IMPAIRMENT OF BROMOSULFOPHTHALEIN HEPATIC TRANSPORT AND CHOLESTASIS INDUCED BY DIETHYL MALEATE IN THE RABBIT

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Abstract—The present study was designed to investigate the effect of hepatic glutathione depletion induced by intraperitoneal administration of diethyl maleate (DEM) on the maximum biliary transport (T_m) and on the biliary excretion of bromosulfophthalein (BSP) in anaesthetized rabbits when the dye was perfused endovenously at doses exceeding T_m.

The T_m of total BSP (BSPt) and that of conjugated BSP (BSPc) were significantly reduced after DEM administration whereas that of unconjugated BSP (BSPu) was markedly increased. A reduction in the biliary excretion of BSPt and BSPc, in the percentage of BSPc, in the cumulative excretion of BSPt and in the percent-dose recovery were also observed. However, no change in hepatic glutathione S-transferase activity was noted after DEM. The cholestasis observed following DEM administration coursed with falls in the biliary secretion of sodium, chloride and bicarbonate.

Cellular glutathione (GSH)* depletion induced by different methods has proved to be a useful tool in detoxification and drug metabolism since disturbances in conjugation may impair the biliary excretion of different xenobiotics such as BSP [1–3]. The most common chemical compound employed to induce cellular GSH depletion is diethyl maleate, an unsaturated α,β-carbonyl compound which reacts with GSH to yield derivatives that are excreted in the bile and which produce a choleretic effect in species such as the rat, the dog and the rabbit [4–7]. In the latter species, unlike what happens in the former, the choleretic response to DEM is less intense; it does not last as long and is followed by intense cholestasis, metabolic acidosis and a pronounced reduction in the biliary excretion of HCO_3^- [7], whose secretion—specially high in this species—plays an important role in bile formation [3, 8]. Moreover, it has been reported that BSP, a xenobiotic with a low T_m in rabbits [9, 10], induces choleresis [9], cholestasis [10] or no effect at all [11]. Additionally, in view of the fact that BSP and DEM conjugation is catalyzed by GSH-S-transferases, whose activity is very low in the rabbit [12], the aim of the present work was to assess the effect of hepatic GSH depletion by DEM administration on biliary BSP excretion and on other biliary, hepatic and blood parameters.

MATERIALS AND METHODS

Animals and experimental procedures. Male New Zealand rabbits weighing between 1.5 and 2.0 kg were used. The animals were housed in environ-mental conditions and fed a standard diet. Food but not water was withheld for 18 hr prior to surgery. After pentobarbital anaesthesia (30 mg/kg i.v.) tracheotomy was performed followed by a median ventral laparotomy. The cystic duct and the pylorus were ligated. The choledocus and the left femoral artery and vein were cannulated as described previously [7]. A cannula was also introduced into the first part of the duodenum. The body temperature of the rabbits, measured with a rectal probe, was kept between 38.5–39.5° with an electric heating pad. After an equilibrium period of 30 min to allow bile flow to stabilize, bile was collected in previously weighed tubes over four hours at intervals of 10 or 15 min. Part of the samples taken (<10%) was frozen at −40° until later assay. An aliquot of bile equivalent to that taken every 10–15 min was later infused into the duodenum.

After collecting four baseline 15 min samples continuous BSP infusion was started at a dose of 0.88 µmol/min/kg and maintained over a period of 60–180 min. In one group of rabbits DEM was administered at 120 min at a dose of 3.2 mmol/kg mixed 1/1 (v/v) with corn oil; another group received corn oil alone and was used as the control group.

Sixty microlitres of blood samples were obtained from the femoral artery at 0, 60, 120 and 180 min for the determination of pO_2, pCO_2, pH and HCO_3^- concentrations. At minutes 5, 10, 15, 20, 40, 60, 90, 120, 150 and 180 after the start of BSP infusion, blood samples were obtained for the analysis of plasma BSP levels.

