Cholesterol crystallization in model biles: effects of bile salt and phospholipid species composition

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Abstract Cholesterol in human bile is solubilized in micelles by (relatively hydrophobic) bile salts and phosphatidylcholine (unsaturated acyl chains at sn-2 position). Hydrophobic tauroursodeoxycholate, dipalmitoyl phosphatidylcholine, and sphingomyelin all decrease cholesterol crystal-containing zones in the equilibrium ternary phase diagram (van Erpecum, K. J., and M. C. Carey. 1997. Biochim. Biophys. Acta. 1345: 269–282) and thus could be valuable in gallstone prevention. We have now compared crystallization in cholesterol-supersaturated model systems (3.6 g/dl, 37°C) composed of various bile salts as well as egg yolk phosphatidylcholine (unsaturated acyl chains at sn-2 position), dipalmitoyl phosphatidylcholine, or sphingomyelin throughout the phase diagram. At low phospholipid contents [left two-phase (micelle plus crystal-containing) zone], tauroursodeoxycholate inhibited, but dipalmitoyl phosphatidylcholine and sphingomyelin all enhanced crystallization. At pathophysiologically relevant intermediate phospholipid contents [central three-phase (micelle plus vesicle plus crystal-containing) zone], tauroursodeoxycholate inhibited, but dipalmitoyl phosphatidylcholine and sphingomyelin enhanced, crystallization. Also, during 10 days of incubation, there was a strong decrease in vesicular cholesterol contents and vesicular cholesterol-to-phospholipid ratios (~1 on day 10), coinciding with a strong increase in crystal mass. At high phospholipid contents [right two-phase (micelle plus vesicle-containing) zone], vesicles were always unsaturated and crystallization did not occur. Strategies aiming to increase amounts of hydrophilic bile salts may be preferable to increasing saturated phospholipids in the system for crystallization behavior. In the case of excess bile salts [PL/(BS + PL) ratios ~≤0.2], crystals precipitate at fast rates, and both various intermediate anhydrous cholesterol crystals (needles, arcs, tubules, and spirals) and mature rhomboid cholesterol monohydrate crystals can be detected by microscopy. In the case of higher amounts of phospholipids, crystal precipitation proceeds at slower rates (with predominant formation of mature cholesterol monohydrate crystals), and large amounts of cholesterol are solubilized in vesicles together with phospholipids. In the case of excess phospholipids [high PL/(BS + PL) ratios], solid cholesterol crystals do not occur, and cholesterol is mainly solubilized in vesicular phases. On the basis of these data, the equilibrium cholesterol-bile salt-phospholipid ternary phase diagram [Fig. 1 (5, 6)] is assumed to contain a one-phase zone (only micelles), a left two-phase (micelle and cholesterol crystal-containing) zone, a central three-phase (micelle, vesicle, and cholesterol crystal-containing) zone, and a right two-phase (micelle and vesicle-containing) zone. The phase diagram describes the occurrence of cholesterol crystals...

Precipitation of cholesterol crystals from supersaturated bile is a prerequisite for gallstone formation (1). The sterol is poorly soluble in an aqueous environment, and is solubilized in bile in mixed micelles by bile salts (BS) and phospholipids (PL). Phosphatidylcholine is the major phospholipid in bile [>95% of total: mainly 16:0 acyl chains at the sn-1 position and mainly unsaturated (18:2 > 18:1 > 20:4) acyl chains at the sn-2 position (2)]. In the case of cholesterol supersaturation, the excess sterol may be contained in vesicles together with phospholipids (3, 4) or precipitated as solid crystals.

