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BILIARY EXCRETION OF BROMSULPHTHALEIN AND GLUTATHIONE CONJUGATE OF BROMSULPHTHALEIN IN RATS PRETREATED WITH DIETHYL MALEATE

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Abstract—In rats diethyl maleate (DEM, 0·7 ml/kg i.p.) decreased the hepatic glutathione level to one tenth of the control value. Owing to the low glutathione level the conjugation of bromsulphthalein (BSP) with glutathione was markedly depressed. DEM-treated rats were given BSP and a glutathione conjugate of BSP (BSP-GSH) intravenously at various dose levels, and their biliary excretion and tissue concentrations were determined. No significant difference between the hepatic transport maxima for BSP (673 μg/min/kg) and for BSP-GSH (689 μg/min/kg) was found. BSP-GSH increased the biliary flow, BSP diminished it. Depending on the dose, 52–83 per cent of the BSP administered was taken up by the liver in 45 min, whereas the BSP-GSH predominantly appeared in extra-hepatic tissues. The half saturation doses for transport maxima were 75 mg/kg for BSP and 31 mg/kg for BSP-GSH. After administration of these doses the hepatic concentration of BSP was approximately ten times as high as the hepatic concentration of BSP-GSH.

BROMSULPHTHALEIN is conjugated with glutathione in the liver.[1–5] This process is catalyzed by glutathione *S*-aryltransferase.[6] In rat bile about 70–85 per cent of the BSP appears in conjugated form.[4,7] Although much work has been done in order to elucidate the importance of conjugation for the hepatic transport of BSP,[2,8,9] no definite conclusion has been reached as yet.[10] The present study was designed to compare the hepatic transfer maxima of BSP and its glutathione conjugate BSP-GSH, and to estimate their affinity for the transport system. This comparison can be done under experimental conditions that prevent the conjugation of BSP with glutathione. Benziodarone was found to be a potent inhibitor of glutathione *S*-aryltransferase, but it also impaired the biliary excretion of BSP-GSH.[9] A protein-free diet does not seem to alter the hepatic transfer system in rats. However, because of the incomplete depletion of liver glutathione,[8] the conjugation was inhibited only moderately.

Boyland and Chasseaud[11] reported that after administration of diethyl maleate (DEM) to rats, the liver glutathione level fell rapidly to 5–10 per cent of the control value. In our preliminary study the hepatic transport of rose bengal and fluorescein was not inhibited by DEM. Rose bengal and fluorescein are not conjugated with glutathione, however, both dyes are thought to have a transport system in common with BSP.[1,12] Therefore we assumed that DEM would not affect the hepatic excretion of BSP or BSP-GSH.

MATERIALS AND METHODS

Materials. The diethyl maleate supplied by Eastman Organic Chemicals, Rochester was redistilled before use. The sodium salt of BSP was purchased from Merck A.G., Darmstadt, and ^{35}S-BSP (16·3 mCi/m-mole) from Radiochemical Center, Amersham–Searle.

Preparation of BSP-GSH from rabbit bile. Chinchilla rabbits of 2·5 to 3·0 kg body wt were anaesthetized with urethane (800 mg/kg i.p. plus 400 mg/kg s.c.). After ligation of the cystic duct, the bile duct was cannulated with PE-50 tubing. BSP (100 mg/kg) was infused into the femoral vein at a rate of 1·5 mg/min/kg, and the bile collected for 3 hr.

