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EFFECT OF DIETHYL MALEATE ON THE BILIARY EXCRETION RATE OF INFUSED SULFOBROMOPHTHALEIN-GLUTATHIONE*

BURTON COMBES‡ and BECKY BACKOF
Liver Unit, Department of Internal Medicine, The University of Texas Health Science Center at Dallas, Dallas, TX 75235, U.S.A.

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Abstract—Diethyl maleate (DEM) was given intraperitoneally to rats in a dose (4.3 mmoles/kg) known to markedly decrease glutathione levels in liver. DEM induced a choleresis previously shown to be due to the osmotic activity of DEM conjugates (DEM-glutathione and subsequent metabolic products) excreted into bile. Coincident with the choleresis, the biliary excretory Tm for the infused glutathione conjugate of sulfobromophthalein (BSP-GSH) was depressed significantly. The data are interpreted as indicating that DEM-GSH conjugates compete with BSP-GSH conjugates for a canalicular carrier mechanism.

Diethyl maleate (DEM) has been used to lower hepatic glutathione (GSH) in experiments designed to study the effect of decreased GSH on the biliary transport of organic anions [1, 2]. DEM lowers the hepatic content of GSH as a result of trapping of GSH during diethyl maleate-glutathione (DEM-GSH) conjugation [3–5]. Recently we demonstrated that DEM conjugates (DEM-GSH and its subsequent metabolic products) are excreted in bile and induce a choleresis in the rat and dog attributed to the osmotic activity of the DEM conjugates in bile [5]. These findings raise the possibility that DEM conjugates compete with biliary excretion of other organic anions, such as sulfobromophthalein sodium (BSP), that are excreted in bile as GSH conjugates. This possibility was tested in the following studies in which the effect of DEM on maximum rates of conjugated BSP excretion into bile was determined in rats during infusion of preformed BSP-glutathione (BSP-GSH).

MATERIALS AND METHODS

Chemicals. Diethyl maleate was obtained from Matheson, Coleman & Bell, Cincinnati, OH; glutathione from the Sigma Chemical Co., St. Louis, MO; and BSP from Hynson, Westcott & Dunning Inc., Baltimore, MD. BSP-GSH was synthesized according to the method of Whelan *et al.* [6]. The material containing 97.4% BSP-GSH and 2.6% of a BSP band considered to be $BSP(GSH)_2$ was utilized without further purification.

Experimental procedures. Male Sprague–Dawley rats, weighing between 200 and 300 g and with liver weights accounting for 4% of total body weight, were anesthetized with pentobarbital (50 mg/kg) administered intraperitoneally (i.p.); fitted with a bile duct cannula (PE-10 polyethylene tubing) through a midline abdominal incision which was then closed by suture; and fitted with internal jugular vein (PE-50) and carotid artery (PE-10) cannulas for administration of fluids and collection of blood respectively. Body temperature was monitored by a Tele-Thermometer connected to a rectal probe (Yellow Springs Instrument Co., Yellow Springs, OH) and was maintained at 37–38° by means of a heating pad placed under the animal. An isotonic saline infusion was begun at a rate of 1.2 ml/hr, and bile was collected for 30 min (−30 to 0 min). At zero time, a priming dose of BSP-GSH (9.5 μmoles in isotonic saline) was administered intravenously, and the saline infusion was replaced by a constant infusion of BSP-GSH (0.6 μmoles BSP-GSH per min per 100 g) in saline (1.2 ml/hr). Bile was collected at 10-min intervals for 120 min and six arterial blood samples (100 μl each) were obtained at 20-min intervals beginning 20 min after BSP-GSH was started. Preliminary experiments demonstrated that maximum excretory rates of BSP conjugates in bile were reached by 50 min and persisted with only a slight fall in excretion for the balance of the experiment. To observe the effect of DEM on maximum conjugated BSP excretion in bile, DEM mixed 1:1 with corn oil was administered i.p. at a dose of 4.3 mmoles/kg at 60 min to six of the rats. Six control animals received a comparable volume of corn oil i.p. at 60 min.

