SEPARATE PATHWAYS FOR BILIARY EXCRETION OF SULFOBROMOPHTHALEIN AND BILIRUBIN IN RATS

by 3785

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TABLE OF CONTENTS

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INTRODUCTION

In spite of a large volume of reports on bilirubin (3) and BSP metabolism (52), the mechanisms of hepatic uptake of anions, their intrahepatic metabolism, and subsequent excretion in bile remain poorly understood. Much to blame for the diversity of data and contrasting conclusions regarding the hepatic aspects of anion excretion into bile is the multiplicity of objectives with which the studies reported in the literature were undertaken.

Clinical interests have lead to development of liver function tests in which BSP is injected intravenously and the rate of dye removal from the bloodstream is measured. Plasma levels of bilirubin have been correlated with various pathologic conditions. Although of great clinical usefulness, measurements of decreasing anion concentrations in plasma following their intravenous injections cannot assess individually the multiple processes responsible for removal from the bloodstream, viz., uptake by liver, storage and metabolism within the liver cell, excretion into bile, and production of bile volume. A disturbance in any one of these processes may lead to decreased rates of dye removal from plasma.

To distinguish between the various processes involved in the transport of a compound from blood to bile, and to derive which process in this chain is rate-limiting, a number of studies have been reported in which the compound was injected, or slowly infused, and the concentrations of that compound and its metabolites were measured in plasma, liver, and bile.

Thus, when BSP was administered to rats (17, 31), dogs (8, 11, 12, 22, 31, 40, 44, 56, 57), sheep (2, 23, 24, 55), rabbits (31, 35, 36), or man (39, 48, 57), the following observations were made: with a small dose rate of BSP, the rate of dye excretion in bile eventually equalled the infusion rate; with increasing dose rate, the rate of excretion leveled off at a maximum value, and dye accumulated in the liver, predominantly in the conjugated form (16, 37, 45). Increased storage of dye in the liver accounted for the finding of continued high disappearance rates of dye from the bloodstream at times when the maximum excretion was reached. The maximal rate at which BSP disappeared from blood was much faster than the maximal rate of excretion in the bile (8, 11, 12, 22, 56); hence, hepatic uptake or conjugation to glutathione (7) did not seem to be rate-limiting. Similar observations have been reported when bilirubin was administered to various animal species (5, 21); uptake did not appear to limit bilirubin transport from blood to bile. Accumulation of conjugated bilirubin in the liver, high efficiency of the glucuronyl transferase system, and other evidence (3), under conditions of maximal bilirubin excretion in bile, eliminated the obligatory conjugation of bilirubin as a rate-limiting process. Hence, at present, the view prevails that under normal conditions in man (49, 51, 54), rat (5, 17, 31), dog (8, 22, 31, 40, 56, 57), guinea pig (21), sheep (23, 24, 55), and rabbit (31, 35, 36), and for a list of compounds eliminated primarily via the bile, including bilirubin and BSP, the process of excretion from hepatocyte into bile

canaliculus is rate-limiting in the overall transport of these compounds from blood to bile.

To gain understanding of the mechanism of the excretory process, a number of investigators have administered combinations of pigments and dyes to animals and then observed if competition for excretion into bile occurred. However, reports vary with respect to a large variety of animal species studied, differences in anesthesia, surgery, and analytical methods employed, differences in dose rates and modes of administration of various compounds, and variations in the time period after administration at which samples were taken for analysis. As a result, there exists considerable confusion regarding mutual influences of various compounds upon each other's excretion into bile (15, 27, 28, 30, 41). Even though there is consensus among authors that BSP is excreted into bile in preference to bilirubin, wide divergence of opinion exists to the degree of preference, and to what extent, if at all, BSP competes with bilirubin for biliary excretion (13, 18, 27, 41). Yet, a detailed quantitative understanding of mutual interactions between BSP and bilirubin in the process of their biliary elimination is essential for two reasons: (a) to decide if multiple excretory processes exist for various anions; and (b) to interpret the results of BSP clearance studies under conditions of elevated plasma bilirubin levels.

In the present report, bilirubin was infused into rats at the lowest rate that effected maximal excretion (E_{max}) into bile within 60 minutes, and sustained E_{max} for an additional

hour. When increasing amounts of BSP were infused simultaneously with the standard dose of bilirubin, increasing amounts of BSP were excreted with only a slight decrease in E_{max} for bilirubin until a combined maximal excretion rate for BSP-plus-bilirubin was reached at 1:1 molar ratio of infused anions. Increasing the dose rate for BSP above the 1:1 level resulted in mole-to-mole competition with bilirubin for biliary excretion. A model is proposed in which bilirubin and BSP compete for biliary excretion via a primary process with a low apparent K_m value for BSP, and BSP infused at higher concentrations can be excreted via a second process that requires a higher BSP concentration for its maximal excretory rate to occur. The capacities of these excretory processes are defined here. Additional evidence for the existence of multiple excretory processes apparent under various experimental and pathologic conditions is discussed, and conflicting reports on competition between BSP and bilirubin for biliary excretion are reconciled on the basis of the model.

MATERIALS AND METHODS

ANIMALS

Male Charles River rats weighing 200 to 450 grams, were fed a pelleted diet (Purina Lab Chow) ad libitum. On the day of an experiment, a rat was anesthetized by intraperitoneal injection of a 6% pentobarbital sodium solution in 0.9% NaCl (0.75 ml per kg rat weight). The bile duct was cannulated with polyethylene tubing, and the abdominal incision was sutured to prevent heat and water losses (47). Throughout the experiment, 15-minute bile samples were collected in tared --ml polyethylene tubes shielded from light; sample weights were recorded. Bilirubin was infused into the left jugular vein; each rat received about 70 μg (0.12 uM) of bilirubin dissolved in 0.07 ml of fluid (see below) per minute per 100 g body weight. BSP was infused into the right femoral vein; the different rates of BSP infusions empoyed in this investigation were obtained by varying the BSP concentrations in the infusates so that the volume of BSP solution given per minute and per 100 g rat weight was constant (0.07 ml per minute per 100 g). When BSP was given sumultaneously with bilirubin, the concentration of the bilirubin and BSP solutions infused were increased so that total volume of the two infusates administered per minute per 100 g rat was about equal to the volume infused when bilirubin or BSP was given alone. Infusion rates were regulated by the use of a Harvard multispeed infusion pump.

BILIRUBIN

Crystalline bilirubin, purchased from Nutritional Biochemicals Corporation, was used without further purification. C^{14}-bilirubin was prepared* by a modification (42) of the method of Barret et al. (6) from bile obtained from a dog that had been injected with delta-aminolevulinic acid-C^{14}. The C^{14}-bilirubin used had a constant specific radioactivity upon recrystallization, and a 8 micromolar solution of the bilirubin in chloroform had a molar extinction coefficient at room temperature of more than 57000 at 450 nm (cf. ref. 9). The labeled bilirubin was stored under vacuum, protected from light, at -15°C for period up to 21 days without noticeable change in either specific activity or molar extinction. For infusion into a rat, a fresh solution of unlabelled and C^{14}-bilirubin of suitable specific activity was prepared. Bilirubin was dissolved in a small amount of 0.25 N NaOH and diluted to a concentration of circa 1 mg/ml with a solution containing 0.5% NaCl and 0.51% Na_2CO_3 (33). The solution was neutralized with 0.1 N HCl, and transferred immediately to the syringe, protected from light, belonging to the infusion apparatus.

Bile and plasma total bilirubin analysis -- The method of Hillmann and Beyer (29) was employed. A diazo-reagent was prepared by storing a mixture of 10 ml of 0.01 M 2,4-dichloranilin in 0.25 N HCl and 0.2 ml of 0.1 N $NaNO_2$ in the dark

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* We are indebted to Dr. A. S. Mia for preparing the labelled bilirubin.

at 6°C for 15 minutes, and adding 50 ml of ethyleneglycol to this mixture. A 0.2 ml aliquot of bile or plasma was mixed with 3 ml of the reagent, and the optical density of the solution was determined in a spectrophotometer (Spectronic 20) at 525 nm.

Plasma direct bilirubin analysis -- Directly reacting bilirubin was determined by the Malloy and Evelyn method (38). A 100-µl plasma sample was mixed with 2 ml distilled water and 2 ml freshly prepared diazotized sulphanilic acid (10 ml 0.1% sulphanilic acid mixed with 0.3 ml 0.5% $NaNO_2$). Exactly 1 minute after mixing, the optical density was read at 560 nm in a Beckman DU Spectrophotometer, against a sulphanilic acid blank.

Liver bilirubin analysis -- The methods of Hargreaves (26) were used for the assays of total and direct hepatic bilirubin. Liver was sliced, 15 ml of a citric acid-phosphate buffer were added per g of liver slices, and the mixture was homogenized in a teflon-glass homogenizer.

Liver total bilirubin -- A diazo reagent was prepared as follows: 10 ml of 1.0% sulphanilic acid in 0.25 N HCl was mixed with 0.3 ml of a 1.5% solution of $NaNO_2$. Three ml of absolute ethanol was layered on 1.1 ml of liver homogenate, and 0.5 ml of freshly-prepared diazo-reagent and 0.1 ml of saturated ammonium sulphate solution were added. After mixing the contents, the tubes were stored at -12°C for at least

30 minutes, and then centrifuged for 10 minutes at 1500 x g. The optical density of the supernatant was determined with a Spectronic-20 colorimeter at 525 nm against a blank containing 0.5 ml of 0.25 N HCl instead of the diazo reagent.

