Sphingomyelin exhibits greatly enhanced protection compared with egg yolk phosphatidylcholine against detergent bile salts[1]

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Abstract **Inclusion of phosphatidylcholine within bile salt micelles protects against bile salt-induced cytotoxicity. In addition to phosphatidylcholine, bile may contain significant amounts of sphingomyelin, particularly under cholestatic conditions. We compared protective effects of egg yolk phosphatidylcholine (similar to phosphatidylcholine in bile), egg yolk sphingomyelin (mainly 16:0 acyl chains) and dipalmitoyl phosphatidylcholine against taurocholate in complementary in vitro studies. Upon addition of taurocholate-containing micelles to sonicated egg yolk phosphatidylcholine vesicles, subsequent micellization of the vesicular bilayer proved to be retarded when phospholipids had also been included in these micelles in the rank order: egg yolk phosphatidylcholine < dipalmitoyl phosphatidylcholine < sphingomyelin. Hemolysis of erythrocytes and LDH release by CaCo-2 cells after addition of taurocholate micelles were strongly reduced by including small amounts of sphingomyelin or dipalmitoyl phosphatidylcholine in these micelles (PL/(PL + BS) ⩾ 0.1), whereas egg yolk phosphatidylcholine provided less protection. Amounts of non-phospholipid-associated bile salts (thought to be responsible for cytotoxicity) in egg yolk phosphatidylcholine-containing micelles were significantly higher than in corresponding sphingomyelin- or dipalmitoyl phosphatidylcholine-containing micelles (tested at PL/(PL + BS) ratios 0.1, 0.15, and 0.2). LDH release upon incubation of CaCo-2 cells with taurocholate simple micelles at these so-called "intermixed micellar-vesicular" concentrations was identical to LDH release upon incubation with corresponding taurocholate-phospholipid mixed micelles. In conclusion, we found greatly enhanced protective effects of sphingomyelin and dipalmitoyl phosphatidylcholine compared to egg yolk phosphatidylcholine against bile salt-induced cytotoxicity, related to different amounts of non-phospholipid-associated bile salts. These findings may be relevant for protection against bile salt-induced cytotoxicity in vivo.**—Moschetta, A., G. P. vanBerge-Henegouwen, P. Portincasa, G. Palasciano, A. K. Groen, and K. J. van Erpecum. **Sphingomyelin exhibits greatly enhanced protection compared with egg yolk phosphatidylcholine against detergent bile salts.** ***J. Lipid Res.*** **2000.** 41: **916–924.**

Supplementary key words bile salts • CaCo-2 cells • erythrocytes • intermixed micellar-vesicular bile salt concentration • micelles • phosphatidylcholine • phospholipids • sphingomyelin • vesicles

Bile salts are amphiphilic compounds that act as detergents above their critical micellar concentration. The cytotoxic effect of bile salts has been shown for hepatocytes (1, 2), erythrocytes (2–4), and mucosa of various organs including stomach (5), intestine (6), and gallbladder (7, 8). The damaging effects of bile salts depend on their degree of hydrophobicity (9) and on the cell membrane composition (10). At physiological concentrations, in bile in the gallbladder and bile ducts and within the intestinal lumen, bile salts are associated with phospholipids and cholesterol in mixed micellar structures. However, significant amounts of bile salts are also present under these conditions as monomers and as "simple" micelles (i.e., without incorporated phospholipids). There is some evidence that this so called "intermixed micellar-vesicular bile salt concentration" (IMC: bile salt monomers + simple micelles) (11) may be responsible for the potentially damaging effects on membrane bilayers (12, 13). At the concentrations occurring in hepatic and gallbladder biles, bile salts could theoretically damage the apical membrane of the hepatocytes and of the cells lining the biliary tract. The absence of such a damaging effect in vivo suggests the existence of cytoprotective mechanisms either at the level

Abbreviations: DMEM, Dulbecco's modified Eagle's minimum essential medium; EYPC, egg yolk phosphatidylcholine; EYSM, egg yolk sphingomyelin; DPPC, dipalmitoyl phosphatidylcholine; IMC, intermixed micellar–vesicular bile salt concentration; LDH, lactate dehydrogenase.

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of the cell membrane or within biliary micelles. Increased concentrations of cholesterol and phospholipids (in particular sphingomyelin) in the hepatocyte canalicular membrane appear to protect against cytotoxic effects of the bile salts within the canalicular lumen (14–16). On the other hand, in in vitro studies, inclusion of egg yolk phosphatidylcholine (PC) within bile salt micelles protects in a concentration-dependent manner against bile salt-induced cytotoxicity (17). In line with these findings, mice with homozygous disruption of the *mdr*2 gene exhibit severe bile salt-induced hepatocyte damage in vivo: as *mdr*2 encoded P-glycoprotein, which normally functions as a "flippase" transporting PC molecules from the inner to the outer leaflet of the hepatocytic canalicular membrane, is absent there is virtually no PC protecting against bile salt-induced hepatotoxicity in bile of these mice (18).

Both PC and sphingomyelin (SM) are the major phospholipids of the canalicular membrane outer leaflet (16, 19). Although PC is the major phospholipid species in gallbladder bile, with minor amounts of SM (20), larger quantities of SM could be present in nascent bile within the canalicular lumen, particularly under pathological conditions such as cholestasis. When, in the rat model, cholestasis is induced by bile salt infusion, there is a considerable increase of SM content, from 3% to ⩽30% of total phospholipids in bile (21). Also, an alkaline sphingomyelinase recently detected in human bile (22) could contribute to the virtual absence of SM in gallbladder bile under non-cholestatic conditions because of reabsorption by the bile duct epithelial cells of the hydrolytic product ceramide, which is more hydrophobic than SM (23). This hypothesis is supported by the fact that sheep, which secrete significant amounts of SM in their biles (20), exhibit no biliary sphingomyelinase activity (22).

In the present study we examined the protective effects of various phospholipids against detergent taurocholate in a number of complementary in vitro studies. We compared the effects of PC from egg yolk (EYPC, mainly 16:0 acyl chains at the *sn*-1 position and mainly unsaturated (18:1 > 18:2 > 20:4) acyl chains at the *sn*-2 position, similar to PC in human bile (24)), SM from egg yolk (EYSM, mainly 16:0 acyl chains, similar to SM in bile (25)) and dipalmitoyl PC (DPPC, similar structure and gel-to-liquid crystalline transition temperature (26) as SM).

