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Dapsone-induced cholestasis and impairment of bile salt output in the rat

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Abstract

To evaluate the effect of dapsone (4,4′-diaminodiphenylsulfone, DDS) on biliary bile salt secretion, we administered the drug to male and female Wistar rats at a dose of 30 mg/kg body wt, twice a day, for 4 days. DDS decreased basal bile flow by about 20% in both male and female rats. In addition, basal biliary bile salt secretion was decreased by the drug in animals from both sexes (about 30% decrease). Bile salt maximum secretory rate, as evaluated by infusing tauroursodeoxycholate at stepwise-increasing rates, was not affected by DDS in either male or female rats, suggesting that the density of canalicular bile salt transporters is preserved. The size of the bile salt pool and the rate of *de novo* synthesis of bile salts, measured in bile salt-depleted animals, were decreased by about 33 and 35%, respectively; there was no difference in response between males and females. The ability of the ileum to reabsorb bile salts, as estimated by analysis of the expression of the ileal apical sodium-dependent bile salt transporter and of sodium taurocholate transport activity in brush border membrane vesicles, was not affected by DDS in either males or females. Overall, our findings suggest that an impairment of *de novo* synthesis mediated by a direct inhibition of CYP3A metabolism, rather than a decreased intestinal reabsorption of bile salts, accounts for the decrease in bile salt pool size. The dissociation between alteration of bile secretory function and the oxidative stress induced by DDS, which is known to be relevant only in male rats, is discussed. © 2002 Published by Elsevier Science Inc.

Keywords: Dapsone; Cholestasis; Bile salt secretion; Bile salt metabolism; Bile salt pool; ASBT

1. Introduction

DDS is a drug that, alone or in combination with other therapeutic agents, is effective in a variety of infectious diseases, including leprosy [1] and malaria [2], as well as infections with *Pneumocystis carinii* and *Toxoplasma gondii* in AIDS patients [3]. DDS is also useful in the treatment of a number of inflammatory conditions, including dermatitis herpetiformis [4]. The mechanism of the antibacterial action of DDS is similar to that of the sulfonamides in that it is an antagonist of *p*-aminobenzoic acid in folate synthesis. In addition, its action as an anti-inflammatory agent and in a variety of skin disorders is due to inhibition of neutrophil adherence and function [5].

DDS is metabolized mainly by two different routes: *N*-acetylation and *N*-hydroxylation. The *N*-hydroxylation is catalyzed either by enzymes such as cytochrome P450 [6], flavin monoxygenase and prostaglandin H-synthetase in liver, or by myeloperoxidase in peripheral polymorphonuclear leukocytes [7]. DDS *N*-hydroxylamine has been suggested to be the causative agent involved in the characterized adverse reactions to DDS [8].

The most common dose-dependent DDS-induced adverse drug reaction is hematologic toxicity, characterized by methemoglobinemia and hemolysis [9]. DDS *N*-hydroxylamine has been shown to be related directly

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Abbreviations: DDS, 4,4′-diaminodiphenylsulfone, dapsone; BS, bile salt(s); ASBT, apical sodium-dependent bile salt transporter; TUDC tauroursodeoxycholate; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; ALP, alkaline phosphatase; BBMV, brush border membrane vesicle(s); *SR*m, maximum secretory rate; HI, hydrophobicity index; TC, taurocholate; β-MC, β-muricholate; UDC, ursodeoxycholate; CDC, chenodeoxycholate; DC, deoxycholate; HDC, hyodeoxycholate; C, cholate.

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to methemoglobin formation in patients [10]. Similarly, in rats, the rank order of DDS-induced methemoglobin formation *in vivo* agreed with the formation of the hydroxylamine metabolite by microsomes *in vitro*. Indeed, only male rats, which have a far higher capability than female rats to metabolize DDS into its hydroxylamine metabolite, show a significant increase in methemoglobin levels following DDS administration [11]. In addition to these dose-dependent adverse effects, DDS was shown to produce a number of adverse reactions unrelated to dosage, i.e. hypersensitivity reactions (reviewed by Medina *et al.* [12]). Among them, there is the so-called "sulfone syndrome," which involves a hepatic dysfunction, among other manifestations. It was suggested that DDS-induced hepatic dysfunction is cholestatic in nature, primarily involving hepatocellular damage [13]. However, the mechanism by which DDS induces cholestasis has not been addressed.

BS are the chief constituents of bile. They play a crucial role in bile formation and, once delivered to the intestine, in lipid absorption by the intestinal tract [14]. BS undergo a very efficient cycling between the intestine and the liver. The enterohepatic circulation of BS is dependent primarily upon their secretion at the canalicular membrane of the hepatocyte, followed by their active absorption at the apical surface of enterocytes in the terminal ileum mediated by the ileal ASBT, their subsequent basolateral transport into the portal venous circulation, and, finally, their uptake at the sinusoidal pole of the hepatocyte. Overall, 95% of the total BS are conserved in each cycle, as a result of the efficiency of the transporters involved. The basal rate of hepatic BS synthesis replenishes the BS loss from enterohepatic circulation via the feces. BS are amphipathic compounds that, due to their detergent properties, can disrupt membrane structure and alter permeability to hepatocellular constituents [15]. Furthermore, BS are able to uncouple oxidative phosphorylation, thus inducing oxidative stress and other cytotoxic damage [16]. BS toxicity depends, at least in part, on their hydrophobicity. The modulation of the hydrophilic-hydrophobic balance of the BS pool has been postulated to play a role in the maintenance of integrity of membranes, biliary lipid secretion, and bile flow [14]. Thus, it is relevant to ascertain whether a hepatotoxic drug may affect liver function as a consequence of alterations in BS metabolism.

