Interactions between organic anions, micelles and vesicles in model bile systems

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Biliary lipid secretion probably involves both ‘micellization’ and ‘vesiculation’ of bile-canalicular membrane lipids. Several hydrophilic organic anions inhibit the secretion of lipids into the bile without altering bile salt secretion [Verkade, Vonk and Kuipers (1995) *Hepatology* **21**, 1174–1189]. Hydrophobic organic anions do not interfere with biliary lipid secretion. We investigated whether the organic-anion-induced inhibition of biliary lipid secretion *in vivo* could be attributed to inhibition of micellization, by the application of *in vitro* models of micellization. Carboxyfluorescein was entrapped in a self-quenching concentration in small unilamellar vesicles (SUV) composed of cholesterol/egg phosphatidylcholine (molar ratios 0, 0.2 and 0.5). Certain organic anions clearly affected the bile-salt-induced release of fluorescence from these SUV, reflecting

interference with micellization. However, the effects of hydrophilic and hydrophobic organic anions did not correspond with their effects on biliary lipid secretion *in vivo*, irrespective of the bile salt species used (taurocholate, taurodeoxycholate or tauroursodeoxycholate) and of the lipid composition of the SUV. Ultracentrifugation and dynamic light-scattering studies indicated that organic anions do interact with bile salt/phosphatidylcholine/cholesterol mixed micelles, but that they do not inhibit micellization, for example by competing with phosphatidylcholine and/or cholesterol for incorporation into mixed micelles. In conclusion, the present *in vitro* data indicate that the *in vivo* mechanism of organic-anion-induced inhibition of biliary lipid secretion is not mediated by inhibition of micellization.

INTRODUCTION

Under physiological conditions, the biliary secretion of phospholipids [mainly phosphatidylcholine (PC)] and cholesterol is coupled to that of bile salts [1–3]. However, certain organic anions are able to inhibit the secretion of phospholipids and cholesterol dose-dependently without influencing the secretion of bile salts [4–11]. This effect of organic anions has become known as ‘uncoupling’, and has been reported in many species, including humans [12]. In a study in rats, we were able to demonstrate that only relatively hydrophilic organic anions induce uncoupling and that this effect is exerted after their secretion into the bile canaliculus [4,10]. The capacity of organic anions to interfere with biliary lipid secretion coincides with a specific elution profile of the organic anions upon gel-filtration chromatography of bile [4,10]. The relatively hydrophilic organic anions, such as ampicillin, sulphated tauroolithocholate (STLC) and bilirubin ditaurate, inhibit biliary lipid secretion and show high affinity for biliary bile salts (micelles) [4,10]. On the other hand, relatively hydrophobic organic anions such as Indocyanin Green (ICG) and Rose Bengal do not affect biliary lipid secretion in rats, and associate preferentially with phospholipid- and cholesterol-containing fractions of bile (vesicles) [4]. These studies provided strong indications that the induction of biliary lipid secretion by bile salts is exerted by bile salts present inside the bile canaliculus [3,10]. Accordingly, an interaction of bile salts with (domains of) the bile-canalicular membrane stimulates phospholipids and cholesterol to enter the bile canaliculus. Studies in a knock-out mouse model support the important role of canalicular bile salts

in the induction of lipid secretion [13,14]. Mice in which the *mdr2* gene is disrupted do not secrete phospholipids into bile, despite normal secretion rates of bile salt [14]. The *mdr2* gene encodes a bile-canalicular membrane P-glycoprotein [15], and present data strongly suggest that this protein functions as a PC-specific flippase [16–19]. Although the *mdr2* protein most likely accounts for the supply of biliary phospholipid to the outer leaflet of the bile-canalicular membrane, the molecular mechanism of the actual segregation of lipid molecules into the canaliculus has not been elucidated.

