SEPARATE PATHWAYS FOR BILIARY EXCRETION OF SULFOBROMOPHTHALEIN AND BILIRUBIN IN RATS

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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