MATERIAL AND METHODS

Materials

Taurocholate was obtained from Sigma Chemical Co. (St. Louis, MO) and yielded a single spot upon thin-layer chromatography (butanol–acetic acid–water, 10:1:1 (vol/vol/vol), application of 200 μg bile salt). Cholesterol (Sigma) was ⩾98% pure by reverse-phase HPLC (isopropanol–acetonitrile 1:1, vol/vol, detection at 210 nm). Phosphatidylcholine from egg yolk (EYPC; Sigma), dipalmitoyl phosphatidylcholine (DPPC; Sigma) and sphingomyelin from egg yolk (EYSM; Avanti Polar-Lipids Inc., Alabaster, AL) all yielded a single spot upon thin-layer chromatography (chloroform–methanol–water 65:25:4 (vol/vol/vol) application of 200 μg lipid). Acyl chain compositions as determined by gas–liquid chromatography (27) were virtually identical to previously published data (23) and showed a preponderance of 16:0 acyl chains for EYSM, similar to sphingomyelin in human bile (25). As shown by reverse-phase HPLC, EYPC contained mainly 16:0 acyl chains at the *sn*-1 position and mainly unsaturated (18:1 > 18:2 > 20:4) acyl chains at the *sn*-2 position, similar to phosphatidylcholine in human bile (24). Dulbecco's modified Eagle's minimum essential medium (DMEM) was obtained from Flow Laboratories (Irvine, GB). All other chemicals and solvents were of ACS or reagent grade quality.

Ultrafilters with a molecular weight cutoff of 5 kDa were obtained from Sartorius (Göttingen, Germany: Centrisart I). The enzymatic cholesterol assay kit was obtained from Boehringer (Mannheim, Germany). A 3α-hydroxysteroid dehydrogenase for the enzymatic measurement of bile salt concentrations (28) and a colorimetric chloride kit were purchased from Sigma. The reverse-phase C18 HPLC column was from Supelco (Supelcosil LC-18-DB, Supelco, Bellefonte, PA).

Preparation of lipid solutions

Lipid mixtures containing variable proportions of cholesterol, phospholipids (both from stock solutions in chloroform) and taurocholate (from stock solutions in methanol) were vortex-mixed and dried at 45°C under a mild stream of nitrogen, and subsequently lyophilized during 24 h, before being dissolved in aqueous 0.15 m NaCl plus 3 mm NaN_3. Tubes were sealed with Teflon-lined screw caps under a blanket of nitrogen to prevent lipid oxidation and vortex-mixed for 5 min followed by incubation at 37°C in the dark. All solutions were warmed up to 45°C for 10 min before use. The final mol percentages of cholesterol, phospholipids, and bile salts did not differ more than 1% from the intended mol percentages.

Lipid analysis

Phospholipid concentrations in solutions were assayed by determining inorganic phosphate according to Rouser (29). Cholesterol concentrations were determined with an enzymatic assay (30) and bile salts with the 3α-hydroxysteroid dehydrogenase method (28).

Preparation of small unilamellar vesicles

Small unilamellar vesicles were prepared by sonication. Lipids, from stock solutions in chloroform, were vortex-mixed, dried under a mild stream of nitrogen, freeze-dried in liquid nitrogen, and subsequently lyophilized for 24 h. The lipid film was dissolved in nitrogen-flushed aqueous 0.15 m NaCl plus 3 mm NaN_3, and thereafter the suspensions were probe-sonicated for 30 min at 45°C (above the main transition temperatures of the phospholipids). After sonication, the suspension was centrifuged for 30 min at 50,000 *g* at 40°C in order to remove potential remaining vesicular aggregates and titanium particles. The resulting small unilamellar vesicles were stored above 40°C, and used within 12 h. Small unilamellar vesicles were prepared with 100% EYPC, or 80% EYPC/20% EYSM, or 60% EYPC/40% EYSM as phospholipid (final phospholipid concentration 4 mm). The hydrodynamic radius (Rh: at 37°C), as determined by quasielastic light scattering spectroscopy, of the small unilamellar vesicles composed with 100% EYPC was 50 ± 1.65 nm. Partial replacement of EYPC with EYSM led to slightly decreased vesicle sizes (mean ± SEM: 43.5 ± 1.23 nm in the case of 40% EYSM).

Resistance of vesicles against taurocholate or taurocholate-phospholipid mixed micelles

Interactions of sonicated small unilamellar vesicles with taurocholate were examined by measuring optical density at 405 nm

every min during 30 min at 37°C in a termostated Benchmark microplate reader (Bio-Rad, Hercules, CA). Solutions were stirred for 2 s prior to each measurement. A decrease of the OD at 405 nm after addition of taurocholate is compatible with micellization of the vesicles. Absorbance measured in control vesicles without addition of taurocholate always remained stable during the experiments. We added taurocholate (final concentration 6 mm) or mixed micellar solutions containing both taurocholate (final concentration 6 mm) and either EYPC or EYSM or DPPC (PL/(PL + BS) ratio = 0.2, 37°C) to EYPC-containing vesicles (final vesicular phospholipid concentration 4 mm). Furthermore, we added taurocholate (final concentration 5 mm) to sonicated vesicles containing 100% EYPC or 80% EYPC/20% EYSM or 60% EYPC/40% EYSM as phospholipids (final phospholipid concentration 4 mm, 37°C).

Last, 40 μL of taurocholate simple micelles or mixed micellar solutions containing both taurocholate and either EYPC or EYSM or DPPC (PL/(PL+BS) ratio = 0.2, (180 mm taurocholate, 37°C) were added to 200 μL of supersaturated model bile (10.5 mm EYPC, 42.3 mm taurocholate, 17.5 mm cholesterol), in order to decrease cholesterol saturation of the resulting model system to unsaturated levels (see inset Fig. 3: final cholesterol saturation index 0.25 and 0.21, respectively (according to Carey's Critical Tables (31, 32), which are based on EYPC-containing systems)). Because micellar cholesterol solubility in SM- or DPPC-containing systems is approximately one-third of micellar solubility in EYPC-containing systems (23), all final systems were unsaturated regardless of phospholipid type. Because the original model bile was composed so that it plotted in the middle three-phase zone of the equilibrium ternary phase diagram (33), and thus contained large quantities of vesicles in addition to micelles and cholesterol monohydrate crystals, a decrease of OD at 405 nm was considered to be consistent with micellization of these vesicles.

Resistance of erythrocytes against taurocholate simple micelles and taurocholate-phospholipid mixed micelles

Fresh human erythrocytes (aliquots of 10 mL human blood from a single volunteer) were sedimented 3 times by centrifugation at 3000 rpm for 15 min; the plasma and buffy coat were discarded and the pellet was resuspended to the original blood volume in TRIS buffer (10 mm TRIS, 130 mm NaCl, and 10 mm glucose, pH 7.4). A constant temperature of 37°C was maintained during the experiment. When these erythrocytes are incubated for 15 min with 50 mm taurocholate, hemolysis amounts to 95–100% (i.e., identical to values after 15 min incubation in distilled water (17)). In order to determine potential protective effects of various phospholipids against detergent bile salts, erythrocytes (0.2 mL) were added to 0.8 mL taurocholate (50 mm) or mixed micellar solutions containing both taurocholate (50 mm) and increasing amounts (5, 10, 15, 20, or 25 mm) of either EYPC or EYSM or DPPC. After incubation for 15 min, 7 mL buffer was added in order to decrease the progress of hemolysis to negligible levels (2). The samples were then centrifuged for 1 min at 10,000 *g* and the extent of lysis was assayed in the supernatant (absorbance at 540 nm).