Analytical methods. Bile samples were collected in pretared vials with bile volume determined gravimetrically assuming a density of 1.0 g/ml. BSP concentration in bile was determined by measuring the absorbance at 580 nm on appropriately diluted bile specimens made alkaline with 0.01 N KOH. BSP concentration in plasma was determined by the method of Gaebler [7]. The distribution of BSP compounds in bile was determined by paper chromatographic analysis described in detail in previous publications [8, 9]. The concentration of bile salts in

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† Address correspondence to: Burton Combes, M.D., Department of Internal Medicine, The University of Texas Health Science Center, Dallas, TX 75235, U.S.A.

bile was determined utilizing the 3-hydroxy-steroid dehydrogenase method described by Barnhart and Combes [10] which permits quantitation of bile salts in the presence of BSP compounds. Sodium and potassium concentrations in bile were determined by flame photometry (IL Flame Photometer, model 143, Instrumentation Laboratory, Inc., Watertown, MA), and chloride concentrations were determined by titration with a silver electrode (Buchler–Cotlove Chloridometer, Buchler Instruments, Inc., Fort Lee, NJ). The concentration of bicarbonate in bile was determined by the number of milliequivalents of HCl neutralized by the sample of bile [11].

Statistical analysis. Significant differences between means were assessed by Student's *t*-test.

RESULTS

Infusion of BSP-GSH resulted in increasing rates of conjugated BSP excretion in bile with maximum rates of excretion noted by 40–60 min after the infusion was begun (Fig. 1). Thereafter, excretion rates were relatively well-maintained, falling approximately 12% over the ensuing hour (60–120 min). Bile flow increased in association with conjugated BSP excretion rate (Fig. 1), reached a peak at 40–60 min, and remained fairly steady thereafter (6% decrease between 60 and 120 min).

Administration of DEM at 60 min induced a choleresis (Fig. 1) which peaked at 70–80 min. This choleresis, in confirmation of earlier findings [5], was not accompanied by or due to an increase in bile salt excretion rate in bile (Fig. 2), and is attributed to the osmotic activity of DEM compounds (DEM-glutathione and its metabolites) excreted in bile. During the height of the choleresis (70–90 min), the conjugated BSP excretion rate in bile fell (Fig. 1). Thereafter, the excretion rate of BSP conjugates and the bile flow rate gradually returned toward those observed in corn oil-treated rats. The average values of bile flow, BSP excretion and bile salt excretion rate during the 20-min intervals prior to administration of DEM (40–60 min), at the height of the DEM-induced choleresis (70–90 min), and at the completion of the experiment (100–120 min) are presented in Table 1. Plasma concentrations of BSP increased above control in DEM-treated rats (Fig. 1).

The results of a chromatographic analysis of the BSP compounds excreted in bile are summarized in Table 2. BSP metabolites are referred to as BSP A, BSP B and BSP C. BSP A is BSP-GSH; BSP B and C contain largely BSP-cysteinyl glycine and BSP-cysteine [12, 13]. Only conjugated BSP compounds were identified in bile of animals infused with BSP-GSH. Excretion rates of BSP A and BSP C were significantly lower in DEM-treated than in control rats at 70–90 min, during the height of the DEM-induced choleresis.

In the absence of a chemical method for measuring DEM compounds in bile, estimates of the DEM excretion rate were derived from measurements of