Liver direct bilirubin -- One ml of liver homogenate was mixed with 0.1 ml of above diazo reagent. After 30 minutes, 0.1 ml of a 5% ascorbic acid solution was added, and 5 minutes later, 0.1 ml of saturated $(NH_4)2SO_4$ and 3 ml ether were added. After mixing and storing at -12°C for at least 30 minutes, the solution was centrifuged, and the optical density of the supernatant was determined as described above.

BSP

A stock solution of 50 mg/ml BSP was purchased from Hyson, Westcott and Dunning, Inc. The stock solution was diluted with saline to desired concentrations for infusion.

Bile and plasma BSP analysis -- Two-tenths ml of bile or plasma was mixed with 3 ml of 0.05 N NaOH. The optical density was determined at 575 nm against a blank containing 1.5% HCl instead of the alkali.

Separation of free and conjugated BSP -- The free and conjugated BSP of bile were separated by means of ascending paper chromatography. One-tenth ml of bile was mixed with 3.9 ml of acetone-water (3:0.9, v/v). The mixture was centrifuged for 15 minutes at 1500 x g. The supernatant was saved and

evaporated to dryness, and 0.2 ml of water was added to the residue. The sample was spotted at the end of a strip of Whatman 3 MM paper. The solvent used was a mixture of tertiary butanol and water (1.73:1, v/v). After 10 hours, the strip was taken out of the jar, dried and sprayed with a 0.25 N NaOH solution. The BSP concentrations in the separated spots were determined with a densitometer provided with a peak area integrator.

Liver BSP analysis -- Approximately 1 gram of liver slices were homogenized in about 10 ml of an acetone-water mixture (3:1, v/v). The homogenate was centrifuged for 10 minutes at 1500 x g, the supernatant was saved, and the precipitate was washed three times with the acetone-water mixture. The supernatants were pooled and evaporated to dryness under reduced pressure. The residue was transferred to a test tube using 3 ml of water and 1 ml of detergent. Turbidity was removed from this mixture by addition of chloroform and storage at 6°C for 24 hours. The optical density of the aqueous layer was determined at 575 nm against a detergent-water (1:3, v/v) blank.

PHYLLOERYTHRIN

The plant pigment was isolated by a modification of the procedures of Rimington and Quin (46). In a mortar, 800 g of sheep feces were ground to a paste with glacial acetic acid. The paste was repeatedly extracted at room temperature with mixtures of ether and small amounts of saturated sodium acetate

solution and centrifuged until no more colored material could be extracted. The supernatants were pooled, transferred to a separatory funnel, and washed with water. The water layers were discarded. The ether solution was washed with 2% HCl four times to remove non-desired porphyrins. Then, a 10% HCl solution was added to the ether layer to extract phylloerythrin from ether into the acid solution; the ether layer was discarded. The 10% HCl fraction was neutralized by addition of a saturated sodium acetate solution, and phylloerythrin was brought back into ether solution. The acid fractionation procedure was repeated twice with ether and four more times with chloroform. By then, the 2% HCl fraction remained colorless. The chloroform solution was evaporated and some warm methanol and a few drops of glacial acetic acid were added to the residue. After cooling, and centrifugation, the supernatant was discarded. The precipitate was repeatedly extracted with chloroform solution, the chloroform solutions were pooled, and phylloerythrin was extracted into 10% HCl and stored at -15°C. One hundred fifty ml of 10% HCl solution containing 540 ug of phylloerythrin per ml were obtained.

The purity of our phylloerythrin was assessed by comparing a light absorption spectrum obtained in a chloroform solution and one obtained in a 10% HCl solution with the corresponding spectra reported in the literature (46); similar peak wavelengths and relative peak heights were found; no peaks other than those reported were observed.

For infusion or injection into rats, the acidic phylloerythrin solution was neutralized with Na_2CO_3 and diluted with water for proper dosage. In the experiments in which phylloerythrin and bilirubin were infused simultaneously -- in a 0.2:1 molar ratio -- the total volume of infusate administered per minute was regulated so that it equalled the volumes of anion solutions infused when either bilirubin or phylloerythrin was given as the sole anion. The high osmolarity of our phylloerythrin infusate (about 15 times the osmolarity of serum) had no effect on bile flow rate and biliary excretion of bilirubin, as judged from control experiments in which HCl-Na_2CO_3 solutions were infused of equally high osmolarity but devoid of phylloerythrin.

CALCULATIONS

Hepatic uptake -- the term "hepatic uptake" is used, as originally proposed by Gartner et al. (21), to denote sum total of bilirubin that was processed by the liver in the course of an experiment, i.e., the sum of conjugated bilirubin found in plasma plus hepatic and biliary total bilirubin contents. A value for hepatic uptake probably represents a minimum estimate of the amount of bilirubin processed by the liver (21) since it ignores the following two processes: (1) distribution of direct-reacting bilirubin in extrahepatic tissues, including excretion in urine; and (2) escape of free bilirubin from liver to plasma.

The hepatic uptake of BSP, calculated as the sum of total biliary excretion and liver storage of BSP, is also a minimum estimate (43).

Concentration and excretion maxima in bile -- After bilirubin infusion was started, the concentration of bilirubin in bile increased steadily and then leveled off at its maximum value. The time at which the maximum concentration was reached was determined by infusing C^{14}-bilirubin and collecting bile over subsequent 2-minute intervals on tared aluminum planchets. After weighing, the bile samples were spread evenly over the planchets by dilution with acetone, evaporated, and counted in an end-window counter (Nuclear Chicago) *V. Maximum concentrations of C^{14}-bilirubin in bile were reached, in all cases, within 45 minutes after infusion was started. The concentration and excretion maxima of bilirubin and BSP in bile reported here are average values from at least three measurements obtained during a 45-minute interval after the plateau had been reached.

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* Though enough counts were collected to stay within 1% probable error (95% level), no differences were detectable between the specific activities of biliary and infused C^{14}-bilirubin. Production and excretion of endogenous (unlabeled) bilirubin was therefore negligible.

EXPERIMENTAL DESIGN

After the left jugular vein and right femoral vein and the bile duct were cannulated, zero time was recorded. During a control period, 0.9% NaCl solution was infused and the excretion of endogenous produced bilirubin in bile was measured. Infusions of BSP or phylloerythrin was started at 15 minutes and bilirubin infusion at 30 minutes, and administration of the anions was continued until 120 minutes after zero time. Then a 3-ml blood sample was obtained by heart puncture and the liver was excised, perfused with 0.9% NaCl solution, and frozen.

Effects of BSP on bilirubin -- Preliminary experimentation showed that a bilirubin infused rate of 70 µg (0.12 uM) per min per 100 g rat sufficed to establish maximal biliary excretion rates for bilirubin. Fourteen rats were infused with this dose of bilirubin and control values for bile flow rate, bilirubin concentration in plasma, liver, and bile, and for biliary bilirubin excretion were obtained. Five groups of rats were infused with the following molar concentrations of BSP relative to the standard dose of bilirubin: 0.2:1, 0.5:1, 1:1, 2:1, and 3:1. The above parameters of bilirubin distribution and bile flow in the mixed-anion-infused groups were compared with those in the control group.

Effects of bilirubin on BSP -- The results of the five groups that had received above ratios of BSP plus bilirubin were

compared with those of five groups of rats that received the same doses of BSP without added bilirubin.

Effects of phylloerythrin on bilirubin -- A group of rats was infused with a 0.2:1 molar ratio of phylloerythrin to the standard dose of bilirubin. The results of this group were compared with (a) the control rats that had received only bilirubin, and (b) rats infused with a hypertonic medium of the same composition as used to administer phylloerythrin.

RESULTS AND CONCLUSIONS

A. Experiments in which only bilirubin was infused; control group

The results of the experiments in which only bilirubin was infused are presented in Table 1. Though the rat weights varied from 260 to 570 grams, no consistent effect caused by the divergence in rat weights could be detected in the results obtained. Probably, this is due to the fact that the amounts of bilirubin infused into the rats was taken in proportion to the body weights, namely about 70 ug per min per 100 g body weight.

The following pieces of evidence are submitted to attest to the fact that maximum biliary excretion (E_{max}) for bilirubin was reached at the above infusion rate:

a. The concentration of bilirubin in bile, after reaching a maximum value within 45 minutes of bilirubin infusion, remained at its maximum over the next 45 minutes;

b. The excretion rate of bilirubin in the bile, also, reached

TABLE 1
BILIRUBIN BALANCE IN THE CONTROL RATS

During a 30-min period of 0.9% NaCl infusion, the concentration of bilirubin in bile was about 0.06 mg/g and the rate of excretion of the endogenously produced bilirubin amounted to about 0.3 μg/min/100 g. All rats were infused with bilirubin at an average rate of 69.8 μg/min/100 g for 90 minutes. Measurements on bile were made during the final 45 minutes when Emax for bilirubin had been attained, and averages of at least three determinations are listed. Terminal liver and plasma samples were analyzed.