In order to examine potential changes of erythrocyte membrane composition due to incubation with mixed phospholipid-taurocholate micelles, after such incubation (PL/(PL + BS) ratio of added mixed micelles = 0.35: complete protection against hemolysis at this ratio), erythrocytes were washed extensively and membrane phospholipids extracted according to Reed (34), separated by thin-layer chromatography (chloroform–methanol–acetic acid–water 50:25:8:4 (vol/vol/vol/vol), and separated spots quantified with the Rouser assay (29).

Finally, we incubated erythrocytes with mixed micellar solutions containing taurocholate (50 mm) and small amounts (total phospholipid concentration 5 mm) of both EYPC and EYSM at various ratios (PC/SM ratios: 100/0, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, and 0/100).

Resistance of CaCo-2 cells against taurocholate simple micelles and taurocholate-phospholipid mixed micelles

CaCo-2 cells were cultured as previously described (35) with minor modification. Briefly, CaCo-2 cells were grown in T-75 plastic flasks in DMEM supplemented with 20% fetal calf serum (Gibco, Grand Island, NY) 50 U/mL penicillin, and 50 U/mL streptomycin (Irvine, GB). Before confluency, the cells were split (split ratio 1:8) as follows: CaCo-2 cells were rinsed twice with Hank's balanced salt solution (Gibco, Grand Island, NY) and incubated for 5 min at 37°C with 1 mL of dissociation solution (Sigma, St. Louis, MO) after DMEM medium supplemented with 20% fetal calf serum was added to the cell suspension. Monolayers were grown in microwell plates in DMEM, which was replaced daily with fresh medium. After 10 days, the postconfluent cultures were washed with phosphate-buffered saline (pH 7.4) and the cells were incubated with taurocholate (bile salt concentration 30 mm) or mixed micelles containing taurocholate plus EYPC or EYSM or DPPC (bile salt concentration 30 mm, PL/(PL + BS) ratio = 0.05, 0.1, 0.15, 0.2, 0.25, and 0.3 at 37°C, pH 7.4). After 30 min incubation, the medium was collected and the cells were treated with 0.4% Triton X-100. Lactate dehydrogenase (LDH) activity, as a sensitive parameter of cell damage, was measured according to Mitchell, Santone, and Acosta (36) in both medium and in Triton X-100 treated cells. Fat-free bovine serum albumine (final concentration 0.6%) was added to prevent interference of bile salts with the spectrophotometric assay of LDH activity. Enzyme activity in each single experiment was normalized as percentage of the total LDH activity (medium + Triton X-100 treated cells).

IMC measurement

Under the conditions of the experiments in this study, in systems containing taurocholate and phospholipids, taurocholate is contained not only in mixed (i.e., bile salt-phospholipid) micelles, but occurs also as non-phospholipid-associated bile salt, either as monomers or, above the critical micellar concentration, in small "simple" micelles. The monomeric plus simple micellar bile salt concentration is referred to as "intermixed micellar-vesicular (non-phospholipid-associated) bile salt concentration," usually abbreviated as "IMC" (11). We determined IMC in micellar model systems containing either EYPC, DPPC, or EYSM as phospholipids and taurocholate as bile salt (PL/(PL + BS) ratio = 0.1, 0.15, and 0.2, bile salt concentration 30 mm, 37°C), using a minor modification of the rapid centrifugal ultrafiltration technique (37).

A 5 kDa Centrisart ultrafilter was rinsed with H_2O and centrifuged for 5 min at 500 *g* in order to remove glycerol remnants from the membrane. The water was removed carefully from both sides of the membrane with a syringe. The filter was preincubated at 37°C for 1 h before usage. A 2 mL aliquot of model system was put into the filter device (in duplicate) and centrifuged at 500 *g* for 5 min in a prewarmed (37°C) centrifuge. The filtrate was carefully collected with a syringe. Filtration was repeatedly performed, adjusting centrifugal speed so as to obtain constant filtrate volumes of approximately 50 μL. Bile salt and chloride concentrations reached stable values in the third filtrate. Slightly lower concentrations in the first and second filtrates resulted from small amounts of water remaining in the membrane after rinsing the ultrafilter (37). We considered the third filtrate to represent the simple micellar + monomeric fraction, and therefore decided to use the third filtrate for measurement of the

IMC (the first two filtrates were added each time to the filtrant) (37). This technique has been validated before (12, 37, 38). During ultrafiltration, Gibbs-Donnan effects occur as a result of uneven distribution across the membrane of non-filterable particles with a highly negative charge (in particular mixed micelles), thus leading to an overestimation of the concentrations of negatively charged monomeric and simple micellar bile salts in the filtrate (11, 37). We corrected the concentrations of bile salts measured in the filtrate for Gibbs-Donnan effects by multiplying the bile salt concentration in the filtrate with the ratio of chloride concentrations in filtrant and filtrate (11, 37, 38).

Statistical analysis

Values are expressed as means ± SEM. Differences between groups were tested for statistical significance by analysis of variance with the aid of NCSS software (Kaysville, UT). When ANOVA detected a significant difference, results were further compared for contrasts using Fisher's least significant difference test as post-hoc test. Statistical significance was defined as two-tailed probability of less than 0.05.

RESULTS

Interaction between phospholipid vesicles and taurocholate or taurocholate-phospholipid mixed micelles

Incubation with detergent taurocholate led to fast micellization of sonicated EYPC vesicles as indicated by decrease of absorbance values (**Fig. 1**). Incorporation of phospholipid in taurocholate micelles delayed micellization of the vesicular bilayer in the rank order: EYPC < DPPC < EYSM. In order to examine whether this protective effect was caused by phospholipid transfer from mixed micelles into the vesicular bilayer, we performed the following experiment: EYPC in vesicles was partially replaced by EYSM (vesicular SM/PC ratios 20/80 or 40/60). As shown in **Fig. 2**, these SM-containing vesicles exhibited increased rather than decreased sensitivity for the detergent action of taurocholate, the magnitude of this effect depending on the amount of vesicular EYPC replaced by EYSM. These results suggest that protective effects of phospholipids such as SM occur because of decreased detergent effects of mixed EYSM-taurocholate micelles compared to simple micelles or EYPC-taurocholate micelles rather than transfer of EYSM from mixed micelles into the vesicular bilayer.

