Biliary Excretion of Conjugated Sulfobromophthalein Sodium (BSP) in Rats Fed a Protein-Free Diet[1] (34292)

GREGORY WHELAN, JANE HOCH, AND BURTON COMBES[2]
(Introduced by J. D. Wilson)

The Liver-Gastroenterology Unit, The Department of Internal Medicine, The University of Texas Southwestern Medical School at Dallas, Dallas, Texas 75235

Until recently, the impression that conjugation of BSP with glutathione is an important determinant of the maximal rate at which the dye is excreted into bile has been based on evidence interpreted as indicating that impaired intrahepatic conjugation, induced by dietary means, results in a decrease in the rate of biliary excretion of BSP (1). Hepatic glutathione content and BSP–glutathione conjugating enzyme activity were depressed 70 and 25%, respectively, in the latter studies conducted in rats by feeding a protein-free diet for 2 days. After intravenous injection of these animals with unconjugated BSP, excretion of BSP into bile was reduced due to a decrease in appearance of conjugated dye. When hepatic glutathione content was maintained at approximately normal levels by supplementing the protein-free diet with 1% cystine, biliary excretion of conjugated and total BSP was restored to control values.

The recent development of a method for harvesting relatively large amounts of synthetic conjugated BSP (BSP–glutathione) (2), provides an opportunity for reexamining the above conclusion. Transport of injected BSP–glutathione from blood to bile will bypass the intrahepatic conjugation step. If impaired conjugation of BSP accounts for decreased biliary excretion of dye in rats fed a protein-free diet who are injected with unconjugated BSP, one would expect no such decrease in excretion of dye into bile when conjugated BSP is injected into a similar group of animals.

Materials and Methods. Female Sprague-Dawley rats 170–220 g were used in these studies. Special rat diets consisting of both a normal protein test diet and a protein-free diet were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Each was supplemented with a vitamin fortification mixture. Unconjugated BSP was purchased from Hynson, Westcott and Dunning Co., Inc. Conjugated BSP was synthesized and harvested by a method which will be described in detail in a separate publication. The salient features are as follows: Reduced glutathione (736 mg) was dissolved in 40 ml of an aqueous solution of BSP (50 mg/ml), in a 250-ml Ehrlenmeyer flask. The solution was brought to pH 10 by addition of approximately 2 ml of concentrated NH_4OH, the flask was covered with parafilm and shaken slowly at 37° for 2 hr. Free BSP, BSP–glutathione, and a third BSP compound, probably BSP–diglutathione, were detected on paper chromatography of the 2-hr incubation mixture. Most of the third BSP compound was removed in a purple "gel-like" material formed by addition, with constant stirring, of 2.5 vol of anhydrous acetone for each volume of aqueous solution. After centrifugation at 2500 rpm in a swinging bucket rotor in an International Centrifuge, model CM, the supernate containing primarily free BSP and BSP–glutathione was decanted and BSP–glutathione was harvested by slow addition during continuous mixing of 4.5 vol of anhydrous acetone for each 3.5 vol of supernate. Centrifugation was carried out as be-

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fore, the supernate was discarded, and the tube walls and surface of the resultant gel-like material were carefully rinsed twice with 87.5% acetone. The rinses were discarded, the gel-like precipitate was dissolved in distilled water and lyophilized. The resultant purple-tinged powder contained two BSP bands by paper chromatography with approximately 95% consisting of BSP–glutathione and 5% probably BSP–diglutathione.

A number of animals previously maintained on a regular pellet diet were weighed and then divided into two groups, one of which was fed the normal protein test diet and the other the protein-free diet for a period of 2 days. The animals were then reweighed on the morning of the study and any change in weight recorded. The common bile ducts of the rats were cannulated with fine polyethylene tubing (PE 10) under light ethyl ether anesthesia, and the midline abdominal incision was closed with sutures. Within each group, some animals were given unconjugated and others synthetic conjugated BSP. In other experiments conducted in rats on a control diet, it was demonstrated that the maximal rate of dye excretion into bile was greater in animals receiving conjugated BSP (2). Moreover, larger doses of conjugated material had to be injected in order to maintain dye excretion rates at maximal values during the course of the study. The doses of 18 μmoles/100 g of body weight for unconjugated BSP and of 30 μmoles/100 g of body weight for conjugated BSP were used in these studies. Both dyes were diluted to the required concentration with sterile isotonic saline and administered into a femoral vein as a single injection. Prior to injection, a control bile sample was collected for 10 min. Bile was then collected into previously tared bottles for four successive 10-min samples following injection. At the end of each study, a blood sample was removed via the aorta into a previously heparinized syringe. The rats were kept lightly anesthesized throughout the study with ether. Body temperature was maintained between 37 and 38° by the use of an electric heating pad beneath the animal and was monitored with the use of a rectal probe (Tele-Thermometer, Yellow Springs Instrument Co.).