Micellar solubilization of bile-canalicular membrane lipids has been suggested as a mechanism for biliary lipid secretion for more than 20 years [20–25]. Since 1984 it has become clear that, in addition to mixed micelles, cholesterol/phospholipid vesicles are present in bile, either transiently during the initial phases of bile formation, such as in unsaturated rat bile [26–28], or permanently, as in supersaturated human bile [29–31]. It has been suggested that bile salts induce the biliary secretion of lipids both by (direct) solubilization of canalicular membrane lipids into mixed micelles (‘micellization’) [32] and by stimulation of a ‘pinching-off’ process from the bile canalicular membrane, which would explain the presence of vesicles in the bile (‘vesiculation’) (for a review, see [25]). Presently available data suggest that these two processes, i.e. micellization and vesiculation, both occur *in vivo* [14,33]. However, the relative contribution of each process under physiological circumstances has not been clarified.

An attractive explanation for the organic-anion-induced uncoupling of biliary lipid secretion [4] involves disturbance of the micellization process. One might hypothesize that incor-

Abbreviations used: BSP, bromosulphthalein; CF, carboxyfluorescein; DBSP, dibromosulphthalein; ICG, Indocyanin Green; PC, phosphatidylcholine; STLC, sulphated tauroolithocholate; SUV, small unilamellar vesicles; TDOC, taurodeoxycholate.

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poration of organic anion molecules into the micelles impairs the solubilization capacity (detergent activity) of these bile salt/organic anion hybrids/micelles. In the present study, we have investigated this hypothesis using two different *in vitro* model systems. First, we analysed the membrane-perturbing effects of bile salts towards phospholipid/cholesterol vesicles in the presence or absence of various organic anions. The apparent detergent activity of bile salts towards (model) membranes has been recognized previously [24,34–38]. We hypothesized that, if the organic anion effect *in vivo* involved inhibition of micellization, one would also expect a decreased detergent activity of bile salt/hydrophilic organic anion mixtures *in vitro*. This hypothesis was tested in model systems that have specifically been demonstrated to mimic micellization [39]. Secondly, a decrease in the detergent (micellization) activity of bile salt micelles in the presence of organic anions would theoretically also have implications for the distribution of PC and cholesterol in micelles and vesicles in model bile. In the presence of an organic anion which inhibits the detergent capacity of bile salts, the distribution of lipids would be expected to shift towards the vesicular form relative to the micellar form. The distribution of lipids in the micellar compared with the vesicular form in the presence or absence of various organic anions was therefore also tested in the present study, using ultracentrifugation and dynamic light-scattering analyses. Our results obtained with *in vitro* systems indicate that inhibition of bile-salt-induced micellization of membrane lipids does not play a major role in the organic-anion-induced inhibition of biliary lipid secretion *in vivo*.

MATERIALS AND METHODS

Chemicals

Egg-yolk PC (type V-E), cholesterol (type CH-S), taurodeoxycholate (TDOC), taurooursodeoxycholate, dibromosulphthalein (DBSP), bromosulphthalein (BSP), Rose Bengal, Methylene Blue, STLC, ICG and Hepes were obtained from Sigma (St. Louis, MO, U.S.A.). Ampicillin was from Beecham Farma B. V. (Amstelveen, The Netherlands), bilirubin ditaurate and carboxyfluorescein (CF) were from Calbiochem (La Jolla, CA, U.S.A.), and Sepharose 4B was from Pharmacia (Woerden, The Netherlands).

Liposomes

For the preparation of liposomes, aliquots of stock solutions of PC and cholesterol in chloroform/methanol (4:1, v/v) were mixed in different molar ratios (cholesterol 0%, 20% or 50%). The solvent was evaporated under a stream of nitrogen and the lipid film was dissolved and lyophilized. To prepare small unilamellar vesicles (SUV), 1.0 ml of Hepes (10 mM)/NaCl (135 mM), pH 7.4, with or without CF (100 mM), was added to 10 mmol of dried lipid and vigorously vortexed. The resulting suspension was sonicated for 15 min with a probe-type sonifier (Branson B12, output 70 W) under a stream of nitrogen. The optically clear suspension was centrifuged for 15 min at 10000 *g* to remove titanium particles and large liposomes. SUV in which CF was entrapped were chromatographed over a (pre-cast) Sephadex-25 column (elution buffer 10 mM Hepes/135 mM NaCl, pH 7.4), and collected in the void fraction in order to separate them from free CF. Fluorescence measurements in the absence and presence of the detergent Triton X-100 (0.05%, v/v) confirmed that the entrapped CF was present in self-quenching concentrations (> 95%).