The animals were killed by exsanguination. Livers were flushed with an ice-cold NaCl solution (0.9%, w/v) injected via the hepatic vein. Following this the livers and bladders were removed. Liver samples were freeze-clamped in liquid nitrogen for later analysis of the amounts BSP and GSH and GSH-S-transferase activity.

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* Abbreviations used: BSPt, BSPc, BSPu, total, conjugated and unconjugated bromosulfophthalein; DEM, diethyl maleate; GSH, glutathione; T_m, maximum biliary transport.

Chemicals and analytical methods. BSP, DEM, 5-5'dithiobis-nitrobenzoic acid, glutathione reductase, β-nicotinamide adenine dinucleotide phosphate and GSH were purchased from Sigma Chemical Co. (St Louis, MO). All other reagents were of the highest quality available commercially. The rate of bile flow was determined gravimetrically. Sodium and potassium concentrations in bile were measured by flame photometry (NaK II Meteor flame photometer, Madrid, Spain). Chloride concentrations in bile were determined by titration with a silver electrode (Analytical Control, chloridometer model 160, Italy). pO_2, pCO_2, and blood pH and HCO_3^- concentrations in blood and bile were measured in an automated gas analytical system (Corning Medical, model 168, Medfield, MA). Bile, plasma and urine BSP concentrations were determined spectrophotometrically at 580 nm after appropriate dilution with 0.1 N NaOH. Liver BSP concentrations were determined according to the method of Whelan *et al.* [13]. BSP was separated from BSP-GSH on Whatman No. 1 filter paper using an ascending system consisting of butan-1-ol : acetic acid : ethanol : water (120/1/20/40, v/v/v/v). The spots were cut, eluted and read at 580 nm after alkalinization. Liver GSH concentrations were estimated as described by Tietze [14] and hepatic GSH-S-transferase activity was determined using BSP as

the substrate, according to the method of Habig *et al.* [15].

Statistics. Values are given as means ± SEM. Statistical analysis of the results was performed by comparing means of two groups of experiments by the pooled *t*-test using a significance level of P = 0.05 to reject the null hypothesis.

RESULTS

Effects of DEM on BSP plasma disappearance, maximum biliary transport and composition of BSP compounds in bile, liver and urine

At all times of the experimental period the mean plasma BSP concentrations in the control and DEM-treated rabbits were similar; in no case was BSPc detected in plasma (data not shown). The T_m values of BSPt, BSPc and BSPu were reached at about 60 min after starting infusion, resulting in similar average values in both kinds of experiments; however, after bolus intraperitoneal doses of 3.2 mmol/kg of DEM the T_m of BSPt and BSPc decreased significantly whereas, contrariwise, that of BSPu increased (Figs 1 and 2).

Moreover, as shown in Fig. 2, both the biliary excretion rate of BSPt and its cumulative biliary excretion values were reduced after DEM administration. The decrease in BSPt excretion was due to
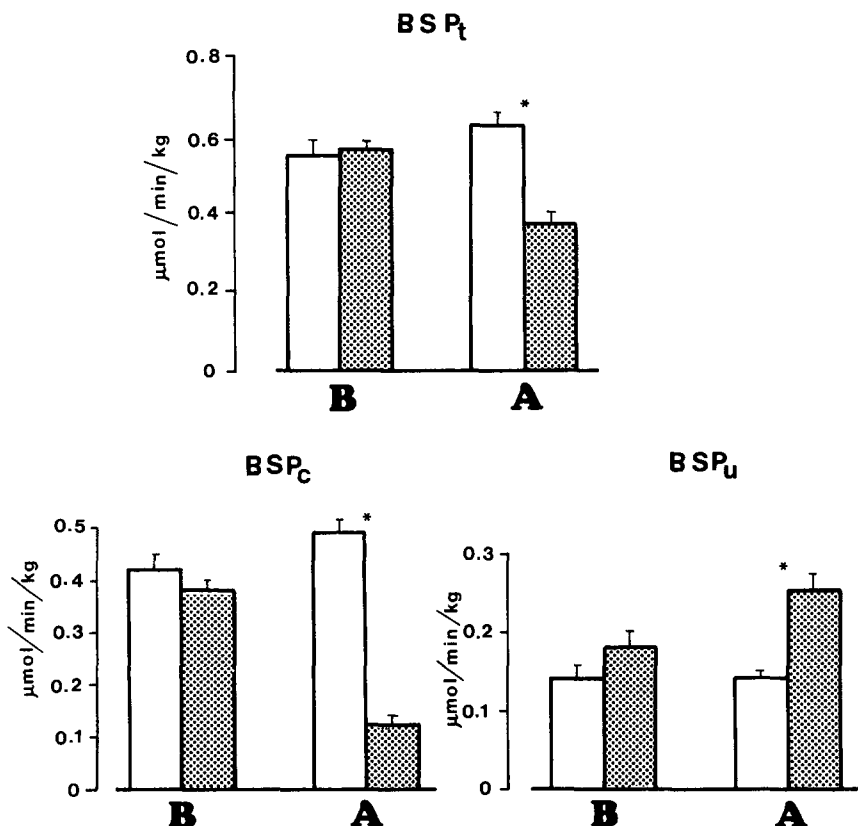