The studies of Wang and Carey (5) have revealed the importance of the relative amounts of bile salts versus phospholipids in the system for crystallization behavior. In the case of excess bile salts [PL/(BS + PL) ratios ~≤0.2], crystals precipitate at fast rates, and both various intermediate anhydrous cholesterol crystals (needles, arcs, tubules, and spirals) and mature rhomboid cholesterol monohydrate crystals can be detected by microscopy. In the case of higher amounts of phospholipids, crystal precipitation proceeds at slower rates (with predominant formation of mature cholesterol monohydrate crystals), and large amounts of cholesterol are solubilized in vesicles together with phospholipids. In the case of excess phospholipids [high PL/(BS + PL) ratios], solid cholesterol crystals do not occur, and cholesterol is mainly solubilized in vesicular phases. On the basis of these data, the equilibrium cholesterol-bile salt-phospholipid ternary phase diagram [Fig. 1 (5, 6)] is assumed to contain a one-phase zone (only micelles), a left two-phase (micelle and cholesterol crystal-containing) zone, a central three-phase (micelle, vesicle, and cholesterol crystal-containing) zone, and a right two-phase (micelle and vesicle-containing) zone. The phase diagram describes the occurrence of cholesterol crystals...

Abbreviations: BS, bile salts; CSI, cholesterol saturation index; DPPC, dipalmitoyl phosphatidylcholine; EYPC, egg yolk phosphatidylcholine; EYSM, egg yolk sphingomyelin; IMC, intermixed micellar-vesicular bile salt concentration; MWCO, molecular weight cutoff; PL, phospholipids; TC, taurocholate; TDC, taurodeoxycholate; TUDC, tauroursodeoxycholate.

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micelles, and vesicles under equilibrium conditions, but accurate quantification of these phases has been hampered by methodological problems. Micelles and vesicles may be separated with the aid of gel filtration with bile salts at intermixed micellar/vesicular concentrations in the eluant buffer in order to avoid artifactual shifts of salts at intermixed micellar/vesicular concentrations in may be separated with the aid of highly selective ultrafilters and dialysis, taking into account the intermixed micellar/vesicular bile salt concentration.

In the present study, we have systematically determined the distribution of lipids into various phases throughout the ternary phase diagram. The hydrophilic bile salt ursodeoxycholate is frequently used in clinical practice to dissolve cholesterol gallstones, and dietary modulation of biliary phospholipid composition has been suggested to prevent gallstone formation (12–15). We therefore also evaluated effects of bile salt species and phospholipid class composition on crystallization.

MATERIALS AND METHODS

Materials

Taurocholate (TC), taurodeoxycholate (TDC), and tauroursodeoxycholate (TUDC) were obtained from Sigma (St. Louis, MO) and yielded a single spot on thin-layer chromatography [butanol–acetic acid–water 10:1:1 (v/v/v)], application of 200 μg of bile salt. Cholesterol (Sigma) was ≥98% pure by reversed-phase HPLC [isopropanol–acetonitrile 1:1 (v/v), detection at 210 nm]. Phosphatidylcholine from egg yolk (EYPC; Sigma), and dipalmitoyl phosphatidylcholine (DPPC; Sigma) and sphingomyelin from egg yolk (EYSM; Avanti Polar-Lipids, Alabaster, AL) yielded a single spot on thin-layer chromatography [chloroform–methanol–water 65:25:4 (v/v/v), application of 200 μg of lipid]. Acyl chain compositions as determined by gas-liquid chromatography (16) were virtually identical to previously published data (6) and showed a preponderance of 16:0 acyl chains for EYSM, similar to trace SM in bile (17). As shown by reversed-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 PC in human bile (2). All other chemicals and solvents were of American Chemical Society or reagent-grade quality.

Ultrafilters with a molecular weight cutoff (MWCO) of 10,000 and 300,000 were purchased from Sartorius (Centrisart I; Göttingen, Germany), and dialysis membranes with an MWCO of 300,000 from Spectrum Laboratories (SpectraPor®; Laguna Hills, CA). The enzymatic cholesterol assay kit was obtained from Boehringer (Mannheim, Germany) and the enzymatic phospholipid kit was purchased from Sopar Biochem (Brussels, Belgium). 3α-Hydroxysteroid dehydrogenase for the enzymatic measurement of bile salt concentrations (18) and a colorimetric chloride kit were purchased from Sigma. The reversed-phase C18 HPLC column was from Supelco (Supelcosil LC-18-DB; Supelco, Bellefonte, PA).

