# Phospholipid molecular species influence crystal habits and transition sequences of metastable intermediates during cholesterol crystallization from bile salt-rich model bile[1]

**Fred M. Konikoff,[2] David E. Cohen, and Martin C. Carey[3]**

Department of Medicine, Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School and Harvard Digestive Diseases Center, Boston, MA 02115

**Abstract** Despite its importance in cholesterol gallstone formation, crystallization of cholesterol from bile is poorly understood, especially with respect to the influences of other biliary lipids. We reported recently (Konikoff et al. *J. Clin. Invest.* 1992. **90:** 1155–1160) that cholesterol can crystallize from model and native biles as filamentous crystals covered by a surface layer of lecithin molecules. During growth, filamentous crystals transformed via metastable intermediates into classical plate-like cholesterol monohydrate crystals. Using the same dilute (1.2 g/dl total lipid) bile salt-rich (97.5 moles %) model bile supersaturated with cholesterol, we have studied the effects of natural egg yolk, soy bean as well as single molecular species of lecithins, other phospholipids and related lipid classes on early filamentous cholesterol crystallization, as well as transformations between crystal habits and their growth to equilibrium cholesterol monohydrate plates. After extraction and derivatization, HPLC analysis revealed that the surfaces of filamentous crystals were enriched preferentially with lecithin molecular species having longer and more saturated *sn*-1 and *sn*-2 acyl chains compared to mixed lecithin species of whole bile. In contrast, the molecular species distribution of lecithins on equilibrium plate-like crystals resembled that of whole bile. After incubation of pre-formed anhydrous cholesterol and cholesterol monohydrate crystals in cholesterol-free lipid solutions, we demonstrated that surface-adsorbed lecithins were not preferentially enriched excluding nonspecific lecithin adsorption. Time-sequences and transformations between metastable crystalline intermediates were altered markedly by specific phospholipid species: model biles composed of saturated short-chain, medium-chain, and polyunsaturated long-chain lecithins induced rapid precipitation of short filamentous crystals that became plate-like slowly by an "arborization pattern." Long-chain saturated lecithins and natural sphingomyelins retarded cholesterol crystallization markedly, and filamentous as well as metastable intermediate crystals made transient appearances only after plate-like crystals had formed. These observations suggest that phospholipid molecular species and class influence the earliest events in cholesterol crystallization from bile salt-rich model bile. Furthermore, as the molecular species of lecithins adsorbed onto filamentous cholesterol crystals were more saturated than in whole bile and essentially identical to those in biliary vesicles, this finding provides chemical evidence for a vesicular origin of the critical cholesterol nucleus. Because biliary phospholipids are amenable to dietary manipulations, and because the basic paradigm of the nucleating bile in the present study has been confirmed in more physiologically relevant model systems, (D. Q-H. Wang, F. M. Konikoff, and M. C. Carey, unpublished results) our observations may provide a scientific basis for new approaches that delay or prevent the earliest events in cholesterol gallstone formation.—**Konikoff, F. M., D. E. Cohen, and M. C. Carey.** Phospholipid molecular species influence crystal habits and transition sequences of metastable intermediates during cholesterol crystallization from bile salt-rich model bile. *J. Lipid Res.* 1994. **35:** 60–70.

**Supplementary key words** gallstones • nucleation • lecithin molecular species • hydrophobicity

Mixed micelles and vesicles, the principal lipid aggregates in bile (1), solubilize and disperse otherwise insoluble cholesterol molecules in the biliary tree and during bile's concentration and storage in the gallbladder (2). Although cholesterol supersaturation is the physical-chemical driving force for crystallization, other promoting and inhibiting factors determine the kinetics such as whether crystallization occurs (3, 4). When supersaturated bile becomes lithogenic, dissolved cholesterol molecules become unstable and begin to precipitate and

Abbreviations: PC, phosphatidylcholine (lecithin); EY, egg yolk; SB, soy bean; TC, taurocholate; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; PE, phosphatidylethanolamine.

[1]Presented in part at the Annual Meeting of the American Gastroenterology Association, San Francisco, CA, 1992 and published as an abstract in *Gastroenterology.* 1992. **102:** A835.