Fig. 1. Comparison of values of maximum biliary transport of total (BSPt), conjugated (BSPc) and unconjugated (BSPu) bromosulfophthalein in corn oil (open bars, control animals) and diethyl maleate-treated rabbits (filled-in bars) before (B) and after (A) corn oil or diethyl maleate administration. Values are given as means ± SEM for 6 experiments. *P < 0.05, significantly different from controls.
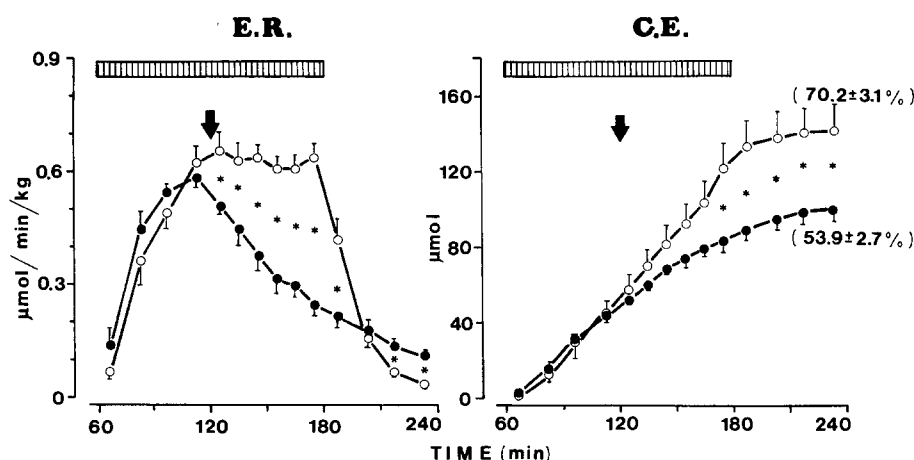
Fig. 2. Biliary excretion rate (E.R.) and cumulative biliary excretion (C.E.) of total BSP during and following constant i.v. infusion of BSP in corn oil (open circles, control animals) and diethyl maleate-treated rabbits (filled-in circles). Numbers in brackets: % recovery of total BSP of dose administered. Values are given as means ± SEM for 6 experiments. ▨▨▨▨▨ BSP infusion (0.88 μmol BSP/min/kg); ↓ corn oil or DEM administration; *P < 0.05, significantly different from controls.

a reduction in the excretion rate of BSPc since the bile output of BSPu was significantly increased. The percent-dose recovery at the end of the assays was smaller in the DEM-treated animals than in the control group (53.9 ± 2.7%, N = 6 and 70.2 ± 3.1%, N = 6, respectively).