CF-release assay

The effects of bile salts and organic anions on the integrity of SUV were determined by measuring the release of CF by means of fluorescence (excitation, 492 nm; emission, 516 nm) using a 3000 fluorimeter (Perkin-Elmer, Gouda, The Netherlands). Stock solutions of TDOC and organic anions were mixed in appropriate amounts with buffer to obtain final TDOC concentrations of 0, 0.1, 0.5, 1.0, 2.0 and 5.0 mM, with a fixed organic anion concentration of 5 mM. After 15 min at room temperature, CF-loaded SUV were added during light vortexing (final lipid concentration approx. 0.5 mM). After another 15 min, fluorescence was determined in a 10-fold diluted sample. This value of fluorescence was divided by that of the same sample after addition of Triton X-100 (final concentration 0.05%, v/v), to obtain the percentage of leakage of CF from the liposomes. All determinations were performed in triplicate.

Similar experiments were performed with SUV which were prepared with phospholipids and cholesterol from rat bile. For this purpose, bile was collected from Wistar rats with a permanent bile fistula (8 days after interruption of the enterohepatic circulation). Bile was protected from light and extensively dialysed against Hepes/NaCl (10 mM/135 mM, pH 7.4) at 4 °C (cut-off 3000 Da). The dialysis procedure was continued until the bile acid concentration was lower than 0.5 mM. CF was added to the dialysed rat bile, which was subsequently sonicated and chromatographed as described above.

The kinetics of the membrane-perturbing effects of bile salts and organic anions were also analysed by a CF-release assay. Fluorescence was measured every 10 s for a period of 600 s, immediately after addition of CF-containing SUV to bile salt/organic anion mixtures. For these experiments, SUV composed of 20 mol% cholesterol/80 mol% PC were used. Final concentrations were 0.5 mM for the bile salts (TDOC), 0 or 5 mM for each organic anion (ampicillin, STLC), and 23 µmol of SUV lipid.

Ultracentrifugation

Ultracentrifugation was applied to study the effects of organic anions on the density distribution of SUV, in the presence or absence of organic anions and with or without preincubation with TDOC. In the first experiment, SUV consisting of PC and cholesterol (molar ratio 4:1) were incubated with ampicillin, STLC or Rose Bengal (final concentration 5 mM) for 15 min at room temperature, and then loaded on top of a NaBr gradient and centrifuged for 24 h, as described in [40]. After centrifugation, the gradient was divided into six fractions of increasing density for determination of PC and cholesterol concentrations. In a second experiment, SUV composed of PC and cholesterol (molar ratio 1:1) were incubated for 15 min with 2 mM TDOC in the absence of organic anions or in the presence of 5 mM ampicillin, STLC or Rose Bengal, loaded on the bottom of a NaBr gradient, and centrifuged for 24 h [40]. After centrifugation, the gradient was divided into six fractions of increasing density for determination of bile salts, PC and cholesterol concentrations.

Dynamic light scattering

Model bile solutions, consisting of taurocholate (20 mM), phospholipid (3 mM) and cholesterol (0.3 mM) in Hepes/NaCl buffer (135 mM/10 mM, pH 7.4), were incubated for 15 min with bilirubin ditaurate, BSP, Rose Bengal, DBSP or STLC. The final concentration of the organic anion upon incubation was

5 mM or 0 mM (control). After incubation, samples were diluted 10-fold with Hepes/NaCl buffer (time zero), after which the mean particle size of the solution was determined at certain time points. The *z*-average particle size was determined by a Malvern 4700 system using a 25 mW He-Ne laser and Automeasure version 3.2 software (Malvern Ltd., Malvern, U.K.). To account for viscosity and refractive index, the dilution buffer (10 mM Hepes/135 mM NaCl, pH 7.4) was used as blank. Apart from the *z*-average particle size, the polydispersity value was also analysed at each time point, as a measure of the size homogeneity of the dispersion.