Preparation of model biles

Lipid mixtures containing variable proportions of cholesterol, phospholipids (both from stock solutions in chloroform), and bile salts (from stock solutions in methanol) were vortex mixed and dried at 45°C under a mild stream of nitrogen, and subsequently hydrolyzed for 24 h, before being dissolved in aqueous 150 mM NaCl plus 3 mM NaN3. 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 to 45°C for 10 min before use. The final mole percentages of cholesterol, phospholipids, and bile salts did not differ by more than 1% from the intended mole percentages. Also, model systems always plotted in the intended zones of the appropriate phase diagrams (5, 6), as inferred by microscopic examination.

Lipid measurement

Phospholipid concentrations in model systems were assayed by determining inorganic phosphate (19). Cholesterol concentrations were determined with an enzymatic assay (20), and bile salts were determined by the 3α-hydroxysteroid dehydrogenase method (18).

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 “simple” micelles. The monomeric plus simple micellar bile salt concentration is referred to as the “intermixed micellar/vesicular (non-phospholipid-associated)
Isolation of various lipid phases

Cholesterol crystals and aggregated vesicles. After 10 and 40 days (in some cases also after 1 day) of incubation at 37°C, various phases were isolated from cholesterol-supersaturated model systems as described (11). In brief, detergent-resistant aggregated vesicles were precipitated by ultracentrifugation for 30 min at 50,000 g and 37°C in a TLA 55 rotor (Beckman, Palo Alto, CA) (22). In the case of coexistent cholesterol crystals and aggregated vesicles [three-phase (micelle, vesicle, and crystal-containing) zone; see Fig. 1], centrifugation of an additional bile sample was also performed 10 min after addition of deoxycholate in quantities sufficient to desaturate the model system [final cholesterol saturation index (CSI) <1]. After such incubation, light microscopy and stability of turbidity measurements [optical density at 405 nm (OD405)] (23) revealed that all vesicular aggregates had been completely micellized. Experiments with isolated cholesterol crystals showed that solubilization of the cholesterol crystals did not occur during the short incubation with deoxycholate. Therefore, cholesterol crystal mass equals cholesterol content in the pellet after addition of deoxycholate, and cholesterol content in vesicular aggregates can be calculated from the difference of cholesterol contents between the pellets without and with added deoxycholate (23). IMC values measured in noncentrifuged model biles were identical to IMC values in the corresponding supernatants. We did not find a bile salt gradient in the supernatant after centrifugation, indicating that the short centrifugation procedure did not cause an inhomogeneous distribution of micelles or unilamellar vesicles in the tube. Furthermore, centrifugation did not influence the content of mixed micelles in the model bile, because lipid contents in micelles obtained by ultrafiltration of supernatant through the 300,000 MWCO filter (see below) were identical to lipid concentrations in ultrafiltrates of corresponding whole model biles.

As model systems plotting in the left-two phase zone (see Fig. 1) contain only micelles and cholesterol crystals at thermodynamic equilibrium, cholesterol crystals were precipitated in this case by ultracentrifugation, without added deoxycholate. Although studies by quasielastic light scattering spectroscopy (5) have suggested that small unilamellar vesicles may occur transiently in supersaturated model systems in the left-two-phase zone before equilibrium is reached, amounts of cholesterol in these vesicles are expected to be minor compared with cholesterol crystal mass. Recovery of cholesterol and phospholipid in various phases was always 95–100%.

Micelles and small unilamellar vesicles. Micelles were isolated from the supernatant by ultrafiltration with a highly selective 300,000 MWCO ultrafilter. Small unilamellar vesicles were obtained by dialysis (500 μl sample, 16 h, 37°C) in a SpectraPor® dialysis device with an MWCO of 300,000, against three times 20 volumes of aqueous 0.15 M NaCl plus 3 mM NaN3 containing the relevant bile salt at concentrations identical to the IMC of the various original model system in order to avoid artificial shifts of lipids between vesicles and micelles (11, 21). The ultrafilters and dialysis membranes were completely impermeable to small unilamellar or aggregated vesicles but completely permeable to simple and mixed micelles [tested with a wide range of micellar compositions (11), including TDC-, TC-, and TUDC-containing mixed micelles at 37°C, at a total lipid concentration of 3.6 g/dl and at PL/(BS + PL) ratios of 0.2–0.55, either without or with cholesterol; with SM or PC as phospholipid]. Recovery of cholesterol and phospholipids in various phases was always 95–100%.

