Role of bile salt hydrophobicity in hepatic microtubule-dependent bile salt secretion

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Abstract Under basal conditions, bile salt secretion by the liver is not affected by microtubule disruption. However, when a bile salt load is imposed on the liver, a microtubule-dependent secretion mechanism is recruited (*J. Lipid Res.* 1988. **29:** 144–156). We tested the hypothesis that recruitment of this microtubule-dependent mechanism is influenced by the relative hydrophobicity of the bile salts being secreted. Intact male rats were depleted of bile salts by overnight biliary diversion, pretreated with colchicine (a microtubule inhibitor) or its inactive isomer, lumicolchicine (control), and reinfused intravenously with bile salts of increasing hydrophobicity (taurodehydrocholate < tauroursodeoxycholate < taurocholate) at 200 nmol/min • 100 g. After 45 min, when steady-state bile salt secretion was achieved, tracer [^{3}H]taurocholate was administered intravenously. The colchicine-insensitive component of bulk bile salt secretion was constant at ~ 130 nmol/min • 100 g, and the colchicine-sensitive component increased from ~ 0 to 35 and 60 nmol/min • 100 g, respectively, with reinfusion of the more hydrophobic bile salts. Retained bile salts accumulated in the liver and serum and were detectable in urine. Peak biliary secretion of [^{3}H]taurocholate in control animals increased linearly from 15.3 to 18.0% administered dose/min with increasing hydrophobicity of the secreted bile salts ($P < 0.002$). In colchicine-pretreated animals, peak secretion rates decreased linearly from 13.8 to 9.2%/min ($P < 0.001$), with maximal inhibition in taurocholate-reinfused animals ($P < 0.01$). Utilization of a microtubule-dependent secretion mechanism increases with increasing bile salt hydrophobicity. This mechanism permits more efficient hepatic secretion of bile salts, but increases the susceptibility of bile salt secretion to microtubule disruption. We postulate that microtubule-dependent insertion of bile salt transporters into the canalicular membrane underlies the enhanced bile salt secretion observed when a bile salt load is imposed upon the liver.—**Crawford, J. M., D. C. J. Strahs, A. R. Crawford, and S. Barnes.** Role of bile salt hydrophobicity in hepatic microtubule-dependent bile salt secretion. *J. Lipid Res.* 1994. **35:** 1738–1748.

Supplementary key words liver • canaliculus • ursodeoxycholic acid • rat

The substantial flux of bile salts through the liver as part of the enterohepatic circulation provides a major driving force for bile formation, and promotes the secretion of phospholipid and cholesterol into bile (1). Abnormalities in bile salt secretion can exacerbate liver dysfunction in cholestatic disorders (2), but may be partially rectified by pharmacologic alterations in the composition of the bile salt pool (3). In particular, ursodeoxycholic acid treatment has been found to ameliorate many effects of cholestatic disorders, and may even delay the progression of disease (4, 5). While the basis of protection appears to be related to the decreased hydrophobicity of ursodeoxycholic acid and its conjugates relative to endogenous bile acids, a clear explanation of the mechanism(s) by which UDCA improves liver function has not emerged.

We have demonstrated that exposure of the liver to increased levels of bile salts recruits a microtubule-dependent mechanism for efficient secretion of the bile salt load (6, 7). In this study, we have examined the hypothesis that recruitment of this microtubule-dependent mechanism is dependent, in part, on the relative hydrophobicity of the bile salts being secreted. Experiments were conducted utilizing a bile salt "depleted/reinfused" rat model, which permits the replacement of the endogenous bile salt pool by the infusion of bile salts of increasing hydrophobicity: taurodehydrocholate, tauroursodeoxycholate, and taurocholate (6). Under these experimental conditions, we demonstrate that microtubule-dependent bile salt stimulation of hepatic bile salt secretion is more efficient as the hydrophobicity of the infused bile salt increases. Marked cholestatic toxicity is encountered during infusion of the

Abbreviations: HPLC, high performance liquid chromatography; HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; DMSO, dimethylsulfoxide; TDHC, taurodehydrocholate ([3,7,12-triketo-5β-cholan-24-oyl]-2-aminoethanesulfonic acid); TUDC, tauroursodeoxycholate ([3α,7β-trihydroxy-5β-cholan-24-oyl]-2-aminoethanesulfonic acid); TC, taurocholate ([3α,7α,12α-trihydroxy-5β-cholan-24-oyl]-2-aminoethanesulfonic acid); TCDC, taurochenodeoxycholate ([3α,7α-trihydroxy-5β-cholan-24-oyl]-2-aminoethanesulfonic acid); [^{3}H]TC, [^{3}H]taurocholate; DMSO, dimethylsulfoxide.

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hydrophobic bile salt, taurochenodeoxycholate, when microtubule function is disrupted. Our findings suggest that the beneficial effects of treatment with hydrophilic bile salts may be explained, in part, by a favorable balance between their stimulation of hepatocellular bile salt secretion into bile and their minimal-to-absent hepatotoxicity.

MATERIALS AND METHODS

Chemical reagents

Colchicine and lumicolchicine were obtained from Sigma Chemical Co., St. Louis, MO. The sodium salts of taurodehydrocholate (TDHC), tauroursodeoxycholate (TUDC), taurocholate (TC), and taurochenodeoxycholate (TCDC) were purchased from Calbiochem, La Jolla, CA. The purity of all bile salts exceeded 99% by high-performance liquid chromatography (HPLC). [^{3}H]taurocholate (2.1 mCi/μmol) and [^{3}H]colchicine (77.4 mCi/μmol) were obtained from New England Nuclear, Boston, MA. 3α-Hydroxysteroid dehydrogenase used in the bile salt assay was obtained from Worthington Diagnostic Systems, Inc., Freehold, NJ. All other reagents were of the highest analytical grade available.

