Role of the hepatocyte microtubular system in the excretion of bile salts and biliary lipid: implications for intracellular vesicular transport

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Abstract The role of the hepatocyte microtubular system in the transport and excretion of bile salts and biliary lipid has not been defined. In this study the effects of microtubule inhibition on biliary excretion of micelle- and non-micelle-forming bile salts and associated lipid were examined in rats. Low-dose colchicine pretreatment had no effect on the baseline excretion of biliary bile salts and phospholipid in animals studied 1 hr after surgery (basal animals), but slightly retarded the excretion of tracer [^{14}C]taurocholate relative to that of lumicolchicine-pretreated (control) rats. However, colchicine pretreatment resulted in a marked reduction in the excretion of 2 μmol/100 g doses of a series of four micelle-forming bile salts of differing hydrophilicity, but had no significant effect on the excretion of the non-micelle-forming bile salt, taurodehydrocholate. Continuous infusion of 0.2 μmol of taurocholate/(100 g · min) following 24 hr of biliary drainage (depleted/reinfused animals) resulted in physiologic bile flow with biliary excretion rates of bile salts, phospholipid, and cholesterol that were markedly inhibited (mean 33, 39, and 42%, respectively) by colchicine or vinblastine pretreatment. Excretion of tracer [^{14}C]taurocholate also was markedly delayed by colchicine in these bile salt-depleted/reinfused animals. In contrast, colchicine did not inhibit bile salt excretion in response to reinfusion of taurodehydrocholate. Thus, under basal conditions, the microtubular system appears to play a minor role in hepatic transport and excretion of bile salts and biliary lipid. However, biliary excretion of micelle-forming bile salts and associated phospholipid and cholesterol becomes increasingly dependent on microtubular integrity as the transcellular flux and biliary excretion of bile salts increases, in both bile salt-depleted and basal animals. We postulate that cotransport of micelle-forming bile salts and lipids destined for biliary excretion, via an intracellular vesicular pathway, forms the basis for this microtubule dependence. — **Crawford, J. M., C. A. Berken, and J. L. Gollan.** Role of the hepatocyte microtubular system in the excretion of bile salts and biliary lipid: implications for intracellular vesicular transport. *J. Lipid Res.* 1988. **29:** 144–156.

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The enterohepatic circulation of bile salts exceeds by more than tenfold the rate of intrahepatic bile salt synthesis (1). Following hepatic uptake (2), it is presumed that bile salts distribute among cytoplasmic proteins, such as the glutathione-S-transferases (3) and the recently characterized bile salt-binding proteins (4), or partition into intracellular membranes (5, 6). Hepatocytes have little storage capacity for bile salts, and under physiologic conditions maintain a low intracellular bile salt concentration (5, 7). However, the substantial flux of bile salts through the liver provides a major driving force for hepatic bile formation (2, 7). In contrast, biliary phospholipid is derived almost completely from within the hepatocyte (8, 9), with little apparent contribution from plasma (10, 11). Phospholipid synthesis occurs predominantly in the endoplasmic reticulum (12), from whence a large hepatic store of phospholipid is available for excretion into bile under both basal and choleretic conditions (13, 14). Although cholesterol is also synthesized in the endoplasmic reticulum (15), hepatocellular cholesterol synthesis generates only a minor fraction of that excreted in bile (16, 17), with the remainder derived from extrahepatic sources (7).

Despite the disparate sources of biliary lipid, the excretion of phospholipid and cholesterol is clearly dependent on bile salt output (7, 18); both phospholipid and cholesterol excretion rates vary curvilinearly as a function of bile salt excretion and tend to reach limiting values at high bile salt outputs (7, 19). Interactions between bile salts, phospholipid, and cholesterol, in the form of biliary vesicles or micelles, have been clearly documented in the

Abbreviations: The following systematic names are given to taurine-conjugated bile salts referred to by trivial names: dehydrocholate, 3,7,12-triketo-5β-cholanoate; ursodeoxycholate, 3α,7β-dihydroxy-5β-cholanoate; cholate, 3α,7α,12α-trihydroxy-5β-cholanoate; chenodeoxycholate, 3α,7α-dihydroxy-5β-cholanoate; deoxycholate, 3α,12α-dihydroxy-5β-cholanoate. TDC, taurodeoxycholate; TCDC, taurochenodeoxycholate; TC, taurocholate; TUDC, tauroursodeoxycholate; TDHC, taurodehydrocholate; ANOVA, analysis of variance.

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canalicular lumen (13, 20, 21). However, we have little understanding of the intracellular mechanisms responsible for transport of bile salts, phospholipid, and cholesterol from plasma or subcellular sites of synthesis and biotransformation to the canalicular membrane for excretion into bile. Studies employing inhibitors of cytoskeletal proteins, such as phalloidin for microfilaments (22, 23) and colchicine for microtubules (13, 20, 23, 24), have provided conflicting conclusions regarding the role of the cytoskeleton in hepatocellular bile salt and biliary lipid transport and excretion.

The present study was undertaken to systematically examine the effects of impaired hepatic microtubule function on the biliary excretion of a series of physiologically relevant bile salts and associated biliary phospholipid and cholesterol. Our findings provide insight into the mechanisms of bile salt translocation in hepatocytes; in particular, the interactions of micelle-forming bile salts with the microtubular system and the putative contribution of microtubule-dependent vesicular transport.

MATERIALS AND METHODS

Chemical reagents

Colchicine and lumicolchicine were obtained from Sigma Chemical Co., St. Louis, MO. Vinblastine sulfate was a gift from Eli Lilly Co., Indianapolis, IN. [^{14}C]Taurocholate (50 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, IL. Sodium bile salts (taurodehydrocholate, tauroursodeoxycholate, taurocholate, taurochenodeoxycholate, and taurodeoxycholate) were purchased from Calbiochem/Behring Diagnostics, LaJolla, CA. The purity of all bile salts exceeded 98% as determined by thin-layer chromatography. 3α-Hydroxysteroid dehydrogenase used in the bile salt assay was obtained from Worthington Diagnostic Systems, Inc., Freehold, NJ, and Cholesterol Reagent Sets™ were from Boehringer Mannheim, Indianapolis, IN. Soluene-350 solvent system and Dimiscent scintillation cocktail were purchased from Packard Instrument Co., Inc., Downers Grove, IL. All other reagents were of the highest analytical grade available.

Preparation of animals

Male Sprague-Dawley rats (Charles River Breeding Laboratories, North Wilmington, MA), weighing 300 ± 12 g (mean ± SD; n = 88) were maintained on Purina rat chow and water ad libitum. Surgery was performed between 8 and 9 AM under light ether anesthesia and was consistently less than 20 min in duration. The left jugular vein was cannulated with a 40-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 was begun at 1.5 ml/hr. The common bile duct was cannulated with a 19-cm length of tubing. Following surgery, the animals were placed in restraining cages, warmed under heating lamps, and allowed access to rat chow and water ad libitum.