Quantification of cholesterol crystals by microscopy

Numbers of various cholesterol crystal shapes (intermediate anhydrous crystals such as arcs, needles, tubules, and spirals: mature rhomboid monohydrate crystals) were determined by daily examinations for 10 days with the aid of a polarizing microscope and KOVA® plastic slides (Hycor Biomedical, Garden Grove, CA) with 10 standardized examination chambers. Each chamber contains one large grid (3 × 3 mm; volume, 0.9 μl), divided into 81 small grids (size, 0.33 × 0.33 mm). Seven microliters from a 10× diluted sample was placed on a KOVA® slide and crystal numbers were counted in nine consecutive small grids at ×100 magnification. In model biles plotting in the left two-phase zone, sizes of cholesterol monohydrate crystals were highly variable and data for small (<10 μm in diameter) and larger monohydrate crystals are given separately. Daily examinations to determine crystal numbers and mass over 10 days were repeated two or three times, and representative curves are shown.

Statistical analysis

Data for lipid distribution into various phases are expressed as means ± SEM of four or five experiments. Differences between groups were tested for statistical significance by ANOVA with the aid of NCSS (Kaysville, UT) software. When ANOVA detected a significant difference, results were further compared for contrasts by using Fisher’s least significant difference test as a post hoc test. Statistical significance is defined as a two-tailed probability of less than 0.05.

RESULTS

Three-phase (micelle, vesicle, and cholesterol crystal-containing) zone

Influence of phospholipid class. Figures 2 and 3 show lipid distribution into various phases after 1, 10, and 40 days of incubation of SM- or EYPC-containing supersaturated model systems, plotting in the three-phase zone (TC as bile salt in all cases; see insets to Figs. 2 and 3). In SM-containing systems, ~90% of all phospholipid was contained in vesicular aggregates (Fig. 2B). By contrast, in EYPC-containing systems with the same relative composition, large amounts of phospholipids also distributed into micelles and small unilamellar vesicles (Fig. 3B). Amounts of cholesterol contained in micelles or small unilamellar vesicles were also significantly greater in EYPC- than in SM-containing systems (Figs. 2A and 3A). After 10 days of incubation, there was a strong decrease in cholesterol content in aggregated or small unilamellar vesicles and—less pronounced—in micelles as compared with 1 day of incubation, coinciding with a strong increase in cholesterol crystal mass (Figs. 2A and 3A). There were only small changes of phospholipid content in various phases during this time period (Figs. 2B and 3B). As a result, vesicular cholesterol-to-phospholipid (chol/PL) ratios that were above 1 on day 1 (particularly in small unilamellar vesicles) decreased to values ~1 by day 10 (Figs. 2C and 3C). Also, chol/PL ratios in micelles (that were slightly supersaturated on day 1), decreased (micellar CSI ~1 on day 10). There were no significant changes after longer (40 days) incubation (Figs. 2 and 3). Results in DPPC-containing systems (not shown) and SM-containing systems of the same relative composition were identical throughout the study period.

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Although with daily examination during the first 10 days, numbers of cholesterol monohydrate crystals were larger in EYPC- than in SM- or DPPC-containing systems (Fig. 4A), crystals were much larger in SM- or DPPC-containing systems. Cholesterol crystal mass was also larger in SM- or DPPC-containing systems both during the 10-day study period (Fig. 4B) and after 40 days of incubation (Figs. 2A and 3A).