Preparation of animals

Male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing 306 ± 17 g (mean ± SD; n = 56) were maintained on Purina rat chow and water ad libitum. Surgery was performed between noon and 2:00 PM under light ether anesthesia, and was consistently less than 30 min in duration. The left jugular vein was cannulated with a 46-cm length of PE-10 polyethylene tubing (Clay Adams Division, Beckton, Dickinson and Co., Parsippany, NJ) and an intravenous infusion of 0.15 M NaCl at 0.15 ml/h was begun. The common bile duct was cannulated with a 19-cm length of PE-10 tubing. After surgery, animals were placed in restraining cages, warmed under heating lamps, and allowed access to rat chow and water ad libitum.

The rats were subjected to biliary diversion overnight, and experiments were begun the following day, 22 h after surgery. Colchicine or its inactive isomer, lumicolchicine, were administered i.v. via the jugular cannula (0.12 mg/100 g body weight, in 0.15 M NaCl containing 1.5% DMSO, v/v). DMSO (0.075 ml) was used to solubilize lumicolchicine and colchicine (3 mg) prior to addition of 0.15 M NaCl (5 ml); preliminary studies demonstrated no effect of this low concentration of DMSO on biliary function. Two h after colchicine/lumicolchicine administration, a loading dose of 2000 nmol/100 g of a selected bile salt was administered as a bolus i.v., followed immediately by continuous infusion of the same bile salt at 200 nmol/min • 100 g in 0.15 M NaCl at 1.5 ml/h. After a further 45 min (2.75 h after colchicine/lumicolchicine administration), a bolus tracer dose of [^{3}H]taurocholate ([^{3}H]TC; 3 nmol in 0.25 ml 0.15 M NaCl; 0.15–0.25 μCi) was administered i.v. (t = 0 min). Bile collection continued for an additional 2 h. Previous studies on similarly prepared animals have shown no effect of colchicine pretreatment on the uptake of [^{3}H]taurocholate from plasma, during unlabeled TC reinfusion (7). In addition, there is no significant hemolysis during reinfusion of TDHC, TUDC, or TC in either lumicolchicine- or colchicine-pretreated animals, when measured by release of hemoglobin into the serum (8; data not shown). At the end of selected experiments, animals were anesthetized (pentobarbital, i.p., 50 mg/kg) for harvesting of urinary bladder urine, aortic blood, and the liver. Such specimens were stored at −20°C and analyzed for bile salt content within 2 weeks.

Analytical methods

Bile was collected sequentially in tared tubes and bile flow was determined gravimetrically, assuming a specific gravity of 1.0. Samples were stored at −20°C overnight prior to measurement of radioactivity, bile salt, and phospholipid content. Total 3α-hydroxy-bile salt output was assayed using 3α-hydroxysteroid dehydrogenase, using TC as the standard (9). Control studies demonstrated that TC, TUDC, and the taurine conjugate of 3α-hydroxy-7-keto-5β-cholanoic acid were detected with equal efficiency in this assay. Phospholipid was assayed as previously described (6). Aliquots (5 μl) of bile samples and the injected radiolabel solution were counted in 5 ml ReadySafe liquid scintillation cocktail (Beckman Instruments, Inc., Fullerton, CA), using a Beckman LS5000 liquid scintillation spectrometer. Internal standards were calibrated with [^{3}H]toluene as an external standard to enable correction of counts per minute to disintegrations per minute.

For routine analysis of the bile salt composition of bile, high-performance liquid chromatography (HPLC) was performed (10), using a Beckman model 330 HPLC system with an isocratic 10 mM KH_2PO_4 methanol–water (75:25, v/v, pH 5.35) buffer (flow rate 0.7 ml/min), and an Altex Ultrasphere ODS column, 250 × 4.6 mm (Altex, Palo Alto, CA) with octadecylsilane 5 μm silica particles as the stationary phase. Eluted bile salts were detected in an on-line spectrophotometer by their absorbance at 340 nm and compared with known standards for identification. The eluant was collected in 0.5 min fractions and radioactivity of 0.25 ml aliquots was determined as described above. For each bile salt, a "hydrophobicity index" (HI) was computed as described by Heuman (11). As bile samples from animals infused with TDHC contained more hydrophobic hydroxylated metabolites, the HI for the mixture was computed from the weighted values of the eluted peaks (11).

Detailed analysis of bile, liver, serum, and urine samples for bile salt composition was performed as follows.

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Bile (10 μl), serum (1 ml), and urine (0.2–1.0 ml) samples were diluted tenfold with water prior to extraction. Frozen liver samples were freeze-dried, ground, and the resulting powder was extracted with water (10 ml) in a tumbling mixer. Solids were removed by centrifugation at 2,500 *g* for 10 min. Bile acids were extracted from each of the samples by passage over an activated Sep Pak C_{18} cartridge equilibrated with water. After washing the cartridge with water (10 ml), the bile acids were eluted with methanol (2 × 2 ml). The methanol extracts were evaporated to dryness under N_2 and reconstituted in 80% aqueous methanol.

