AN ABSTRACT OF THE THESIS OF

Dale G. Hoyt for the degree of Master of Science in Pharmacy presented on April 13, 1984 .

Title: Characterization of Cholestasis Induced by 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) in Rats.

Abstract approved: Redacted for privacy

Robert E. Larson, Ph.D.

BCNU caused severe cholestasis in rats after a single intraperitoneal injection. The cholestasis was characterized by a selective reduction of the bile salt independent bile flow to 11% of control. Decreased food or water consumption by the treated rats apparently did not contribute to cholestasis.

Measurements of plasma Na^+ and K^+ concentrations were consistent with current concepts regarding the role of Na^+/K^+ ATPase in the formation of bile salt independent bile flow. Nevertheless, the possibility that the permeability of water to the canalicular space may be reduced in treated rats remains to be evaluated.

Although bile salt excretion rates were slightly reduced in treated rats during cholestasis, bile salt concentrations in bile were elevated. Therefore, bile salt excretion was limited by cholestasis, not by a defect in concentrative transport. This increase in biliary bile salt concentration and increases in bile:plasma osmolality ratios tend to discount a breakdown of the bile:plasma permeability barrier as a cause of cholestasis in BCNU treated rats.

In contrast to the effect of BCNU on bile salt excretion, treated rats failed to concentrate the xenobiotic organic anion, sulfobromophthalein (BSP), in bile to a normal extent. This was evident prior to the onset of cholestasis and, therefore, may be reflective of the lesion that causes cholestasis.

Because BSP excretion depends largely upon conjugation of the dye with glutathione (GSH), hepatic GSH and BSP metabolites in bile were monitored. BCNU caused an increase in hepatic GSH before its effects on BSP excretion and bile flow were evident. The effect on GSH may be a result of stimulated synthesis, inhibited enzymatic degradation, inhibited efflux from the hepatocyte, or a combination of these. In any case, the decreased BSP excretion was not due to a lack of substrate for conjugation. BCNU, besides inhibiting BSP excretion, produced a qualitative change in BSP metabolites found in bile. This effect may be due to an alteration of GSH-S-transferase activity in treated rats. However, decreased canalicular excretion or basolateral uptake may acount for the results.

CHARACTERIZATION OF CHOLESTASIS INDUCED BY 1,3-bis-(2-CHLOROETHYL)-1-NITROSOUREA IN RATS

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHARACTERIZATION OF CHOLESTASIS INDUCED BY 1-3-bis-(2-CHLOROETHYL)-1-NITROSOUREA IN RATS

Introduction

Cholestasis is simply defined as a reduction in bile flow. The phenomenon may be divided into two classes based on the anatomical site of the primary lesion. Thus, extrahepatic cholestasis is caused by lesions at sites other than hepatocytes and bile canaliculi (1, 2). This category includes cholestasis due to mechanical obstruction of the bile ducts or changes in bile duct structure and function. Intrahepatic cholestasis results from an alteration in hepatocyte function or from changes in canalicular structure (1, 2). Many agents cause intrahepatic cholestasis and many of these agents are used therapeutically. The wide spectrum of compounds with definitive actions on hepatic function has inspired the development of several theories that seek to explain cholestasis (1). This discussion will be confined to theories of intrahepatic cholestasis.

The first theory to be considered deals with the function of the smooth endoplasmic reticulum (SER) of the hepatocyte. It was proposed that cholestasis could be caused by the development of a hyperplastic, hypoactive, smooth endoplasmic reticulum (HHSER) (1, 3). This theory is basically concerned with the activity of the mixed function oxygenase enzymes associated with the SER. Hyperplasia of the organelle may be functionally irrelevant in that the products of altered enzyme activity are believed to be toxic. It is known that bile acids are synthesized from cholesterol along biochemical pathways

that include the SER and mitochondria. Ring hydroxylations are performed by mixed function monooxygenases of the SER while the cholesterol side chain is oxidatively shortened by mitochondrial enzymes (4, 5). It is also known that certain bile acids, (the allo-bile acids and mono- and dihydroxy bile acids) cause cholestasis when infused while cholic acid and taurocholic acid are choleretic (6, 7, 8, 9). Because 12-alpha-hydroxylation does not occur after side chain oxidation, and because the potential primary synthesis of lithocholate, a monohydroxy bile acid, has been demonstrated, it has been proposed that agents which alter the relative activity of mitochondrial and SER enzymes could cause cholestasis by an increase in the synthesis of cholestatic bile acids (1, 5). Because cholestasis affects microsomal mixed function oxygenases, an alteration in bile salt metabolism should be demonstrated prior to the onset of cholestasis if this mechanism is relevant (10).

A second theory proposes that the activity of the enzymes sodium, potassium-adenosine triphosphatase (Na/K-ATPase) and magnesium-adenosine triphosphatase (Mg-ATPase) are important in the transduction of chemical energy for the formation of the bile salt independent (BSIF) and bile salt dependent flows (BSDF) respectively (1, 11, 12, 13). The BSIF, as estimated by a single linear regression of bile flow or erythritol clearance on bile salt excretion rate, has been found to correlate with the activity of Na/K-ATPase of various hepatocyte membrane fractions (1, 14, 15, 16, 17, 18, 19). The BSIF has also been found to depend on bicarbonate, chloride and sodium in the perfusate of isolated perfused rat livers (15, 20, 21, 22, 23). These ion fluxes may ultimately be driven by or coupled to an established

Na^+ gradient and the movement of these solutes could be responsible for the movement of water into canalicular bile (11, 20, 23).

The BSDF has been interpreted to be a result of the osmotic activity of bile salts in bile. In this discussion, it will be defined as the slope of the relationship between erythritol clearance and bile salt excretion. Although not proven conclusively, the high concentration of bile salts in bile would suggest the presence of active transport of these compounds which could be driven by ATPase. Transport could also be driven either by a tendency to form micelles which decreases the free concentration in bile, or by a membrane potential (11, 24). The uptake of certain bile salts is sodium dependent (25). An agent that affects the movement of bile salts into bile would be expected to simply reduce bile flow while an agent that affects the composition of micelles in bile would both alter the BSDF (volume of bile per bile salt molecule excreted) and bile flow.

A third theory postulates that disruption of canalicular microfilaments can cause cholestasis. It was developed to explain the cholestatic effects of compounds known to interact with actin of the hepatocellular cytoskeleton (1, 26, 27, 28). Recently it has been shown that canaliculi regularly open and close in isolated, bicellular hepatocyte complexes, and that cytochalasin B interferes with this process (28, 29). A canalicular pumping action is implied by these results but its contribution to bile flow has not been evaluated at present.

Finally, the maintenance of the structure of the tight junctions that isolate the canaliculi from plasma has been viewed as important

in bile formation. Microfilaments may play a role here as well since phalloidin has been shown to interfere with their function and to increase permeability between bile and plasma (26). That an increase in bile to plasma permeability can cause cholestasis is supported by the acute effect of alpha-napthylisothiocyanate (ANIT), although the mechanism here may or may not involve the microfilaments (30). ANIT causes distortion of canaliculi prior to the development of cholestasis. Diverticuli are observed under the electron microscope and these can penetrate to the Space of Disse (31). Functionally this effect is confirmed by increased bile: plasma sucrose and phosphate ratios in the isolated, perfused rat liver (30). In addition there appears to be a decrease in the number of microfilamentous strands in the junctional complexes (30). All of these effects imply that there is an increase in permeability between bile and plasma after ANIT treatment, prior to cholestasis. This results in an inability to concentrate solutes in bile and cholestasis develops (30).

The studies described herein are concerned with the development of cholestasis due to the antineoplastic drug, 1,3-Bis(2-Chloroethyl) -1-Nitrosourea (BCNU or carmustine). Previously BCNU had been identified as a hepatotoxic agent in rats and humans (32). The toxicity of BCNU in rats after a single dose was recognized as a delayed effect resulting in retention of BSP and elevated serum bilirubin. Also, livers were distorted, taking on a hob-nailed appearance under gross examination. Histologically, there was pericholangitis and a distortion of the lobular architecture. These effects are similar to those produced by chronic administration of ANIT (1, 32). Over a

delayed time course, BCNU causes progressive but differential effects on hepatic mixed function oxygenases and their response to induction (33, 34, 35). These effects and as yet unidentified acute effects on mixed function oxygenases may have relevance within the HHSER theory of cholestasis.

Therefore, in the present study, a characterization of the nature of the cholestasis and its time course of development was undertaken. It was felt that a determination of the time course of the effect of BCNU would provide a reference for other mechanistic studies. One philosophical framework used in the study for the elucidation of mechanism is that the pathogenesis is characterized by a temporal ordering of critical primary and secondary lesions. Changes in function that occur after the development of cholestasis cannot be considered as causes of cholestasis. This philosophy has been applied to the development of cholestasis due to ANIT (30, 36). It cannot be assumed that all changes in function that occur prior to cholestasis are necessarily causative.

It is hoped that this study will be valuable for at least two reasons. First, it may be hoped that elucidation of the mechanisms of cholestasis could lead to its mitigation in cases of drug induced cholestasis. This might be accomplished through optimal dosing for the desired drug effects relative to the cholestatic effect or through the wise use of antagonism. The second purpose is to expand man's knowledge concerning the physiology of bile formation. This knowledge has been limited by technological considerations to this date (11).

Materials and Methods

Materials. Polyethylene tubing (PE 10, PE 50, PE 90) was purchased from Clay Adams, Parsippany, NJ. The following reagents were obtained from Sigma Chemical Co., Saint Louis, Mo.: Sulfobromophthalein, sodium taurocholate, 3-alpha-hydroxysteroid dehydrogenase (EC 1.1.1.50), and beta-nicotinamide adenine dinucleotide (oxidized). Ethylenediaminetetraacetic acid (EDTA), glycine, hydrazine sulfate, methanol, microcrystalline cellulose and monobasic, dibasic and tribasic sodium phosphate were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ. Sodium toluene-p-sulfonate was obtained from Eastman Kodak Co., Rochester, NY. [^{14}C]-erythritol was purchased from the Radiochemical Centre, Amersham, England. Dimilume-30 fluor was purchased from Packard Instrument Companies Inc., Downer's Grove, IL. Metaphosphoric acid was obtained from Mallinckrodt Inc., Paris, KY. Ortho-phthalaldehyde was purchased from Nutritional Biochemicals Corporation, Cleveland, OH, and reduced glutathione was obtained from Boehringer Mannheim GmbH, West Germany. All chemicals and enzymes were of the highest reagent grade available and were used without further purification.

Treatment. Male Sprague-Dawley rats, weighing 250 to 350 g, were given either 15 mg/kg or 20 mg/kg BCNU in corn oil at 1.0 ml/kg i.p. in one dose. Animals were housed in pairs until the time of analysis, with food and water ad lib. Control rats, which received 1.0 ml/kg corn oil, were included with each group of treated rats. Experiments were carried out at the indicated time after dosing.

Where surgery was required, rats were anaesthetized with pentobarbital sodium in water: propylene glycol: ethanol (7:2:1) which was administered intraperitoneally at a dosage of 60 to 90 mg/kg.

For the determination of bile flow a midline abdominal incision was made, and the bile duct was isolated and cannulated with PE 10 polyethylene tubing. Bile was collected in plugged 1.0 ml glass syringe barrels (minimum graduation = .01 ml) and the time of appearance of bile at the tip of the cannula was noted. All surgery was performed between 8 am and 1 pm.

The Effect of BCNU on Bile Flow, Sulfobromophthalein (BSP) Biliary Excretion and Plasma Retention. For the analysis of BSP excretion, BSP in distilled water was given intravenously (50 mg/kg, 1 ml/kg) in the posterior vena cava. When BSP appeared in the bile collection tube, the cannula was transferred to a new tube. Blood samples (150 μl) were taken 15 and 30 minutes after BSP injection from a cut in the tail for determination of BSP retention (32).

Bile flow was calculated as total volume bile, collected both before and after BSP injection, divided by the time interval, divided by either wet liver weight or animal weight.

BSP concentrations in bile and plasma were determined spectrophotometrically (37, 38). Twenty μl of bile was added to 1.0 ml H_2O. One hundred ul of this dilution was added to 3.0 ml of 0.9% NaCl and a drop of 0.1 N HCl or 0.1 N NaOH was added. Two samples from each collection, one to which HCl was added and the other to which NaOH was added, were prepared. The concentration of BSP was calculated as,

$$[BSP] = \frac{A_{580}\ \text{sample}}{A_{580}\ \text{standard}} \times [BSP]\ \text{standard} \times \text{dilution factor}$$

where A_{580} is the difference in absorbance at 580 nm between base and acid. The dilution factor is 51 relative to the standard. The extinction coefficients of BSP and its metabolites (cBSP) were assumed to be equal for the analysis of bile and plasma (38). Thus, this method quantitates total BSP (free plus metabolized). A Coleman Junior II, model 6120 spectrophotometer was used for these determinations.

The mass of BSP excreted was calculated from bile volume x [BSP] in the sample. The excretion rate was determined as this mass divided by the time interval divided by wet liver weight or animal weight.

A procedure that minimized sample volume was used for the determination of plasma BSP (37). Blood samples (in heparinized capillary tubes) were centrifuged (2000 rpm x 20 min.) to obtain plasma. A volume of plasma was added to 0.5 ml of alkaline buffer[1] and the absorbance at 578 nm was obtained on a Bausch and Lomb Spectronic 500 spectrophotometer to give $A_{(base)}$. 100 µl of acid buffer[2] was added and absorbance retaken to give $A_{(acid)}$. The concentration of BSP was obtained as,

$$[BSP] = \frac{A(sample)}{A(standard)} \times [BSP]_{standard},$$

where A is the difference between $A_{(base)}$ and $A_{(acid)}$.

[1] Alkaline buffer, Na_2HPO_4 229 mM, Na_3PO_4 10.2 mM, Na toluene-p-sulfonate 10.2 mM, pH = 10.6.

[2] Acid buffer, 2M NaH_2PO_4

Data Analysis At each analysis time point after dosing, the mean values of bile flow, concentration of BSP in bile, BSP excretion rate and plasma concentration of BSP at 30 minutes after BSP injection in treated and control groups were compared. When the variance of the means were equal (F test where $F = (S_1/S_2)^2$, alpha = .05), a pooled variance was obtained and the null hypothesis of equality was analyzed by the independent Student's t-test. When the group variances were not equal in the F test, the statistic t' was calculated as $t' = (x_1-x_2)/(s_1^2/n_1 + s_2^2/n_2)^{1/2}$ with degrees of freedom $v' = (u_1 + u_2)^2/(u_1^2/v_1 + u_2^2/v_2)$ where $u_m = s_m^2/n_m$, $v_m = n_m-1$ and X_m is a mean. When v' is rounded down to the nearest integer, it can serve as the degrees of freedom where t' behaves as a t-distribution (39). This will be referred to as the t' test.

Plasma Clearance The right femoral artery and vein of 2 control rats under pentobarbital anesthesia were cannulated with PE 50 tubing. BSP, 50 mg/kg, was then administered via the venous cannula. Blood samples of 150 μl were taken from the arterial cannula from 30 sec. to 45 min. The concentration of BSP in plasma was determined as described earlier (37).

The data were analyzed under the assumptions of a 2 compartment open model with instantaneous input. No statistical analysis was performed. The purpose of this experiment was only to provide an idea of the plasma concentrations of BSP that could be attained after a 50 mg/kg injection.

The Effect of BCNU on Erythritol Clearance (CE) and Bile Salt Excretion. The procedure used for this analysis has been described by Layden and Boyer (16). The analysis was performed at 1 hr., 48 hours and 13 days after dosing with corn oil, 20 mg/kg BCNU or 15 mg/kg BCNU respectively. Maximal reductions in bile flow were previously observed at these times. Surgery was performed between 9:30 am and 1 pm.

At the analysis time, rats were anaesthetized and the following surgery was performed. After a midline abdominal incision, the renal pedicles were ligated to preclude loss of erythritol. The dorsal aorta was cannulated for blood sampling with PE 90 tubing at the bifurcation to the external iliac arteries. The bile duct was isolated and cannulated with PE 10 tubing. The right jugular vein was cannulated with PE 10 tubing for injection of the radioisotope. Bile was allowed to flow into a plugged 1.0 ml syringe barrel for an interval of approximately 15 minutes. The flow rate per mass rat was calculated. Only treated rats with a flow less than or equal to the lowest initial control group flow rates received radioisotope. After this period, 1.8 μCi of [^{14}C]-erythritol was injected via the jugular vein as .4 ml of an isotonic saline solution with cold erythritol (8.33 mg/ml). After a 15 minute equilibration period, bile was collected as before at approximately 15 minute intervals for up to 90 minutes. Blood samples (0.5 ml) were collected from the arterial cannula at the midpoint of the bile collection intervals. Samples were immediately centrifuged (2000 rpm x 20 min.) to obtain plasma. Body temperature was maintained at 37°C with heating pads thermostatically controlled via a rectal probe (Yellow Springs Instrument Co.).

At the end of sample collection, rats were sacrificed and livers were excised and weighed.

Five to 50 µl of bile or 50 µl plasma were added to 5 ml of fluor and counted for 20 min. or to an error less than or equal to 1.5% on a Packard Instruments Tri Carb model 3385 Liquid Scintillation Spectrometer. Quenching was corrected by automatic external standardization. Counting efficiency in individual biological samples was obtained from a plot of the volume of bile or plasma vs. counting efficiency of 1 µl of 4.5 µCi/ml [^{14}C]-erythritol added to a volume of unlabeled control bile or plasma. Erythritol clearance was calculated as the disintegrations per minute (dpm) in bile x dpm^{-1} in plasma x bile flow x gram liver^{-1}.