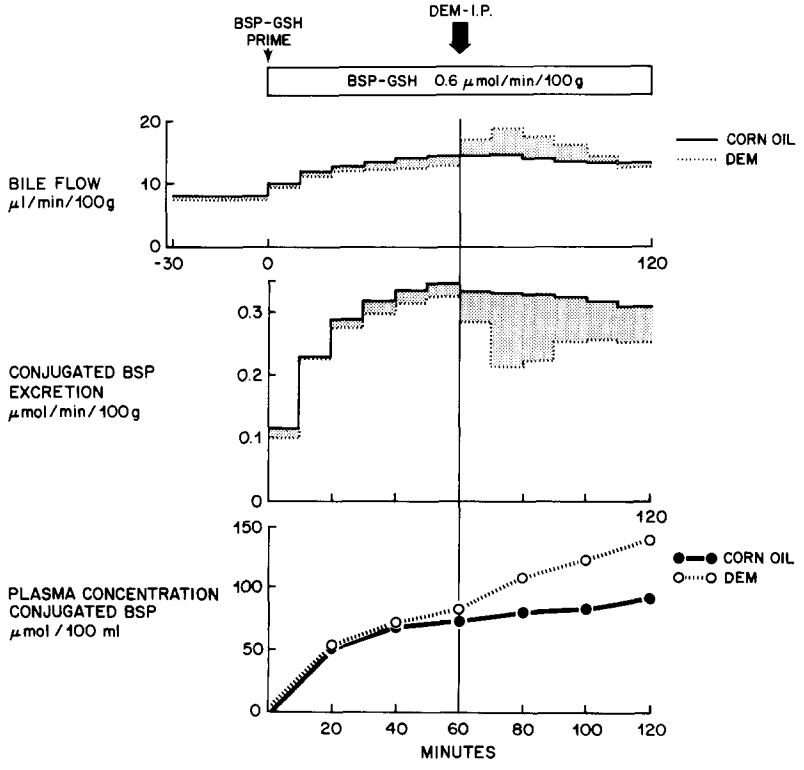

Fig. 1. Effect of DEM on bile flow rate, biliary excretion rate of infused BSP-GSH, and plasma concentration of conjugated BSP compounds. Twelve rats received a priming dose and then a constant infusion of BSP-GSH beginning at zero time. At 60 min, six rats received DEM 1:1 in corn oil at a dose of 4.3 mmoles/kg, i.p. The other six rats received a comparable volume of corn oil i.p. The data shown represent mean values for the six DEM- and six corn oil-treated rats.

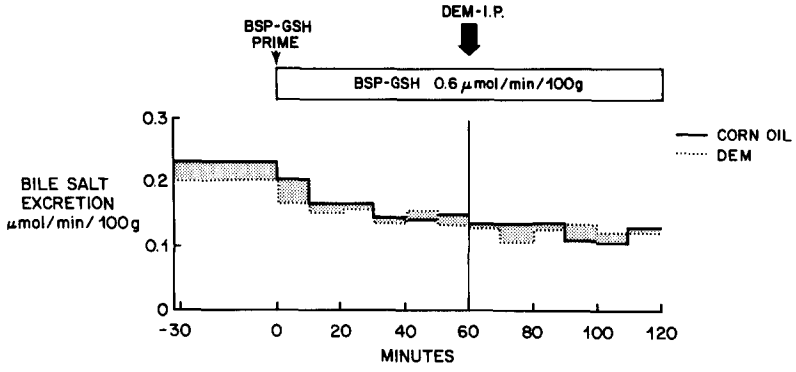

Fig. 2. Bile salt excretion rate in bile during a control period, during BSP-GSH infusion, and following administration of DEM or corn oil i.p. The data shown represent mean values for the six DEM- and six corn oil-treated rats.

Table 1. The effect of DEM on rates of bile flow, BSP excretion, and bile salt excretion in rats

		Minutes 40–60*	70–90†	100–120†
Bile flow (μl/min/100 g)	Corn Oil	14.3 ± 2.8‡	14.2 ± 2.9	13.4 ± 2.9
	DEM in corn oil	12.8 ± 2.3	18.2 ± 3.5	13.6 ± 2.2
			P < 0.05	
BSP excretion (μmoles/min/100 g)	Corn oil	0.34 ± 0.077	0.32 ± 0.087	0.30 ± 0.084
	DEM in corn oil	0.32 ± 0.063	0.22 ± 0.062	0.26 ± 0.081
			P < 0.05	
Bile acid excretion (μmoles/min/100 g)	Corn oil	0.15 ± 0.042	0.14 ± 0.039	0.12 ± 0.035
	DEM in corn oil	0.15 ± 0.063	0.12 ± 0.059	0.12 ± 0.057

* Before DEM.
† After DEM.
‡ Values are mean ±S.D. for the six corn oil- and the six DEM-treated rats (corn oil and DEM in corn oil were injected at 60 min).

Table 2. The effect of DEM on distribution of conjugated BSP compounds excreted into bile

BSP compounds in bile (μmoles/min/100 g)		Minutes 40–60*	70–90†	100–120†
BSP A	Corn oil	0.17 ± 0.05‡	0.14 ± 0.03	0.12 ± 0.03
			P < 0.02	
	DEM in corn oil	0.13 ± 0.05	0.08 ± 0.04	0.10 ± 0.05
		0.1 > P > 0.05		
BSP B	Corn oil	0.13 ± 0.03	0.14 ± 0.04	0.14 ± 0.05
	DEM in corn oil	0.16 ± 0.03	0.12 ± 0.03	0.13 ± 0.03
		P < 0.02		
BSP C	Corn oil	0.03 ± 0.01	0.04 ± 0.02	0.04 ± 0.02
			P < 0.02	
	Dem in corn oil	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01
		P = 0.01		

* Before DEM.
† After DEM.
‡ Values are mean ±S.D. for the six corn oil- and the six DEM-treated rats (corn oil and DEM in corn oil were injected at 60 min).