To the pooled bile an equal volume of acetone was added, the mixture kept at a temperature of 5° for 6 hr and the precipitate removed by centrifugation. To the supernatant an equal volume of acetone was added again, the precipitate centrifuged and the supernatant decanted. The combined precipitates were extracted with 75% aqueous acetone (v/v) as described above. The combined supernatants were then evaporated to dryness under reduced pressure in a water bath at 40–50°. The residue was dissolved in 5–7 ml of distilled water, the solution adjusted to pH 6 with acetic acid and centrifuged. The clear solution was loaded on to a Sephadex G-10 column (1·5 × 120 cm) and the BSP-GSH eluted with distilled water at a rate of 10–12 ml/hr. From the appearance of the first coloured drop, the effluent was collected in 10 ml fractions and each fraction was analyzed. Five to ten μl samples were applied on Whatman No. 1 filter paper, and the chromatograms were then developed in a descending system consisting of *n*-propanol–acetic acid–water (10 : 1 : 5, by vol.).[4] The paper was exposed to ammonia vapor, the spots cut out and the dye eluted with 0·5 M NaOH solution. The absorbance was measured in a Beckman DU spectrophotometer at 580 nm. On the chromatograms of fractions 1 to 8–10 only one spot (R_f = 0·40) was detected by ammonia vapor. This spot was ninhydrin-positive. The first and second fractions were contaminated with bile pigments and lipids. These two fractions were discarded. Fractions 3 to 8–10 were combined and evaporated *in vacuo*. The dry residues, prepared by this procedure from the bile of several rabbits, were combined and analyzed. The chenodeoxycholic acid content of the preparation was less than 0·6 per cent. A sample of the preparation was hydrolyzed and the hydrolysate analyzed by paper chromatography according to Javitt *et al.*[5] The proportion of glutamic acid to glycine was 1 : 0·92. No amino acids other than alanine were detected. Alanine appears to be the degradation product of cystein.[2] The extinction coefficients ($E^{1cm}_{1\%}$ at 580 nm) were 602 for the BSP-GSH preparation and 797 for BSP. The molecular weights of BSP-GSH and BSP are 1020 and 794, respectively. Assuming that the molecular extinction coefficients of BSP and BSP-GSH are identical, the isolated BSP-GSH concentrate contained 97% BSP-GSH. Based on the amount of BSP administered to the rabbits, the yield of the isolation procedure was 13 per cent. Several rabbits were given ^{35}S-BSP (100 μCi to 100 mg unlabelled BSP). The isolation of labelled BSP-GSH was performed as above.

Depletion of hepatic glutathione. DEM was diluted with an equal volume of sunflower oil. The hepatic glutathione level, as determined by the method of Grunert and Phillips,[13] averaged 173 ± 12 μg/g in ten control rats. Half an hour after administration of DEM (0·7 ml/kg i.p.) the glutathione concentration in the liver decreased to 18 ± 5 μg/g in eight rats, remained at this level for 2–3 hr and then began

to increase. In response to doubling the dose of DEM the additional fall of the glutathione concentration was insignificant.

Animals and collection of bile. Male Wistar rats of 150-250 g body wt were anaesthetized with sodium pentobarbital (30 mg/kg i.p.). The common bile duct was cannulated with PE-10 tubing. Half an hour following the administration of DEM (0·7 ml/kg i.p.), BSP or BSP-GSH was injected into the femoral vein and the bile collected for 1 hr. Body temperature was maintained at 37 by means of a heat lamp. The bile samples collected between 30 and 60 min after administration of the dye were used for determination of BSP or BSP-GSH excretion. At the end of the experiment the animals were killed by bleeding from the carotid artery, and the liver and some other tissues removed and weighed.

Dose of BSP and BSP-GSH and determination of their tissue concentrations. ^{35}S-BSP (10–20 μCi/kg) and ^{35}S-BSP-GSH (10–20 μCi/kg) were diluted with unlabelled BSP and BSP-GSH, respectively. The dose of BSP-GSH was expressed in terms of equivalent amounts of BSP, calculated from optical density at 580 nm.

The plasma and tissue samples were digested with hyamine hydroxide, and the bile samples (5–10 μl) were added without digestion to Kinard's[14] liquid scintillation solution. Radioactivity was measured in a liquid scintillation spectrometer (Tri-Carb Spectrometer, Model 3380, Packard) using an external standard. After administration of BSP to DEM-treated rats a small fraction (10–16%) of BSP appearing in the bile consisted of a mixture of conjugates (Table 1). Most (80–90%) of the conjugates proved to be BSP-GSH. After administration of BSP-GSH to DEM-treated rats approximately 9 per cent of the total BSP-GSH appearing in the bile was a metabolite of BSP-GSH. Based on the R_f values, this material seems to be identical with Combes'[4] metabolite B and with Krebs'[15] compound II. In most experiments the metabolite of BSP and of BSP-GSH were not separated from their parent compounds. Hereafter the total amount of the drug administered and its metabolites will be indicated in terms of dye concentration.

