Asymmetric distribution of phosphatidylcholine and sphingomyelin between micellar and vesicular phases: potential implications for canalicular bile formation[1]

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Abstract Both phosphatidylcholine (PC) and sphingomyelin (SM) are the major phospholipids in the outer leaflet of the hepatocyte canalicular membrane. Yet, the phospholipids secreted into bile consist principally ($>$95%) of PC. In order to understand the physical–chemical basis for preferential biliary PC secretion, we compared interactions with bile salts (taurocholate) and cholesterol of egg yolk (EY)SM (mainly 16:0 acyl chains, similar to trace SM in bile), buttermilk (BM)SM (mainly saturated long ($>$20 C-atoms) acyl chains, similar to canalicular membrane SM) and egg yolk (EY)PC (mainly unsaturated acyl chains at *sn*-2 position, similar to bile PC). Main gel to liquid-crystalline transition temperatures were 33.6°C for BMSM and 36.6°C for EYSM. There were no significant effects of varying phospholipid species on micellar sizes or intermixed-micellar/vesicular bile salt concentrations in taurocholate-phospholipid mixtures (3 g/dL, 37°C, PL/BS + PL = 0.2 or 0.4). Various phases were separated from model systems containing both EYPC and (EY or BM)SM, taurocholate, and variable amounts of cholesterol, by ultracentrifugation with ultrafiltration and dialysis of the supernatant. At increasing cholesterol content, there was preferential distribution of lipids and enrichment with SM containing long saturated acyl chains in the detergent-insoluble pelletable fraction consisting of aggregated vesicles. In contrast, both micelles and small unilamellar vesicles in the supernatant were progressively enriched in PC. Although SM containing vesicles without cholesterol were very sensitive to micellar solubilization upon taurocholate addition, incorporation of the sterol rendered SM-containing vesicles highly resistant against the detergent effects of the bile salt. These findings may have important implications for canalicular bile formation.—Eckhardt, E. R. M., A. Moschetta, W. Renooij, S. S. Goerdayal, G. P. van Berge-Henegouwen, and K. J. van Erpecum. **Asymmetric distribution of phosphatidylcholine and sphingomyelin between micellar and vesicular phases: potential implications for canalicular bile formation.** *J. Lipid Res.* 1999. **40:** 2022–2033.

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The hepatocyte plasma membrane is functionally divided into a canalicular (or apical) region adjacent to the lumen of the bile canaliculus and a sinusoidal (or basolateral) region in close contact with sinusoidal blood (1). Although the canalicular region comprises only 10–15% of the total plasma membrane, it plays a crucial role in the process of nascent bile formation and biliary secretion of bile salts, phosphatidylcholine (PC), and cholesterol.

In recent years, there has been considerable progress in understanding transport mechanisms of these lipids over the canalicular membrane: *mdr* (multi drug resistance) 2 P-glycoprotein functions as a "flippase" translocating PC molecules from the inner to the outer leaflet of the canalicular membrane (2) and the so-called "Sister P-glycoprotein" appears to mediate bile salt transport (3). Nevertheless, only limited data are available on the events occurring during nascent canalicular bile formation and the physical–chemical mechanisms involved. With the aid of ultrarapid cryofixation and electron microscopic imaging, Crawford et al. (4) could visualize significant amounts of unilamellar vesicles within the canalicular lumen, consistent with a vesicular mode of cholesterol and PC secretion. Nevertheless, these findings do not exclude the possibility that detergent bile salts, after their secretion into the canalicular lumen, could micellize considerable amounts of cholesterol and PC from the outer leaflet of the canalicular membrane.

Another matter of debate is why PC is the predominant phospholipid in bile: both sphingomyelin (SM) and PC are the major structural phospholipids in the outer leaflet of the hepatocyte canalicular membrane (in an average

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ratio of 0.7) (1, 5), but PC is the major (~95%) phospholipid species in bile (6). It has long been well known that biliary PC contains mainly 16:0 acyl chains at the *sn*-1 position and predominantly unsaturated (18:2 > 18:1 > 20:4) acyl chains at the *sn*-2 position (7). Recent data from Nibbering and Carey (8) indicate that the trace amounts of SM in rat bile contain mainly 16:0 acyl chains, whereas canalicular membrane SM contains predominantly long (>20 C-atoms) saturated acyl chains amide-linked to the sphingosine moiety. Similar predominance of SM with long saturated acyl chains has previously been reported for hepatocyte plasma membrane (9). Contents of SM and cholesterol are higher in the canalicular than in the sinusoidal membrane (1, 10), with decreased fluidity and increased resistance against detergent effects of bile salts as a result. Extensive physical–chemical and cell–biological studies indicate that cholesterol has a higher affinity for natural SM than for PC (11–17) and may be tightly bound in laterally segregated SM domains in the hepatocyte canalicular membrane (14, 18).

The present study aims to increase insight into the process of canalicular bile formation by means of studying interactions of bile salts with SM and PC, with or without cholesterol, in a number of complementary in vitro systems. We used PC from egg yolk (mainly 16:0 acyl chains at the *sn*-1 position, and mainly unsaturated acyl chains at the *sn*-2 position, similar to PC in bile (7)), SM from egg yolk (mainly 16:0 acyl chains, similar to trace SM in bile (8)) and SM from buttermilk (mainly long saturated acyl chains, similar to SM in the canalicular membrane (8, 9)).

MATERIALS 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–acetonitril 1:1, vol/vol, detection at 210 nm). Phosphatidylcholine from egg-yolk (EYPC; Sigma), sphingomyelin from egg-yolk (EYSM; Avanti Polar-Lipids Inc., Alabaster, AL), and sphingomyelin from buttermilk (BMSM; Matreya Inc., Pleasant Gap, PA) all yielded a single spot on thin-layer chromatography (chloroform–methanol–water 65:25:4, vol/vol/vol, application of 200 μg lipid). Methylated fatty acids (16:0, 18:0, 18:1, 18:2, 20:0, 21:0, 22:0, 23:0, 24:0, 24:1, 25:0), used as standards for gas–liquid chromatography, were purchased from Sigma. Acyl chain compositions as determined by gas–liquid chromatography (19) were virtually identical to previously published data (20) and showed mainly saturated long (≥20 C-atoms) acyl chains for BMSM, similar to acyl chain composition of SM in hepatocytic plasma membranes (8, 9), and a preponderance of 16:0 acyl chains for EYSM. 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 (21), similar to phosphatidylcholine in human bile (7). All other chemicals and solvents were of ACS or reagent grade quality.

Ultrafilters with a molecular mass cut-off of 10 kDa and 300 kDa were purchased from Sartorius (Göttingen, Germany: Centrisart I), dialysis membranes with a molecular mass cut-off of 300 kDa from Spectrum Laboratories (Laguna Hills, CA, USA: SpectraPor). Sephacryl S400 gel filtration material was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). The enzymatic cholesterol assay kit was obtained from Boehringer (Mannheim, Germany), and the enzymatic phospholipid kit from Sopar Biochem (Brussels, Belgium). 3α-Hydroxysteroid dehydrogenase for the enzymatic measurement of bile salt concentrations (22) 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 model systems

Lipid mixtures containing variable proportions of cholesterol, phospholipids (both from stock solutions in chloroform), or 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. The final mol percentages cholesterol, phospholipid, and bile salt did not differ more than 2.5% from the intended mol percentages.