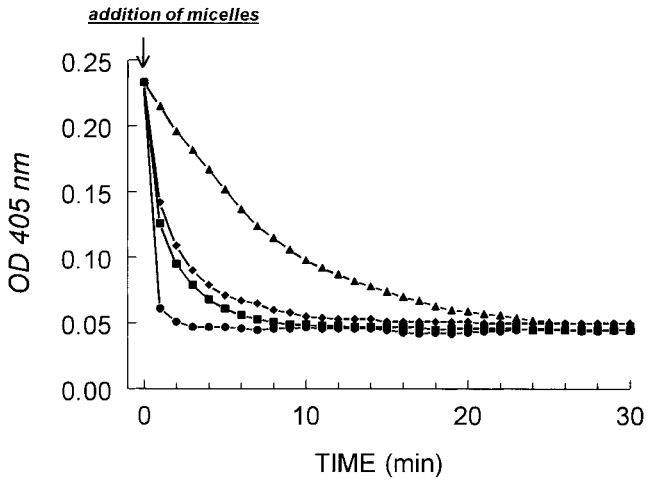

Fig. 1. Effects of taurocholate (final concentration 6 mm) or taurocholate plus phospholipid-containing mixed micelles (final taurocholate concentration 6 mm, PL/(PL + BS) ratio = 0.2, 37°C) on sonicated EYPC vesicles (final vesicular EYPC concentration 4 mm). Compared to taurocholate alone, decrease of absorption was delayed by incorporation of phospholipid in the micelles in the rank order: EYPC < DPPC < EYSM, consistent with delayed micellization of the vesicle bilayers. SEMs are contained within the symbols (n = 4). (●) taurocholate; (■) EYPC-taurocholate; (◆) DPPC-taurocholate; (▲) EYSM-taurocholate.

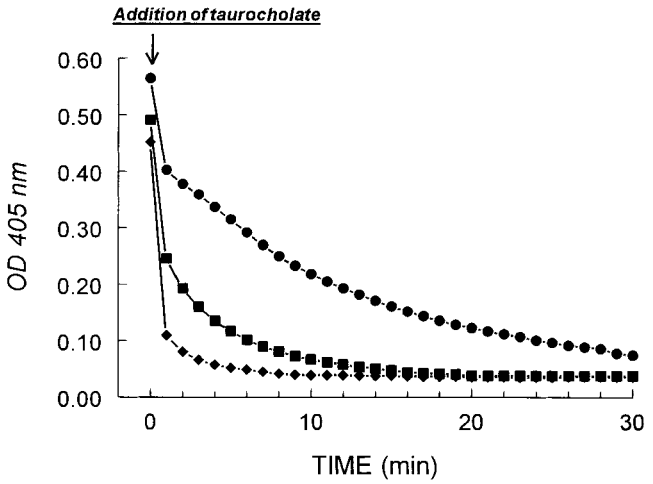

Fig. 2. Effect of taurocholate (final concentration 5 mm) on vesicles (final vesicular phospholipid concentration 4 mm, 37°C). Vesicles were composed with 100% EYPC or with 20 or 40% of the EYPC replaced by EYSM. Decrease of absorption values was enhanced by inclusion of SM in the vesicular bilayer, depending on the amount of vesicular SM. These findings indicate faster micellization in case of SM-containing vesicles. SEMs are contained within the symbols (n = 4). (●) 100% EYPC; (■) 80% EYPC/20% EYSM; (◆) 60% EYPC/40% EYSM.

Micellization of vesicles in supersaturated EYPC-containing model bile after addition of taurocholate simple micelles in quantities sufficient to decrease CSI to unsaturated levels was delayed by incorporation of phospholipid in these micelles in the rank order: EYPC < DPPC < EYSM (**Fig. 3**).

Effect of various phospholipids on taurocholate-induced hemolysis

When erythrocytes were incubated with 50 mm taurocholate, cytolysis amounted to 95–100% (virtually identical to values after incubation with distilled water). Whereas incorporation of EYPC in quantities ⩾20 mm (PL/(PL + BS) ratio ⩾0.29) in taurocholate micelles reduced cytolysis appreciably, the same protective effect occurred for taurocholate-DPPC and taurocholate-EYSM micelles already at phospholipid concentrations ⩾5 mm (PL/(PL + BS) ratio ⩾0.1, **Fig. 4**). When the erythrocytes were incubated with mixed micelles containing both EYPC and EYSM (bile salt concentration 50 mm, total phospholipid concentration 5 mm), hemolysis was pro-

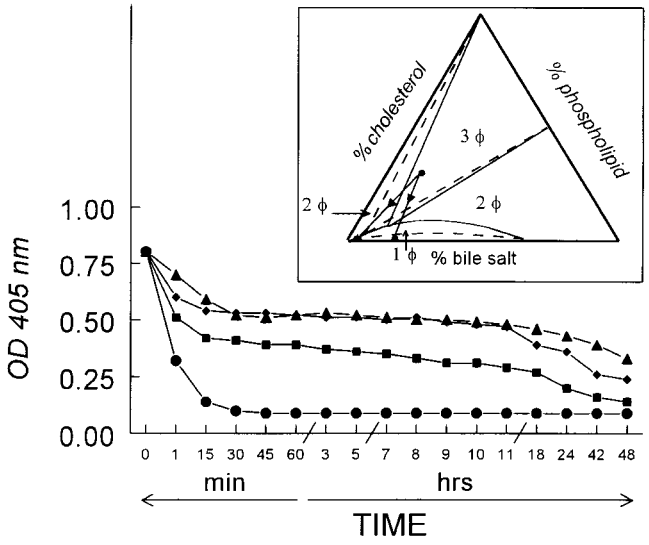

Fig. 3. Effect of taurocholate simple micelles or mixed micelles containing taurocholate and either EYPC or EYSM or DPPC (PL/(PL + BS) ratio = 0.2, 37°C) on supersaturated model bile (42.3 mm taurocholate, 10.5 mm EYPC, 17.5 mm cholesterol, total lipid concentration 3.6 g/dL), plotting in the middle three-phase zone of the ternary equilibrium phase diagram (33), and containing large amounts of vesicles besides mixed micelles and cholesterol monohydrate crystals. Inclusion of phospholipids in the taurocholate micelles reduced decrease of absorption values in the rank order EYPC < DPPC < EYSM, indicating decreased micellization of vesicular bilayers. (●) taurocholate; (■) EYPC-taurocholate; (◆) DPPC-taurocholate; (▲) EYSM-taurocholate. Note non-linear scale for x-axis. Inset: equilibrium taurocholate-phospholipid-cholesterol ternary phase diagram for EYPC- (continuous lines (33)) as well as DPPC- or EYSM- (interrupted lines (23)) containing systems. Arrows indicate changes in relative lipid composition after addition of simple or mixed micelles. 1ϕ = 1-phase zone; 2ϕ = 2-phase zone; 3ϕ = 3-phase zone.