Influence of bile salt species. Figure 5 shows lipid distribution into various phases after 10 days of incubation of supersaturated model systems composed of TDC, TC, or TUDC and plotting in the central three-phase zone (same relative lipid composition as in Fig. 2). Various phases were isolated after 1 day (open columns), 10 days (cross-hatched columns), and 40 days (solid columns) of incubation. There is a decrease in cholesterol and (less pronounced) phospholipid content in micelles and vesicles after 10 days, coinciding with an increased crystal mass and decreased chol/PL ratios (C). MIC, Micelles; SUV, small unilamellar vesicles; AGG, aggregated vesicles; CRY, cholesterol crystal mass. Inset: Equilibrium bile salt-phospholipid-cholesterol ternary phase diagram. Continuous line, phase diagram for EYPC (5). Interrupted line, decreased one-phase micellar zone and extension of right two-phase zone in the case of DPPC or SM as phospholipid (6). Dot indicates model bile plotting in three-phase zone.

Fig. 2. Distribution of cholesterol (A) and phospholipid (B) into various phases in supersaturated model biles composed of SM and TC, plotting in the central three-phase zone (total lipid concentration = 3.6 g/dl, PL/(BS + PL) ratio = 0.2, 24.8 mol% cholesterol, 37°C). Various phases were isolated after 1 day (open columns), 10 days (cross-hatched columns), and 40 days (solid columns) of incubation. There is a decrease in cholesterol and (less pronounced) phospholipid content in micelles and vesicles after 10 days, coinciding with an increased crystal mass and decreased chol/PL ratios (C). MIC, Micelles; SUV, small unilamellar vesicles; AGG, aggregated vesicles; CRY, cholesterol crystal mass. Inset: Equilibrium bile salt-phospholipid-cholesterol ternary phase diagram. Continuous line, phase diagram for EYPC (5). Interrupted line, decreased one-phase micellar zone and extension of right two-phase zone in the case of DPPC or SM as phospholipid (6). Dot indicates model bile plotting in three-phase zone.

Fig. 3. Distribution of cholesterol (A) and phospholipid (B) into various phases in supersaturated model biles composed of EYPC and TC, plotting in the central three-phase zone (same relative lipid composition as in Fig. 2). Various phases were isolated after 1 day (open columns), 10 days (cross-hatched columns), and 40 days (solid columns) of incubation at 37°C. There is a decrease in cholesterol content of micelles and vesicles after 10 days, coinciding with an increased crystal mass and decreased chol/PL ratios (C). MIC, Micelles; SUV, small unilamellar vesicles; AGG, aggregated vesicles; CRY, cholesterol crystal mass. Inset: Equilibrium bile salt-phospholipid-cholesterol ternary phase diagram. Continuous line, phase diagram for EYPC (5). Interrupted line, decreased one-phase micellar zone and extension of right two-phase zone in the case of DPPC or SM as phospholipid (6). Dot indicates model bile plotting in three-phase zone.

Fig. 5 shows lipid distribution into various phases after 10 days of incubation of supersaturated model systems composed of TDC, TC, or TUDC and plotting in the three-phase zone (EYPC as phospholipid in all cases; see inset to Fig. 5). There were increased amounts of phospholipids and cholesterol contained in aggregated and small unilamellar vesicles in the case of more hydrophilic bile salts, whereas solubilization in micelles was decreased. Micellar CSI as well as chol/PL ratios in small unilamellar or aggregated vesicles (Fig. 5C) were ~1 in all cases. Results were essentially the same after 40 days.
of incubation (not shown). With daily examination during the first 10 days, numbers of (mainly cholesterol monohydrate) crystals were much larger in the case of more hydrophobic bile salts (TDC > TC > TUDC; Fig. 6A). Cholesterol crystal mass was also significantly higher in the case of more hydrophobic bile salts (TDC > TC > TUDC; Fig. 6B).

We also examined effects of increasing contents of one of the three lipids by 5 mol%, keeping the ratio between the other two lipids constant (model biles 1–4 in Fig. 1). Despite changed relative lipid composition, all model biles plotted in the central three-phase zone of the appropriate phase diagram (5). As predicted by the “phase rule” (24), after 40 days of incubation, micelles are of one invariant composition, represented by the micellar apex of the three-phase zone [for TC-containing systems: PL/(BS/PL) ratio of 0.148, i.e., point b in Fig. 1]. Location of the micellar apex depends on the hydrophobicity of the bile salts incorporated in the system, with a leftward shift in the case of TUDC-containing systems [point b1 in Fig. 1: PL/(BS + PL) ratio of 0.127] and a rightward shift in the case of TDC-containing systems [PL/(BS + PL) ratio of 0.169]. In all model systems, a micellar CSI of 1 and chol/PL ratios of ~1 in (unilamellar and aggregated) vesicles (represented by point c in Fig. 1) indicate thermodynamic equilibrium after the prolonged (40-day) incubation.