The bile acids were analyzed by gradient elution HPLC on a 10 cm × 2.1 mm i.d. C_8 reversed-phase column at a flow rate of 0.2 ml/min. The gradient was 30–99% methanol in a background of 1% (v/v) acetic acid over 10 min, followed by 99% methanol–1% acetic acid for a further 2 min. The HPLC eluate was split 1:1 with 0.1 ml/min being passed into the IonSpray™ interface of a PE-Sciex API III mass spectrometer operating in the negative ion mode. The negative ions entering the mass spectrometer were scanned from *m/z* values of 300 to 700. Reconstructed selected ion chromatograms were created from the overall data set using the manufacturer's software package. Quantitative data were derived by comparing the area of individual peaks in the unknown samples with those in a standard curve obtained by injecting known amounts of the taurine and glycine conjugates of cholic acid, ursodeoxycholic acid, chenodeoxycholic acid, deoxycholic acid, dehydrocholic acid, and β-muricholic acid, yielding a sensitivity down to 10 pmol. Only the linear parts of the standard curves were used for these calculations. Samples were diluted as necessary to bring them into this range.

Data presentation and statistical analysis

The data consisted of sequential measurements of *a)* biliary secretion indices (bile flow and rates of bile salt and phospholipid secretion per 100 g body weight); and *b)* radiolabel secretion as a function of time after [³H]taurocholate injection. All measurements are expressed as mean ± SD. Biliary secretion indices are presented in abbreviated form for clarity of analysis. Specifically, steady-state values for bile flow and bile salt and phospholipid secretion for the 2-h interval immediately after colchicine or lumicolchicine pretreatment are denoted as "D" (depleted; see Fig. 1). Steady-state values achieved during the 45 min immediately after unlabeled bile salt reinfusion are denoted as "R," for the 30 min after [³H]taurocholate injection as "I," and for the 30 min at the end of the experiment as "E." These results are shown in Fig. 2.

The biliary secretion curves for ³H radiolabel (Fig. 3) consisted of a steep rise followed by a gradual fall to zero, and can be characterized by the maximal secretion rate (% of the administered dose secreted per min), time to achieve maximal output (T_{max}; min), and cumulative output (% of administered dose). These parameters were calculated for each rat in each experimental group, and were compared using one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple range tests for pairwise comparisons (12). The parameters and the results of comparison testing are shown in Table 1.

RESULTS

Experimental protocol

Figure 1 shows the effect of overnight biliary diversion on bile salt secretion rate and the changes subsequent to colchicine or lumicolchicine pretreatment and taurocholate (TC) reinfusion in a preliminary set of animals. Bile salt secretion remained at initial levels of ~250 nmol/min • 100 g for 3 h, and then declined to levels less than 10% of initial values by 20 h. The size of the bile salt pool (43 ± 5 μmol/100 g, calculated according to Hardison et al., 13) is comparable to reported values of 43–47 μmol/100 g for adult male rats (13, 14). There was no change in the low levels of bile salt secretion after colchicine or lumicolchicine pretreatment. Upon reinfusion of TC, steady-state output was achieved by the second 15-min interval and was maintained for the remainder of the experimental period. In colchicine-pretreated animals, bile salt output reached only 60% of that in control animals.

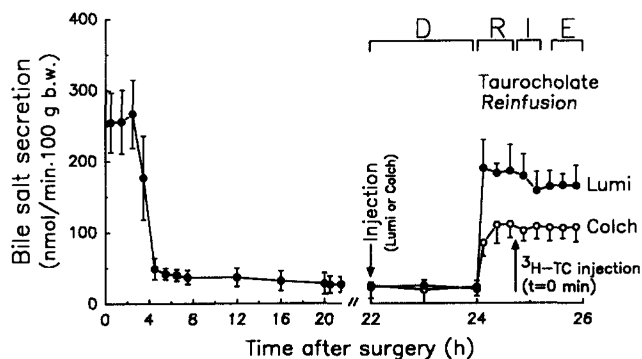

Fig. 1. Bile salt secretion during overnight biliary diversion and reinfusion of taurocholate. Intact rats were surgically fitted with biliary and i.v. jugular catheters (see Materials and Methods). Twenty-two hours after surgery, colchicine (○) or its inactive isomer, lumicolchicine (●, 0.12 mg/100 g body weight) was injected i.v., followed 2 h later by reinfusion of taurocholate at 200 nmol/min • 100 g body weight (with a loading dose of 2000 nmol/100 g at the start of reinfusion). Bile salt secretion rate (nmol/min • 100 g) is shown. In the experiments to follow, tracer [³H]taurocholate was injected 45 min after initiation of bile salt reinfusion, and biliary secretion of radiolabel monitored over the subsequent 2 h. The letters D, R, I and E denote the intervals Depleted, Reinfused, Injection, and End, for which biliary secretion indices are expressed in Fig. 2. Lumi, lumicolchicine; Colch, colchicine, ³H-TC, [³H]taurocholate. Mean ± SD, n = 4 for each group.

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As measurement of bulk bile salt secretion is a relatively coarse indicator of hepatocyte secretory function, our protocol included monitoring the biliary secretion of [^{3}H]TC (3 nmol) injected i.v. during the reinfusion period. This maneuver permits evaluation of the transhepatic transport of a tracer dose of bile salt on a minute-to-minute basis (6, 7, 15). Figure 1 shows the time at which tracer [^{3}H]taurocholate was administered in subsequent experiments, 45 min after initiation of bile salt reinfusion.

Biliary secretion indices

The bile salts selected for reinfusion in this study were a series of taurine-conjugated cholyl-derivatives: the synthetic triketo taurodehydrocholate (TDHC), dihydroxy tauroursodeoxycholate (TUDC), and trihydroxy taurocholate (TC), listed in increasing order of hydrophobicity, as measured by reversed-phase HPLC (see Methods). The compiled biliary secretion indices for animals infused with these bile salts are shown in **Fig. 2** for the four intervals ("D," "R," "I," and "E") denoted in Fig. 1. Bile flow increased in all lumicolchicine-pretreated animals upon reinfusion of bile salt. Colchicine effectively inhibited the TC-induced choleresis, with gradual diminution in bile flow over the final hour of the experimental period in these animals.