Bile Salt Excretion. Total bile salts in bile were determined in the bile samples mentioned above by a modification of the method of Talalay (40). The concentration of bile salts in bile samples was estimated in duplicate where sample volume allowed. Bile was diluted with methanol (usually 9 volumes). Standards were constructed with sodium taurocholate in methanol. Fifty µl of dilute bile or standard was added to a cuvette containing 0.75 ml G.E.H. buffer[3] and 0.1 ml of 5.32 µmol/ml beta-NAD$^+$ at pH = 7 to 7.5. The reaction was initiated at room temperature by the addition of 0.100 ml of 3-alpha-hydroxysteroid dehydrogenase (EC 1.1.1.50.), 0.7 units/ ml. As the 3-alpha-hydroxyl on the steroid ring A is oxidized to the ketone, NAD$^+$

[3] G.E.H. buffer, 1.3M glycine, 7.1mM EDTA, .51M hydrazine sulfate, adjusted to pH=9.4 with solid NaOH.

is reduced stoichiometrically. The conditions of the reaction medium facilitate completion of the forward reaction:

$$\text{Bile salt (reduced)} + NAD^+ \rightarrow \text{bile salt (oxidized)} + NADH + H^+.$$

The increase of absorbance at 340 nm, for NADH was monitored on an Aminco DW 2A spectrophotometer. The concentration of bile salt in samples was obtained from the standard relation of concentration of sodium taurocholate vs. the net change in absorbance at 340 nm (A_{net}) where $A_{net} = A_{340}(\text{sample}) - A_{340}(\text{blank})$ and A_{340} is the final minus the initial aborbance. The final absorbance value was taken when the absorbance failed to change more than .002 in 15 minutes. This occured 60 to 90 minutes after initiation of the reaction. The A_{340} (blank) was determined from a sample constructed with methanol in place of bile. The bile salt excretion rate was calculated as the concentration bile salt in sample multiplied by sample volume divided by the collection time interval and by the liver mass giving the final rate as nmol/min/g liver.

Determination of Bile Salt Independent Bile Flow (BSIF) and Bile Salt Dependent Bile Flow (BSDF). All the data obtained for the treatments were fitted to the model:

$$y = B_0 + B_1x_1 + B_2x_2 + B_3x_3 + B_{13}x_1x_3 + B_{23}x_2x_3,$$

where y is erythritol clearance, x_1 = 0 if control or 15 mg/kg BCNU or = 1 if 20 mg/kg BCNU, x_2 = 0 if control or 20 mg/kg BCNU or = 1 if

15 mg/kg, and x_3 = bile salt excretion rate. Three least squares regression lines result from this model, one for each treatment group. Regression analysis of this model allows comparison of the slopes (reflective of BSDF) and y intercepts (taken to equal BSIF) of the treated groups vs. control with an increased number of degrees of freedom relative to the degrees of freedom for each group alone (41). The Statistical Interactive Programming System (SIPS) was used to construct the model and evaluate hypotheses comparing slopes and intercepts on the OSU CYBER 70/73 computer using analysis of variance (41, 42). A difference from control was considered to be significant at alpha = .05 in F-test of the null hypothesis that a given $B_n = 0$.

A regression of CE vs. bile flow was performed over all the 15 minute bile observations taken. The slope is an estimation of the bile: plasma erythritol ratio (16).

In addition, the experimental group averages of bile salt excretion rate (nmol/min/g liver), bile salt concentration in bile and bile: plasma erythritol concentration ratio were compared by the independent, 2 tailed Student's t-test or t'-test (39).

The Effect of BCNU on Bile and Plasma Osmolality, Sodium, and Potassium Concentrations. Rats were pretreated with BCNU as before and blood and bile samples were taken at pre- and post-cholestatic time points. Bile was collected as previously described and a blood sample was taken by cardiac puncture at the midpoint of the bile collection interval. Blood samples were centrifuged at 2000 RPM for 20 minutes to obtain plasma. Care was taken to collect blood in

siliconized rather than heparinized Vacutainer^{R} tubes to preclude contamination with sodium.

Osmolality of both bile and plasma samples was determined in undiluted samples on a Wescor 5100 Vapor Pressure Osmometer. The instrument was calibrated with a sodium chloride standard (280 mOsmol/kg).

Bile and plasma samples (25 μl) were diluted with 5.00 ml water for determination of potassium (K^+). Five hundred μl of this dilution was added to 10.00 ml water for the sodium (Na^+) determination. Concentrations were established by atomic absorbtion spectroscopy on a Varian Techtron AA6 spectrometer which uses the respective elemental hollow cathode lamps. Absorbance was recorded at 766.5 nm for K^+ and 589.0 nm for Na^+. The slit width was 0.2 nm. Samples and standards were aspirated into an acetylene-air flame. Three readings of absorbance, each integrated over 3 seconds of aspiration, were made followed by a reading of distilled water. The net absorbance was calculated as the mean of three sample readings minus the blank reading. Concentrations were determined from standard curves of absorbance vs. concentration for each element. Standard curves were constructed each time the instrument was used and were calculated by linear regression (least squares method) using the OSU CYBER 70/73 computer (42). Concentrations were expressed as meq/l in the original sample.

The data were analyzed by the Student's t-test of means when variances of control and treated values were not different in a 2-tailed F-test at an alpha value of .05. When variances were

different by this criterion, the t'-test was used (39). A P-value less than .05 was considered significant.

Thin Layer Chromatography (TLC) of BSP in Bile. The same rats used for measurement of bile and plasma osmolality, Na^+ and K^+ were used in this experiment. They received a 50 mg/kg intravenous injection of BSP after samples were collected for that experiemnt.

Bile samples containing BSP and metabolites were applied to thin layer chromatography plates according to the method of Whelan and Plaa (43). The TLC plates were prepared from 16 g microcrystalline cellulose blended with 100 ml water. The resulting slurry was spread (approximately 275 μm thickness) on glass plates, air dried and then oven dried at 120°C for 30 minutes. Samples of bile (1-5 μl) were spotted across the plates. Standard BSP was spotted on each plate for reference. Plates were developed in the organic phase of n-butanol: glacial acetic acid: water (40:10:50). In addition, a beaker of 6% acetic acid was placed in the tank to aid equilibration. After developement, the plates were dried and viewed over a light source in ammonia fumes. The location of the purple spots, indicative of BSP, was marked on the reverse of the plate with a glass marker. R_f values were calculated from these markings. Plates were then sprayed with 0.25% ninhydrin in 95% ethanol, heated at 125°C for 5 minutes and reexamined. Ammonia reactive spots which also reacted with ninhydrin were noted. To standardize the plates, sample R_f values were divided by R_f BSP to give R_m. Spots were numbered 1 to 4 from top to bottom on the plate. The ranges of R_m values for each spot among all the rats are distinct (Table 2).

Bile Flow and Food Consumption. Rats were given corn oil or 20 mg/kg BCNU in a volume of 1.0 ml/kg intraperitoneally. Three experimental groups were constructed. One received corn oil injections and food ad libitum. One received corn oil injections and no food. The third group received the BCNU injections and these rats were provided with food. All rats were provided with water ad libitum. Food and water consumption and bile flow were determined 48 hours after the injections. This represents an early time point where maximally reduced bile flow was observed after the 20 mg/kg dosage.

Mean values of food and water consumption and bile flows were tested for equality using the Student's t-test when the variances of the means were not significantly different and using the t'-test when the variances were different in a 2 tailed F-test at an alpha value of .05 (39). Mean values were assumed to be different when P was less than .05.

The Effect of BCNU on the Concentration of Reduced Glutathione in the Liver. Rats were sacrificed at 3, 6, 12, 24, and 48 hours after an intraperitoneal dose of corn oil or 20 mg/kg BCNU. Treated and control rats were paired for feeding. Four rats were treated with BCNU for each time point and food consumption was monitored daily or at the time of sacrifice. Four controls were dosed with corn oil 24 hours later and these rats were provided with an amount of food equal to that consumed by their respective paired, treated rat. This procedure was used to reduce the effect that differing food consumption might have on concentration of reduced glutathione (GSH) in the liver (44).

GSH was determined by the method of Hissin and Hilf (45). At the time of sacrifice, the liver was removed and weighed in isotonic saline. A sample of liver, about 750 mg, was removed and homogenized in 3.0 ml of 25% metaphosphoric acid and 11.25 ml of .005 M EDTA-.1M sodium phosphate buffer previously adjusted to pH 8.0 (EDTA-phosphate buffer). The resulting 5% metaphosphoric acid solution precipitates protein and suppresses GSH oxidation (46, 47). The homogenate was centrifuged at 100,000 x g for 30 minutes. Duplicate 0.1 ml samples of the supernatant were diluted to 5.0 ml with EDTA-phosphate buffer. One hundred μl of this dilute supernatant was added to 3.8 ml of EDTA-phosphate buffer. For blanks, 0.1 ml water was used. Then 0.1 ml of 2 mg/ml ortho-phthalaldehyde in acetone-free methanol was added and fluorescence at 420 nm due to 350 nm excitation was measured 15 minutes later in a Turner fluorometer (model #111). For the standard, 1.0 ml of .20 μg/ml GSH in EDTA-phosphate buffer was added to 2.9 ml EDTA-phosphate buffer. Reaction was initiated with othro-phthalaldehyde and the fluorescence was measured at 15 minutes. Two standards were analyzed each time the assay was attempted. The average fluorescence of two runs was used in each calculation after subtraction of the blank. The data were expressed in μg GSH/g liver. Also, the average difference between control and treated rats in a pair was obtained. Data were analyzed by the paired Student's-t-test on the hypothesis that the average difference in a pair is zero. A P value less than .05 was considered significant.

Results

The Effect of BCNU on Bile Flow and BSP Excretion. The 20 mg/kg dosage of BCNU reduced bile flow significantly at 48 and 96 hours post dose (Fig. 1). The control values (mean ± S.E.) were 32.7 ± 3.4 µl/min/kg rat at 48 hours and 44.0 ± 3.8 µl/min/kg at 96 hours compared to treated means of 12.1 ± 3.4 µl/min/kg and 13.0 ± 1.3 µl/min/kg at those respective times. At both times the difference between treated and control was significant at $P < 0.005$.

The effect was not as definitive after a dose of 15 mg/kg BCNU (Fig. 2). The largest difference occurred at 13 days post dose at which time controls exhibited a mean flow (±S.E.)of 52.0 ± 7.0 µl/min/kg rat (or 1.3 ± .16 µl/min/g liver). The mean for treated rats at that time was 30 ± 9.3 µl/min kg (or .69 ± .25 µl/min/g). the P value was about 0.1 for a difference of means in a t-test. Bile flow was more normal on days 14 and 15. After the high dose (20 mg/kg) no signs of recovery were seen from 2 through 4 days after treatment.

A 50 mg/kg injection of BSP produced an initial plasma concentration of 100 mg/100 ml which fell to about 3 mg/100 ml by 45 min in controls (Fig. 3). The concentration of BSP in plasma 30 minutes after injection was significantly elevated in rats which had received 20 mg/kg BCNU 96 hours previously. Plasma levels were consistently higher in BCNU treated rats starting at 24 hours (Fig. 4). After 15 mg/kg BCNU, the 30 minute plasma BSP concentration was elevated at all times from 4 to 15 days post dose. The differences were significant at 10 and 13 days ($P < .05$, Fig. 5). These results extend the

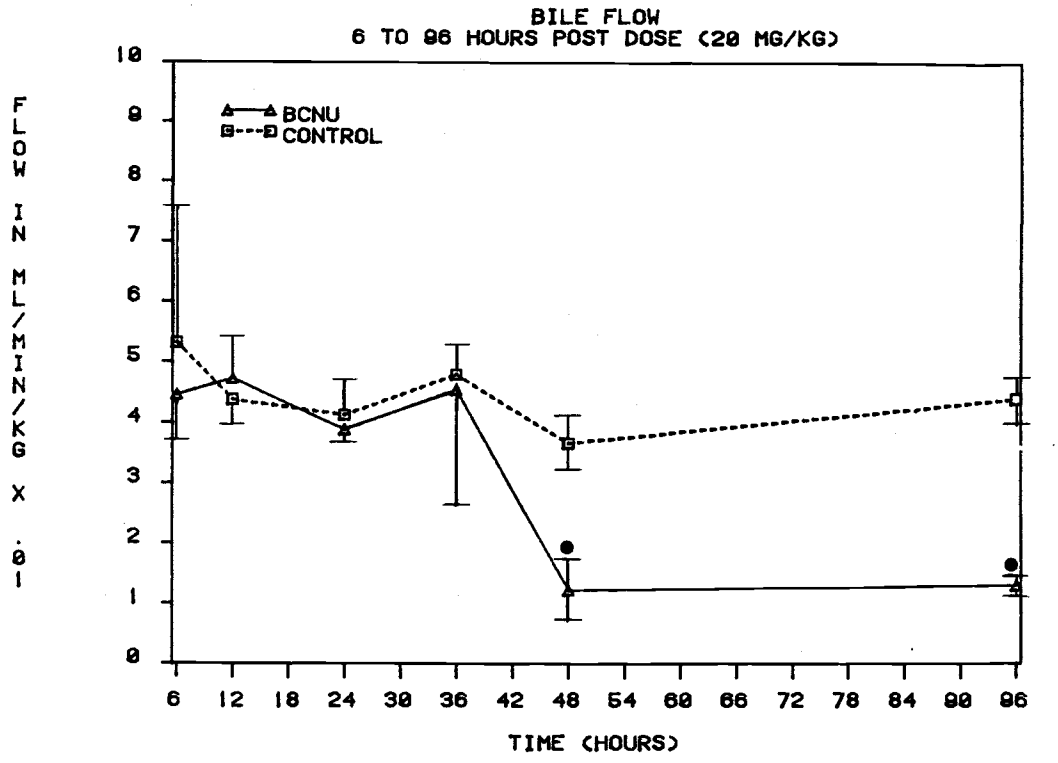

Figure 1 The effect of 20 mg/kg BCNU on bile flow. BCNU in corn oil was administered i.p. and bile flow was determined at the indicated time after dosing. The mean and standard error are indicated. Bile flow was significantly reduced 48 to 96 hours after dosing. (● ,P <.05)

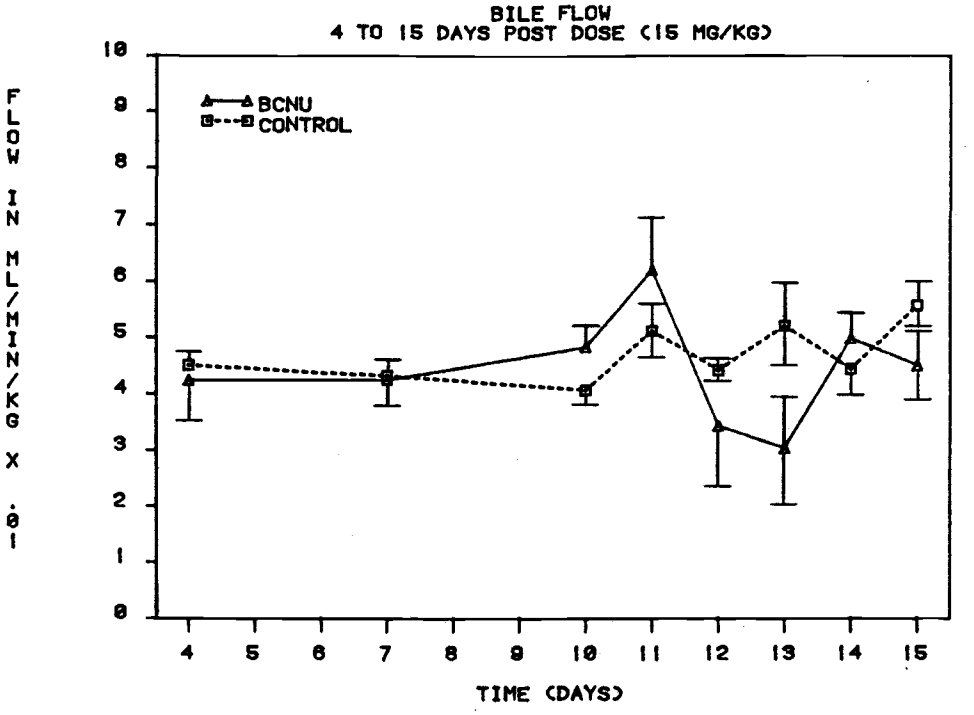

Figure 2 The effect of 15 mg/kg BCNU on bile flow. BCNU in corn oil was administered i.p. and bile flow was determined at the indicated time after dosing. The mean and standard error are indicated. No significant differences were observed.

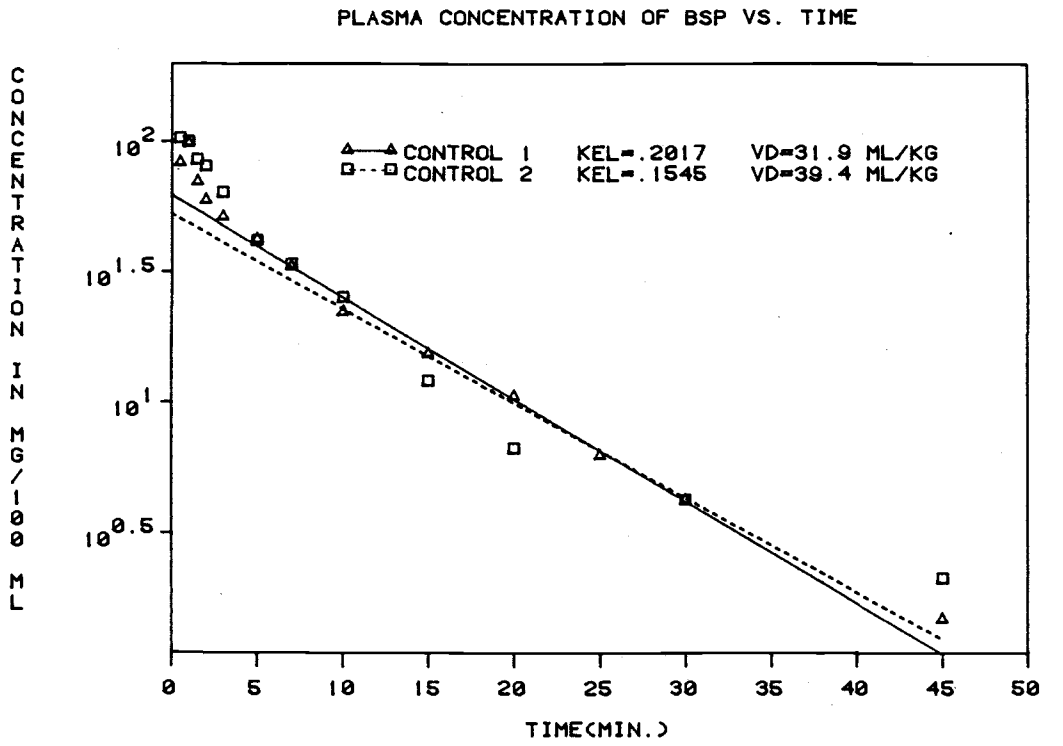

Figure 3 The plasma disappearance of BSP in control rats. BSP (50 mg/kg) was administered i.v. to anaesthetized rats and the concentration of BSP in plasma was determined at the indicated times after injection. (KEL, elimination rate constant; VD, volume of distributuion).