Rat No.	Rat Wt.	Bilirubin Infused Per 90 min	Bile*			Liver Storage		Plasma Level			Total Recovery over 90 Minutes % of Infused					
			Flow&	Cmax	Emax	Total	Direct	Free	Direct	Total	Bile@	Plasma Free	Plasma Direct	Liver¢ Stored	Uptake@	Total@
			g/15 min 100g rat	mg/g	μg/min 100g rat	μg/g	% of total	μg/ml	μg/ml	μg/ml						
1	260	16.4	0.086	9.47	53.5	90	-	-	9.1	-	60.0	-	0.67	5.12	65.8	-
2	360	22.0	0.064	10.74	44.8	163	-	-	14.5	-	50.7	-	0.95	7.81	59.5	-
3	375	23.6	0.093	6.93	44.2	87	32	121	13.8	135	46.1	7.66	0.89	5.27	54.2	61.9
4	300	18.6	0.079	9.05	49.0	74	44	-	5.1	-	57.7	-	0.32	4.18	62.2	-
5	335	21.1	0.091	7.65	47.3	126	-	133	15.3	149	54.8	8.48	0.95	6.70	62.5	70.2
6	370	23.3	0.065	12.25	50.9	136	43	105	71.2	176	57.2	6.69	4.54	6.20	67.9	74.7
7	410	28.4	0.091	8.13	49.3	136	54	90	20.3	110	55.1	5.17	1.18	5.63	61.9	67.1
8	365	22.0	0.066	9.30	39.8	139	75	110	40.7	151	41.7	7.30	2.70	6.25	50.7	58.2
9	370	23.9	0.091	7.76	43.8	161	-	96	81.0	177	49.1	5.93	5.01	6.81	60.9	66.9
10	480	29.7	0.087	7.65	42.6	151	-	172	4.5	176	48.5	11.41	0.29	9.35	58.2	69.3
11	540	33.8	0.088	8.76	48.3	143	50	138	15.8	154	56.1	8.86	1.01	9.82	67.0	55.8
12	430	26.8	0.063	8.96	39.7	173	61	163	26.7	190	42.3	10.48	1.71	9.20	53.2	63.7
13	540	32.5	0.074	8.36	37.9	154	57	76	47.7	124	46.6	5.05	3.17	8.80	58.5	66.7
14	570	36.1	0.071	10.39	49.6	178	57	114	29.4	143	56.3	7.17	1.86	9.95	68.1	75.3
AV.	407	25.6	0.079	8.96	45.76	137	53	120	28.2	153	51.59	7.65	1.80	7.22	60.8	66.3
SD.$	90	5.6	0.011	1.38	4.51	31	12	28	23.0	24	5.73	1.94	1.45	1.85	5.3	5.9

* Excretion of endogenously produced bilirubin is included in the values for maximum concentration (Cmax) and excretion (Emax).
& Average flow rates during the entire 120 minutes period of the experiment are listed.
@ Excretion of endogenously produced bilirubin is not included in these figures.
¢ Stored = Amount of bilirubin recovered in the liver.
Uptake = Calculated figure, namely the sum of the recoveries in liver plus bile plus plasma-direct.
$ Standard deviation

its plateau within the first 45 minutes and remained at this level;

c. During the second 45-minute interval, while bilirubin excretion remained at the same (maximum) level, the concentration of free bilirubin in plasma was still slowly rising;

d. Infusing smaller doses of bilirubin resulted in lower excretion rates; and

e. Infusion of larger amounts of bilirubin did not increase the excretion rate. Moreover, larger amounts of bilirubin proved to be toxic: the bile flow rate dropped precipitously. Such an effect on bile flow rate was not encountered in the present study, signifying that a subtoxic, yet high enough rate of infusion was chosen to establish E_{max} for bilirubin in the bile.

Variations in weight of bile collected per 15-minute interval and per 100 g rat weight were small, considering the large range of rat weights included in the experiment. An average bile flow rate of about 0.08 g per 15 min per 100 g was observed. The small value of the standard deviation (0.01 g/15 min/100 g) was helpful in the evaluation of effects of other anion infusions upon bile flow. Bile flow rates were not influenced by the chosen rate of bilirubin infusion: the flow rates monitored over the two 15-minute control periods remained constant during the ensuing 90-minute interval over which bilirubin was infused.

During the final 45 minutes of bilirubin infusion, the concentration of bilirubin in bile as well as the rate of biliary excretion of bilirubin has stabilized at their maximum levels of about 9 mg/g and 46 μg per min per 100 g, respectively. These values are similar to those reported in the literature (1, 20, 27). At E_{max} the rate of excretion of bilirubin was about two-thirds of the infusion rate.

Over our entire 90-minute period of bilirubin infusion about half of the bilirubin infused was excreted in the bile; another 16% of the infused dose was recovered in terminal plasma and liver samples. About one-third of the infused dose was not recovered as bilirubin in plasma plus liver plus bile. Bilirubin uptake by extrahepatic tissues, as evidenced by the yellow color of adipose stores, may have constituted the major portion of the non-recovered bilirubin. Also, conversion of bilirubin to non-diazo-position products may be considered.

From the recoveries of direct-reacting bilirubin in plasma and total bilirubin in liver and bile it follows that liver processed about 60% of our infused dose. Accumulation of a small, but significant, amount of direct-reacting bilirubin in plasma suggests that the liver could take up more bilirubin than it could excrete into the bile. It has been proposed in the literature that the excretion of anions into the bile is the limiting step in the transport from blood to bile, and the present findings would support this proposal.

B. Effects of BSP on bilirubin

The bilirubin infusion rate of 0.12 μM per min per 100 g used for the control rats was maintained for the experiments in which BSP was administered simultaneously in various molar ratios relative to bilirubin. The individual results of the bilirubin-plus-BSP trials are compared with the average results of the bilirubin-alone trials (control group) in Tables 2 and 3.

The simultaneous infusion of BSP caused a significant ($p<0.05$) increase in bile flow over that of the control group (Table 2). This increase varied considerably from rat to rat and was not clearly related to the molar ratio of anions infused. The complicated relationship between dose rate and choleretic effect of BSP has been reported (2, 53).

The maximum concentration (C_{max}) of bilirubin in bile was depressed by the simultaneous infusion of BSP (Table 2). A small amount of BSP (0.2:1) already caused a significant drop ($p<0.005$) in biliary C_{max} of bilirubin. With larger amounts of BSP, the resulting depressions of C_{max} of bilirubin were proportional to the molar ratios of infused anions. The relatively steep drop in C_{max} for bilirubin occurring between the 0:1 and 0.2:1 infusion ratios may be related to the effect of BSP on bile flow. Namely, since the amount of bilirubin infused was not far in excess over the requirement to establish E_{max}, increased bile volume due to BSP decreased the bilirubin concentration in the bile below C_{max}. When this dilution effect is taken into account for rat number 17 (Table 2),

TABLE 2

RESULTS OF THE BILIRUBIN AND BSP INFUSION EXPERIMENTS

After a 15-minute control period, BSP was infused. Bilirubin infusion (70 µg/min/100 g) was started 15 minutes later, and infusion of the two compounds was continued over 90 minutes. Measurements in bile were made during the final 45 munutes when Emax had been reached. Liver and plasma assays were performed on terminal samples.

Rat No.	Rat Wt.	Infused Molar Ratio BSP/Bili.	Bile Data: Flow Rate g/15 min 100g rat	Bile Data: Bilirubin Cmax mg/g	Bile Data: Bilirubin Emax µg/min 100g rat	Bile Data: Bilirubin Emax (%)*	Bile Data: BSP Cmax mg/g	Bile Data: BSP Emax µg/min 100g rat	Bile Data: BSP Emax (%)*	Liver Storage: Bilirubin Total µg/g liver 100 g rat	Liver Storage: Bilirubin Direct % of total	Liver Storage: BSP µg/g liver 100 g rat	Plasma: Bilirubin Total µg/ml	Plasma: Bilirubin Direct µg/ml	Plasma: BSP µg/ml
AV.&407			0.079	8.96	45.8	(65.3)	-	-		33.8	53	-	120	28	-
SD.& 90		0:1	0.011	1.38	4.5	(6.3)	-	-		6.5	12	-	28	23	-
(n)&(14)			(14)	(14)	(14)	(14)	-	-		(14)	(9)	-	(11)	(14)	-
15	350	0.2:1	0.069	7.3	33	(48)	3.0	14 (70)		33.6	-	8.6	106	18	0
16	360	0.2:1	0.074	7.9	42	(60)	2.9	15 (75)		35.3	-	8.0	154	32	0
17	330	0.2:1	0.100	6.7	45	(64)	2.9	18 (90)		39.5	45	7.7	133	16	0
18	400	0.2:0	0.079	-	-	(-)	3.8	20 (100)		-	-	6.3	-	-	0
19	405	0.2:0	0.080	-	-	(-)	3.6	19 (93)		-	-	6.3	-	-	6
20	345	0.5:1	0.123	6.4	55	(79)	4.4	37 (73)		23.3	-	10.1	154	15	10
21	330	0.5:1	0.085	6.1	36	(51)	7.8	45 (88)		48.4	-	31.7	172	30	33
22	330	0.5:1	0.071	8.5	42	(60)	8.0	40 (80)		44.1	44	15.6	173	24	17
23	305	0.5:0	0.116	-	-	(-)	5.9	51 (100)		-	-	9.7	-	-	5
24	430	0.5:0	0.084	-	-	(-)	8.6	49 (99)		-	-	10.2	-	-	7
25	375	1:1	0.091	6.1	38	(54)	12.3	77 (77)		56.7	52	77	231	25	160
26	475	1:1	0.088	6.5	39	(56)	11.2	67 (66)		46.2	-	61	199	15	173
27	365	1:0	0.070	-	-	(-)	12.0	54 (54)		-	-	99	-	-	260
28	400	1:0	0.105	-	-	(-)	11.7	83 (83)		-	-	39	-	-	43
29	380	2:1	0.118	4.1	31	(44)	12.2	91 (45)		64.5	52	123	212	37	260
30	470	2:1	0.096	3.4	22	(31)	14.0	90 (44)		65.1	-	90	231	15	213
31	280	2:0	0.089	-	-	(-)	13.5	85 (43)		-	-	291	-	-	441
32	443	3:1	0.100	3.1	19	(27)	16.0	105 (35)		69.7	55	336	217	43	608
33	295	3:1	0.149	3.1	32	(46)	12.0	125 (42)		80.7	57	273	189	45	490
34	410	3:1	0.076	2.4	11	(16)	14.0	67 (22)		77.5	-	352	127	21	889
35	350	3:0	0.076@ 0.033@	-	-	(-)	15.0	77@(25) 33@(11)		-	-	540	-	-	857
36	305	3:0	0.106@ 0.044@	-	-	(-)	13.0	89@(29) 39@(13)		-	-	745	-	-	800
37	435	3:0	0.100@ 0.034@	-	-	(-)	13.0	90@(30) 29@(10)		-	-	504	-	-	1084

* Percent of infusion rate.
& Control data; average values, standard deviations, and numbers of rats.
@ With the 3:0 infusion experiment, the bile flow decreased sharply; the data presented here are high and low values.