In this study, we investigated the effect of DDS on biliary secretion of BS, liver integrity, and intestinal absorption of BS, as well as the relationship of BS handling to DDS-induced hematotoxic effects, in both male and female rats. Since methemoglobinemia induced by the *N*-hydroxylated derivative of DDS is almost absent in female rats, comparison of DDS effects between males and females may be useful to associate any potential alteration in biliary BS secretion with the *N*-hydroxylated derivative of DDS. The data provide evidence that the changes induced by DDS in bile secretory function are associated with a decrease in biliary BS secretion, without affecting liver integrity or ileal absorption of BS. These effects are unlikely to be mediated by the *N*-hydroxylated metabolite of DDS.

2. Materials and methods

2.1. Chemicals

DDS and 3α-hydroxy-steroid dehydrogenase were obtained from the Sigma Chemical Co. Sodium TUDC was a gift from Prodotti Chimici e Alimentari S.p.A. This BS was more than 98% pure when examined by HPLC. [^{14}C]TC (46.40 mCi/mmol; 98% purity) was obtained from New England Nuclear. A specific anti-rat ASBT antibody was used in western blot studies [17]. All other reagents were of the highest analytical grade available from commercial sources.

2.2. Animals and treatments

Adult male (300–350 g) and female (200–250 g) Wistar rats were used throughout. Before the experiments, the animals were maintained on a standard diet and water *ad lib.*, and housed in a temperature- (21–23°) and humidity- (45–50%) controlled room under a constant 12-hr light, 12-hr dark cycle.

In preliminary experiments, we analyzed the changes in basal bile flow in response to DDS, when administered at different doses and for different periods of time. In an initial experimental set, male or female animals were randomly divided into eight experimental groups. Four of them received two daily doses (10:00 a.m. and 6:00 p.m.) of 30 mg/kg of DDS, i.p., for 1, 2, 4, or 6 consecutive days; the remaining groups (controls) received only the vehicle (propylene glycol). To analyze the dose-dependent effect of DDS on basal bile flow, new sets of male and female rats were divided into four experimental groups, three of them receiving 15, 30, and 60 mg/kg body wt of DDS, i.p., twice a day for 4 consecutive days; the other one received only the vehicle. Since the maximal effect was reached at the dose of 30 mg/kg body wt, administered for 4 consecutive days, this dosage was used in the remaining experiments, irrespective of the sex (see below).

2.3. Surgical procedures

Animals were anesthetized with a single dose of sodium pentobarbital (50 mg/kg body wt, i.p.), and maintained under this condition throughout the experiment. The femoral vein was cannulated using PE-50 polyethylene tubing (Intramedic, Clay Adams). Next, a middle abdominal incision was made, and the common bile duct was also cannulated (PE-10, Intramedic, Clay Adams). Bile

collection was started between 9:00 and 11:00 a.m. to minimize the influence of circadian variations. Tracheal cannulation was performed systematically to remove bronchial secretion induced by the anesthetic. Body temperature was maintained at 37.5 to 38.0° with a warming lamp to prevent hypothermic alterations of the bile flow. At the end of each experiment, animals were killed by exsanguination, in order to obtain blood samples for methemoglobin content and ASAT (EC 2.6.1.1), ALAT (EC 2.6.1.2), and ALP (EC 3.1.3.1) activity determinations. Finally, the liver was removed and weighed.

To evaluate the effect of DDS on intestinal BS transport activity and ASBT expression, ileal BBMV were prepared. A middle abdominal incision was made, and two consecutive segments close to the ileocecal valve (about 25 cm each) were cut and removed. These segments more likely correspond to the proximal and distal regions of the ileum, respectively, which represent the main place for active transport of TC and expression of ASBT in the intestine [17]. The segments were flushed with and placed in saline at 4° until preparation of the mucosal tissue.

2.4. Experimental procedure

2.4.1. Basal bile flow studies

The effect of DDS on basal bile flow was analyzed in all the experimental groups, irrespective of the dose or time period of DDS administration. After a 30-min period of stabilization, spontaneously secreted bile was collected for another 30 min in preweighed vials.

2.4.2. TUDC infusion studies

TUDC was selected for BS *SR*m assessment due to its extremely low toxicity. In contrast to other naturally occurring BS, whose apparent maximum transport is largely determined by the cytotoxic nature of each BS, the secretory transport maximum of TUDC is thought to reflect actual maximal canalicular transport activity, rather than cytotoxicity [18]. The *SR*m of TUDC was assessed by infusing this BS i.v., dissolved in 2% (w/v) serum bovine albumin in saline, at stepwise-increasing rates (2.3, 3.1, 4.2, 5.8, 7.7, 14.0, and 20.6 μmol/min per 100 g body wt). Each infusion rate was maintained for 20 min, and bile samples were collected every 10 min for 140 min. *SR*m was calculated as the mean of the three highest, consecutive 10-min secretory rates recorded over the whole infusion period [18].