RESULTS

CF-release assays

Figure 1 shows CF release from SUV at different concentrations of TDOC in the absence or presence of various organic anions. In the absence of organic anions, TDOC induced CF release as a function of its concentration (Figure 1, upper panel). The sigmoid-shaped relationship between TDOC concentration and

CF release is similar to relationships described previously between bile salt concentration and cell membrane damage in erythrocytes [34] and CaCo-2 cells [35]. The inclusion of increasing amounts of cholesterol into the SUV membranes led to less release of CF upon incubation with TDOC (Figure 1, upper panel). Virtually identical values for CF release were observed when the TDOC solutions were preincubated with 5 mM ampicillin (results not shown). Ampicillin is a relatively hydrophilic organic anion which is able to inhibit the secretion of PC and cholesterol *in vivo* [4]. Preincubation with a relatively hydrophobic organic anion, ICG, which does not interfere with biliary lipid secretion *in vivo* [4], dramatically altered CF release: in SUV with 0% or 20% cholesterol, more than 90% of CF was released at a TDOC concentration of 0.1 mM. Additional experiments showed that the same percentage of CF leakage was observed in the absence of TDOC (results not shown). SUV composed of 50% cholesterol were much more resistant to ICG, both in the presence and in the absence of TDOC (Figure 1, lower panel). The effects of STLC, a relatively hydrophilic uncoupling organic anion, resembled those of ICG (results not shown). When the various results with 50%-cholesterol SUV are compared (Figure 1, lower panel), it becomes apparent that ICG and STLC induce more CF release at TDOC concentrations below 1 mM ($P < 0.001$), but at TDOC concentrations above 1 mM these organic anions protect the SUV membrane to some extent.

The effects of the various organic anions appeared to be independent of the bile salt used, since similar results were obtained in studies in which either taurocholate or urso-deoxycholate was employed instead of TDOC. The only major difference observed in these studies was the expected shift towards the need for higher concentrations of the bile salt for CF release, in accordance with the differences in critical micellar concentration of these bile salts (results not shown).

In order to evaluate whether the observed divergence between the earlier *in vivo* data (rats) and the present *in vitro* data is attributable to differences between bile and SUV with respect to lipid composition, similar experiments were performed with SUV composed of rat bile lipids (Figure 2). The effects of TDOC and organic anions on CF release from these bile-type SUV were

Figure 1 Sensitivity of SUV to TDOC in the absence and presence of organic anions

CF-loaded SUV consisting of PC (■) or PC plus cholesterol (Chol) (△, 4:1 molar ratio; ○, 1:1 molar ratio) were incubated for 15 min at room temperature with TDOC in the absence of organic anions (upper panel). Lower panel: effects of various organic anions (5 mM) on CF leakage from SUV composed of PC plus cholesterol (1:1 molar ratio): ■, absence of organic anions; ○, ampicillin; ▲, STLC; △, ICG. The effects of the organic anions on SUV composed of PC and of PC plus cholesterol (4:1 molar ratio) are described in the Results section. Membrane perturbation was monitored by determining CF leakage, which was expressed as the fraction of the maximal leakage obtained after addition of Triton X-100 (final concentration 0.05%). Data are means $\pm$ S.D. from three independent SUV preparations.

Figure 2 Sensitivity of SUV consisting of rat bile lipids to TDOC in the absence and presence of organic anions

CF-loaded SUV consisting of rat bile lipids were incubated for 15 min at room temperature with TDOC in the absence of organic anions (■) or in the presence of 5 mM ampicillin (○), STLC (▲) or ICG (△). Membrane perturbation was monitored by determining CF leakage, which was expressed as a fraction of the maximal leakage obtained after addition of Triton X-100 (final concentration 0.05%). Data are means $\pm$ S.D. from three experiments.