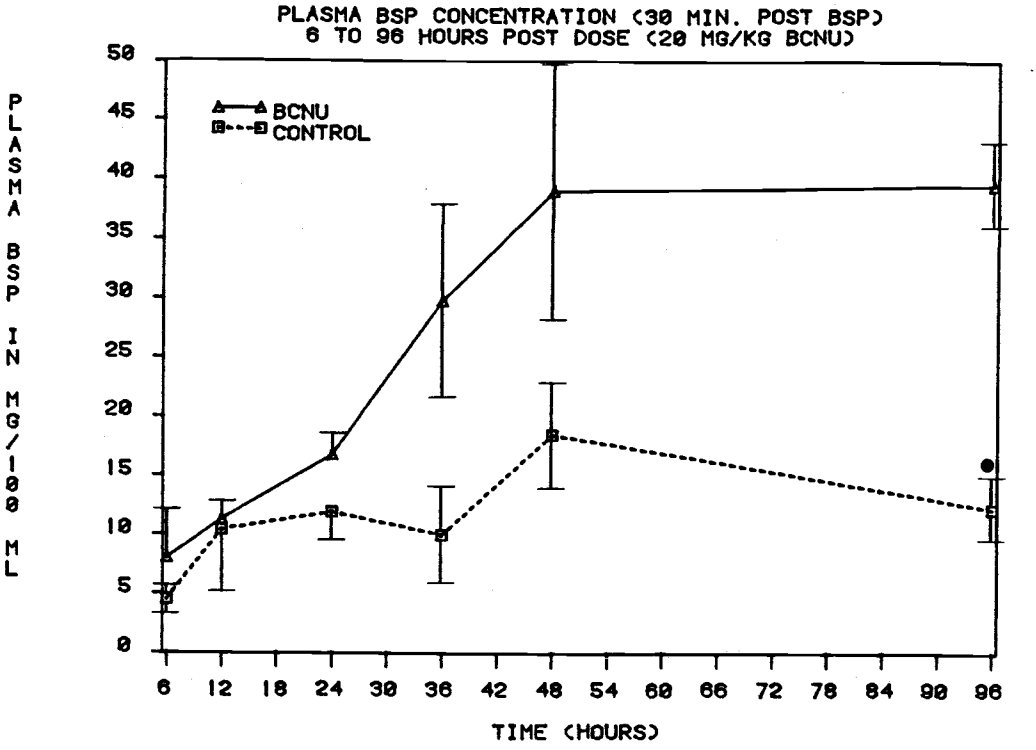

Figure 4 The effect of 20 mg/kg BCNU on the plasma concentration of BSP 30 minutes after BSP administration. BCNU was administered i.p. and at the time indicated after dosing, BSP (50 mg/kg) was injected i.v. in anaesthetized rats. A blood sample was taken 30 minutes later and the BSP concentration in plasma was determined. The mean and standard error are indicated. A significant difference was noted at 96 hours after treatment (● , $p < .05$).

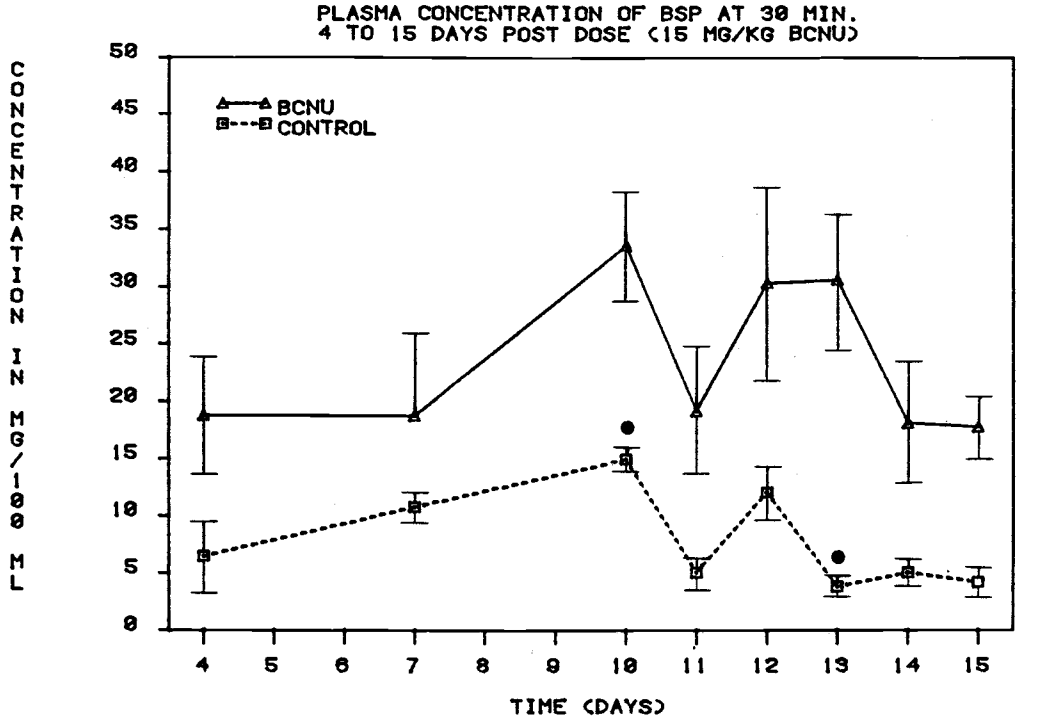

Figure 5 The effect of 15 mg/kg BCNU on the plasma concentration of BSP 30 minutes after BSP administration. BCNU was administered i.p. and at the time indicated after dosing, BSP (50 mg/kg) was injected i.v. in anaesthetized rats. A blood sample was taken 30 minutes later and the BSP concentration in plasma was determined. The mean and standard error are indicated. Significant increases were apparent at 10 and 13 days after treatment (● , $p < .05$).

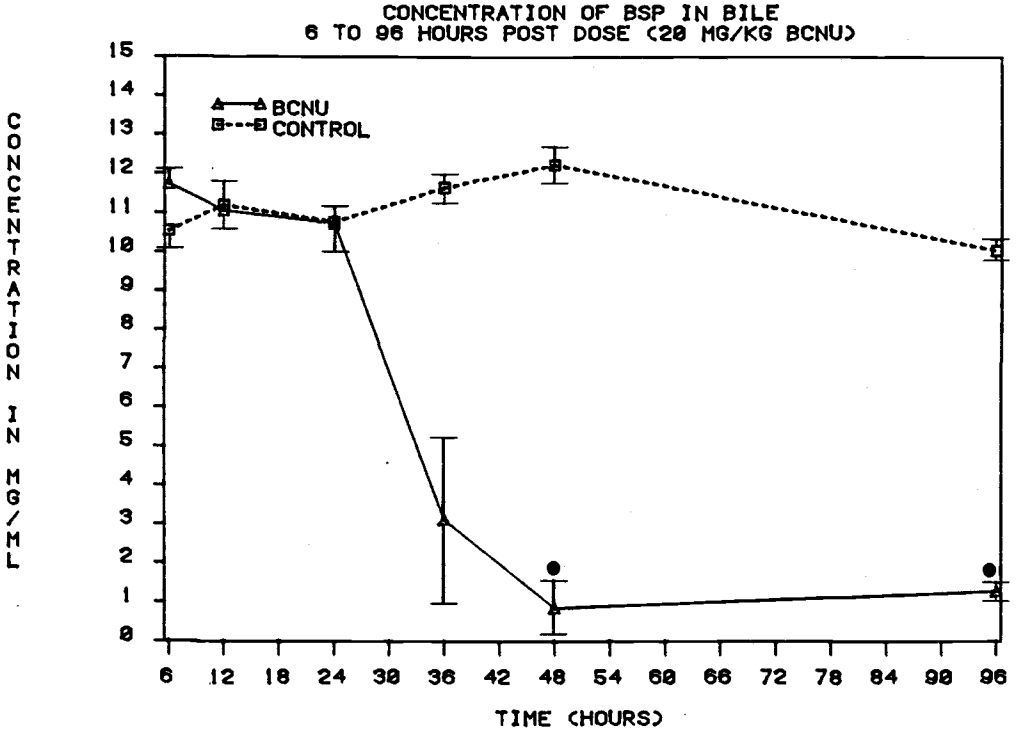

Figure 6 The effect of 20 mg/kg BCNU on the concentration of BSP in bile. 20 mg/kg BCNU was administered i.p. and at the time indicated after dosing BSP (50 mg/kg) was injected i.v. in anaesthetized bile-fistula rats. The mean and standard error are indicated. The concentration was significantly reduced 48 and 96 hours after treatment (● , $p < .05$).

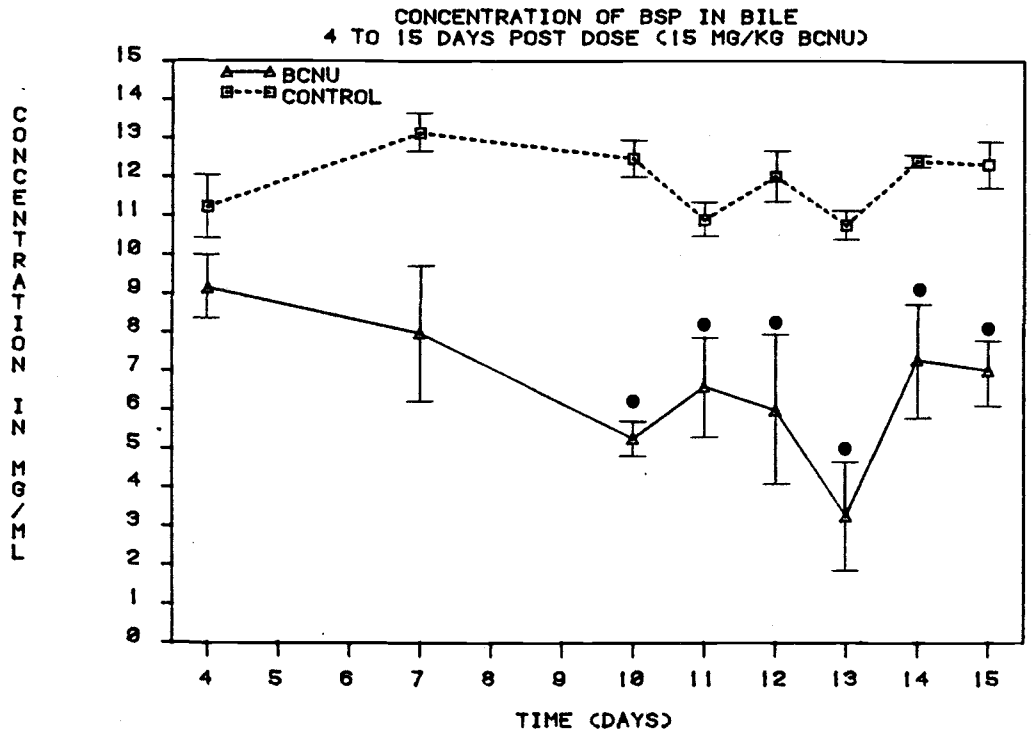

Figure 7 The effect of 15 mg/kg BCNU on the concentration of BSP in bile. 15 mg/kg BCNU was administered i.p. and at the time indicated after dosing BSP (50 mg/kg) was injected i.v. in anaesthetized bile-fistula rats. The mean and standard error are indicated. Concentrations were significantly reduced from 10 to 15 days after treatment (● , $p < .05$).

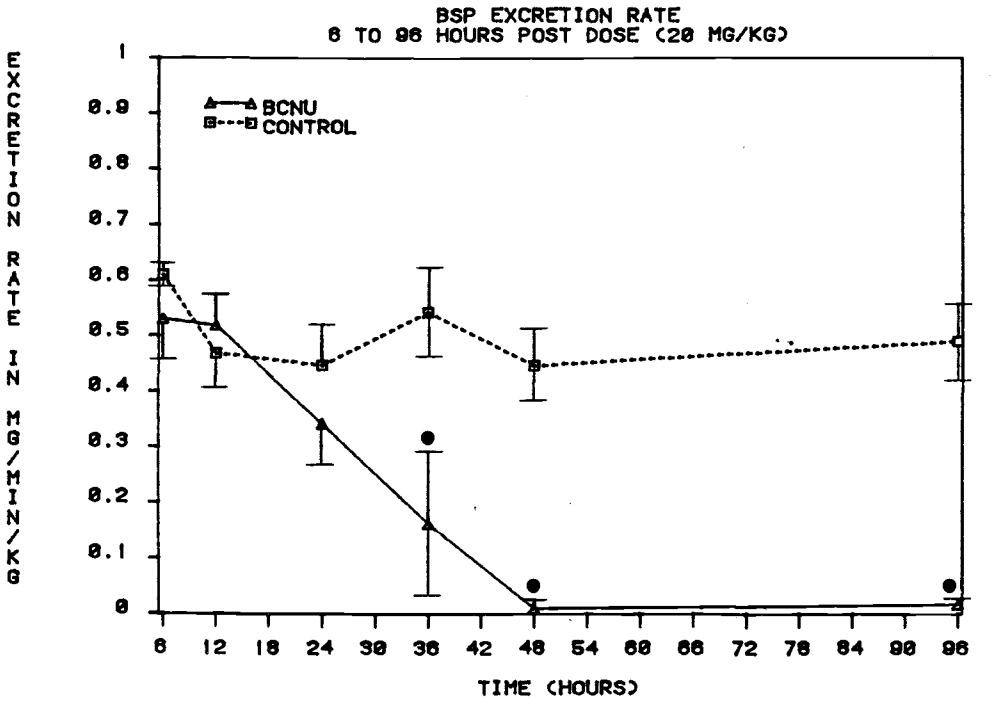

Figure 8 The effect of 20 mg/kg BCNU on BSP excretion rate. 20 mg/kg BCNU was administered i.p. and at the time indicated after dosing BSP (50 mg/kg) was injected i.v. in anaesthetized bile-fistula rats. The mean and standard error are indicated. The excretion rate was significantly reduced 36 to 96 hours after treatment (● , $p < .05$).

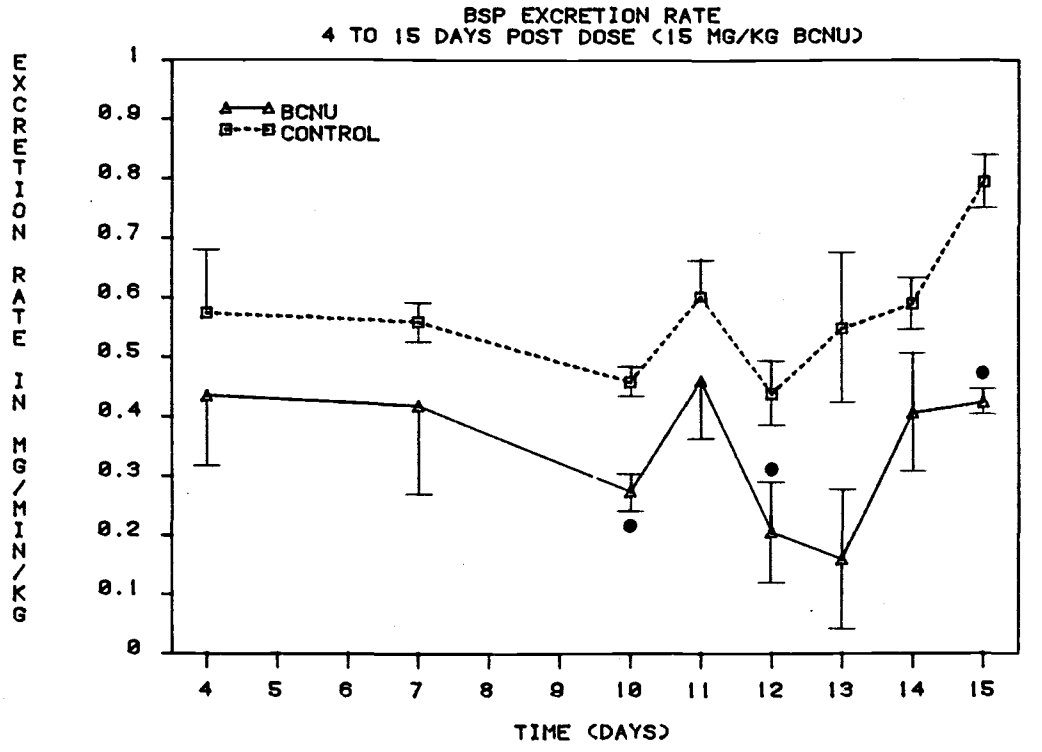

Figure 9 The effect of 15 mg/kg BCNU on BSP excretion rate. 15 mg/kg BCNU was administered i.p. and at the time indicated after dosing BSP (50 mg/kg) was injected i.v. in anaesthetized bile-fistula rats. The mean and standard error are indicated. The excretion rate was significantly reduced 10, 12 and 15 days after treatment (● , $p < .05$).

observation of Thompson and Larson that BSP retention occurs before toxicity (32).