2.4.3. BS depletion studies

These studies were performed to estimate the size, composition, HI, and the ''circulation frequency'' (number of enterohepatic cycles/24 hr) of the BS pool, as well as the *de novo* hepatic BS synthesis rate. The chronic bile fistula model provides a means for rapidly depleting the body of BS by eliminating the primary source of these compounds, i.e. the enterohepatic circulation [19]. Animals were put in restraining cages and subjected to continuous biliary drainage for 12 hr. Losses of water, electrolytes, and proteins were compensated for by i.v. infusion of a Krebs–Henseleit-bicarbonate solution (pH 7.4) containing 3 mg/mL of glucose and 2 mg/mL of serum bovine albumin. No further decrease in BS output was observed after 8 hr, indicating that a complete BS pool washout was reached at this time, as reported by others [19]. Thus, the BS excreted during the first 8 hr of bile drainage, when a complete BS pool washout was reached, represent the BS pool size; BS output after this period estimates the *de novo* hepatic BS synthesis rate [20].

2.4.4. Intestinal BBMV studies

These studies were made to evaluate the expression of ASBT and the ileal BS transport. For BBMV preparation, the intestinal segments were opened lengthwise, the mucus layer was removed carefully, and the mucosa was obtained by scraping [21]. Total homogenates were prepared from mucosa samples, as previously described [22]. BBMV were prepared from total homogenates by using a divalent cation precipitation method [23], with some modifications [22]. Aliquots of the homogenates and BBMV preparations were used for alkaline phosphatase activity determination. Apical membrane enrichment was estimated by calculating the ratio of the alkaline phosphatase activity in the BBMV to the alkaline phosphatase activity in the homogenate. The remaining BBMV preparation was used for western blotting (to assess ASBT expression), or for TC transport activity determination. Protein concentration in the homogenate and the BBMV preparations was measured, using bovine serum albumin as the standard [24].

2.5. Analytical procedures

The volume of bile was determined by weight, assuming a density of 1.0 g/mL. The bile flow was calculated as the relationship between the volume of bile secreted and the period of collection.

Total BS concentration in bile was measured by the 3α-hydroxy-steroid dehydrogenase procedure [25]. BS output was calculated by multiplying BS concentration and bile flow. Biliary BS composition was assessed by HPLC (Waters), as reported previously [26]. Individual BS were identified by using appropriate standards. An estimate of the BS pool size is obtained by measuring the BS excreted during the first 8 hr of bile drainage, when a complete BS pool washout was reached, and subtracting the contribution due to *de novo* hepatic BS synthesis rate [20]. Since the daily BS secretion is the product of the pool size and the number of times the pool circulates per day, and since both the daily secretion and the pool size may be calculated, the ''recirculation frequency'' (number of enterohepatic cycles/24 hr) may be obtained by dividing the daily BS secretion by the pool size.

The HI of the BS pool, described by Heuman [27], was calculated according to the equation:

$$HI = \sum HI_x F_x$$

where HI_x represents the hydrophobicity index, and F_x the molar fraction of each individual BS in the BS pool. The value of HI_x for each individual BS was obtained from a previous report [27].

Serum activities of ASAT and ALAT were assessed spectrophotometrically by measuring NADH consumption at 340 nm. ALP was assessed using *p*-nitrophenyl phosphate as a substrate. In all cases, commercial kits were used (Wiener Lab.).

Methemoglobin content in blood was assayed spectrophotometrically, using the method of Evelyn and Malloy [28].

Western blotting for ASBT was performed in ileal BBMV, by loading samples containing a fixed amount of protein (15 μg) in the gels. This amount was found to give a densitometric signal in the linear range of the response curve for the anti-ASBT antibody (data not shown). BBMV samples were loaded onto a 10% SDS-polyacrylamide gel [29], and subjected to electrophoresis. After electrotransfer onto nitrocellulose membranes (Protran, Schleicher & Schuell), the blots were blocked overnight at 4° with Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk, and then incubated for 1 hr with the primary anti-ASBT antibody (1:2000). The immune complex was detected by incubation with horseradish peroxidase-linked secondary antibody (1:2000) (Amersham Pharmacia Biotech, Inc.) for 1 hr. Immunoreactive bands were detected using a chemiluminescence kit (ECL + Plus, Amersham Pharmacia Biotech, Inc.), exposed to Bio-Max MR-2 film (Sigma Chemical Co.) for 5 min, and quantified by densitometry (Shimadzu CS-9000, Shimadzu Corp.).

Sodium-dependent TC uptake was assayed by a rapid filtration technique as previously described [30]. Briefly, a 20-μL aliquot of BBMV suspension (40–60 μg protein), prepared from proximal and distal regions of the ileum, was preincubated for 2 min at 37° in a water bath. Uptake was initiated by the addition of 80 μL of incubation solution (125 mM NaCl or KCl, 50 mM mannitol, 10 mM HEPES-Tris, pH 7.5, 0.2 mM $CaCl_2$, and 1 mM $MgCl_2$) containing [^{14}C]TC. Unlabeled TC was added to the incubation medium to reach the desired, final BS concentration (10, 25, 50, 125, and 250 μM). Uptake was halted at different intervals by the addition of 3 mL of ice-cold stop solution (100 mM NaCl or KCl, 100 mM mannitol, 10 mM HEPES-Tris, pH 7.5). The samples were immediately filtered through a 0.45 μm Millipore filter (Millipore), and the filter was washed with additional stop solution containing a 1 mM concentration of unlabeled TC. The filters were dissolved in 5 mL of OptiPhase "Hisafe" 3 (Wallac Scintillation Products), and radioactivity was assessed in a liquid scintillation counter (Rack Beta 1214, Pharmacia). All determinations were performed routinely in duplicate. Na^+-dependent uptake of TC was calculated as the difference between the uptake in the presence and absence of a Na^+ gradient. K_m and V_{max} were calculated by a nonlinear curve-fitting algorithm for Na^+-dependent TC uptake.