Figure 3 Time-dependent release of CF from SUV induced by TDOC in the absence and presence of organic anions

CF-loaded SUV consisting of PC plus cholesterol (molar ratio 4:1) were added at time zero to 135 mM Hepes/10 mM NaCl buffer (pH 7.4) containing 0.5 mM TDOC in the absence (○) or presence of 5 mM STLC (▲) or 5 mM ampicillin (▽). CF-derived fluorescence (arbitrary units) was determined every 10 s.

very similar to those observed with the 20%-cholesterol SUV composed of commercially obtained lipids. This finding indicates that lipids from the rat bile used (89% phospholipids, predominantly PC, and 11% cholesterol) behaved in accordance with their gross lipid composition when assembled in the form of CF-loaded SUV and that potentially co-isolated biliary-lipid-associated protein(s) do not interfere under the conditions used.

We then studied whether the membrane curvature of the liposomes affected the release of entrapped CF. A set of studies was performed using identical concentrations of (biliary) lipids, organic anions and TDOC, but employing multilamellar instead of unilamellar vesicles. The molecular packing of lipids in multilamellar vesicles is more constrained than in SUV, in which the curvature of the outer lipid layer more readily allows a (partial) intercalation of other molecules. However, similar CF-release curves were obtained with CF-loaded multilamellar vesicles and SUV (results not shown).

In the studies described so far, bile salts and organic anions were incubated with the SUV for 15 min, after which fluorescence was analysed in a 10-fold-diluted sample. *In vivo*, the 'incubation time' of bile salts/organic anions with the canalicular membrane is more likely of the order of seconds. We therefore investigated whether a difference existed in the rate at which the various bile

salts/organic anions perturbed the SUV membranes (Figure 3). When SUV were added to 0.5 mM TDOC, a hyperbolic increase in the fluorescence signal was observed in the subsequent 10 min, reflecting a gradual perturbation of the available SUV membranes. The additional presence of 5 mM ampicillin did not significantly affect the rate at which the fluorescence signal increased. However, the combination of 0.5 mM TDOC and 5 mM STLC led to a very rapid release of fluorescence, reaching a plateau within 90 s. The plateau level reached was identical with the fluorescence measured after addition of Triton X-100 (final concentration 0.05%, v/v) to the reaction mixture, reflecting complete perturbation of the available membranes. Similar results were obtained when taurocholate or tauroursodeoxycholate was used instead of TDOC.

Ultracentrifugation

In order to explore the mechanism by which (some of) the organic anions induced and/or promoted the release of CF from liposomes, SUV were ultracentrifuged in the absence or presence of the organic anions and in the absence of bile salts (Table 1). When vesicles containing 20% cholesterol were layered on top of a NaBr solution [40] and centrifuged for 24 h, no redistribution of the lipid components in the direction of higher densities was observed. A very similar result was obtained when 5 mM ampicillin was present in the original vesicle fraction, but in the case of STLC the mass of vesicular PC and cholesterol was recovered in the bottom fractions of the gradient (Table 1).

The effects of TDOC and organic anions on lipid vesicles were also studied by ultracentrifugation. SUV containing 20% cholesterol were incubated with 2 mM TDOC in the absence or presence of organic anions, and loaded at the bottom of a NaBr gradient to ensure a good separation of micelles and vesicles (Table 2). In the absence of organic anions, ultracentrifugation revealed a distribution of TDOC, PC and cholesterol over the vesicular and micellar fractions. In accordance with previous studies on the ultracentrifugation of (model) biles, the vesicular fraction was relatively enriched in cholesterol compared with the micellar fraction [40]. A very similar lipid distribution was observed when ampicillin was present during the initial incubation of TDOC and liposomes. However, upon co-incubation with either STLC or Rose Bengal, TDOC and a considerable fraction of the cholesterol and phospholipids were recovered in the fractions with the highest densities (Table 2). Light-microscopic analysis of these fractions revealed the presence of amorphous precipitates (results not shown). Incubation of SUV (either 0% or 50% cholesterol) with 5 mM ICG for 15 min at room temperature did not induce precipitation (results not shown).

Table 1 Density-gradient ultracentrifugation of SUV in the absence or presence of organic anions

SUV, composed of PC plus cholesterol (4:1 molar ratio), were incubated for 2 h at 37 °C without organic anions (control) or with ampicillin or STLC, layered on top of a NaBr gradient, and centrifuged for 24 h [40]. After centrifugation, the gradient was divided in six fractions of increasing density, and the concentrations of PC and cholesterol were determined in each fraction.