Two experimental subsets of rats were employed. "Basal" rats were pretreated with intravenous colchicine, an inhibitor of microtubule assembly (25), or its inactive isomer, lumicolchicine (26), 0.5–1 hr prior to surgery. Animals were allowed a 1.25- to 0.75-hr postoperative recovery period, respectively, prior to the experiments (see Figs. 1 and 2). "Bile salt-depleted/reinfused" rats were subjected to continuous biliary drainage for 22 hr, and colchicine or lumicolchicine was administered intravenously via the jugular cannula; this was followed 2 hr later by the reinfusion of unlabeled bile salts (see Figs. 3, 4, and 6). Alternatively, these rats were pretreated with vinblastine, which inhibits microtubule disassembly (27), or an equal volume of saline (see Fig. 5).

Experiments were conducted with paired animals that were prepared in an identical manner and pretreated with colchicine or lumicolchicine. These compounds were prepared as 0.6 mg/ml solutions in 0.15 M NaCl and injected intravenously in a dose of 0.12 mg/100 g body weight. This relatively low dose of colchicine has previously been shown to impair hepatocellular microtubule assembly and to inhibit the secretion of plasma proteins (28). In other experiments, vinblastine sulfate (5 mg/ml) was administered in a dose of 1 mg/100 g body weight. Experiments were initiated (t = 0 min) by the intravenous administration of labeled or unlabeled bile salts (see Results for experimental details).

Analytical methods

Bile was collected sequentially in tared tubes and bile flow was determined gravimetrically. Samples were then processed for measurement of radioactivity or stored under nitrogen at −70°C for subsequent assay.

Total 3α-hydroxy bile salt output in bile aliquots was determined enzymatically using the 3α-hydroxysteroid dehydrogenase procedure (29). Taurodehydrocholate, a synthetic, non-micelle-forming triketo-bile acid (30, 31) is converted predominantly to 3α-hydroxy bile salt derivatives by reductive hepatic metabolism (31), and thus the same assay was employed for analysis of bile from animals infused with this bile salt. Phospholipid concentration in bile was determined by the method of Bartlett (32). Biliary cholesterol content was measured enzymatically using the Worthington Cholesterol Reagent Set™, which is based on a modification of the cholesterol oxidase–ester hydrolase method (33).

Radioactivity in bile samples collected following injection of [^{14}C]taurocholate was measured in 10–40-μl aliquots which were bleached under incandescent light for 2 hr following the addition of 0.5 ml of Soluene-350, 0.1 ml of isopropyl alcohol, and 0.1 ml of 30% hydrogen peroxide.

Dimiscint (5 ml) was then added and the vials were counted in a liquid scintillation spectrometer (Beckman LS7500). Internal standards were calibrated with [^{14}C]toluene as an external standard to enable correction of counts per minute to disintegrations per min. Quadruplicate 5-μl aliquots of [^{14}C]taurocholate, obtained directly from the barrel of the syringe following injection, were counted in order to provide an accurate measure of the administered radioactivity. Scintillation vials were counted for 5 min or 20,000 counts to ensure an error of less than 2%.

Statistical analysis

The data consisted of sequential measurements of bile flow and bile salt, phospholipid, cholesterol, and radioactivity excretion in bile under a variety of experimental conditions. Tests for normality revealed a normal distribution of values at each time point, permitting their representation as mean ± SD versus time (see Figs. 1–6). Differences between curves obtained for different experimental subgroups were evaluated using analysis of variance (ANOVA) (34). In experiments employing either bolus injection of bile salts (see Fig. 2) or injection of tracer radiolabeled bile salt (see Figs. 1 and 6), the data were analyzed with respect to the maximum rate of excretion (excretory rat max; E_{max}), time after injection to achieve maximum excretion (t_{max}), and total recovery (Cumulative Recovery). These curve parameters, calculated for each rat in all experimental subgroups (see Figs. 1, 2, and 6), were found to have a normal distribution, and were simultaneously compared using Hotelling's multivariate *t*-test. Individual comparisons of parameters were performed using appropriate ANOVA followed by multiple sample comparison tests. Results from experiments utilizing steady reinfusion of bile salts (see Figs. 3, 4, and 5) were also compared using appropriate ANOVA and multiple sample comparison tests. The relationship of bile salt excretion rate to bile flow (see Fig. 7) was examined using linear regression analysis, and statistical comparisons of subgroups were performed using ANOVA.

RESULTS

Effects of colchicine pretreatment on biliary excretion of bile salts (basal rats)

Biliary excretion of bile salts was examined initially in intact animals receiving either colchicine or its inactive stereoisomer, lumicolchicine, prior to surgery (i.e., "basal" rats; see Methods). In preliminary experiments (n = 6) bile salt and phospholipid excretion was shown to remain stable for 2–3 hr following surgery, with no significant difference between the animals pretreated with lumicolchicine (control) or colchicine (bile salt excretion 305 ± 3 vs. 288 ± 28 nmol/(100 g · min), and phospholipid excretion 26.5 ± 3.0 vs. 28.3 ± 4.3 nmol/(100 g · min), respectively). This absence of a colchicine effect on baseline bile salt excretion rate was observed consistently in all subsequent basal rats examined (n = 50).

To further investigate the effects of microtubule dysfunction in "basal" rats, a tracer dose (3 nmol) of [^{14}C]taurocholate was administered intravenously as a bolus 2.75 hr after colchicine or lumicolchicine pretreatment (1.5–2.0 hr after surgery), and biliary excretion of radiolabel was measured. Maximal output of radiolabel occurred 2.5 min after injection in both experimental groups (**Fig. 1A**). Cumulative recovery of radiolabel at 90 min was comparable (93.2 ± 0.9 vs. 93.6 ± 0.5% of the administered dose, respectively; Fig. 1B). However, a statistically significant lag was consistently apparent in cumulative recovery of radiolabel in the bile of animals pretreated with colchicine (ANOVA curve comparison; $P < 0.0001$). This was the result of decreased excretion of radiolabel during the initial 8 min following [^{14}C]taurocholate injection (comparison of curves from 0–8 min; $P < 0.0001$) and increased excretion from 9 to 40 min ($P < 0.0001$), relative to control animals. The lag in excretion was not attributable to altered bile flow: thus, bile flow was comparable in colchicine- and lumicolchicine-pretreated rats (9.3 ± 0.9 vs. 10.1 ± 0.7 μl/(100 g · min), respectively), and a similar effect of colchicine was evident when radiolabel excretion was plotted as a function of cumulative volume rather than time.

Effects of colchicine pretreatment on biliary excretion of bile salts of different hydrophilicity (basal rats)

In the next phase of the study, the effects of colchicine on biliary excretion of a series of taurine-conjugated, micelle-forming bile salts were examined in basal rats. We postulated that the hepatocellular transport and biliary excretion of the more hydrophobic bile salt species (with an increased tendency to partition into intracellular lipid assemblies (35, 36)) would be most likely influenced by colchicine-induced microtubule dysfunction. In these experiments, a modest bile salt load (2 μmol/100 g) was administered intravenously to basal rats; the dose employed was well below the reported maximal biliary secretory transport rates of the infused bile salts (37), and approximated the bile salt load that might occur in the postprandial state (38, 39).