[2]Present address: Department of Gastroenterology, Ichilov Hospital, Tel Aviv Medical Center, 6, Weizman Street, Tel Aviv 64239, Israel.

[3]To whom correspondence should be addressed at: Gastroenterology Division, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.



Downloaded from www.jlr.org by guest, on June 18, 2012

crystallize by processes that are not yet understood (3, 4). The current paradigm is that cholesterol molecules nucleate and crystallize from cholesterol-rich vesicles after their aggregation (5) and possible fusion (3, 4, 6). Crystallization ultimately leads to the formation of micrometer-sized cholesterol monohydrate crystals that become agglomerated via an organic glycoprotein matrix to form cholesterol gallstones (7). In actively nucleating biles of patients with cholesterol gallstone disease (8, 9), cholesterol monohydrate crystals are observed as thin plate-like parallelograms with angles of 79.1° and 100.8° and frequently with a single notched corner (10, 11). Although, the phase behavior and crystallography of anhydrous cholesterol and cholesterol monohydrate (11–13) are well known, the physical–chemical nature of the earliest nucleated cholesterol crystals in bile is only beginning to be understood (14). Because the end-point of earlier crystallization studies is the detection time of plate-like cholesterol monohydrate crystals (15), efforts to date have focused on factors such as proteins (16, 17), major and minor lipids (18), and the ionic environment (19, 20) that influence the time of appearance of these crystals in bile.

In a recent study (14) we found that cholesterol can crystallize from model as well as native biles as thin filamentous crystals that grow slowly through a sequence of intermediate microcrystalline structures before becoming classic cholesterol monohydrate plates. Fluorescence microscopy and chemical analysis revealed that the earliest cholesterol filaments were covered by a surface layer of lecithin (phosphatidylcholine, PC) molecules (14). This raised the possibility that, of the major biliary lipids, the molecular species of phospholipids could, in part, control the cholesterol crystallization process. In the present study, we demonstrate that early cholesterol crystallization events are strongly influenced by both lipid class and molecular species of phospholipids in a bile salt-rich model bile.

## MATERIALS AND METHODS

### Chemicals

Taurocholate (TC) (sodium salt, Sigma Chemical Co., St. Louis, MO) was recrystallized by the method of Pope (21), and was >99% pure by HPLC and TLC (14). Cholesterol (Nu-Chek Prep Inc., Elysian, MN) was > 99% pure by GLC and TLC (14). Grade I egg yolk phosphatidylcholine (EYPC), egg yolk phosphatidylethanolamine (EYPE), and soy bean phosphatidylcholine (SBPC) (Lipid Products, South Nutfield, Surrey, U.K.), were > 99% pure by HPLC and TLC. Synthetic palmitoyl-eicosapentaenoyl (16:0–20:5) PC and palmitoyl-docosahexaenoyl (16:0–22:6) PC were generous gifts from Dr. John S. Parks (Bowman Gray School of Medicine, Winston-Salem, NC). Cardiolipin (bovine heart), sphingomyelin (egg and bovine brain), PC from bovine heart and brain, and all other synthetic molecular species of PC including monooleoyl-(lyso)-PC were from Avanti Polar Lipids (Birmingham, AL). Oleic acid and monooleoylglycerol were obtained from Nu-Chek Prep Inc. (Elysian, MN), and all other chemicals and solvents were ACS or reagent grade quality (Fisher Scientific Co., Medford, MA). NaCl was roasted for 3 h at 600°C to oxidize and remove organic impurities. Pyrex brand glassware was alkali-alcohol washed (EtOH–2 M KOH 1:1, v/v), and then acid-washed (1 M $HNO_3$) for 24 h each, and rinsed thoroughly with purified water prior to drying. Water was filtered, ion-exchanged, and glass-distilled (Corning Glass Works, Corning, NY).

### Model biles

*Nucleating biles.* TC, phospholipids, or other acyl chain lipids and cholesterol were co-precipitated from stock solutions ($CHCl_3$ and/or MeOH) in a molar ratio of 97.5:0.8:1.7 (14), dried under $N_2$ followed by reduced pressure for 12 h, and then flushed with argon, sealed, and stored at −20°C. Micellar biles were composed by dissolving the dried lipid film in 0.15 M NaCl, (pH 6–7 and pH 6.5 for oleoyl "acid-soap") containing 3 mM $NaN_3$ as an antimicrobial agent and incubating for 1 h at 60°C. According to the relative lipid composition plotted on appropriate phase diagrams (22), sixfold dilution induced cholesterol supersaturation and initiated nucleation (14). Throughout all experiments (if not stated otherwise), nucleating biles were flushed with argon and incubated in sealed glass tubes at 37°C.