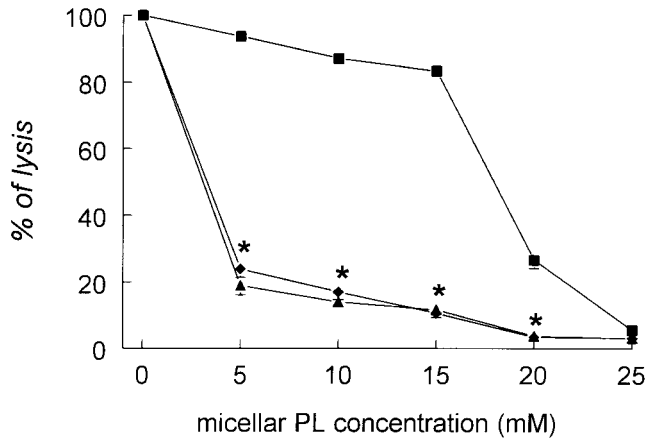

Fig. 4. Effect of phospholipids on taurocholate-induced lysis of human erythrocytes. Red blood cells were incubated for 15 min at 37°C with 50 mm taurocholate (100% lysis) or with mixed micelles containing taurocholate (50 mm) and progressive amounts of phospholipids. EYSM and DPPC had enhanced protective effects compared with EYPC. (■) EYPC-taurocholate; (◆) DPPC-taurocholate; (▲) EYSM-taurocholate. * $P < 0.05$ compared to EYPC-taurocholate.

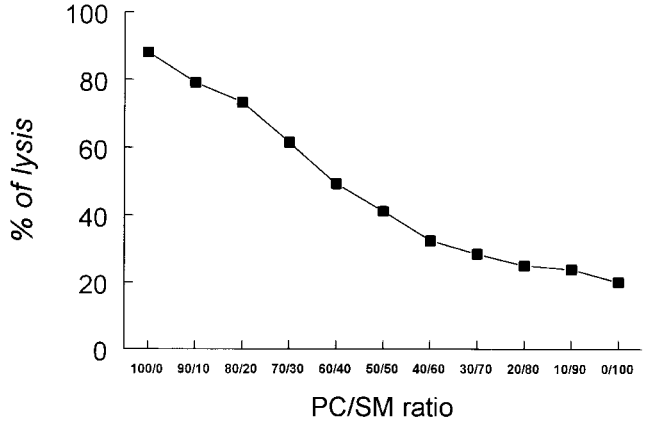

Fig. 5. Effects of phospholipids on taurocholate-induced lysis of human erythrocytes. Red blood cells were incubated for 15 min at 37°C with mixed micelles (taurocholate concentration 50 mm, total phospholipid concentration 5 mm) that contained variable amounts of both EYPC and EYSM. Hemolysis was progressively inhibited at increasing SM contents.

gressively inhibited at increasing SM contents (**Fig. 5**). Light microscopical examination of red blood cells after incubation with each micellar solution revealed no differences in sizes or shapes compared with control erythrocytes. Moreover, no changes in phospholipid composition or SM/PC ratio in erythrocyte membranes were found after incubation with EYPC-taurocholate or EYSM-taurocholate mixed micelles: 25% of total phospholipids consisted of PE, 14% of PI-PS, 25% of SM, and 36% of PC; and SM/PC ratios were 0.68, 0.65, and 0.69 for control erythrocytes, after incubation with EYPC-taurocholate micelles and EYSM-taurocholate micelles, respectively. It should be noted that these data were obtained after incubation with micelles at a micellar PL/(PL + BS) ratio of 0.35 in order to ensure complete protection against hemolysis.

Effect of various phospholipids on taurocholate-induced damage of CaCo-2 cells and on intermixed micellar-vesicular bile salt concentrations

As shown in **Fig. 6A**, incorporation of increasing amounts of phospholipids (up to PL/(BS+PL) ratio 0.3) in taurocholate micelles (bile salt concentration 30 mm) progressively reduced LDH release upon incubation with CaCo-2 cells. Protective effects were significantly higher for EYSM or DPPC than EYPC. Intermixed micellar-vesicular (i.e., monomeric plus simple micellar) bile salt concentrations (IMC) in EYPC-containing micellar solutions were significantly higher than in corresponding EYSM- or DPPC-containing solutions at all ratios examined (PL/(BS + PL) ratios 0.1, 0.15, and 0.2). For all taurocholate-phospholipid systems, there was a decrease of IMC at increasing phospholipid contents (**Table 1**).

Because the intermixed micellar-vesicular (IMC) bile salts have been suggested to be responsible for the membrane-damaging effects (12, 13), we also incubated CaCo-2 cells with taurocholate simple micelles (i.e., without phospholipids) at concentrations identical to IMC values of Table

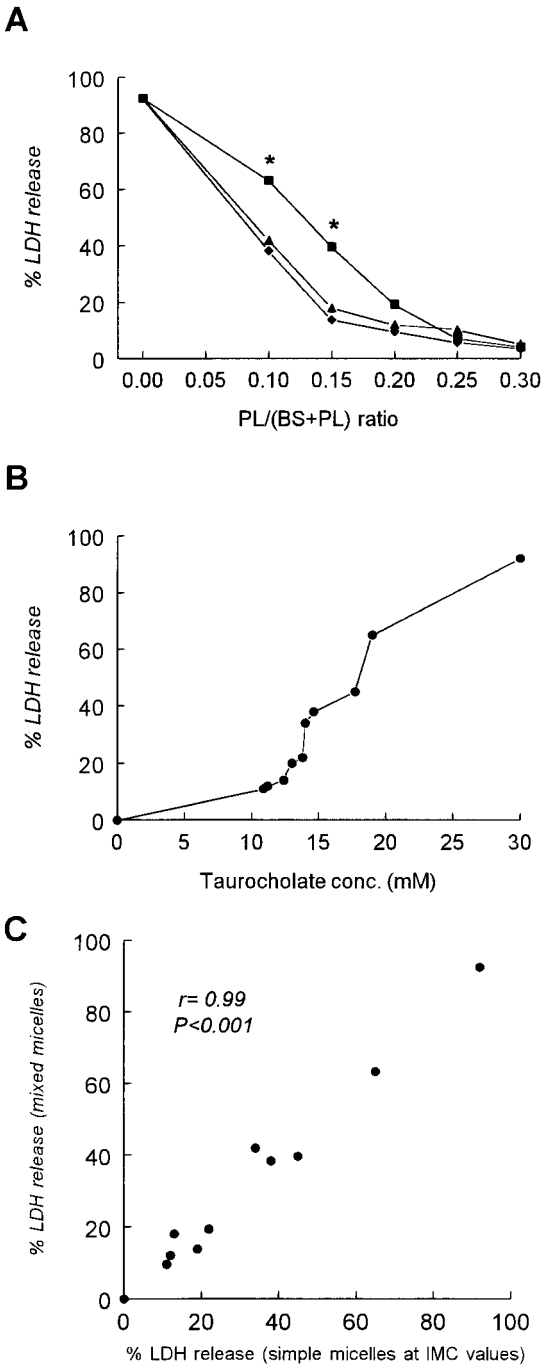

Fig. 6. A: Effect of phospholipids on taurocholate-induced LDH release by CaCo-2 cells. CaCo-2 cells were incubated for 30 min at 37°C with taurocholate simple micelles (30 mm) or mixed micelles containing taurocholate (30 mm) and progressive amounts of phospholipids. Protective effects of SM and DPPC were greater than protective effects of EYPC. (■) EYPC-taurocholate; (◆) DPPC-taurocholate; (▲) EYSM-taurocholate. * $P < 0.05$ compared to EYSM- and DPPC-taurocholate. B: LDH release by CaCo-2 cells upon incubation with taurocholate simple micelles at concentrations identical to IMC-values of mixed micelles used in A (given in Table 1). C: Identical LDH release upon incubation of CaCo-2 cells with taurocholate-phospholipid mixed micelles or taurocholate simple micelles at corresponding IMC concentrations.