At the end of dye administration (min 180) both the concentration and excretion of BSPt decreased rapidly in the control group but slower and to a lesser degree in the DEM experiments, such that during the 220–240 min period the concentrations and excretion rate of BSP were significantly higher than in the controls (Fig. 2). The percentage of BSPc excreted in bile before administration of corn oil or DEM was similar in both groups (74 ± 3%, 69 ± 4%, respectively); this percentage dropped markedly after DEM but not after corn oil, reaching a minimum value of 29%.

Table 1 shows the amounts and composition of BSP in the livers of the two experimental groups; in rabbits treated with DEM almost twice the amounts found in the controls were observed, mainly in the form of unmetabolized BSP. The small quantity of dye excreted in the urine (<3% of the dose) was not statistically different between groups, either regarding the amount or the percentage of conjugated BSP.

Effect of DEM on hepatic GSH content and GSH-S-transferase activity

Liver glutathione concentrations were significantly

reduced in DEM-treated rabbits as compared to the controls. However the in vitro conjugating activity of the liver, expressed per g of liver was unaffected by DEM administration (Table 2).

Effect of DEM on biliary secretion during BSP infusion

Figure 3 illustrates the changes in bile flow rates in the control and DEM-treated rabbits. Biliary flow rates before corn oil or DEM administration were similar in both groups, both in basal conditions (0–60 min) and during the first hour of BSP infusion (60–120 min); additionally, as described above, the cholestatic effect of BSP was observed. However, the choleresis induced by DEM administration in the absence of BSP [7] was abolished by infusion of the dye; from 150 min onwards, bile flow fell slowly and irreversibly in the DEM experiments whereas in the controls it was seen to return progressively to basal levels.

Regarding the secretory rates of the inorganic electrolytes assayed, during the 0–120 min period similar changes were observed in both types of experiments; however, from this moment onwards BSP impaired the stimulatory effects of DEM on the biliary output of these components. Thirty minutes after DEM injection a progressive reduction in the biliary excretion rate of all of them took place

Table 1. Comparison of total BSP and unconjugated BSP in livers of control (corn oil) and diethyl maleate-treated rabbits (DEM)

	Total BSP (μmol/g liver)	Unconjugated BSP (% of total BSP)
Control	0.21 ± 0.03	9.8 ± 0.8
DEM	0.39 ± 0.02	78.7 ± 4.1
Significance	P < 0.05	P < 0.05

Values are given as means ± SEM for 4 to 5 animals.

Table 2. Comparison of hepatic concentration of total glutathione (GSH) and GSH-S-transferase activity in liver homogenates of control (corn oil) and diethyl maleate-treated rabbits (DEM)

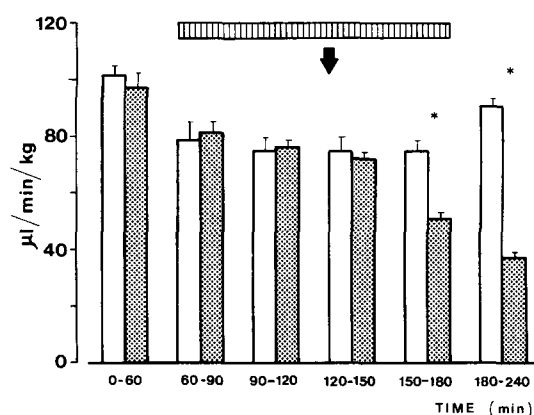|	GSH (μmol/g liver)	GSH-S-transferase activity (μmol BSPc/g liver/min)
Control	3.12 ± 0.31	0.93 ± 0.05
DEM	1.15 ± 0.07	0.98 ± 0.06
Significance	P < 0.05	N.S.

Values are given as means ± SEM for 4 to 5 animals.

Fig. 3. Comparison of mean values ($\pm$SEM) of bile flow in corn oil (open bars, control animals) and diethyl maleate-treated (filled-in bars) rabbits, before, during and after constant infusion of BSP. ▬▬▬ BSP infusion (0.88 μmol BSP/min/kg); ↓ Corn oil or DEM administration; *P < 0.05, significantly different from controls.

whereas a recovery towards basal levels was observed in the control animals (Fig. 4).