2.6. Statistical analysis

Results are expressed as means ± SD. Means of two groups were compared with Student's *t*-test, after testing the equality of variances with an *F*-test. Multiple means were compared by using one-way ANOVA followed by the Newman–Keuls test for pairwise comparisons. *P* values lower than 0.05 were judged to be significant.

3. Results

3.1. Basal bile flow, serum biochemical markers, and liver to body weight ratio

Fig. 1 shows the effect of DDS administration on basal bile flow in male rats. DDS, administered at two daily doses of 30 mg/kg body wt for 4 days, induced a decrease of bile flow of about 20%, with respect to controls. A similar cholestatic effect was observed in female rats receiving DDS at the same dose (data not shown). Consequently, the dose of 30 mg of DDS/kg body wt, administered twice a day for 4 days, was selected to analyze the effect of the drug on bile secretory function in animals from both sexes. DDS, at this dose, did not increase serum biochemical markers of hepatocellular injury, i.e. ASAT, ALAT, and ALP, in either male or female rats (data not shown). DDS administration induced a decrease in body weight in both males and females (Table 1). Liver weight was decreased only in male rats, so that no change occurred in the liver-to-body weight ratio in these animals.

3.2. DDS-induced methemoglobinemia

It has been demonstrated previously that DDS induces a marked increase in methemoglobinemia in male rats but not in female rats [11], a differential effect associated with the gender-dependent rate of synthesis of the *N*-hydroxylated derivative of DDS [10]. Concordingly, we observed that the percentage of methemoglobin in blood was increased by DDS far more in male rats (from 2.0 ± 0.2 to $38.1 \pm 5.4\%$, $N = 4$, $P < 0.05$) than in female rats (from 3.9 ± 0.3 to $5.9 \pm 1.4\%$, $N = 4$, $P < 0.05$).

3.3. BS secretion under basal conditions

As shown in Table 2, treatment of the animals with DDS induced a decrease in total BS output in both male and female rats (−30 and −37%, respectively). A careful

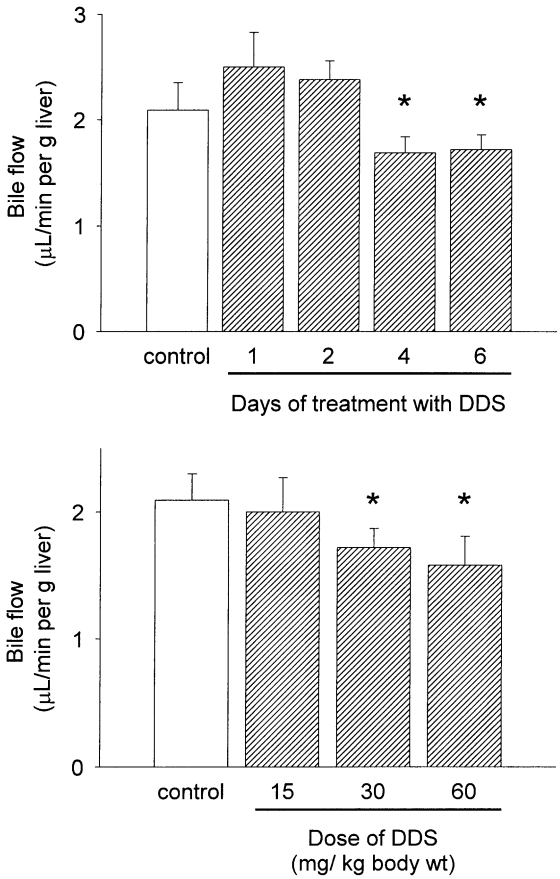

Fig. 1. Effect of DDS on basal bile flow. Basal bile flow was assessed in male rats following the administration of two daily, i.p. doses of 30 mg/kg body wt of DDS for 1, 2, 4, or 6 days (upper panel). The lower panel shows the basal bile flow evaluated after administration of different doses of DDS (15, 30, or 60 mg/kg body wt) twice daily for 4 days. The corresponding controls received only the vehicle (open bar). Because there was no differences among the groups treated with different doses of the vehicle or with the same dose of the vehicle for different time periods, the data on bile flow were all included in the same experimental group. Values are expressed as means ± SD of 12 control animals, and 4 animals in each DDS group. Key: (∗) significantly different from the control group ($P < 0.05$).

analysis of the basal biliary output of individual BS in male and female rats revealed that the decrease in total BS output induced by DDS was accounted for mainly by the primary BS, β-MC, although lower outputs of UDC and CDC also were recorded. A significant decrease in the secondary BS, DC, and an increase in HDC was observed only in male rats.

3.4. SRm studies

As can be seen in Fig. 2, the *SR*m of the model BS, TUDC, was not influenced by DDS pretreatment in male rats (772 ± 154 and 756 ± 168 nmol/min per g liver for DDS-treated and control rats, respectively, $N = 4$) or in female rats (558 ± 75 and 580 ± 84 nmol/min per g liver for DDS-treated and control rats, respectively, $N = 4$).

3.5. Pool size and de novo synthesis of BS

The size of the endogenous BS pool was decreased by 34% in male rats and by 32% in female rats after DDS administration (Table 3). In control rats, the mean number of enterohepatic cycles of the BS pool per day was similar between males (8.3 ± 1.6, $N = 4$) and females (8.7 ± 1.0, $N = 4$). DDS administration did not affect this parameter in either male or female rats (data not shown). The relative contribution of each individual BS to the total BS pool was assessed by HPLC. The data, summarized in Table 3, indicate that the decrease of the size of the BS pool induced by DDS was due to a selective decrease in β-MC and UDC. Consequently, the HI of the BS pool of male and female rats increased (about 40% on the average over the respective controls). BS output after 8 hr of BS pool depletion, a parameter which reflects *de novo* BS synthesis rate [21], was decreased by DDS in males (from 11.8 ± 2.1 to 7.8 ± 0.8 nmol/min per g liver, $P < 0.05$, $N = 4$) and in females (from 8.6 ± 1.0 to 5.3 ± 0.9 nmol/min per g liver, $P < 0.05$, $N = 4$).