**Right two-phase (micelle and vesicle-containing) zone**

*Influence of phospholipid class.* We examined lipid distribution into various phases after 10 days of incubation of EYPC-, SM-, or DPPC-containing systems plotting in the right-two phase zone [TC as bile salt in all cases: total lipid concentration = 3.6 g/dl, PL/(BS + PL) ratio = 0.3, 25 mol% cholesterol, 37°C]. Various phases were isolated after 10 days of incubation. Distribution of phospholipids and cholesterol into vesicles is increased in the case of hydrophilic bile salts (TDC < TC < TUDC), with a reciprocal decrease in micelles. Crystal mass is significantly lower in the case of hydrophilic bile salts (TDC > TC > TUDC). Chol/PL ratios in small unilamellar and aggregated vesicles are ~1 in all cases (C). Open columns, TDC; cross-hatched columns, TC; solid columns, TUDC. MIC, Micelles; SUV, small unilamellar vesicles; AGG, aggregated vesicles; CRY, cholesterol crystal mass. Inset: Equilibrium bile salt-phospholipid-cholesterol ternary phase diagram. Continuous line, phase diagram for hydrophobic bile salts (TDC > TC > TUDC). Interrupted line, decreased one-phase micellar zone and extension of right two-phase zone in the case of hydrophilic bile salts (5). Dot indicates model bile plotting in three-phase zone.

### Figure 4
Numbers of cholesterol monohydrate crystals (A) and crystal mass (B) during 10 days of incubation in supersaturated model systems containing EYPC, SM, or DPPC and plotting in the central three-phase zone (TC as bile salt in all cases: for relative lipid composition see Figs. 2 and 3). Although crystal numbers are larger in the case of EYPC, crystal mass is higher in the case of SM or DPPC, related to greater crystal sizes. EYPC, solid diamonds; SM, solid circles; DPPC, solid squares. Please note logarithmic scale for (A).

### Figure 5
Distribution of cholesterol (A) and phospholipid (B) into various phases in supersaturated model biles containing TDC, TC, or TUDC and plotting in the central three-phase zone [EYPC as phospholipid in all cases: total lipid concentration = 3.6 g/dl, PL/(BS + PL) ratio = 0.3, 25 mol% cholesterol, 37°C]. Various phases were isolated after 10 days of incubation. Distribution of phospholipids and cholesterol into vesicles is increased in the case of hydrophilic bile salts (TDC < TC < TUDC), with a reciprocal decrease in micelles. Crystal mass is significantly lower in the case of hydrophilic bile salts (TDC > TC > TUDC). Chol/PL ratios in small unilamellar and aggregated vesicles are ~1 in all cases (C). Open columns, TDC; cross-hatched columns, TC; solid columns, TUDC. MIC, Micelles; SUV, small unilamellar vesicles; AGG, aggregated vesicles; CRY, cholesterol crystal mass. Inset: Equilibrium bile salt-phospholipid-cholesterol ternary phase diagram. Continuous line, phase diagram for hydrophobic bile salts. Interrupted line, decreased one-phase micellar zone and extension of right two-phase zone in the case of hydrophilic bile salts (5). Dot indicates model bile plotting in three-phase zone.
systems, distribution of phospholipids into aggregated vesicles was lower (~40%), with larger amounts in micelles or small unilamellar vesicles (~35% and ~25%, respectively) compared with SM- or DPPC-containing systems. Chol/PL ratios in aggregated and small unilamellar vesicles were far below 1 in all cases. Results were essentially the same after 40 days of incubation.