TABLE 3

EFFECTS OF BSP ON BILIRUBIN BALANCE

For experimental conditions see Table 2. All bilirubin recoveries listed are expressed as percentages of the amounts of bilirubin infused over the entire 90-min period.

Rat* No.	Rat Wt.	Infused Molar Ratio BSP/Bili	Measured Value: Bile&	Liver	Plasma@ Free	Plasma@ Direct	Total Recovered	Derived Data: Uptake¢	Other$ Sites
15	350	0.2:1	39.4	6.8	5.7	1.2	53.0	47.3	47.0
16	360	0.2:1	43.9	7.4	7.7	2.1	61.0	53.3	39.0
17	330	0.2:1	50.7	6.8	7.4	1.0	67.9	60.5	32.1
20	345	0.5:1	61.3	5.7	8.8	1.0	76.8	68.0	23.2
21	330	0.5:1	38.0	10.4	9.1	1.9	59.3	50.2	40.7
22	330	0.5:1	44.0	10.3	9.5	1.5	65.4	55.9	34.6
25	375	1:1	38.8	12.7	13.0	1.6	66.1	53.1	33.9
26	475	1:1	40.1	11.0	11.6	1.0	63.8	52.1	36.2
29	380	2:1	34.3	16.0	11.1	2.3	63.7	52.6	36.3
30	470	2:1	24.0	17.1	14.1	1.0	56.2	42.1	43.8
32	443	3:1	23.1	20.6	11.4	2.8	58.0	46.5	42.0
33	295	3:1	36.2	18.5	9.1	2.9	66.7	57.6	33.3
34	410	3:1	12.2	20.2	6.9	1.4	40.7	33.8	59.3
Control Data		(see Table 1)							
Av.	407	0:1	51.6	7.22	7.65	1.80	66.3	60.8	33.7
SD.	90		5.7	1.85	1.94	1.45	5.9	5.3	6.2
(n)	(14)		(14)	(14)	(11)	(14)	(11)	(14)	(11)

* The rat numbers listed in this table correspond to the numbers in Table 2.
& Total recovered in bile during the entire 90-min infusion period.
@ Plasma volume was estimated as 4% of the body weight.
¢ Uptake = Sum of recoveries in bile plus liver plus plasma-direct.
$ Other sites = 100% minus total bilirubin recovered in liver, plasma, and bile.

whose bile flow rate was strongly influenced by BSP and whose bilirubin C_{max} was lowest, the mean value for C_{max} of the 0.2:1 group is only slightly, but still significantly different from the control value. The dilution effect of increased bile flow upon C_{max} is discounted in the value for E_{max} -- the product of bile flow and C_{max} (Table 2). A decrease in E_{max} with increasing ratio of BSP to bilirubin was observed over the entire range of molar ratios studied.

Since excretion of bilirubin in bile is depressed by simultaneous infusion of BSP, the question arises where the "excess" bilirubin is stored. There was a slight increase in the levels of free and direct-reacting bilirubin in plasma with the higher molar ratios infused (Table 2). However, as expressed in Table 3, the control group excreted an average of 51.6% of the infused dose of bilirubin in the bile, while the 3:1 group excreted 23.8%, leaving an excess of about 27.8%, of the infused bilirubin to be stored. Increased storage in plasma accounted for only 2% of the infused dose. Increased storage in sites other than liver, plasma, and bile accounted for about 13% of the infused dose (Table 3). The liver increased its bilirubin storage by as much as 13% of the infused dose (Table 3); hence, the liver is the chief bilirubin storage compartment under the condition of impaired biliary excretion.

The information presented in Tables 2 and 3 allows us to pinpoint the step in the chain of hepatic bilirubin uptake, conjugation and excretion at which BSP interferes. Bilirubin

uptake by liver, though slightly reduced (Table 3), remained effective enough to establish greatly increased levels of bilirubin in the liver. Also, the process of bilirubin conjugation in the liver was maintained at a high level so that the same proportion of the bilirubin in liver and plasma was present in the direct-reacting form despite the increase in these tissues (Table 2). These findings narrow the location of the interference by BSP upon bilirubin excretion down to an excretory step beyond the process of conjugation. Dilution of excreted bilirubin by the choleretic effect of BSP may explain the lowering of C_{max} for bilirubin in the bile of 0.2:1 group as compared to the 0:1 controls. But, as stated above, in the range of infused molar ratios of BSP to bilirubin from 0.2:1 to 3:1 no consistent differences in bile flow were observed and hence, the decreases in values of E_{max} and C_{max} are parallel. This finding indicates a direct effect by BSP upon the concentrating mechanism establishing C_{max} for excretion of bilirubin.

C. Effects of bilirubin on BSP

To assess the effects of bilirubin infusion upon the BSP balance, the results of rats infused simultaneously with both anions -- in variable molar ratios relative to the standard dose of bilirubin (0.12 μMoles/min/100 g) -- were compared with the data obtained from rats infused with BSP alone; e.g., the 0.5:1 group was compared with the 0.5:0 group. Rats infused with BSP alone will be referred to as BSP controls.

At an infusion rate of 0.12 μMoles of BSP/min/100 g, Cmax and Emax for BSP in bile were established in the BSP controls; the same molar infusion rate as was needed to reach Emax for bilirubin sufficed to attain Emax for BSP (Table 2, the 1:0 group). The control values obtained for Cmax (12-15 mg BSP per g bile) and Emax (80-90 μg/min/100 g) are similar to those reported by others (27, 31).

Over a range of BSP infusion rates from 0.2 to 2 times the amount needed to establish Emax, no consistent differences in effect on bile flow rate were observed between the mixed-anion-infused rats and their BSP controls (Table 2). In the 3:0 rats, however, the bile flow decreased sharply during the course of a 2-hour experiment. Interestingly enough, this situation was reversed when bilirubin was infused simultaneously (3:1 rats, Table 2).

When low concentrations of BSP were infused in the BSP controls (0.2:0 and 0.5:0, Table 2), the maximal excretion rate of BSP into bile kept pace with the infusion rate. Simultaneous administration of bilirubin, establishing lopsided bilirubin to BSP ratios of 5:1 and 2:1, decreased the excretion efficiency for BSP (Table 2). With equimolar amounts and with 2:1 molar ratios of BSP to bilirubin infused, no differences between the experimental rats and their BSP controls were observed with respect to either Cmax or Emax of BSP in bile. The high efficiency for BSP excretion in bile, observed in the 3:1 group compared with its BSP control (Table 2), resulted from the restoration of bile flow rate caused by the

simultaneous infusion of bilirubin. In fact, as shown by the E_{max} values for the 3:1 group, the simultaneous infusion of bilirubin may elevate the excretion efficiency of BSP into bile above control values.

Over the entire concentration range of BSP infused, alone or in combination with bilirubin, the storage of BSP in liver was inversely related to the rate of BSP excretion in bile (Table 2). It seemed that whatever BSP is not excreted in bile is stored to a large extent in the liver, and that a limit in hepatic storage capacity for BSP had not been reached even at our highest BSP infusion rates. Little or no BSP was detectable in plasma at the lower rates of BSP infusion, but with increased amounts of BSP infused, the level of BSP in plasma reflected that in liver (Table 2); there was about a constant ratio between BSP concentrations in plasma and liver.

Table 4 lists the total BSP recoveries in bile over the entire infusion period, as well as calculated values for uptake of BSP by liver and the sum of extrahepatic sites. The data contain the following pieces of evidence with regard to the rate-limiting step in the process of BSP excretion:

a. When increasing amounts of BSP were infused, without simultaneous bilirubin administration, larger percentages of the higher BSP doses were concentrated by the liver. When bilirubin was given simultaneously, a progressive decrease in biliary excretion of BSP was balanced by progressive increases in hepatic storage. The similar percentage uptake figures for BSP by liver in the 2:1 and

TABLE 4

EFFECT OF BILIRUBIN ON BSP BALANCE

For experimental conditions, see legends to Table 2. All BSP recoveries listed are expressed as percentages of the amounts of BSP infused over the entire 105-min period.

