. BSP excretion rate in control rats, BCNU-treated rats, controls treated with cholestyramine and BCNU-treated rats treated with cholestyramine. Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Cholestyramine-treated rats received 1.0 g/kg orally 72 h later. Bile was collected under pentobarbital anaesthesia 4 h after treatment with cholestyramine. 50 mg/kg BSP was injected i.v. after a basal period (15 min) of bile collection.

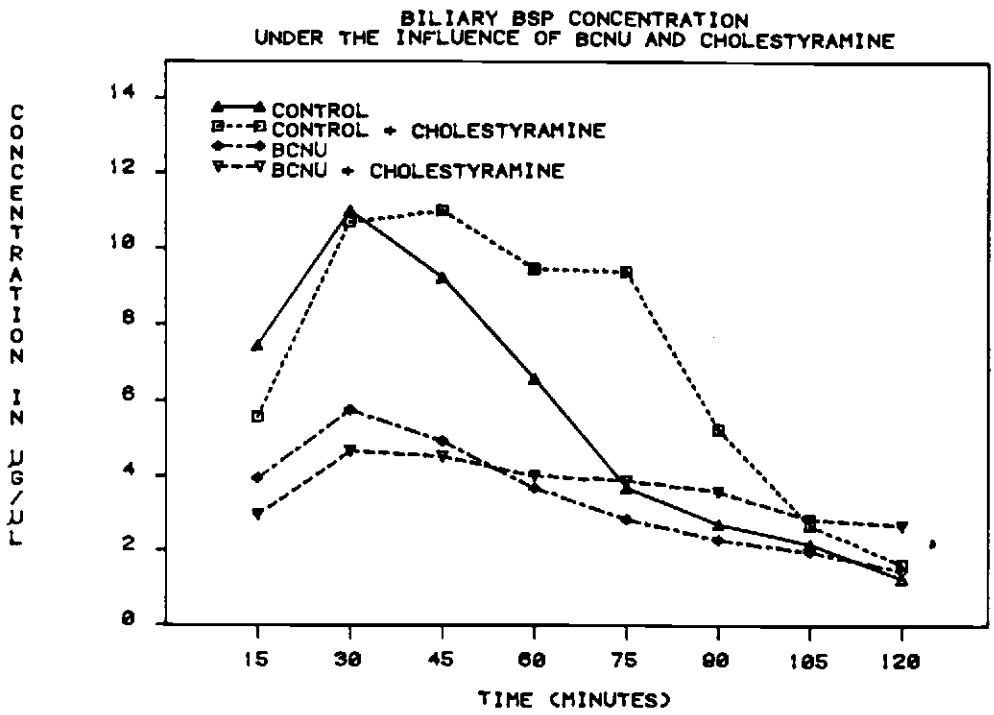


Figure 16. Biliary [BSP] in control rats, BCNU-treated rats, controls treated with cholestyramine and BCNU-treated rats treated with cholestyramine. Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Cholestyramine-treated rats received 1.0 g/kg orally 72 h later. Bile was collected under pentobarbital anaesthesia 4 h after treatment with cholestyramine. 50 mg/kg BSP was injected i.v. after a basal period (15 min) of bile collection.

individual rats varied slightly in the interval in which the highest biliary concentration was attained. When the mean of the highest concentrations attained by individual rats in a group regardless of time after BSP injection were compared, the differences between the controls and BCNU-treated rats were more pronounced. The means and standard errors of highest concentrations were 13.64 $\pm$ 1.28 µg/µl in control, 14.00 $\pm$ 1.46 µg/µl in controls treated with cholestyramine, 5.12 $\pm$ 0.56 µg/µl in BCNU-treated rats that received cholestyramine ($P < 0.001$ vs. control) and 5.79 $\pm$ 1.34 µg/µl in rats treated with BCNU alone ($P < 0.005$ vs. control). Cholestyramine did not increase the ability of BCNU-treated rats to concentrate BSP in bile. Additionaly, the 2 BCNU-treated rats that were not affected by the cholestyramine treatment only concentrated BSP to the levels of 5.6 and 6.1 µg/µl which was similar to the others in the group. The lack of correlation between the biliary concentrations of bile salts and maximum concentrations of BSP is depicted in figure 17.

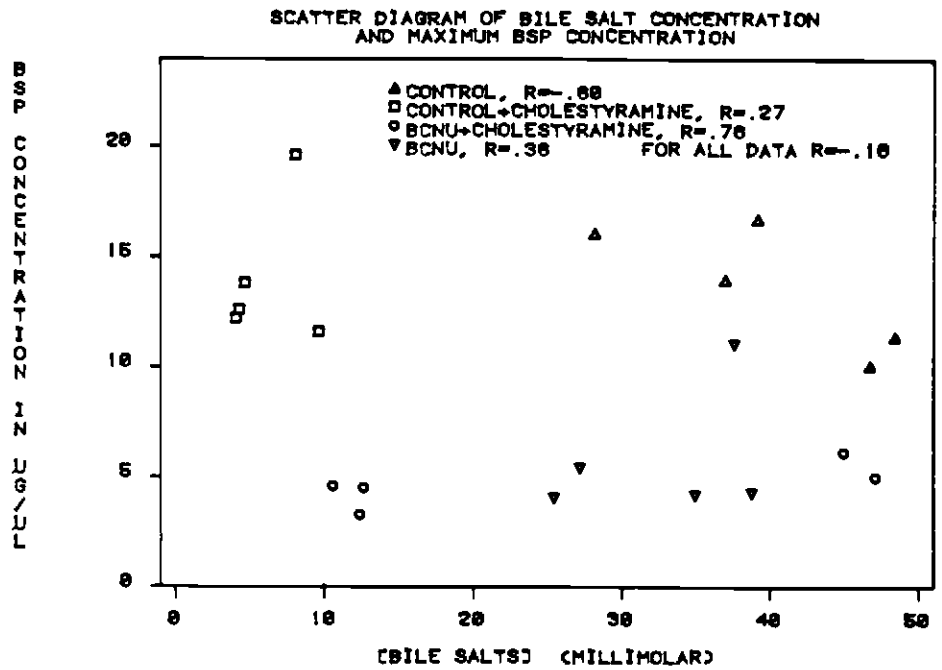


Figure 17. Relationship between biliary BSP and bile salt concentrations in control rats, BCNU-treated rats, controls treated with cholestyramine and BCNU-treated rats treated with cholestyramine. Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Cholestyramine-treated rats received 1.0 g/kg orally 72 h later. Bile was collected under pentobarbital anaesthesia 4 h after treatment with cholestyramine. 50 mg/kg BSP was injected i.v. after a basal period (15 min) of bile collection.

DISCUSSION

The administration of cholestyramine failed to reverse the impaired BSP excretion caused by BCNU. The highest average concentration of BSP in bile was the same as in rats that received BCNU alone even though biliary bile salt concentrations were lowered by cholestyramine in 3 of the 5 BCNU-treated animals. In this experiment 25 mg/kg BCNU did not cause apparent cholestasis 72 h after administration, except in the basal period. Bile salt concentration in bile of rats that received only BCNU was normal but BSP excretion was decreased. The ability to concentrate BSP was depressed in those rats that received BCNU alone even though bile salt concentration was normal in this experiment.

O'Maille et al. (1966) demonstrated the possibility that bile salts and BSP could compete for excretion at the canalicular membrane. Other reports indicated that bile salts might inhibit glutathione transferases (Vessey and Zakim, 1981) and bind to the enzymes (Lawrence et al., 1980) possibly influencing the storage function. The lack of effect of bile salt depletion seen here argues against the importance of these possibilities for inhibition of BSP excretion by BCNU.

Depletion of bile salts in control rats lowered

the BSP excretion rate but highest average biliary concentrations of BSP attained were unaffected which suggests that bile salts were not important for maintaining concentrative transport of BSP as well. This is in agreement with results suggesting that BSP excretion is not driven by association with micelles in bile (Ritt and Combes, 1967; Delage et al., 1976; Binet et al., 1979).

The cholestasis caused by cholestyramine in both control and BCNU-treated rats was probably due to the depletion of bile salts and resultant decrease in biliary concentration. Bile flow is divided into 2 components. These are bile salt independent and bile salt dependent bile flow (Boyer and Klatskin, 1970; Erlinger, 1982). These fractions are estimated by relating bile flow to the bile salt excretion rate over a range of excretion rates (fig. 18). The relation can be approximated by a line at all but very low bile salt concentrations (Balabaud et al., 1977). Extrapolation to zero bile salt excretion generally indicates a positive y-intercept which is taken as an estimate of bile salt independent bile production. This latter fraction is generated by the osmotic activity of particles other than bile salts in bile (Boyer, 1980). The bile salt dependent bile flow is related to the osmotic activity of bile salts in bile so that when bile

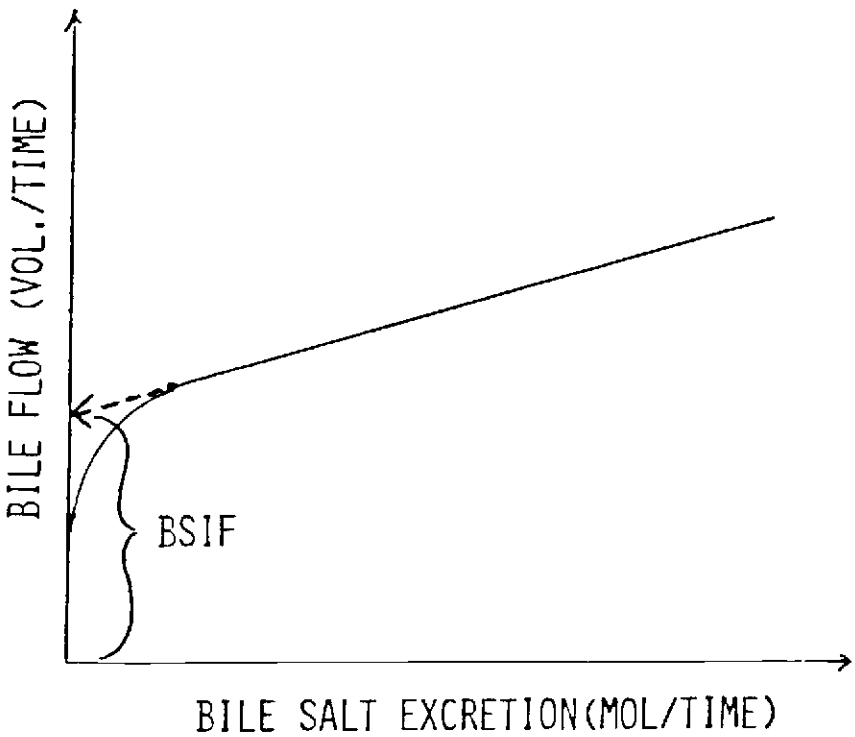


Figure 18. Estimation of BSIF and BSDF. BSIF is estimated by extrapolation to zero bile salt excretion rate. The slope is the BSDF.

salt concentration decreases, movement of water into bile decreases (Boyer, 1980). In effect, the flow is determined at a point further to the left in figure 18 (excretion rate=flow x concentration), and where the slope may be increased (so that the effect of decreasing bile salt excretion is increased).

In conclusion, bile salt depletion did not alter the ability of control or the inability of BCNU-treated rats to concentrate BSP in bile. Also, in this experiment BCNU-treated rats with normal biliary bile salt concentrations were unable to concentrate BSP. Therefore, general bile salt accumulation in the hepatocytes of BCNU-treated rats probably does not play a role in the impairment of BSP excretion described in these studies.

VI. The Effect of Pentobarbital on Impaired Organic Anion Excretion and Cholestasis Caused by BCNU

INTRODUCTION

BCNU-induced cholestasis was due to selective depression of bile salt independent bile flow (BSIF) while bile salt dependent bile flow (BSDF) was normal (Hoyt, 1984). It was hypothesized that BCNU-induced cholestasis could be reversed by a treatment that stimulates BSIF. In the previous study, BSIF and BSDF were determined by relating the biliary clearance of intravenously administered erythritol to the bile salt excretion rates (Boyer and Klatskin, 1970; Layden and Boyer, 1976; Balabaud et al., 1977). BSIF was estimated by extrapolation to zero bile salt excretion as in figure 18. The unaltered BSDF, defined as the slope of the linear approximation in units of nmol bile salt/µl bile formed, suggested that the osmotic effect of bile salts in bile was not affected by the drug and that movement of water into the canalicular space was not restricted in treated rats (Balabaud et al., 1977; Hoyt, 1984). However, bile osmolality and the ratio of bile:plasma osmolality were increased slightly prior to the onset of cholestasis, but not during cholestasis, suggesting that movement of water might have been

impeded earlier (Hoyt, 1984).