**Influence of bile salt species.** We also determined lipid distribution into various phases after 10 days of incubation of supersaturated model biles containing TDC, TC, or TUDC and plotting in the right-two phase zone [EYPC as phospholipid in all cases: total lipid concentration = 3.6 g/dl, PL/(BS + PL) ratio = 0.5, 17 mol% cholesterol, 37°C]. Distribution of cholesterol and phospholipids into aggregated vesicles increased in the rank order: TDC > TC > TUDC-containing systems, with reciprocal decreases in micellar solubilization. Chol/PL ratios in small unilamellar or aggregated vesicles were far below 1 in all cases. Results were essentially the same after 40 days of incubation.

**Left two-phase (micelle and vesicle-containing) zone**

In model systems plotting in the left-two phase zone, vesicles could not be detected. With daily examination during the first 10 days, cholesterol crystal mass as well as numbers of small cholesterol monohydrate crystals were always higher in SM- or DPPC-containing systems than in EYPC-containing systems (Fig. 7). Anhydrous crystal forms occurred more frequently in EYPC-containing systems.

In contrast to results in the three-phase zone, cholesterol crystal masses and numbers of cholesterol monohydrate crystals were higher in the case of more hydrophilic bile salts (TDC > TC > TUDC; Fig. 8). Reciprocal effects were found for cholesterol solubilization in micelles (TDC > TC > TUDC). Anhydrous crystal forms occurred more frequently in the case of hydrophobic bile salts.

**DISCUSSION**

Biliary cholesterol supersaturation has traditionally been considered the major factor determining precipitation of cholesterol crystals and gallstone formation. The studies of Wang and Carey (5) have revealed the importance of relative amounts of bile salts versus phospholipids in the system for the crystallization process. In the case of excess bile salts, precipitation of cholesterol (intermediate anhydrous and mature monohydrate) crystals occurs at fast rates. At higher phospholipid contents, cholesterol-phospholipid vesicles are formed, with the result that precipitation of cholesterol crystals is diminished (three-phase zone), or even completely prevented (right two-phase zone). We have evaluated in the present study lipid distribution into various phases throughout the phase diagram.
We also determined, in supersaturated three-phase model systems that contained cholesterol, EYPC, SM or DPPC, and TC, lipid distribution into various phases as a function of time (after 1, 10, and 40 days of incubation: Figs. 2 and 3). After 1 day, small unilamellar and aggregated vesicles were supersaturated (cholesterol/PL ratios $>1$). During prolonged incubation, and coinciding with progressive cholesterol crystallization, vesicular cholesterol contents and cholesterol/PL ratios decreased, approaching equilibrium (cholesterol/PL ratio $\sim 1$) on day 10. Data obtained by video-enhanced contrast microscopy have suggested that precipitation of cholesterol crystals occurs from aggregated vesicular phases (25). In the present study, the magnitudes of shifts of cholesterol between various phases (large increase in crystal mass; large decrease in cholesterol contained in aggregated vesicles, particularly in the case of SM; Fig. 2) also provide indirect evidence of crystal precipitation from vesicular aggregates. In the right two-phase zone, cholesterol/PL ratios in (unilamellar and aggregated) vesicles were always less than 1, thus explaining the absence of cholesterol crystallization.

We also examined effects of varying phospholipid class. In model systems plotting in the left two-phase or central three-phase (crystal-containing) zone, speed, and extent of crystallization was enhanced in the case of DPPC or SM as compared with EYPC. In contrast, previous studies (12–14) have indicated that disaturated PC species inhibit crystallization, and PC species with unsaturated acyl chains at the sn-2 position promote crystallization progressively at increasing unsaturation. We have previously developed the equilibrium ternary phase diagram for cholesterol, TC, and SM or DPPC-containing systems (6). Compared with EYPC-containing systems under the same conditions (5), the right two-phase (vesicle and micelle-containing) zone is greatly expanded to the left at the expense of the central three-phase and left two-phase zones. In previous studies (12–14), the position in the phase diagram was probably changed from the central three-phase zone to the right two-phase zone in the case of more saturated PC species, thus explaining suppressed crystallization. However, with careful attention (as in the present study) given to ensure that model systems, with identical relative lipid composition, are composed so that they all plot in the central three-phase zone of the appropriate ternary phase diagram (5, 6), more saturated phospholipids apparently promote crystallization. Dietary modification toward more saturated biliary phospholipids has been proposed to prevent gallstone formation in humans (15). Nevertheless, effects of dietary modification are expected to be relatively small, and insufficient to induce a change from central three-phase to right two-phase zone position. Indeed, no changes in biliary cholesterol crystallization or lipid solubilization could be induced by such a dietary modification in humans (15).