Differential scanning calorimetry

BMSM or EYSM (10 mg), from stock solutions in chloroform, were dried at 45°C under a mild stream of nitrogen and dissolved in 1 mL H$_2$O followed by 5 cycles of freeze-thawing. Main gel to liquid–crystalline phase transition temperatures (melting temperatures: T$_m$) were measured with a Perkin-Elmer DSC-4 differential scanning calorimeter (Perkin-Elmer, Norwalk, CT), at a scan rate of 5°C/min.

Quasi-elastic light-scattering (QLS) spectroscopy

QLS measurements of micelles in mixtures of taurocholate and phospholipids were performed on a home-built apparatus, the details of which were published elsewhere (23). Measurements were performed with the argon laser tuned to 514.5 nm at a scattering angle of 90°. Vesicular sizes were measured with a Malvern 4700c QLS spectrometer (Malvern Ltd., Malvern, UK), equipped with an argon laser (Uniphase Corp., San Jose, CA) at a wavelength of 488 nm. All samples were maintained at a constant temperature of 37°C by means of a Peltier thermostatic block or a water bath. To remove dust, tubes were first centrifuged for 10 min at 10000 *g.* Data are given as hydrodynamic radius (Rh: means of at least 3 measurements).

Lipid analysis

Phospholipid concentrations in model systems were assayed by determining inorganic phosphate according to Rouser, Fleischer, and Yamamoto (24), but in serial fractions from gel-filtration experiments with an enzymatic assay. Cholesterol concentrations were determined with an enzymatic assay or by reverse-phase HPLC (acetonitril–isopropanol 1:1, vol/vol, detection at 210 nm), and bile salts with the 3α-hydroxysteroid dehydrogenase method (22) or by HPLC (25). In systems containing both EYPC and SM, the phospholipids were extracted according to Bligh and Dyer (26), separated by thin-layer chromatography (chloroform–methanol–acetic acid–water 50:25:8:2, vol/vol/vol/vol), and quantified by determination of phosphorus contents of separated phospholipid spots. In order to determine the fatty acid profiles of sphingomyelin, 1 μmol sphingomyelin was hydrolyzed in 1 mL nitrogen-flushed HCl–methanol–H$_2$O (8.3:80.6:11.1, vol/vol/vol, 16 h, 70°C) (19). The methylated fatty acids were extracted three times with 1 mL hexane; the pooled hexane phase was washed with 3 mL H$_2$O, dried over Na$_2$SO$_4$, and concen-

trated under nitrogen. The fatty acid methylesters were dissolved in 30 μl hexane, 2 μL of which were injected in a GC14-A gas-chromatograph (Shimadzu, Kyoto, Japan), equipped with a bonded FSOT capillary column (length 30 m, diameter 0.32 mm).

IMC measurement

Apart from mixed (i.e., phospholipid–bile salt) micelles, model bile systems also contain non-phospholipid–associated bile salts, either as monomers or, above their critical micellar concentration, associated 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 (27). We determined the IMC in micellar model systems with relatively low or relatively high amounts of either EYSM, BMSM, or EYPC as phospholipid and taurocholate as bile salt (PL/(PL + bile salt) ratio = 0.2 and 0.4; total lipid concentration 3 g/dL, 37°C). The effect of incorporating small (3 mol %) amounts of cholesterol on the IMC in these systems was also explored.

A 10 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 during 30 min 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 pre-warmed (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 (28). 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) (28, 29). No phospholipids were detectable in the filtrates (detection limit of the assay: 0.048 mm), indicating that no mixed micelles had passed through the filter. 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 (27, 28). We corrected the concentrations of bile salts measured in the filtrate for Gibbs-Donnan effects by multiplying the bile salt concentration in the filtrate by the ratio of chloride concentrations in filtrant and filtrate (27–29).

Separation of vesicular and micellar phases

Two independent procedures were used to separate vesicular and micellar phases: ultrafiltration of the whole system or ultracentrifugation with subsequent ultrafiltration and dialysis of the supernatant. With the first procedure (ultrafiltration), micellar phases were isolated from model systems containing both EYPC and SM as phospholipid, taurocholate, and various amounts of cholesterol (10 mm EYPC, 6.6 mm EYSM or BMSM, 16.6 mm taurocholate, and 1.6, 3.2, or 6.4 mm cholesterol: cholesterol/phospholipid ratios 0.1, 0.2, or 0.4). These model systems all plot in the right two-phase zone of the equilibrium ternary phase diagram (20, 30) and contain micelles and vesicles of various compositions. We used one additional model system (57 mm taurocholate, 19 mm EYPC, 19 mm EYSM, 46 mm cholesterol) plotting in the middle three-phase (micelles, vesicles, and solid cholesterol crystals containing) zone of the equilibrium ternary phase diagram (20, 30). According to the phase rule, all three phases of this system should have one invariant composition at equilibrium (31). The ultrafilter had a molecular mass cut-off of 300 kDa, and had previously been rinsed with aqueous 0.15 m NaCl plus 3 mm NaN_3, containing taurocholate at concentrations identical to the IMC of the original model systems, in order to avoid artifactual shifts of lipids between vesicles and micelles (27–29). These filters were completely permeable not only to simple micelles but also to mixed taurocholate/phospholipid micelles (tested with mixed micelles at 37°C, at total lipid concentrations of 2, 5, and 10 g/dL and at PL/(BS+PL) ratios of 0.55, 0.5, 0.4, and 0.3, either without or with small amounts (0.25 mol%) cholesterol: either SM, EYPC, or both SM and EYPC (SM/PC ratio 0.37) as the phospholipid), but were completely impermeable to small unilamellar or aggregated vesicles. The membrane-containing inner tube of the filter device (placed membrane-down on top of 2 mL of model system) was allowed to sink slowly into the filtrant by gravity, thus producing approximately 200 μL micelle-containing filtrate within 2 h. In order to purify the vesicles, 200 μL of the remaining filtrant then was diluted 10 times with aqueous 0.15 m NaCl/3 mm NaN_3 containing taurocholate according to the IMC (in order to avoid artifactual shifts of lipids between various phases), followed by ultrafiltration until approximately 90% of the volume was contained within the filtrate. This procedure was repeated twice in order to wash out the remaining mixed micelles.

With the second procedure, aggregated vesicles in 2 mL of model system were precipitated by ultracentrifugation during 30 min at 50,000 g and 37°C in a TLS 55 rotor (Beckman, Palo Alto, CA) (32). The pellet was resuspended in a final volume of 2 mL isopropanol. A Tyndall effect was generally visible in the supernatant, consistent with the presence of small unilamellar vesicles, as confirmed with quasi-elastic light scattering spectroscopy. Micelles were isolated from the supernatant by ultrafiltration with the aid of the 300 kDa filter described above (identical micellar compositions had been obtained by ultrafiltration of the corresponding whole model system, indicating that the short ultracentrifugation procedure did not induce artifactual shifts between phases). Small unilamellar vesicles were isolated from the supernatant by dialysis (500 μL sample, 16 h, 37°C) in a SpectraPor® dialysis device with a molecular mass cut-off of 300 kDa, against two times 20 volumes of aqueous 0.15 m NaCl plus 3 mm NaN_3 containing taurocholate at concentrations identical to the IMC of the original model system. The dialysis membrane was completely permeable for the same micelles used to validate the 300 kDa ultrafilter (see above), but not for small unilamellar vesicles or for aggregated vesicles. Recovery of cholesterol and phospholipids in separated micellar, unilamellar and aggregated vesicular phases was 95–100% of lipids in the corresponding whole model system.