The formation of BSIF is related to the excretion of sodium, bicarbonate and chloride (Hardison and Wood, 1978; Scharschmidt et al., 1982; Van Dyke et al., 1982b; Anwer and Hegner, 1983; Scharschmidt and Van Dyke, 1983; Garcia-Marin et al., 1985). Replacement of sodium in the perfusate of the isolated, perfused rat liver by monovalent cations other than lithium abolishes BSIF (Hardison and Wood, 1978; Van Dyke et al., 1982b; Anwer and Hegner, 1983). Removal of HCO_3^- decreases BSIF by about 50% (Van Dyke et al., 1982b; Anwer and Hegner, 1983). The contribution of Cl^- transport to the formation of BSIF was observed only after HCO_3^- was removed first and BSIF fell an additional 30% (Anwer and Hegner, 1983).

Numerous studies have also correlated BSIF with the activity of plasma membrane sodium/potassium adenosine triphosphatase (Na^+/K^+ ATPase) under the influence of various agents including phenobarbital (Heikel and Lathe, 1970; Gumucio and Valdivieso, 1971; Layden and Boyer, 1976; Reichen and Paumgartner, 1977; Simon et al., 1977; Wannagat et al., 1978; Simon et al., 1980). Since Na^+/K^+ ATPase appears to be localized to the sinusoidal membrane (Latham and Kashgarian, 1979), it was proposed that sodium enters bile through the junctional complexes between hepatocytes that isolate

the canaliculus from the sinusoid (Layden et al., 1978; Boyer, 1980).

The initial goal of this experiment was to reverse or prevent the action of BCNU on bile flow. Because BCNU selectively depressed BSIF, it was decided to use an agent known to stimulate BSIF selectively (Capron et al., 1977). Pentobarbital-pretreatments stimulated BSIF selectively in rats but failed to induce microsomal cytochrome content (Capron et al., 1977). Because phenobarbital- pretreatments also affect organic anion excretion (Klaassen and Plaa, 1968; Hart et al., 1969; Klaassen, 1970), the effect of pentobarbital on BSP excretion was investigated as well. If BCNU inhibited the activity of Na^+/K^+ ATPase (or other enzymes involved in bile production and organic anion excretion) or reduced their numbers, then it might be possible to reverse cholestasis and the excretory deficit by inducing the number of enzymes, as is the case with phenobarbital (Simon et al., 1977). Activity might be directly increased by altering membrane fluidity also (Simon et al., 1980).

It would have been advantageous to use phenobarbital since much was known about its actions (Berthelot et al., 1970; Klaassen, 1971; Capron et al., 1977; Simon et al., 1977). However, phenobarbital-

pretreatments greatly increase the hepatic metabolism of BCNU causing a 9-fold decrease in bioavailability via the intraperitoneal route in rats by increasing the metabolism of the drug (Levin et al., 1979). Although data were unavailable regarding the effect of pentobarbital on the pharmacokinetics of BCNU, it represented a better choice than phenobarbital.

MATERIALS and METHODS

Polyethylene tubing (PE 10) was puchased from Clay Adams, Parsippany, NJ. Pentobarbital sodium was obtained from City Chemical Corp., NY. Sulfobromophthalein (BSP) was from Sigma Chemical Co., St. Louis, MO. Sodium phosphate was from J.T. Baker Chemical Co., Phillipsburg, NJ. Sodium toluene-p-sulfonate was obtained from Eastman Kodak Co., Rochester NY.

Control rats received corn oil and 25 mg/kg BCNU was administered to another group. One half of animals in each of control and BCNU-treated groups also received pentobarbital injections twice daily for 4 days prior to surgery to determine bile flow. The pentobarbital treatment schedule was modified from the procedure of Capron et al. (1977) such that 120 mg/kg instead of 140 mg/kg total pentobarbital was given on the 4th day. The BCNU and control groups that did not receive pentobarbital received the vehicle instead. Pentobarbital was dissolved in propylene glycol: ethanol: water, 4:1:5 at a concentration of 70 mg/ml.

On day one, rats received an injection of 40 mg/kg pentobarbital at 8:00 a.m. and 5:00 p.m., or an equivalent volume of vehicle i.p. On day 2, rats

received vehicle or 50 mg/kg pentobarbital at 8:00 a.m. When the pentobarbital-treated rats had regained the righting reflex and began to move in a coordinated way, BCNU or corn oil was injected i.p. At 5:00 p.m. the rats received 50 mg/kg pentobarbital or vehicle again. On days 3 and 4, rats received 60 mg/kg pentobarbital or vehicle twice per day. On day 5, all rats were anaesthetized with pentobarbital and bile flow and the excretion of a single i.v. injection of 50 mg/kg BSP were determined. Data were analyzed by analysis of variance (Snedecor and Cochran, 1980).

RESULTS

Pretreatment with pentobarbital prevented the effects of 25 mg/kg BCNU on bile flow and BSP excretion. Three days after treatment with BCNU, cholestasis was evident the rats that received BCNU alone (fig. 19; $P < 0.05$ for comparison with the other groups). No other groups were affected in any way.

The effect of BCNU on BSP excretion rate was also prevented by pentobarbital (fig. 20). The BSP excretion rate was reduced by BCNU alone with respect to controls ($P<0.005$), pentobarbital-treated controls ($P<0.01$), and rats treated with both agents ($P<0.025$). There were no differences among controls, pentobarbital-treated controls and rats treated with pentobarbital and BCNU.

By itself, pentobarbital slightly decreased the concentration of BSP in bile attained after the single i.v. injection of the dye (fig. 21; $P < 0.025$). But the concentration in the bile of rats treated with pentobarbital and BCNU was not different from control. BCNU alone caused the characteristic reduction of biliary concentration of BSP. Contrasting BCNU-treated rats with each of the other groups indicated significant inhibition ($P < 0.005$ vs. control, $P < 0.01$ vs. pentobarbital-treated controls, $P < 0.005$ vs. rats

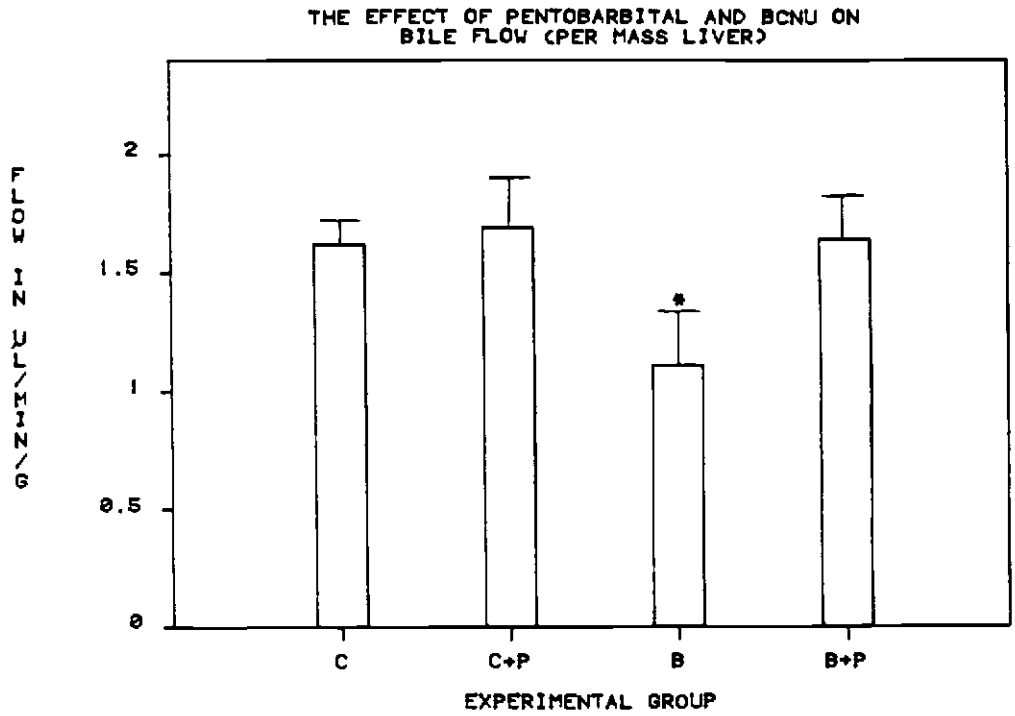


Figure 19. Bile flow in control rats (C), BCNU-treated rats (B), controls pretreated with pentobarbital (C+P) and BCNU-treated rats pretreated with pentobarbital (B+P).Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia 72 h after treatment with corn oil or BCNU. 50 mg/kg BSP was injected i.v. after a basal period (30 min) of bile collection. The mean and standard error are indicated (*, $P < 0.05$).

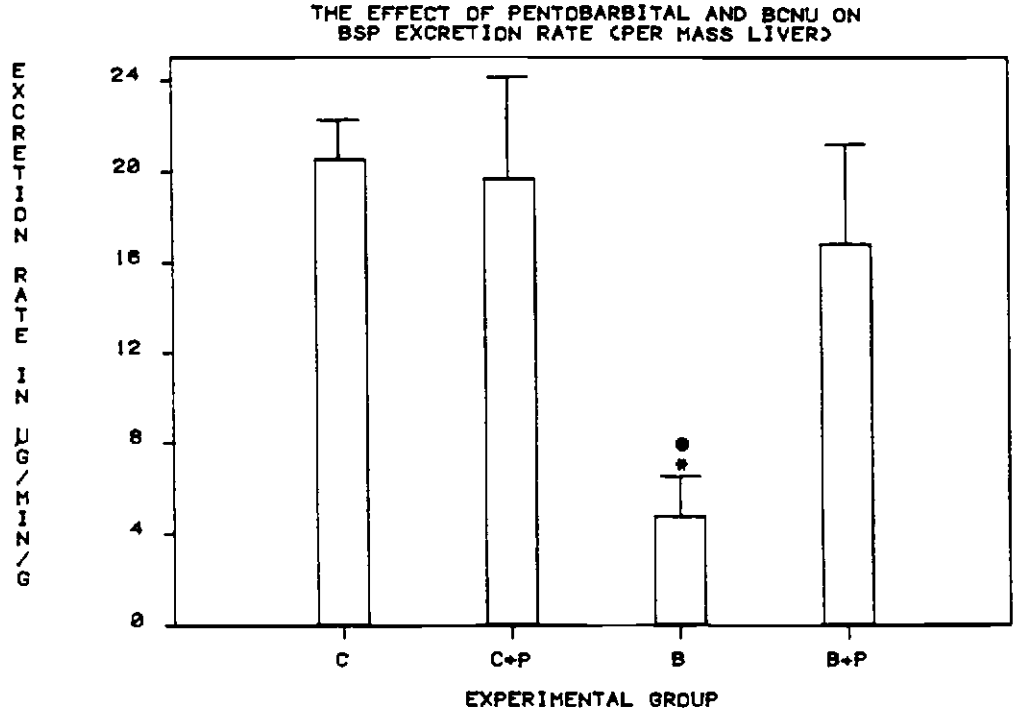


Figure 20. BSP excretion rate in control rats (C), BCNU-treated rats (B), controls pretreated with pentobarbital (C+P) and BCNU-treated rats pretreated with pentobarbital (B+P).Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia 72 h after treatment with corn oil or BCNU. 50 mg/kg BSP was injected i.v. after a basal period (30 min) of bile collection. The mean and standard error are indicated (✱, $P < 0.05$ vs. control; ●, $P < 0.05$ vs. B+P).