The increased retention of BSP in plasma was paralleled by decreased appearance of the dye in the bile of treated rats (Fig. 6, 7). After the high dosage (20 mg/kg) the concentration of BSP in the bile was reduced significantly at 48 and 96 hours. By 36 hours, the mean concentration was obviously reduced, although the difference from control was not quite significant (Fig. 6). After 15 mg/kg BCNU, the concentration of BSP in bile was significantly reduced from 10 through 15 days (Fig. 7).

The high dosage of BCNU caused significant reductions in BSP excretion rate, in mg/min/kg rat, as measured at 36, 48, and 96 hours (Fig. 8). After the low dosage, BSP excretion rate was reduced significantly on days 10, 12, and 15. In addition the mean BSP excretion rates were depressed at all other times between 4 and 15 days (Fig. 9).

The Effect of Food and Water Consumption on Bile Flow. Because BCNU caused decreases in body weight (data not shown) the effect of fasting on bile flow was investigated (32, 33). Fasting of rats for 48 hours had no effect on bile flow. The 20 mg/kg dosage of BCNU again caused significant cholestasis relative to control ($P < .05$), and relative to fasted rats ($P < .001$ (Table 1)). There was no significant difference between control and fasted rats when flow was expressed either on the basis of body weight or liver weight ($P > .5$, $P = .4$ respectively).

TABLE 1. The Effect of Fasting or BCNU Treatment on Bile Flow and Food and Water Consumption by Male Rats in a 48 Hour Period [a]

Group [b]	Bile flow (µl/min/g liver)	Food Consumed/48 h (g)	Water Consumed/48 h (ml)	Food/Water Ratio (g/ml)
Control	1.02 ± .199	71.3 ± 4.05	73.3 ± 2.87	.972 ± .035
Fasted	1.25 ± .103	---	19.8 ± 3.73 [c]	---
20 mg/kg BCNU	.191 ± .0379 [c]	32.0 ± 3.81 [c,d]	38.5 ± 3.40 [c,e]	.834 ± .075

a) Values are mean ± standard error

b) n = 4 in each group

c) P < .001 for a comparison with control

d) P < .005 for a comparison with zero

e) P < .01 for a comparison with fasted rats

The normal flow rate in fasted rats occurred despite a reduction in food and water consumption relative to control or BCNU treated rats (Table 1). A comparison of food consumption among the groups, revealed a decrease in the BCNU treated rats relative to control ($p < .001$). Food consumption in the BCNU treated rats was significantly greater than zero ($P < .005$). Water consumption was reduced in the BCNU rats ($P < .001$) and in the fasted rats ($P < .001$). Water consumption was higher in BCNU treated rats with respect to fasted rats ($P < .01$). The ratio of food to water consumption was not significantly altered by BCNU despite the significant reduction in total consumption (Table 1).

The Effect of BCNU on BSP Metabolites in Bile. It should be noted that spot 1 may be non-metabolized BSP (Table 2). Only one rat produced ninhydrin reactivity in spot one. There was no ninhydrin reactivity in spots 2 and 3, which were obtained from treated rats. All rats produced spot 4 in ammonia and in all cases this spot contained amino acid.

Comparing the groups, rats given 20 mg/kg BCNU 24 or 48 hours previously produced spots 1 and 4 as did controls. In addition, 2 ammonia reactive, but not ninhydrin reactive spots were observed (spots 2 and 3). These same results were seen in rats 11 and 12 days after 15 mg/kg BCNU. However, 13 days after this dosage, the pattern was identical with control.

BCNU treatment altered the pattern of BSP metabolites produced by rats. There was an increase in the number of non-ninhydrin reactive

metabolites. This effect was noted at times that were not associated with cholestasis and was similar after both dosages of BCNU.

The Effect of BCNU on the Hepatic Concentration of Reduced Glutathione (GSH). BSP excretion is enhanced by conjugation with GSH, but BCNU produced effects on hepatic GSH levels at times when reduced BSP excretion was not observed. GSH was increased significantly with respect to control at 24 and 48 hours after 20 mg/kg BCNU. The P values for the paired t-test were less than .01 and .05 for the 24 and 48 hour time points respectively. The time course of changes in hepatic GSH concentration is illustrated in Figure 10. The time course of changes in the average differences between paired rats is seen in Figure 11. A paired difference is the value from a treated rat subtracted from a control rat that received the amount of food consumed by that treated rat.

The Relationship of Erythritol Clearance to Bile Flow. Erythritol clearance was used to estimate canalicular bile production. It was expected that any effect of BCNU on the reabsorption of bile at the bile ducts would be discernable with this technique. The analysis was performed at the time of maximum reduction in BSP excretion and bile flow. In these studies only those rats which were apparently cholestatic were used. All of the rats receiving 20 mg/kg BCNU were cholestatic. Therefore, selection of rats based on initial bile flow (prior to erythritol injection) was confined to those rats which received the lower dosage of BCNU.

TABLE 2. Thin Layer Chromatography of BSP Metabolites in Bile From Control and BCNU Treated Rats

Treatment	Spot Number [a] 1	2	3	4
Control	4/5 (0) [b]	--	--	5/5 (5)
20 mg/kg BCNU				
-24 hours	4/4 (0)	4/4 (0)	4/4 (0)	4/4 (4)
-48 hours	3/3 (1)	2/3 (0)	2/3 (0)	3/3 (3)
15 mg/kg BCNU				
-11 Days	5/5 (0)	5/5 (0)	5/5 (0)	5/5 (4)
-12 Days	4/4 (0)	3/4 (0)	3/4 (0)	4/4 (4)
-13 Days	2/2 (0)	--	--	2/2 (2)

a) Spots are defined by Rm Range (Rm = R_f sample/R_f BSP standard)
Spot 1: .81-.98
Spot 2: .64-.82
Spot 3: .43-.49
Spot 4: .27-.41

b) Fraction of rats producing spots containing BSP (ammonia reactive) and amino acid (ninhydrin reactive): Number with spot/number tested; number in parentheses is the number of spots also containing amino acid

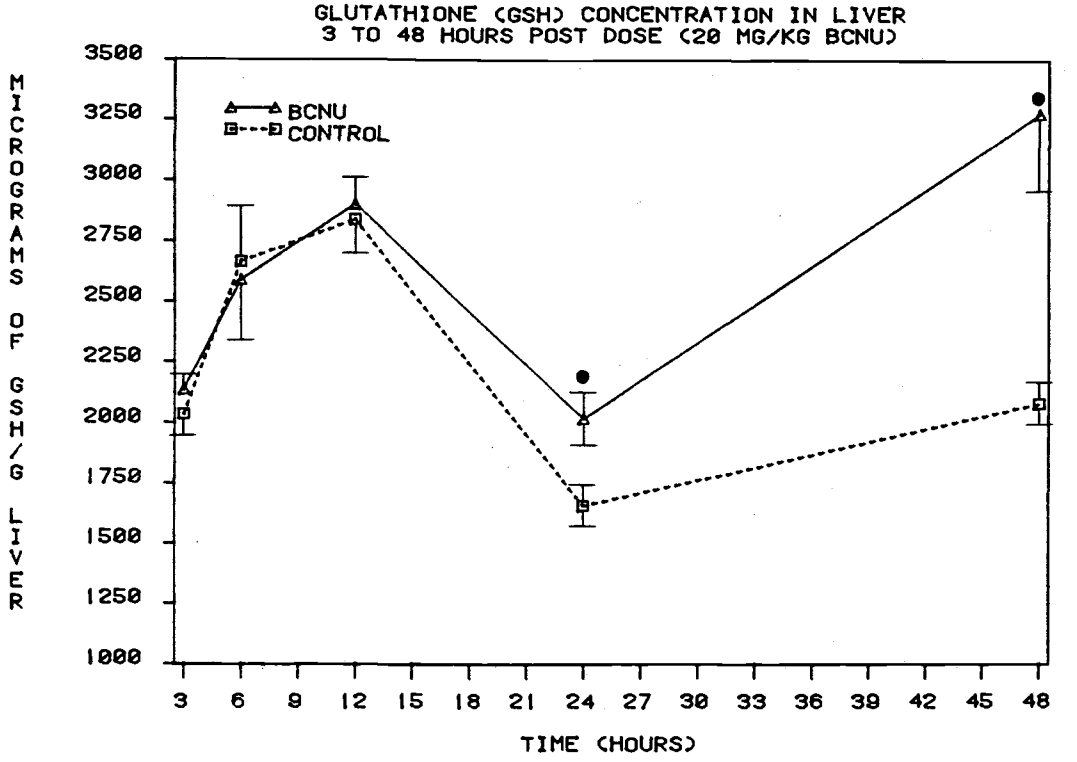

Figure 10 The effect of 20 mg/kg BCNU on hepatic GSH. BCNU (20 mg/kg, i.p.) was administered. Vehicle (corn oil) was administered to a pair fed control rat. At the time indicated after treatment, rats were sacrificed and hepatic GSH was determined. The experimental group mean and standard error are indicated. Hepatic GSH was significantly increased 24 and 48 hours after treatment (● , $p < .05$).

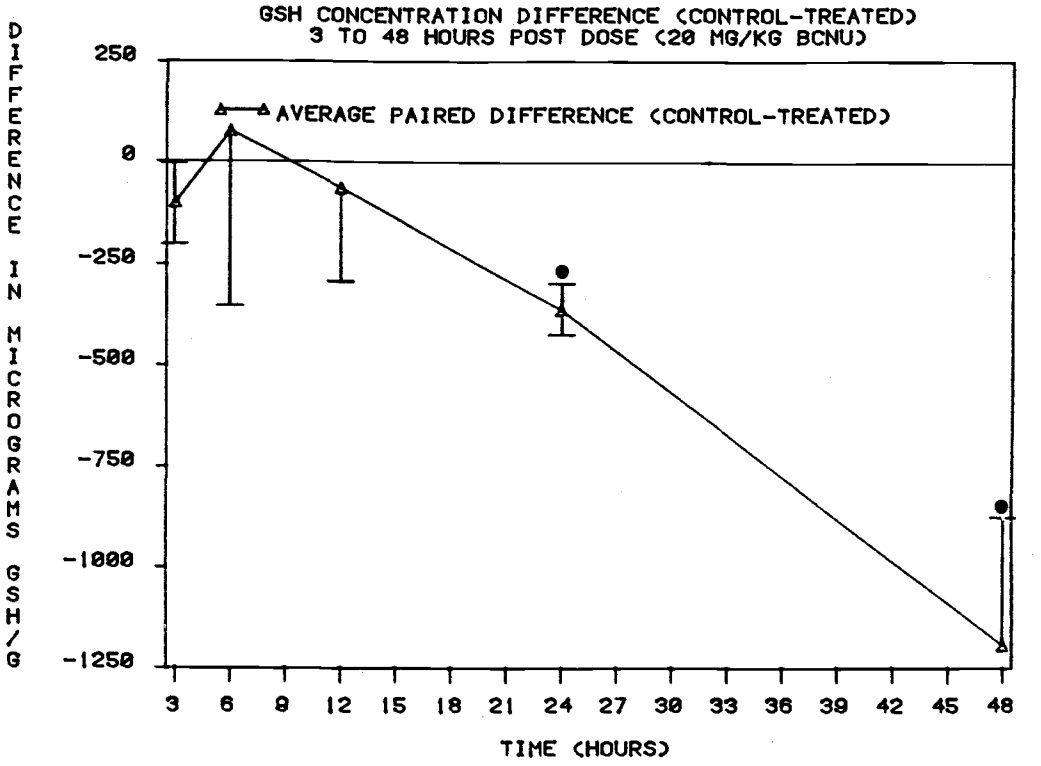

Figure 11 The effect of 20 mg/kg BCNU on the paired difference in hepatic GSH. BCNU (20 mg/kg, i.p.) was administered and vehicle (corn oil) was administered to a pair-fed control rat. At the time idicated after treatment, rats were sacrificed and hepatic GSH was determined. The mean difference between paired rats (control-treated) and the standard error of the difference are indicated. Hepatic GSH was significantly increased in treated rats 24 and 48 hours after treatment. (● , $p < .05$).

Regression of erythritol clearance (EC) and bile flow rate for all data obtained gave the relationship (R = .97):

$$y = 1.299(x)-.023,$$

where y is CE in µl/min/g liver and x is bile flow in µl/min/g liver. The hypothesis that the slope is 1.0 was rejected since $P < .005$. This slope is one estimation of the bile:plasma erythritol concentration ratio (Fig. 12). Also, the mean bile: plasma erythritol ratios for each treatment group were compared (Fig. 13). Each mean was found to be significantly greater than 1.0 again ($P < .001$ in each case). There was no significant difference between the group of rats which received the high dosage and the controls, but the rats treated with 15 mg/kg BCNU exhibited a slight but statistically significant reduction in the bile: plasma erythritol ratio relative to control ($P< .025$). This suggests that less water was absorbed from bile at the level of the bile ductular epithelium in that treatment group.

The Relationship of CE to Bile Salt Excretion Rate. The determination of CE and bile salt excretion was used to estimate the BSIF and BSDF and thus to investigate which of these were affected by BCNU (Fig. 14, 15). Analysis of variance revealed that the slopes of the relationships for the three groups were not significantly different at alpha = .05 in an F-test. The intercepts at zero bile salt excretion, or BSIF, in the treated groups were depressed at both dosages relative to control because at alpha = .01, the F-test was significant for both groups of treated rats. When each group was treated separately in regression analyses of CE and bile salt excretion rates, correlation coefficients of .5489, .8213, and .9298 were obtained for controls, 20

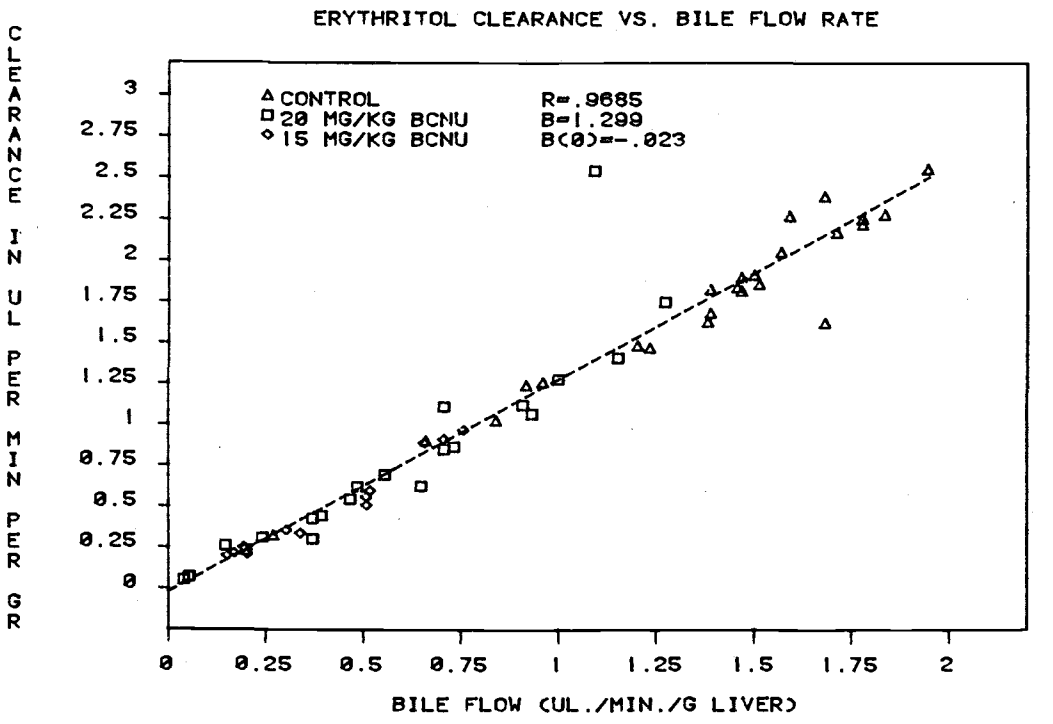

Figure 12 The relationship of erythritol clearance to bile flow. Clearance and flow were determined in control, 20 mg/kg BCNU (48 hours post dose) and 15 mg/kg BCNU (13 days post dose) rats (R, correlation coefficient; B, slope estimate; (B(Ø), estimated y-intercept).

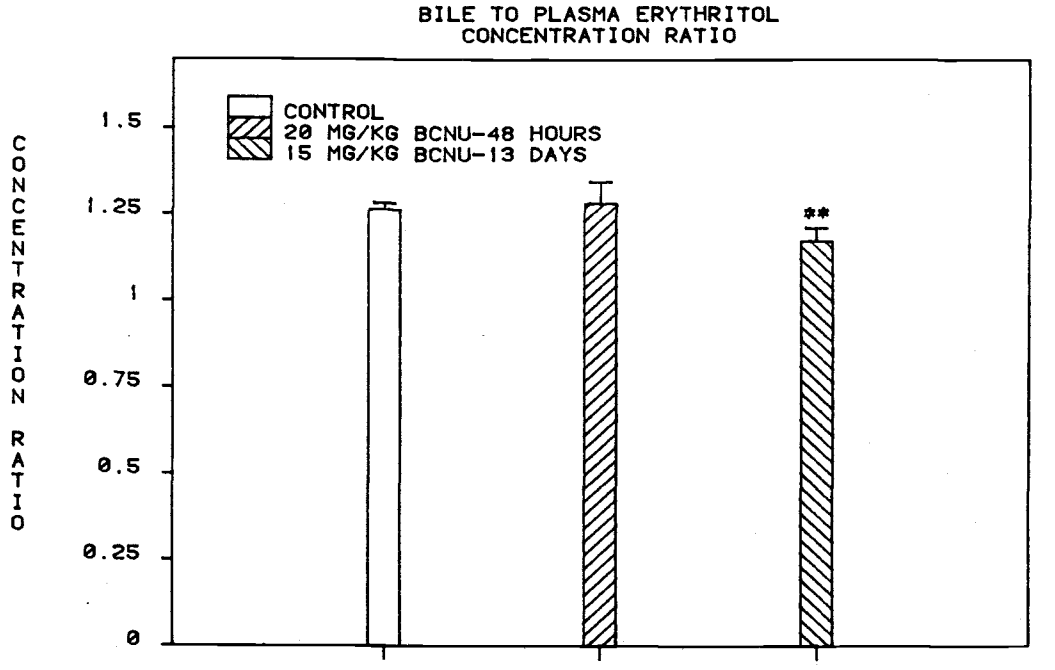

Figure 13 The effect of BCNU on the bile to plasma erythritol ratio. Rats recieved corn oil, 20 mg/kg BCNU or 15 mg/kg BCNU i.p. At the indicated time after dosing [^{14}C]-erythritol was administered to anesthetized bile-fistula rats and its equilibrium concentration in bile and plasma was determined. All ratios were significantly greater than 1.0 ($p < .001$ in each case). The ratio in rats treated with 15 mg/kg BCNU was less than control (** , $P < .025$).