*Biles with pre-formed cholesterol crystals.* Biliary lipids without cholesterol were co-precipitated and dried from stock solutions as described above. Micellar solutions were composed with TC and EYPC, whereas vesicular solutions were prepared with EYPC alone. Dried lipid films were resuspended in 0.15 M NaCl and 3 mM $NaN_3$ to achieve the same concentrations of TC and/or EYPC as in the nucleating bile (see above). In the case of TC-containing biles, complete micellization was accomplished by incubation at 60°C for 1 h. Multilamellar vesicles of EYPC without TC were produced by at least 10 min vortex mixing. Crystals of pure anhydrous cholesterol and cholesterol monohydrate were prepared from glacial acetic acid and 95% ethanol, respectively (11). Approximately 7 mg of pre-formed cholesterol crystals was then introduced into 50 ml of either micellar or vesicular lipid solutions and incubated at 37°C under argon.

### Analytical procedures

*Light microscopy.* At frequent intervals during incubation small ($\approx 5\ \mu l$) samples of model bile were placed on a glass slide (Fisher Scientific Co.) at room temperature



Downloaded from www.jlr.org by guest, on June 18, 2012

(≈23°C), and observed unperturbed using a polarizing microscope (Zeiss Photomicroscope III). Samples were then compressed with a cover slip, and examined further using phase contrast optics. The relative numbers of different crystalline habits of cholesterol were expressed on a semiquantitative scale representing numbers of crystals per microscopic field (× 63 magnification): 0 (no crystals), 1 (< 5 crystals), 2 (5–10 crystals), 3 (> 10 crystals).

*HPLC analysis of surface-adsorbed lecithins.* Cholesterol crystals were sedimented from 50–100 ml of nucleating biles by ultracentrifugation (210,000 *g*, 60 min), followed by retention of crystals on microfilters (0.22 μm Nuclepore membranes), and water washing (× 100 vol). After extraction into chloroform, the surface-absorbed lecithins were analyzed by HPLC of their benzoyl-diglyceride derivatives as described previously (23). The phospholipids were separated from cholesterol by preparative HPLC (Hibar 5 μm 250 × 4.6 mm LiChrospher Si-100 column, EM Science, Cherry Hill, NJ). The total lecithin fraction was dried, hydrolyzed by phospholipase C, and the resulting diglycerides were derivatized with benzoyl chloride. The benzoyl diglyceride derivatives were then fractionated by reverse phase HPLC (Ultrasphere 5 μm ODS 250 × 2.5 mm column, Beckman, Fullerton, CA). Peaks were identified according to capillary gas chromatography of their fatty acid methyl esters following preparative HPLC (23). Relative peak areas at 230 nm represented mole fractions of individual lecithin species in the original sample. For PC molecular species comprising 1% or more of total lecithin, peak areas were accurate to ± 2% (23). Statistically significant differences in lecithin molecular species between whole biles and crystal surfaces were examined according to Student's *t*-test.

## RESULTS

The upper panel of **Fig. 1 (a–c)** provides a semiquantitative demonstration of crystal number and crystallization habits (corresponding to photomicrographs a–c, lower panel) as functions of time in days after initiation of nucleation in the bile salt-rich model bile consisting of TC, EYPC, and cholesterol. The crystallization sequence (14) demonstrates filaments (Fig. 1a, upper and lower panels) at 0–5 days, metastable intermediates, i.e., spirals, ribbons, helices, and tubes (Fig. 1b) at 1–7 days, and equilibrium plates (Fig. 1c, upper and lower panels) at 2+ days. This crystallization sequence (Fig. 1, upper panel) was highly reproducible (n > 50) and served as a frame of reference for comparison with all subsequent experiments.