In some experiments, small unilamellar vesicles were also separated from micelles in model systems by gel filtration of the supernatant on a Sephacryl S400 column (gel bed 30 cm, diameter 1.5 cm, flow-rate 0.5 ml/min, 1 mL fractions), equilibrated with aqueous 0.15 m NaCl plus 3 mm NaN_3 containing taurocholate at concentrations identical to the IMC of the original model system (27–29). Combined dialysis and ultrafiltration yielded the same amounts of vesicles and micelles as gel filtration, but was less time-consuming, required smaller amounts of aqueous bile salt solution, and did not lead to dilution of the sample. Therefore, combined dialysis and ultrafiltration was considered to be the preferable method to isolate micelles and small unilamellar vesicles from the model system.

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, and subsequently lyophilized

during 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 during 30 min at 50°C (above the main transition temperatures of the phospholipids). After sonication, the suspension was centrifuged during 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 at temperatures above 40°C, and used within 24 h. Small unilamellar vesicles were prepared with 100% EYPC, 100% (EY or BM)SM, 80% EYPC/20% (EY or BM)SM, or 60% EYPC/40% (EY or BM)SM as the phospholipid. Final phospholipid concentrations were 4 mm. Vesicles were prepared without or with cholesterol (cholesterol/phospholipid ratio 0, 0.2 or 0.4).

Interactions of small unilamellar vesicles with bile salts

Interactions of small unilamellar vesicles with taurocholate were examined by measuring absorbance at 405 nm every min during 80 min at 37°C, in a thermostatted Benchmark microplate reader (Bio-Rad, Hercules, CA). The solutions were stirred for 2 sec prior to each measurement. A decrease of the OD 405 after addition of taurocholate is compatible with micellization of the vesicles, whereas an increase can be attributed to growth, fusion, or aggregation of the vesicles (33). Absorbance measured in control vesicles without taurocholate always remained stable during the experiment. In the case of cholesterol-containing vesicles, after the experiment the mixtures were observed by polarizing light microscopy, in order to examine whether liquid or solid cholesterol crystals had formed.

In additional experiments, we added taurocholate to sonicated EYPC, BMSM, and cholesterol containing vesicles (final composition of the system: 16 mm taurocholate, 10 mm EYPC, 6.6 mm BMSM, and 6.4 mm cholesterol) and determined SM/PC ratios in micelles (obtained by ultrafiltration) at 10 min, 1 h, 4 h, and 12 h, in order to obtain further information on extent of asymmetric phospholipid distribution as a function of time.

Statistical analysis

Values are expressed as mean ± SEM. Differences between groups were tested for statistical significance by analysis of variance with the aid of SPSS software, version 7.5. 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 a two-tailed probability of less than 0.05.

RESULTS

Main gel to liquid crystalline phase transition temperatures of BMSM and EYSM

Main gel to liquid crystalline phase transition temperatures as determined by differential scanning calorimetry were 33.6°C for hydrated BMSM (maximum ΔH/sec at 29°C) and 36.6°C for hydrated EYSM (maximum ΔH/sec at 33°C).

Micellar sizes and intermixed micellar/vesicular bile salt concentrations of taurocholate–phospholipid systems

The hydrodynamic radius (Rh) of taurocholate–phospholipid mixed micelles (3 g/dL, 37°C) was 2.1 ± 0.1 nm at low phospholipid content (PL/(PL + BS) ratio = 0.2) but increased to 2.9 ± 0.1 nm at higher phospholipid content (PL/(PL + BS) ratio = 0.4). There were no sig-

nificant differences in sizes between EYPC-, EYSM-, or BMSM-containing mixed micelles at these ratios. Intermixed micellar/vesicular (i.e. monomeric + simple micellar) bile salt concentrations (IMC) of the same systems also strongly depended on phospholipid content: the IMC was 17.5 ± 0.3 mm at PL/(PL + BS) ratio 0.2, but 8.4 ± 0.3 mm at PL/(PL + BS) ratio 0.4 (37°C, 3 g/dL). Again, there were no significant differences between systems containing EYPC, EYSM, or BMSM as the phospholipid. Inclusion of small amounts (3 mol %) of cholesterol in the system led to turbidity due to formation of vesicles in EYSM- or BMSM-containing systems, whereas systems containing EYPC remained clear under these circumstances. However, the IMC did not change by inclusion of the sterol in EYSM-, BMSM-, or EYPC-containing systems.

Distribution of cholesterol and phospholipid between micellar and vesicular phases

Model systems composed with taurocholate, both EYPC and sphingomyelin as phospholipid, and variable amounts of cholesterol (16.6 mm taurocholate/10 mm EYPC/6.6 mm EYSM or BMSM/1.6, 3.2, or 6.4 mm cholesterol, see also Materials and Methods) were studied after 2 weeks of incubation at 37°C. These systems were highly turbid and microscopic examination revealed the presence of large amounts of aggregated vesicles. Solid cholesterol crystals, however, were not observed. In a first series of experiments, micelles were isolated from model systems by ultrafiltration. As shown in **Fig. 1**, with increasing cholesterol content of the model systems, micellar phospholipid solubi-
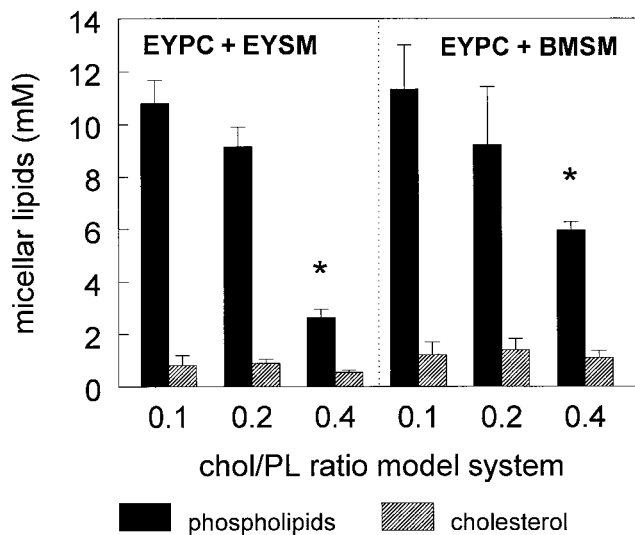

Fig. 1. Micellar solubilization of phospholipids (solid bars) and cholesterol (hatched bars) as determined by ultrafiltration of model systems after 2 weeks incubation at 37°C (16.6 mm taurocholate, 10 mm EYPC, 6.6 mm EYSM or BMSM, 1.6, 3.2, or 6.4 mm cholesterol, cholesterol/phospholipid ratios 0.1, 0.2, and 0.4). The amount of phospholipid solubilized in micelles decreased significantly at higher cholesterol content of the systems, whereas the amount of cholesterol solubilized in micelles did not change at varying cholesterol contents. There were no significant differences between EYSM- and BMSM-containing systems. An asterisk (*) indicates significant difference from chol/PL ratios 0.1 and 0.2.

lization decreased strongly, whereas micellar cholesterol solubilization remained constant. The decrease of micellar phospholipid occurred both in EYSM- and BMSM-containing systems.