The concentration of BSP in bile was measured colorimetrically in a Beckman DU spectrophotometer set at 575 mμ, on appropriately diluted bile specimens (5 μl of bile to which 4 ml of 0.1 *N* KOH was added) against a reagent blank consisting of 0.1 *N* KOH. The concentration of BSP was then calculated from a previously constructed standard curve and recorded as μmoles of dye/100 ml bile. Total BSP excretion in any sample was calculated as the product of bile volume (considered equivalent to the weight of bile collected) and BSP concentration and is expressed as μmoles of BSP/100 g of body weight/10 min. The rate of dye excretion into bile for each rat was calculated by averaging the values for each period, once stable maximal rates were attained. This usually included data from the last three bile collection periods.

Aliquots of bile were applied to Whatman No. 1 filter paper and subjected to descending chromatography as previously described (3) in a solvent system consisting of *n*-propyl alcohol, water, and glacial acetic acid in a 10:5:1 (v/v) ratio. The presence of BSP compounds was ascertained by exposing the dried chromatograms to ammonia vapor and the distribution of BSP compounds was determined as described in detail in previous publications (3, 4). The BSP concentration in plasma was measured by the method of Gaebler (5).

Results and Discussion. In confirmation of previous studies conducted in this laboratory (1), feeding a protein-free diet for 2 days resulted in a decrease in the maximal rate of dye excretion into bile when free BSP was administered intravenously (Table I). The fall was attributed completely to a decrease in appearance of conjugated dye in bile. Excretion of free BSP increased, but the increment was insufficient to maintain the maximal rate of dye delivery into bile at control levels. Bile volume was depressed prior to and after injection of free BSP in the rats on a protein-free diet. The concentration of dye in bile was similar in the control and experimental animals. Plasma concentration of dye

TABLE I. Dye Excretion into Bile in Rats on Control and Protein-Free Diets.

Material injected (μmoles /100 g)	Diet	No. of animals	Liver wt /body wt (%)	BSP conc in plasma at 40 min (μmoles /100 ml)	Bile vol (ml/100 g/10 min)		Dye in bile			
					Control	During dye excretion	Conc (μmoles /100 ml)	Maximal excretion (μmoles/ 100 g/10 min)	Distribution (μmoles/100 g/10 min)	
									Free	Conjugated
Free BSP, 18	Control	9	4.0 ± 0.3[a]	49.5 ± 9.7	0.082 ± 0.013	0.072 ± 0.015	2043 ± 179	1.45 ± 0.34	0.46 ± 0.07	0.99 ± 0.31
	Protein-free	8	3.6 ± 0.2	67.8 ± 14.6	0.062 ± 0.007	0.055 ± 0.012	2012 ± 238	1.07 ± 0.19	0.65 ± 0.13	0.42 ± 0.13
		p:	<0.02[b]	<0.01	<0.01	<0.05	>0.8	<0.02	<0.01	<0.001
Conjugated BSP, 30	Control	7	3.9 ± 0.4	48.9 ± 17.6	0.089 ± 0.008	0.119 ± 0.017	2587 ± 203	3.09 ± 0.53	0	—[c]
	Protein-free	6	3.4 ± 0.6	44.5 ± 19.5	0.075 ± 0.008	0.122 ± 0.013	2592 ± 156	3.14 ± 0.26	0	—[c]
		p:	<0.10	>0.7	<0.01	>0.8	>0.8	>0.8		

[a] Values reported represent mean ± standard deviation.
[b] Significance of difference from respective control group, *t* test.
[c] No significant difference in distribution of conjugated BSP compounds excreted in bile in both groups of animals injected with conjugated BSP.

at the conclusion of the study was elevated in the rats on the protein-free diet.

By contrast, the maximal rate of dye delivery into bile was the same in animals on protein-free or control diets when conjugated BSP was injected (Table I). Bile volume, although depressed during the preinjection period in the rats on a protein-free diet, was comparable to that in the appropriate control animals during the period of dye excretion. The concentrations of dye in bile and in plasma were similar in the control and experimental animals. As reported previously (2), the maximal rate of dye excretion into bile was approximately doubled when conjugated rather than free BSP was administered intravenously.

The observations that control values of dye excretion into bile are maintained in animals fed a protein-free diet when conjugated BSP is injected, and that dye excretion falls because of decreased appearance of conjugated dye in bile when free BSP is injected into similarly fed rats provide strong evidence that impaired intrahepatic conjugation of BSP was responsible for decreased dye excretion in the latter situation. The validity of this conclusion which has been questioned recently (6) is further supported by earlier studies in which it was demonstrated that the maximal rate of excretion into bile of 3, 6-dibromphthalein disulfonate, a compound related structurally to BSP, and which is transported into bile as the unconjugated material, is comparable in rats fed control and protein-free diets (7).

The combination of findings presented in the current and other studies from this laboratory (1, 2), namely that impaired intrahepatic conjugation of BSP results in a fall in dye excretion into bile when free BSP is administered, and that the rate of dye excretion is considerably enhanced when conjugated BSP is injected, demonstrate that conjugation is an important determinant of biliary transport of BSP.

Summary. Biliary excretion of injected conjugated BSP was not affected by feeding rats a protein-free diet for 2 days. The same diet was previously shown to result in decreased dye excretion into bile accounted for

by a fall in appearance of conjugated dye when unconjugated BSP was injected. The present findings support earlier conclusions that impaired intrahepatic conjugation results in a decreased rate of biliary excretion of BSP.

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