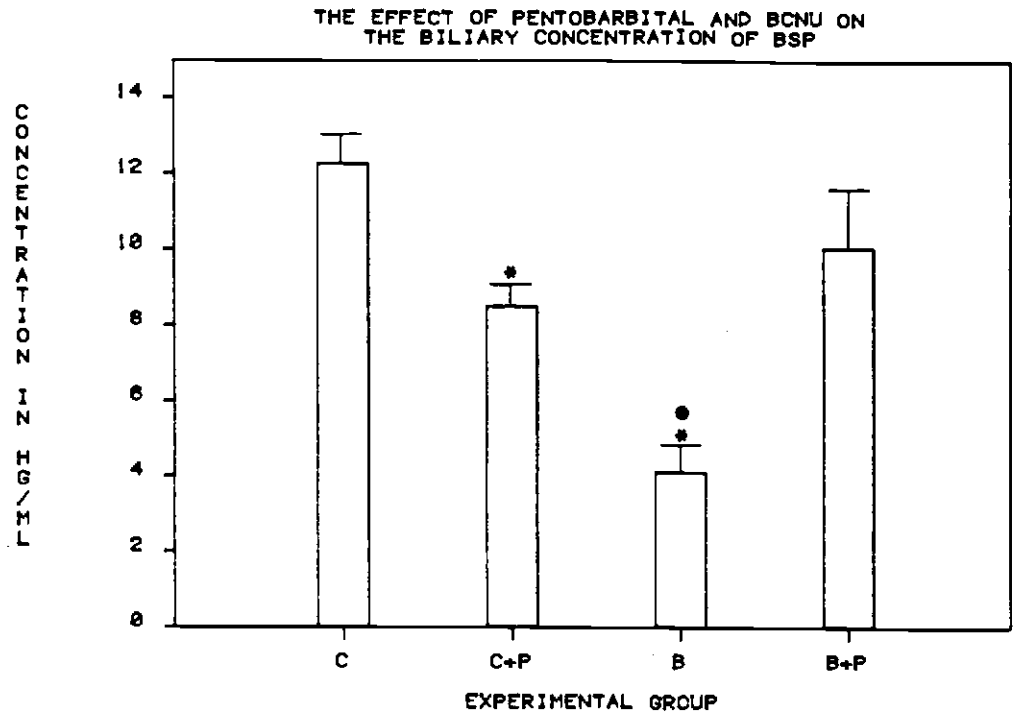


Figure 21. Biliary [BSP] in control rats (C), BCNU-treated rats (B), controls pretreated with pentobarbital (C+P) and BCNU-treated rats pretreated with pentobarbital (B+P).Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia 72 h after treatment with corn oil or BCNU. 50 mg/kg BSP was injected i.v. after a basal period (30 min) of bile collection. The mean and standard error are indicated (✱,P <0.05 vs. control; ●,P < 0.05 vs. B+P).

receiving both drugs).

DISCUSSION

Pentobarbital prevented the effect of BCNU on bile flow and BSP excretion. These effects were measured 3 days after BCNU administration and 5 days after the start of pentobarbital-treatment.

The presence of pentobarbital in the liver may have altered the interaction of BCNU with critical sites or may have influenced the development of changes that normally lead to cholestasis and inhibition of BSP excretion in BCNU-treated rats. Pentobarbital might also act on hepatic structures to stimulate bile production and organic anion excretion in BCNU-treated rats. Phenobarbital-pretreament may increase the BSIF by increasing the hepatic activity of Na^+/K^+ ATPase (Simon et al., 1977). Again, however, bile flow was not stimulated in controls although the inducibility of such a system may be increased in the treated rats. Wilson and Larson (1982) have observed a similar phenomenon with respect to phenobarbital induction of P-450 in BCNU-treated mice.

The effect of pentobarbital on BSP excretion was similar to that on bile flow except that the concentration of BSP in bile was reduced slightly in controls treated with the drug. The fact that the biliary concentration of BSP in rats treated with BCNU

and pentobarbital was normal is interesting. Phenobarbital induces GSH-S-transferase (Ernster and Orrenius, 1965; DePierre et al., 1984) and pentobarbital may have increased BSP metabolism by some increment, facilitating excretion, although this activity was not inhibited by 20 mg/kg BCNU at 48 h (chapter III).

Phenobarbital acutely stimulates GSH efflux into bile independently from choleresis (Kaplowitz et al., 1983). GSH excretion may be largely due to passive diffusion (Kaplowitz et al., 1983; Lauterberg et al., 1984). The action may be due to an effect of the barbiturate on the canalicular membrane itself that may also occur in the case of pentobarbital, normalizing the ability to concentrate BSP. Likewise, the uptake of BSP by the non-saturable mechanism that predominates at high plasma concentrations of BSP may be facilitated by a membrane action of pentobarbital in BCNU-treated rats.

Very recently the acute effects of pentobarbital-anaesthesia on bile flow were examined by comparison with surgically prepared, awake rats (Kuipers et al., 1985). These researchers found that pentobarbital (about 60 mg/kg i.p.) reduced bile flow for about 1 h, but then selectively increased BSIF 160% as time passed (Kuipers et al., 1985). The acute stimulation of BSIF was related to the excretion of pentobarbital and its metabolites (Kuipers et al.,

1985). The pentobarbital-treatment did not significantly stimulate flow in the control rats in this experiment (fig. 19). This argues against a significant effect of drug excretion the morning after the last dosage. In addition, although BSP excretion was slightly depressed in controls that also received pentobarbital-pretreatment, there was no inhibition of the ability to concentrate BSP in the rats treated with BCNU and pentobarbital. This argues against the hypothesis that the effects of pentobarbital- pretreatment are strictly related to its biliary excretion and implies some other action on the cellular structures associated with BSIF and BSP excretion.

The effect of pentobarbital pretreatment on the actions of BCNU may have been related to effects on the pharmacokinetic behavior of BCNU. One way to be sure would be to directly investigate this possibility by determining plasma concentrations of the nitrosourea as in the study of Levin et al. (1979). It may have been possible to administer BCNU before the start of pentobarbital treatments. Four days of pentobarbital treatments were used here. Under this schedule, BCNU could not be given until after the second or third injection to analyze bile production at an early time. Although absorption of the drug might have been altered by pentobarbital, care was taken to allow rats to awaken

on the morning of day 2 before administration of BCNU to preclude direct interaction of the 2 agents in the abdominal cavity.

The metabolism of BCNU appears to involve denitrosation to bis(2-chloroethyl)urea by a micrsomal enzyme (Hill, 1975). The mechanism may be similar to that for 1-chloroethyl,3-cyclohexyl-1-nitrosourea which is catalyzed by NADPH-cytochrome P-450 reductase, a phenobarbital-inducible enzyme (DePierre et al., 1981; Potter and Reed, 1983). Increased metabolism appears to be the mechanism by which phenobarbital reduces the i.p. bioavailability of BCNU (Levin et al., 1979). One must consider that BCNU was administered after the third injection of pentobarbital so that induction of BCNU metabolism would have been due to a cumulative dosage of 130 mg/kg pentobarbital and over an elapsed time of 26 h from the first injection. A single injection of 100 mg/kg doubled the hepatic reductase content in rats in the studies of Ernster and Orrenius (1965). As mentioned, it might be revealing to administer BCNU before starting the pentobarbital treatments. If BCNU exerts its effects in relation to rapid breakdown to reactive compounds and uptake of BCNU and/or degradation products by the liver, one might rule out pharmacokinetic considerations. Capron et al. (1977) showed that pentobarbital-pretreatments stimulated bile

flow in the absence of induction of microsomal cytochrome. Perhaps pentobarbital is also not able to increase the reductase-mediated metabolism of BCNU. The induction of hepatic enzymes by phenobarbital is not completely non-specific and NADPH-cytochrome P-450 reductase is increased by about the same factor and over the same time course as the cytochrome (Ernster and Orrenius, 1965; DePierre, 1981). Perhaps the lack of effect of pentobarbital on cytochrome content (Capron et al., 1977) reflects a muted induction of reductase also.

In conclusion, pentobarbital-treatments over 4 days prevented the impairment of biliary excretory function normally seen after BCNU-treatment. An attempt was made to prevent the interaction of BCNU and pentobarbital in the abdomen but chemical antagonism cannot be ruled out elsewhere in the animal. Pentobarbital may have altered the pharmacokinetic behavior of BCNU. Decreased absorption, increased excretion or altered distribution may have decreased the amount of BCNU reaching the liver. This could be important given that small changes in dosage alter the effects of BCNU drastically (Hoyt, 1984). Pentobarbital may or may not be very effective in stimulating metabolism of BCNU. Indirect considerations suggest it may not be effective. Bile flow and BSP excretion were

not increased in controls treated with pentobarbital alone suggesting that the result in BCNU-treated rats that also received pentobarbital-pretreatment was not just the addition of 2 opposite actions (physiologic antagonism), although direct stimulation of excretory systems may be more pronounced when they are not functioning at a high rate. The actions of pentobarbital were not related simply to the biliary excretion of pentobarbital. It is more likely that pentobarbital altered the interaction of BCNU with critical sites or that the drug may have influnced the development of cholestasis after critical interaction.

VII. The Effect of Theophylline and Glucagon on BCNU-induced Cholestasis

INTRODUCTION

The formation of BSIF dependends on the transport of sodium (Na^+), chloride (Cl^-) and bicarbonate (HCO_3^-) which exert an osmotic effect once in the canaliculus (Hardison and Wood, 1978; Van Dyke et al., 1982b; Anwer and Hegner, 1983). Advances have been made over the past few years in defining the mechanisms by which these inorganic electrolytes are excreted in bile. It has been an extremely difficult task for investigators to unravel the interaction of electrolyte transport systems, membrane permeability to ions and the activity of Na^+/K^+ ATPase mostly because one cannot sample canalicular bile (unmodified by ductular cells downstream from the site of initial formation) in vivo (Boyer, 1980).

The hepatocyte is a polar system with at least 2 grossly different membrane domains, the sinusoidal (basolateral) and the canalicular (apical) membranes (Boyer, 1980). there are usually 2 schemes for trasncellular transport of an electrolyte that are possible which involve systems located at either the canalicular or sinusoidal membrane. The question

invariably arises as to the location of a particular transport function (Scharschmidt and Van Dyke, 1983). If this question was more easily answered, the mechanism of bile formation might be better understood.

The activtiy of Na^+/K^+ ATPase as a factor in bile formation has been mentioned before. Its participation may be relatively indirect in that the enzyme is located mainly on the basolateral membrane which represents about 87% of the cell surface (Latham and Kashgarian, 1979; Boyer, 1980). It cannot directly inject osmotic activity in the form of Na^+ into bile. Instead, the enzyme maintains the cell membrane potential of around -30 mV inside and provides a Na^+ gradient that may drive transcellular inorganic electrolyte transport into bile (Boyer, 1980). Na^+ may then enter the canaliculus by a paracellular path via the junctional complexes to maintain biliary electroneutrality (Boyer, 1980; Erlinger, 1982). This is consistent with the concept that the junctional complex possesses an anion barrier (Bradley and Herz, 1978), although Cl^- can enter bile via junctions also (Boyer, 1980).

The sinusoidal membrane contains a Na^+/H^+ antiporter inhibited by amiloride (Arias and Forgac, 1984), and canalicular membrane vesicles contain a Cl^-/HCO_3^- antiporter inhibited by 4,4'-diisothiocyano-

2,2'-disulfonic acid stilbene and a Cl^-/OH^- (for example, pH sensitive) antiporter (Meier et al., 1984a; Warnock et al., 1984). In addition the bile salt, ursodeoxycholate, can stimulate HCO_3^- excretion in rat bile to a concentration above that in plasma, where the basal ratio is 1:1. This stimulation was inhibited by acetozolamide implying the involvement of carbonic annhydrase (Garcia-Marin et al., 1985). Thus, one sequence of events may involve the extrusion of protons into blood utilizing the energy of the ATPase-generated Na^+ gradient. This could increase intracellular HCO_3^- which could then exchange with biliary Cl^- resulting in HCO_3^- transport to bile. Some evidence indicates an alternative possibility that the canalicular membrane has a proton-ATPase which would increase biliary pH thereby facilitating HCO_3^- excretion directly (Oertle et al., 1983).