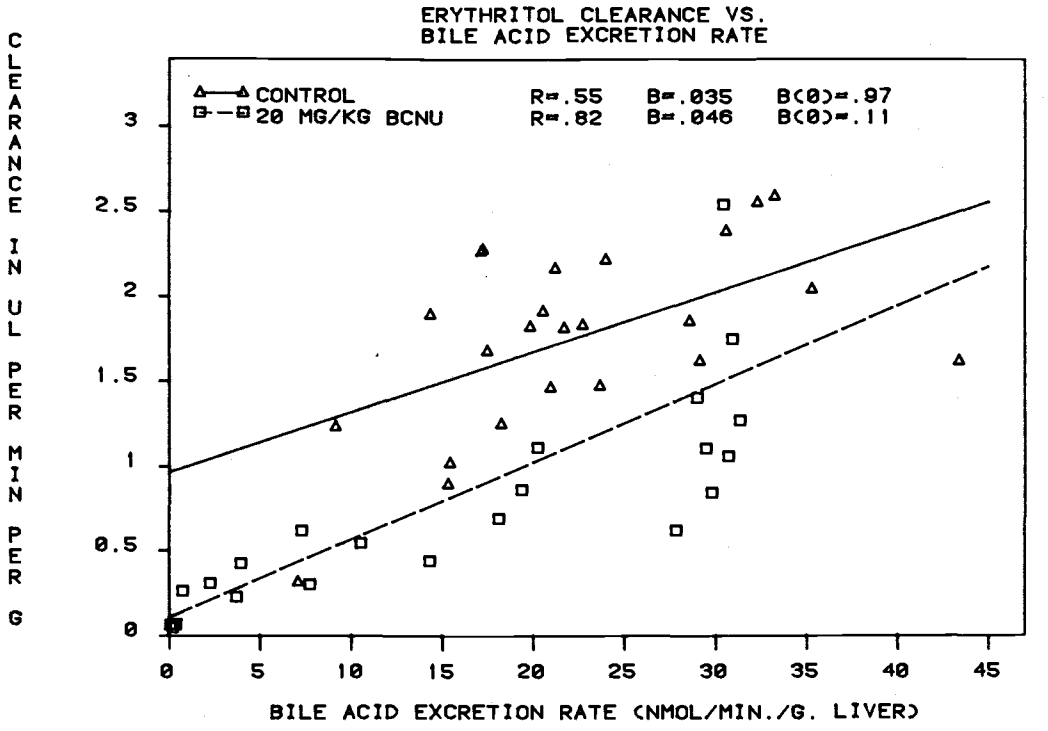

Figure 14 The relationship between erythritol clearance and bile salt excretion rate in control rats and rats treated with 20 mg/kg BCNU, i.p.(48 hours post dose). No significant difference in slopes of the regression lines was found while the y-intercepts were different. (R, correlation coefficient B, estimated slope; B(Ø), estimated y-intercept).

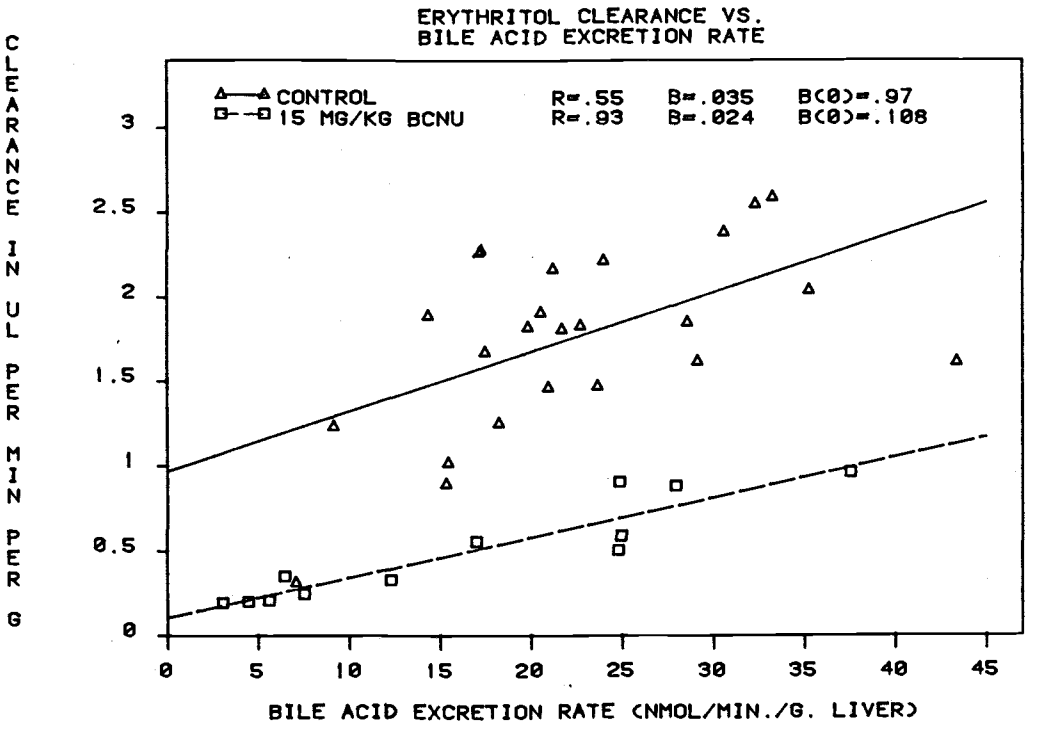

Figure 15 The relationship between erythritol clearance and bile salt excretion rate in control rats and rats treated with 15 mg/kg BCNU, i.p. (13 days post dose). No significant difference in slopes of the regression lines was found while the y-intercepts were different. (R, correlation coefficient; B, estimated slope; B(Ø), estimated y-intercept).

mg/kg BCNU and 15 mg/kg BCNU respectively. Therefore, BCNU pretreatment reduced the BSIF without affecting the BSDF.

When the average concentration of bile salts in bile were compared among the groups with each 15 minute bile sample as an observation, it was found that rats treated with either 20 or 15 mg/kg BCNU maintained elevated concentrations with respect to control ($P < .05$ and .001 respectively). The values obtained, as mean ± standard error, were 16.8 ± .99 mM in controls, 22.8 ± 2.7 mM for 20 mg/kg BCNU, and 35.8 ± 3.0 mM for 15 mg/kg BCNU. Rats treated with 15 mg/kg BCNU attained a significantly higher mean than the rats treated with 20 mg/kg BCNU ($P < .005$, Fig. 16).

A similar analysis of the bile salt excretion rates gave means ± standard error in nmol/min/g liver of 22.4 ± 1.7, 15.8 ± 2.6, and 16.4 ± 3.3 for control, high and low dose rats respectively. In the 2 tailed t-test, only the high dose rats had significantly reduced bile salt excretion relative to control ($P < .05$). P was between .05 and .10 when the mean bile salt excretion rate in the low dose rats was compared to control (Fig. 17).

The Effect of BCNU on the Osmolality, Sodium (Na^+) and Potassium (K^+) Concentrations of Bile and Plasma. In agreement with the high biliary bile salt concentrations caused by BCNU at the time of cholestasis, bile osmolalities were increased after both dosages (Table 3). In general, bile: plasma osmolality ratios were increased also. This result indicated that BCNU treated rats could maintain a concentration gradient between bile and plasma before and during periods of reduced bile flow. Changes in osmolality were probably not

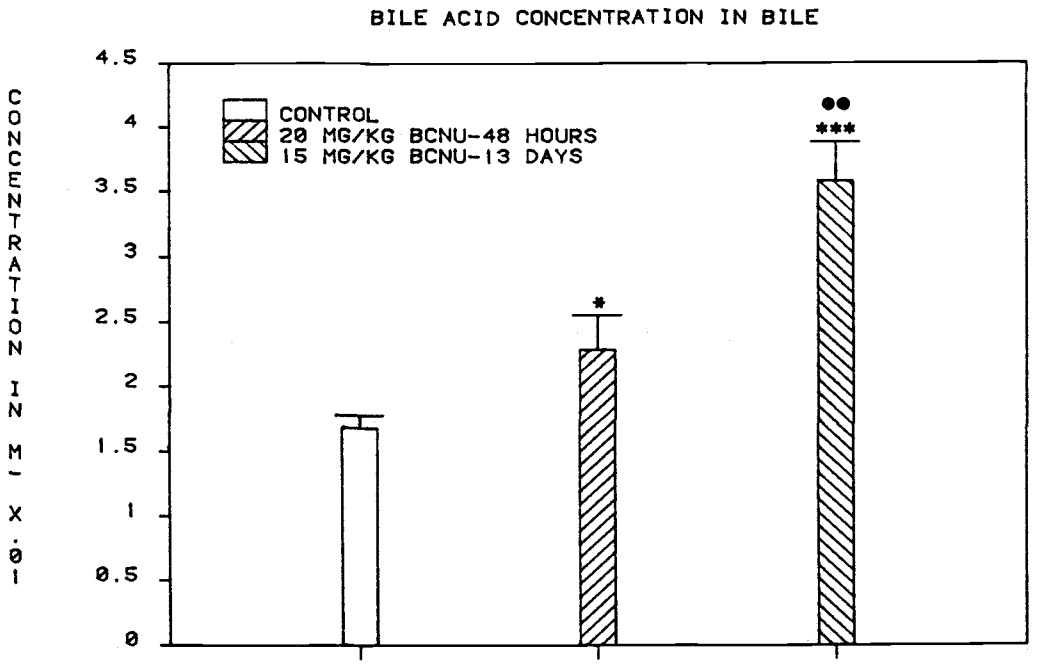

Figure 16 The effect of BCNU on the biliary concentration of bile acid. Bile acids were determined in the bile of anaesthetized bile-fistula rats at the indicated times after treatment with 20 or 15mg/kg BCNU i.p. The mean and standard error are indicated. The concentration in 15 mg/kg BCNU treated rats was significantly increased relative to both of the other groups and the concentration in 20 mg/kg BCNU treated rats was significantly greater than control.
(* , comparison with control; * ,$p < .05$;***, $p < .025$; ●● , comparison with 20 mg/kg BCNU, $p < .025$).

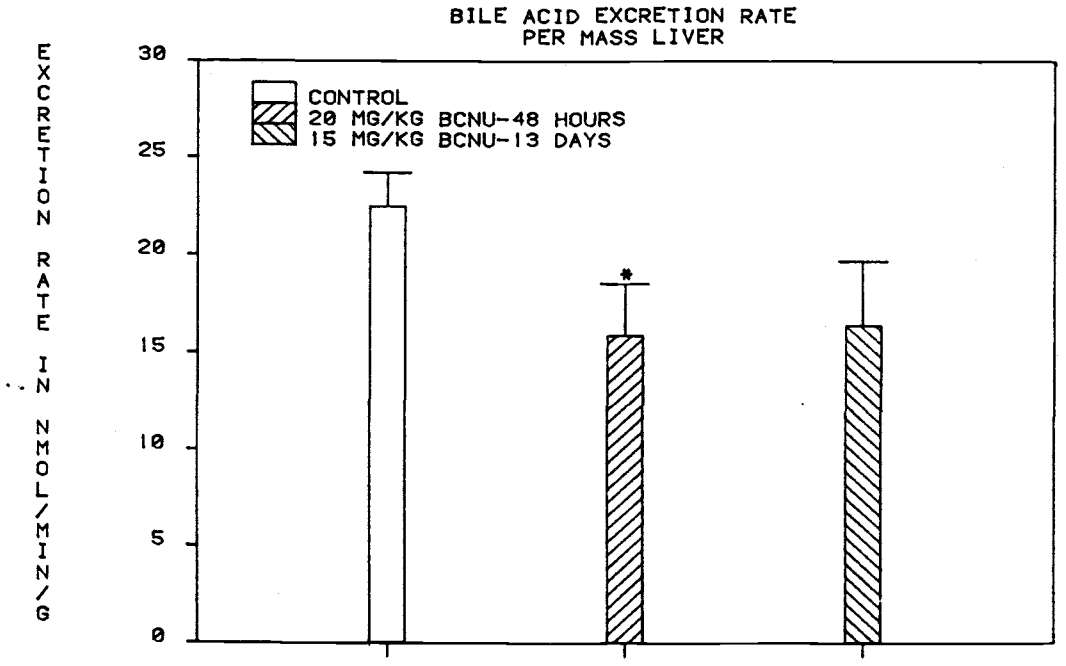

Figure 17 The effect of BCNU on bile acid excretion rate. The excretion rate was determined in anaesthetized bile-fistula rats at the indicated times after i.p. administration of 20 or 15 mg/kg BCNU. The excretion rate is expressed in nmol/min/g liver. It was significantly reduced in rats treated with 20 mg/kg BCNU(*, $p < .05$).

TABLE 3 The Effect of BCNU Treatment on the Osmolality of Bile and Plasma of Male Rats [a]

Treatment	Time	Bile Flow (µl/min/g liver)	Bile Osmolality (mOsmol/kg)	Plasma Osmolality (mOsmol/kg)	Bile/Plasma Osmolality Ratio
Control	--	1.39± .19	280.8± 5.2	292.4± 2.5	.960± .016
20 mg/kg BCNU	24 h	1.35± .31	276.4± 5.1	269.8± [d] 5.6	1.025± [c] .014
	36 h	.981± .33	303.2± [c] 1.8	292.4± 2.8	1.037± [d] .013
	48 h	.650± [b] .23	293.0± 4.2	296.6± 8.0	.989± .017
15 mg/kg BCNU	11 d	.733± .26	288.4± .79	287.8± 2.9	1.002± .026
	12 d	1.13± .16	306.8± [e] 2.6	301.0± [b] 2.0	1.019± [c] .0098
	13 d	.346± [d] .099	298.0± [b] 1.5	300.5± 3.4	.992± .0067

a) mean value ± standard error
b) $P < .05$
c) $P < .025$
d) $P < .01$
e) $P < .005$

related to changes in the ratio of food: water consumed since no significant difference in this value was observed between control rats and rats treated with 20 mg/kg BCNU in an earlier experiment (Table 1).

BCNU caused large increases in bile and plasma K^+. The increases were larger after the low dose (15 mg/kg). In contrast to the bile: plasma osmolality ratios, bile: plasma K^+ ratios were not greatly affected (Table 4).

In contrast to the effect on plasma K^+, BCNU caused statistically significant reductions in plasma Na^+ prior to measurable decreases in bile flow (Table 5). Bile:plasma Na^+ ratios were generally unaffected.

TABLE 4 The Effect of BCNU Treatment on the Potassium Concentrations of Bile and Plasma of Male Rats [a]

Treatment	Time	Bile Flow (µl/min/g liver)	Bile $[K^+]$ (meq/l)	Plasma $[K^+]$ (meq/l)	Bile/Plasma $[K^+]$ Ratio
Control	--	1.39± .19	5.26± .207	5.13± .330	1.058± .129
20 mg/kg BCNU	24 h	1.35± .31	5.13± .428	4.66± .109	1.095± .0706
	36 h	.981± .33	6.33± [d] .246	6.57± [c] .332	.977± .0735
	48 h	.650± [b] .23	5.99± [c] .114	7.42± [e] .315	.814± .0412
15 mg/kg BCNU	11 d	.733± .26	8.47± [e] .556	8.51± [f] .454	1.001± .0603
	12 d	1.13± .16	7.49± [e] .459	7.74± [e] .511	.970± .0286
	13 d	.346± [d] .099	8.48± [c] .712	9.14± 1.55	.970± .100

a) mean value ± standard error
b) $P < .05$
c) $P < .025$
d) $P < .01$
e) $P < .005$
f) $P < .001$

TABLE 5 The Effect of BCNU Treatment on the Sodium Concentrations of Bile and Plasma of Male Rats[a]

Treatment	Time	Bile Flow (µl/min/g liver)	Bile $[Na^+]$ (meq/l)	Plasma $[Na^+]$ (meq/l)	Bile/Plasma $[Na^+]$ Ratio
Control	--	1.39± .19	161.5± 2.06	159.7± 1.33	1.018± .0368
20 mg/Kg BCNU	24 h	1.35± .31	166.8± 1.44	156.5± 1.89	1.066± .0096
	36 h	.981± .33	160.3± 8.44	140.3± [e] 3.67	1.142± .0526
	48 h	.650± [b] .23	166.6± 10.3	145.1± [f] 1.67	1.146± .0614
15 mg/Kg BCNU	11 d	.733± .26	148.2± 6.03	139.9± [f] 2.63	1.063± .0563
	12 d	1.13± .16	144.6± [c] 5.33	139.2± [f] 2.31	1.038± .0236
	13 d	.346± [d] .099	147.4± [c] 3.77	140.8± [e] 3.63	1.049± .0344

a) mean value ± standard error
b) $P < .05$
c) $P < .025$
d) $P < .01$
e) $P < .005$
f) $P < .001$

Discussion

The data indicated that BCNU caused cholestasis and that this effect was due to an inhibition of the movement of water into bile. Cholestasis did not appear to be due to increased water reabsorption at ductular sites. It also seemed that the inhibition of water movement into bile was not a result of an increase in biliary or canalicular permeability to solutes which would inhibit the maintainance of an osmotic gradient between bile and plasma. Cholestasis might be due to decreased water permeability or to an effect on bioenergetic and/or ion transport systems that may be responsible for a portion of bile formation.