Table 3. Excretion in bile of electrolytes, bile salts, and BSP compounds and estimates of DEM compounds

Time (min)	Injection at 60 min	1 Bile flow (μl/min/100 g)	2 Sodium (μEq/ml)	3 Potassium (μEq/ml)	4 Chloride (μEq/ml)	5 Bicarbonate (μEq/ml)	6 Anion gap (μEq/ml)	7 Bile salt (μmoles/ml)	8 Anion gap—bile salt (μEq/ml)	9 (μEq/min/100 g)	10 BSP compounds (μmoles/min/100 g)	11 (μEq/μmole)	12 (μEq/min/100 g)	13 DEM compounds (μEq/min/100 g)
−30 to 0*	Corn oil	8.1 ± 2.0†	155.9 ± 8.5	6.4 ± 0.5	102.7 ± 6.3	28.6 ± 2.6	31.0 ± 15.0	29.5 ± 6.0	1.4 ± 5.1					
	DEM‡	7.3 ± 0.6	161.8 ± 12.4	6.1 ± 0.5	110.8 ± 13.8	30.7 ± 4.4	26.4 ± 7.3	27.0 ± 3.9	−0.6 ± 2.2	−0.004				
40 to 60‡	Corn oil	14.3 ± 2.8	173.2 ± 6.7	7.3 ± 0.8	92.0 ± 4.0	28.9 ± 2.4	59.6 ± 10.6	10.8 ± 4.6	48.7 ± 9.3	0.70 ± 0.19	0.34	2.1	0.70 ± 0.19	0
	DEM‡	12.8 ± 2.3	184.2 ± 13.6	7.4 ± 0.5	95.4 ± 5.7	32.3 ± 6.9	64.0 ± 9.3	10.9 ± 3.4	53.1 ± 6.3	0.69 ± 0.19	0.32	2.2	0.69 ± 0.19	0
70 to 90‖	Corn oil	14.2 ± 2.9	171.3 ± 6.8	7.2 ± 0.6	94.1 ± 3.7	29.7 ± 1.8	54.7 ± 9.9	10.2 ± 4.3	44.5 ± 11.4	0.64 ± 0.21	0.32	2.0	0.64 ± 0.19	0
	DEM‡	18.2 ± 3.5	173.1 ± 2.0	6.9 ± 0.9	89.2 ± 5.8	21.4 ± 5.1	69.5 ± 8.0	6.4 ± 1.6	63.0 ± 6.8	1.16 ± 0.31	0.22		[0.44]¶	0.72**
		P < 0.05				P < 0.01	P < 0.02	P < 0.05	P < 0.01	P < 0.01				
100 to 120‖	Corn oil	13.4 ± 2.9	171.0 ± 7.3	6.7 ± 0.4	95.9 ± 4.8	29.1 ± 4.2	52.4 ± 12.4	8.8 ± 2.3	43.6 ± 12.1	0.59 ± 0.21	0.30	2.0	0.59 ± 0.21	0
	DEM	13.6 ± 2.2	176.6 ± 10.6	7.6 ± 0.8	91.1 ± 5.2	22.1 ± 6.9	71.3 ± 11.6	9.0 ± 3.2	62.3 ± 7.6	0.84 ± 0.13	0.26		[0.52]¶	0.32**
						0.1 > P > 0.05	P < 0.02		P < 0.01	P < 0.05				

* Prior to BSP-GSH infusion.
† Values are means ± S.D.s for six corn oil- and six DEM-treated rats.
‡ In corn oil.
§ During BSP-GSH infusion, prior to DEM.
‖ During BSP-GSH infusion and after DEM or corn oil given i.p. at 60 min.
¶ Calculated by multiplying values in column 10 by 2.0.
** Calculated by subtracting values in column 2 from those in column 9.