3.6. Expression of ASBT protein and TC transport in ileal BBMV

To explore the possibility that DDS affects the BS pool size by affecting ileal BS absorption, we studied ASBT protein expression and TC transport activity in BBMV;

Table 1
Effect of dapsone (DDS) treatment on body and liver weights

	Initial body wt (g)	Final body wt (g)	Change in body wt (g)	Liver wt (g)	Final liver/body wt ratio (%)
Male					
Control	298 ± 11	306 ± 10	7 ± 3	13.2 ± 2.2	4.3 ± 0.4
DDS	300 ± 9	278 ± 10*	−22 ± 8*	12.0 ± 1.2*	4.3 ± 0.4
Female					
Control	229 ± 9	228 ± 7	−2 ± 5	8.9 ± 1.0	3.9 ± 0.4
DDS	228 ± 8	204 ± 12*	−24 ± 11*	8.9 ± 0.4	4.4 ± 0.3*

Animals received two daily, i.p. doses of 30 mg/kg body wt of DDS for 4 days, or only vehicle in the controls. Results are the means ± SD for 12–15 experiments.

* Significantly different from the control group ($P < 0.05$).

Table 2
Effect of DDS on basal bile flow and BS output

	Bile flow (μL/min per g liver wt)	Total BS output (nmol/min per g liver wt)	Individual BS output (nmol/min per g liver wt)					
			β-MC	UDC	HDC	C	CDC	DC
Male								
Control	2.01 ± 0.25	64.4 ± 9.2	34.6 ± 4.9	5.0 ± 07	2.7 ± 0.4	15.0 ± 2.1	3.1 ± 0.5	4.1 ± 0.6
DDS	1.62 ± 0.14	45.3 ± 5.4	15.6 ± 1.9*	2.3 ± 0.3*	4.8 ± 0.6*	17.5 ± 2.1	2.2 ± 0.3*	2.9 ± 0.4*
Female								
Control	1.98 ± 0.21	57.0 ± 7.4	26.1 ± 3.4	3.0 ± 0.4	3.7 ± 0.5	16.3 ± 2.1	5.8 ± 0.7	2.0 ± 0.3
DDS	1.60 ± 0.14	35.6 ± 5.6	11.3 ± 1.7*	1.6 ± 0.2*	3.5 ± 0.6	13.8 ± 2.1	3.8 ± 0.6*	1.5 ± 0.3

Bile flow and BS output were measured in basal bile of animals treated with two daily, i.p. doses of DDS (30 mg/kg body wt) for 4 days, or in control animals receiving only the vehicle. Abbreviations: β-MC: β-muricholate; UDC: ursodeoxycholate; HDC: hyodeoxycholate; C: cholate, CDC: chenodeoxycholate; and DC: deoxycholate. Results are the means ± SD for 6 experiments.

* Significantly different from the control group ($P < 0.05$).

these parameters estimate the density and the activity of sodium-dependent TC transporters located at the apical membrane of the enterocyte. The results indicate that DDS treatment did not affect ASBT protein levels in either male (Fig. 3) or female (data not shown) rats. The effect of DDS administration on [^{14}C]TC uptake by BBMV prepared from proximal and distal regions of the ileum in male rats is shown in Fig. 4. Neither the V_{max} nor the K_m constants were affected by DDS treatment. Similar results were obtained with female rats (data not shown).

4. Discussion

In this study, we examined several effects induced by DDS on bile secretory function in male and female rats. We