TABLE 1. CONJUGATED BSP IN BILE OF CONTROL AND DIETHYL MALEATE PRE-TREATED RATS EXPRESSED AS A PERCENTAGE OF THE TOTAL BSP*

Dose of BSP (mg/kg i.v.)	%Conjugated BSP	
	Control†	Diethyl maleate†
25	81·8 ± 1·16	14·8 ± 0·70
50	72·6 ± 1·29	16·6 ± 2·54
100	73·4 ± 3·12	10·2 ± 1·14

* Diethyl maleate (0·7 ml/kg i.v.) was given 30 min prior to the administration of BSP. The determinations were performed in bile specimens collected 30–60 min after administration of BSP.

† Mean value ± S.E. of five rats.

Statistical analyses. Hepatic transport maxima (T_m) and half saturation doses (K_m) for BSP and BSP-GSH were calculated by the method of Wilkinson.[16] Student's *t*-test was used for statistical analysis.

RESULTS

Percentage of conjugated BSP in bile. In the bile of the control rats conjugated BSP amounted to 73–82 per cent of the total BSP, while in the bile of DEM-treated rats

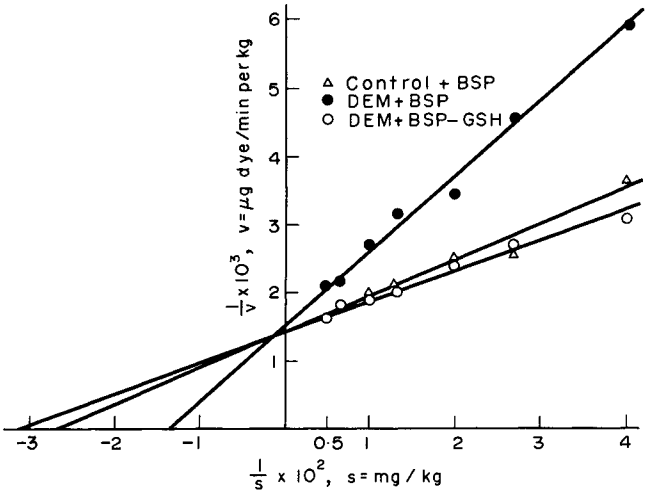

FIG. 1. Lineweaver-Burk[27] plots of BSP and BSP-GSH doses [S] vs biliary excretion rate v. Each point represents the mean value of six rats.

it was only to 10–16 per cent of the total (Table 1). On increasing the dose of DEM from 0·7 ml/kg to 1·5 ml/kg, the ratio of conjugated BSP decreased to an average of 8 per cent. However, several rats succumbed to this dose within 1 hr.

Biliary excretion of BSP and BSP-GSH. Figure 1 represents the Lineweaver–Burk[27] plot of the BSP and BSP-GSH doses vs the rates of biliary excretion. In DEM-treated rats at a dose of 25 mg/kg of BSP-GSH almost twice as much dye was excreted as after the same dose of BSP. On increasing the dose of BSP, the rate of dye excretion tended to approach that observed after the same dose of BSP-GSH. Calculating by Wilkinson's[16] method, the apparent T_m for BSP was 673 ± 108 μg/min/kg, and that for BSP-GSH 689 ± 73 μg/min/kg. The T_m values do not differ significantly ($P > 0{\cdot}05$) either from each other, or from the T_m value of the control animals (704 ± 116 μg/min/kg). These data lend further support to the assumption that DEM does not alter the transport system for BSP and BSP-GSH. In the DEM-treated rats the half saturation doses for transport maxima were 75·3 ± 8·7 mg/kg for BSP and 31·1 ± 10·8 mg/kg for BSP-GSH. The difference between the two values is significant ($P < 0{\cdot}05$). In these experiments it was remarkable that BSP-GSH increased the bile flow, while BSP depressed it (Table 2). BSP-GSH also enhanced the bile flow in the control rats. The increase of bile flow could not be accounted for by the chenodeoxycholic acid content of the BSP-GSH preparation, since chenodeoxycholic acid did not show significant choleretic action even at a dose of 10 mg/kg.

