Effects of Benziodarone on the Metabolism and Biliary Excretion of Sulfobromophthalein and Related Dyes[1] (34328)

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A variety of methods have been used in attempts to elucidate the importance of conjugation with glutathione (GSH) for the hepatic excretion of sulfobromophthalein (BSP). Substrate (GSH) depletion studies, either diet-induced (1, 2) or chemically-induced (3), indicate that interference with conjugation can impair the biliary excretion of BSP under circumstances where general excretory function of the liver is not disturbed.

Another approach to this problem has been reported by Boyland and Grover (4). They found that 2-ethyl-3-(4-hydroxy-3, 5-diiodobenzyl) benzofuran (benziodarone), a potent inhibitor of an *S*-arylglutathione transferase derived from rat liver, could produce BSP retention in rabbits, but that the plasma clearance of phenoldibromphthalein disulfonate (DBSP), an analog which is excreted without conjugation, was unaffected. Boyland and Grover (4) concluded that benziodarone was acting by specifically inhibiting the conjugation of BSP with GSH. The present communication describes some observations we have made on dye excretion in benziodarone-treated rats.

Methods. Male albino rats (350–400 g) obtained from Holtzman Co. (Madison, Wisconsin) were used in these studies. The rats were fed (Purina laboratory chow 5001) and watered *ad libitum*. Sodium pentobarbital (50 mg/kg) was used as an anesthetic in all surgical procedures. Body temperature was maintained at 37° (measured with a rectal probe) by means of a heat lamp.

Benziodarone, dissolved in corn oil, was injected intraperitoneally (50 mg/kg) into rats 1 hr prior to commencement of a 60-min dye infusion. BSP (Dade Reagents Inc.) and DBSP were diluted with saline so that when infused at a rate of 0.037 ml/min from a Harvard infusion pump, a dye infusion rate of 2.5 mg/min/kg was obtained. Synthetically conjugated BSP was prepared by reacting 600 mg of reduced glutathione (Nutritional Biochemicals Corp.) in 10 ml of BSP solution (50 mg/ml) for 24 hr at pH 7–8; the pH of the reaction mixture was adjusted to 7.4 prior to infusion into rats. The composition of the reaction mixture was determined using thin-layer chromatography (TLC) and was found to be 4 ± 1% (SE) unchanged BSP, 42 ± 2% BSP–GSH and 55 ± 2% of a third component, chromatographically similar to the disubstituted conjugate [BSP–$(GSH)_2$] (5).

Biliary excretion studies were performed in rats in which the bile duct was cannulated with polyethylene tubing (PE10) and a femoral vein cannulated with PE50 tubing for infusion. Bile volume was determined gravimetrically; the density of the bile was 1.0.

Dye concentrations were determined colormetrically after dilution with 0.01 *M* NaOH and the absorbance was measured at 580 mμ for BSP and its conjugates, and 575 mμ for DBSP. Estimation of BSP–GSH and unchanged BSP in bile was made after the components in 5 μl of bile were separated on cellulose plates by TLC using the upper phase of *n*-butanol:acetic acid:water (4:1:5) as the developing solvent (6).

Hepatic glutathione concentrations were measured by the fluorimetric method of Cohn and Lyle (7), modified to use 50 μl of supernatant diluted with 0.45 ml of 5% *m*-phosphoric acid and 2 ml of 0.2 *M* Tris buffer, giving a final pH of 8.0. The BSP conjugat-

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ing enzyme activity in 1 g of liver was determined by the method of Goldstein and Combes (8) using optimal conditions described by Klaassen and Plaa (9). A Unicam S.P. 800 spectrophotometer with a constant wavelength attachment was used to record changes in absorbance at 330 mμ. In some experiments, benziodarone was added *in vitro* dissolved in acetone and a similar volume of acetone (1–5 μl) was added to controls.

Statistical analysis of the results was performed by comparing means of treated groups with their respective controls by a two-tailed t test using a significance level of $p = 0.05$ to reject the null hypothesis.

Results. Preliminary studies showed that the dosage regimen for benziodarone used by Boyland and Grover (4) in the rabbit (5 mg/kg/day, ip for 4 days) produced no alterations in BSP plasma disappearance or biliary excretion in the rat. However, it was established that treatment of rats with benziodarone 50 mg/kg ip in corn oil 1 hr prior to BSP injection (60 mg/kg) produces significant plasma retention of BSP. This dosage regimen for benziodarone was used in subsequent experiments.