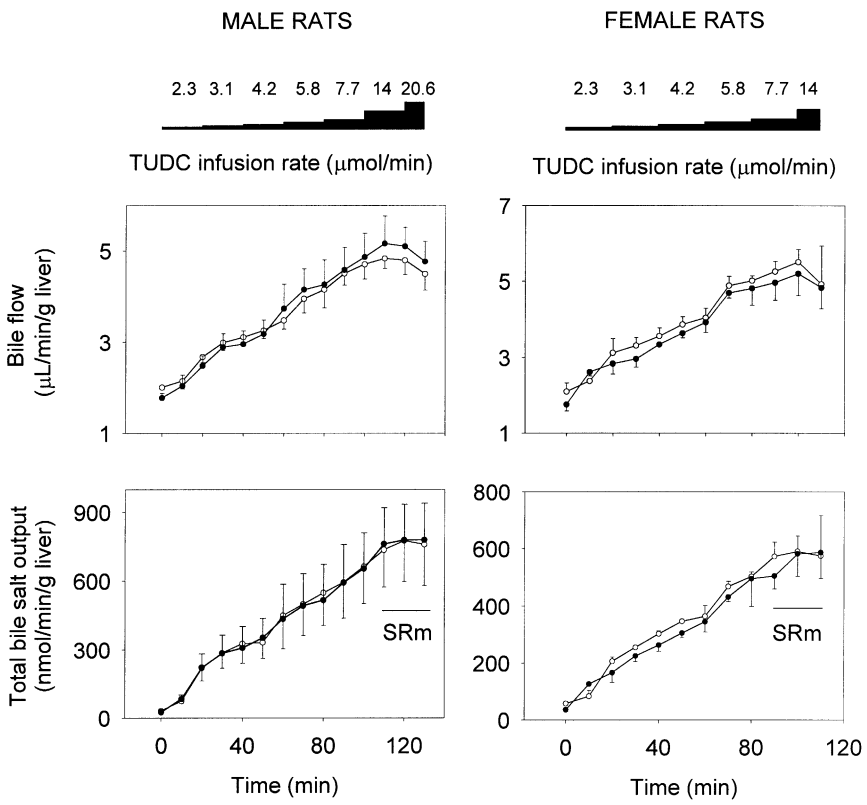

Fig. 2. Effect of DDS on the *SR*m of TUDC. Shown are sequential changes in bile flow and total BS output in male (left panel) and female (right panel) rats treated with DDS (30 mg/kg body wt) twice a day, for 4 days (●), or in control rats receiving only the vehicle (○). Animals were infused i.v. with TUDC at stepwise-increasing rates (2.3, 3.1, 4.2, 5.8, 7.7, 14, and 20.6 μmol/min). *SR*m of TUDC, calculated as the mean of the three highest, consecutive secretory rates, turned out to be 772 ± 154 and 756 ± 168 nmol/min per g liver for DDS-treated and control male rats, respectively, and 558 ± 75 and 580 ± 84 nmol/min per g liver for DDS-treated and control female rats, respectively. DDS did not affect the *SR*m of TUDC in any case. All values are means ± SD of 4 experiments per group.

Table 3
Effect of DDS on endogenous BS pool size, composition, and hydrophobicity index (HI)

	BS pool size (μmol/kg body wt)	Individual BS amount in the BS pool (μmol/kg body wt)						HI
		β-MC	UDC	HDC	C	CDC	DC	
Male								
Control	333 ± 64	180.6 ± 26.6	22.8 ± 13.6	14.2 ± 3.5	80.4 ± 17.2	15.6 ± 14.5	18.6 ± 10.1	−0.42 ± 0.09
DDS	218 ± 38*	72.0 ± 9.9*	10.6 ± 3.3*	21.1 ± 7.5	89.9 ± 18.6	10.1 ± 2.9	13.4 ± 5.3	−0.26 ± 0.05*
Female								
Control	272 ± 28	124.6 ± 17.0	14.6 ± 4.1	17.8 ± 2.9	77.8 ± 14.2	27.6 ± 7.9	9.6 ± 3.0	−0.35 ± 0.04
DDS	186 ± 30*	59.0 ± 8.2*	8.7 ± 2.0*	18.7 ± 8.7	72.0 ± 9.7	19.5 ± 9.5	8.0 ± 6.5	−0.21 ± 0.07*

BS pool size, composition, and HI were measured in bile-drained animals pretreated with two daily, i.p. doses of DDS (30 mg/kg body wt) for 4 days, with the vehicle in the controls. Abbreviations: β-MC: β-muricholate; UDC: ursodeoxycholate; HDC: hyodeoxycholate; C: cholate; CDC: chenodeoxycholate; and DC: deoxycholate. Results are the means ± SD for 4 experiments.

* Significantly different from the control group ($P < 0.05$).

observed an impairment in bile flow and BS secretion associated with the alteration of the BS pool. We complemented the study by performing experiments aimed to assess whether decreased ileal absorption of BS may account for the decrease in the size of the BS pool. The potential relationship between hematologic toxicity of DDS and the altered bile secretory function was also evaluated.

Previous reports analyzing the toxicity of DDS, e.g. hematologic toxicity, have been conducted mainly in the rat as an experimental model. The rat was also found to be an appropriate model for the study of DDS efficacy in the treatment of *Pneumocystis carinii* infections [31]. In these studies, DDS was administered at a dose of 25 mg/kg body wt per day, with time periods of administration varying from one to several days. In preliminary experiments, we tested the effect of different protocols of DDS administration on basal bile flow, according to the range of dosage described previously. Based on these results (see Fig. 1), which were similar for both male and female rats, we decided to study the effect of DDS on bile secretory function, using the dose of 30 mg/kg body wt, twice a day, for 4 days.

We observed that no significant changes in plasma activity of ASAT, ALAT, or ALP occurred in response to DDS treatment, despite a significant reduction in bile flow in DDS-treated animals. These results suggest that, whereas DDS has little or no impact on liver integrity in this experimental setup, the drug induces a pure cholestatic, i.e. nonhepatotoxic, effect. Although ALP is commonly regarded as a marker of cholestasis, its increased level in plasma in cholestatic diseases is associated with hepatocellular BS accumulation, which produces an induction of intracellular ALP synthesis [32]. The dissociation between the cholestatic effect of DDS and the lack of increase of plasma ALP levels is somewhat expected, since