There was a marked and consistent effect of colchicine pretreatment on the biliary excretion of injected taurodeoxycholate, taurochenodeoxycholate, taurocholate, or tauroursodeoxycholate (listed in order of decreasing hydrophobicity (7)), as shown in **Fig. 2** and **Table 1**. The primary effect of microtubule disruption was a diminution of the maximal rate of bile salt excretion (mean 48%, Fig. 2, first four panels in column A, and Table 1, E_{max}) and a decrease in total bile salt recovery at 10 min (mean 44%, Fig. 2, first four panels in column B, and Table 1, Cumulative Recovery). In contrast, colchicine pretreatment produced no significant change in the rate of biliary

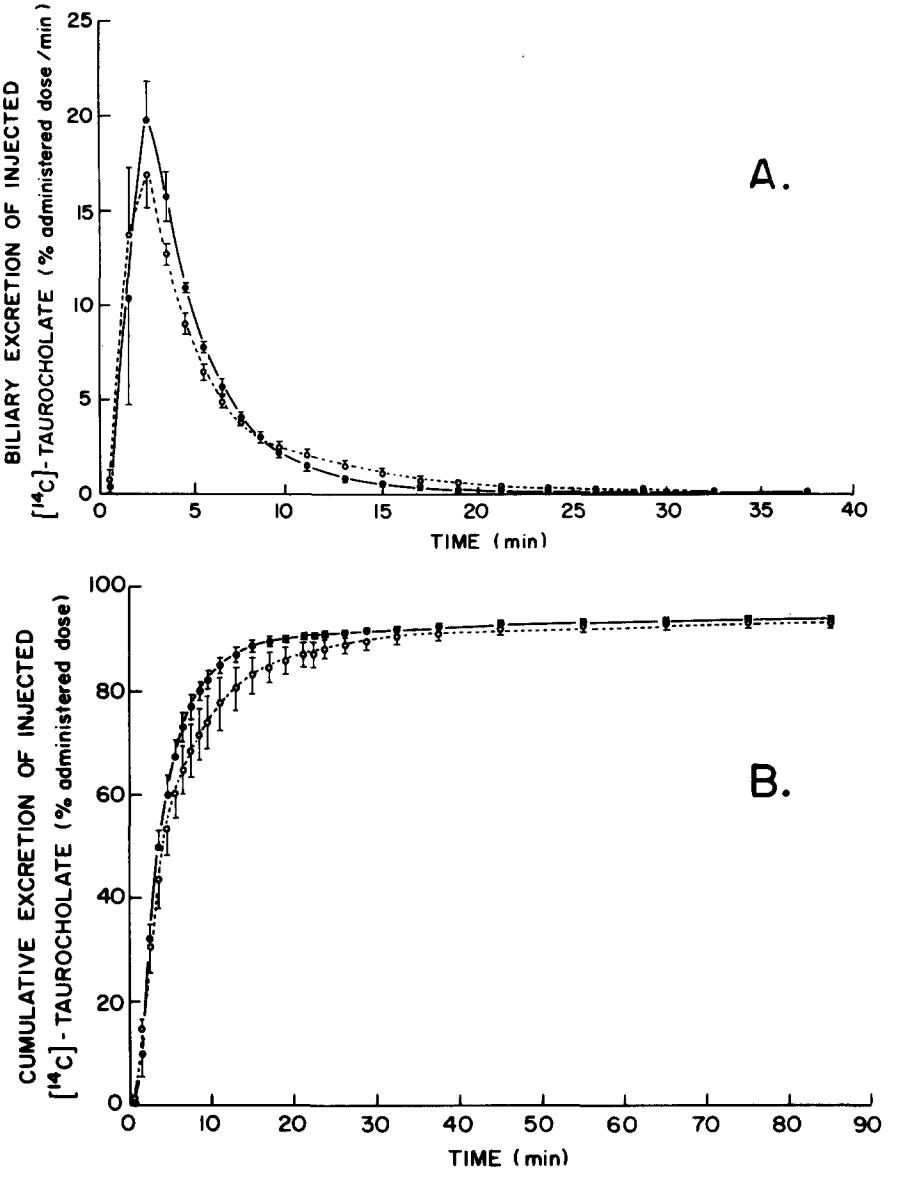

Fig. 1. Effect of colchicine pretreatment on biliary excretion of intravenous [^{14}C]taurocholate under basal conditions. [^{14}C]Taurocholate (3 nmol; 0.15 μCi) was administered i.v. to rats pretreated with colchicine (n = 4) or lumicolchicine (n = 4), and bile was collected continuously for 90 min (see Methods). A) Biliary excretion of injected ^{14}C radioactivity over the initial 40 min, shown as % administered dose/min. B) Biliary excretion of injected radioactivity plotted as cumulative recovery of the administered dose over the 90-min experimental period; (●—●) lumicolchicine-pretreated rats (control); (○- - -○) colchicine-pretreated animals. Results are expressed as mean ± SD.

excretion or total recovery of the synthetic non-micelle-forming bile salt, taurodehydrocholate (Fig. 2, bottom panels in columns A and B, and Table 1). The time elapsed to maximal biliary excretion rate was not statistically significant between colchicine-pretreated and control groups for any given bile salt (Table 1, t_{max}).

There were no significant differences between the bile salt excretion curves following taurochenodeoxycholate, taurocholate, or tauroursodeoxycholate injection in control animals, and no differences were evident between the colchicine-pretreated rats injected with these bile salts (Fig. 2A and B, Table 1). However, bile salt excretion following administration of the most hydrophobic bile salt, taurodeoxycholate (35), differed from that of the other micelle-forming bile salts, both in control and colchicine-pretreated animals. This was based on both curve comparison by ANOVA ($P < 0.005$) and the time to maximal excretion (t_{max}, Table 1, $P < 0.01$). In addition, there