anion excretion unaccounted for by chloride, bicarbonate, bile salts and BSP compounds. From −30 to 0 min, i.e. prior to BSP-GSH infusion, the mean anion gap in bile (column 6 in Table 3 = concentrations of sodium + potassium − chloride − bicarbonate) was accounted for by bile salts (column 7) in bile. During BSP-GSH infusion and prior to administration of DEM or corn oil (40–60 min), the anion gap not accounted for by bile salts but attributed to BSP conjugates in bile increased to approximately 50 μEq per ml (column 8). This value times bile flow (column 1) yields an excretion rate of approximately 0.7 μEq per min per 100 g (column 9). Following administration of DEM, the anion gap not accounted for by bile salts (now attributed to BSP and DEM compounds) increased even further, and the excretion rate of this anion fraction increased to 1.16 μEq per min per 100 g at 70–90 min and to 0.84 μEq per min per 100 g at 100–120 min. BSP compounds excreted in bile throughout the study in control animals, and prior to administration of DEM in DEM animals, accounted for approximately 2 μEq for each μmole of BSP conjugates (column 11). Assuming that this ratio applied after administration of DEM, calculated rates of BSP conjugate excretion were 0.44 μEq per min per 100 g at 70–90 min and 0.52 μEq per min per 100 g at 100–120 min (column 12). These latter values subtracted from the excretion rates for the anion fraction attributed to BSP and DEM compounds yield rates of DEM compound excretion of 0.72 and 0.32 μEq per min per 100 g at 70–90 and 100–120 min respectively (column 13).

DISCUSSION

The salient finding of the present study is that the maximum rate of biliary excretion of infused conjugated BSP was depressed during the choleresis induced by DEM. Any linkage of these phenomena should consider events that accompany DEM administration, which include its uptake in liver, conjugation with GSH resulting in a fall in hepatic GSH levels [3–5], and excretion of DEM compounds (DEM-GSH and its subsequent metabolic products) in bile.

Interference with canalicular transport of BSP conjugates by DEM conjugates, which themselves are transported in bile, is a likely explanation for our findings. First, maximum suppression of the rate of conjugated-BSP excretion involving the various BSP conjugates occurred during the height of the conjugated DEM excretion rate (at 70–90 min). Second, return of the rate of conjugated BSP excretion toward the control rate occurred simultaneously with a decrease in the rate of conjugated DEM excretion (100–120 min). The implication is that DEM and BSP conjugates share a common canalicular transport mechanism. It is uncertain whether one or more transport mechanisms is responsible for excretion of DEM conjugates, however, since the combined DEM and BSP conjugate excretion rate was greater than the maximum rate of conjugated BSP excretion alone. The present studies do not critically address the problem of whether BSP conjugate excretion interferes with the biliary excretion of DEM con-

jugates. In an earlier study [5], in which rats received the same dose of DEM but no BSP-GSH, approximately 0.72 μEq of DEM conjugates was excreted in bile per min per 100 g over the first 30 min after DEM administration accompanied by an increase in bile flow of 9.6 μl per min per 100 g. In the present study in which animals received both DEM and BSP-GSH, a similar rate of DEM conjugate excretion (0.72 μEq per min per 100 g) was measured 10–30 min after DEM was given and was accompanied by an increase in bile flow of approximately 7.2 μl per min per 100 g. Comparable rates of DEM excretion and increments of bile flow suggest relatively little suppression of excretion of DEM conjugates by BSP conjugates. Thus, if one transport mechanism is responsible for excretion of both DEM and BSP conjugates, it appears to have a greater affinity for the DEM compounds and a greater capacity than that exhibited for BSP conjugates alone.

Decreased hepatic GSH *per se* is an unlikely cause of decreased excretion of infused BSP-GSH since lowering of hepatic GSH in rats either by feeding a protein-free diet for 2 days [14, 15] or by administration of iodomethane [16, 17] does not affect biliary excretion of infused BSP-GSH. Moreover, in the present study, the conjugated BSP excretion rate was returning toward control levels 50–60 min after administration of DEM, whereas hepatic GSH remains markedly depressed for at least 3 hr after the dose of DEM given to our animals [5].