The involvement of Cl^- excretion is not well defined. One hypothesis is that Cl^- may be taken up from blood with the Na^+ gradient and enter bile along the favorable electrical gradient (Scharschmidt and Van Dyke, 1983). One consideration is that Cl^- removal from the perfusate of the isolated, perfused rat liver did not reduce BSIF unless HCO_3^- was removed first (Anwer and Hegner, 1983). Theophylline stimulated BSIF in the rat and in the isolated, perfused rat liver (Knodell,

1978; Anwer et al., 1984). Anwer et al. (1984), demonstrated that the action in the isolated rat liver was dependent on the presence of Cl^- in the perfusate and that dibutyryl cyclic adenosine monophosphate (db cAMP) produced a similar action although Cl^--dependence was not investigated. The concept was consistent with the ability of high doses of theophylline to raise intracellular cAMP however and indicated a role for Na^+-coupled Cl^- transport in bile formation (Scharschmidt and Van Dyke, 1983; Anwer et al., 1984).

The purpose of the following experiments was to stimulate BSIF directly in rats that were made cholestatic with BCNU-pretreatment. Pentobarbital may have stimulated the conversion of ATP to energy for bile production by stimulating ATPase activity. Theophylline and glucagon both stimulated BSIF in the rat and in the isolated rat liver could increase intracellular cAMP, and db cAMP stimulated BSIF (Thomsen and Larsen, 1981; Anwer et al., 1984). The actions of theophylline were dependent on the presence of Na^+ and Cl^- suggesting that stimulation of Na^+-coupled Cl^- transport in conjunction with elevated cAMP was the mode of action (Anwer et al., 1984). The question was whether agents known to stimulate BSIF could reverse the effect of BCNU which was to depress the BSIF (Hoyt, 1984). Theophylline and

glucagon were infused intravenously in an attempt to reverse the cholestasis caused by BCNU. Reversal by these agents might implicate cAMP or reduced transcellular electrolyte transport in the mechanism of BCNU-induced cholestasis.

MATERIALS and METHODS

Theophylline and glucagon were purchased from Sigma Chemical Co., St. Louis, MO. Bovine serum albumin was obtained from Calbiochem, San Diego, CA.

Rats were treated with 25 mg/kg BCNU or corn oil i.p. Three days after injection rats were anaesthetized with pentobarbital and the bile ducts were cannulated. The right femoral vein was cannulated with PE 50 tubing for infusion of theophylline or glucagon. Bile was collected for 90 min in 10 min intervals. After 3 intervals, infusions were initiated. Theophylline (8 mg/ml or 44.4 mM in 0.9% NaCl) was infused over 10 min starting 30 min after the beginning of bile collection. The total volume infused was 7.5 ml/kg (rate=.75 ml/min/kg) yielding a total dosage of 0.33 mmol/kg. Glucagon was dissolved in 0.9% NaCl/1.0% albumin and was infused constantly from 30 min using a Gilson Minipuls 2 pump at a rate of 0.5 µg/min/kg and 30 µl/min. The infusion continued for the rest of the test period. The average bile flows in each interval were used to compare the treatment groups using Student's t-test (Snedecor and Cochran, 1980). In each group, the post-infusion bile flows in each interval were compared with the average of the 3 pre-infusion intervals.

RESULTS

Theophylline and glucagon each stimulated bile flow in rats treated with 25 mg/kg BCNU 3 d earlier. Prior to infusion of theophylline, the BCNU-treated rats were cholestatic (fig. 22). The average bile flow for the 3 intervals before infusion was 2.01 $\pm$ 0.1323 µl/min/g of liver in controls and 1.31 $\pm$ 0.1098 µl/min/g in the treated rats (mean $\pm$ standard error; $P < 0.001$). Interval by interval comparisons with control and comparisons of bile flow after the start of infusion of theophylline with the average bile flows before infusion are depicted in figure 22. The bile flow at 20 min in BCNU-treated rats was not different from control statistically , but it is safe to say that BCNU depressed flow based on the comparison of the pre-infusion averages and the comparison of values at 10 and 30 min. Theophylline (0.33 mmol/kg) did not stimulate bile flow above the pre-infusion average in the control rats but its action on bile flow was pronounced in the BCNU-treated rats. In the latter group, flow was increased above the pre-infusion average from 50 min to the end of the experiment at 90 min (theophylline was infused between 30 and 40 min). Also, bile flow was stimulated to a value equal to control from the end of infusion at 40 min through 90 min.

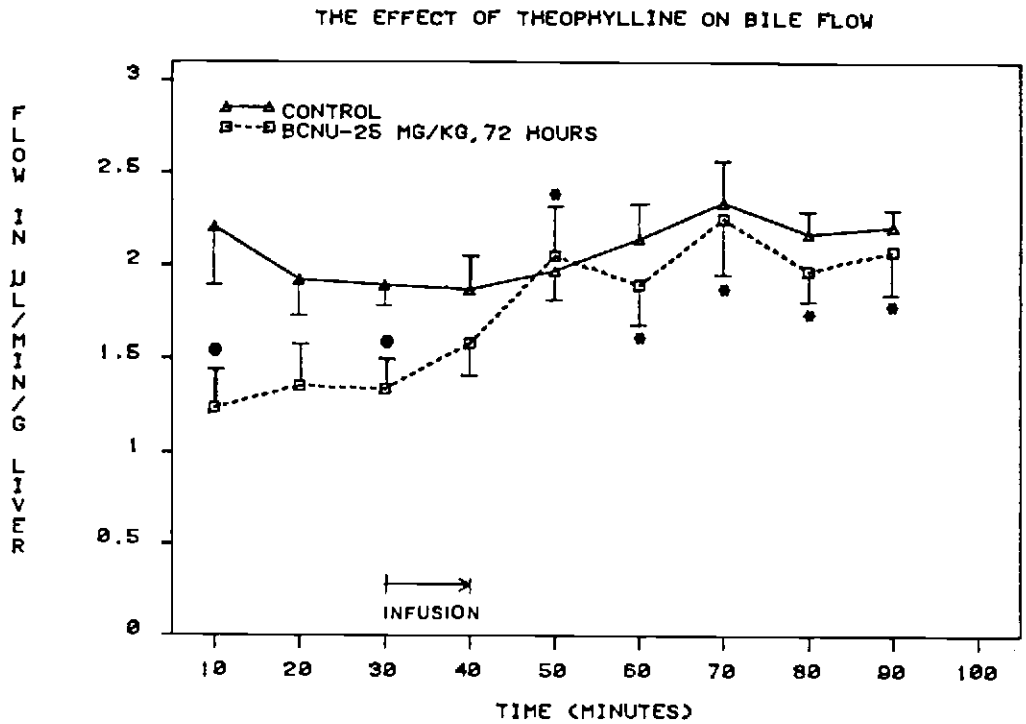


Figure 22. Bile flow in control rats and BCNU-treated rats, before and after administration of theophylline (0.33 mmol/kg i.v.) Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia 72 h after treatment. The mean and standard error are indicated (●, $P < 0.05$ vs. control; ✱, $P < 0.05$ vs. preinjection average).

The pattern was repeated under constant infusion of glucagon (0.5 µg/min/kg). Bile flow was depressed relative to control from 10 to 50 min into the experiment (fig. 23). The pre-infusion averages for bile flow were 1.86 $\pm$ 0.1131 µl/min/g of liver in control and 0.850 $\pm$ 0.1385 µl/min/g in BCNU-treated rats ($P < 0.001$). The infusion of glucagon did not increase bile flow in the control rats. However, flow was increased significantly above the pre-infusion average in rats treated with BCNU at 80 and 90 min (infusion started at 30 min) to a level equal to control.

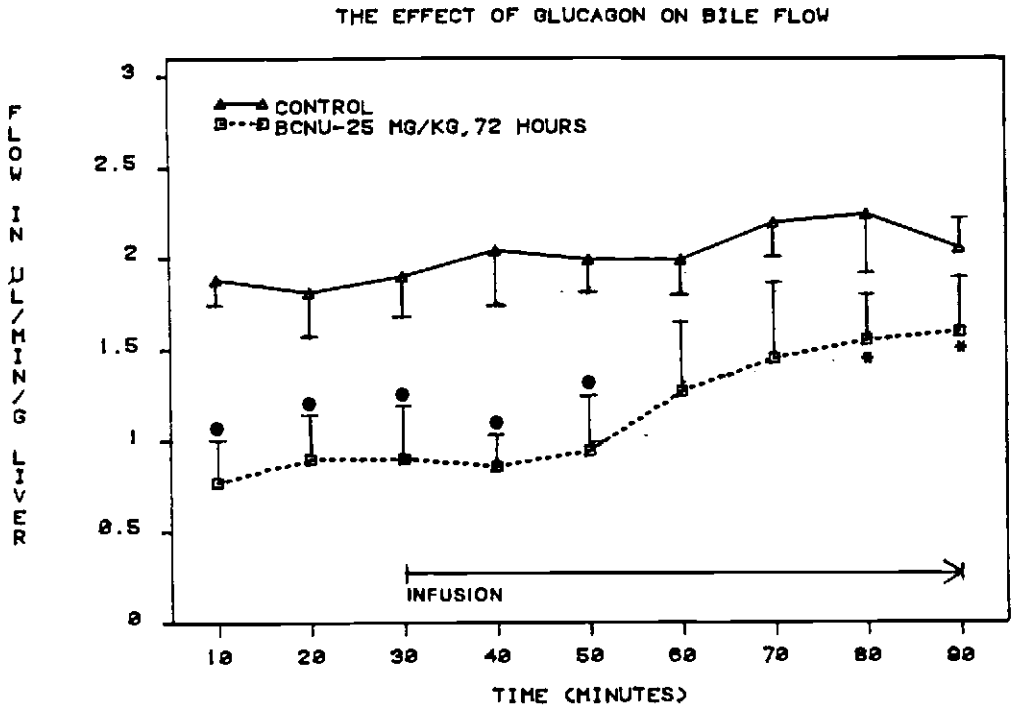


Figure 23. Bile flow in control rats and BCNU-treated rats, before and during infusion of glucagon (0.5 µg/min/kg i.v.) Rats received corn oil or 25 mg/kg BCNU in corn oil (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia 72 h after treatment. The mean and standard error are indicated (●, $P < 0.05$ vs. control; ✱, $P < 0.05$ vs. preinfusion average).

DISCUSSION

Theophylline and glucagon both increased bile flow in the BCNU-treated rats. Bile flow was not stimulated significantly in the controls although there was a tendency for an increase. The bile flow was stimulated to control levels in both cases although the dosage of theophylline employed increased flow a little more than did glucagon.

Because the choleretic effect of theophylline seems to be dependent on the presence of Na^+ and Cl^- (Anwer et al., 1984). The simplest conclusion from these results is that Cl^- transport was stimulated in the cholestatic rats resulting in choleresis. These results imply that the system for Cl^- transport was intact in BCNU-treated rats and, although it was not supporting normal flow, the capacity for stimulation was present. The results also indicated that water could enter bile at increased rates. Because osmolality was not determined, one cannot say whether there was an increased osmotic gradient. Normally bile is iso-osmotic with plasma (Boyer, 1980; Hoyt, 1984). Water flow could have been stimulated in treated rats by excessive solute concentrations. If osmolality was still high, restriction of water movement would be implied. Osmolality was not increased during cholestasis and bile

salt dependent bile flow, thought to indicate the osmotic action of biliary bile salts, was not altered previously (Balabaud et al., 1977; Hoyt, 1984). In this experiment, an attempt was made to administer about the same total amount of theophylline to the liver of the rat as was perfused into the isolated liver in the experiments of Anwer et al. (1984). They indicated that such a dosage (0.1 mmol/liver) would not produce sufficient biliary excretion of the drug to stimulate flow (Anwer et al., 1984). A determination of biliary theophylline concentration would be needed to verify this.

The action of theophylline could involve inhibition of phosphodiesterase intracellularly to increase cAMP since a large dosage was administered. The initial plasma concentration would have approached 7 mM if the injection was given instantaneously, assuming a plasma volume 4.8% of body weight. Inhibition of the adenosine receptor that inhibits adenyl cyclase occurs in the μM range (Daly et al., 1981).