In subsequent experiments, various cholesterol-containing phases were separated by ultracentrifugation followed by ultrafiltration and dialysis of the supernatant (see Materials and Methods). After precipitation of aggregated vesicles by ultracentrifugation, the supernatant displayed a typical Tyndall effect, indicating the presence of small
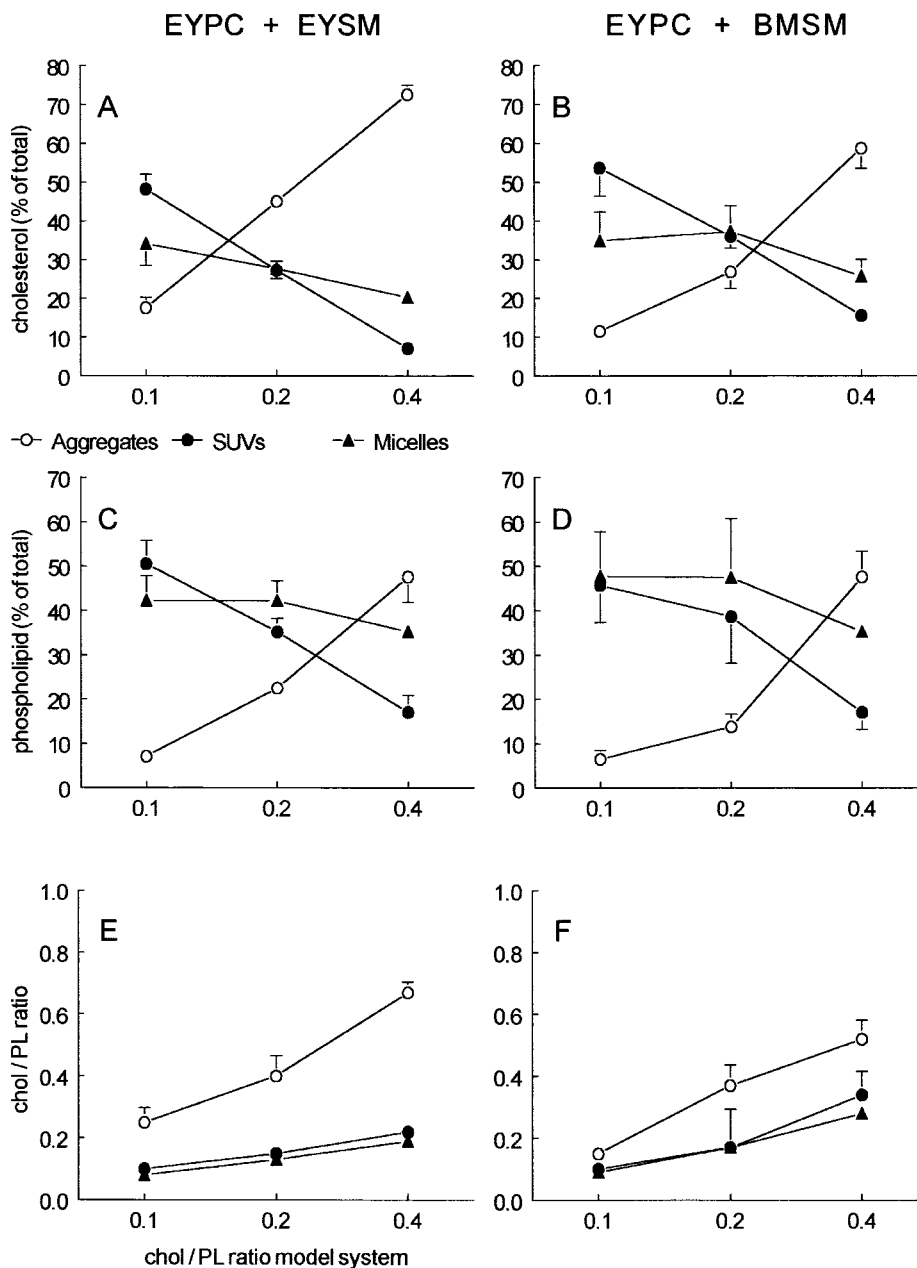
Fig. 2. Solubilization of cholesterol and phospholipids in aggregated vesicles (open circles), unilamellar vesicles (closed circles), and mixed micelles (triangles) in model systems after 1 week incubation at 37°C. Lipid composition of the model systems was the same as in Fig. 1. The phases were separated by ultracentrifugation and subsequent ultrafiltration and dialysis of the supernatant (see Materials and Methods). Figures 2A and B show cholesterol solubilization, 2C and D phospholipid solubilization, and 2E and F cholesterol/phospholipid ratios. Figs. 2A, C, E apply to EYSM-containing systems and Figures 2B, D, F to BMSM-containing systems. At increasing cholesterol contents of the system, solubilization of cholesterol and phospholipids in aggregated vesicles increased strongly, whereas solubilization of these lipids in small unilamellar vesicles decreased strongly (significant differences between all chol/PL ratios). The amount of mixed micellar cholesterol and phospholipid also tended to decrease at increasing cholesterol content in the system. Cholesterol/phospholipid ratios increased significantly at increasing cholesterol contents in the system, particularly in aggregated vesicles. There was no significant influence of SM-type on lipid solubilization in the various phases, although effects of cholesterol inclusion tended to be more pronounced in the case of EYSM.

unilamellar vesicles. Indeed, quasi-elastic light scattering spectroscopy of the supernatant revealed the presence of particles with a hydrodynamic radius of 43 ± 12 nm, consistent with small unilamellar vesicles.

As shown in **Fig. 2 A–B**, at increasing cholesterol content of the system, the excess cholesterol was mainly distributed into the detergent-insoluble pelletable fraction consisting of aggregated vesicles. Distribution of cholesterol into small unilamellar vesicles and in mixed micelles decreased. Although the total amount of phospholipids was kept constant in the system, distribution of phospholipids into aggregated vesicles increased at higher cholesterol contents, whereas the amount of phospholipids in small unilamellar vesicles and mixed micelles decreased (Fig. 2C–D). Whereas the cholesterol/phospholipid ratios in micelles and small unilamellar vesicles increased only slightly at higher cholesterol content of the system, there was a strong increase of cholesterol/phospholipid ratio in aggregated vesicles (Fig. 2E, F). The effects of cholesterol tended to be more pronounced in systems containing EYSM (Fig. 2A, C, E) than in systems composed with BMSM (Fig. 2B, D, F).