In addition, organic anion excretion was decreased while bile acid excretion was relatively unaffected when the biliary concentrations of the substrates were used as criteria of function. As discussed below, it appears that the two excretory systems are relatively separate in their function. However, there may be some degree of overlap in substrate specificity. Therefore, increased biliary bile salt concentrations may be explained by a relative insensitivity of bile salt transport to BCNU. Organic anion transport may be inhibited by virtue of a direct sensitivity to BCNU treatment, as a consequence of increased biliary bile salt concentrations or by a decrease in bile salt output.

It appears that increasing the dosage of BCNU decreases the time to onset of cholestasis and increases the severity and duration of the effect. The 20 mg/kg dosage of BCNU caused a clear reduction in bile

flow by 48 hours after a single intraperitoneal injection (Fig. 1). The lower dosage, 15 mg/kg, exerted a much less definitive effect. The maximal effect at 15 mg/kg occurred at 13 days (Fig. 2). Rats appeared to begin a recovery on day 14 after the low dosage.

It was verified in a separate experiment that the effect was not secondary to any effect of BCNU on food or water consumption (Table 1). Fasted rats consumed less food, obviously, and less water over a 48 hour time period relative to controls or rats treated with 20 mg/kg BCNU. Despite this, bile flow was not different from fed controls. The treated rats consumed less food and water than did fed controls and again exhibited significant cholestasis.

The excretory function of the liver was assessed using bromosulfophthalein (BSP) because of its diagnostic value and high degree of biliary excretion (15).

BSP excretion into bile is a rate-limited process consisting of 2 to 3 steps (38, 49, 50). The first is uptake by the hepatocyte from plasma across the basolateral (sinusoidal) membrane. The second is binding to glutathione-S-transferases and conjugation with reduced glutathione (GSH) (38, 49, 51). This step does not always occur in a net manner since free BSP is excreted also (38, 43). Free BSP and the products of BSP conjugation (cBSP) appear in bile after flux across the canalicular membrane. Overall transport is concentrative (49, 52, 53). BCNU caused significant reductions in BSP excretion rate and in the ability to concentrate BSP in bile (Fig. 6-9). These effects developed prior to a measurable decrease in bile flow and therefore are not a result of reductions in bile flow.

If BCNU acts on basolateral uptake of BSP, then its effect must be independent of the sodium gradient from plasma to cell, cell membrane potential, and utilization of metabolic energy. Schwenk et al. demonstrated that at low BSP concentrations (i.e. 10 μM), uptake into isolated hepatocytes followed Michaelis-Menten kinetics (54). However, at higher concentrations, uptake was non-saturable. At concentrations above 50 μM, uptake was sodium-independent. In addition, uptake was independent of metabolic energy as demonstrated with rotenone or antimycin A, and lowering of membrane potential with gramicidin had no effect. These results indicated that basolateral uptake at BSP is diffusional in nature, but facilitated transport could not be completely ruled out (54). The plasma concentrations of BSP in controls in the present study were greater than 10 μM (.84 mg/100 ml) in the range of non-saturable uptake, for 45 minutes after injection (Fig. 3).

If basolateral uptake of BSP is carrier mediated, then BCNU might inhibit uptake by a direct or indirect action on the hypothesized carrier system. Because BCNU caused a decrease in bile salt excretion rate at the time of reduced bile flow and BSP excretion, the possibility of inhibition of BSP uptake by bile salts accumulated in plasma cannot be excluded because high doses of bile salts inhibit the uptake of dibromosulfophthalein, indocyanine green, and N-acetylprocainamide ethobromide in isolated hepatocytes (55).

The possibility that bile salts inhibited BSP uptake is unlikely because the bile salt uptake and BSP uptake systems are relatively separate. Anwer and Hegner showed that cholate and taurocholate, the predominant bile salts, were taken up by isolated hepatocytes by both sodium-dependent and independent processes (56). This has been

confirmed by others (25). In their studies BSP and bilirubin competitively inhibited the sodium-independent component suggesting a common carrier for uptake of bile salts and other organic anions. The sodium-dependent bile salt uptake was non-competitively inhibited by BSP and bilirubin which suggested the presence of 2 distinct uptake mechanisms. Anwer and Hegner suggested that the sodium-independent uptake system for bile salts is a common carrier for bile salts and BSP while sodium-dependent uptake is specific for bile salts (56). Later it was shown that taurocholate competitively blocks a fraction of BSP uptake as revealed by Inui-Cristensen plots (57). This inhibition was significant at BSP concentrations less than 10 uM, which is much lower than the peak plasma concentrations observed in controls in the present study (Fig. 3). It was indicated by Laperche et al. that taurocholate sensitive uptake of BSP is probably identical with the sodium-independent bile salt uptake of Anwer and Hegner since the K_i for taurocholate inhibition of the taurocholate sensitive component of BSP uptake was approximately equal to the K_m of taurochoalte for sodium-independent bile salt uptake (54, 56, 57).

Therefore, the importance of a basolateral carrier shared by taurocholate and BSP is probably of little consequence with regard to the effect of BCNU in this study since initial BSP concentrations in plasma after a 50 mg/kg intravenous injection are approximately 100 mg/100 ml plasma (1.2 mM) in controls (Fig. 3). Since 10 μM (.84 mg/100 ml) is about the BSP concentration where such shared transport becomes obvious, competition would not become important until 45 minutes after injection (Fig. 3, 57). Therefore, it is unlikely that a BCNU-induced accumulation of bile salts could inhibit the uptake of

50 mg/kg BSP. Also, despite inhibition of BSP uptake by exogenous taurocholate, overall BSP excretion may actually increase (58). However, the possibility exists that BCNU could increase the levels of the more toxic, less hydroxylated bile salts, according to the HHSER theory of cholestasis, and these might inhibit organic anion uptake and excretion (1, 3). Any effects of BCNU on basolateral uptake may be academic with respect to BSP excretion since this is not the rate limiting excretory step (49, 53). To summarize, decreased excretion due to inhibited uptake from plasma represents an unlikely mechanism for the effect of BCNU on BSP excretion, by virtue of the high initial plasma BSP concentrations attained and because plasma uptake is not a rate limiting step in normal rats. But, this mechaism cannot be completely excluded by the present data.

The second step in BSP excretion is the binding of BSP to the glutathione-S-transferases in the cytoplasm (51, 59). This binding may be important in reducing the intracellular activity of BSP and therefore in facilitating uptake (49, 51, 59). These enzymes constitute about 10% of hepatic cytoplasmic protein which implies a significant capacity for the facilitation of uptake (51). BCNU could reduce BSP excretion by interfering with this process.

BSP is also metabolized by glutathione-S-transferases to the conjugated form (cBSP) (60). Both BSP and cBSP are concentrated in bile after intravenous infusion and they compete for the same secretory system with cBSP apparently having a higher affinity for the system (49). Conjugation appears to be a requirement for maximal biliary excretion of BSP (38, 61). Therefore, BCNU could reduce BSP excretion by inhibiting BSP conjugation. Because BSP is conjugated

with GSH, the metabolites of BSP were qualitatively characterized by cellulose thin layer chromatography as described by Whelan and Plaa (43). As a point of comparison, controls in this study produced only one spot associated with amino acid and one spot corresponding to free BSP (Table 2). Controls in the study of Whelan and Plaa exhibited several BSP-amino acid metabolites (43). The difference may be due to the dosage of BSP used. Whelan and Plaa administered 100 mg/kg BSP while 50 mg/kg was used in the present study (43). The assay may not be sensitive enough to detect the lower concentration of metabolites that would be expected after the lower dosage of BSP.

BCNU at both doses affected the excretory pattern of BSP before and during cholestasis. In general there was an increase in the number of BSP spots that were not associated with amino acid (Table 2). It can be concluded that there was a qualitative change in the metabolism of BSP due to BCNU treatment. BCNU might directly interfere with the GSH conjugation reaction. Alternatively, BCNU may exert an indirect effect. For example, it is known that bile salts bind GSH-S-transferase B with the less hydroxylated forms showing the highest affinity (62). It has also been demonstrated that bile salts inhibit conjugation of several xenobiotics by glutathione-S-transferases and that 50% inhibition occurred in the physiological range of bile salt concentrations (63). The 50% inhibitory concentration decreased with decreasing hydroxylation of the steroid ring system. These considerations are important since BCNU increased biliary bile salt concentrations at the time of a maximal decrease in BSP excretion. Other indirect effects are certainly possible.

Inhibition of BSP conjugation could explain the decreased BSP excretion rate. It has been shown that cBSP is excreted much more rapidly than BSP despite the fact that cBSP is taken-up from plasma at a slower rate than BSP (38).

Because BSP is extensively metabolized via GSH conjugation, an assay of some index of GSH status in the liver was required. The hepatic content of reduced glutathione, GSH, was chosen because of its role in conjugation, detoxication, and cell protection (51, 64). The 20 mg/kg dosage of BCNU caused an accumulation of GSH in the liver which was significant prior to the effect on bile flow and the effect on BSP excretion (Fig 10-11). Since rats were pair-fed, the effect of food intake on GSH content and turnover can be excluded from the BCNU mechanism (44).

The concentration of GSH in liver depends on the balance between synthesis, degradation, and efflux of GSH. GSH is synthesized from glutamate, cysteine, and glycine. The enzyme, gamma-glutamylcysteine synthetase, which forms gamma-glutamylcysteine from glutamate and cysteine is subject to feedback inhibition by GSH. GSH undergoes conjugation reactions via glutathione-S-transferases. Both conjugated and unconjugated reduced glutathione (GS-X and GSH) are degraded by gamma-glutamyl transpeptidase. GSH can be peroxidized to oxidized glutathione (GSSG) via glutathione peroxidase in a reaction which protects cells from free radical and oxygen toxicities. GSH is recycled from GSSG via glutathione reductase utilizing reducing equivalents from NADPH (64). Both GSH and GSSG undergo efflux from hepatocytes into plasma and bile (47). Arguments have been made that efflux of GSH from hepatocytes is the most important process that

decreases hepatic GSH concentrations, and that enzymatic mechanisms are of lesser importance (47, 65). The possible sites of action of BCNU in this system will be discussed as they relate to the elevated hepatic GSH observed.

One documented effect of BCNU is the irreversible and stoichiometric inhibition of glutathione reductase (GSSG-R) in solution (66). In addition, GSSG-R is inhibited in a number of cell types (67). It has also been shown that in the mouse 50 μg/kg of BCNU given parenterally causes an 88% inhibition of GSSG-R activity in the liver 10 minutes after dosing (67). This effect would serve to decrease hepatic GSH concentrations. However, there is evidence that synthesis of GSH is increased in the erythrocytes of humans with familial GSSG-R deficiency (68). A compensatory increase in GSH after BCNU is plausible considering that there is a feedback inhibition by GSH at gamma-glutamylcysteine synthetase and this may explain the increased hepatic GSH observed in the present study (64).

It is conceivable that inhibition of glutathione-S-transferases by BCNU could result in increased liver GSH and/or decreased uptake of BSP by the hepatocyte. The magnitude of the increase of GSH at a certain time would depend on the basal level of activity of these enzymes. Such inhibition might explain the decreased BSP excretion rates and changes in BSP metabolism as mentioned. However, GSH accumulation was evident at 24 hours after 20 mg/kg BCNU and BSP excretion was not reduced until 36 hours after that dosage. One would expect decreased BSP excretion and GSH accumulation to be coincident if transferase function is rate limiting for BSP excretion and if it is important for GSH homeostasis. If glutathione-S-transferase activity

is significantly inhibited by BCNU at 24 hours, this would imply that BSP excretion does not depend on conjugation. This is unlikely since cBSP is excreted more rapidly than free BSP (61). It is still possible that transferase inhibition accounts for the BCNU effect on BSP.

If GSH efflux is important, then its inhibition by BCNU could explain the increase in hepatic GSH. GSH efflux occurs across the basolateral and canalicular membranes (47, 69). GSSG also crosses both membranes (47, 65). GSH is not taken up from plasma (47). In contrast to BSP, GSH moves into bile down its concentration gradient (46). Biliary efflux is proportional to hepatic GSH concentration, but facilitated transport cannot be ruled out (46, 47). GSSG appears to be concentrated in bile relative to liver suggesting energy coupled transport (46). GSH and GSSG apparently do not compete for biliary excretion and it is GSH that is the predominant form in bile (46, 47). It would seem then that interactions between GSSG and GSH in biliary excretion are not important. Therefore, any increases in hepatic GSSG that might occur due to an inhibition of GSSG-R by BCNU, should not affect the efflux of GSH. Also, GSH and GSSG effluxes are not inhibited by cBSP in the anesthetized rat, implying that there are three separate routes of biliary excretion for these compounds (47). It is therefore unlikely that increased GSH levels (or possibly GSSG levels) could result in an inhibition of BSP excretion after BCNU treatment.

The increase in GSH caused by BCNU may be explained by an inhibition of carrier mediated transport or a decrease in membrane permeability to GSH. In a general sense basolateral and canalicular fluxes of GSH may be decreased. Kaplowitz et al. found that although GSH

efflux into bile was independent of bile acid excretion rate, efflux did decrease after prolonged taurocholate infusion (47). At the time of cholestasis, BCNU had caused significant increases in biliary bile salt concentration (Fig. 16). If there were early increases in bile salt levels or more limited increases in the more toxic, less hydroxylated bile salts, this could explain the hepatic accumulation of GSH. A single mechanism common to the BCNU-induced effects on hepatic GSH and BSP excretion would not be supported because of the temporal separation of these effects unless GSH excretion was more sensitive to BCNU than was BSP excretion at 24 hours.

In summary, BCNU may alter enzymatic synthesis and/or degradation of GSH, but efflux seems to be a more important factor determining hepatic GSH levels. Therefore, inhibition of efflux by BCNU could explain the increased hepatic GSH. Inhibition of glutathione-S-transferases might explain the altered BSP metabolism, but this does not seem to account for the early increase in hepatic GSH. Increased GSH could also result from stimulation of synthesis, possibly as a compensatory response to GSSG-R inhibition. Although possible, a single mechanism explaining the hepatic accumulation of GSH and decreased BSP excretion does not appear likely because of the temporal separation of the effects.

The third step in the excretion of BSP is the movement of BSP and cBSP across the canalicular membrane into bile (49). In normal rats, dogs, and man, secretion of BSP into bile after uptake from blood is regarded as the rate limiting step because the transport maximum (T_m) for BSP is less than the rate of uptake (49). Because cBSP appears in plasma or in the perfusate of the isolated, perfused, rat liver after

administration of BSP and because conjugation is extensive, canalicular flux has been assumed to be a rate controlling step, along with conjugation, in the excretion process (38, 49). Therefore, this may be an important site at which BCNU could inhibit BSP excretion.

Transport of BSP from cell to bile has been proposed to be driven by a combination of an electrical potential difference across the canalicular membrane and binding of solutes to mixed micelles in bile (11, 24). The so-called "micellar sink" is supposed to reduce the activity of solutes in bile and thus facilitate concentrative transport. BSP has been shown to self-aggregate in solution and to associate with bile salt-cholesterol-phospholipid mixed micelles in bile (24). The importance of the latter mechanism for concentrating BSP in bile is questionable because infusion of both the micelle forming and non-micelle forming bile salts is known to increase BSP T_m in dogs and hamsters (70, 71). In addition, infusion of the micelle forming bile acid analogue, sodium glyco-24, 25-dihydrofusidate decreases BSP T_m in the hamster (72). Therefore, it is unlikely that BCNU inhibits BSP excretion by altering the association with micelles since such an association does not seem to be important. Also, a lack of effect on the BSDF (Fig.14 & 15) implies that bile salt micelles are unaffected in any case (14, 73).

Curtis and Mehendale demonstrated that, in rats, transport of phenolpthalein glucuronide (a non-metabolized anion that is excreted into bile) correlates well with the activity of a fraction of magnesium adenosine triphosphatase (Mg^{2+} ATPase) in the liver plasma membrane fraction enriched in bile canaliculi (the BCEF) (13). These results would suggest the presence of some form of an active anion

secretory mechanism at the canaliculus. Nevertheless, the evidence for carrier mechanisms involved in organic anion transport across the canalicular membrane is not conclusive. In the case of carrier mediated transport, BCNU may directly affect the synthesis, degradation or function of the carrier. Indirect effects on carrier function are also possible (i.e. by decreasing membrane fluidity or producing inhibitory substances). In the case of active transport, energy transduction or energy supply may be reduced by BCNU. In the case of passive diffusion, permeability may be decreased or membrane surface area may be reduced.

It was demonstrated by O'Maille et al. that BSP and taurocholate compete for overall excretion in the dog when taurocholate was first infused above its T_m (53). However, taurocholate induced choleresis of canalicular origin and increased BSP T_m while decreasing the concentration of BSP (total) in bile when a BSP T_m was first established. The conclusion was that the bile flow rate, up to a point, can influence the BSP excretion rate. In other words, the concentration of BSP in bile alone can limit excretion (53). Solvent drag (i.e. bile flow) also increases dibromosulfopthalein (DBSP) excretion (74). DBSP differs from BSP in that there are 2 less bromine atoms in DBSP. Its excretion is similar to that of BSP except that DBSP is not metabolized (58). O'Maille et al. pointed out that, for concentrative transport of BSP, there is an intrinsic energy barrier at the T_m associated with the dissociation of the anion from hypothetical transport structures into bile. Solvent drag facilitates transport below this level (53). Certainly, such an effect implies a mechanism consistent with countertransport in a carrier mediated organic anion

transport hypothesis as alluded to by Vonk et al (74). The presence of a T_m implies the presence of a carrier, but membrane flux could theoretically be limited by membrane surface area or other physical characteristics such as viscosity.