The action of glucagon probably involved the stimulation of adenyl cyclase from the external surface of the cell (Rall and Sutherland, 1958; Pohl et al., 1971; Rodbell et al., 1971a; Rodbell et al., 1971b; Rodbell et al., 1971c; Herberg et al., 1984), although certain actions involving calcium homeostasis may occur

(Lotersztajn et al., 1984; Akatsuka et al., 1985). Theophylline may also influence intracellular calcium (Rall, 1980). With respect to bile formation, it is known that removal of calcium from the perfusate of an isolated liver and that excessive elevation intracellular calcium both cause cholestasis (Owen, 1977; Cho and Anwer, 1983). On the other hand it is apparent that dibutyryl cAMP is choleretic (Thomsen and Larsen, 1981; Anwer et al., 1984).

If the actions of theophylline and glucagon were mediated by cAMP, then the results suggest that the ability of cAMP to act on bile flow was not irreversibly inhibited by BCNU-pretreatment. The results may also indicate that glucagon can stimulate cAMP formation in BCNU-treated rats. This implies that the components of the adenyl cyclase system were functional under stimulation and possibly that the sinusoidal membrane, where the complex is located, was not sufficiently altered to prevent stimulation. The results also imply that electrolytes may enter bile when stimulated in BCNU-treated rats. However, they do not rule out the possibility that excessive stimulation was required to overcome effects of BCNU on electrolyte excretion.

Anwer and Hegner (1983) showed that the importance of Cl^- in the formation of BSIF could only be demonstrated in the absence of HCO_3^-. This

means that HCO_3^- supported BSIF completely when Cl^- was absent. This could explain the lesser effect of the 2 drugs in controls, in which it is assumed that HCO_3^- related mechanisms are normal. If this were true, then biliary HCO_3^- transport in BCNU-treated rats should be investigated. A lesser impact of HCO_3^- replacement on the basal bile flow of an isolated, perfused liver from a BCNU-treated rat might be expected.

The electrolyte dependence of glucagon-induced choleresis has not been determined. Glucagon stimulates Na^+/K^+ ATPase acutely and this may be responsible for choleresis (Fehlmann and Freychet, 1981). However, the increased ATPase may be secondary to stimulation of Na^+ influx via cotransport systems such as the alanine-preferring amino acid uptake (or Na^+- coupled Cl^- transport as postulated here) since the enzyme responds on a rapid time scale to maintain low intracellular Na^+ (Freychet and Le Cam, 1978; Van Dyke et al., 1983, Scharschmidt and Van Dyke, 1983).

The mechanism of choleresis in relationship to cAMP is not understood and all the discussion so far is simply circumstantial speculation (Scharschmidt and Van Dyke, 1983; Anwer et al., 1984). However, other epithelia increase electrogenic Cl^- transport in response to intracellular cAMP and/or calcium (Frizzell

et al., 1979).

In summary, glucagon and theophylline reversed the cholestasis caused by treatment with 25 mg/kg BCNU 3 days earlier. The results implied that the systems involved in bile formation were capable of functioning but that they were operating at a lower level than controls until stimulated. The controls were not significantly stimulated by theophylline or glucagon, perhaps because capacity for stimulation was greater when bile flow was low. The action of theophylline and glucagon may be related to increased cellular cAMP (Thomsen and Larsen, 1981; Anwer et al., 1984) which suggests a mechanistic link between cAMP and the cholestatic actions of BCNU.

VIII. Identification of Bile Salts Excreted in Bile During BCNU-induced Cholestasis

INTRODUCTION

Cholestasis is an enigmatic toxicologic response. It is difficult to define the mechanism(s) by which cholestasis develops, mainly because no one understands how bile formation takes place in detail. The ideas about causes of cholestasis developed from what was known about the cellular actions of the chemicals that caused it.

There are two types of cholestasis, extrahepatic and intrahepatic (Plaa and Priestly, 1977). Extrahepatic cholestasis is caused by an obstruction of the bile duct such as by ligation. Some may use this term to indicate a problem located exclusively in the grossly visable bile ducts. The liver first forms bile in small spaces between hepatocytes called canaliculi (Boyer, 1980). As bile moves from there to the small intestine, it encounters a different cell type, the ductular epithelium. Ductular epithelium is not inactive. It may absorb or secrete water and is the site of action of the hormone secretin, which causes the addition of extra water and HCO_3^- to bile (O'Maille *et al.*, 1966; Boyer, 1980). The previous investigations heavily

suggested that BCNU caused cholestasis by acting on hepatocytes since this is the site of excretion of BSP and because the bile:plasma ratio of the canalicular clearance probe, erythritol was not elevated by BCNU (Hoyt, 1984). The small bile ductules are visable by light microscopy within the organ (Elias, 1949). Their location may be the reason that some use the term intrahepatic cholestasis for cholestasis that does not involve the grossly visable ducts external to the liver. Intrahepatic cholestasis will be defined here as cholestasis involving hepatocyte function exclusively. The rat lacks a gall bladder but it was recently discovered that rats have an extensive anastamosing network of bile ductules in the portal tract not found in the dog, guinea pig or rabbit, but found in a less developed way in humans and monkeys (Yamamoto and Phillips, 1984; Yamamoto et al., 1985). Functions of storage and modification of bile in the rat have been proposed for the system but the function is unknown (Yamamoto and Phillips, 1984). The finding that BCNU causes intrahepatic cholestasis as defined here is not affected by this discovery however.

One proposal for a cause of intrahepatic cholestasis is inhibition of sinusoidal Na^+/K^+ ATPase (Plaa and Priestly, 1977). The activity of this enzyme in isolated membranes has been correlated with bile flow

under the influence of agents such as phenobarbital and thyroid hormone which are choleretic (Layden and Boyer, 1976; Simon et al., 1977) and ethinyl estradiol which is cholestatic (Heikel and Lathe, 1970; Gumucio et al., 1971). The interperetation of such a correlation between isolated membranes and the function of the liver has been questioned because of the rapid adjustments the enzyme makes to maintain intracellular homeostasis in isolated hepatocytes (Scharschmidt and Van Dyke, 1983). In this connection, ethinyl estradiol was shown to depress [86]-rubidium uptake by isolated hepatocytes (Berr et al., 1984). Other ATPases at the canalicular could be important as well (Curtis and Mehendale, 1981; Oertle et al., 1983).

Another proposal suggests that cytoskeletal microfilament function is important. Microfilaments surround the canaliculi and cause the canaliculi to contract rhythmicly (Oshio and Phillips, 1981). Taurocholate, a choleretic, stimulates contractions while the microfilament inhibitors phalloidin and cytochalasin B cause cholestasis and inhibit contractions (Watanabe et al., 1983; Phillips et al., 1983; Miyairi et al., 1984).

The maintainance of a semi-permeable barrier between bile and plasma may be important. Palloidin and alpha-napthylisothiocyanate cause cholestasis and

increase permeability (Elias et al., 1980; Krell et al., 1982; Jaeschke et al., 1983). This would prevent the maintainance of a concentration gradient between bile and plasma for substances such as bile salts that are concentrated in bile and the osmotic potential would dissipate resulting in cholestasis (Krell et al., 1982).

The proposal to be evaluated in the experiment that follows holds that an agent may cause cholestasis by inducing the synthesis of endogenous compounds that are known to inhibit bile production when infused. These might act by one or more of the aforementioned mechanisms. Specifically, the more hydrophobic bile salts are known to cause cholestasis (Fisher et al., 1971; Plaa and Priestly, 1977). Bile salts are synthesized from cholesterol, with the initial steps of ring hydroxylation occurring on the smooth endoplasmic reticulum (SER). Oxidation of the side chain of cholesterol is a function of mitochondria (Elliott and Hyde, 1971).

The first hydroxylation is mediated by cholesterol 7-alpha hydroxylase (Mitropoulos and Balasubraniam 1971). The enzyme is a NADPH- and cytochrome-dependent monooxygenase that is induced in rats with phenobarbital but more selectively by biliary drainage or oral cholestyramine (Danielsson et al., 1967; Boyd and Percy-Robb, 1971; Hansson and Wikvall,

1979; Hansson and Wikvall, 1980). It is a specific form of cytochrome P-450 with a shorter half-life than other hepatic monooxygenases (Danielsson and Wikvall, 1981). The presence of bile salts in the intestine normally maintains the 7-alpha hydroxylase at a low level so that new synthesis is not undertaken in the face of an efficient enterohepatic circulation (Boyd and Percy-Robb, 1971; Packard and Shepard, 1982). The mechanism is not by direct feedback inhibition of bile salts on the enzyme as originally assumed (Shefer et al., 1970), because addition of bile salt to the enzyme in vitro has no effect (Davis et al., 1983; Kubaska et al., 1985). Instead, it was demonstrated by Dos Santos et al. (1982) that the jejunum and ileum release a factor when bile salts are not present in the digestive tract that stimulates 7-alpha hydroxylase. The enzyme is also regulated by phosphorylation via cAMP-dependent protein kinase (Goodwin et al., 1982; Sundaram et al., 1983).

After 7 alpha-hydroxylation, the 3-beta hydroxyl of cholesterol is oxidized by the sequential action of a microsomal NAD-dependent dehydrogenase giving delta 4,7 alpha hydroxycholest-3-one (Elliott and Hyde, 1971). The double bond at C-4 allows the addition of a 5-beta hydrogen from NADPH by the action of delta 4,3-ketosteroid reductase, recently purified from a

100,000 x g supernatant of rat liver (Elliott and Hyde, 1971; Okuda and Okuda, 1984). This is an important point in the pathway since the introduction of a 5-alpha hydrogen generates the allo-bile salts which appear to be more toxic than the 5-beta bile salts (Vonk et al., 1981). The carbonyl left at C-3 is reduced ultimately by 3 alpha-hydroxysteroid dehydrogenase utilizing NADPH (Elliott and Hyde, 1971).

The synthesis of the predominant bile salt, cholate (3 alpha, 7 alpha, 12 alpha trihydroxy-5 beta-cholan-24-oate) requires the introduction of a hydroxyl at C-12 by the action of an NADPH dependent, cytochrome dependent, microsomal, 12 alpha hydroxylase which is a P-450 isozyme separate from the 7 alpha-hydroxylase (Elliot and Hyde, 1971; Bostrom and Wikvall, 1982). The enzyme is stimulated by a 60,000 dalton microsomal protein. Stimulation requires GSH, not GSSG, and the protein has GSH-S-transferase activity toward 1-chloro-2,4- dinitrobenzene (Danielsson et al., 1983). This could be an example of a pyhsiological function of the microsomal GSH-S-transferase (Morgenstern et al., 1980). Twelve alpha hydroxylation is important because it may not occur after the cholesterol side chain is cleaved by beta-oxidation to give the final C-24 carboxylic acids (Elliott and Hyde, 1971; Plaa and Priestly, 1977). The initiation of side

chain oxidation appears to mediated by a mitochondrial cytochrome P-450 monooxygenase that hydroxylates C-27 and appears important in keeping the ratio of cholate:chenodeoxycholate (3 alpha, 7 alpha dihydroxy- 5 beta- cholan-24-oate) at 4:1 in normal rat bile (Atsuta and Okuda, 1982). The hypothesis is that if side chain oxidation takes place before hydroxylation of the steroid nucleus, then the more hydrophobic bile salts will result (Schaffner and Popper, 1969; Plaa and Priestly, 1977). In fact, Mitropoulos and Myant (1967a and 1967b) showed that the cholestatic bile salts, lithocholate (3 alpha hydroxy-5 beta-cholan-24-oate) and chenodeoxycholate (Fisher et al., 1971) could result from cholesterol in vitro in the presence of rat liver mitochondria.

One further point concerning bile salt synthesis is that rodents, in contrast to other species, can make beta-muricholate (3 alpha, 6 beta, 7 beta trihydroxy-5 beta-cholan-24-oate) from lithocholate and chenodeoxycholate (Mitropolous and Myant, 1967a and 1967b). This may represent a rodent-specific detoxification based on the trend for decreased toxicity with increased hydrophilicity (Fisher et al., 1971; Plaa and Priestly, 1977). Liver injury secondary to bile duct ligation appears to be delayed in rats due to this capability (Thomas et al., 1964; Greim et al., 1972).

Although beta-muricholic acid is not found in man, humans can perform an analogous alpha hydroxylation of chenodeoxycholate and lithocholate at C-6 (Bremmelgaard and Sjovall, 1980).