Rat* No.	Rat Wt.	Infused Molar Ratio BSP/Bili.	Measured Value				Derived Data	
			Bile	Liver	Plasma	Total	Uptake&	Other Sites@
15	350	0.2 : 1	57.6	5.2	0	62.8	62.8	37.2
16	360	0.2 : 1	57.9	4.8	0	62.7	62.7	37.3
17	330	0.2 : 1	73.1	5.0	0	78.1	78.1	21.9
18	400	0.2 : 0	79.5	3.6	0	83.1	83.1	16.9
19	405	0.2 : 0	76.4	4.1	1.1	81.6	80.5	18.4
20	345	0.5 : 1	59.7	2.9	0.7	63.3	62.6	36.7
21	330	0.5 : 1	73.0	8.0	2.3	83.3	81.0	16.7
22	330	0.5 : 1	58.3	4.1	0.3	62.7	62.4	37.3
23	305	0.5 : 0	79.3	2.2	0.3	81.8	81.5	18.2
24	430	0.5 : 0	75.9	3.0	0.5	79.4	78.9	20.6
25	375	1 : 1	57.4	10.0	6.1	73.5	67.4	26.5
26	475	1 : 1	49.2	8.7	5.7	63.6	57.9	36.4
27	365	1 : 0	45.3	13.1	9.6	68.0	58.4	32.0
28	400	1 : 0	64.9	6.7	2.3	73.9	71.6	26.1
29	380	2 : 1	32.8	8.7	4.9	46.1	41.5	53.6
30	470	2 : 1	29.4	6.2	3.8	39.4	35.6	60.6
31	280	2 : 0	34.7	15.3	8.2	58.2	50.0	41.8
32	443	3 : 1	28.4	18.8	7.5	54.7	47.2	45.3
33	295	3 : 1	33.7	12.3	6.1	52.1	46.0	47.9
34	410	3 : 1	17.7	17.4	15.5	46.2	35.1	53.8
35	350	3 : 0	15.8	27.7	10.7	54.2	43.5	45.8
36	305	3 : 0	18.9	27.8	10.0	56.7	46.7	43.3
37	435	3 : 0	16.6	24.4	13.5	54.5	41.0	45.5

* The rat numbers listed in this Table correspond to the numbers in Table 2.

& Uptake by liver = Sum of recoveries in bile plus liver, whereby it is assumed that no BSP, once taken up by the liver, is put back into the bloodstream.

@ Other sites = 100% minus total BSP recovered in liver, plasma, and bile.

3:1 infusion trials indicate that hepatic uptake and concentration of BSP had not reached its limit under our experimental conditions.

b. In spite of vastly different BSP concentrations in livers of rats infused with various BSP concentrations, no differences were observed in the proportions of BSP that were present as conjugates. We conclude, therefore, that hepatic uptake, concentration, and conjugation of BSP have ample capacities, but that the process of excretion into bile is the rate-limiting step in the over-all pathway from blood to bile.

D. Effects of phylloerythrin on bilirubin

Preliminary experiments showed that hypertonic, Na_2CO_3-neutralized 10% HCl solutions, infused at the same rate at which the phylloerythrin-containing solutions were administered, had no effect of bile flow rate. In a control group (0:1; Table 5), that received 0.12 μMoles of bilirubin per minute per 100 g rat in addition to the above hypertonic solution, the bilirubin balance was in no respect different from the 0:1 controls presented in Table 1. The simultaneous infusion of phylloerythrin in a 0.2:1 molar ratio to bilirubin (Table 5) caused an increase in bile flow rate and a depression in the C_{max} for bilirubin in bile, thereby leaving the value for maximum bilirubin excretion in bile unaffected. The levels of bilirubin in liver and plasma, too, remained within control limits when 0.2:1 phylloerythrin was infused.

TABLE 5

BILIRUBIN AND PHYLLOERYTHRIN INFUSION EXPERIMENTS

Phylloerythrin was stored in a 10% HCl solution. Immediately before infusion, the solution was neutralized by addition of Na_2CO_3. The control (0:1) group was infused with 0.12 μM of bilirubin per minute per 100 g rat weight and with the same amount of soda-neutralized 10% HCl solution as was given in the phylloerythrin infusion trials.

Rat No.	Rat Wt.	Infused molar ratio Phylloerythrin / Bilirubin	Bile flow rate	Bilirubin recovered in bile	
				Conc. max.	Excretion max.
			g/15 min	μg/g	μg/min	% of infusion rate
38	465	0:1	0.087	7.8	48	69
39	405	0:1	0.086	8.5	52	74
40	310	0.21:1	0.115	6.2	45	64
41	320	0.21:1	0.114	6.3	47	67
42	335	0.21:1	0.086	7.2	46	66

There was a difference in the effects of phylloerythrin and BSP upon bilirubin balance when the compounds were infused in 0.2:1 molar ratios relative to bilirubin. In either case, the bile flow rate increased, but BSP depressed the E_{max} for bilirubin in bile while phylloerythrin did not.

DISCUSSION

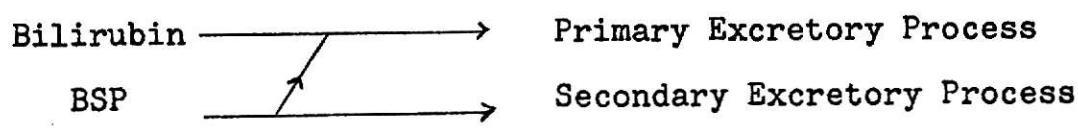
Based on our data, we will try to answer the question: does the liver discriminate between bilirubin and BSP in the process of biliary excretion?

When either bilirubin or BSP was infused alone, some striking similarities in their biliary excretion were observed: (a) the same molar concentration in the infusate (0.12 μMoles of either compound infused per min per 100 g rat weight) were required to establish E_{max}; (b) the values for C_{max} in bile were similar, namely 8.96 mg of bilirubin and about 13 mg of BSP per g bile (Table 2) which equalled 15.6 and 15.5 μMoles per g bile, respectively; and, (c) the values derived for hepatic uptake (Table 3 and 4) were similar for bilirubin and BSP in the 0:1 and 1:0 experiments.

When combinations of BSP and bilirubin were infused, however, the liver seemed to excrete BSP into bile in preference over bilirubin. The proportions of infused BSP excreted in bile were slightly larger than those for bilirubin (Table 6, last column). Cantarow et al. (13) injected either bilirubin or BSP alone, or combinations of these compounds into dogs and found that the biliary excretion of bilirubin, but not that of

BSP, was depressed in the combined-compound experiments. Hargreaves (27) infused large amounts of BSP and bilirubin into rats and reported that BSP was excreted in preference to bilirubin.

Striking as the similarities between biliary excretions of BSP and bilirubin may be when either compound is given alone, the evidence obtained from experiments in which combinations of the two compounds were administered points to a difference in the liver's processing of BSP and bilirubin. In the following discussion, the manner in which the liver discriminates between BSP and bilirubin will be probed. Based on our experimental data, evidence will be presented for the following concept:



Bilirubin, in competition with BSP, is excreted by one process, arbitrarily designated primary. A second excretory process, available only to BSP, becomes more active when the dye is administered in high concentrations because of its high K_m value for BSP relative to that of the primary process. The two excretory processes do not necessarily have different anatomical locations.

To facilitate discussion of the two excretory processes, average values for the biliary excretion of BSP and bilirubin, expressed as Moles times 10^{-8} (octomoles) per min per g rat weight, are presented in Table 6. Though the E_{max} for bilirubin

TABLE 6

AVERAGE MOLAR AMOUNTS OF BSP AND BILIRUBIN EXCRETED AT E_{max} IN BILE WHEN BOTH COMPOUNDS WERE INFUSED SIMULTANEOUSLY

verages of the biliary excretory values listed in Table 2 and of infusion rates are presented as oles x 10^{-8} per min per 100 g.

SP/Bilirubin Infusion olar atio	Moles x 10^{-8}	Biliary Excretion Maxima Bilirubin	BSP Plus Bilirubin	BSP	BSP Excreted Primary* Process	Secondary& Process	BSP/Bilirubin Excreted in Bile Primary Process	Total Excretion	Difference of Excreted Minus Infused Ratios
0:1	0 :12	8.0	8	0	0	0	-	-	-
.2:1	2.4:12	7.0	8.9	1.9	1.0	0.9	0.14	0.27	+ 0.07
.5:1	6 :12	7.7	12.6	4.9	0.3	4.6	0.03	0.71	+ 0.21
1:1	12 :12	6.7	15.3	8.6	1.3	7.3	0.19	1.28	+ 0.28
2:1	24 :12	4.6	15.4	10.8	3.4	7.4	0.74	2.35	+ 0.35
3:1	36 :12	3.6	15.4	11.8	4.4	7.4	1.22	3.27	+ 0.27

Depression in bilirubin excretion from control value of 8 (column 3) equals BSP excreted by primary process.

: BSP excreted by primary plus secondary processes equals total BSP excreted (column 5).

was 8 and that for BSP only 10 octomoles when either compound was infused alone, in the mixed-compound infusion trials a maximum excretion of 15.4 octomoles for bilirubin plus BSP was observed. This combined E_{max} value was approached when the 1:1 molar ratio of compounds was infused, and maintained at the 2:1 and 3:1 levels (Table 6, column 4). At the high levels of infusion, increased excretion of BSP was exactly balanced by decreased bilirubin excretion (Table 6). Hence, when excretion of BSP is plotted against the decrease in bilirubin excretion (Fig. 1), a linear correlation is obtained for the 1:1, 2:1, and 3:1 trials, and the slope of this line indicates that there is mole-to-mole substitution of bilirubin by BSP in the bile after a combined E_{max} of 15.4 octomoles has been reached.