More hydrophilic bile salts such as TUDC reduced crystallization in model biles plotting in the central three-phase zone, in agreement with previous data (26). In contrast, in model biles plotting in the left two-phase zone, crystallization was enhanced at increasing bile salt hydrophilicity, in the rank order TDC < TC < TUDC. Apparently, solubilization of cholesterol in vesicular phases (i.e., position in the central three-phase zone) is a prerequisite for reduced crystallization by TUDC. Enhanced crystallization in TUDC-containing model biles that do not contain vesicles (i.e., plot in the left two-phase zone) can easily be explained by the decreased micellar cholesterol solubility in the case of more hydrophilic bile salts (5). Although most cholesterol-supersaturated human biles are assumed to plot in the central three-phase zone, some may be located in the left two-phase zone, on the basis of crystallization sequences (27) and absence of vesicular phases (28, 29). These data would suggest potential adverse effects of ursodeoxycholate therapy (frequently used in clinical practice to dissolve cholesterol gallstones) at...
the local level in bile. However, the major effects of ursodeoxycholate in humans are a decrease in intestinal cholesterol absorption (30) and a lowering biliary cholesterol secretion, with the result that bile becomes unsaturated. Indeed, we found that cholesterol crystals decreased in size or even disappeared during prolonged ex vivo incubation of gallbladder bile obtained from gallstone patients treated with ursodeoxycholate (31).

Different effects of bile salt hydrophilicity versus phospholipid acyl chain saturation on crystallization behavior in the three-phase zone (i.e., inhibition vs. promotion) may relate to different effects on micellar cholesterol solubilization. Whereas TUDC decreases solubilization of the sterol to a relatively minor degree (5), there is a 70% reduction of micellar solubility limits for SM- or DPPC-containing systems as compared with EYPC-containing systems (6). Apparently, such strongly reduced micellar solubilization cannot be compensated for by enhanced vesicular solubilization. One should also realize that our data on lipid distribution with various phospholipid classes (Figs. 2–4) cannot be compared in a quantitative way with data obtained by modulation of bile salt species (Figs. 5 and 6) because lipid composition could not be completely identical because of limitations of the phase diagram (5, 6).

The present study increases insight in physical-chemical interactions between bile salts, phospholipids, and cholesterol and in the process of crystallization. Nevertheless, several limitations apply to the (patho)physiological relevance of our findings. Obviously, residence time of bile in the gallbladder and bile ducts in vivo is much shorter than our prolonged in vitro model bile incubation times. Also, composition of our model systems was far from physiological: proteins were absent, only one bile salt was incorporated instead of a mixture of various bile salts, and there is virtually no SM in human bile. Furthermore, although large amounts of aggregated vesicles form within a few hours of ex vivo incubation of human biles, as observed by video-enhanced microscopy (25), vesicle aggregation may be particularly extensive in model bile (10). These aggregated vesicles preclude accurate separation of vesicular and micellar phases with the aid of gel filtration (10). We therefore included an initial ultracentrifugation step in our protocol. We obtained in our three-phase model biles at equilibrium, vesicular chol/PL ratios of ~1 and micellar CSI values of ~1, as expected from theoretical considerations (24). Nevertheless, we cannot definitely exclude that our multistep protocol could induce some artifactual shifts between various lipid phases.

In summary, we found enhanced lipid distribution into vesicular phases, and reciprocal decreases in micellar lipid solubilization in the case of more hydrophilic bile salts or more saturated phospholipids. Whereas EYPC decreases crystallization compared with SM or DPPC in all crystall-containing zones, formation of vesicular phases is a prerequisite for inhibition of crystallization by the hydrophilic bile salt TUDC.  

REFERENCES