Although the effect of DEM on hepatic uptake of BSP-GSH was not measured in our study, Whelan [2] found no impairment of initial uptake of BSP-GSH and higher concentrations of conjugated BSP in the liver at the completion of his study in guinea pigs pretreated with DEM prior to infusion of BSP-GSH. Thus, interference with BSP-GSH uptake as a primary event is unlikely to account for decreased excretion of BSP conjugates induced by DEM. The increase in plasma concentration of conjugated BSP in the present study in DEM-treated rats can be accounted for adequately by the decrease in the rate of biliary excretion of the BSP conjugates.

A transient toxic phenomenon due to DEM cannot be excluded but seems unlikely since the rates of both overall organic anion excretion (DEM plus BSP compounds) and bile flow increased following administration of DEM. Not assessable is whether unconjugated DEM in liver inhibits biliary transport of BSP-GSH.

We have not ascertained whether the depressant effect of DEM is specific for glutathione conjugates excreted in bile or whether this effect also involves transport of other organic anions excreted into bile unchanged or as glucuronides. Varga *et al.* [1] reported that DEM did not inhibit biliary transport of either rose bengal, which is excreted unchanged, or fluorescein, which is excreted in part as a glucuronide [18]. On the basis of these latter results, Varga *et al.* assumed that DEM would not affect the hepatic excretion of BSP or BSP-GSH.

A number of studies have demonstrated that dye excretion rates in bile are higher when BSP-GSH rather than unconjugated BSP is infused [6, 15, 17, 19, 20]. When unconjugated BSP is administered, dye taken up in liver cells is partially conjugated with glutathione to form BSP-GSH, which is converted to BSP-cysteinyl glycine and BSP-cysteine. Both conjugated and unconjugated BSP are subsequently transported into bile. When BSP-GSH is infused, only conjugated BSP compounds are present in liver and bile. The same range of concentrations of conjugated dye was reached in liver when either BSP or BSP-GSH was infused. Thus, conjugation did not limit transport of dye. The results suggest that BSP-GSH excretion is higher when the liver contains only conjugated dye than when both unconjugated and conjugated BSP are present in liver. Unconjugated BSP in liver appears to depress transport of conjugated dye from liver cells into bile [19, 20].

Lowering hepatic glutathione concentrations by feeding rats a protein-free diet does not affect maximum dye excretion in animals infused with BSP-GSH but does cause a fall in rats infused with unconjugated BSP [15, 21]. In the latter instance, decreased excretion of dye is due to decreased excretion of conjugated BSP, despite hepatic levels of conjugated BSP equal to those attained during infusion of BSP-GSH, but in association with a decrease in the proportion of conjugated BSP relative to unconjugated BSP in liver [15]. Presumably excess unconjugated dye in livers of protein-free animals was the result of the fall in GSH in these livers and was responsible for the increased inhibition of conjugated BSP excretion in these rats.

The current studies demonstrate that DEM impairs excretion of infused conjugated BSP and propose a mechanism by which this occurs. They also suggest that DEM will impair dye excretion when unconjugated BSP is infused by (1) lowering hepatic GSH and thereby reduplicating the effects observed in rats fed a protein-free diet, as well as by (2) the additional depression of conjugated BSP excretion induced by DEM. Studies carried out by Whelan [2] in guinea pigs yield such results. In his studies, the maximum rates of dye excretion in bile were decreased from 2.03 to 1.23 μmoles per 10 min per 100 g when BSP-GSH was infused and from 0.84 to 0.50 μmole per 10 min per 100 g when unconjugated BSP was administered to DEM-treated animals. In the latter instance, the decrease in dye excretion was due to a decrease in excretion of BSP conjugates. By contrast, Varga *et al.* [1] found no difference in maximum rates of dye excretion when unconjugated BSP was given to rats pretreated with DEM and found similar maximum excretion rates when BSP-GSH or BSP was infused in DEM-treated animals. In a subsequent study from the same laboratory, Gregus *et al.* [22] found that dye excretion in DEM-treated rats was much higher when BSP-GSH rather than BSP was infused. The reasons for these discrepant results in the same laboratory are unclear. The design of the experiments in each study was somewhat different. Moreover, BSP-GSH utilized in the first study [1] was extracted from the bile of rabbits infused with BSP, whereas in the second study [22] BSP-GSH was synthesized by the method [6] we have used in the current work. Since Gregus *et al.* [22] used only DEM-treated rats, however, no assessment was made of the effect of DEM on dye

excretion when only BSP or BSP-GSH was administered to their animals.

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