The metabolic acidosis previously observed after DEM administration [7] was also found in our series; from 45 to 60 min after injection blood pH, pCO_2 and the HCO_3^- concentration fell, whereas the pO_2 level rose (data not shown).

DISCUSSION

The unchanged plasma elimination of BSP after DEM injection suggests that DEM does not affect either the initial distribution volume of BSP nor translocation of the dye across the sinusoidal plasma membrane in the rabbit liver. This is not surprising since in spite of the reduction in GSH levels, and

hence the conjugation possibilities of BSP, this reaction apparently does not affect hepatic uptake of the dye [2, 4]. Neither does the existence of competition between BSP and DEM in the uptake process seem likely. Indeed, whereas the uptake of BSP in isolated hepatocytes is a saturable and carrier-mediated process [3, 16] lipophilic xenobiotics, such as DEM, may pass through the membrane by diffusion [3, 17]. In the same way, we think it unlikely that the origin of the changes observed in our experiments could be due to competitive or non-competitive inhibition of GSH-S-transferase activity by DEM since liver GSH-S-transferase activity was the same in both groups of animals and although both xenobiotics are conjugated with GSH the reactions are catalyzed by two different GSH-S-transferases [12].

The conjugation of BSP with GSH is not obligatory for its biliary excretion but does exert an important positive effect on excretion, as has been reported for the rat, guinea pig and dog [4, 13, 18–20]. Thus, the reduction in GSH reserves in the livers of DEM treated animals would explain the reduction in the excretion of both conjugated and total BSP. Contrariwise, the biliary excretion and T_m of non-metabolized BSP were increased after DEM administration. This pattern was similar to that observed in rats treated with benziodarone—an inhibitor of GSH-S-transferase [1]—and also similar to that reported for animals subjected to a protein-free diet [18–21] or chemical treatment [1, 4, 22].

It has been reported that conjugation with GSH facilitates the excretion of BSP because the conjugated form has a higher excretion rate than BSPu [13, 23] and because BSPu in the liver competes with and depresses the transport of BSPc into bile [23, 24]. These findings, together with our results relating to hepatic GSH concentrations, suggest that the greater depletion of GSH in DEM-treated rabbits caused by the simultaneous administration of DEM during BSP
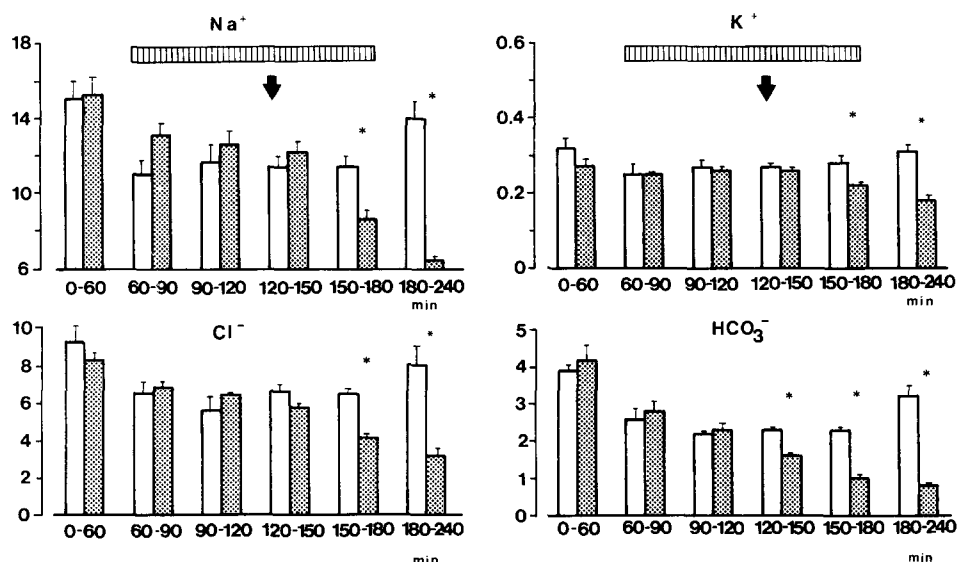