Bile salt secretion increased substantially upon reinfusion of TDHC, TUDC, and TC. Colchicine had no effect on bile salt output during TDHC reinfusion, but inhibited bile salt output in TUDC-reinfused rats, and to a greater extent in those reinfused with TC. Interestingly, the level of colchicine-insensitive bile salt secretion in the three groups was approximately constant at ~ 130 nmol/min • 100 g. In accordance with previous reports (6, 16), there was little enhancement of phospholipid output upon reinfusion of TDHC, and this was blocked by colchicine. TUDC and TC both promoted phospholipid secretion; this was inhibited by colchicine in both groups, but to a greater extent with reinfused TC. Routine HPLC on representative bile samples obtained during the reinfusion period demonstrated that reinfused TC and TUDC were secreted intact, while reinfused TDHC was converted quantitatively to slightly more hydrophobic metabolites. By HPLC–electrospray ionization–mass spectrometry (see Methods), these metabolites were identified as a mixture of di-keto/mono-hydroxy, mono-keto/di-hydroxy, and trihydroxy taurine-conjugates.

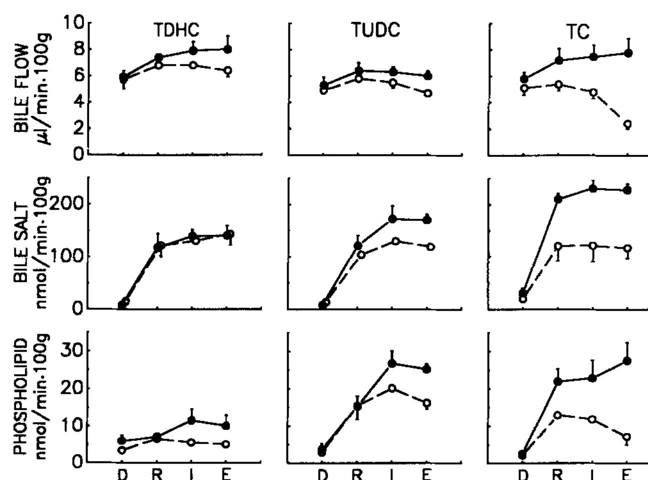

Fig. 2. Biliary secretion indices for rats pretreated with colchicine (○) or lumicolchicine (●) and injected with [^{3}H]taurocholate (see Figs. 3 and 4). Steady state bile flow and biliary bile salt and phospholipid secretion rates are shown for the four intervals denoted in Fig. 1. These are: Depleted (the 2-h period after colchicine or lumicolchicine pretreatment), Reinfused (the 45-min period after initiation of bile salt reinfusion), Injection (the 30-min period after injection of [^{3}H]taurocholate), and End (the last 30 min of the experimental period, 90–120 min after injection of radiolabel). The reinfused bile salt is shown at the top of each column. TDHC, taurodehydrocholate; TUDC, tauroursodeoxycholate; TC, taurocholate. Mean ± SD, n = 4 for each group.

As only control TUDC- and TC-reinfused animals appeared to be eliminating the infused bile salts effectively, urine, serum, and liver samples from an animal in each experimental group were obtained 45 min after initiation of bile salt reinfusion, i.e., the time at which steady-state secretion rates were well-established. Similar samples also were obtained from a basal animal not subjected to bile salt depletion and from a depleted animal not subjected to bile salt reinfusion. Concentrations and bile salt composition were analyzed by HPLC–electrospray ionization–mass spectrometry (**Table 1**). In the basal and depleted animals, there were no detectable urinary bile salts, and a mixture of di- and trioxygenated (keto- and hydroxy-) taurine and glycine conjugates in serum and the liver. For TDHC-reinfused animals, taurine-conjugated bile salts were detected in the urine of both lumicolchicine- and colchicine-pretreated animals, and were a mixture of TDHC and a diketo/monohydroxy taurine derivative (presumably the 3-hydroxy metabolite; 17). The bile salts present in the serum and livers of both TDHC reinfused animals consisted of a mixture of triketo (unmetabolized), diketo/monohydroxy, and monoketo/dihydroxy metabolites. Substantial amounts of trihydroxy TC were detected in the livers of both animals. In the colchicine-pretreated TUDC- and TC-reinfused animals, unmetabolized bile salts were detected in the urine, and slight (TUDC) or markedly (TC) elevated levels of the unmetabolized bile salts were present in the serum and liver.

Biliary secretion of [^{3}H]TC

Measurement of the bulk secretion of bile salts provided information about the steady-state hepatic processing of bile salts in the experimental groups. A "pulse-chase" of tracer radiolabeled bile salt was used to provide minute-to-minute information about transhepatic transport, but a dilemma existed regarding the choice of radiolabel. Use of a tracer dose of radiolabeled TDHC, TUDC, or TC in the respective reinfusion

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TABLE 1. Urine, serum, and liver concentrations of bile salts