Hepatic concentrations of BSP and BSP-GSH. In the DEM-treated rats BSP was found predominantly in the liver, which however, contained only a small fraction of the BSP-GSH dose given (Fig. 2). The difference in hepatic BSP and BSP-GSH concentration cannot be explained solely by the fact that more BSP-GSH than BSP was excreted (Fig. 1), since the hepatic concentration of BSP was higher than that of BSP-GSH also in the bile duct-ligated rats (Table 3). Hepatic dye concentration averaged 609 μg/g at a BSP dose level of 25 mg/kg. The total amount of dye in the livers of

TABLE 2. EFFECT OF BSP AND BSP-GSH ON BILIARY FLOW IN CONTROL RATS AND IN RATS TREATED WITH DEM

	Dose of BSP or BSP-GSH (mg/kg i.v.)	Biliary flow* (μl/min/kg) after administration of BSP	BSP-GSH
Control	50	63·7 ± 5·7	64·5 ± 6·9
	100	59·3 ± 6·8	70·3 ± 7·6
	200	47·5 ± 5·4	71·4 ± 7·7†
DEM	50	44·5 ± 4·8	73·4 ± 3·2†
	100	35·6 ± 3·7	66·3 ± 5·8†
	200	34·5 ± 2·1	60·8 ± 7·0†

* The results represent the mean value ± S.E. of six rats. Spontaneous bile flow in the control rats was 52·5 ± 5·3 μl/min/kg and in the DEM-treated rats 49·7 ± 4·0 μl/min/kg.

† Significantly different from the respective values of the BSP-group ($P < 0·05$).

these rats was, on the average, 83 per cent of the dose. At a BSP dose level of 100 mg/kg only 52 per cent of the BSP was taken up by the liver. This dose-related accumulation of BSP may be attributed to a limited storage capacity of the liver (see also Fig. 2). In an additional experiment two groups of DEM-treated rats, five animals in each group, were given, respectively, 75·3 mg/kg BSP and 31·1 mg/kg BSP-GSH intravenously. Forty-five minutes later the hepatic dye concentrations were found to be 855 ± 45 μg/g after administration of BSP, and 63 ± 5 μg/g after that of BSP-GSH.

DISCUSSION

The "uphill" transport of drugs has much in common with enzyme reactions. For this reason, Michaelis–Menten kinetics are often applied to characterize transport processes.[17] Recently, doubt has been shed upon the validity of enzyme kinetics in

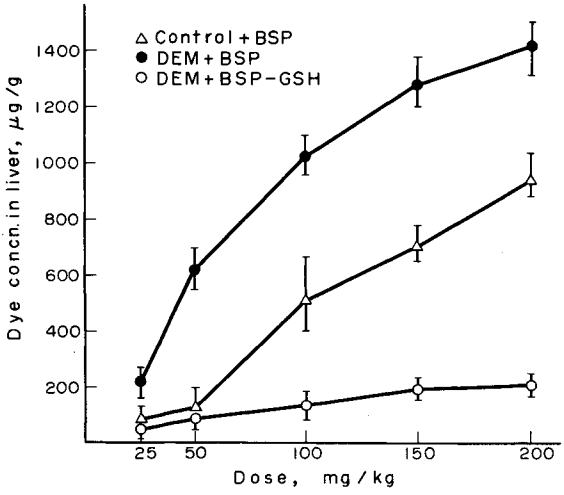

FIG. 2. Hepatic dye concentration in control and DEM-treated rats 60 min after intravenous administration of BSP or BSP-GSH. Each point represents the mean value ± S.E. of six rats.