Distribution of sphingomyelin and egg yolk phosphatidylcholine between vesicles and micelles

Figure 3 shows the distribution of SM and EYPC into vesicles (small unilamellar and aggregated combined) and micelles quantified after separation of the phases by means of ultrafiltration (see Materials and Methods). Micellar enrichment with PC and vesicular enrichment with SM is evident, particularly at high cholesterol contents of the system. A similar pattern of phospholipid distribution was observed when various phases were separated by ultra-
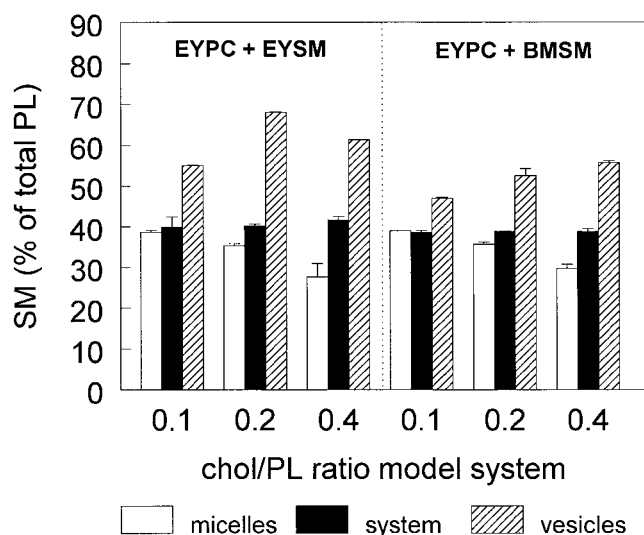
centrifugation with subsequent dialysis and ultrafiltration of the supernatant. Apart from data on phospholipid solubilization in micelles, this procedure also yielded separate information on phospholipid distribution into small unilamellar and into large aggregated vesicles (**Fig. 4**). SM was highly enriched in aggregated vesicles. In contrast, PC preferentially distributed into micelles and into small unilamellar vesicles, particularly at high cholesterol contents of the system. Asymmetric distribution of phospholipids tended to be more pronounced in EYSM-containing systems (Fig. 4A) than in BMSM-containing systems (Fig. 4B), but significance was not reached.

As shown in **Fig. 5**, in systems composed with BMSM, there was a preferential distribution of SM with long (>21 C-atoms) saturated acyl chains into aggregated vesicles, and a preferential distribution of SM with shorter or unsaturated acyl chains in micelles or small unilamellar vesicles, provided that the systems also contained cholesterol. No such asymmetric distribution occurred in systems com-
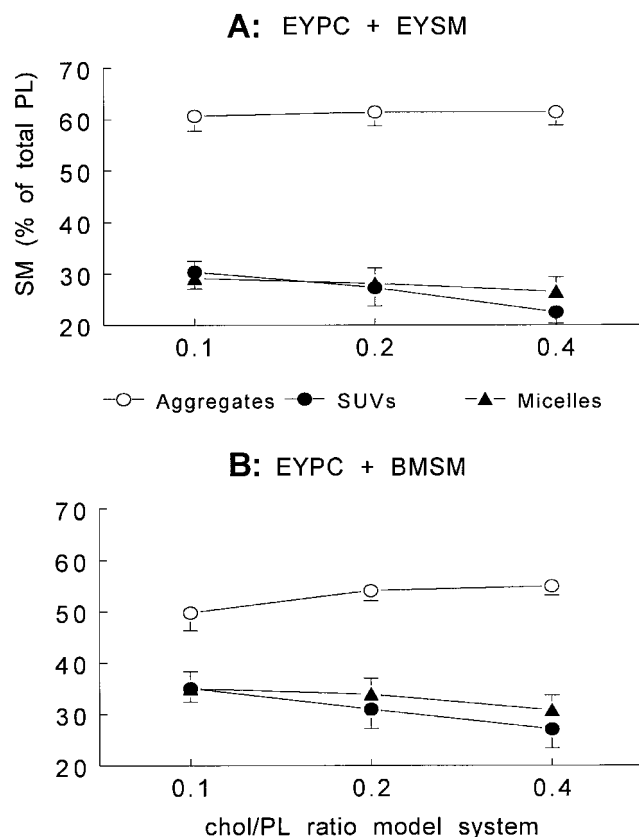

Fig. 4. Distribution of SM and PC between aggregated vesicles (open circles), small unilamellar vesicles (closed circles), and micelles (triangles), as determined by ultracentrifugation (see Materials and Methods) of model systems after 1 week incubation at 37°C. Lipid composition of the model systems was the same as in Fig. 1. SM solubilization in micelles and small unilamellar vesicles decreased and SM solubilization in aggregated vesicles increased significantly at increasing cholesterol content of the system (significant differences between all chol/PL ratios). Reciprocal results were obtained for PC solubilization. There was no significant influence of SM-type on the distribution of phospholipids between the various phases, although effects of cholesterol inclusion tended to be more pronounced in the case of EYSM.


Fig. 3. Distribution of SM and PC between vesicles and micelles, as determined by ultrafiltration of model systems after 2 weeks incubation at 37°C. Lipid composition of the model systems was the same as in Fig. 1. Micellar SM solubilization decreased, and vesicular SM solubilization increased at increasing cholesterol content of the system (differences significant between all chol/PL ratios). Reciprocal results were obtained for PC solubilization.
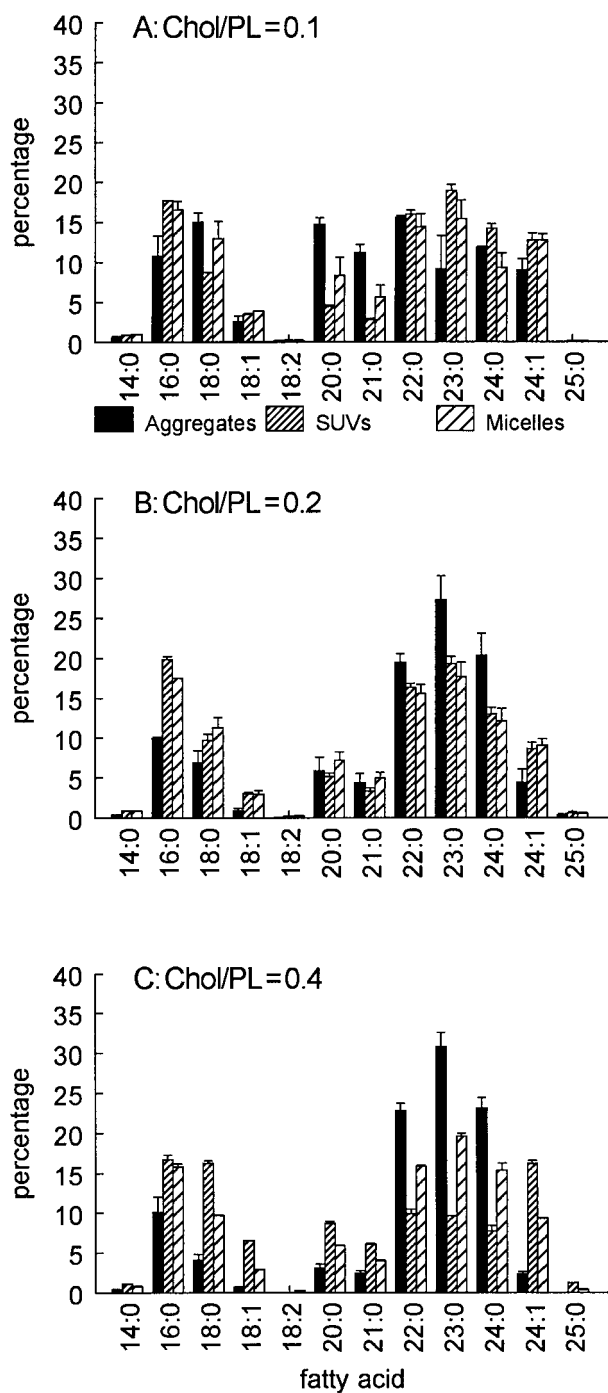
Fig. 5. Distribution of SM species between aggregated vesicles, small unilamellar vesicles, and micelles in model systems after incubation at 37°C (16 mm taurocholate, 10 mm EYPC, 6.6 mm BMSM, 1.6, 3.2, or 6.4 mM cholesterol, cholesterol/phospholipid ratios 0.1, 0.2, or 0.4). At high cholesterol content, there is a preferential distribution of SM with long (>21 C-atoms) saturated acyl chains into aggregated vesicles and a preferential distribution of SM with shorter or unsaturated acyl chains in micelles and small unilamellar vesicles (significant effects for % long and % saturated acyl chains: ANOVA).