Anion	Component	Fraction ...	Concentration (mmol/l)					
			1 (top)	2	3	4	5	6 (bottom)
Control	PC		1.20	0.02	0.02	0.01	0.01	0.01
	Cholesterol		0.13	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Ampicillin	PC		1.04	0.08	< 0.01	0.01	0.02	0.08
	Cholesterol		0.13	0.01	< 0.01	< 0.01	< 0.01	0.01
STLC	PC		0.05	0.02	0.02	0.05	0.55	0.97
	Cholesterol		< 0.01	0.01	0.03	< 0.01	0.08	0.13

Table 2 Density-gradient ultracentrifugation of SUV, after preincubation with TDOC in the absence or presence of organic anions

SUV, composed of PC plus cholesterol (1:1 molar ratio), were incubated for 15 min with 2 mM TDOC in the absence of organic anions (control) or in the presence of 5 mM ampicillin, STLC or Rose Bengal, loaded on the bottom of a NaBr gradient, and centrifuged for 24 h [40]. After centrifugation, the gradient was divided in six fraction of increasing density, and the concentrations of bile salts, PC and cholesterol were determined in each fraction.

Anion	Component	Fraction ...	Concentration (mmol/l)					
			1 (top)	2	3	4	5	6 (bottom)
Control	Bile salts		< 0.01	< 0.01	0.10	1.12	0.28	0.20
	PC		0.38	0.30	0.06	0.40	0.03	0.02
	Cholesterol		0.29	0.39	0.05	0.06	< 0.01	< 0.01
Ampicillin	Bile salts		< 0.01	< 0.01	0.06	1.10	0.32	0.21
	PC		0.45	0.39	0.08	0.49	0.04	0.02
	Cholesterol		0.32	0.41	0.07	0.07	< 0.01	0.15
STLC	Bile salts		< 0.01	< 0.01	0.08	0.73	0.62	0.90
	PC		0.33	0.20	0.13	0.35	0.13	0.23
	Cholesterol		0.22	0.20	0.04	0.07	0.09	0.37
Rose Bengal	Bile salts		< 0.01	0.02	0.01	0.03	0.05	1.57
	PC		0.29	0.18	0.01	0.01	0.04	0.47
	Cholesterol		0.17	0.20	0.01	0.01	0.01	0.12

Figure 4 Change in mean particle size of model bile solutions, as determined by dynamic light scattering, in the presence and absence of organic anions after 10-fold dilution at time zero

Model bile solutions, consisting of taurocholate (20 mM), phospholipid (3 mM) and cholesterol (0.3 mM) in 135 mM Hepes/10 mM NaCl buffer (pH 7.4), were incubated for 15 min with bilirubin ditaurate (BDT), BSP, Rose Bengal (RB), DBSP or STLC. The final concentration of the organic anion upon incubation was 5 mM or 0 mM (control). After incubation, samples were diluted 10-fold with Hepes/NaCl buffer (time zero), after which the mean particle size in the solution was determined at the time points indicated. For methodological details, see the Materials and methods section.

Dynamic light scattering

The effects of organic anions on lipid transfer from micelles to vesicles was studied by means of dynamic light scattering (Figure 4). The generation of vesicles from an equilibrated mixed micellar solution was induced by 10-fold dilution [41,42]. The process was monitored by repeated estimation of the mean particle size in the solution for up to 48 h after dilution. The mean particle size of control bile increased in a hyperbolic fashion from below 10 nm at time zero to 65 nm at 300 min. The presence of either ampicillin (results not shown) or bilirubin ditaurate in the model bile solution did not affect this increase. However, the increase in mean particle size was completely prevented by BSP, DBSP and

Rose Bengal. Values after 300 min were similar to those 24 and 48 h after dilution.