At the lower BSP infusion levels (0.2:1 and 0.5:1), competition with bilirubin for excretion into bile was noticeable, but not on a mole-to-mole basis (Table 6, Fig. 1). In fact, if we extrapolate the linear portion of figure 1 to the ordinate, we find that 7.4 octomoles of BSP are excreted in addition to undiminished E_{max} for bilirubin. In view of such a large excretory capacity for BSP in addition to 8 octomoles of bilirubin, how could the following findings be explained: (a) that in the 0.2:1 and 0.5:1 trials, the bilirubin excretion was depressed relative to the 0:1 control; (b) that in these trials, the BSP excretion was depressed relative to the 0.2:0 and 0.5:0 controls (Table 2); (c) that the lowering in BSP excretion, expressed as a percentage of the excretion in the

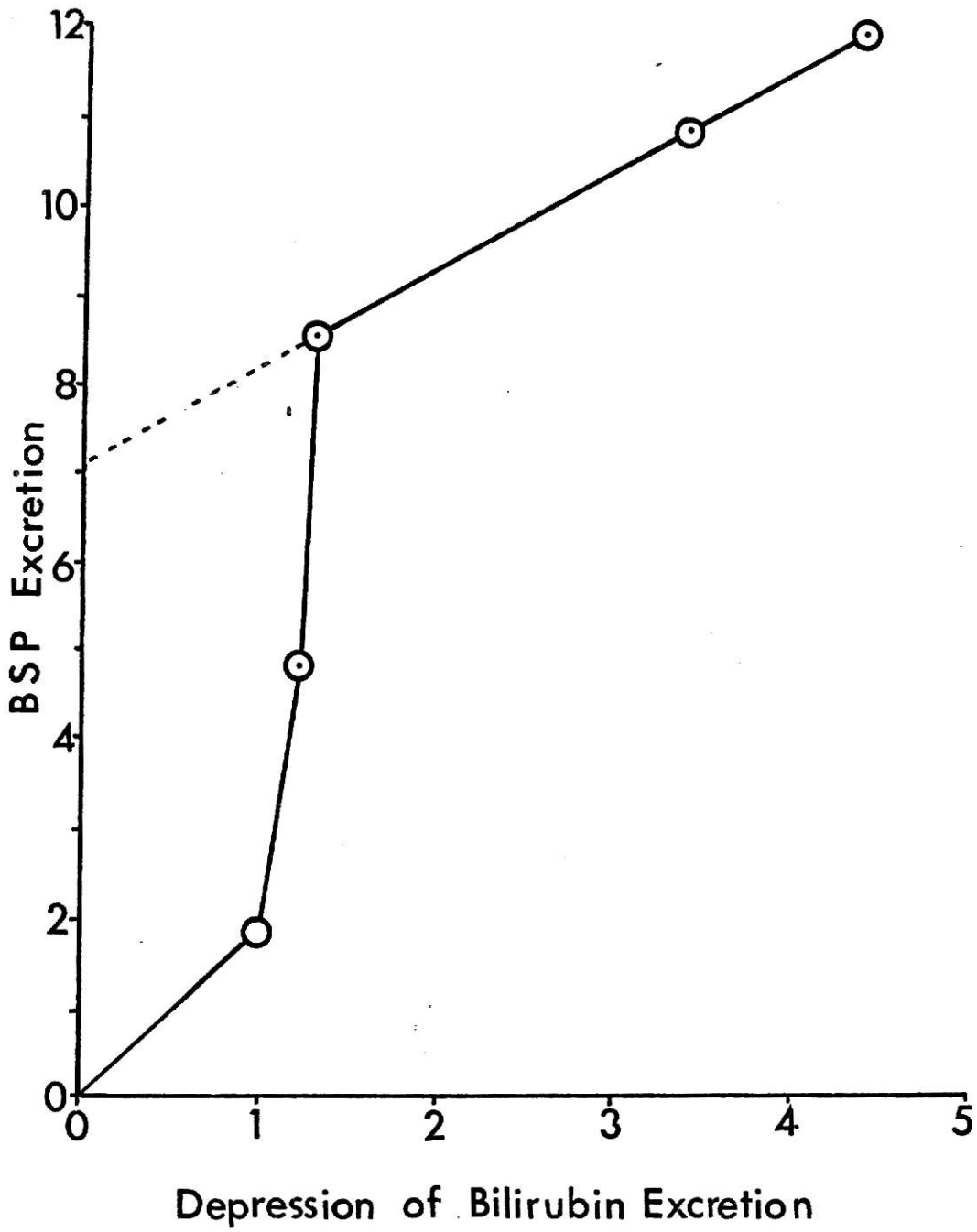


Fig. 1. Relation between depression in biliary bilirubin excretion and elimination of BSP. All data are expressed in octomoles/min/100 g; see Table 6.

BSP controls, was not greater in the 0.5:1 trials than it was in the 0.2:1 experiments (Table 2); (d) that when the submaximal amounts of BSP were infused in combination with bilirubin, BSP accumulated in the liver to a larger extent than when the submaximal levels of BSP were infused alone? Apparently, in the 0.2:1 and 0.5:1 trials, the total excretion of the two compounds was limited to submaximal levels, and competition between BSP and bilirubin for excretion into bile occurred.

To explain the limitation in combined excretion, as well as the competition between BSP and bilirubin, observed when submaximal levels of the combined compounds were infused, we must assume that the mole-to-mole competition in the process of bilirubin excretion obtained also at the submaximal levels of combined infusion. The process of bilirubin excretion, which we introduced above as the primary excretory process, had an E_{max} of 8 octomoles. The observed depressions in bilirubin excretion from the value of 8 octomoles can then be equated with equimolar amounts of BSP excreted by the primary process (Table 6, column 6). The balance of the total BSP excretion took the route of a secondary excretory process (Table 6, column 7) by which BSP, but not bilirubin, can be excreted. BSP excretions by primary and secondary processes were related to the infused molar ratios of BSP over bilirubin, as shown in Fig. 2. When small amounts of BSP were infused (0.2:1), the primary and secondary processes were of about equal importance for BSP excretion. With increasing ratios

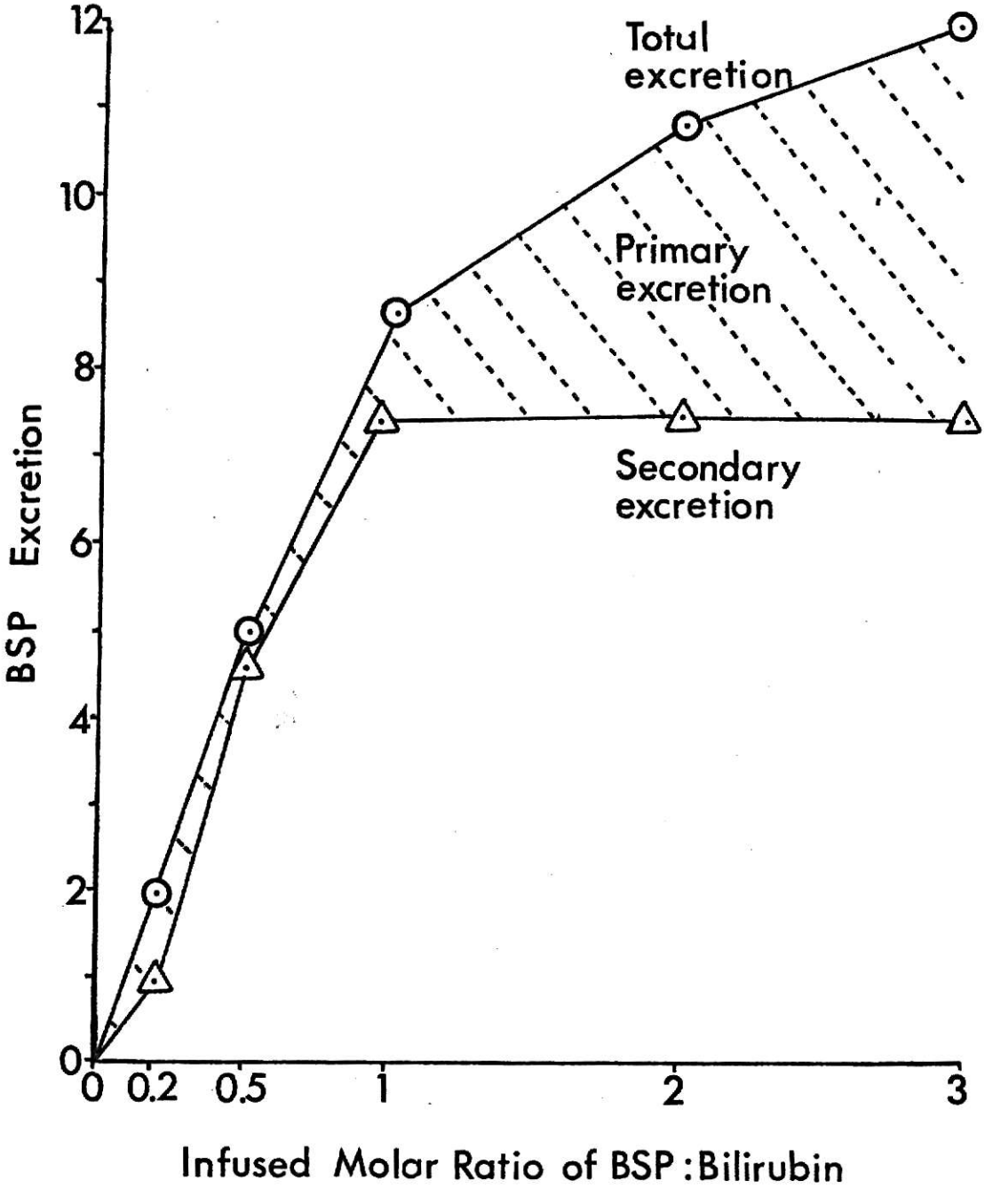


Fig. 2. Biliary BSP excretions by primary and secondary processes. BSP excretions are expressed in octomoles/min/100 g. The difference between total and secondary excretions, cross-hatched area, represents excretion by the primary process.

of BSP infused (0.5:1), the secondary process rapidly became the main route for BSP excretion. When the 1:1 molar ratio was infused, the secondary process for BSP excretion reached saturation (7.4 octomoles/min/100 g); therefore, infusion of more BSP (2:1 and 3:1) led to increased use of the primary excretory route for BSP in competition with bilirubin (Fig. 2). Since, the level of BSP in the liver increased with increasing molar ratios infused (Table 2), excretion of BSP by the two processes was considered in its relation to hepatic BSP concentration. The relationship between secondary BSP excretion and hepatic dye concentration (Fig. 3) does not show first order kinetics in its approach to saturation. The curvilinear relation observed (Fig. 3) is the characteristic of a process in which 2.661 molecules of BSP react with one active excretory site with a K_m of 5.546 octomolar $\overset{*}{V}$. The relationship between primary BSP excretion and hepatic BSP levels is more difficult to evaluate because two different processes compete with primary BSP excretion, namely, bilirubin excretion by the primary process and secondary BSP excretion. If the secondary

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*The Michaelis - Menten theory applied to the system, n BSP (liver) + 1 Active site (A) $\rightleftharpoons$ A (BSP)n $\longrightarrow$ n BSP (bile), yields the following relation between secondary BSP excretion rate (E_S) and hepatic BSP concentration (S):

$$E_S = \frac{E_{max}}{1 + K_m/S^n}$$

in which E_{max} equals 7.4 octomoles/min/100 g.