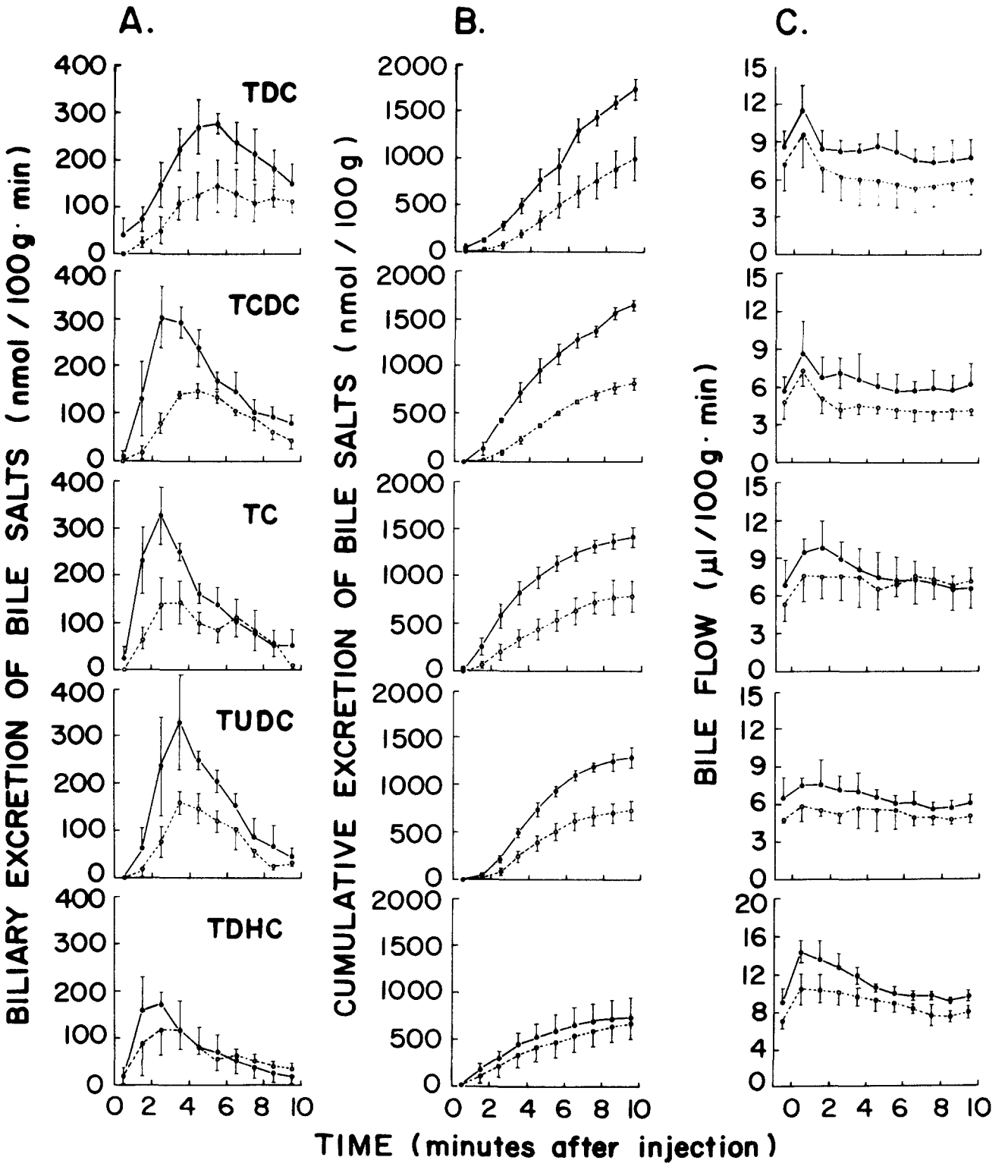

Fig. 2. Effects of colchicine pretreatment on bile salt excretion and bile flow following i.v. administration of bile salts of different hydrophobicity. Following low-dose (0.12 mg/100 g) i.v. colchicine (n = 20) or lumicolchicine (n = 20), a 2 µmol/100 g bolus of bile salt was administered (n = 4 for each subgroup), and bile was collected for 1-min periods for 10 min (see Methods). A) Bile salt output is shown as the increase relative to baseline excretion rate (nmol/(100 g · min)). B) Bile salt output plotted as the cumulative increase in excretion over the 10-min experimental period (nmol/100 g). C) Bile flow rate (µl/100 g). Bile salts are listed in order of decreasing hydrophobicity. TDC, taurodeoxycholate; TCDC, taurochenodeoxycholate; TC, taurocholate; TUDC, tauroursodeoxycholate; TDHC, taurodehydrocholate (non-micelle-forming bile salt); (●—●) lumicolchicine-pretreated rats (control); (○- - -○) colchicine-pretreated animals. Baseline rates of bile salt excretion were determined in the 5 min prior to injection of bile salts. Numerical parameters for these data are shown in Table 1.

TABLE 1. Bile flow and biliary excretion of bile salts in colchicine- and lumicolchicine-treated rats following intravenous injection (2 μmol/100 g body weight) of bile salts of different hydrophilicity

Bile Salt	n	E_{max}	t_{max}	Cumulative Recovery	Peak Bile Flow
		nmol/(100 g · min)	*min*	*nmol/100 g*	*μl/(100 g · min)*
TDC					
Lumi[a]	4	290 ± 35	5.0 ± 0.6	1723 ± 111	2.82 ± 1.20
Colch[a]	4	152 ± 45*,[b]	5.8 ± 1.9	990 ± 237*	2.28 ± 1.27
TCDC					
Lumi	4	332 ± 30	3.0 ± 0.6†	1564 ± 55	2.92 ± 1.46
Colch	4	156 ± 13*	4.0 ± 0.6†	831 ± 73*	2.51 ± 1.79
TC					
Lumi	4	329 ± 60	2.8 ± 0.5†	1421 ± 113†	3.16 ± 1.09
Colch	4	162 ± 53*	3.0 ± 0.6†	783 ± 179†,*	3.52 ± 1.50
TUDC					
Lumi	4	282 ± 30	3.5 ± 0.0†	1306 ± 109†	1.77 ± 0.04
Colch	4	162 ± 26*	3.5 ± 0.0†	745 ± 105†,*	1.48 ± 1.13
TDHC					
Lumi	6	190 ± 53	2.3 ± 0.6†	732 ± 243‡	5.54 ± 1.71
Colch	6	137 ± 52	2.3 ± 0.8†	671 ± 170‡	3.79 ± 1.32

Numerical parameters for the experiments shown in Fig. 2 are presented. Baseline excretion of bile salts was 171 ± 76 nmol/(100 g · min), and bile flow was 6.6 ± 2.0 μl/(100 g · min). E_{max}, maximal increment in bile salt excretory rate relative to baseline; t_{max}, time after injection to achieve maximal increment in bile salt excretory rate; Cumulative Recovery, cumulative increment in bile salt excretion over 10 min relative to baseline; peak bile flow, maximal increase in bile flow relative to baseline. Bile salt abbreviations are presented in Fig. 2. Data are expressed as mean ± SD.

[a] Lumi, lumicolchicine; colch, colchicine.

[b] *$P < 0.01$ versus lumicolchicine control rats in same group; †$P < 0.01$ versus similarly treated taurodeoxycholate-infused rats; ‡$P < 0.01$ versus similarly treated rats infused with micelle-forming bile salts (TDC, TCDC, TC, TUDC).

was a tendency for decreased recovery of bile salts in bile as the hydrophobicity of the injected species decreased ($P < 0.01$, Cumulative Recovery, Table 1). This decreased recovery of taurocholate and tauroursodeoxycholate may reflect alternate routes of elimination for bile salts, or more efficient hepatocellular transport mechanisms for the more hydrophobic bile salts.

In this set of 44 basal animals, baseline bile flow just prior to injection of bile salts was modestly reduced in colchicine-pretreated rats (5.67 ± 1.66 μl/(100 g · min) vs. 7.54 ± 1.97 μl/(100 g · min) in control animals; $P < 0.005$). However, the choleretic response to all injected bile salts, expressed as the peak increase in bile flow over baseline (Table 1), was not significantly different between colchicine-pretreated and control animals. In addition, the choleretic response to the four micelle-forming bile salts in control or colchicine-pretreated animals was comparable. The enhanced, albeit transient, choleresis observed following taurodehydrocholate administration ($P < 0.05$) is consistent with literature reports of the marked choleretic properties of taurodehydrocholate (30, 31).