The data obtained in this experiment demonstrate that BCNU decreases BSP excretion rate prior to the onset of cholestasis at both doses. Since the excretion rate is calculated as the biliary concentration multiplied by the bile flow rate, it is the biliary concentration of total BSP that was depressed more severely as the data do indicate.

Because of the temporal order of the effect with respect to cholestasis, excretion rate was not limited by reduced solvent drag initially. Therefore, a reduced net flux of free and/or conjugated BSP through the canalicular membrane must be a characteristic of the reduced excretion rate following BCNU treatment. Such a decrease in canalicular flux could result from aforementioned alterations in uptake and/or conjugation of BSP. These effects would simply reduce the rate of presentation of free BSP and/or cBSP to the canalicular membrane. Inhibition of conjugation with GSH would cause more free BSP to be presented to the canalicular membrane. If cBSP is excreted more rapidly than free BSP at canalicular sites, the excretion rate for total BSP would fall. Alternatively, an effect of BCNU on the canalicular membrane could explain the reduction in BSP excretion and increased cell GSH. As discussed earlier, GSH efflux must be measurably reduced prior to the reduction in BSP excretion. This is possible if GSH efflux normally occurs at a sufficiently high rate. This mechanism, inhibition of the final step in BSP and cBSP

excretion, could also explain the observed change in BSP metabolites isolated by TLC. BSP and glutathione metabolites of BSP might be subject to further metabolic activity in this case.

One additional point to consider lies at the extrahepatic level. BCNU could affect organic anion excretion by altering blood distribution. Substances that are subject to high hepatic extraction can exhibit perfusion limitation in excretion (75). Chlorpromazine causes BSP retention apparently as a result of reduced hepatic perfusion (76). Additionally, changes in the flow of blood within the liver (i.e. portacaval shunting) would alter excretion rate if there is heterogeneity in the ability of hepatocytes to excrete xenobiotics. For instance, it has been shown by immunohistochemical techniques that glutathione-S-transferases are not uniformly distributed across the lobular organizational unit (77). They appear to be more highly localized in the centrilobular regions. Thus, a BCNU effect on blood flow or distribution could reduce BSP excretion and alter BSP metabolism.

Past observations indicate that bile formation can be divided into two fractions (11, 14, 15, 73). These are termed the bile salt independent flow (BSIF) and the bile salt dependent flow (BSDF). The concept was developed from the relationship of bile flow and bile salt excretion rate. The BSIF was taken to be the y-intercept of a single regression line with bile salt excretion rate as the dependent variable. The BSDF was represented as the slope. The BSDF was supposed to be an index of the osmotic effect of bile salts in bile (the slope units are volume bile/mole bile salt). It was found that a single line cannot describe such data as well as one might expect. The slope

increases as the bile salt concentration decreases. This might be due to the dissociation of bile salts from micelles at low concentration resulting in an increased osmotic potential per mole bile salt (48). It might even be argued that no BSIF exists if the curve was to bend toward the origin at zero bile salt excretion. However, experiments that greatly deplete bile salts in rats show that bile flow is substantial at low bile salt concentrations (14, 73). It has been shown that all changes in bile flow cannot be explained by changes in bile acid excretion after various treatments (78). It has also been argued that bile flow totally dependent on bile salt excretion would be maladaptive (15). Therefore, it does seem that two fractions of bile flow exist. It has been found, however, that taurocholate increases the BSIF (19, 79). This calls into question the true "independence" of this fraction of flow.

Despite these problems, the analysis of BSDF and BSIF seems useful. In this experiment canalicular bile production was estimated with [^{14}C]-erythritol. Its use for this purpose rests on the assumptions that it is metabolically inert, distributes passively in total body water, and that it is impermeable to the bile ducts. The latter assumption has been disproven. It appears that erythritol can move by a polar route between bile duct epithelial cells (80).

BCNU treatment caused a reduction of the BSIF to about 11% of control. There was no significant change in the BSDF indicating no overt change in the molar osmotic potential of biliary 3-alpha-hydroxysteroids, in the concentration range observed (Fig. 14-15). A regression of erythritol clearance (CE) on bile flow for all data as one group produced a linear relationship with a high

correlation coefficient (.97) and a slope greater than 1.0 (1.29). This slope is an estimation of the bile: plasma erytritol ratio for all rats (Fig. 12). It indicates that water is reabsorbed at the bile ducts and that there exists some degree of erythritol impermeability at that site. A comparison of the bile: plasma erythritol ratios for the three separate groups revealed that water was reabsorbed slightly differently in each group but there was no consistent effect of BCNU (Fig. 13). The only significant effect was at the low dose where the ratio was decreased. This indicated a decrease in water reabsorption at the ductular sites or a decrease in plasma to bile erythritol flux. A decrease in water reabsorption would antagonize cholestasis. It is implied, then, that BCNU cholestasis is characterized by a lack of water movement into bile at the canalicular level.

The conclusion that the permeability to solutes is not increased is supported by the presence of high biliary bile salt concentrations at cholestasis (Fig. 16). Elevations of 1.4 and 2.1 fold were observed for the high and low doses respectively. This indicated that the bile to plasma permeability was not increased at canalicular or ductular sites since such a change would not allow the maintanance of concentration gradients between bile and plasma. The argument assumes that the plasma concentration of bile salts was not greatly increased by BCNU.

These ideas are reinforced by an examination of the effects of another cholestatic agent, alpha-napthylisothiocyanate (ANIT). Scanning electron microscopy has revealed that ANIT distorts the canaliculi and actually causes the development of diverticuli that penetrate to the Space of Disse (31). Such penetration would seem to

allow the communication of bile and plasma. Also, in the isolated, perfused rat liver ANIT increased the permeability between bile and perfusate as measured by the distribution of sucrose or phosphate. In addition, ANIT almost completely inhibited the ability of livers to concentrate BSP and taurocholate in bile relative to perfusate or relative to control bile (30). This result is expected if permeability is increased.

BCNU inhibits the ability of rats to concentrate BSP in bile, but bile salt concentrations are greatly increased. These results are therefore not consistent with increased permeability between bile and plasma at canalicular or ductular sites, unless the increase is selective for the more negatively charged and higher molecular weight solute (BSP). This is unlikely since permeability decreases with increased molecular weight, distribution across a cell membrane increases with the concentration of the nonionized form, and the junctional complexes which form the canaliculi present a barrier to anion movement between bile and plasma (80, 81, 83).

It should be noted that the average bile salt excretion rates were reduced in BCNU treated rats (Fig. 17). In view of the large increases in bile salt concentrations, this effect is probably a function of reduced solvent drag and not a result of an impairment in the bile salt secretory mechanism. This is in contrast to the effect of BCNU on BSP excretion. As was mentioned earlier, O'Maille et al. observed that BSP and taurocholate compete for overall excretion in the dog when taurocholate is first infused above its T_m (53). Reduced organic anion excretion due to BCNU might therefore be a result of increases in bile salt concentrations if these substances share

canalicular transport systems and if bile salt concentrations are elevated early enough.

A correlation between the activity of sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase) both of the liver plasma membrane fraction enriched in bile canalicular membranes (BCEF) and other surface membrane fractions, and the BSIF has been established (1, 11, 15, 16, 18, 19, 83, 84, 85). The concept that canalicular Na^+/K^+-ATPase is responsible for the BSIF has been questioned (11, 13, 15, 86). Boyer has pointed out that the canalicular membrane makes up a very small portion of the hepatocyte surface (11). Moreover, the enzyme is primarily localized on the basolateral membrane (87). The activity of Na^+/K^+-ATPase is less than that of magnesium adenosine triphosphatase (Mg^{2+} ATPase) in the BCEF as well (12). The correlations of Na^+/K^+-ATPase activity with BSIF may be explained by the demonstrated contamination of BCEF preparations with basolateral membrane (13). These considerations have led to the concept of a paracellular movement of water and electrolytes through junctional complexes into canalicular bile (11, 88). Junctions may therefore represent a site where BCNU could reduce water flux into bile in accord with the conclusions drawn from the erythritol clearance data.

Basolateral Na^+/K^+-ATPase may also present a site of action for BCNU since, within the concept of paracellular electrolyte flux, this enzyme is responsible for the transduction of chemical energy into a sodium (Na^+) gradient which ultimately results in the movement of water into the canaliculi (11). In the isolated, perfused rat liver (IPRL), substitution Na^+ with choline in the perfusate causes a

reduction in BSIF (20, 23). The Na^+ gradient from plasma to cell may be important as well. For example, the basolateral uptake of the choleretic bile salt, taurocholate, is Na^+ dependent (25).

It has been established that several electrolytes are important in the maintainance of the BSIF (20, 23). Ion substitution in the perfusate of the IPRL was the technique applied. Hardison and Wood demonstrated that total substitution of bicarbonate with tricine reduced BSIF 50% (21). This level of effect has been confirmed by others (20, 22, 23). Very recently evidence has been obtained that a fraction of the BSIF is chloride dependent. However, chloride dependence is not observed unless bicarbonate has been removed from the perfusate as well, indicating that the chloride system may be partially compensated for with the bicarbonate system (20). An important observation is that lithium (Li^+) can replace Na^+ and support the BSIF (20, 23). Anwer and Hegner concluded that Li^+ distributes in an extracellular /intracellular ratio greater than 1.0 while inside membrane potential was about -8 mV as measured by $^{36}Cl^-$ equilibrium potential (20). Potassium cyanide, but not ouabain, decreased the Li^+ ratio to less than 1.0. Apparently, Li^+ can substitute for Na^+ in some active system other than Na^+/K^+-ATPase. The authors indicated the possibility of an ill-defined Na^+/Cl^- coupled transport system, possibly involving a Na^+/H^+ exchange mechanism at one cellular pole or the other to account for bicarbonate transport as well. It was indicated that bicarbonate accounts for 50% or more of the BSIF and the Cl^- system accounts for 30% of the BSIF (20).

BCNU could completely inhibit these 2 systems, then for a substantial reduction in the BSIF if such systems are present in the

anaesthetized rat. Specific effects that BCNU may have on whatever components of BSIF that actually exist cannot be deduced from the present data however.

In vivo, the electrolyte systems described would appear to be related to the Na^+ gradient across the cell membrane. The efficacy of Li^+ in supporting the BSIF is not inconsistent. Therefore, basolateral Na^+/K^+-ATPase may be hypothesized to transduce at least some chemical energy for the BSIF and its inhibition may be a critical step in the pathogenesis of BCNU-induced cholestasis. Any combination of effects could result in the substantial reduction seen, but it is unlikely that any single component is specifically affected when one considers the magnitude of the BCNU effect on the BSIF.

On the extrahepatic level, it is known that various hormones affect the BSIF (53, 89, 90, 91). BCNU could alter the release of any of these substances and therefore have some effect on the BSIF.

Also, it has been demonstrated that changes in hepatic perfusate flow specifically affect the BSIF, and reduced perfusate flow has been shown to be a component of the cholestasis due to scillaren and choloropromazine (22, 92, 93, 94). It was demonstrated in the isolated, perfused rat liver that a three-fold reduction in perfusate flow (from 5.5 to 6.5 ml/min/g liver down to 1.7 to 2.3 ml/min/g liver) caused only an 18% decrease in BSIF without altering the BSDF. Any perfusate flow above approximately 3 ml/min/g did not affect the BSIF (92). BCNU reduced the BSIF to 11% of control. This degree of reduction cannot be explained on the basis of altered hepatic perfusion alone. It is certainly possible, if not probable, that

there are changes in hepatic perfusion rates and this may be responsible for some component of BCNU-induced cholestasis in vivo.

To explore the idea that basolateral Na^+/K^+-ATPase is involved in the energy transduction for the BSIF, it was decided to measure bile and plasma osmolality, Na^+ and K^+ concentrations. Consideration of plasma perturbations after treatment should include other possible systemic effects including those on the kidneys. It should be noted that extrahepatically induced changes in plasma: hepatocyte ratios of solute concentration might also affect the generation of BSIF if it ultimately depends on a Na^+ gradient. The IPRL might be used to eliminate such extrahepatic effects that may occur. Osmolality, Na^+ and K^+ were determined before and during cholestasis to decide whether any changes might be a cause of cholestasis.

It was noted that all bile: plasma ratios (B/P) were either statistically unchanged or increased in the treated rats relative to control (Table 3-5). Significant increases in B/P osmolality occurred at both doses of BCNU prior to cholestasis (Table 3). These changes support the idea that solute permeability is not increased at the canalicular or ductular sites. The erythritol clearance data support the idea that increased water reabsorption at the ductular site is not responsible for BCNU-induced cholestasis. Thus, it would appear that increased bile osmolality results from decreased water movement into canalicular bile. The early increases in B/P osmolality may represent the development of this change, and increased biliary bile salt concentrations may account for it. Bile flow may be maintained at early times by compensatory increases in solute movement into canalicular bile. In any case, increases in water or solute permeability would be

expected to dissipate concentration gradients, and this was not observed. A BCNU-induced decrease in permeability to water is consistent with these results, and it may be a cause of cholestasis.

Significant decreases in plasma Na^+ were observed prior to the onset of cholestasis, at both doses (Table 5). The most significant changes observed were the large increases in plasma K^+ levels. This was most severe at the low dose, where mean values above 8 meq/l were seen (Table 4). Changes such as these are consistent with a decrease in the activity of membrane Na^+/K^+-ATPase. However, there is no reason to expect that this effect is confined to the liver. The effects on Na^+ and K^+ are also consistent with increases in permeability of cells to Na^+ and K^+ or cell lysis, which might be considered as the ultimate sort of permeability. Along this line, it is known that humans deficient in glucose-6-phosphate dehydrogenase that receive BCNU are very susceptible to oxidative hemolysis which is probably due to the ability of BCNU to inhibit GSSG-R (67). This known effect of BCNU could elicit the release of K^+ from many cellular sites. It is also possible that increases in plasma bile salts occur early, in view of the high biliary concentrations seen at the time of BCNU-induced cholestasis. The bile salts possess a well known detergent character which could elicit K^+ release (95). Other factors such as acidosis can result in the release of cellular K^+ stores (81). In view of the importance of the kidneys in the maintenance of electrolyte homeostasis, the renal system should be acknowledged as a possible site of action of BCNU responsible for increased plasma K^+ (81). Regardless of whether BCNU induces changes in electrolytes by

an action on hepatic or extrahepatic Na^+/K^+-ATPase, such alterations would be expected to reduce the BSIF as was observed.

In conclusion, BCNU-induced cholestasis can be characterized as a specific reduction in the so-called BSIF. The reduction in BSIF is most likely due to a decrease in the movement of water into canalicular bile rather than an increase in the reabsorption of water at other sites in the biliary tree. Changes in plasma Na^+ and K^+ concentrations are consistent with this conclusion. Because biliary bile salt concentrations and bile:plasma osmolality ratios are increased, BCNU-induced cholestasis is not due to increased permeability between bile and plasma as has been proposed for agents such as alpha-napthylisothiocyanate and phalloidin (26, 30). This leaves open the possibility that the permeability of the system to water is decreased. The permeability of junctional complexes can be assessed in more detail with a determination of sucrose clearance (26).

BCNU inhibits organic anion (BSP) excretion prior to the development of cholestasis while the transport of bile salts is sustained. This change is most likely due to an effect on xenobiotic metabolism (i.e. GSH-S-transferase) a decrease in canalicular membrane transport of BSP (active, facilitated or passive), or both. In conjunction with this, reduced glutathione concentration in the liver was found to increase prior to effects on BSP excretion and bile flow. Therefore, the increase in GSH cannot be completely explained by a postulated inhibition of GSH-S-transferase. This change could be due to an effect of BCNU on enzymatically controlled glutathione homeostasis,

hepatocellular efflux, or both. A single mechanism explaining the BCNU-induced effects on GSH and BSP is not possible unless there is a difference in sensitivity to a primary lesion induced by BCNU. For example, BCNU could exert an effect on the canalicular membrane to reduce BSP excretion and GSH efflux. This idea is consistent with the conclusion that the permeability of the biliary system is not increased by BCNU. However, if this is true GSH efflux must be measurably reduced earlier than BSP excretion. This is not to say that the effect on GSH is not the sum of increased synthesis and reduced degradation and/or efflux.

Because a linear relationship between hepatic GSH concentration and its biliary excretion rate exists, it should be possible to determine whether BCNU reduces the canalicular efflux of GSH by measuring those parameters (47). The analysis of the canalicular excretion of BSP is more problematic. However, it should be possible to measure cellular uptake and GSH-S-transferase activity separately (54, 63). Inferences regarding the canalicular step might then be made by a process of elimination.

Bibliography

1. Plaa, G.L., Priestly, G.B., Intrahepatic cholestasis induced by drugs and chemicals. Pharmacological Reviews 28(3):207-273, 1977.

2. Sherlock, S.: Patterns of hepatocyte injury in man. Lancet, 782-786, Apr. 3, 1982.

3. Schaffner, F., Popper, H.: Cholestasis is the result of hypoactive, hypertrophic smooth endoplasmic reticulum in the hepatocyte. Lancet, ii: 355-359, 1969.

4. Boyd, G.S. and Percy-Robb, I.W.: Enzymatic regulation of bile acid synthesis. American Journal of Medicine 51: 580-587, 1971.

5. Elliot, W.H. and Hyde, P.M.: Metabolic pathways of bile acid synthesis. American Journal of Medicine 51: 568-579, 1971.

6. Fisher, M.M., Magnusson, R., Miyai, K.: Bile acid metabolism in mammals, I. bile acid induced intrahepatic cholestasis. Lab. Invest., 21:88-91, 1971.

7. Javitt, N.B., Emerman, S.: Effect of sodium taurolithocholate on bile flow and bile acid excretion. J. Clin. Invest., 47: 1002-1014, 1968.

8. Kakis, G., Yousef, I.M.: Pathogenesis of lithocholate and taurolithocholate-induced intrahepatic cholestasis in rats. Gastroenterology 75: 595-607, 1978.