DISCUSSION

We describe an *in vitro* approach to unravelling the molecular mechanism by which organic anions inhibit bile-salt-induced biliary lipid secretion, making use of knowledge obtained from *in vivo* studies [3,4,10,13,43]. As discussed in the Introduction section, biliary lipid secretion seems to involve both micellization and vesiculation. In the present study, we designed an *in vitro* model which primarily involves micellization. Using this model, we investigated whether the organic-anion-induced inhibition of biliary lipid secretion *in vivo* might be attributed to inhibition of micellization. We rationalized that, if disturbance of micellization represents the major mechanism of organic-anion-induced inhibition of biliary lipid secretion *in vivo*, hydrophilic organic anions would inhibit the membrane-perturbing (detergent) effects of bile salts *in vitro*. Similarly, hydrophobic organic anions would not interfere with membrane perturbation *in vitro*, since these anions do not inhibit bile-salt-induced lipid secretion *in vivo* [3,4].

As a first experimental model to study the effects of bile salts on membranes, the technique of CF-loaded liposomes was used. Walde et al. [39] concluded that taurocholate-induced release of CF from PC liposomes coincided with the formation of PC/taurocholate mixed micelles. This phenomenon was only observed at concentrations of taurocholate at or above its critical micellar concentration. It therefore seems reasonable to regard bile-salt-induced release of CF from liposomes as an *in vitro* model for micellization of membrane components.

In the present study we concentrated on those organic anions whose *in vivo* effects have been described and which are known to be secreted into bile predominantly in unmetabolized form [4,10]. A dose-dependent effect of TDOC on membrane perturbation was observed, in accordance with previous data on the effects of bile salts on membranes (liposomes, erythrocytes, Caco-2, bile-canalicular membranes) [24,34–38] and with the micellization of membrane lipids at TDOC concentrations above its critical micellar concentration (Figure 1). When either ursodeoxycholate or taurocholate was used, CF release was also closely related to the critical micellar concentrations of these bile

salt species (results not shown). If organic anions such as ampicillin and STLC exert their effects *in vivo* by inhibition of bile-salt-induced micellization, inhibition of bile-salt-induced CF release would be expected on co-incubation with these organic anions. However, preincubation of either the liposomes or the bile salt with ampicillin did not affect the TDOC-induced CF release. Although STLC partially prevented CF release from liposomes with high cholesterol content at high TDOC concentrations, it stimulated CF release at low TDOC concentrations and, more importantly, also in liposomes made from bile-derived lipids. In addition, ICG (which, in contrast with STLC, does not influence biliary lipid secretion *in vivo*) behaved similarly to STLC in the various *in vitro* models used.

Discrepancies between effects observed *in vivo* and those with the *in vitro* model system may be due to our model system being inappropriate, despite previously published indications as to its validity (see discussion above, and [39]). To investigate this possibility, we performed a number of control experiments in which various characteristics of the *in vivo* process were included into our *in vitro* model. We focused on the lipid composition and structures of the liposomes used. However, the observed *in vitro* effects could not be accounted for by the PC/cholesterol molar ratio of the membranes. In accordance with earlier data on cellular membranes [36,44,45], liposomes in which the cholesterol content was increased appeared to be more resistant to TDOC. However, even up to equimolar PC/cholesterol ratios, the effects of the various organic anions *in vitro* did not correspond with those observed *in vivo*. The strongly curved bilayer structure of SUV was apparently not responsible for the discrepancy either, as similar data were obtained with multilamellar vesicles. In another control experiment, rat bile was dialysed against a bile-salt-free buffer (molecular mass cut-off 3000 Da) to obtain bile-type lipids and potentially important factors which would be co-isolated, such as vesicle-associated proteins [46–49]. However, CF-loaded SUV prepared from this dialysate reacted in a similar fashion to bile salts and organic anions as did the 20 %-cholesterol liposomes, suggesting similar phenomena in liposomes prepared from commercially obtained lipids or from rat bile lipids. Since the composition of the lipids that are secreted into the bile is not identical with that of the bile-canalicular membrane [3,25], the theoretical possibility that the model system used is inappropriate cannot be fully excluded. However, the results of the various control experiments do not support this possibility.