Condition	Urine (µM)		Serum (µM)		Liver (nmol/g)	
Basal	ND		4.0	0.7 dihydroxy 0.1 diketo/monohydroxy 1.6 monoketo/dihydroxy 1.6 trihydroxy	72	12 dihydroxy 12 diketo/monohydroxy 32 monoketo/dihydroxy 16 trihydroxy
Depleted	ND		0.1	0.05 dihydroxy 0.09 trihydroxy	15	2 dihydroxy 13 trihydroxy
Depleted/Reinfused						
TDHC						
Lumi	0.5	0.2 triketo (TDHC) 0.3 diketo/monohydroxy	12.7	10.9 triketo (TDHC) 1.2 diketo/monohydroxy 0.6 monoketo/dihydroxy	74	1 diketo/monohydroxy 37 monoketo/dihydroxy 26 trihydroxy (TC)
Colch	10.9	10.6 triketo (TDHC) 0.3 diketo/monohydroxy	5.3	1.8 dihydroxy 0.4 diketo/monohydroxy 2.2 monoketo/dihydroxy 0.9 trihydroxy (TC)	124	5 diketo/monohydroxy 72 monoketo/dihydroxy 45 trihydroxy (TC)
TUDC						
Lumi	ND		3.6	dihydroxy (TUDC)	50	31 dihydroxy (TUDC) 19 trihydroxy (TC)
Colch	0.6	dihydroxy (TUDC)	6.5	dihydroxy (TUDC)	50	39 dihydroxy (TUDC) 11 trihydroxy (TC)
TC						
Lumi	ND		3.2	trihydroxy (TC)	54	2 dihydroxy 52 trihydroxy (TC)
Colch	1.0	trihydroxy (TC)	49	trihydroxy (TC)	448	2 dihydroxy 31 monoketo/dihydroxy 415 trihydroxy (TC)

Bile salt concentrations were determined by HPLC-electro-spray ionization-mass spectrometry (see Methods) in samples harvested from one animal in each group as follows: Basal, not subjected to experimental manipulation; Depleted, overnight biliary diversion only; Depleted/reinfused, overnight biliary diversion and bile salt reinfusion for 45 min. For each animal, the concentrations of the predominant bile salt species are given, expressed in terms of the hydroxylation status of the steroid ring. In the basal and depleted animals, these were a mixture of taurine- and glycine-conjugates. The bile salt species in depleted/reinfused animals were almost entirely taurine conjugates. Assignment of isomers, when possible, is given in parentheses; ND, not detectable.

groups would have provided direct information on the hepatic processing of each bile salt. There were two reasons for not pursuing this experimental avenue: *a)* the limited availability and high cost of commercial radiolabeled TDHC and TUDC; and *b)* the possibility that impurities in "home-made" preparations would invalidate experimental results. Rather, we chose to use one readily available radiolabeled bile salt, [^{3}H]TC, for all studies based on the following reasoning. *a)* All animals would be receiving the identical radiolabeled probe from the same synthetic batch, so that there would be no chemical differences in the administered probe. *b)* The observed hepatic secretion of radiolabel into bile would reflect the ability of the liver to process a tracer dose of the same bile salt species under different ambient conditions, without concern about differences in the biophysical properties of the bile salt probe itself. It is possible that competition between administered tracer [^{3}H]TC and bulk reinfused with TC may have impaired the hepatic secretion of [^{3}H]TC per se. However, in control animals we found the opposite to be true, in that [^{3}H]TC was secreted most effectively in the TC-reinfused group. *c)* Tracer [^{3}H]TC was eliminated completely ($>$ 95%) in bile in all experimental groups, so that incomplete biliary secretion of radiolabel was not a confounding factor in the interpretation of differences in biliary secretion patterns.

Figure 3A shows the biliary secretion of radiolabel after intravenous injection of tracer [^{3}H]TC into the same animals as in Fig. 2, 45 min after reinfusion with TDHC, TUDC, or TC was begun. The numerical parameters derived from the secretion curves are presented in **Table 2.** In all animals, maximal secretion of radiolabel occurred ~3-4 min after radiolabel administration. In lumicolchicine-pretreated animals, the maximal secretion rate of radiolabel increased slightly with increasing hydrophobicity of the reinfused bile salt, with the highest values for maximal secretion rate observed during TC reinfusion (see Table 2). Colchicine pretreatment caused a progressive diminution in maximal secretion rate as infused bile salt hydrophobicity increased; this diminution was 10% in the TDHC-reinfused group, but was 33% ($P < 0.01$) and 49% ($P < 0.01$) in the TUDC- and TC-

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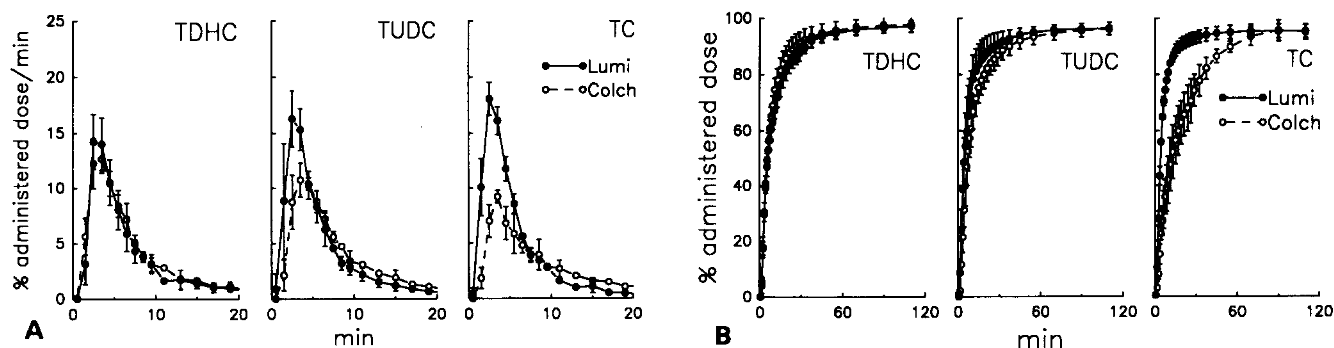