9. Vonk, R.J., Tuchweber, B., Masse, D., Perea, A., Audet, M., Roy, C.C., Yousef, I.M.: Intrahepatic cholestasis induced by allomonohydroxy bile acid in rats. Gastroenterology, 81: 242-249, 1981.

10. Hutterer, F., Bacchin, P.G., Denk, H., Schenkman, J.B., Schaffner, F., Popper, H.: Mechanism of cholestasis; 1. Effect of Bile acids on the microsomal cytochrome P-450 dependent biotransformation system in vitro. Life Sciences, 9: 877-887, 1970.

11. Boyer, J.L.: New concepts of hepatocyte bile formation. Physiological Reviews 60(2): 303-326, 1980.

12. Boyer, J.L., Reno, A.: Properties of (Na^+ + K^+)-activated ATPase in rat liver plasma membranes enriched with bile canaliculi. Biochimica et Biophysica Acta 401: 59-72, 1975.

13. Curtis, L.R., Mehendale, H.M.: Hepatobiliary dysfunction and inhibition of adenosine triphosphatase activity of bile canaliculi enriched fractions following in vivo mirex, photomirex, and chlorodecone exposures. Tox. and Appl. Pharm. 61: 429-440, 1981.

14. Boyer, J.L., Klatskin, G.: Canalicular bile flow and bile secretory pressure, evidence for a non-bile salt dependent fraction in the isolated perfused rat liver. Gastroenterology 59(6): 853-859, 1970.

15. Erlinger, S.: Does Na^{+}-K^{+}-ATPase have any role in bile secretion? Am. J.of Physiol., 243(4): G243-G247, 1982.

16. Layden, T.J., Boyer, J.L.: The effect of thyroid hormone on bile salt-independent bile flow and Na+, K+-ATPase activity in liver plasma membranes enriched in bile canaliculi. J. Clin. Invest. 57: 1009-1018, 1976.

17. Reichen, J., Paumgartner, G.: Relationship between bile flow and Na+, K+ adenosinetriphosphase in liver plasma membranes enriched in bile canaliculi. J. Clin. Invest. 60: 429-434, 1977.

18. Simon, F.R., Sutherland, E., Accatino, L: Stimulation of hepatic sodium and potassium-activated adenosine triphosphatase activity by phenobarbital. J. Clin. Invest. 59: 849-861, 1977.

19. Wannagat, F-J., Adler, R.D., Ockner, R.K.: Bile acid induced increase in bile acid-independent flow and plasma membrane NaK-ATPase activity in rat liver. J. Clin. Invest. 61: 297-307, 1978.

20. Anwer, M. Sawkat, Hegner, D.: Role of inorganic electrolytes in bile acid-independent canalicular bile formation. Am. J. Physiol. 244(2): G116-G124, 1983.

21. Hardison, W.G.M., Wood, C.A.: Importance of bicarbonate in bile salt independent fraction of bile flow. Am. J. Physiol. 235: E158-E164, 1978.

22. Miyai, K., Hardison, W.G.M.: Cholestasis induced by scillaren administration, bicarbonate deprivation, or reduced hepatic blood flow. Experimental and Molecular Pathology 36: 333-346, 1982.

23. Van Dyke, R.W., Stephens, J.E., Scharshmidt, B.F.: Effects of ion substitution on bile acid-dependent and independent bile formation by rat liver. J. Clin. Invest. 20: 505-517, 1982.

24. Scharschmidt, B.F., Schmid, R.: The micellar sink, a quantitative assessment of the association of organic anions with mixed micelles and other macro-molecular aggregates in rat bile. J. Clin. Invest. 62: 1122-1131, 1978.

25. Van Dyke, R.W., Stephens, J.E., Scharshmidt, B.F.: Bile acid transport in cultured rat hepatocytes. Am. J. Physiol. 243(6): G484-G492, 1982.

26. Elias, E., Hruban, Z., Wade, J.B., Boyer, J.L.: Phalloidin induced cholestasis: a microfilament mediated change in junctional complex permeability. Proc. Soc. Nat. Acad. Sci. 77(4): 222-2233, 1980.

27. Phillips, M.J., Oda, M., Mak, E., Fisher, M.M., Jeejeebhoy, K.N.: Microfilament dysfunction as a possible cause of intrahepatic cholestasis. Gastroeneterology 69: 48, 1975.

28. Phillips, M.J., Oshio, C., Miyairi, M., Smith, C.R.: Intrahepatic cholestasis as a canalicular motility disorder, evidence using cytochalasin. Lab. Invest. 48(2): 204-211, 1983.

29. Oshio, C., Phillips, M.J.: Contractility of bile canaliculi, implications for liver function. Science 212: 1041, 1981.

30. Krell, Hoke, H., Pfaff, E.: Development of intrahepatic cholestasis by α-napthyliosothiocyanate in rats. Gastroenterology 82, 507-514, 1982.

31. Yoshino, K.: Scanning electron microscopy on the rat liver with alpha-napthylisothiocyanate-induced cholestasis. Gastroenterology Japan 15(6): 550-563, 1980, Abstract.

32. Thompson, G.R., Larson, R.E.: The hepatotoxicity of 1,3-BIS (2-Choloroethyl)-1-Nitrosourea (BCNU) in rats. J. Pharmacol. Exp. Ther. 166: 104-112, 1969.

33. Lu, I., Larson, R.E.: Hepatic oxidative metabolism of pentobarbital following intoxication with 1,3-BIS (2-Chloroethyl) -1-Nitrosourea (BCNU). Proc. Western Pharmacol. Soc. 13, 78-81, 1970.

34. Wilson, V.L., Larson, R.E.: Delayed alterations in hepatic mixed function oxygenase enxymes in carmustine treated mice. Proc. Amer. Assoc. Cancer Res. 21: 38, 1981.

35. Wilson, V.L., Larson, R.E.: Responsiveness of carmustine treated mice to induction and inhibition of mixed function oxygenases. Research Communications in Chemical Pathology and Pharmacology 36(3): 439-448, 1982.

36. Drew, R., Priestly, B.G.: Microsomal drug metaoblism during alpha-napthylisothiocyanate-induced cholestasis. Tox. and Appl. Pharm. 35: 491-499, 1976.

37. Richterich, R.: Clinical Chemistry: Theory and Practice. pp 444-445, Academic Press, New York, 1969.

38. Whelan G., Hoch, J., Combes, B.: A direct assessment of the importance of conjugation for the biliary transport of sulfobromophthalein sodium. J. Lab. Clin. Med. 75(4): 542-557, 1970.

39. Snedecor, G.W., Cochran, W.G.: Statistical Methods, 7th ed. pp 96-97, Iowa State University Press, Ames, Iowa, 1980

40. Talalay, P.: Enzymic analysis of steroid hormones. Methods of Biochemical Analysis 8: 132-137, 1960.

41. Neter, J., Wasserman, W.: Applied Linear Statistical Models. pp. 294-320, Richard D. Irwin, Inc., Homewood, Illinois, 1974.

42. Rowe, K., Brenne, R.: Statistical Interactive Programming System (SIPS) Command Reference Manual for Cyber 70/73 and Honeywell 440, Statistical Computing Report No. 8. Oregon State University, Jan. 1982.

43. Whelan, F.J., Plaa, G.L.: The application of thin layer chromatography to sulphobromophthalein metabolism studies. Tox. and Appl. Pharm. 5: 457-463, 1963.

44. Lauterberg, B.H., Mitchell, J.R.: Regulation of hepatic glutathione turnover in rats in vivo and evidence for kinetic homogeneity of the hepatic glutathione pool. J. Clin. Invest. 67: 1415-1424, 1981.

45. Hissin, P.J., Hilf, R.: A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74: 214-226, 1976.

46. Eberle, D., Clarke, R., Kaplowitz, N.: Rapid oxidation in vitro of endogenous and exogenous glutathione in bile in rats. J. Biological Chem. 256: 2115-2117, 1981.

47. Kaplowitz, N., Eberle, D.E., Petrini, J., Touloukian, J., Corvasce, M.S., Kuhlenkama, J.: Factors influencing the efflux of hepatic glutathione into bile in rats. J. Pharmacol. Exp. Ther. 224(1): 141-147, 1983.

48. Doull, J., Klaassen, C.D., Amdur, M.O., Editors, Casarett and Doull's Toxicology, The Basic Science of Poisons. Second Edition, pp. 45, 47, MacMillan, New York, 1980.

49. Forker, E.L.: Mechanisms of hepatic bile formation. Ann. Rev. Physiol., 39: 323-347, 1977.

50. Wheeler, H.O. Meltzer, J.I., Bradley, S.E.: Biliary transport and hepatic storage of sulfobromophthalein sodium in the unanesthetized dog, in normal man and in patients with hepatic disease. J. Clin. Invest. 39: 1131-1141, 1960.

51. Kaplowitz, N.: Physiological significance of glutathione-S-transferases. Am. J. Physiol. 239: G439-G444, 1980.

52. Klaassen, C.D., Roberts, R.J., Plaa, G.L.: Maximal biliary excretion of bilirubin and sulfobromophthalein during various rates of infusion in rats of different weights and strains. Tox. and Appl. Pharm. 15: 143-151, 1969.

53. O'Maille, E.R.L., Richards, T.G., Short, A.H.: Factors determining the maximal rate of organic anion secretion by the liver and further evidence on the hepatic site of action of the hormone secretin. Journal of Physiology 186: 424-438, 1966.

54. Schwenk, M., Burr, R., Schwarz, L., Pfaff, E.: Uptake of bromosulfophthalein by isolated liver cells. Eur. J. Biochem. 64: 189-197, 1976.

55. Vonk, R.J., Jekel, P.A., Meijer, D.K.F., Hardonk, M.J.: Transport of drugs in isolated hepatocytes, the influence of bile salts. Biochemical Pharmacology 27: 397-405, 1978.

56. Anwer, M. Sawkat, Hegner, D.: Effect of organic anions on bile acid uptake by isolated rat hepatocytes. Hoppe-Seyler's Z. Physiol. Chem. 359: 1027-1030, 1978.

57. Laperche, Y., Preaux, A.M., Berthelot, P.: Two systems are involved in the sulfobromophthalein uptake by rat liver cells: one is shared with bile salts. Biochemical Pharmacology 30(11), 133-1336, 1981.

58. Gregus, Z., Fischer, E.: Effect of sodium taurocholate on hepatic uptake and biliary excretion of organic anions in rats. Arch. Int. Pharmacodyn. 240: 180-192, 1979.

59. Levi, A.J., Gatmaitan, Z., Arias, I.M.: Two hepatic cytoplasmic protein fractions, Y and Z, and their possible role in the hepatic uptake of bilirubin, sulfobromophthalein, and other anions. J. Clin. Invest. 48: 2156- , 1969.

60. Combes, B., Stakelum, G.S.: A liver enzyme that conjugates sulfobromophthalein sodium with glutathione. J. CLin. Invest. 40: 981-988, 1961.

61. Gregus, Z., Klaasen, C.D.: Comparison of biliary excretion of organic anions in mice and rats. Tox. and Appl. Pharm. 63: 13-20, 1982.

62. Lawrence, R.A., Baker, P.R., Cuschieri, A.: Bile salt binding to hepatic ligandin and serum albumin of the rat. Biochemical Society Transactions 8(3): 372, 1980.

63. Vessey, D.A., Zakim, D.: Inhibition of glutathione-S-transferase by bile acids. Biochemical Journal 197, 321-325, 1981.

64. Meister, A.: Selective modification of glutathione metabolism. Science 220 (4596): 472-477, 1983.

65. Sies, H., Bartoli, G.M., Burk, R.F., Waydhas, C: Glutathione efflux from perfused rat liver after phenobarbital treatment, during drug oxidations, and in selenium deficiency. Eur. J. Biochem. 89, 113-118, 1978.

66. Babson, J.R., Reed, D.J.: Inactivation of glutathione reductase by 2-choloroethyl nitrosourea-derived isocyanates. Biochemical and Biophysical Research Communications: 83(2): 754-762, 1978.

67. Frischer, H., Ahmad, T.: Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU (1,3-Bis(Choloroethyl)-1-Nitrosourea). J. Lab. Clin. Med. 89(5):1080-1091, 1977.

68. Loos, H., Roos, D., Weeining, R., Houwerzijl, J.: Familial deficiency of glutathione reductase in human blood cells. Blood 48, 53-62, 1976.

69. Tate, S., Meister, A.: Interactions of gamma-glutamyltranspeptidase with amino acids, dipeptides, and derivatives and analogs of glutathione. J. Biological Chem. 249: 7593, 7602, 1974.

70. Binet, S., Delage, Y., Erlinger, S.: Influence of taurocholate, taurochenodeoxycholate, and taurodehydrocholate on sulfobromophthalein transport into bile. Am. J. Physiol. 236(1): E10-E14, 1979.

71. Ritt, D.J., Combes, B.: Enhancement of apparent excretory maximum of sulphobromophthalein sodium (BSP) by taurocholate and dehydrocholate. J. Clin. Invest. 46: 1108-1109, 1967.

72. Delage, Y., Dumont, M., Erlinger, S.: Effect of glycodihydrofusidate on sulfobromophthelein transport maximum in the hamster. Am. J. Phsyiol. 231(6): 1875-1878, 1976.

73. Balabaud, C., Kron, K.A., Gumucio, J.J.: The assessment of the bile salt-nondependent fraction of canalicular bile water in the rat. J. Lab. Clin. Med. 89(2): 393-399, 1977.

74. Vonk, R.J., Danhof, M., Coenraads, T., Van Doorn, A.B.D., Keulemans, K., Scaf, A.H.J., Meijer, D.K.F.: Influence of bile salts on hepatic transport of dibromosulphthalein. Am. J. Physiol. 237(6): E524-E534., 1979.

75. Pang, K.S.: Hepatic clearances of drugs and metabolites. Trends in Pharmaceutical Sciences: p 247-251, June, 1980.

76. Eckhardt, E.T., Plaa, G.L.: The effect of phenothiazine derivatives on the disappearance of sulfobromophthalein from mouse plasma. J. Pharmacol. Exp. Ther. 138, 387-391, 1962.

77. Redick, J.A., Jakoby, W.B., Baron, J.: Immunohistochemical localization of glutathione S-transferases in livers of untreated rats. J. Biological Chem. 257(24): 15200-15203, 1982.

78. Klaassen, C.D.: Does bile acid secretion determine canalicular bile production in rats? Am. J. Physiol. 220(3): 667-673, 1971.

79. Baker, A.L., Wood, R.A.B, Moosa, A.R., Boyer, J.L.: Sodium taurocholate modifies the bile acid independent fraction of canalicular bile flow in the rhesus monkey. J. Clin. Invest. 64: 312-320, 1979.

80. Smith, N.D., Boyer, J.L.: Permeability characteristics of bile duct in the rat. Am. J. Physiol. 242(5): G52-G57, 1982.

81. Gilman, A.G., Goodman, L.S., Gilman, A.: Goodman and Gilman's The Pharmacological Basis of Therapeutics. p. 2-5, p 872, p 848-892. Sixth Ed. MacMillan Publishing Co., Inc., New York, 1980.

82. Bradley, S.E., Herz, R.: Permselectivity of biliary canalicular membrane in rats: clearance probe analysis. Am. J. Physiol. 235-III: E570-E576, 1978.

83. Gumucio, J.J., Valdivieso, V.D.: Studies on the mechanism of the ethinylestradiol impairment of bile flow and bile salt excretion in the rat. Gastroenterology, 61(3): 339-344, 1971.

84. Heikel, T.A.J., Lathe, G.H.: The effects of 17- -ethinyl-substitued steroids on adenosine triphosphateses of rat liver plasma membrane. Biochemical Journal 118: 187-189, 1970.

85. Simon, F.R., Gonzalez, M., Sutherland, E., Accatino, L., Davis, R.A.: Reversal of ethinyl estradiol-induced bile secretory failure with Triton WR-1339. J. Clin. Invest. 65: 851-860, 1980.

86. Meijer, D.K.F., Vonk, R.J., Weitering, J.G.: The influence of various bile salts and some cholephilic dyes on Na+, K+ and Mg++ - activated ATPase of rat liver in relation to cholestatic effects. Tox. and Appl. Pharm., 43, 597-612, 1978.

87. Latham, P.S., Kashgarian, M., The ultrastructural localization of transport ATPase in the rat liver non-bile canalicular plasma membranes. Gastroenterology, 76: 988-996, 1979.

88. Layden, T.J., Elias, E., Boyer, J.R.: Bile formation in the rat, the role of the paracellular shunt pathway. J. Clin. Invest. 62: 1375-1385, 1978.

89. Hanks, J.B., Kortz, W.J., Andersen, D.K., Jones, R.S.: Somatostatin supression of canine fasting bile secretion. Gastroenterology 84, 130-137, 1983.

90. Jones, R.S., Meyers, W.C.: Regulation of hepatic biliary secretion. Ann. Rev. Physiol. 41, 67-82.

91. Thomsen, O.Ø, Larsen, J.A.: The effect of glucagon, dibutyryl cyclic AMP and insulin on bile production in the intact rat and the perfused rat liver. ACTA Physiol. Scand. 111: 23-30, 198.

92. Tavoloni, N., Reed, J.S., Boyer, J.L.: Hemodynamic effects on determinants of bile secretion in isolated rat liver. Am. J. Physiol. 234: E584-E592, 1978.

93. Tavoloni, N., Reed, J.S., Boyer, J.L.: Effect of chlorpromazine on hepatic clearance and excretion of bile acids by the isolated perfused rat liver. Proc. Soc. Exp. Biol. Med., 170: 486-492, 1982.

94. Tavoloni, N., Reed, J.S., Hruban, Z., Boyer, J.L.: Effect of cholorpromazine on hepatic perfusion and bile secretory function in isolated perfused rat liver. J. Lab. Clin. Med. 94: 726-741, 1979.

95. Yousef, I.M., Fisher, M.M.: In vitro effect of free bile acids on the bile canalicular membrane phospholipids. Canadian Journal of Biochemistry 54: 1040-1046, 1976.