TABLE 1. Intermixed micellar-vesicular bile salt concentration (IMC) in systems containing taurocholate and various phospholipids

	IMC (mm)		
PL / (PL + BS) ratio	0.1	0.15	0.2
TC + EYPC	19 ± 0.6	17.7 ± 0.8	14 ± 1.1
TC + SM	14.6 ± 0.6	12.9 ± 0.8	10.9 ± 0.4
TC + DPPC	13.8 ± 0.7	12.4 ± 0.4	11.2 ± 0.6

Monomeric plus simple micellar bile salt concentration (IMC) measured in model systems containing constant taurocholate concentration (30 mm) plus increasing amounts of EYPC or SM or DPPC. Values are expressed as means ± SEM of three different experiments in duplicate. There was a significant difference at all PL / (PL + BS) ratios between EYPC-containing systems and SM- or DPPC-containing systems. Moreover, for all phospholipids there was a significant decrease of IMC values at increasing phospholipid contents.

1. While there was only minor LDH release below 15 mm bile salt concentrations, there was a steep increase of LDH release at higher concentrations (Fig. 6B). LDH release after incubation with mixed (phospholipid-containing) taurocholate micelles was virtually the same as LDH release after incubation with taurocholate simple micelles (without phospholipids) at concentrations identical to corresponding IMC values (Fig. 6C).

DISCUSSION

Bile salts play a pivotal role in cholesterol and phospholipid secretion in bile and in intestinal lipid absorption. Nevertheless, in particular hydrophobic bile salts may also have adverse effects: they may damage the canalicular membrane of the hepatocyte in cholestasis, and promote formation of adenomas and cancer in the colon (39).

The main finding in the present study was that incorporation of phospholipids in taurocholate micelles protected against detergent effects of the bile salts, with a greater protection offered by EYSM or DPPC than by EYPC. Similar results were obtained in a large variety of in vitro systems such as sonicated vesicles, supersaturated model systems, human erythrocytes, and the CaCo-2 cell line. In the experiments with sonicated EYPC vesicles (Fig. 1), including phospholipids within taurocholate micelles retarded micellization. Nevertheless, at the end of the experiment, absorbance values were very low regardless of whether simple or mixed (phospholipid-containing) micelles had been added. These findings are consistent with complete micellization of all vesicles and thermodynamic equilibrium reached during the experiment. These results can easily be explained as, in the absence of cholesterol, final compositions of all model systems plot in the (one-phase) micellar zone of the appropriate equilibrium phase diagrams (23, 33). In contrast, in the experiments with cholesterol-supersaturated EYPC-containing model systems (Fig. 3), absorbance values at the end of the experiment were much higher after addition of phospholipid-containing mixed micelles (rank order EYPC < DPPC < EYSM) than after addition of simple taurocholate micelles,

indicating persistence of significant amounts of vesicles. For all these systems, cholesterol saturation after addition of micelles was far below 1. Therefore, all final systems were unsaturated and should have contained only micelles at equilibrium, even if one takes into account the considerably reduced micellar cholesterol solubility in SM- or DPPC-containing systems compared to EYPC-containing systems (23). The progressive decreases of absorbance after addition of phospholipid-containing micelles, as well as the high absorbance values at the end of the experiment, indicate that thermodynamic equilibrium was not reached during the experiment. The slower decrease of absorbance after addition of EYSM- (or DPPC-) containing mixed micelles than after addition of EYPC-containing mixed micelles can easily be explained by the well-known prolonged metastability of SM-containing systems (40). One should realize in interpreting results in cholesterol-supersaturated model systems displayed in Fig. 3, that decreases of turbidity are supposed to reflect a (partial) shift of lipids from vesicles to mixed micelles, but that we cannot exclude the possibility that the disaggregation of larger into smaller vesicular clusters or precipitation of cholesterol crystals could have contributed to decreased absorbance values under these circumstances.

In principle, protective effects could result from transfer of phospholipids from mixed micelles into the membrane bilayer or from a local effect of the phospholipids within the mixed micelles. There were no changes in phospholipid composition or SM/PC ratio of the erythrocyte membrane after incubation with EYPC- or EYSM-containing taurocholate micelles. Also, we found no changes of erythrocyte morphology after incubation with these micelles. Even small changes in phospholipid composition of the erythrocyte membrane should have had a profound impact on erythrocyte shape (41). Furthermore, incubation of sonicated EYPC vesicles with EYSM-containing taurocholate micelles resulted in delayed micellization of the vesicular bilayer compared to incubation with taurocholate micelles. In contrast, partial replacement of vesicular EYPC by EYSM increased rather than decreased sensitivity to taurocholate, in line with previous studies (42). Taken together, these findings suggest that protective effects of including phospholipids such as EYSM within the micelles are not caused by transfer of these phospholipids from the micelles into the vesicular bilayer, but rather by a local effect at the level of the micelles.

The so-called intermixed micellar-vesicular bile salt concentration (IMC: bile salt monomers + simple micelles (11)) has been proposed to exert the damaging effects on the membrane bilayer (12, 13). Indeed, LDH release upon incubation of CaCo-2 cells with the various mixed (phospholipid-containing) taurocholate micelles was identical to LDH release upon incubation with taurocholate simple micelles at corresponding IMC values (Fig. 6C). The enhanced protective effect of incorporating EYSM or DPPC compared to EYPC within taurocholate micelles can thus be explained by the much lower intermixed micellar-vesicular bile salt concentrations found in the present study for EYSM- and DPPC-containing micelles. Similar magnitude of protective effects of including EYSM or DPPC within taurocholate micelles may relate to similarity in molecular structure and acyl chain composition of both phospholipids: whereas DPPC contains 16:0 acyl chains at both *sn*-1 and *sn*-2 positions, in case of EYSM, 16:0 is also the major acyl chain amidated to the sphingosine backbone. Also, main gel to liquid–crystalline transition temperatures are similar for DPPC and EYSM (42°C vs. 37°C; (26)). EYPC offered less protection against detergent effects of taurocholate than EYSM or DPPC. EYPC has a molecular structure quite different from both other phospholipids, due to the presence of *cis*-unsaturated acyl chains at the *sn*-2 position. Also, main gel to liquid–crystalline transition temperature is much lower (<0°C; (26)).