In experiments where BSP was infused, excretion of total BSP in the bile was markedly impaired after benziodarone treatment (Fig. 1). The decrease in total BSP concentration was due to a decrease in the BSP–GSH component, while the biliary ex-

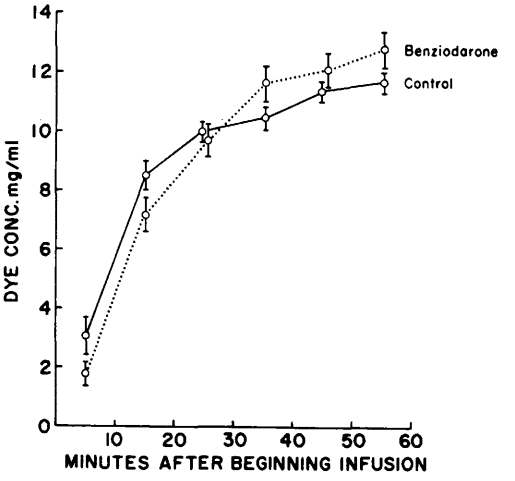

FIG. 2. Biliary concentration of DBSP in four control and five benziodarone-treated (50 mg/kg ip 1 hr) rats infused with DBSP at a rate of 2.5 mg/min/kg for 1 hr; each point represents the mean ± SE.

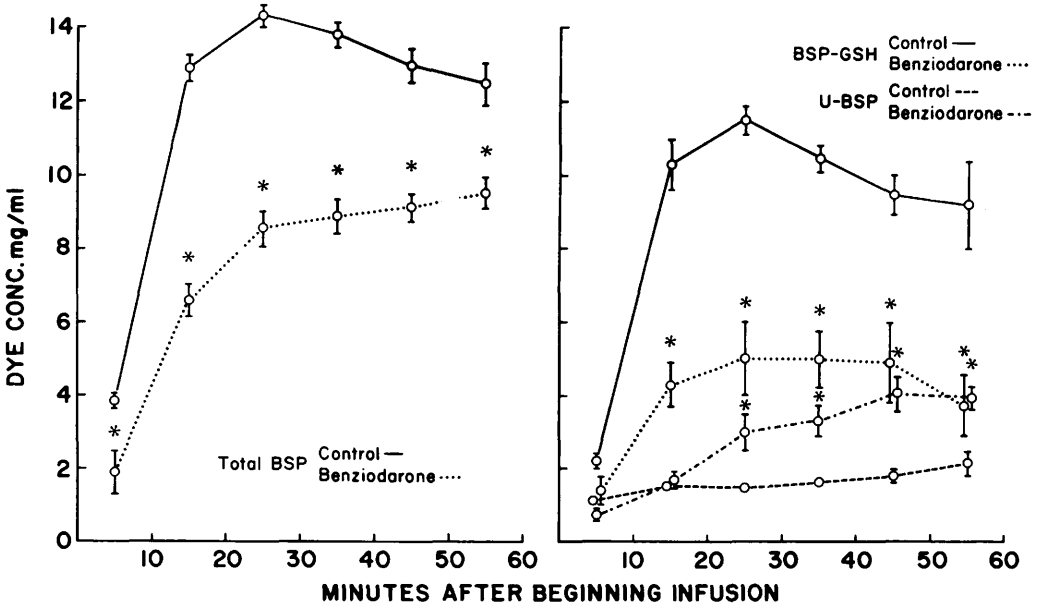

FIG. 1. Biliary concentration of total BSP, unconjugated BSP (U-BSP) and BSP–GSH in rats infused with BSP at a rate of 2.5 mg/min/kg for 1 hr. Benziodarone (50 mg/kg ip) was injected 1 hr prior to infusion of BSP; the points represent the mean ± SE of six control and five treated rats; an asterisk denotes a value significantly different ($p < 0.05$) from its respective control.