### Molecular species of surface-adsorbed lecithins

In preliminary experiments (data not displayed), 50-ml volumes of nucleating biles (see Methods) were composed

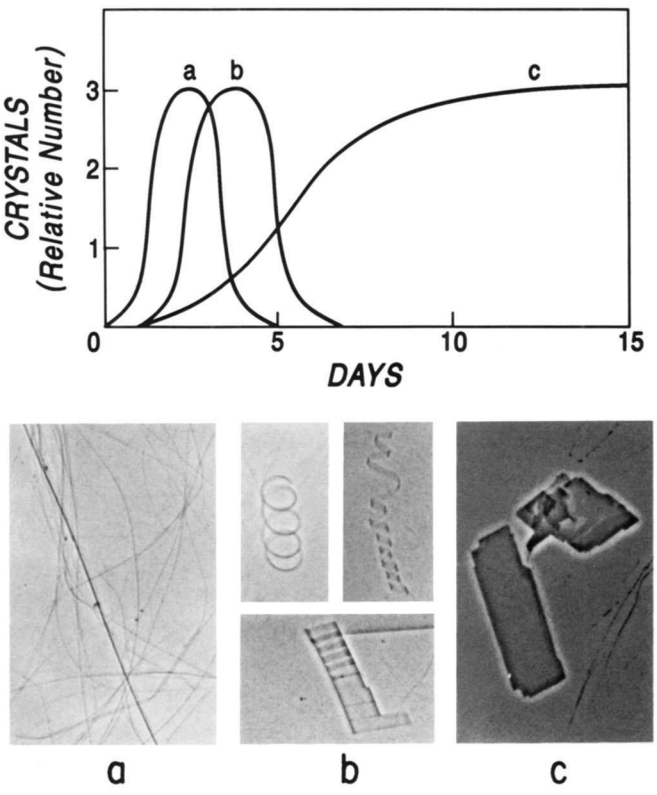


**Fig. 1.** Upper panel: a schematic diagram of cholesterol crystallization from bile salt-rich model bile (37°C) depicting relative numbers of crystals of different habits (a–c) as functions of time in days. Lower panel: phase contrast photomicrographs of cholesterol crystals (a–c, corresponding to those in the upper panel) over the same time period; (a) earliest crystal habit (filaments); (b) intermediate crystal habits (spirals, helical ribbons, tubes); (c) equilibrium crystal habit (plates). The relative numbers of crystals (upper panel) are depicted semiquantitatively on a scale from 0 (least number) to 3 (greatest number) (see Materials and Methods).

with equimolar amounts of 18:0–18:1 PC and either 16:0–18:1 or 16:0–18:2 PC, the latter representing the more hydrophilic molecular species of PC in human biles (24). After harvesting at 14 h, the surfaces of filamentous cholesterol crystals were found to be preferentially enriched with 18:0–18:1 PC species (by 19% and 30%, respectively).[4] This preferential enrichment of crystals was more marked in biles containing the more unsaturated native PC species 16:0–18:2 (30%) than in the one containing the more saturated 16:0–18:1 PC species (19%).

During nucleation from biles composed of natural PC mixtures (EYPC and SBPC), crystals were harvested and the surface-adsorbed PCs were extracted from filamentous cholesterol crystals at 14 h of incubation, and from cholesterol monohydrate plates at 14 days. **Table 1** lists

[4]Enrichment (%) of a species is calculated as 100 × [moles% on crystal surface − moles% in original bile]/moles% in original bile.



Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 1. Distribution and enrichment/depletion of egg yolk phosphatidylcholine molecular species on nucleating cholesterol crystal surfaces compared with whole bile