The differences in IMC values found in the present study suggest different micellar structure and a smaller coexistence (mixed + simple micelles containing) region (43) for EYSM- or DPPC-taurocholate micelles compared to EYPC-taurocholate micelles: more taurocholate molecules may be required to dissolve a molecule of EYSM or DPPC than a molecule of EYPC. As a result, in a mixed micellar solution, the proportion of taurocholate associated with phospholipid would be greater in case of EYSM or DPPC than EYPC, with reciprocal decrease of taurocholate concentration in the IMC.

Our findings may be relevant for canalicular bile formation. Upon addition of bile salts to isolated canalicular membranes (44, 45) or erythrocytes (46), there is a considerable release of PC but not of SM from the membranes. In line with these in vitro findings, PC is the major phospholipid species in gallbladder bile, with minor amounts of SM (20). Nevertheless, these data do not exclude the possibility that larger amounts of SM could be present in nascent bile within the canalicular lumen, due to a putative cholehepatic shunt (23) after hydrolysis of SM to more hydrophobic ceramide by an alkaline sphingomyelinase recently detected in bile (22). Also, under pathological condition such as cholestasis, large amounts of SM may appear in bile (21). Under such cholestatic conditions, SM within micelles in the canalicular lumen could protect against further bile salt-induced liver injury.

The protective effects of SM may also be relevant at the level of the intestinal tract. In the small intestine, intraluminal bile salts exert a negative feedback control on cholecystokinin release and gallbladder contraction (47). We have found in duodenal infusion experiments in humans that the extent of the negative feedback control is decreased by including phospholipids in bile salt micelles that are infused, with stronger effects for DPPC or EYSM than EYPC, supposedly due to different IMC values (unpublished data).

Phospholipids may also be relevant at the level of the colon, in particular by decreasing IMC for hydrophobic bile salts such as deoxycholate, a well-known promotor of colon cancer (39). It has indeed been shown recently, that rats fed a high sphingomyelin diet exhibit a significantly reduced susceptibility for colon cancer on experimental carcinogenic diet (48, 49).

In conclusion, our data reveal greatly enhanced protective effects of sphingomyelin and dipalmitoyl phosphatidylcholine compared to egg yolk phosphatidylcholine against bile salt-induced cytotoxicity, which may relate to different intermixed micellar-vesicular (i.e., non-phospholipid associated) bile salt concentrations. These findings may be relevant at the level of the hepatocyte canalicular membrane and intestinal lumen.

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REFERENCES

1. Barnwell, S. G., P. J. Lowe, and R. Coleman. 1983. Effect of taurochenodeoxycholate or tauroursodeoxycholate upon biliary output of phospholipids and plasma-membrane enzymes, and the extent of cell damage, in isolated perfused rat livers. *Biochem. J.* **216:** 107–111.
2. Heuman, D. M., W. M. Pandak, P. B. Hylemon, and Z. R. Vlahcevic. 1991. Conjugates of ursodeoxycholate protect against cytotoxicity of more hydrophobic bile salts: in vitro studies in rat hepatocytes and human erythrocytes. *Hepatology.* **14:** 920–926.
3. Coleman, R., S. Iqbal, P. P. Godfrey, and D. Billington. 1979. Membranes and bile formation. Composition of several mammalian biles and their membrane-damaging properties. *Biochem. J.* **178:** 201–208.
4. Lowe, P. J., and R. Coleman. 1981. Membrane fluidity and bile salt damage. *Biochim. Biophys. Acta.* **640:** 55–65.
5. Davenport, H. W. 1968. Destruction of the gastric mucosal barrier by detergent and urea. *Gastroenterology.* **54:** 175–181.
6. Chadwick, V. S., T. S. Gaginella, G. L. Carlson, J. C. Debognie, S. F. Phillips, and A. Hoffmann. 1979. Effect of molecular structure on bile acid-induced alterations in absorptive function, permeability, and morphology in the perfused rabbit colon. *J. Lab. Clin. Med.* **94:** 661–674.
7. Ostrow, J. D. 1969. Absorption by the gallbladder of bile salts, sulfobromophthalein and iodipamide. *J. Lab. Clin. Med.* **74:** 482–494.
8. Ostrow, J. D. 1967. Absorption of bile pigments by the gallbladder. *J. Clin. Invest.* **46:** 2035–2052.
9. Coleman, R. 1987. Bile salts and biliary lipids. *Biochem. Soc. Trans.* **15:** 68S–80S.
10. Coleman, R., P. J. Lowe, and D. Billington. 1980. Membrane lipid composition and susceptibility to bile salt damage. *Biochim. Biophys. Acta.* **599:** 294–300.
11. Donovan, J. M., N. Timofeyeva, and M. C. Carey. 1991. Influence of total lipid concentration, bile salt:lecithin ratio, and cholesterol content on inter-mixed micellar/vesicular (non-lecithin-associated) bile salt concentrations in model bile. *J. Lipid Res.* **32:** 1501–1512.
12. Donovan, J. M., A. A. Jackson, and M. C. Carey. 1993. Molecular species composition of inter-mixed micellar/vesicular bile salt concentrations in model bile: dependence upon hydrophilic-hydrophobic balance. *J. Lipid Res.* **34:** 1131–1140.
13. Narain, P. K., E. J. DeMaria, and D. M. Heuman. 1998. Lecithin protects against plasma membrane disruption by bile salts. *J. Surg. Res.* **78:** 131–136.
14. Amigo, L., H. Mendoza, S. Zanlungo, J. F. Miquel, A. Rigotti, S. Gonzalez, and F. Nervi. 1999. Enrichment of canalicular membrane with cholesterol and sphingomyelin prevents bile salt-induced hepatic damage. *J. Lipid Res.* **40:** 533–542.
15. Schachter, D. 1988. The hepatocyte plasma membrane: organization and differentiation. *In* The Liver: Biology and Pathobiology. I. M. Arias, W. B. Jakoby, H. Popper, D. Schachter, and D. A. Shafritz, editors. Raven Press, Ltd., New York, NY. 131–140.
16. Kremmer, T., M. H. Wisher, and W. H. Evans. 1976. The lipid composition of plasma membrane subfractions originating from the three major functional domains of the rat hepatocyte cell surface. *Biochim. Biophys. Acta.* **455:** 655–664.
17. Velardi, A. L. M., A. K. Groen, R. P. Oude Elferink, R. van der Meer, G. Palasciano, and G. N. Tytgat. 1991. Cell type-dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology.* **101:** 457–464.
18. Smit, J. J., A. H. Schinkel, R. P. J. Oude Elferink, A. K. Groen, E. Wagenaar, L. van Deemter, C. A. Mol, R. Ottenhoff, N. M. van der Lugt, and M. A. van Roon. 1993. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell.* **75:** 451–462.
19. Higgins, J. A., and W. H. Evans. 1978. Transverse organization of phospholipids across the bilayer of plasma membrane subfractions of rat hepatocytes. *Biochem. J.* **174:** 563–567.
20. Alvaro, D., A. Cantafora, A. F. Attili, C. S. Ginanni, C. De Luca, G. Minervini, A. Di Biase, and M. Angelico. 1986. Relationships between bile salts hydrophilicity and phospholipid composition in bile of various animal species. *Comp. Biochem. Physiol. B.* **83:** 551–554.
21. Barnwell, S. G., B. Tuchweber, and I. M. Yousef. 1987. Biliary lipid secretion in the rat during infusion of increasing doses of unconjugated bile acids. *Biochim. Biophys. Acta.* **922:** 221–233.
22. Duan, R. D., E. Hertervig, L. Nyberg, T. Hauge, B. Sternby, J. Lillienau, A. Farooqi, and A. Nilsson. 1996. Distribution of alkaline sphingomyelinase activity in human beings and animals. Time and species differences. *Dig. Dis. Sci.* **41:** 1801–1806.
23. van Erpecum, K. J., and M. C. Carey. 1997. Influence of bile salts on molecular interactions between sphingomyelin and cholesterol: relevance to bile formation and stability. *Biochim. Biophys. Acta.* **1345:** 269–282.
24. Hay, D. W., M. J. Cahalane, N. Timofeyeva, and M. C. Carey. 1993. Molecular species of lecithins in human gallbladder bile. *J. Lipid Res.* **34:** 759–768.
25. Nibbering, C. P., and M. C. Carey. 1999. Sphingomyelins of rat liver: biliary enrichment with molecular species containing 16:0 fatty acids as compared to canalicular-enriched plasma membranes. *J. Membr. Biol.* **167:** 165–171.
26. Koynova, R., and M. Caffrey. 1995. Phases and phase transitions of the sphingolipids. *Biochim. Biophys. Acta.* **1255:** 213–236.
27. Hakomori, S. 1983. Chemistry of Glycosphingolipids. *In* Handbook of Lipid Research. D. J. Hanahan, editor. Plenum Press, New York, NY. 37–39.
28. Turley, S. D., and J. M. Dietschy. 1978. Reevaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J. Lipid Res.* **19:** 924–928.
29. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids.* **5:** 494–496.
30. Fromm, H., P. Hamin, H. Klein, and I. Kupke. 1980. Use of a simple enzymatic assay for cholesterol analysis in human bile. *J. Lipid Res.* **21:** 259–261.
31. Carey, M. C. 1978. Critical tables for calculating the cholesterol saturation of native bile. *J. Lipid Res.* **19:** 945–965.
32. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *J. Clin. Invest.* **61:** 998–1026.
33. Wang, D. Q. H., and M. C. Carey. 1996. Complete mapping of crystallization pathways during cholesterol precipitation from model bile: influence of physical-chemical variables of pathophysiologic relevance and identification of a stable liquid crystalline state in cold, dilute and hydrophilic bile salt-containing systems. *J. Lipid Res.* **37:** 606–630.
34. Reed, C. F., S. N. Swisher, G. V. Marinetti, and E. G. Eden. 1960. Studies of the lipids of the erythrocyte. I. Quantitative analysis of the lipids of normal human red blood cells. *J. Lab. Clin. Med.* **56:** 281–289.
35. Mohrmann, I., M. Mohrmann, J. Biber, and H. Urer. 1986. Sodium-dependent transport of Pi by an established intestinal epithelial cell line (CaCo-2). *Am. J. Physiol.* **250:** G323–G330.
36. Mitchell, D. B., K. S. Santone, and D. Acosta. 1980. Evaluation of cytotoxicity in cultured cells by enzyme leakage. *J. Tissue Cult. Methods.* **6:** 113–116.
37. Donovan, J. M., and A. A. Jackson. 1993. Rapid determination by centrifugal ultrafiltration of inter-mixed micellar/vesicular (non-