posed with EYSM, which can be explained by the small amounts of long acyl chains in EYSM.

The model systems that we used above all plotted in the right two-phase zone of the equilibrium ternary phase diagram (20, 30) and contained micelles and vesicles of various compositions. In addition, we determined composition and SM/PC ratio of micelles from a model system (57 mm taurocholate, 19 mm EYPC, 19 mm EYSM, 46 mm cholesterol), plotting in the middle three-phase (micelles, vesicles, and solid cholesterol crystals containing) zone of the ternary phase diagram (20, 30), after 50 days incubation at 37°C. According to the phase rule, all micelles in this three-phase system should have the same, invariant composition at thermodynamic equilibrium (31). Microscopic examination of the model system revealed aggregated vesicles and solid cholesterol crystals. Composition of micelles (obtained by ultrafiltration of the supernatant) was 41 mm (77 mol %) taurocholate, 11 mm (21 mol %) phospholipids and 1.2 mm (2 mol %) cholesterol and micellar SM/PC ratio was 0.33 (versus 1.0 in the whole system).

We also determined micellar SM/PC ratios at various time points after addition of taurocholate to sonicated EYPC-, BMSM-, and cholesterol-containing vesicles (final composition of the system: 16.6 mm taurocholate, 10 mm EYPC, 6.6 mm BMSM, 6.4 mm cholesterol). Micellar SM/PC ratio at 10 min after addition of taurocholate was 0.67 (identical to the SM/PC ratio of 0.66 in the whole system) but decreased to 0.61 after 1 h, 0.55 after 4 h, and 0.41 after 12 h.

Resistance of phospholipid vesicles against detergent bile salts

As shown in **Fig. 6A**, vesicles without cholesterol and containing EYPC as the sole phospholipid tended to be rather resistant against the detergent effects of taurocholate, as indicated by the slow decrease of absorption values during the time period studied (conditions: vesicular phospholipid 4 mm final concentration: addition of taurocholate at 5 mm final concentration, 37°C).

In contrast, vesicles without cholesterol and containing (EY or BM)SM as the sole phospholipid were extremely sensitive, with a virtual instantaneous drop of absorbance to 0 upon addition of taurocholate. Also, partial replacement of vesicular EYPC by EYSM or BMSM (vesicular EYPC/SM ratios 80/20 or 60/40), without inclusion of cholesterol, led to significant vesicular destabilization, as evidenced by absorption values upon addition of taurocholate. The vesicular destabilization depended on the amount of EYPC replaced by SM and was strongest in the case of partial replacement by EYSM (Fig. 6A). Essentially the same results were obtained upon addition of taurocholate at 4, 6, and 7 mm final concentrations (results not shown).

As shown in Fig. 6B, incorporation of cholesterol in SM-containing EYPC vesicles prevented the destabilizing effect of SM (conditions: vesicular phospholipid 4 mm final concentration; vesicular EYPC/SM 80/20 or 60/40; vesicular cholesterol/phospholipid ratio 0.4; addition of taurocholate at 5 mm final concentration; 37°C): Absorbances by spectrophotometry of these cholesterol-enriched vesicles were stable in the case of EYPC as the sole vesicular phospholipid but increased in the case of incorporation of SM in the vesicles. The extent of increase depended on the
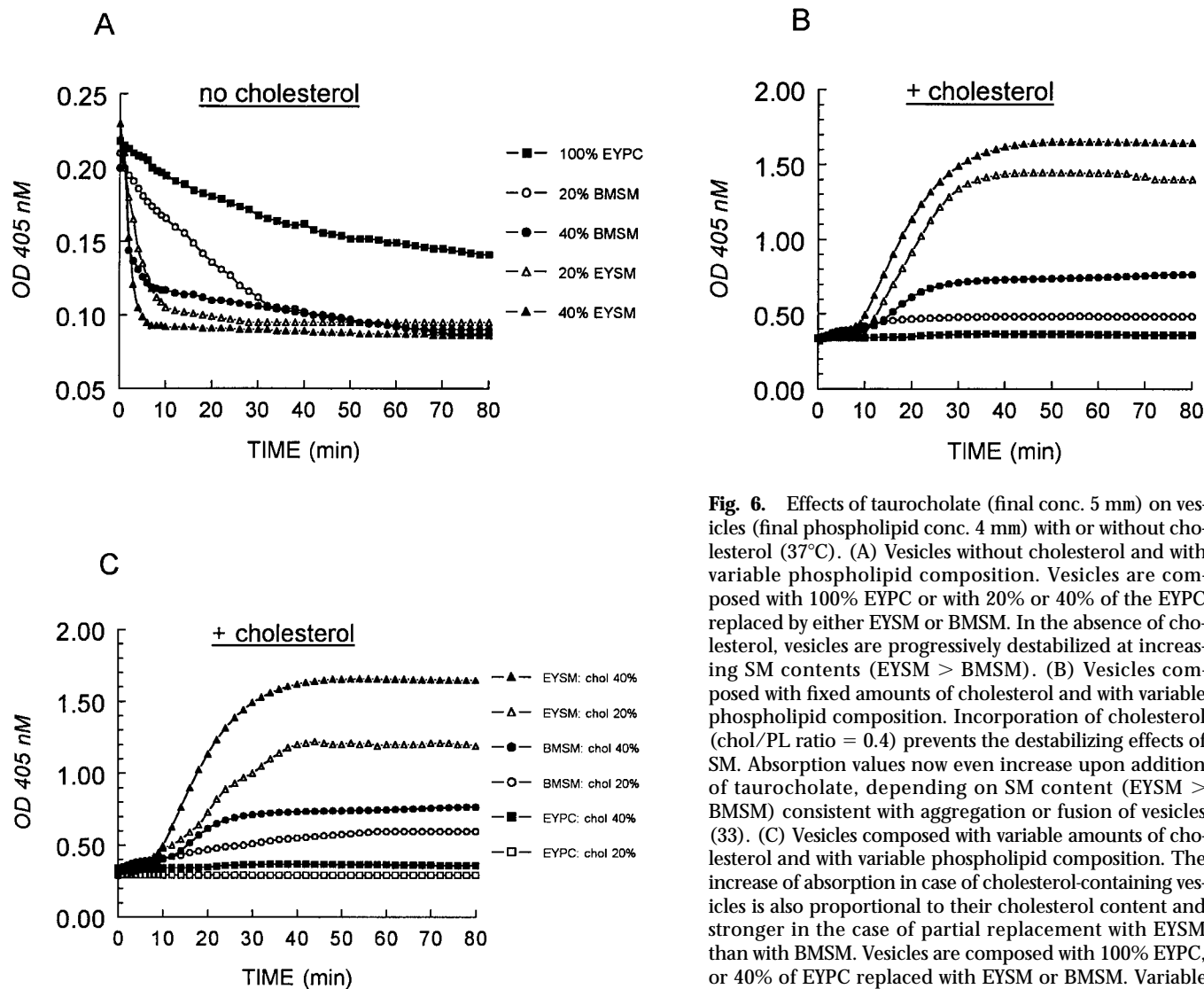
A