Fig. 3. Biliary secretion of radiolabel after i.v. administration of [^{3}H]taurocholate to depleted/reinfused rats. Tracer [^{3}H]taurocholate (3 nmol; 0.15–0.25 μCi) was administered i.v. to bile salt-depleted rats pretreated with colchicine (Colch, ○) or lumicolchicine (Lumi, ●) and reinfused with TDHC, TUDC, or TC at 200 nmol/min • 100 g. Biliary secretion of radiolabel was monitored for 120 min. A: Radiolabel secretion, expressed as percentage of the administered dose secreted per min. Secretion progressively declined beyond 20 min and hence is not shown. B: Cumulative radiolabel secretion, expressed as percentage of the administered dose. Data are plotted as the mean ± SD for the mid-point of each measured time interval (n = 4 for each group). Abbreviations as in Fig. 2.

reinfused animals, respectively. The progressive effect of colchicine pretreatment is also evident when the data is plotted as cumulative recovery of radiolabel in bile over the entire 120-min experimental period (Fig. 3B). Recovery of administered radiolabel was >95% by 120 min after injection in all groups. However, the time taken to achieve this level of recovery was markedly delayed in the colchicine-pretreated TC-reinfused group.

The striking correlation of colchicine inhibition to bile salt hydrophobicity is shown in **Fig. 4,** which shows the values for maximal radiolabel secretion rate plotted against the hydrophobicity index (HI) of the bulk bile salt species being secreted into bile (as measured by HPLC; see Methods). This provides graphic illustration of the near-linear correlation between bile salt hydrophobicity (10, 11) and: *a)* the enhancement of radiolabel secretion rate in lumicolchicine-pretreated animals ($P < 0.002$ by linear regression analysis); and *b)* the inhibition of radiolabel secretion in colchicine-pretreated animals ($P < 0.001$). When these data were plotted using the HI of unmetabolized TDHC, a more curvilinear relationship between HI and maximal secretion rate was obtained (not shown).

Analysis of bile aliquots obtained during the maximal secretion period (3–5 min after radiolabel injection) and during the lag phase of biliary secretion (25–30 min after

TABLE 2. Biliary secretion of radiolabel in depleted-reinfused rats

Reinfused Bile Salt	n	T_{max}	Maximal Secretion Rate	Cumulative Output
		min	*% administered dose/min*	*% administered dose*
TDHC				
Lumi	4	3.0 ± 0.6	15.3 ± 2.1	97 ± 2
Colch	4	3.0 ± 0.6	13.8 ± 1.1	98 ± 3
TUDC				
Lumi	4	2.8 ± 0.5	16.7 ± 1.8	97 ± 2
Colch	4	4.0 ± 1.0	11.1 ± 1.4[a]	96 ± 2
TC				
Lumi	4	2.5 ± 0.0	18.0 ± 1.7[b]	96 ± 3
Colch	4	3.5 ± 0.8	9.2 ± 0.5[a,c]	95 ± 3

The numerical parameters for the experiments shown in Fig. 3 are summarized. Tracer [^{3}H]taurocholate was administered to lumicolchicine- (Lumi) or colchicine- (Colch) pretreated rats reinfused with taurodehydrocholate (TDHC), tauroursodeoxycholate (TUDC), or taurocholate (TC); n, number of animals in each experimental group; T_{max}, time elapsed in minutes between injection of [^{3}H]taurocholate and maximal rate of secretion of radiolabel in bile; Maximal Secretion Rate, maximum rate of radiolabel secretion in bile, expressed as % of the administered dose of radiolabel per min; Cumulative Output, cumulative recovery of injected radiolabel over the 120 min experimental period, expressed as % of administered dose of radiolabel. All values are expressed as mean ± SD.

[a] $P < 0.01$ versus lumicolchicine-pretreated animals.

[b] $P < 0.05$ versus similarly pretreated TDHC-reinfused animals.

[c] $P < 0.01$ versus similarly pretreated TDHC-reinfused animals.

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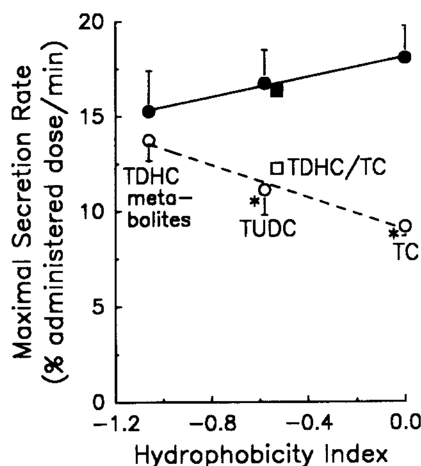

Fig. 4. Maximal secretion rate of radiolabel in bile in lumicolchicine (●) and colchicine (○) pretreated rats, as a function of the hydrophobicity index of the secreted bile salts (determined by HPLC elution time, see Methods). Maximal Secretion Rate, obtained from Table 1 for the experiments shown in Fig. 3, is expressed as percentage of the administered dose of radiolabel secreted per min (± SD, n = 4 for each group). This plot also shows the maximal secretion rate of radiolabel in depleted rats reinfused with an equimolar mixture of TDHC and TC (100 nmol/min • 100 g for each bile salt) and pretreated with lumicolchicine (■) or colchicine (□), plotted against the hydrophobicity index of the secreted bile salt mixture (−0.53; n = 2 for each group, differences in values are less than the size of the symbol). Lines were obtained by linear regression. *, $P < 0.01$ versus lumicolchicine-pretreated control.

injection) demonstrated that there was no metabolic alteration of [^{3}H]TC during transit through the liver. This finding held true for every animal in each treatment group.