In addition to the primary synthesis of bile salts, gut microflora can dehydroxylate bile salts. This causes the secondary synthesis of lithocholate from chenodeoxycholate and of deoxycholate from cholate (Elliott and Hyde, 1971).

The bile salts exist mainly in conjugated form (see RESULTS). Bile salts may be conjugated at the carboxylic acid with glycine or taurine and sulfated at the hydroxyl groups (Bremmelgaard and Sjovall, 1980). However, sulfated bile salts are absent or insignificant in the liver of rats and man after biliary obstruction (Greim et al., 1972). After injection of chenodeoxycholate , deoxycholate or cholate in humans with intrahepatic cholestasis, about 5% of the bile salts are excreted in urine and 80% of this is sulfated (Bremmelgaard and Sjovall, 1980). Bile salts may be conjugated to a limited extent with glucuronic acid at the 3-hydroxyl (Oelberg et al., 1984).

The original hypothesis suggesting that agents might cause cholestasis by altering bile salt synthesis was proposed by Schaffner and Popper (1969). They suggested that the toxic hydrophobic bile salts were

increased in conjunction with decreased metabolic activity and morphologic hypertrophy of the SER. The idea was plausible given the realization that 12 alpha hydroxylation did not occur after side chain cleavage of cholesterol and that primary synthesis of lithocholate and chenodeoxycholate was possible (Mitropolous and Myant, 1967a and 1967d; Elliott and Hyde, 1971; Plaa and Priestly, 1977). Side chain cleavage is carried out mainly by mitochondrial systems while hydroxylation is an activity of the SER (Schaffner and Popper, 1969; Elliott and Hyde, 1971; Plaa and Priestly, 1977; Atsuta and Okuda, 1982). Thus a decrease in the activity of the monooxygenases of the SER relative to mitochondrial activity could cause primary synthesis of toxic bile salts. Increased action of intestinal microflora could generate them as well.

The attractiveness of this theory is that the mono- and dihydroxy bile salts and the allo- bile salts are cholestatic when administered i.v. (Javitt and Emerman, 1968; Fisher et al., 1971; Kakis and Yousef, 1978; Vonk et al., 1981; Mathis et al., 1983; Oelberg et al., 1984). Various mechanisms for the induction of cholestasis by bile salts have been proposed. The cholestatic bile salts inhibit Na^+/K^+ ATPase (Hepner and Hofmann, 1973; Meijer et al., 1978; Kakis and Yousef, 1980). The actual mechanism of lithocholate-induced

cholestasis involves the canalicular membrane in that canaliculi are "dilated and displayed bizarre lamellar transformation" when observed by electron microscopy (Kakis and Yousef, 1980). Lithocholic acid infusion caused a 5 to 6-fold increase in canalicular membrane cholesterol (Kakis and Yousef, 1980). These findings suggest that membrane compostion, and possibly fluidity, affect the ability to secrete bile. Membrane composition and fluidity are determinants of the activity of membrane enzymes (Sandermann et al., 1978). The ability of bile salts to damage membranes is attenuated by low fluidity (Lowe and Coleman, 1981). Perhaps the incorporation of cholesterol during lithocholate infusion represents a protective action by the cell.

The purpose of the following experiment was to determine whether BCNU caused the production of cholestatic bile salts. Bile was analyzed by HPLC to separate 4 bile salts and their glycine and taurine conjugates.

MATERIALS and METHODS

HPLC grade methanol and reagent grade acetic acid were purchased from J.T. Baker Chemical Co., Phillipsburg NJ. Sodium salts of bile acids were used as standards. Cholate (C), litho- cholate (LC), deoxycholate (DC), chenodeoxycholate (CDC) and glyco-lithocholate (GLC)were purchased from Calbiochem, La Jolla, CA. Taurine conjugates of cholate (TC), deoxycholate (TDC), chenodeoxycholate (TCDC) and lithocholate (TLC) and glycine conjugates of cholate (GC), deoxycholate (GDC) and chenodeoxycholate (GCDC) were obtained from Sigma Chemical Co., St. Louis, MO. All bile salts were 97 to 99% pure by manufacturer specifications and were used without further purification.

Rats were treated with 25 mg/kg BCNU or corn oil i.p. After 72 h, rats were anaesthetized and a blood sample (1.0 ml) was taken by cardiac puncture. Plasma was obtained by centrifugation of blood placed in heparin-coated conical tubes at 4000 x g x 15 min. Bile flow was determined as usual after blood sampling.

Bile and plasma samples were added drop by drop to 20 volumes of boiling ethanol with simultaneous ultrasonication ot disperse the material. The denatured

samples were filtered and evaporated to dryness under vacuum (Eneroth and Sjovall, 1971). Methanol (an equal volume as the amount of sample processed) was added and this material was analyzed by HPLC.

The HPLC method used was that of Reid and Baker (1982). A Waters Intelligent Sample Processor, model 710B was used to inject the samples and standards. The pump was the Waters M 6000 model which was connected in series to a pre-column packed with C_{18} resin and a Waters Radial Compression Separation System (Z Module) containing a C_{18} cartridge. The bile salts were detected with a Waters R401 differential refractometer. Bile salt standards were cholate (C), deoxycholate (DC), chenodeoxycholate (CDC), lithocholate (LC), and the taurine (T) and glycine (G) conjugates of these four bile salts. The mobile phase was methanol:water 75:25 with acetic acid at a concentration of 2.5% v/v. The pH was adjusted to 5.25 with 10 M NaOH before use. The flow rate was 1.0 ml/min. Standard curves were constructed by plotting peak height vs. nmol of bile salt injected.

RESULTS

The HPLC method was successful in resolving 6 of 8 bile salt standards for quantitation (table 10). The correlation coefficient for linear regression of peak height and nmol bile salt was .999 or greater for TC, TCDC, TDC, GC and GCDC and .9983 for TLC. GDC and C were not resolved so the peak at about 21 min retention was characterized only qualitatively. Unconjugated chenodeoxycholate, deoxycholate and lithocholate were not detected in any samples.

The 25 mg/kg dosage of BCNU reduced bile flow to 63% of control 72 h after administration (table 11). TC was slightly but insignificantly increased in the the bile of BCNU-treated rats. Biliary concentrations of TCDC, GC and GCDC were decreased. The mean concentration of TDC was 25% of control. GCDC was not detected in the bile of treated rats. On a qualitative level, a peak corresponding to GDC and/or C was present in the bile of all but one control rat and was not detected in any treated rat. Conversely, this peak was present in the plasma of every treated rat and not in the plasma of control rats.

Table 10. HPLC Retention Times for Bile Salt Standards.[a]

Bile Salt	Retention Time (min)
Taurocholate (TC)	7.5
Taurochenodeoxycholate (TCDC)	10.0
Taurodeoxycholate (TDC)	10.8
Glycocholate (GC)	12.2
Taurolithocholate (TLC)	15.6
Glycochenodeoxycholate (GCDC)	18.3
Glycodeoxycholate (GDC)	21.0
Cholate (C)	21.4

a) the mobile phase was 75:25, methanol:water, with 2.5% acetic acid, adjusted to pH=5.25; flow rate was 1.0 ml/min; glycodeoxycholate and cholate were not sufficiently resolved for quantitation

Table 11. Biliary Bile Salt Concentrations in Cholestatic Rats.[a]

			Bile Salt Concentration					
Group	N	Bile Flow	TC	TCDC	TDC	GC	GCDC	GDC & C[b]
Control	5	1.99 (.12)	8.29 (.62)	2.02 (.08)	1.03 (.28)	3.01 (.45)	0.56 (.15)	4/5
BCNU	4	1.25[d] (.19)	10.16 (1.19)	0.11[f] (.11)	0.24[c] (.091)	0.28[e] (.13)	N.D.[g]	0/4

a) rats received corn oil or BCNU (25 mg/kg, i.p.) and bile was collected 72 hours later; bile flow is expressed as µl/min/g liver; bile salt concentration is in mM; values are means, the standard error is in parentheses

b) GDC and C were not resolved, the number of rats with a peak at 21 min retention/rats in the group is given

c) $P < 0.05$

d) $P < 0.025$

e) $P < 0.005$

f) $P < 0.001$

g) no GCDC was detected, $P < 0.025$ comparing control with zero

DISCUSSION

The most significant observation was that there was a decrease in the biliary content of the more hydrophobic bile salts. No derivatives of lithocholic acid were observed in BCNU-treated rats. These changes are similar to those just recently reported for ethinyl estradiol-induced cholestasis in women. Van der Werf et al., (1985) found a 25% increase in total cholates in duodenal bile of women , a 25% decrease in deoxycholates, and a 13% decrease chenodeoxycholates. In addition, the ratio of total glycine conjugates to taurine conjugates declined by 52%. A shift toward trihydroxy bile salts in rat liver was observed during alpha-napthyl isothiocyanate-induced cholestasis (Schaffner et al., 1973), and after bile duct ligation in rats (Kinugasa et al., 1981). These comparisons imply that the changes in biliary bile salt composition observed in BCNU-induced cholestasis are secondary to the reduced bile flow rate and are not reflective of the pathogenesis of cholestasis. One explanation may be that a shift toward the more hydrophilic bile salts and taurine conjugates serves to protect the organism in the compromised state. One possible mechanism might involve the not widely recognized fact that the jejunum and ileum may produce a factor that stimulates cholesterol-7

alpha-hydroxylase when intestinal bile salt content is decreased (Dos Santos et al., 1982). Stimulation of hydroxylase might generally facilitate the rate of hydroxylation of the steroid nucleus allowing more complete hydroxylation prior to side chain oxidation. The net result would be a shift to more completely hydroxylated bile salts.

Although it is possible that toxic bile salts were accumulated in liver of BCNU-treated rats this would not seem to be the case. Lithocholate causes cholestasis but is rapidly excreted in bile (Javitt and Emerman, 1968). The induction of cholestasis by lithocholate also precedes a reduction in bile salt excretion so that relatively high biliary concentrations of lithocholates are observed (Kakis and Yousef, 1978; Layden et al., 1977). Thus, it seems reasonable to expect that cholestatic bile salts should have been detectable in bile if they were elevated in response to BCNU. This is consistent with the fact that the ability to excrete bile salts in general was not greatly reduced by BCNU during cholestasis (Hoyt, 1984). The bile salt content of hepatocytes should be examined to rule this out definitely however. One detail that should also be explored further is the nature of the peak at 21 min retention on the HPLC, which appeared to be GDC or C. It seemed to be present in the plasma of treated rats and

not controls, and absent from the bile of treated rats but not controls.

The action of lithocholate to increase canalicular membrane cholesterol may indicate one mechanism of cholestasis that should be evaluated with respect to BCNU (Kakis and Yousef, 1980). It was also demonstrated that ethinyl estradiol (EE) causes an increase in cell membrane cholesterol esters by stimulating cholesterol:acyl-Coenzyme A transferase. Moreover, the detergent, Triton WR-1339, reverses the cholestasis and inhibition of Na^+/K^+ ATPase possibly by inhibiting cholesterol esterification (Davis et al. 1978; Simon et al., 1980). Triton WR-1339 might also increase membrane fluidity by its insertion into the membranes (Simon et al., 1980). The actions of EE are very similar to those of BCNU in many respects. The ability to concentrate bile salts was not compromised by either agent during cholestasis (Simon et al., 1980; Hoyt, 1984). Both agents inhibited BSIF (Gumucio et al., 1971; Hoyt, 1984), and neither one increased biliary permeability (Jaeschke et al., 1983; Hoyt, 1984). The major difference is that the ability to concentrate BSP is not impaired by EE, where this was a consistent action of BCNU (Forker, 1969; Hoenig, 1981; Hoyt, 1984; chapter I). The effect of BCNU on membrane composition should be investigated in the future.

The results imply that accumulation of toxic bile salts cannot explain the effects of BCNU on organic anion excretion either. However, sulfated bile salts were not quantitated in this study. Although they represent a minor constituent of bile, they do appear in the urine of cholestatic subjects and chenodeoxycholate-3-sulfate is a potent inhibitor of BSP excretion (Bremmelgaard and Sjovall, 1980, Eng and Javitt, 1983).