no cholesterol

—■— 100% EYPC

—○— 20% BMSM

—●— 40% BMSM

—△— 20% EYSM

—▲— 40% EYSM

B

+ cholesterol

C

+ cholesterol

—▲— EYSM: chol 40%

—△— EYSM: chol 20%

—●— BMSM: chol 40%

—○— BMSM: chol 20%

—■— EYPC: chol 40%

—□— EYPC: chol 20%

Fig. 6. Effects of taurocholate (final conc. 5 mm) on vesicles (final phospholipid conc. 4 mm) with or without cholesterol (37°C). (A) Vesicles without cholesterol and with variable phospholipid composition. Vesicles are composed with 100% EYPC or with 20% or 40% of the EYPC replaced by either EYSM or BMSM. In the absence of cholesterol, vesicles are progressively destabilized at increasing SM contents (EYSM > BMSM). (B) Vesicles composed with fixed amounts of cholesterol and with variable phospholipid composition. Incorporation of cholesterol (chol/PL ratio = 0.4) prevents the destabilizing effects of SM. Absorption values now even increase upon addition of taurocholate, depending on SM content (EYSM > BMSM) consistent with aggregation or fusion of vesicles (33). (C) Vesicles composed with variable amounts of cholesterol and with variable phospholipid composition. The increase of absorption in case of cholesterol-containing vesicles is also proportional to their cholesterol content and stronger in the case of partial replacement with EYSM than with BMSM. Vesicles are composed with 100% EYPC, or 40% of EYPC replaced with EYSM or BMSM. Variable amounts (20% or 40%) of cholesterol are also incorporated in the vesicles. Symbols for (A) also apply to (B).

amount of EYPC replaced by SM and was strongest in the case of replacement by EYSM. Microscopic examination revealed formation of aggregated vesicles under the latter circumstances, whereas solid cholesterol crystals were absent.

As shown in Fig. 6C, the increase in absorption observed in the case of SM-containing vesicles was proportional to their cholesterol content (vesicular phospholipid 4 mm final concentration, vesicular EYPC/SM ratio 60/40; vesicular cholesterol/phospholipid ratio 0.2 and 0.4, addition of taurocholate at a final concentration of 5 mm, 37°C). The increase of absorption was stronger in the case of EYSM- than BMSM-containing vesicles.

The hydrodynamic radius (Rh) as determined by quasi-elastic light scattering spectroscopy of the small unilamellar vesicles composed with 100% EYPC and without cholesterol was 50 ± 1.65 nm. Vesicles with only SM as phospholipid were smaller, with an Rh of 38.0 ± 2.1 nm (EYSM) and 34.2 ± 1.9 nm (BMSM). Partial replacement of EYPC with SM also led to slightly decreased vesicle sizes (43.5 ± 1.23

nm in case of 40% EYSM, 43.9 ± 0.97 nm in case of 40% BMSM). Vesicle sizes increased slightly with inclusion of cholesterol (67.2 ± 0.9 nm, 49.6 ± 1.6 nm, and 49.6 ± 2.6 nm for 100% EYPC, EYSM, and BMSM, respectively; 62.1 ± 1.3 nm and 63.2 ± 1.9 nm in the case of partial replacement of EYPC with 40% EYSM and 40% BMSM, respectively, all at cholesterol/phospholipid ratio 0.4).

DISCUSSION

The major finding of the present study was the asymmetric distribution of PC and SM between vesicular and micellar phases, provided that the system also contained cholesterol. Similar results have been reported after incubation with bile salts of isolated vesicular hepatocyte canalicular membrane subfractions (34, 35) or erythrocytes (36).

In most experiments, we have exploited the approach

of Schroeder, London, and Brown (32) to pellet detergent-insoluble material with the aid of ultracentrifugation. These authors aptly state that "although it is likely that any sedimentable lipids are not solubilized, the inability to sediment does not guarantee solubilization." Nevertheless, they considered "for convenience the pelletable material as insoluble and material that remains in the supernatant as soluble" (32). We found evidence that the supernatant was not homogeneous but contained small unilamellar vesicles in addition to mixed micelles. We further separated these phases with the aid of ultrafiltration and dialysis, or gel filtration techniques, taking into account the intermixed micellar/vesicular bile salt concentration (IMC), in order to avoid artifactual shifts between phases (27–29). In the supernatant, both mixed micelles and small unilamellar vesicles proved to be enriched in PC. In contrast, the pelletable fraction, consisting of aggregated vesicles, was enriched in SM.

The 300 kDa ultrafilter that we used was completely permeable for mixed micelles of a wide range of compositions but completely impermeable for vesicles. We also found strong micellar depletion of SM in a model system plotting in the middle (micelles, vesicles, and solid cholesterol crystal containing) three-phase zone of the ternary phase diagram (20, 30) after 50 days incubation (assumed thermodynamic equilibrium). According to the phase rule (31), at thermodynamic equilibrium, micelles in this system should be of one invariant composition. Therefore, these findings definitely exclude preferential passage of phosphatidylcholine-enriched micelles through the ultrafilter as the explanation of micellar SM depletion.

We also determined micellar SM/PC ratios as a function of time after addition of taurocholate to sonicated SM, EYPC, and cholesterol-containing vesicles. The fact that micellar SM depletion was not present immediately after taurocholate addition but occurred at later stages excludes artifacts during preparation of the model systems as the explanation of our findings.

The present data on asymmetric distribution of (EY)PC and (EY or BM)SM between micellar and vesicular phases are in agreement with equilibrium ternary phase diagrams of (EY or BM)SM, taurocholate and cholesterol (37°C, 3 g/dL) (20): compared to EYPC-containing systems under the same conditions (30), one-phase micellar zones are strongly reduced in SM-containing systems, and there is a marked expansion of the right two-phase (micelles + vesicles-containing) zone (20). The fact that ternary phase diagrams for systems composed with (disaturated) dipalmitoyl (DP)PC (T_m 41°C) are identical to phase diagrams of SM-containing systems (20) points to the importance of acyl chain composition and/or physical state of the phospholipid. Also, after separation of micellar and vesicular phases from analogous EYPC-containing model systems with the aid of gel filtration, PC species with unsaturated acyl chains distribute preferentially into micelles, whereas vesicles are enriched in disaturated PC species, supposedly due to packing constraints with more efficient packing of disaturated PC species in vesicles (37). Similarly, differences in packing constraints in small unilamellar versus

aggregated vesicles (38) may explain their different phospholipid composition.