TABLE 3. CONCENTRATION OF DYE IN TISSUES OF DEM-TREATED, BILE DUCT LIGATED RATS 45 min AFTER INTRAVENOUS ADMINISTRATION OF BSP OR BSP-GSH*

	Dose (mg/kg)	Dye concn (μg/g)†							
		Plasma	Liver	Kidney	Muscle	Skin	Fat	Lung	Heart	Intestine
BSP	25	88	609	51	3	1	1	17	6	9
	100	702	1625	338	18	20	14	112	33	96
BSP-GSH	25	85	53	106	23	33	15	25	16	24
	100	598	196	384	72	97	29	94	40	69

* Common bile duct was ligated prior to the injection of BSP or BSP-GSH.
† Mean value of four rats.

the interpretation of excretion data of cholephilic agents.[18–20] However, as a result of our failure to understand hepatic transport mechanisms at a molecular level[20,21] a more appropriate concept cannot be offered at present.

Combes[8] reported that at approximately equal hepatic BSP and BSP-GSH concentrations, BSP-GSH was preferentially excreted by the liver. Our results corroborate this finding: after the administration of equivalent doses of BSP and BSP-GSH to the DEM-treated rats more BSP-GSH was excreted than BSP (Fig. 1). The difference in excretion rate between these two compounds was more marked at low than at high dose levels, but the apparent T_m values did not differ significantly from each other. However, the affinities of BSP and BSP-GSH for the transport system were different. The determination of affinity ran into difficulties. The half saturation doses for transport maxima were 75 mg/kg for BSP and 31 mg/kg for BSP–GSH. Because of the obvious dissimilarity in distribution of the two drugs the estimation of affinity in terms of doses can be criticized (Table 3). A more reliable approach to the determination of affinity may be calculation of the hepatic dye concentrations at half maximum velocity of excretion. By plotting hepatic dye concentration (Table 2) against hepatic excretion (Fig. 1) the apparent K_m was calculated with the method of Lineweaver and Burk[27] and found to be 740 μg/g for BSP, and 72 μg/g for BSP-GSH. In addition, after administration of half saturation doses, i.e. 75·3 mg/kg BSP or 31·1 mg/kg BSP-GSH, the hepatic dye concentrations were 855 μg/g and 63 μg/g, respectively. Thus, BSP-GSH seems to have a 10–13 times higher affinity for the transport system than does BSP. In the interpretation of these data it must not be neglected that BSP has a greater affinity for plasma proteins than BSP-GSH.[22,23] It has been shown that BSP-GSH is less readily bound also by the proteins of rat liver.[8] Therefore the possibility exists that at a similar transport rate the concentrations of unbound BSP and BSP-GSH are similar at the intracellular site of the transport system. In this case the apparent K_m calculated from the data on hepatic concentration indicates only the fact that BSP is bound to proteins more avidly than BSP-GSH.

Most cholephilic substances are also strongly bound by plasma proteins.[24] It has been shown that specific, anion-binding proteins may have a role in the hepatic uptake and storage of cholephilic agents.[25] Some recent observations, however, show that there may be no direct relationship between affinity for plasma proteins and hepatic excretion rates.[24] Our study indicates that no parallel exists between affinity for hepatocytes and excretion rates, either. This does not seem to be a particular case

restricted to BSP and BSP-GSH. The T_m for biliary excretion of amaranth, an azo dyestuff used as a food colour, is approximately twelve times as high as that of rose bengal. The hepatic concentration of amaranth is approximately 12 per cent of that of rose bengal after administration of equimolar doses (our unpublished observation).

It has been reported,[26] and also observed by the present authors that in normal rats biliary flow is increased by low doses of BSP and depressed by high ones. The present investigations suggest that the choleretic action may be attributed to BSP-GSH, and the inhibitory effect to BSP itself.

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