Fig. 4. Biliary excretion rate (μEq/min/kg) of sodium, potassium, chloride and bicarbonate in corn oil (open bars, control animals) and diethyl maleate-treated (filled-in bars) rabbits before, during and after constant infusion of BSP. Values are given as means $\pm$ SEM for 6 experiments. ▬▬▬ BSP infusion (0.88 μmol BSP/min/kg); ↓ Corn oil or DEM administration; *P < 0.05, significantly different from controls.

infusion may satisfactorily account for the reduction in T_m and for the concentration and biliary excretion of BSPc. They might also explain the high levels of the dye found in liver, mainly in the form of unchanged BSP.

It has been described that cholephilic dyes with a low hepatic T_m, such as the case of BSP in the rabbit [9, 10], are accumulated in the hepatocytes, particularly in the unconjugated form, because their binding with ligandins is stronger than the binding taking place between these latter and the conjugated form [2] and also because their affinity for canalicular transporters is 10–15-fold smaller than that of BSPc [22]. All this is in agreement with our results concerning BSP in the liver since the amount of BSPt in the livers of the DEM-treated rabbits, significantly higher than in the controls, is mainly found in the form of BSPu. Finally, it is known that both the transfer of BSP from blood to bile [2, 24] and bile formation [3] are energy-dependent processes and also that BSPu and other dyes inhibit mitochondrial oxidative phosphorylation and the transport of electrons in the liver [25, 26]. Thus, general toxic influences on liver cells, via intracellular action, due to elevated BSPu concentrations might also be involved in the reduction of biliary BSP excretion after GSH depletion. Nevertheless other mechanisms cannot be ruled out.

With respect to the changes observed in bile flow, our results have shown that intraperitoneal DEM administration simultaneous to i.v. BSP infusion does not induce the typical choleretic response that has been described in the rat, dog and rabbit [4–7]. In our experiments, the cholestasis induced by BSP observed in both groups prior to the administration of corn oil or DEM was maintained during the 60 min period following DEM injection with parallel decreases in the biliary outputs of the inorganic electrolytes assayed. In principle, the absence of a choleretic response to DEM when BSP is being excreted could be due to the fact that DEM is not excreted into the biliary tree. Although biliary DEM excretion was not determined, it was probably reduced since, as pointed out by Barnhart and Combes [5], BSP-GSH could compete with DEM-GSH for transport into bile.

The results concerning bile flow and the secretion of electrolytes point to the idea that the absence of a choleretic response to DEM and the delayed cholestasis in the DEM group are initially due to an inhibition of the bile acid independent flow—BAIF. As has been indicated above, BSPu inhibits some enzymes and carrier proteins involved in BAIF formation [3, 26]. Moreover, it is well known that BAIF formation depends on the active secretion to the biliary tree of inorganic electrolytes [3, 7, 8]. Therefore, the higher acute toxic effects of the dye after DEM (due to the higher levels of BSPu as a result of the depletion of the GSH pool) could not only account for the absence of a choleretic response and the presence of cholestasis in DEM-treated animals but also the delayed recovery of biliary secretion in the control animals, precisely when hepatic levels of BSP become negligible. However, in the case of the cholestasis of the DEM-treated animals, apart from the toxic effects of BSP itself, the toxic effects of

DEM would also be involved; these have already been reported in mice [27], rats [4] and rabbits [7].

In summary, the depletion of hepatic GSH levels and the toxic effects of BSP and/or DEM in the rabbit could account for the BSP-induced cholestasis preceding corn oil or DEM administration and the absence of hypercholeresis and the delayed progressive cholestasis in the DEM-treated group. These changes would be more intense than those described for BSP and DEM administered separately [7] and would become apparent earlier owing to the simultaneous presence of DEM and high intracellular levels of BSPu, the latter in turn secondary to the hepatic GSH depletion induced by DEM.

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