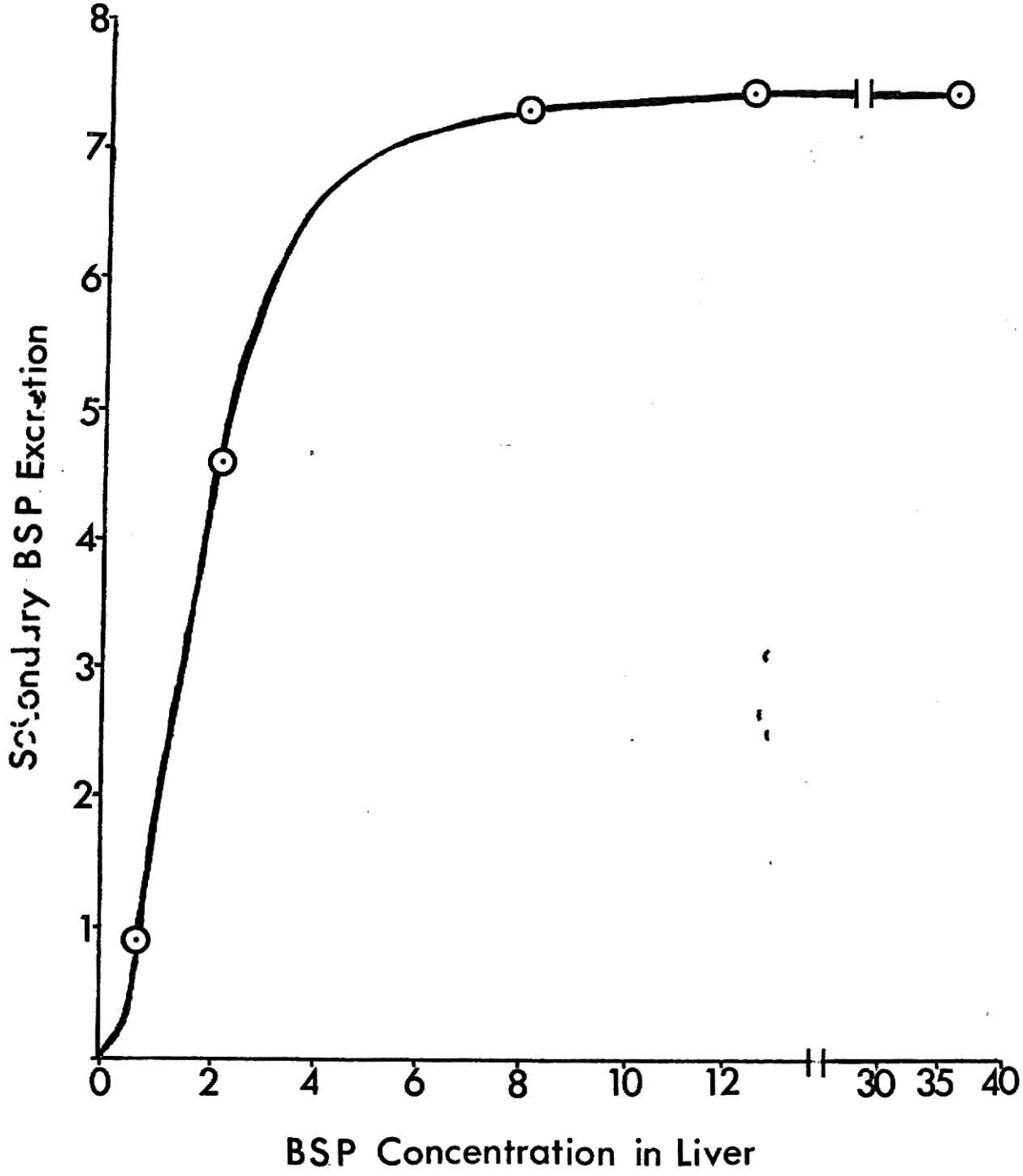

Fig. 3. Relation between BSP concentration in liver and biliary excretion by the secondary process. BSP concentrations and excretions are expressed in their respective octomolar units.

excretory process is available to BSP but not to bilirubin, as was postulated above, then secondary BSP excretion, as a function of hepatic dye concentration, should be similar in experiments when BSP was infused alone or in combination with bilirubin. Hence, for the BSP-alone trials (Table 2), the amounts of BSP excreted by the secondary process can be calculated from the observed hepatic BSP levels on the basis of the relationship contained in Fig. 3. Subtraction of the secondary BSP excretion from the total BSP excretion observed (Table 2) yields values for primary BSP excretion under conditions when no bilirubin was infused. Under these conditions, a maximal value for primary BSP excretion of about 4.4 octomoles/min/100 g was observed when BSP concentration in liver was about 6 octomolar. (A similar value for primary E_{max} of BSP appeared to be approached in the mixed-anion infusion trials; Table 6.) From this calculation, an order of magnitude value for K_m of BSP for the primary excretory process around 0.5 octomolar may be postulated. With the apparent K_m value for the primary excretory process an order of magnitude smaller than the apparent K_m for the secondary excretory process, we can understand that in the 0.2:1 infusion trial the low dose of BSP had to be excreted mostly by the primary process, i.e., in competition with the high dose of bilirubin; hence, bilirubin, not able to pass by the secondary excretory process, accumulated in the liver. In the 0.5:1 trials, the BSP levels in liver had increased so that greatly increased amounts of BSP were excreted by the secondary process, and

diminished competition with bilirubin (compared with the 0.2:1 trials) was observed. The molar ratios of BSP over bilirubin excreted by the primary process (Table 6) were heavily weighted towards bilirubin excretion when the smaller ratios were infused, and BSP was forced towards the secondary excretory process. But when ratios larger than 1:1 were infused, and the secondary process was saturated with BSP, increased molar ratios were excreted by the primary process. The net result was that the molar ratio excreted in bile reflected the infused ratio rather closely but showed, in all cases, a slight preference towards BSP excretion (Table 6)

The above apparent Km values may be related to rate-limiting excretory enzyme activities towards BSP. Alternatively, the Km value may represent affinities for a common energy source, or the critical micellar concentrations for BSP in the two excretory processes. The present findings confirm the choleretic effect of BSP reported in the literature; the increased rates of bile flow were not related to the dosage rate of BSP. Hence, a small amount, but not a proportional amount, of the infused BSP may be excreted in a molecular form and increase the bile volume by an osmotic effect; but the major part of the infused BSP seems to be excreted in an osmotically inactive form, i.e., in the form of micelles.

When either BSP or bilirubin was infused alone, the Emax for BSP was 10 and that for bilirubin was 8 octomoles. The combined Emax was 15.4 octomoles. Hence, in combination, the excretions of BSP and bilirubin were neither simply additive,

nor simply competitive: there was a synergistic interaction between the two compounds in their combined excretion. BSP did not appear to aid in the excretion of bilirubin for, even in the 0.2:1 infusion trials, less bilirubin was excreted and bilirubin accululated in the liver. But bilirubin did promote the excretion of BSP. It is conceivable that formation of micelles by the primary excretory process requires the presence of either endogenously-formed or administered bilirubin. The facilitation of BSP excretion by forced bilirubin excretion may be compared with the observation by Callahan and Schmid (10) that biliary excretion of unconjugated bilirubin in Gunn rats was enchanced by concomitant forced excretion of conjugated pigment. The authors (10) explained this phenomenon by postulating complex formation between the two pigments.

The following set of observations is intriguing: when 2:0 BSP was infused, bile flow was within the expected range; infusion of ane additional "molar unit" of 12 octomoles/min/100 g (3:0) resulted in severely impaired bile flow; but the bile flow rate was repaired by additional infusion of one "molar unit" of bilirubin (3:1 trials). It should be borne in mind that BSP infusions were started 15 minutes prior to the bilirubin infusions. Hence, in spite of pre-loading the liver with BSP, uptake of bilirubin was not impaired, and neither was conjugation of either compound. Since, furthermore, the concentrating mechanism for biliary BSP excretion was unaffected in the 3:0 trials (Table 2), the diminished BSP excretion was the result of an effect on bile flow rate.

It is tempting to speculate that the impairment in bile flow rate was caused by the osmotic effect of BSP accumulation in the liver, and that the additional "molar unit" of infused bilirubin relieved the situation by allowing BSP excretion via the primary process (as discussed above), resulting in subtoxic hepatic levels of BSP (Table 2).