Supplemental experiments

To test the hypothesis that the HI of the ambient bile salt pool is a primary determinant of bile salt secretion by a microtubule-dependent mechanism, an equimolar mixture of TDHC and TC was infused into depleted rats (loading dose of 1000 nmol/100 g followed by infusion at 100 nmol/min • 100 g for each bile salt), and biliary secretion of radiolabel after intravenous administration of tracer [^{3}H]TC was measured. The HI of the secreted bile salt mixture, −0.53, was midway between that obtained with reinfusions of pure TDHC and TC. The results of this experiment are plotted in Fig. 4 (denoted by squares); two animals were used in each group, and experimental variance is less than the size of the symbols. The values of maximal secretion rate for both lumicolchicine (closed square) and colchicine-pretreated (open square) animals approximate those predicted from studies with pure bile salt reinfusions.

We attempted to evaluate the effect of colchicine on bulk bile salt and [^{3}H]TC secretion in depleted animals reinfused with taurochenodeoxycholate (TCDC; 200 nmol/min • 100 g), a more hydrophobic, naturally occurring bile salt with an HI of 0.58. While lumicolchicine-pretreated animals tolerated the experimental regimen, the combination of colchicine pretreatment and reinfusion of pure TCDC proved to be too toxic to the animals, as frank cessation of bile flow with evidence of intravascular hemolysis developed within 30 min of initiating TCDC reinfusion. These experiments were therefore discontinued.

Although colchicine was used to disrupt microtubular function, it is possible that its effects were due to other influences on hepatobiliary function. One possibility, inhibition of hepatic bile salt uptake, does not appear to be operative under these experimental conditions, as we have shown in identically prepared rats reinfused with TC that colchicine pretreatment has no effect on the hepatic uptake of [^{3}H]TC (7). Second, colchicine might act as an organic anion competing for transport at the canalicular membrane (18). To test this possibility, we pretreated depleted animals with [^{3}H]colchicine, rather than unlabeled colchicine, prior to TC reinfusion. Biliary secretion of ^{3}H occurred only during the first 90 min after [^{3}H]colchicine administration, with no further secretion for the remainder of the experimental period. Cumulative recovery of ^{3}H radiolabel in bile was 47 ± 14% (± SD, n = 4). Thus, biliary secretion of colchicine had ceased well before unlabeled bile salt reinfusion was initiated and radiolabeled bile salt was administered. Third, although colchicine has been shown to increase paracellular permeability (19), any leakage of [^{3}H]taurocholate secreted into the canalicular space through paracellular junctions would result in an apparent retention of radioactivity in plasma, which we have not observed (7). We cannot exclude the possibility that colchicine is interacting with soluble cytosolic binding proteins in the hepatocyte and influencing bile salt delivery to the canalicular membrane. However, as control animals were exposed to lumicolchicine, which is a closely related structural isomer of colchicine, this possibility seems unlikely. Thus, the observed inhibition of bile salt secretion by colchicine pretreatment is most likely mediated by an inhibitory effect on microtubular function.

DISCUSSION

Bile salts may traverse hepatocytes from plasma to bile within seconds, although some minutes are required for complete biliary secretion of an intravenous bolus dose (6). The predominant mechanism for movement of bile salts across hepatocytes appears to be diffusion through the cytosol, presumably while bound to soluble proteins such as 3α-hydroxysteroid dehydrogenase and glutathione S-transferase (20, 21). Nevertheless, bile salts interact with intracellular membranes during transit through the hepatocyte, particularly those of the endoplasmic reticulum and Golgi apparatus (22–30). These membrane interactions facilitate biliary secretion of phos-

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phatidylcholine. Biliary phosphatidylcholine is recruited from intracellular stores (31, 32), and its trafficking from the endoplasmic reticulum to the canalicular membrane via phosphatidylcholine transfer protein (PC-TP) is stimulated by bile salts (33). Once delivered to the internal hemileaflet of the canalicular membrane, biliary phosphatidylcholine is presumably translocated from the internal to the external hemileaflet by the action of a phospholipid translocator (34). Bile salts themselves are secreted as monomers across the canalicular membrane by bile salt transport systems (35). Once within the canalicular lumen, they then appear capable of "back-extracting" biliary lipid from the canalicular membrane (36–38). What remains unknown is how bile salt interactions with subcellular structures influence their own secretion from the hepatocyte.

Microtubules play a key role in the intracellular trafficking of membrane components and are necessary for vesicular insertion of membrane proteins and lipid into plasma membrane domains (39–42). The three-dimensional architecture of the endoplasmic reticulum and Golgi apparatus is also dependent on microtubular integrity (43, 44). We and others have shown that a bile salt load recruits a microtubule-dependent mechanism for efficient hepatic secretion of bile salts into bile (6, 7, 15, 27, 28, 42). Although we originally postulated that this process involves microtubule-dependent vesicular trafficking of bile salts per se (6, 15), more recent evidence suggests that bile salts stimulate microtubule-dependent insertion of bile salt transporters into the canalicular membrane (45–49). A potential basis for bile salt recruitment of a microtubule-dependent secretion mechanism is bile salt interactions with intracellular membranes.

Studies with model membrane systems have shown that less hydrophobic bile salts are less likely to partition into membranes (32). Thus, decreasing the relative hydrophobicity of the ambient bile salt pool is likely to reduce bile salt interactions with membranes (50), and hence may reduce the ability of bile salts to stimulate up-regulation of their own secretion. This study was designed to test the prediction that decreasing the hydrophobicity of the bile salt pool would decrease the sensitivity of hepatic bile salt secretion to microtubule disruption.