Effects of microtubule inhibitors on biliary excretion of bile salts, phospholipid, and cholesterol (bile salt depleted/reinfused rats)

The role of microtubules in bile salt excretion was further investigated using an experimental model in which rats were depleted of bile salts by overnight biliary diversion, injected with colchicine or lumicolchicine 22 hr after surgery, and 2 hr later given a loading dose (2 μmol/100 g) followed by a steady infusion (200 nmol/(100 g · min)) of either taurocholate or taurodehydrocholate. In this manner the bile salt pool was largely replaced by a known micelle- or non-micelle-forming bile salt, while maintaining a biliary bile salt excretion rate comparable to that of normal (basal) rats. Bile flow and biliary excretion of bile salts, phospholipid and cholesterol were then measured sequentially (**Fig. 3** and **Fig. 4**). Preliminary washout experiments (n = 6) indicated that colchicine pretreatment just prior to surgery did not alter the pattern of biliary excretion of bile salts or phospholipid over the ensuing 24 hr. Both parameters fell to less than 10% of baseline excretion rates; after 24 hr, bile salt excretion was 27 ± 13 nmol/(100 g · min) in colchicine-pretreated animals versus 27 ± 17 nmol/(100 g · min) for controls, and phospholipid excretion was 0.5 ± 0.3 versus 0.7 ± 0.3 nmol/(100 g · min), respectively.

Biliary excretion of bile salts, phospholipid, and cholesterol reached its nadir of approximately 25, 3, and 4 nmol/(100 g · min), respectively, after 24 hr of biliary diversion (Figs. 3 and 4, n = 16). Reinfusion of either taurocholate or taurodehydrocholate resulted in a rapid increase in bile salt output, which restored a steady-state excretion rate within the physiologic range (150–200 nmol/(100 g · min); Figs. 3A and 4A). Colchicine pretreatment resulted in a marked reduction in bile salt excretion during taurocholate infusion (Fig. 3A; $P < 0.0001$), but had no effect during infusion of taurodehydrocholate (Fig. 4A; $P = 0.882$). These findings were corroborated by measurements of bile flow (Figs. 3D and 4D); the increase in bile flow associated with taurocholate infusion was effectively eliminated by colchicine ($P < 0.0001$), whereas no inhibition of the taurodehydrocholate-induced choleresis was observed ($P = 0.792$).

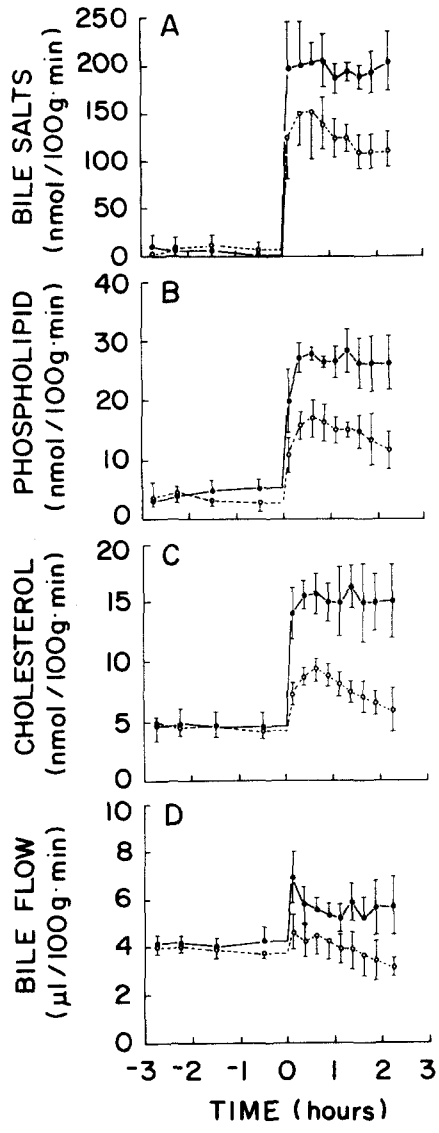

Fig. 3. Effect of colchicine pretreatment on biliary excretion of bile salts, phospholipid, and cholesterol during infusion of taurocholate in bile salt-depleted rats. Colchicine or lumicolchicine were injected i.v. into rats that had been subjected to 22 hr of biliary drainage. Two hours later, 2 μmol/100 g loading dose of taurocholate was administered (t = zero), followed by a continuous physiological infusion of taurocholate at 0.2 μmol/(100 g · min). Bile was collected continuously beginning 1 hr prior to colchicine or lumicolchicine injection (t = −3 hr). Bile flow and biliary excretion of bile salt, phospholipid, and cholesterol were measured; A) bile salt output; B) phospholipid output; C) cholesterol output; D) bile flow; (●—●) lumicolchicine-pretreated rats (n = 4); (○- - -○) colchicine-pretreated rats (n = 4).

Biliary phospholipid and cholesterol excretion increased dramatically with reinfusion of taurocholate (Figs. 3B and 3C) and, in the case of phospholipid, returned to measured baseline rates of excretion. As with bile salt excretion, colchicine pretreatment significantly impaired both phospholipid and cholesterol output ($P < 0.0001$). In contrast, there was only a minimal increase in phospholipid and cholesterol excretion following taurodehydrocholate infusion, and this was effectively inhibited by colchicine pretreatment (Figs. 4B and 4C; $P < 0.0001$).

To address the possibility that the effects of colchicine may have been attributable to impairment of hepatocyte processes (40) other than inhibition of microtubule assembly (25), we examined the effect of vinblastine (which inhibits microtubule dissembly (27)) on taurocholate-induced biliary lipid excretion. An experimental protocol identical to that shown in Fig. 3 was employed, substituting vinblastine for colchicine and using saline as a control (see Methods). The results obtained (**Fig.5**) were essen-

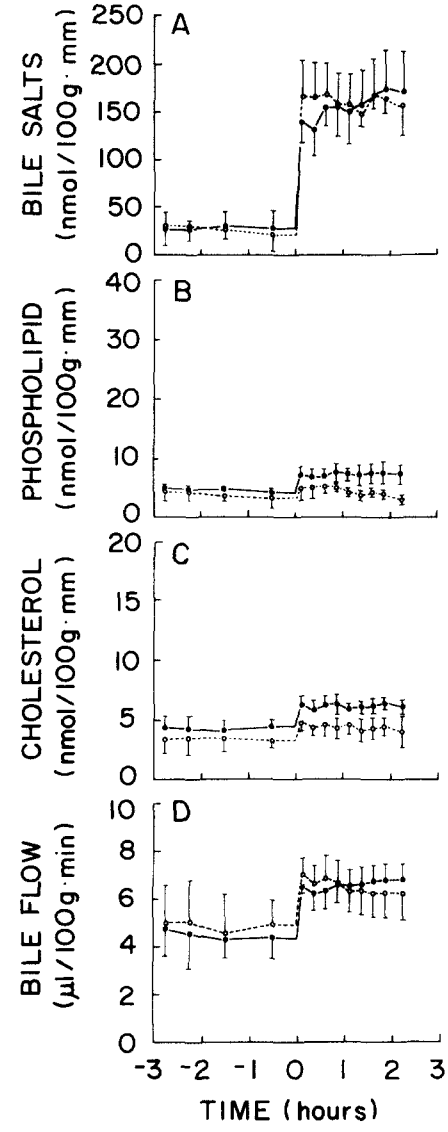

Fig. 4. Effects of colchicine pretreatment on biliary excretion of bile salts, phospholipid, and cholesterol during infusion of taurodehydrocholate in bile salt-depleted rats. Rats were subjected to the same experimental protocol described in the legend to Fig. 3, with infusion of the non-micelle-forming bile salt taurodehydrocholate at t = zero. A) Bile salt output; B) phospholipid output; C) cholesterol output; D) bile flow; (●—●) lumicolchicine-pretreated rats (n = 4); (○- - -○) colchicine-pretreated rats (n = 4).