Although organic anions did not influence bile-salt-induced CF release by interfering with micelle formation, a clear interaction was nevertheless observed. The more hydrophobic organic anions such as ICG and Rose Bengal are probably incorporated into the vesicle membrane, as indicated by the leakage of CF induced in the absence of bile salt. At high organic anion/lipid ratios the hydrophobic organic anions induced the formation of high-density aggregates. It is tempting to speculate that this form of precipitation can be of pathophysiological importance, as cholestasis has been described *in vivo* after injection of these organic anions [4,50].

Although our *in vitro* model of micellization showed significant differences from the *in vivo* effects of bile salts and organic anions, the presence of organic anions clearly affected the interaction of bile salts with vesicles. Cholesterol-rich vesicles incubated with high concentrations of TDOC became more resistant to membrane perturbation in the presence of ICG or STLC. It could be that the organic anions are more readily incorporated into cholesterol-rich membranes, resulting in a decreased susceptibility towards bile salt micelle-induced perturbation. Theoretically, the interaction of the organic anion molecules with the bile salt micelles could reduce the affinity of

the latter for cholesterol-rich vesicles. According to this explanation, the results of the experiments using the 100 %-PC and the 20 %-cholesterol/80 %-PC vesicles could reflect the relative affinities of various organic anions towards bile salt micelles or phospholipid (-cholesterol) vesicles. Alternative explanations are also possible, including the theoretical inappropriateness of the model system as discussed above.

A second approach to studying the hypothetical inhibition of micellization by hydrophilic organic anions involved determination of the lipid distribution in the vesicular and micellar phases in the presence or absence of various organic anions. With an organic anion/bile salt ratio of 2.5 (Table 2), no shift of lipids towards the vesicular fraction was induced by the hydrophilic organic anions ampicillin and STLC, as determined by ultracentrifugation. *In vivo*, the inhibitory effect on biliary lipid secretion is observed at biliary organic anion/bile salt ratios greater than 0.10 [4]. Vesicle formation can be induced by dilution of a micellar solution [41,42]. The presence of micelles and vesicles can be inferred by measuring the mean particle size and the polydispersity of a solution by dynamic light scattering [26,27,51–53]. If hydrophilic organic anions inhibit the micellization of bile salt micelles, we would expect the rate of vesicle formation on dilution of a micellar solution to be increased, or at least to be similar to that on dilution of a micellar solution in the absence of organic anions. Interestingly, dilution of an initially micellar solution in the presence of organic anions such as BSP, Rose Bengal or STLC completely inhibited the formation of vesicles, as demonstrated by dynamic light scattering (Figure 4). Dilution of control bile resulted in a time-dependent increase in mean particle size to approx. 60–75 nm, in accordance with the vesicle size described in studies *in vivo* and *in vitro* using bile-type lipids [26,41,42]. However, with both hydrophilic (DBSP, STLC) and hydrophobic (Rose Bengal, BSP) organic anions, no increase in particle size was observed in the time period studied. For STLC and Rose Bengal, the results are in accordance with those obtained in the ultracentrifugation studies (Table 2), and again indicate that these organic anions are capable of forming small-sized, high-density aggregates with cholesterol and phospholipids. Although these observations do not elucidate the mechanism of the organic-anion-induced inhibition of biliary lipid secretion *in vivo*, the data do indicate that organic anions interfere with bile salt–lipid complexes under non-steady-state conditions, and may actually interfere with vesicle formation.

In conclusion, the present experiments indicate that the effects of organic anions on biliary lipid secretion *in vivo* are unlikely to be based on the inhibition of micellization of bile-canalicular membrane lipids. Rather, additional factors must be operational *in vivo* that are responsible for the apparently specific regulation of lipid secretion. Although the present data only give indirect indications, it seems reasonable to suggest that both biliary lipid secretion and its inhibition by organic anions *in vivo* predominantly involve vesiculation. The present data also indicate that several organic anions interact with bile-type lipids and that they influence the (re-)distribution of lipids in vesicle and micelle fractions in non-equilibrated model bile.

We are indebted to Bart Goldhoorn and Ingrid Zijlstra for their invaluable assistance with the experiments. H.J.V. is a Clinical Research Fellow of the Dutch Foundation for Scientific Research (N.W.O., grant K.W.O. 900-716-028). F.K. is an established investigator of the Netherlands Heart Foundation.

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