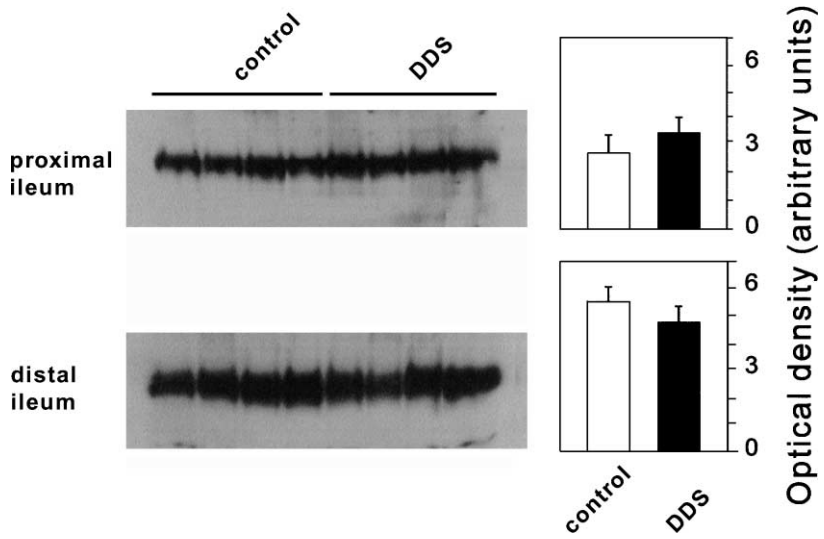

Fig. 3. Effect of DDS on ASBT expression in ileal BBMV. The expression of ASBT was evaluated in male rats treated with DDS (30 mg/kg body wt) twice a day for 4 days, or in control rats receiving only the vehicle. Western blot analysis of ASBT expression was performed in proximal and distal ileum. The transporter was present exclusively as a monomeric form. Data on densitometry represent means ± SD of BBMV prepared from 4 male rats per group. DDS did not affect the ASBT expression determined by densitometry. Similarly, DDS did not affect the expression of ASBT in female rats (data not shown).

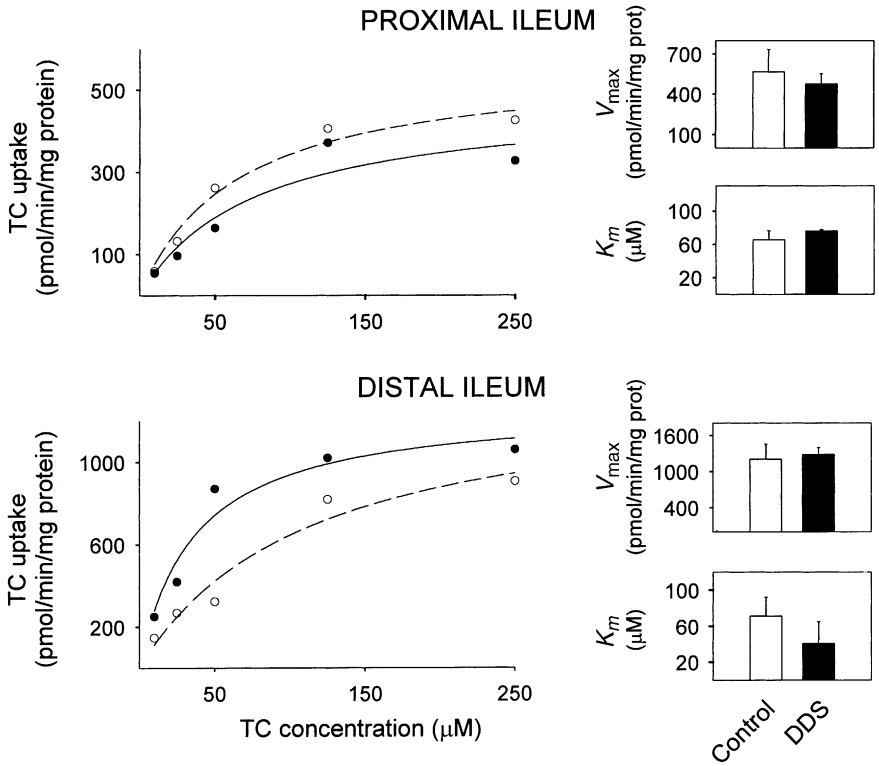

Fig. 4. Effect of DDS on the uptake of [^{14}C]TC by ileal BBMV. The uptake of [^{14}C]TC in ileal BBMV was evaluated in male rats treated with DDS (30 mg/kg body wt) twice a day for 4 days (●), or in control rats receiving only the vehicle (○). [^{14}C]TC uptake was determined in BBMV by rapid filtration methodology in proximal and distal ileum. [^{14}C]TC concentration in the incubation medium ranged from 10 to 250 μM. Carrier-mediated [^{14}C]TC uptake was calculated as the difference between the Na^+ and K^+ (passive diffusion) gradients. The Michaelis–Menten constants V_{max} and K_m were determined for initial [^{14}C]TC uptake velocity (i.e. following a 15-sec incubation period). Data on [^{14}C]TC uptake kinetics represent mean values of 3 experiments per group (left panel). Data on V_{max} and K_m represent means ± SD of 3 experiments per group (right panel). DDS treatment did not affect the Michaelis–Menten constants. Similar experiments performed with female animals also showed no difference between the DDS and control groups (data not shown).

a decrease rather than an increase in BS hepatocellular level likely occurs as the result of the decrease in the overall BS pool induced by DDS treatment (see Table 3). Moreover, there was no difference in the frequency of enterohepatic circulation of the BS pool in rats from either sex, as expected from the finding that the impairments in BS output and BS pool size induced by DDS were of a similar magnitude. The parallel change in these parameters strongly suggests that the decrease in the size of the BS pool accounts for the impairment in the basal BS secretory rate and, consequently, in the BS-dependent bile flow. We also observed that DDS induced a decrease in biliary BS output measured under BS pool depletion conditions, a parameter that reflects the *de novo* BS synthesis rate [20]. Consequently, the decrease in the BS pool size may be explained at least partially by a decrease in BS synthesis. To confirm the contention that BS pool depletion was the only factor involved in the reduction of basal BS output, we performed *SR*m experiments to evaluate the influence of the drug on the maximum canalicular transport of BS. This transport step is mediated mainly by the bile salt export pump, and represents the rate-limiting step in the handling of BS by the liver [33]. Since *SR*m for the model BS, TUDC, was not affected by DDS in rats of either sex (see Fig. 2), it is unlikely that an alteration in the number of canalicular transporters of BS accounts for the impairment in the biliary secretory function induced by the drug.