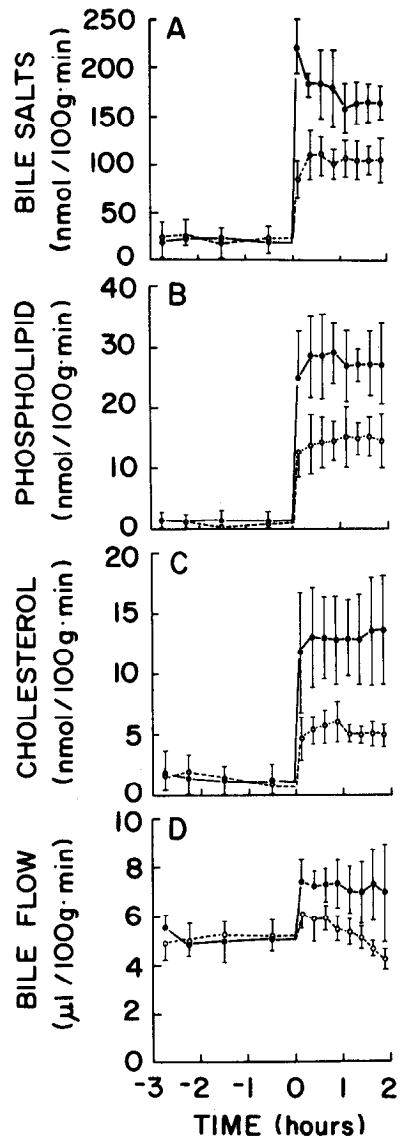

Fig. 5. Effects of vinblastine pretreatment on biliary excretion of bile salts, phospholipid, and cholesterol during infusion of taurocholate in bile salt-depleted rats. Vinblastine sulfate (1 mg/100 g) or saline was administered i.v. to rats that had been subjected to 22 hr of biliary drainage. Two hours later, a 2 μmol/100 g loading dose of taurocholate was administered (t = zero), followed by an infusion of taurocholate at 0.2 μmol/(100 g · min). Bile was collected continuously beginning 1 hr prior to vinblastine or saline injection (t = −3 hr). Bile flow and biliary excretion of bile salt, phospholipid, and cholesterol were measured. A) Bile salt output; B) phospholipid output; C) cholesterol output; D) bile flow; (●—●) saline-pretreated rats (n = 4); (○---○) vinblastine-pretreated rats (n = 4).

tially identical to those for colchicine (Fig. 3). Thus it is unlikely that the effects of colchicine are due to nonspecific effects on cellular function.

Having demonstrated a delay in the excretion of tracer taurocholate in colchicine-pretreated rats in the basal state, we reexamined the effect of colchicine on [^{14}C]taurocholate excretion in 24 hr-depleted rats, reinfused with a physiologic dose of unlabeled taurocholate. The same experimental protocol described in Fig. 3 was used to prepare the animals. Forty-five min after the infusion of unlabeled taurocholate wsa initiated, when rates of bile salt excretion had achieved steady-state in the physiologic range (Fig. 3), a bolus of [^{14}C]taurocholate was administered and biliary excretion of radiolabel was measured. In contrast to the modest effect of colchicine in basal rats (Fig. 1), colchicine pretreatment had a marked effect on the biliary excretion of radiolabel in these depleted/reinfused animals (**Fig. 6**). Maximal excretion rate was reduced from 17.1 ± 1.1 to 8.9 ± 1.2% administered dose/min ($P < 0.001$), and the time to maximal excretion was delayed from 2.5 ± 0 to 3.5 ± 0 min ($P < 0.01$; Fig. 6A); based on ANOVA the curves in Figs. 6A and 6B were significantly different ($P < 0.0001$). However, the cumulative recovery of radiolabel in bile 90 min after injection was identical in colchicine-pretreated and control animals (92 ± 2% administered dose; Fig. 6B); thus, delayed excretion of radiolabel from 8 to 90 min after administration of [^{14}C]taurocholate compensated for the decreased output during the initial excretory period (Fig. 6A).

Relationship of bile salt output and bile flow

Further verification that the influence of colchicine on bile salt excretion was not due to other effects on hepatocyte function emerged following analysis of the relationship of bile salt output to bile flow for all animals infused with a micelle-forming bile salt (**Fig. 7**). Specifically, we examined the possibility that colchicine altered either the underlying relationship of bile flow to bile salt excretion rate, or bile salt-independent bile flow mechanisms. All points shown in Fig. 7 are from time periods during which colchicine-induced inhibition of micelle-forming bile salt excretion was pronounced. A linear plot was obtained [bile flow (μl/(100 g · min)) = 13 × bile salt output (μmol/(100 g · min)) + 2.08; $r = 0.87$, n = 40]. There was no significant difference between the two groups [i.e., slope and y-intercept were comparable for lumicolchicine-pretreated (bile flow = 11 × bile salt excretion + 2.92; $r = 0.80$, n = 20) and colchicine-pretreated rats (bile flow = 14 × bile salt excretion + 1.58; $r = 0.86$, n = 20)]. This relationship was evident over the entire range of observations, for bile flow rates ranging from 2.3 to 10.2 μl/(100 g · min), and bile salt excretion rates from 115 to 560 nmol/(100 g · min). It is thus apparent that the effects of colchicine on the biliary excretion of micelle-forming bile salts are not associated with a change in the overall relationship of bile flow to bile salt excretion.