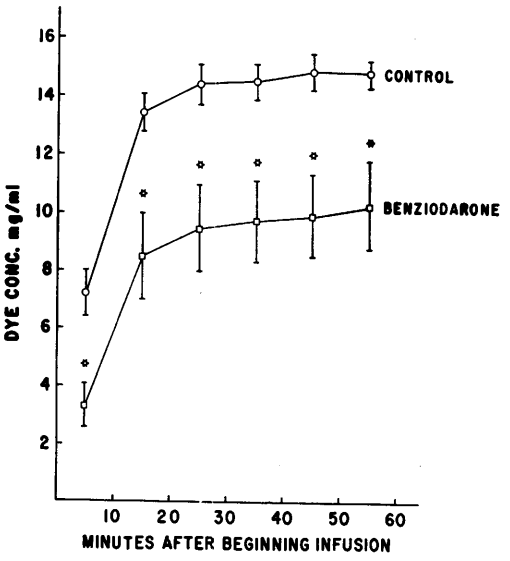

FIG. 3. Biliary concentration of total BSP in four control and five benziodarone-treated (50 mg/kg ip, 1 hr) rats infused with synthetic BSP–GSH conjugates one hr at a rate equivalent to 2.5 mg/min/kg of BSP; each point represents the mean ± SE; an asterisk denotes a value significantly different ($p < 0.05$) from its respective control.

cretion of unchanged BSP was significantly increased. When DBSP was infused at the same rate, no changes were seen in biliary excretion patterns in benziodarone-treated rats (Fig. 2). However, when synthetic conjugates of BSP were infused at a rate equivalent to 2.5 mg/min/kg BSP, significant impairment of biliary excretion was observed (Fig. 3). The effects of benziodarone on the apparent maximal transport rate (T_m) of the dyes paralleled those on biliary concentration, except that the decrease in BSP T_m was augmented by a significant reduction in bile flow (Table I).

Estimates of hepatic parameters of conjugation in livers of rats treated with benziodarone are also shown in Table I. One hr after treatment with benziodarone, neither the hepatic concentration of the substrate GSH nor the *in vitro* conjugating activity of the liver were significantly different from untreated controls.

Benziodarone was found to be a potent inhibitor of the *in vitro* enzyme-catalyzed conversion of BSP to BSP–GSH. Quantitative analysis of the enzyme kinetics by a Dixon-type plot, as used by Boyland and Grover (4) to show noncompetitive inhibition of *S*-arylglutathione transferase with substrate 1,2-dichloro-4-nitrobenzene, was not possible with our data. However, approximately 50% inhibition of the enzyme activity was consistently produced in the presence of benziodarone in concentrations of 0.02–0.03 m*M* (Table II).

Discussion. The effects of benziodarone on the biliary excretion of BSP in the rat are

TABLE I. Parameters of Excretion and Conjugation.[a]

Parameter	Control	Benziodarone[b]
Hepatic GSH concentration (μg/100 mg of liver)	207 ± 9 (4)	213 ± 5 (4)
In vitro conjugating activity (mg of BSP conj/g of liver/5 min)	12.6 ± 0.5 (4)	12.3 ± 0.7 (4)
T_m during dye infusion (mg/min/kg)		
BSP	0.92 ± 0.09 (6)	0.27 ± 0.03[c] (5)
Synthetic BSP–GSH	1.29 ± 0.08 (4)	0.78 ± 0.15[c] (5)
DBSP	0.81 ± 0.07 (4)	0.61 ± 0.10 (5)
Mean bile flow during infusion (μl/min)		
BSP	21 ± 2 (6)	12 ± 1[c] (5)
Synthetic BSP–GSH	32 ± 1 (4)	28 ± 2 (5)
DBSP	25 ± 2 (4)	19 ± 3 (5)

[a] Values are means ± SE; nos. of rats in the various groups are given in parentheses.

[b] Benziodarone (50 mg/kg) was injected intraperitoneally 1 hr prior to infusion of dye, or removal of liver samples.

[c] Significantly different from control ($p < 0.05$).

TABLE II. Inhibition of Enzyme-Catalyzed BSP Conjugation by Benziodarone Added *in Vitro*.[a]

Benziodarone concentration (mM)	226 μM BSP	113 μM BSP
0	12.5	12.3
0.01	8.0	9.8
0.02	4.3	6.4
0.03	3.9	4.5
0.04	4.3	3.9
0.05	2.35	3.5

[a] Values are mg BSP conjugated/g liver/5 min. The same enzyme preparation was used to obtain all of the above figures. Benziodarone was dissolved in acetone, and aliquots of 1–5 μl were added to the incubation tubes to give the above final concentrations. The addition of 1–5 μl acetone to control tubes had no effect on the enzyme activity. Values were calculated from the slope of the linear portion of the absorbence vs. time curve, using the equation of Goldstein and Combes (8).

consistent with the hypothesis that the conjugation of BSP with GSH is inhibited. Biliary concentration of BSP–GSH was dramatically reduced, while biliary concentration of unconjugated BSP was elevated after treatment with benziodarone. The biliary excretion patterns were similar to those seen in rats treated with iodomethane, in which the changes were attributed to chemically-induced depletion of the hepatic substrate GSH, thus leading to impairment of conjugation (3). However, it was shown that unlike iodomethane benziodarone does not alter hepatic GSH concentrations.