Another factor of potential relevance may be the much higher gel to liquid crystalline phase transition temperature (T_m) for SM compared to EYPC: whereas EYPC has a T_m below 0°C (39), we found T_m of hydrated EYSM to be 36.6°C, similar to previous data (39). The slightly lower T_m (33.6°C) for hydrated BMSM (not studied before) is not unexpected, as in general, T_m decreases with increasing chain length (39). Pure phospholipids exist in a solid ordered gel phase below a melting temperature (T_m) that is characteristic of each lipid, and in a liquid disordered (also called "liquid crystalline") phase above T_m. In contrast, in the presence of cholesterol, lipids with a high T_m in the pure state (e.g., disaturated PC species and sphingomyelins) may form a so-called "liquid-ordered" phase around T_m (40–42). This liquid-ordered phase has properties intermediate between the gel and liquid–crystalline phases: Like the gel phase, the liquid-ordered phase is characterized by tight acyl-chain packing and relatively extended acyl chains. On the other hand, like lipids in the liquid–crystalline phase, lipids in the liquid-ordered phase exhibit relatively rapid lateral mobility within the bilayer. Recent data indicate that in bilayers containing more than one phospholipid, in the presence of cholesterol, phase separation of the phospholipids with the higher T_m (such as SM) into cholesterol-rich liquid-ordered domains occurs, and that such a phase separation is a prerequisite for detergent resistance (41, 42). We propose that SM in cholesterol-rich liquid ordered domains is relatively resistant to the micellizing effects of detergent bile salts, thus explaining asymmetric PC and SM distribution as found in the present study.

There is some evidence that monomeric and simple micellar rather than mixed micellar (i.e., phospholipid-associated) bile salts exert the detergent effect on membrane bilayers (43). However, we did not find an effect of varying phospholipid species on intermixed micellar/vesicular (IMC: i.e., simple micellar + monomeric) bile salt concentrations or micellar sizes. In agreement with previous data, micellar sizes were larger (44) and IMC values were lower (27) at increasing phospholipid contents. The absence of any change in IMC values when vesicles were formed by inclusion of cholesterol in the system is in agreement with a previous report of Donovan, Timofeyeva, and Carey (27). These findings suggest that enhanced resistance to bile salt dissolution for cholesterol-enriched membranes as reported in this and previous studies (45) is due to intrinsic properties of the membranes rather than to alterations of the IMC (43).

The pivotal role of cholesterol is also dramatically illustrated by the incubation experiments shown in Fig. 6. In agreement with previous data (46), in the absence of cholesterol, vesicles composed with SM were highly sensitive to detergent bile salts. Incorporation of cholesterol restored membrane resistance against detergent bile salts and even led in some cases to vesicle aggregation (33). Vesicles without cholesterol were only slightly smaller than vesicles with cholesterol. Nevertheless, we cannot defi-

nitely exclude the possibility that reduced interactions between phospholipid molecules, due to increased curvature strain (16, 17, 47, 48), could explain decreased stability of the vesicles without cholesterol.

In the present study, effects of cholesterol inclusion on vesicle aggregation or asymmetric distribution of PC and SM tended to be more pronounced in EYSM- than in BMSM-containing systems, possibly related to different acyl chain composition and/or to subtle differences in physical state due to different T_m of EYSM and BMSM. We (20) and others (15) have found in vesicle-transfer experiments evidence for a higher affinity of cholesterol for EYSM than for SM that contains long-chain fatty acids such as BMSM.

Pathophysiological correlations

The findings in the present study may be relevant for epithelial cells in general, which contain large amounts of glycosphingolipids in the apical membrane (49). We were particularly interested to increase insight into some puzzling events occurring at the canalicular membrane during nascent bile formation. As the hepatocyte, another highly polarized cell, is enriched at the canalicular side in cholesterol and SM with long saturated acyl chains (1, 8–10), our data on BMSM with similar acyl chain composition appear to be most relevant. The fact that PC is the major phospholipid secreted into bile, with only trace amounts of SM (6), despite the presence of large quantities of both phospholipids in the outer leaflet of the canalicular membrane (1, 5), may relate to a high lateral pressure due to translocation of PC molecules by *mdr*2 P-glycoprotein (2). No such protein is known to be present for SM. The present study indicates that the physical–chemical state of phospholipids in the canalicular membrane could also contribute to preferential PC secretion. The high cholesterol content of SM domains that are laterally segregated, perhaps in conjunction with disaturated PC species, might impede bile salt-dependent biliary secretion of SM. Furthermore, recent data from Nibbering and Carey (8) indicate that trace SM in bile contains mainly 16:0 acyl chains, despite the predominance of long acyl chains in canalicular membrane SM (8, 9). Using ultracentrifugation, we found a strong preferential distribution of SM with long acyl chains in the "pelletable insoluble fraction" (according to the terminology of Schroeder et al. (32)) and a preferential distribution of 16:0 SM in the "soluble supernatant", which proved to contain mixed micelles and small unilamellar vesicles. By using electron microscopic techniques, Crawford et al. (4) has demonstrated the presence of significant amounts of such small unilamellar vesicles within the canalicular lumen, consistent with a vesicular mode of cholesterol and phospholipid secretion. Nevertheless, the finding that infusion of the hydrophobic bile salt taurodeoxycholate restores cholesterol secretion in the *mdr*2 "knockout" mice to normal levels (50) would suggest a micellar mode of cholesterol secretion as PC is still virtually absent in this situation. When one considers our present data, there is a distinct possibility that vesicular and micellar modes of lipid secretion could coexist during nascent bile formation. As recently discussed by Oude Elferink, Tytgat, and Groen (51) and Smith et al. (52), the non-linear relationship between phospholipid and cholesterol secretion as recently found in mice with various expressions of the *mdr*2 gene is consistent with a micellar (or alternatively combined micellar + vesicular) but not with a pure vesicular mechanism of biliary lipid secretion.

Last, the inhibiting effects of cholesterol within the membrane on the amounts of phospholipids solubilized in micelles (Fig. 1) would lead to the hypothesis that cholesterol in the canalicular membrane could exert a modulating role in regulation of biliary lipid secretion. For example, biliary secretion of NBD-sphingomyelin infused in isolated livers of *mdr*2 "knockout" mice is strongly decreased compared to *mdr*2 (+/+) mice, although SM should be the predominant phospholipid on the outer leaflet of the canalicular membrane in the absence of PC translocating activity in *mdr* "knockout" mice (C. Frijters, A. K. Groen, and R. J. Oude Elferink, personal communication). This puzzling finding would be easily understood if one assumes inhibiting effects on biliary phospholipid secretion of an increased cholesterol content in the outer leaflet.

In conclusion, our data reveal preferential distribution of lipids and enrichment of SM with long saturated acyl chains in vesicular aggregates at increasing cholesterol content. In contrast, there is preferential distribution of PC into mixed micelles and small unilamellar vesicles under these circumstances. These findings may be relevant for canalicular bile formation.

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