The consequences of cholestasis for the rest of the body may still involve bile salt accumulation despite the fact that there seemed to be a shift to less toxic species. Most bile salts disrupt cell membranes in high concentrations and other cells of the body may be less resistant than hepatocytes to this action (Lowe and Coleman, 1981). Bile and bile salts are toxic to the cardiovascular system of dogs (Wakim et al., 1940). Additionally, the jaundiced serum from bile duct-ligated rats is toxic to cultured rat heart cells and the effect is mimicked by deoxycholate (Better and Harari, 1983). A prolonged cholestasis and the possible shift to extrahepatic obstruction (Thompson and Larson, 1969) would be expected to cause general bile salt accumulation. This could be a cause for some delayed actions of BCNU on hepatic drug-metabolizing systems (Hutterer et al., 1970; Stolzenbach, 1984).

In conclusion, BCNU did not appear to cause cholestasis by inducing the production of toxic bile salts. Instead, the pattern of change in biliary bile salt composition was similar to that produced by ethinyl estradiol, alpha-napthyl isothiocyanate and bile duct ligation. This implied that such changes were secondary to cholestasis.

SUMMARY

BCNU inhibited the ability of rats to maximally concentrate BSP in bile. The single most likely site of action was the canalicular membrane. Under conditions of maximal transport the rate limiting step(s) should have been saturated. Uptake is not considered to be rate-limiting for BSP excretion. In addition, Thompson and Larson (1969) showed that conjugated bilirubin is the major component of total serum bilirubin soon after exposure of rats to BCNU. This suggested that uptake of unconjugated bilirubin from plasma was not greatly inhibited if at all. Since bilirubin and BSP share the same uptake mechanism, it is unlikely that uptake of BSP from plasma was inhibited.

BCNU inhibited the excretion of the non-metabolized dye, ICG. This demonstrated that the inhibitory actions of BCNU were not confined to intracellular metabolism of xenobiotics. Again, the canalicular side of the hepatocyte was indicated as a site of action for inhibition of organic anion excretion. Direct measurement of cytosolic GSH-S-transferase activity indicated that the conjugation of BSP with GSH, which facilitates excretion, was not inhibited in BCNU-treated rats. Neither was the hepatic content of the enzyme activity

reduced by BCNU-pretreatment. Verification of previous findings showed that the hepatic content of GSH was increased, so that there was no lack of substrate for conjugation.

Recent studies by others indicated that GSSG might compete with GSH conjugates for excretion, although the results were inconclusive regarding BSP-SG. Hepatic GSSG was increased 48 h after 20 mg/kg BCNU but was normal at 72 h, a time when BSP excretion was probably still inhibited. This suggested that elevated GSSG was not responsible for the inhibition of canalicular excretion of BSP. The same was true for hepatic GSH. The 20 mg/kg dosage of BCNU inhibited the excretion of both GSH and GSSG into bile further confirming the canalicular effects of the drug.

The inhibition of BSP excretion did not appear to be due to bile salt accumulation. Depletion of total bile salts with oral cholestyramine failed to increase the biliary concentration of BSP in BCNU-treated rats.

The effects of BCNU could be altered by administration of agents known to stimulate excretory function. Pentobarbital-pretreatments prevented cholestasis, possibly by stimulating BSIF, and allowed BCNU-treated rats to concentrate BSP in bile. The action of pentobarbital could possibly have involved an alteration of the pharmacokinetic behavior of BCNU,

reducing the amount of BCNU reaching the liver. Pentobarbital may have altered hepatocellular structures so that critical interactions with BCNU were reduced. Alternatively, pentobarbital may have altered the response of the liver to critical interactions, for example by influencing membrane composition.

Theophylline and glucagon also stimulated bile flow in BCNU-treated rats. The action of these agents probably involves elevation of cAMP. This may stimulate sodium-coupled chloride transport and increase BSIF. It is possible that cAMP was depressed in BCNU-treated rats and that infusion of theophylline or glucagon restored it to a satisfactory level. Alternatively, the system was stimulated to such an extent that effects of BCNU on other systems involved in bile formation were overcome. The effectiveness of glucagon may have indicated that the capacity to produce cAMP was present in BCNU-treated rats.

Analysis of the biliary bile salt composition suggested that BCNU did not induce the production of cholestatic bile salts. Instead, the composition shifted to less toxic, hydrophilic bile salts as was observed in cholestasis induced by alpha-napthyl isothiocyanate, ethinyl estradiol and bile duct ligation. The changes observed were probably a result of cholestasis rather than associated with the cause.

BCNU may exert effects on many systems involved with excretory function such as the cytoskeleton and basolateral transport systems. However,if one was to choose a single site of action for BCNU to account for its inhibition on hepatic excretory function, it would have to be the canalicular membrane. At the very least, BCNU caused a delayed action that affected that side of the hepatocytes.

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APPENDIX

The Effect of Fasting on BSP Excretion

INTRODUCTION

Earlier experiments showed that BCNU-treated rats lose weight (Thompson and Larson, 1969). The effect of fasting on bile flow was investigated previously but BSP excretion was not studied in that experiment (Hoyt, 1984). It was decided to test the effect of a 48 h fast on the excretion of a single i.v. injection of BSP. It should be noted that BSP excretion is inhibited by BCNU at this time (Hoyt, 1984).

METHODS

Control rats were allowed free access to food and water. Fasted rats received no food but had free access to water for 48 h prior to determination of bile flow and the biliary excretion of a single i.v. injection of 50 mg/kg BSP during pentobarbital anaesthesia.

RESULTS

Previous experiments showed that fasting for 48 h did not affect bile flow (Hoyt, 1984). The experiments were repeated in control rats to examine a possible effect on BSP excretion. Fasting again did not affect bile flow (table 12). Neither did a 48 h fast

Table 12. The Effect of Fasting (48 h) on Bile Flow and BSP Excretion[a]

Group	N	Bile Flow (µl/min/g liver)	[BSP] in Bile (µg/µl)	BSP Excretion Rate (µg/min/g liver)
Control	3	1.71 ± 0.1826	12.29 ± 1.056	22.52 ± 7.474
Fasted	3	1.85 ± 0.2766	9.65 ± 0.737	17.95 ± 2.276

a) values are mean ± standard error

significantly alter the BSP excretion rate or the biliary concentration of BSP.

DISCUSSION

Fasting for 48 h had no effect on bile flow or BSP excretion. The action of BCNU cannot be explained by anorexia.

The Effect of BCNU on Liver and Body Weight

RESULTS

BCNU at a dosage of 20 or 25 mg/kg did not alter liver weight. Liver weight in the 15 mg/kg group at 12 d was increased (table 13). Liver to body weight ratios were increased in all groups except 24 h after 20 mg/kg BCNU. This is indicative of drug-induced weight loss at these times. The weight loss is probably due to the anorexic effect of BCNU (Hoyt, 1984).

Table 13. The Effect of BCNU on Liver Weight and Liver/Body Weight Ratio[a]

Group	Time after treatment	N	Liver Weight[b] (g)	Liver/Body Weight[c] (g/100 g)
Control	----	5	10.57	3.57
BCNU				
15 mg/kg	11 d	5	11.74	4.34 ***
	12 d	4	12.68 *	4.43 ***
	13 d	4	11.88	4.82 ***
20 mg/kg				
	24 h	5	10.18	3.85
	36 h	5	11.10	4.13 **
	48 h	5	11.90	4.13 **
	14 d	7	9.35	4.31 ***
25 mg/kg				
	72 h	7	9.93	4.27 ***

a) values are mean
b) MSE= 2.001 g
c) MSE= 1.4087 g/100 g
*) P < 0.025 for comparison with control
**) P < 0.01 for comparison with control
***) P < 0.001 for comparison with control

The Effect of BCNU on Hepatic Blood Flow as Determined by LASER Doppler Veolcimetry

INTRODUCTION

Because blood flow affects the excretion of materials that are efficiently extracted from blood by the liver (Pang, 1980; Klaassen and Watkins, 1984), a determination of the effect of BCNU-pretreatment on hepatic blood flow was attempted.

METHODS

Relative hepatic blood flow was measured in controls and rats treated with 20 mg/kg BCNU. The treated rats were analyzed 24 h, 36 h, 48 h and 13 d after administration of BCNU. At the designated times rats were anaesthetized with pentobarbital and an abdominal incision was made. Blood flow was measured by LASER Doppler Velocimetry using a Medpacific LD 5000 LASER Doppler Capillary Perfusion Monitor. The device projects light at a wavelength of 632.8 nm, generated by a 5 mW helium-neon LASER, from an optical fiber to approximately one mm into the tissue under examination. Reflected light is transmitted back to a photodiode by another optical fiber. The broadening of

the wavelength spectrum is converted to a potential difference which is recorded on a chart. The broadened spectrum results from the reflection of photons by moving particles, erythrocytes in this case (Holloway et al., 1977; Stern et al., 1977; Bonner et al., 1981; Feld et al., 1982).

The signal displayed was in millivolts. The signal cannot be absolutely related to flow without an independent determination, but it is sufficient for a comparison with control rats. The left and right lobes of the liver were probed at one site each and the median lobe was probed at two sites, one within 1 mm of the edge of the liver. Measurements were performed in triplicate at each site and were averaged for a reading. There appeared to be no trend among the sites so all readings were averaged for each experimental group. The function of the instrument was ascertained by transient occlusion of the portal vein in several rats. Also, the effect of the technique of BSP injection into the inferior vena cava was determined in one control and one treated rat (48 h after 20 mg/kg BCNU).

RESULTS

No decrease in hepatic blood flow, relative to control, could be detected (fig. 24). Flow was slightly

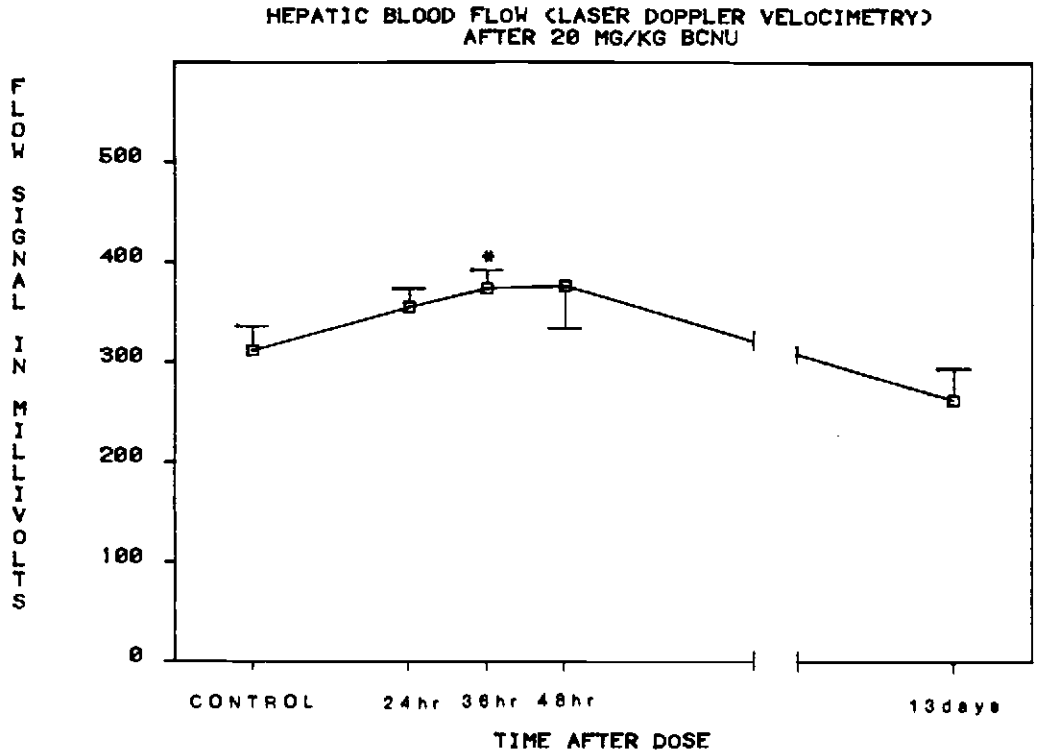


Figure 24. Hepatic blood flow in control rats, and BCNU-treated rats. Rats received corn oil or 20 mg/kg BCNU in corn oil (1.0 ml/kg). The mean and standard error are indicated (*, $P < 0.05$ vs. control).

but significantly higher in rats treated with 20 mg/kg BCNU 36 h prior to measurement.

