Abstract  Cholesterol in human bile is solubilized in micelles by (relatively hydrophobic) bile salts and phosphatidylcholine (unsaturated acyl chains at sn-2 position). Hydrophilic 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, 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 bile, because the latter may enhance crystallization. —Moschetta, A., G. P. vanBerge-Henegouwen, P. Portincasa, G. Palasciano, and K. J. van Erpecum. Cholesterol crystallization in model biles: effects of bile salt and phospholipid species composition. J. Lipid Res. 2001. 42: 1273–1281.

Supplementary key words crystals • dialysis • intermixed micellar/vesicular bile salt concentration • micelles • phosphatidylcholine • sphingomyelin • taurocholate • tauroursodeoxycholate • tauroursodeoxycholate • ultrafiltration • vesicles

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,
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 (from stock solutions in methanol) were vortex mixed and dried at 45°C under a mild stream of nitrogen, 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)
with an MWCO of 300,000, against three times 20 volumes of

mixed micelles [tested with a wide range of micellar compositions

aggregated vesicles (11, 21). The ultrafilters and dialysis

in the model system to avoid artifactual shifts of lipids be-

terol crystal mass. Recovery of cholesterol and phospholipid in

siently in supersaturated model systems in the left two-phase

have suggested that small unilamellar vesicles may occur tran-

namic equilibrium, cholesterol crystals were precipitated in this

1) contain only micelles and cholesterol crystals at thermody-

obtained by ultrafiltration of supernatant through the 300,000

centrifugation procedure did not cause an inhomogeneous dis-

The supernatant after centrifugation, indicating that the short

centrifugation did not influence the content of mixed

Micelles and small unilamellar vesicles. Micelles were isolated from

as micelles or unilamellar vesicles in the tube. Furthermore,

ture of cholesterol contents between the pellets without

with added deoxycholate (23). IMC values measured in non-

centrifuged model biles were identical to IMC values in the cor-

responding 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 dis-

tribution of micelles or unilamellar vesicles in the tube. Further-

more, centrifugation did not influence the content of mixed

Micelles and small unilamellar vesicles. After 10 and 40 days

(in some cases also after 1 day) of incubation at 37°C, various

phases were isolated from cholesterol-saturated model sys-

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 TLS 55 rotor (Beckman, Palo Alto, CA)

(22). In the case of coexistent cholesterol crystals and aggre-

gated 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 quanti-

ties sufficient to desaturate the model system [final cholesterol

saturation index (CSI) <1]. After such incubation, light micro-

scopy and stability of turbidity measurements [optical density at

405 nm (OD<sub>405</sub>) (23) revealed that all vesicular aggregates had

been completely micellized. Experiments with isolated choles-

terol crystals showed that solubilization of the cholesterol crys-

tals did not occur during the short incubation with deoxycho-

late. Therefore, cholesterol crystal mass equals cholesterol

content in the pellet after addition of deoxycholate, and choles-

terol 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 non-

centrifuged model biles were identical to IMC values in the cor-

responding supernatants. As model systems plotting in the left-two phase zone (see Fig.

1) contain only micelles and cholesterol crystals at thermody-

micelles as compared with 1 day of incubation, coinciding

therefore, the supernatant after centrifugation, indicating that the short

centrifugation procedure did not cause an inhomogeneous dis-

tribution into various phases after 1, 10, and 40 days of in-

cubation 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 sys-

tems, ~90% of all phospholipid was contained in vesicular

aggregates (Fig. 2B). By contrast, in EYPC-containing sys-
tems with the same relative composition, large amounts of

phospholipids also distributed into micelles and small uni-

lamellar vesicles (Fig. 3B). Amounts of cholesterol con-

tained in micelles or small unilamellar vesicles were also

significantly greater in EYPC- than in SM-containing sys-
tems (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 DPPG-containing systems (not shown) and SM-

containing systems of the same relative composition were

identical throughout the study period.

Quantitation of cholesterol crystals by microscopy

Numbers of various cholesterol crystal shapes (intermediate

anhydrous crystals such as arcs, needles, tubules, and spirals: ma-
ture rhomboid monohydrate crystals) were determined by daily

examinations for 10 days with the aid of a polarizing microscope

and KOVA<sup>®</sup> plastic slides (Hyco 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<sup>®</sup> 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 monohy-

drate crystals are given separately. Daily examinations to deter-

mine 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 con-

trasts by using Fisher’s least significant difference test as a post-

hoc test. Statistical significance is defined as a two-tailed prob-

ability 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 dis-

tribution into various phases after 1, 10, and 40 days of in-

cubation 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 sys-
tems, ~90% of all phospholipid was contained in vesicular

aggregates (Fig. 2B). By contrast, in EYPC-containing sys-
tems with the same relative composition, large amounts of

phospholipids also distributed into micelles and small uni-

lamellar vesicles (Fig. 3B). Amounts of cholesterol con-
tained in micelles or small unilamellar vesicles were also

significantly greater in EYPC- than in SM-containing sys-
tems (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 DPPG-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 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. 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.](image1)

![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.](image2)
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/H11001 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), with a reciprocal decrease in micelles. Crystal mass is significantly lower in the case of hydrophobic 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.

Fig. 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).

Fig. 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.

Fig. 6. Influence of phospholipid class. Numbers of (mainly 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).
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 (chol/PL ratios >1). During prolonged incubation, and coinciding with progressive cholesterol crystallization, vesicular cholesterol contents and chol/PL ratios decreased, approaching equilibrium (chol/PL ratio ~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, chol/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 crystal-containing (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 toward right two-phase zone position. Indeed, no changes in biliary cholesterol crystallization or lipid solubility 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 the 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

**Fig. 8.** Numbers of large cholesterol monohydrate (ChM) crystals (A), small cholesterol monohydrate crystals (B), anhydrous cholesterol crystals (arcs, needles, tubules, and spirals; C) and crystal mass (D) during 10 days of incubation in supersaturated model systems containing TDC, TC, or TUDC and plotting in the left two-phase zone (EYPC as phospholipid in all cases: total lipid concentration = 3.6 g/dl, PL/(BS + PL) ratio = 0.04, 8 mol% cholesterol, 37°C). Crystal mass is larger in the case of more hydrophilic bile salts throughout the observation period. TDC, solid circles; TC, solid diamonds; TUDC, solid squares. Please note logarithmic scale for (A–C).
the local level in bile. However, the major effects of ursodeoxycholate in humans are a decrease in intestinal cholesterol absorption (30) and a lower 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 biles: influence of physical-chemical variables of pathophysiological relevance and identification of a stable liquid crystalline state in cold, dilute and hydrophilic bile salt-containing systems. J. Lipid Res. 37: 606–630.


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REFERENCES


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