Because active transport of BS by the distal ileum is a key mechanism for conservation of the BS pool, it was also of interest to evaluate the effect of DDS on the ability of the small intestine to absorb BS. This transport is mediated by the apical Na^+-dependent transport system in the ileum, i.e. ASBT [34]. We observed that DDS did not affect ASBT expression or activity, in either male (see Figs. 3 and 4, respectively) or female (data not shown) rats. Taken together with the findings on liver transport and metabolism of BS, this result indicates that impairment in BS synthesis induced by DDS is the main, if not the only, factor affecting the BS pool size in animals receiving the drug.

When individual decrements of the amount of each BS in the BS pool are considered (see Table 3), it becomes apparent that a decreased content of β-MC and UDC, two BS derived from CDC, had occurred. As a result, the CDC group (β-MC + UDC + HDC + CDC)/cholate (C) group (C + DC) ratio in the BS pool decreased from 2.36 to 1.10 in male rats, and from 2.11 to 1.32 in female rats, suggesting a preferential inhibition of the CDC synthetic pathway

over that of C. Therefore, it is unlikely that the microsomal enzyme cholesterol 7α-hydroxylase, the initial, rate-limiting enzyme controlling the overall (C plus CDC) BS synthesis mediated by the classical pathway [35,36], is affected by DDS. Inhibition of the alternative biosynthetic pathway of CDC involving oxidation of the side chain prior to changes in the steroid nucleus cannot be excluded, although the quantitative importance of this pathway to the overall BS synthesis is thought to be minor in the rat [36].

Whereas DDS treatment did not lead to a modification of CDC content in the BS pool, the BS derived from its further conversion to more hydrophilic compounds via 6β-hydroxylation, i.e. β-MC [35], was decreased considerably (−60 and −53% in male and female rats, respectively). This suggests that 6β-hydroxylating activity is inhibited by DDS. The content of UDC, a BS that can be formed either in the liver (from cholesterol) or in the intestine (by bacterial 7α-hydroxysteroid dehydrogenation of CDC and further hepatic reduction of the 7-oxo acid [35,37]), was also decreased (−54 and −40% in male and female rats, respectively). It is known that the constitutive enzymes CYP2C6 and/or CYP2C11, as well as nonconstitutive CYP3A1, are capable of catalyzing the hydroxylation of DDS and the acetylated metabolite [38,39] and that 6β-hydroxylating activity is mediated by isoforms of family 3 of the P450 gene superfamily [40]. Therefore, it is possible that the decrease in 6β-hydroxylation of bile acids induced by DDS results from a direct inhibition of a CYP3A isoform by the drug.

Most of the studies reporting adverse reactions of DDS and other sulfonamides such as sulfamethoxazole support the hypothesis that bioactivation is critical in the pathogenesis of these reactions. For example, oxidative metabolism produces reactive hydroxylamine metabolites that are cytotoxic, and able to bind to cellular macromolecules [41]. Studies with DDS link hydroxylamine formation to the development of hemotoxicity [42] and idiosyncratic blood dyscrasias [43]. The *N*-hydroxylated derivative of sulfamethoxazole was demonstrated to induce *in vitro* cytotoxicity toward peripheral blood mononuclear cells, which correlates well with the development of sulfonamide-induced hypersensitivity reactions [41]. We observed that administration of DDS to male Wistar rats resulted in marked methemoglobinemia, in agreement with previous studies [38]. Methemoglobin formation is a consequence of the *N*-hydroxylation of DDS, which has been established to be toxic toward erythrocytes [44]. DDS causes only a slight methemoglobinemia in female rats when compared to males, agreeing well with a previous study in which a very low synthesis of the hydroxylamine derivative of the drug in female animals was also reported [11]. This sex difference in the bioactivation of DDS in the rat has been ascribed to the sex-dependent expression of the cytochrome P450 enzymes, and in particular to CYP2C11 and CYP3A1 [38], which are expressed only in male rats [45]. Interestingly, in the current study, we observed that DDS impaired bile secretory function in male and female rats in a similar way, thus supporting a role for intact DDS on bile acid metabolism, through an action that is likely independent of the formation of the *N*-hydroxylated derivative.

In summary, our results indicate that DDS administration to male and female rats selectively inhibits BS excretion by reducing the endogenous BS pool, which is most likely due to a decreased synthesis of BS via the CDC, classical pathway; decreased BS output accounts for the cholestatic effect of the drug. These effects are unlikely to be associated with its hematotoxic side-effect induced by its *N*-hydroxylated derivative.

Human hepatic CYP3A4 was thought previously to be the major isoform involved in the hydroxylation of DDS [46]. The authors proposed DDS as a noninvasive *in vivo* probe of CYP3A4. However, recent evidence indicates that more than one cytochrome P450 enzyme is involved in DDS hydroxylamine formation using human liver microsomes [47]. Pretreatment with ketoconazole, an inhibitor of CYP3A, had no effect on DDS hydroxylation in human subjects, indicating that CYP3A may not be involved significantly in DDS hydroxylation *in vivo* [48]. Although CYP3A is not identical in the rat and human, it would be of interest to determine whether DDS may also induce alterations in BS secretion and metabolism in humans, as a potential mechanism responsible for the hepatic dysfunction reported in susceptible patients.

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