A continuous recording during injection of BSP into the abdominal vena cava showed that hepatic blood flow was lowered immediately after the injection. It returned to the preinjection value within 2 min in a control rat and within 5 min in a rat treated with BCNU 48 h previously (data not shown).

This analysis cannot completely rule out changes in the distribution of blood flow within the liver considering the small depth to which the LASER light penetrates. Changes in perfusion could still contribute to effects of BCNU on hepatic excretory function.

The Effect of 1,3-bis(4-hydroxycyclohexyl)-1-nitrosourea on Bile Flow and BSP Excretion

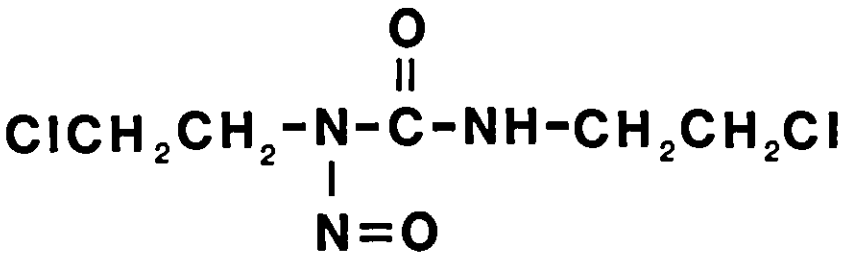
INTRODUCTION

The actions of BCNU may be related to its ability to alkylate and carbamylate cellular molecules. THe following experiment was designed to examine which effect might be more important. The effect of 1,3-bis(4-hydroxycyclohexyl)-1-nitrosourea (BHCNU) on bile production and BSP excretion was determined. This nitrosourea does not alkylate (Tew et al., 1985). The hypothesis was that if BHCNU caused hepatotoxicity, carbamylation might be implicated in the action of BCNU. If toxicity was not caused by BHCNU, then alkylation might be more important. The structures of BCNU and BHCNU are in figure 25.

METHODS

BHCNU was dissolved in propylene glycol:ethanol, 80:20. Rats received 27 mg/kg i.p. or the vehicle at a volume of 1.0 ml/kg. 27 mg/kg is 93.4 µmol/kg and is equimolar to 20 mg/kg BCNU. Determinations of bile flow and biliary excretion of a single i.v. injection of 50 mg/kg BSP were made 7, 14 and 28 d after treatment with

BCNU



BHCNU

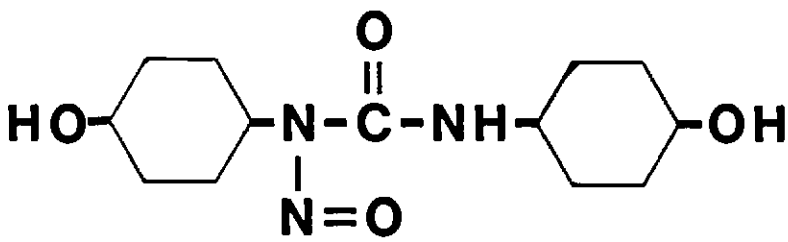


Figure 25. The structures of BCNU and BHCNU.

BHCNU in rats anaesthetized with pentobarbital.

RESULTS

BHCNU at a dosage of 27 mg/kg (approximately a molar equivalent dose to 20 mg/kg BCNU) did not inhibit bile flow or BSP excretion (figs. 26, 27, 28). In fact bile flow was elevated slightly 14 d after treatment. The BSP excretion rate and biliary concentration of BSP were unchanged although there was a trend toward increases at 14 and 28 d.

DISCUSSION

BHCNU exerted no effect on bile flow or BSP excretion. These results suggest that BCNU might cause its effects primarily by alkylation. However, BHCNU is more soluble in polar solutions than BCNU. It was dissolved in porpylene glycol:water because it is insoluble in corn oil in contrast to BCNU. One might expect the pharmacokinetic behavior to differ on this basis. Also, the functional group resulting from breakdown of BHCNU (4-hydroxycyclohexyl isocyanate) is larger than the chloroethyl isocyanate produced by BCNU and may interact in qualitatively and quantitatively different ways with the cells of the organism. Both BCNU

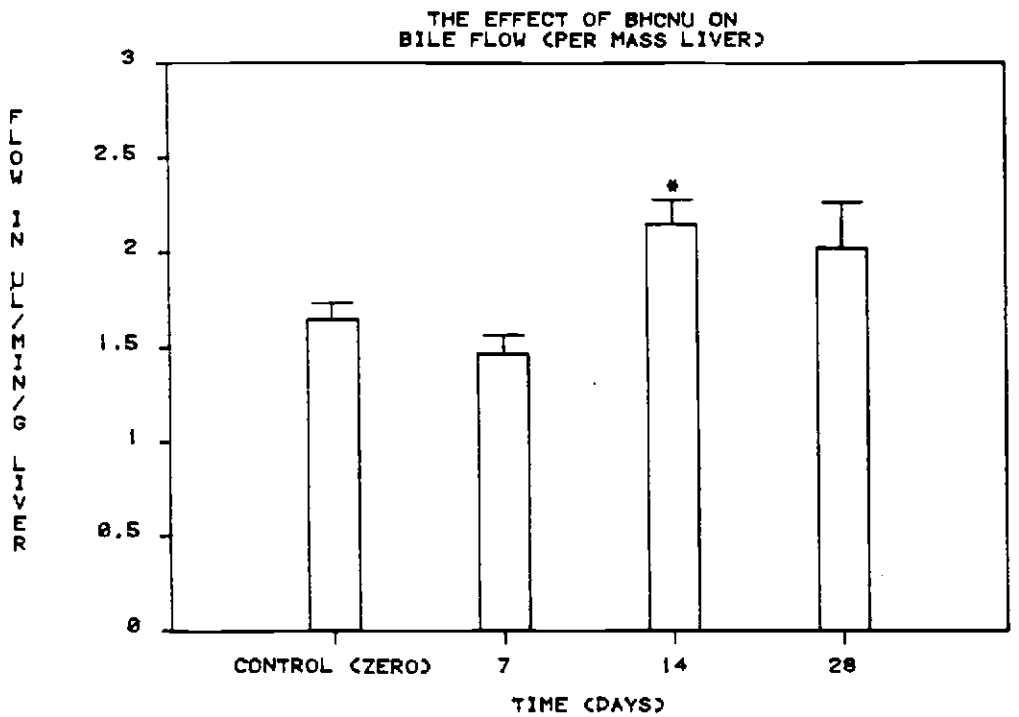


Figure 26. Bile flow in control rats and BHCNU-treated rats. Rats received propylene glycol:water or or 27 mg/kg BHCNU in propylene glycol:water (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia at the indicated times after treatment. 50 mg/kg BSP was injected i.v. after a basal period (30 min) of bile collection. The mean and standard error are indicated (* ,P <0.05).

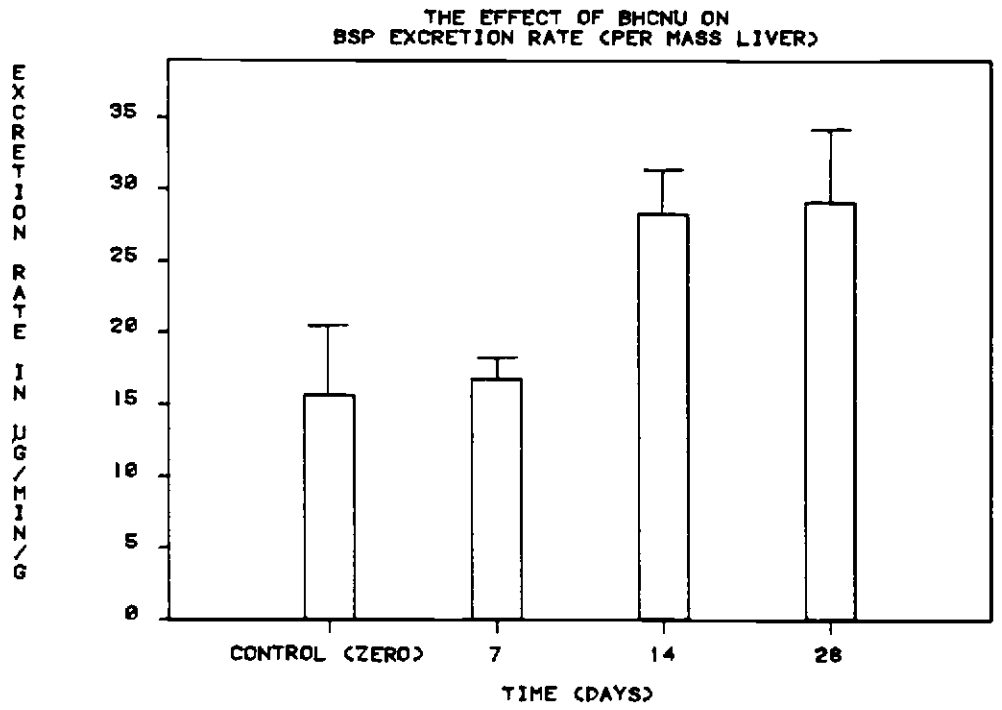


Figure 27. BSP excretion rate in control rats and BHCNU-treated rats. Rats received propylene glycol:water or or 27 mg/kg BHCNU in propylene glycol:water (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia at the indicated times after treatment. 50 mg/kg BSP was injected i.v. after a basal period (30 min) of bile collection. The mean and standard error are indicated.

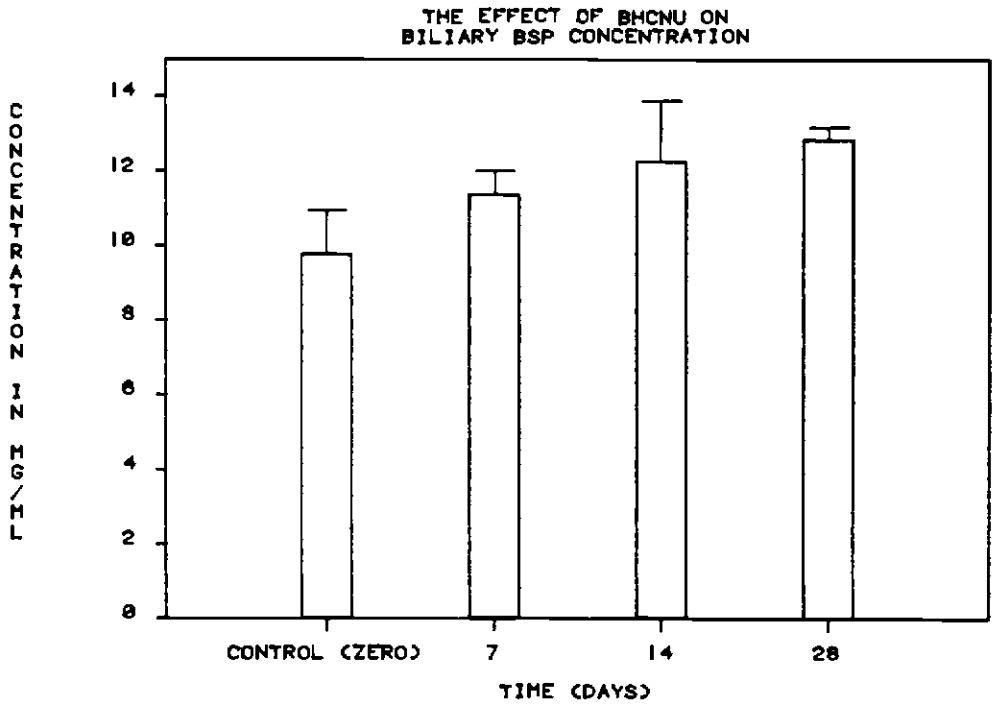


Figure 28. Biliary [BSP] in control rats and BHCNU-treated rats. Rats received propylene glycol:water or or 27 mg/kg BHCNU in propylene glycol:water (1.0 ml/kg). Bile was collected under pentobarbital anaesthesia at the indicated times after treatment. 50 mg/kg BSP was injected i.v. after a basal period (30 min) of bile collection. The mean and standard error are indicated.

and BHCNU can inactivate glutathione reductase however (Babson and Reed, 1978; Tew et al., 1985).