Conflicting reports on competition between BSP and bilirubin for biliary excretion can be reconciled on the basis of the model proposed here. Dragstedt and Mills (18) observed in dogs that artificially induced hyperbilirubinemia caused retention of BSP. Cantarow et al. (13), working with dogs, injected small primer doses (2 mg/kg) of bilirubin and BSP, and continued infusing the two anions at the submaximal dose rate of 25 μg/min/kg. When the results of these anion combination trials are compared with the control data obtained from dogs that had received the same doses of only one of the anions, the biliary excretion of bilirubin was reduced to about 50% of its control value, and that of BSP to about 60%. Hence, their conclusion that artificially induced hyperbilirubinemia does not retard BSP excretion seems unfounded. Mendeloff et al. (41) observed that a small dose of bilirubin (3 mg/kg), intravenously injected into human subjects, failed to decrease the rate of disappearance from plasma of a small dose (3 mg/kg) of BSP injected seven minutes later or infused continually. In these experiments, the combined E_{max} for the two compounds was not reached. Hargreaves (27) infused, over a period of 30 minutes, 0.41 micromoles of bilirubin per minute

per 100 g rat weight into his bilirubin controls (cf. 0.12 μM/min/100 g in our 0:1 control), and used comparable overdoses of BSP to establish molar infusion ratios similar to the ones used in the present study. In our hands, a 30-minute infusion period was too short for E_{max} to be established; moreover, when we administered bilirubin at dose rates in excess of 0.18 μM/min/100 g, the bile flow rate dropped sharply after about 30 minutes. Hence, Hargreaves' results could not be compared with findings reported here. Though his data, obtained on liver slices and homogenates, indicated that BSP decreased the rate of bilirubin conjugation, the in vivo demonstration that BSP infusion sharply diminished biliary excretion of simultaneously infused conjugated bilirubin indicated an effect of BSP upon the excretion of the conjugated pigment. Also, Hargreaves' conclusion that the liver excreted BSP in preference to bilirubin agrees with the findings reported here.

Many reports can be found (see below) on the existence of multiple excretory processes for different compounds under normal, various experimental, and pathologic conditions. For bile salt excretion, the evidence for a separate pathway is compelling. However, the conclusion as to diverse pathways for biliary excretion of a variety of organic anions is questionable as it is generally based on data obtained under conditions when vast quantities of the anions were administered and effects were measured upon excretion of the small amounts

of endogenous bilirubin or upon excretion of a disproportionate amount of a simultaneously administered anion.

Under normal conditions, when small amounts of organic anions were infused simultaneously in approximately equimolar concentrations, molecular competition between various anions for biliary excretion was found (30). These findings, and the apparent similarity with the organic anion elimination (53), have led to the proposal that the organic anions share a common process for excretion into bile. When large dose rates and uneven proportions of the anions were used, preferential excretion of one compound relative to another was encountered (15, 27, 28), but in this case, competition for binding to plasma proteins and for various hepatic processes may complicate the interpretation, as discussed by Alpert et al. (2). In the present experiments with BSP and bilirubin, except when the highest dose rate of BSP was used, the combined levels of BSP and bilirubin in plasma did not exceed the molar concentration of plasma albumin. Hence, competition for binding to plasma albumin could not be responsible for the observation of two distinct excretory processes. For phylloerythrin, also, a secondary pathway may exist since it was observed here that phylloerythrin, like BSP, caused an increase in bile flow rate, but, unlike BSP, it left E_{max} for bilirubin unaffected. Hargreaves (27) concluded to a distinct pathway for indocyanine green, and Combes (16) suggested that several hepatic transport systems for the individual BSP metabolites may exist under normal conditions. Various experimental

conditions influence the biliary excretions of organic anions in a selective manner, suggesting that different pathways for these anions exist. In dogs (58) and human subjects (12) carbon tetrachloride poisoning, though severely depressing BSP excretion in the bile, did not affect bilirubin excretion of endogenous bilirubin. Choleresis induced by injection of bile salts into dogs (13) suppressed biliary excretion of BSP but not that of bilirubin. Selective influences of different drugs upon biliary excretion of various organic anions has been reported by Hargreaves and Lathe (28). Treatment with anabolic steroids produced retention of conjugated BSP before abnormalities in bilirubin metabolism were evident (14, 19, 32, 34). Discrimination between organic anions for biliary excretion, suggesting the presence of multiple excretory process, has been observed under pathological conditions whereby the liver was affected. In human patients, extrahepatic biliary ligation caused BSP conjugates to accumulate in serum before effects on bilirubin metabolism were detectable (14). Patients with Gilbert's disease, a congenital defect in bilirubin metabolism, excreted BSP normally (50). The Dubin-Johnson syndrome in man is characterized by elevated serum levels of conjugated bilirubin, indicating a hepatic excretory defect for the conjugated pigment. Though in these patients the biliary excretion of conjugated BSP was also impaired (25, 39), taurocholate was excreted normally (25). Mutant Corriedale sheep, with a disorder identical to the Dubin-Johnson syndrome

in man, showed a defect in the biliary excretion of a number of organic anions, including BSP and conjugated bilirubin (4), but taurocholate excretion was not affected.

The extents of uptake and conjugation of BSP and bilirubin by the liver, found when the compounds were infused alone, remained unaffected when combinations of them were administered. Also, the extent of reflux from liver back to plasma remained constant, as indicated by lack of variation in the levels of conjugated bilirubin and BSP in plasma. Hence, the increases in the hepatic levels of these compounds, observed when the various combinations were infused, reflected effects upon biliary excretion. In this respect, a qualitative difference between BSP and bilirubin was observed: whereas the concentration of bilirubin in bile decreased steadily when the larger proportions of BSP were infused simultaneously, the concentrating mechanisms for BSP excretion into bile appeared to remain unaffected by the simultaneous infusion of bilirubin (Table 2). The presence of a primary excretory process, available to both compounds, and a secondary excretory process, not open to bilirubin, provides an explanation for both qualitative and quantitative differences observed in biliary excretion of BSP and bilirubin.

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SEPARATE PATHWAYS FOR BILIARY EXCRETION OF SULFOBROMOPHTHALEIN AND BILIRUBIN IN RATS

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Injection of sulfobromophthalein (BSP) is used to test liver function in man and animal, under normal and pathological conditions. Often, when liver function is impaired, elevated levels of bilirubin are found in the bloodstream. In spite of numerous reports on the effects of elevated serum bilirubin levels upon rate of removal of injected BSP from the bloodstream, the mechanisms of hepatic uptake, metabolism, and excretion in bile of the organic anions remain poorly understood. Contributing causes to the inconclusiveness of studies reported have been the use of large doses of anions, failure to monitor effects on flow rate and composition of bile, and short time intervals of observation.

In the present study, male Charles-River rats fed a standard rat chow, were anesthetized with pentobarbital, teflon tubes were inserted into a jugular vein and in the bile duct, and incisions were sutured to prevent water and heat losses. Over the next 30 minutes, bile flow rate and biliary excretion of endogenous bilirubin were measured. Solutions containing various concentrations of bilirubin, freshly isolated phylloerythrin, BSP, or combinations of these compounds were infused at a constant rate into the jugular cannula during a 90-minute period, while bile flow rates and biliary concentrations of infused anions were monitored. The final concentrations of anions and their metabolites in plasma and liver were determined.

The design of the present study was based on preliminary findings: with increasing rates of bilirubin infusion, biliary

excretion of pigment was elevated, until, at an infusion rate of 0.12 μMoles/min/100 g rat, a maximal rate of pigment excretion (E_{max}) was attained; constant E_{max} was observed over the ultimate 45 minutes of the bilirubin infusion period. In the present study, the above dose rate of bilirubin was infused in 14 rats, and accurate values were established for bile flow rate and concentration of free and conjugated bilirubin in bile, liver, and plasma of these bilirubin control rats. Similar control data were obtained for BSP and phylloerythrin. Then, a 0.2:1 molar ration of phylloerythrin relative to above dose rate for bilirubin was infused, and the effects on bilirubin metabolism were determined. BSP was infused, simultaneously with the standard dose rate of bilirubin, in the following molar proportions: 0.2:1, 0.5:1, 1:1, 2:1, and 3:1. Effects of BSP upon the bilirubin control data, as well as influences upon the BSP control data caused by bilirubin, were evaluated.

The results indicated that excretion from hepatocyte into bile canaliculus was rate-limiting for overall transport of anions from blood to bile. When conbinations of BSP and bilirubin were infused, the maximal biliary excretion of the anions was less than the sum of the individual E_{max} values for the BSP and bilirubin control rats, indicating competition between the anions for biliary excretion. Yet, the combined E_{max} was higher than the individual E_{max} control values. A kinetic treatise of the experimental results led to the following model: BSP, in competition with bilirubin, was excreted

by a primary process with a low K_m value for BSP; at higher hepatic BSP concentrations, a second excretory path for BSP (not available to bilirubin) became increasingly more active. Hence, when small doses of BSP were infused simultaneously with the standard dose of bilirubin (0.2:1 trials), limited inhibition of bilirubin excretion was apparent. In the 0.5:1 and 1:1 trials, this inhibition in bilirubin excretion hardly increased. But then -- with the secondary path for BSP excretion saturated -- increasing the dose rate for BSP 2:1 and 3:1 led to mole-to-mole competition with bilirubin excretion.

In the literature, evidence for an excretory process for taurocholate distinct from that for other anions is compelling, but conclusions regarding multiple processes for biliary excretions of various anions are based on questionable evidence. With the two excretory processes for BSP proposed here, the experimental data observed were quantitatively interpreted, and conflicting reports were reconciled.