Existing methods for the determination of BSP–GSH in hepatic tissue suffer from incomplete and variable recovery, therefore it was not possible to confirm that conjugation of BSP was impaired *in vivo*. However, the *in vitro* results confirm those of Boyland and Grover (4); furthermore, the inhibition of conjugation with substrate BSP appears to be as marked as that seen with substrate 1,-2-dichloro-4-nitrobenzene. It was important to establish the substrate specificity since it has been shown previously that norethandrolone inhibits the conjugation of 1,2-dichloro-4-nitrobenzene with GSH (4), but that it stimulates the conjugation of BSP with GSH (10). On the basis of our *in vitro* data we were not able to establish whether the inhibition is competitive or noncompetitive. Neither were we able to demonstrate impaired BSP conjugating activity in the livers of rats treated with benziodarone *in vivo*, although such inhibition has been shown previously in rats treated with carbon tetrachloride (11). The hepatic concentration of benziodarone in our rats was not determined, but since the enzyme preparation used in the *in vitro* assay is a 1–80 dilution of the liver, the concentration of benziodarone in the incubation tubes might be expected to be very low. This indirect evidence suggests that benziodarone inhibition of BSP conjugation may be competitive, or if noncompetitive, is readily reversible.

While the *in vivo* effects of benziodarone on the biliary excretion of BSP are compatible with a hypothesis of impaired conjugation, there is obviously another factor involved; that of impaired dye transport. Our results are in agreement with those of Boyland and Grover (4) in that benziodarone does not affect the hepatic excretion of DBSP, a dye which is excreted without biotransformation in the rat (12). However, we have shown that benziodarone does impair the biliary excretion of synthetic BSP conjugates; a process which also appears to lack a metabolic step. This brings to light the intriguing possibility that there are different hepatic transport processes for unconjugated and conjugated dyes. It should be noted that the biliary excretion of unconjugated BSP is apparently not affected by benziodarone, and shows an increase comparable to that seen after chemically-induced impairment of BSP–GSH excretion (3).

If one compares the inhibitory effects of iodomethane (3) and benziodarone on BSP biliary excretion, there are some similarities. In both cases, the biliary concentration of unconjugated BSP was increased, but not sufficiently to restore total BSP biliary concentration. Neither iodomethane nor benziodarone affected the biliary excretion of DBSP. However, there were some marked differences between the effects of the two chemicals. Although neither influenced *in vit-*

ro BSP conjugation in rats treated *in vivo* with the compounds, benziodarone had a marked effect when added *in vitro*, whereas iodomethane had no effect. On the other hand, iodomethane significantly reduced hepatic GSH concentration, whereas benziodarone had no effect on this parameter. Total BSP excretion after infusion of synthetic BSP–GSH conjugates was impaired after benziodarone treatment, but was not affected by iodomethane treatment. No plasma retention was observed after injection of 60 mg/kg of BSP into iodomethane-treated rats, but significant retention was observed in benziodarone-treated rats. Therefore, while it appears that impairment of BSP conjugation is common to both treatments, the inhibition is probably produced by different mechanisms.

Although our results show that the effects of benziodarone cannot be explained simply by an effect on BSP conjugation alone, but rather by a combination of effects on conjugation and dye transport, they do suggest that the biliary excretion mechanisms for unconjugated moieties of BSP and its analogs may differ in some aspects from those for conjugated moieties.

Summary. Treatment of rats with benziodarone (50 mg/kg ip) 1 hr prior to infusion of BSP resulted in marked impairment of the biliary excretion of BSP. Total BSP concentration was decreased and this was due to a decrease in the BSP–GSH component, while unconjugated BSP concentration was increased. Benziodarone is a potent inhibitor of the *S*-arylglutathione transferase enzyme system which catalyses BSP conjugation with GSH. Benziodarone was found to inhibit the biliary excretion of synthetic BSP–GSH conjugates, although not that of DBSP, a BSP analog excreted without conjugation. It is proposed that benziodarone exerts its influence on BSP excretion by a combination of effects on conjugation and BSP–GSH transport, and that the results have uncovered the possibility that separate mechanisms may exist for the biliary excretion of conjugated and unconjugated dyes.

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