We used rats in which the enterohepatic circulation was interrupted by overnight biliary diversion. This maneuver causes depletion of the endogenous bile salt pool (51, 52); by reinfusing bile salts to produce physiological rates of secretion, the composition of bile salts moving through the liver can be readily manipulated. The bile salts selected for reinfusion, listed in order of decreasing hydrophobicity, were the sodium salts of the following bile acids: taurocholate (TC), a trihydroxy bile acid that is a major constituent of normal rat bile (1); tauroursodeoxycholate (TUDC), a dihydroxy bile acid with octanol:water partitioning similar to the much more hydrophobic taurochenodeoxy- and taurodeoxycholic acids but with detergent-like properties falling between those of TC and taurodehydrocholate (53); and taurodehydrocholate (TDHC), a synthetic non-micelle-forming hydrophilic bile acid that interacts minimally with membrane systems (54). Taurine-conjugates of the bile salts were used in this study to avoid the confounding influences of metabolic processing of unconjugated bile salts (7, 55) and potential depletion of the taurine pool during prolonged reinfusion (56), and to better reflect the fact that orally administered unconjugated bile salts are conjugated upon their entry into the enterohepatic circulation (57).

The results shown in Figs. 1 and 2 demonstrate that overnight bile salt-depleted animals appear capable of secreting reinfused bile salts at the rate of ~ 130 nmol/min • 100 g, and that further enhancement of bile salt secretion depends on intact microtubular function. Notably, such enhancement occurs only during reinfusion of the more hydrophobic bile salts, TUDC and TC, and not during TDHC reinfusion. That incomplete elimination of bile salts leads to corporal retention is confirmed in Table 1, which documents both urinary excretion and serum and hepatic retention of bile salt species, particularly in colchicine-pretreated animals.

The rapid biliary secretion of [^{3}H]TC also was influenced by the choice of reinfused bile salt, as evident in the biliary secretion curves (Figs. 3) and upon comparison of numerical parameters derived from these curves (Table 2 and Fig. 4). Among control rats, [^{3}H]TC was eliminated least effectively in TDHC-reinfused rats, corroborating the findings of Fig. 2 indicating that TDHC-reinfused animals have the lowest capacity for elimination of bile salts. The fact that colchicine does not substantially influence [^{3}H]TC secretion in TDHC-reinfused rats also indicates that TDHC does not recruit a microtubule-dependent mechanism for bile salt secretion. However, the maximal secretion rate for [^{3}H]TC in the colchicine-pretreated TDHC group is greater than that in the TUDC or TC groups, despite the relative constancy of bulk bile salt secretion (Fig. 2). This observation suggests that TDHC metabolites are partially secreted into bile by alternate transport mechanisms, possibly including the multispecific organic anion carrier (MOAT; 58). The diminution in [^{3}H]TC secretion rate in colchicine-pretreated TC-reinfused animals may be the result of a dilutional effect, given the massive accumulation of TC in the serum and liver of these animals (Table 1). However, the very fact that TC accumulated in the liver also points to canalicular membrane bile salt transport as the key step that is altered by microtubule disruption.

These findings support the concept that the hepatic capacity for bile salt secretion is regulated in parallel with the load of bile salts moving through the liver. The secretory capacity decreases following overnight biliary diversion, in keeping with published reports (14, 59–64) and

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contrasting with the documented ease with which livers in bile salt-replete animals can secrete bile salt loads (65-67). The down-regulation in canalicular transport encountered during interruption of the enterohepatic circulation is likely to have a substantial physiological impact, as the decreased bile salt SRm can produce an increased susceptibility of the liver to the cholestatic or cytotoxic effects of hydrophobic bile salts or other compounds normally secreted in bile (60). Our current data indicate that restoration of hepatic secretion capacity is dependent on intact microtubular function, and appears to occur within 15 min of reinfusion of bile salts. Most importantly, we now demonstrate that the up-regulation is dependent in part on the relative hydrophobicity of the bile salts moving through the liver, with more hydrophobic bile salts appearing to stimulate bile salt secretion more effectively.

A paradox is encountered, however, when the well-documented toxicity of more hydrophobic bile salts intervenes, thereby limiting hepatic bile secretory capacity (65, 67). In our hands, lumicolchicine-pretreated animals depleted and reinfused with TCDC effectively secrete this bile salt and the tracer doses of [^{3}H]TC. However, the combination of microtubule disruption and TCDC reinfusion is so toxic as to lead to complete bile secretory failure (data not shown). In published reports, bile salts of low hydrophobicity (TUDC, tauro-α-muricholate, tauro-β-muricholate, and glycoursodeoxycholate) are capable of reducing or ameliorating the cholestatic toxicity and stimulating the secretion of more hydrophobic bile salts (TC and TCDC) from both isolated hepatocytes and the intact liver (68-75). We postulate that such hydrophilic bile salts are beneficial precisely because they are capable of up-regulating bile salt secretion capacity without themselves being directly cytotoxic. While the magnitude of the up-regulation may not be as great as for a more hydrophobic bile salt such as TCDC, it is clear from clinical studies that direct bile salt toxicity, when encountered, dominates the physiological outcome (76).

In conclusion, our findings indicate that the imposition of a modest bile salt load on the liver of animals subjected to bile salt depletion recruits a microtubule-dependent mechanism for efficient bile salt secretion, possibly on the basis of their interactions with intracellular membranes. This mechanism, which may involve vesicular insertion of bile salt transporters into the canalicular membrane, is more effectively recruited as the relative bile salt hydrophobicity increases. However, cholestatic toxicity intervenes during administration of increasingly hydrophobic bile salts, particularly when hepatobiliary function is compromised by microtubule disruption. Thus, an optimal balance between beneficial and detrimental effects appears to be maintained when the liver is exposed to bile salts of lower hydrophobicity. Our findings may explain, in part, the beneficial effects obtained by the pharmacological administration of such bile salts as TUDC, tauro-α-muricholate, and tauro-β-muricholate to patients with cholestatic disorders.

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