lecithin-associated) bile salt concentrations in model bile: influence of Donnan equilibrium effects. *J. Lipid Res.* **34:** 1121–1129.
38. Eckhardt, E. R. M., B. J. M. van de Heijning, K. J. van Erpecum, W. Renooij, and G. P. vanBerge-Henegouwen. 1998. Quantitation of cholesterol-carrying particles in human gallbladder bile. *J. Lipid Res.* **39:** 594–603.
39. Hori, T., K. Matsumoto, Y. Sakaitani, M. Sato, and M. Morotomi. 1998. Effect of dietary deoxycholic acid and cholesterol on fecal steroid concentration and its impact on the colonic crypt cell proliferation in azoxymethane-treated rats. *Cancer Lett.* **124:** 79–84.
40. Estep, T. N., W. I. Calhoun, Y. Barenholz, R. L. Biltonen, G. G. Shipley, and T. E. Thompson. 1980. Evidence for metastability in stearoylsphingomyelin bilayers. *Biochemistry.* **19:** 20–24.
41. Kuypers, F. A., B. Roelofsen, W. Berendsen, J. A. Op den Kamp, and L. L. van Deenen. 1984. Shape changes in human erythrocytes induced by replacement of the native phosphatidylcholine with species containing various fatty acids. *J. Cell Biol.* **99:** 2260–2267.
42. Schubert, R., and K-H. Schmidt. 1988. Structural changes in vesicle membranes and mixed micelles of various lipid compositions after binding of different bile salts. *Biochemistry.* **27:** 8787–8794.
43. Mazer, N. A., G. B. Benedek, and M. C. Carey. 1980. Quasielastic light-scattering studies of aqueous biliary lipid systems. Mixed micelle formation in bile salt-lecithin solutions. *Biochemistry.* **19:** 601–615.
44. Yousef, I. M., and M. M. Fisher. 1976. In vitro effect of free bile acids on the bile canalicular membrane phospholipids in the rat. *Can. J. Biochem.* **54:** 1040–1046.
45. Gerloff, T., P. J. Meier, and B. Stieger. 1998. Taurocholate induces preferential release of phosphatidylcholine from rat liver canalicular vesicles. *Liver.* **18:** 306–312.
46. Billington, D., R. Coleman, and Y. A. Lusak. 1977. Topographical dissection of sheep erythrocyte membrane phospholipids by taurocholate and glycocholate. *Biochim. Biophys. Acta.* **466:** 526–530.
47. Thimister, P. W. L., W. P. M. Hopman, C. E. J. Sloots, G. Rosenbusch, A. Tangerman, H. L. Willems, C. B. H. W. Lamers, and J. B. M. J. Jansen. 1994. Effect of bile salt binding or protease inactivation on plasma cholecystokinin and gallbladder responses to bombesin. *Gastroenterology.* **107:** 1627–1635.
48. Dillehay, D. L., S. K. Webb, E. M. Schmelz, and A. H. J. Merrill. 1994. Dietary sphingomyelin inhibits 1,2-dimethylhydrazine-induced colon cancer in CF1 mice. *J. Nutr.* **124:** 615–620.
49. Parodi, P. W. 1997. Cows' milk fat components as potential anticarcinogenic agents. *J. Nutr.* **127:** 1055–1060.