DISCUSSION

Hepatocellular transport and excretion of bile salts and biliary lipid is poorly understood and may involve complex interactions with hepatocyte components such as cytosolic proteins (3, 4), intracellular and plasma mem-

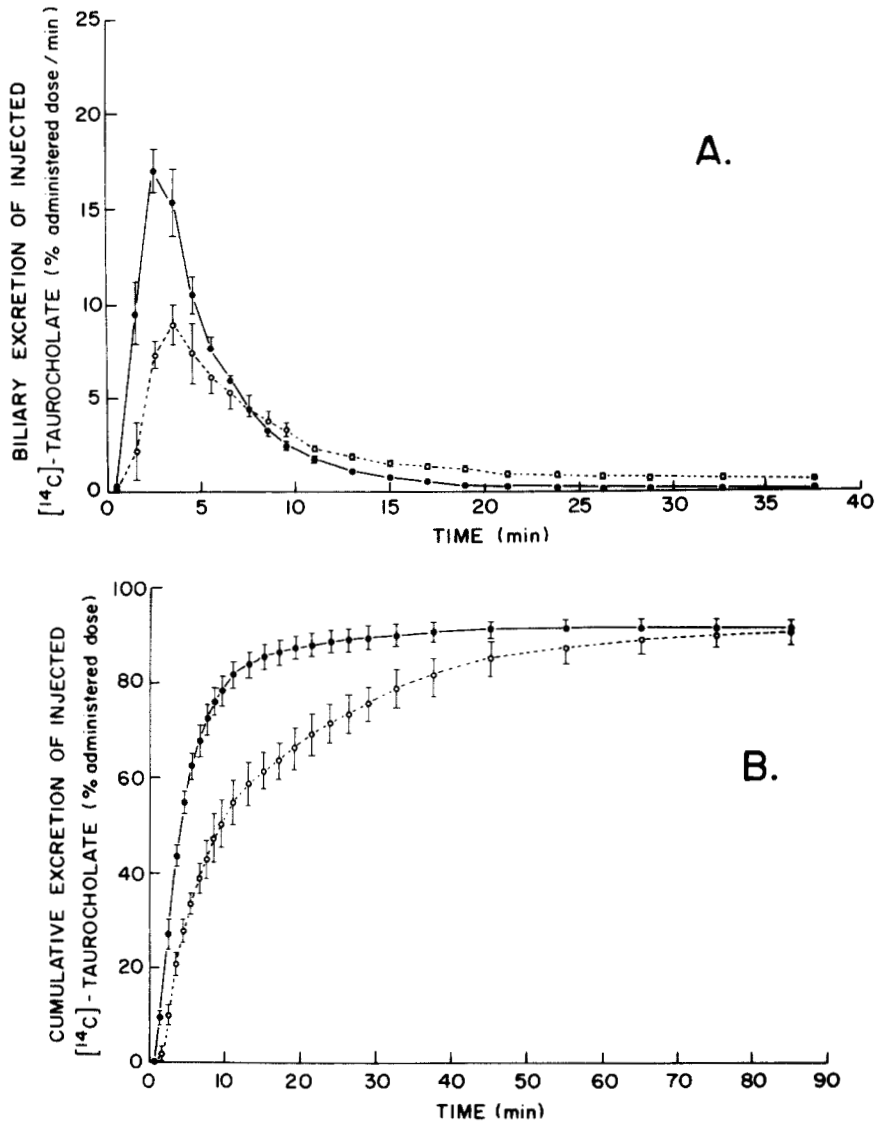

Fig. 6. Effect of colchicine pretreatment on biliary excretion of tracer [^{14}C]taurocholate during steady-state infusion of taurocholate in bile salt-depleted rats. Rats were subjected to 22 hr of biliary drainage, followed by an i.v. injection of colchicine (n = 4) or lumicolchicine (n = 4). Two hours later, a 2 μmol/100 g loading dose of taurocholate was administered i.v., followed by a physiologic infusion of taurocholate at 0.2 μmol/100 g · min (see Methods). Forty-five minutes after commencement of unlabeled taurocholate infusion (t = 0 min), 3 nmol of [^{14}C]taurocholate (0.15 μCi) was administered as a bolus, and bile was collected continuously for 90 min. Total bile salt excretion rates were comparable to those reported in Fig. 3 (data not shown). A) Biliary excretion of ^{14}C radioactivity plotted as % administered dose/min over the initial 40 min after injection; B) biliary excretion of radioactivity plotted as cumulative recovery of administered dose over the 90-min experimental period; (●—●) lumicolchicine-pretreated animals (control); (○- - -○) colchicine-pretreated animals.

branes (4, 41), and the cytoskeleton (13, 20, 23, 42, 43). It has been reported that pretreatment with the microtubule inhibitor, colchicine (25), does not affect the biliary excretion of tracer doses of taurocholate (23) or of phospholipid and cholesterol (43) in the basal state. In contrast, bile flow, and bile salt and lipid excretion are reduced by colchicine pretreatment in the presence of a taurocholate load (13, 20, 23). Biliary excretion of taurodehydrocholate, a non-micelle-forming bile salt (31), appears to be unaffected by colchicine, although inhibition of cholesterol excretion has been reported (13). Investigators have variously concluded that microtubules play either no role (13) or a contributory role (20, 23) in the delivery of bile salts to the canaliculus.

The present study provides a systematic documentation of the effects of microtubule dysfunction on the hepatic transport and biliary excretion of a series of physiologic bile salts and associated biliary lipid. The low dose of col-

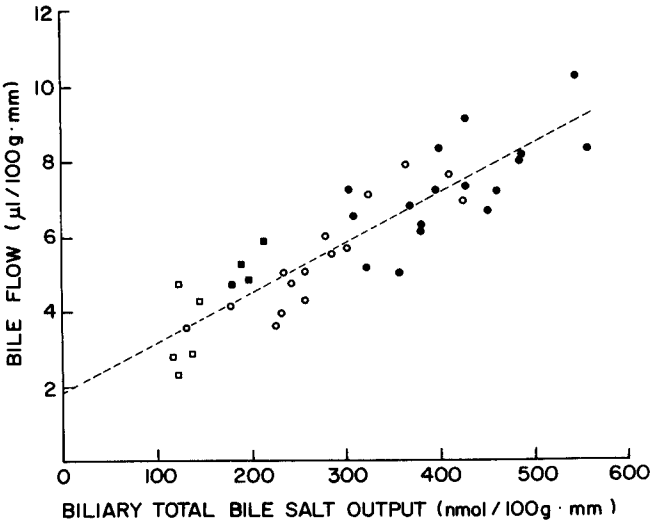

Fig. 7. Relationship of bile flow to total bile salt output in colchicine- and lumicolchicine-pretreated rats. Bile flow is plotted against bile salt output for all animals pretreated with lumicolchicine (closed symbols) or colchicine (open symbols) and administered a micelle-forming bile salt. Circles depict data from animals shown in Fig. 2, for samples obtained 1 min after peak bile salt output (typically 4–5 min after bile salt administration). Animals administered TDC, TCDC, TC, or TUDC are shown; lumicolchicine- (n = 16) and colchicine- (n = 16) pretreated animals. Squares indicate the steady-state values for bile flow and bile salt excretion following reinfusion of taurocholate into bile salt-depleted animals, for rats shown in Fig. 3; lumicolchicine- (n = 4) and colchicine- (n = 4) pretreated animals.

chicine employed to perturb microtubule function was well below maximal doses tolerated by intact rats (40), and is comparable to that employed in previous studies of bile salt/microtubule interactions (13, 20, 23, 42, 43). The virtually identical results obtained with vinblastine in selected experiments support the concept that these effects are due to microtubule disruption. Our findings indicate that the microtubular system plays a significant role in the hepatocellular transport of micelle-forming bile salts and of associated biliary lipid, particularly under conditions of enhanced bile salt flux through the liver.

Colchicine pretreatment had no measurable effect on the steady-state output of bile salts or phospholipid in bile under basal conditions. However, a slight but significant delay in the biliary excretion of tracer [^{14}C]taurocholate was observed consistently in basal rats (Fig. 1), suggesting that taurocholate transport and excretion is dependent, in part, on the microtubular system under basal conditions. In contrast, colchicine pretreatment had a marked effect on bile salt excretion in animals injected with 2 µmol/100 g of a series of micelle-forming bile salts (Fig. 2 and Table 1), a dose presumed to achieve portal blood levels similar to those in the postprandial state (38, 39). Interestingly, biliary excretion of all micelle-forming bile salts examined (taurodeoxycholate, taurochenodeoxycholate, taurocholate, and tauroursodeoxycholate) was impaired to a similar extent by colchicine pretreatment, despite graded differences in their hydrophobic/hydrophilic properties (35). On the other hand, the effect of colchicine pretreatment on excretion of the non-micelle-forming bile salt taurodehydrocholate was minimal and did not achieve statistical significance. Hence, micelle-forming ability per se was a dominant factor in the colchicine-induced inhibition of bile salt excretion under conditions of enhanced bile salt excretion (as would occur in the postprandial state).

In complementary studies, the importance of micelle-forming capability was strikingly demonstrated when the bile salt pool was largely replaced by overnight biliary drainage and reinfusion of either taurocholate or taurodehydrocholate (depleted/reinfused rats; Figs. 3, 4, and 5). The steady-state rate of bile salt excretion attained in lumicolchicine-pretreated (control) animals approximated that in basal rats, yet microtubule dysfunction induced by either colchicine (Fig. 3) or vinblastine (Fig. 5) impaired bile salt excretion only during infusion of taurocholate. Bile flow and phospholipid and cholesterol excretion appeared to be linked closely to taurocholate excretion, as reflected by their inhibition with colchicine pretreatment. However, bile salt excretion following taurodehydrocholate infusion was unaffected by colchicine, as was the observed choleresis (Fig. 4). The slight increase in phospholipid and cholesterol excretion associated with taurodehydrocholate infusion and its inhibition by colchicine pretreatment is consistent with the limited hydroxylation of taurodehydrocholate and formation of more hydrophobic derivatives which occur in vivo (31). These effects of microtubule disruption were observed at bile salt excretion rates that were comparable to those in basal animals, indicating that efficient excretion of a bile salt load superimposed on either the basal (Fig. 2) or bile salt-depleted state (Figs. 3 and 5) requires an intact microtubular system. This concept is supported by the marked colchicine-induced lag in the excretion of tracer [^{14}C]taurocholate in depleted/reinfused animals after attaining steady-state biliary function during bile salt reinfusion (Fig. 6). These findings also suggest that microtubule-independent mechanisms for bile salt excretion are markedly reduced in the hepatocyte by overnight biliary drainage.

The plot of bile flow and bile salt excretion rate for colchicine- and lumicolchicine-pretreated animals (Fig. 7) showed that all data points fell on the same line of identity with a y-intercept of 2 µl/(100 g·min) (i.e., presumed bile salt-independent flow). The derived slope of 12–13 µl of bile produced/µmol of bile salt excreted is consistent with reported literature values (44, 45). Thus, colchicine pretreatment does not influence the relationship of bile salt-dependent bile flow to bile salt excretion rate.

On the basis of these findings we conclude that microtubules are involved in the excretion of bile salts to only a minor extent under basal conditions, but assume increasing importance in the hepatocyte transport of a bile

salt load administered in the bile salt-depleted or in the basal state. The relative lack of effect of colchicine on delivery of both bile salts and phospholipid to bile in basal rats suggests that monomeric transport of bile salts predominates (13). However, the consistent, albeit slight, delay in excretion of radiolabeled taurocholate in basal rats pretreated with colchicine indicates at least some interaction with the microtubular system under such conditions. The delay in excretion is unlikely to be due to altered hepatic uptake, since only tracer amounts (3 nmol) of radiolabeled taurocholate were administered and the sinusoidal uptake capacity is reported to be 10- to 20-fold greater than the secretory rate maximum for bile salts (30, 46). Moreover, at comparable bile salt infusion rates in perfused rat liver there was no impairment of bile salt uptake by colchicine (20).

The data obtained from rats given a bile salt load, either in the basal state or after overnight biliary drainage, indicate that more complex interactions with the microtubule system become operative under these conditions. These effects of microtubular dysfunction are determined by the micelle-forming capabilities of the administered bile salts. O'Maille (30) has demonstrated that taurocholate and taurodehydrocholate compete effectively with one another during biliary excretion when infused at supramaximal rates, thus indicating that efficient excretion of these biles salts into bile does not depend on the micelle-forming capability per se. More recent evidence indicates that these two bile salts are excreted by a common canalicular transport system (31). In light of these observations, the fact that microtubule inhibitors only impair the delivery of micelle-forming bile salts to bile implicates an intracellular interaction of micelle-forming bile salts with the microtubular system. The possibility that colchicine-induced disruption of lipoprotein processing (28, 43) may affect intracellular availability of lipid cannot be excluded, and hence such effects may conceivably act in concert. However, the tight coupling of biliary lipid to bile salt excretion and their simultaneous inhibition by colchicine under these conditions supports the concept of intracellular cotransport of these moieties to the canaliculus.

These observations provide a rational basis for understanding the apparent dissociation between bile salt and biliary lipid excretion reported by some investigators. Poupon et al. (47) showed that cholesterol and phospholipid excretion in dogs was linearly related to that of bile salts at high infusion rates, but deviated from this relationship at low rates of infusion or in the basal state. Lowe, Barnwell, and Coleman (24) reported a delay in peak excretion of phospholipid and cholesterol relative to that of bile salts following a bolus of taurocholate in the perfused rat liver, while Rahman et al. (14) described an apparent threshold of bile salt excretion required to switch on the delivery of phospholipid to bile. Our data suggest that at least two mechanisms are functional in the hepatocellular transport of micelle-forming bile salts. Under basal conditions or following bile salt depletion, when microtubule-independent pathways predominate, biliary lipid is unlikely to be cotransported with bile salts. However, as the bile salt load increases, the microtubule-dependent pathway becomes increasingly operative, and cotransport of phospholipid and cholesterol becomes more apparent.

We speculate that the formation and microtubule-dependent translocation of intracellular vesicles may constitute the means by which micelle-forming bile salts interact with biliary lipid en route to canalicular excretion. The partitioning of bile salts into membranes is well documented (5, 6), and has potentially profound effects on the physical properties of membranes (48, 49). Indeed, bile salts may initiate the partial dissolution of intracellular membranes (50) and thereby provide the driving force for codelivery of bile salts and biliary lipid to the canalicular membrane for subsequent excretion into bile (14, 20). Morphologic studies documenting a marked increase in the density of pericanalicular vesicles during bile salt infusion (51) imply that bile salt-derived vesicles result from this process. The exact origin of these putative vesicles is uncertain, but may involve either the endoplasmic reticulum or the Golgi apparatus (5, 41, 51, 52). The present study better defines interactions between bile salts, biliary lipid, and microtubules, and thus contributes a functional framework for exploring the structural components involved in hepatocellular bile formation.

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