| Species[a] | Whole Bile | Filaments[b] | | Plates[c] | |
|---|---|---|---|---|---|
| *sn*-1, *sn*-2 | Distr[d] (± SD) | Distr (± SD) | Enr[e]/Depl[f] +/− | Distr (± SD) | Enr/Depl +/− |
| 16:0 22:6 | 4.1 ± 0.1 | 3.0 ± 0.4[g] | −26.8 | 3.7 ± 0.2[g,h] | −9.8 |
| 16:0 20:4 | 5.0 ± 0.3 | 3.2 ± 0.5[g] | −36.0 | 4.6 ± 0.3[h] | −8.0 |
| 16:0 18:2 | 23.3 ± 0.3 | 20.7 ± 0.5[g] | −11.2 | 23.5 ± 1.6[h] | +0.9 |
| 18:1 18:2 | 2.3 ± 0.3 | 0.8 ± 0.5[g] | −65.2 | 1.0 ± 0.6[g] | −56.5 |
| 16:0 18:1 | 32.8 ± 0.7 | 36.5 ± 0.8[g] | +11.3 | 34.9 ± 1.4[g] | +6.4 |
| 18:0 20:4 | 7.5 ± 0.2 | 6.2 ± 0.8[g] | −17.3 | 6.5 ± 0.6[g] | −13.3 |
| 18:0 18:2 | 10.8 ± 0.3 | 12.4 ± 0.6[g] | +14.8 | 10.5 ± 0.7[h] | −2.8 |
| 18:0 18:1 | 8.7 ± 0.4 | 12.6 ± 1.4[g] | +44.8 | 9.3 ± 0.4[g,h] | +6.9 |

[a] Arabic numeral to left of colon represents number of carbons in respective acyl chain: numeral to right of colon represents number of *cis* double bonds.
[b] Filamentous crystals as in photomicrograph a of Fig. 1.
[c] Plate-like crystals as in photomicrograph c of Fig. 1.
[d] Distr, distribution (%).
[e] Enr, enrichment (%).
[f] Depl, depletion (%).
[g] Significantly different from whole bile ($P < 0.05$).
[h] Significantly different from filaments ($P < 0.05$).

and **Fig. 2** displays molar percentages and relative enrichments of the principal PC molecular species of EYPC on both crystal habits compared with whole bile. Filamentous crystals were significantly enriched with PC molecular species containing more saturated acyl chains in either the *sn*-1 or *sn*-2 positions: 16:0–18:1 (+11.3%), 18:0–18:2 (+14.8%), and 18:0–18:1 (+44.8%) PC, respectively (Table 1). Conversely, filamentous crystals were relatively deficient in PC molecular species containing more unsaturated acyl chains, 16:0–18:2 (−11.2%), 16:0–20:4 (−36.0%), 16:0–22:6 (−26.8%), 18:0–20:4 (−17.3%), and 18:1–18:2 (−65.2%) PCs (Table 1). For all molecular species except 18:1–18:2 PC, the preferential enrichment/depletion was less marked on equilibrium plate-like crystals, but still differed appreciably from that of whole bile (Table 1, Fig. 2). In biles composed with the more unsaturated SBPC, these patterns were amplified as shown in **Table 2** and **Fig. 3.** Both filamentous and plate-like crystals were enriched preferentially with PC molecular species containing longer and more saturated acyl chains as is shown dramatically for the *sn*-1 18:0 species (Table 2). Of the principal SBPC molecular species, both crystalline forms were enriched with PCs containing saturated *sn*-1 acyl groups (16:0–18:2, 18:0–18:1, 18:0–18:2), with the exception of 16:0–18:1 PC which was depleted on plates. Both crystalline forms were deficient in PCs containing unsaturated *sn*-1 acyl groups in proportion to increasing hydrophilicity (18:1–18:2, 18:2–18:2, 18:2–18:3) with 18:3:18:3 PC being the major exception in that it was significantly enriched on plate-like crystals (Table 2).

To exclude preferential adsorption of certain PC species on crystals solely on the basis of crystal habit, pre-formed anhydrous cholesterol (needle-like habit, 11) and cholesterol monohydrate crystals (plate-like habit, 11) were incubated with either micellar or vesicular lipid solutions composed of biliary lipids without cholesterol (see Methods). Cholesterol was purposely omitted in order to avoid the influence of its well-known ordering effects on the most unsaturated lecithin molecular species in EYPC mixtures. After 14 h of incubation at 37°C, the adsorbed PC species were extracted from the harvested cholesterol crystals and analyzed as described above. This experiment did not reproduce the asymmetric distribution of adsorbed PC species on filamentous crystals observed in the nucleation experiments. Instead, the adsorbed molecular species of lecithin was not significantly different on either crystal habit from that of the whole bile (data not displayed).



Downloaded from www.jlr.org by guest, on June 18, 2012

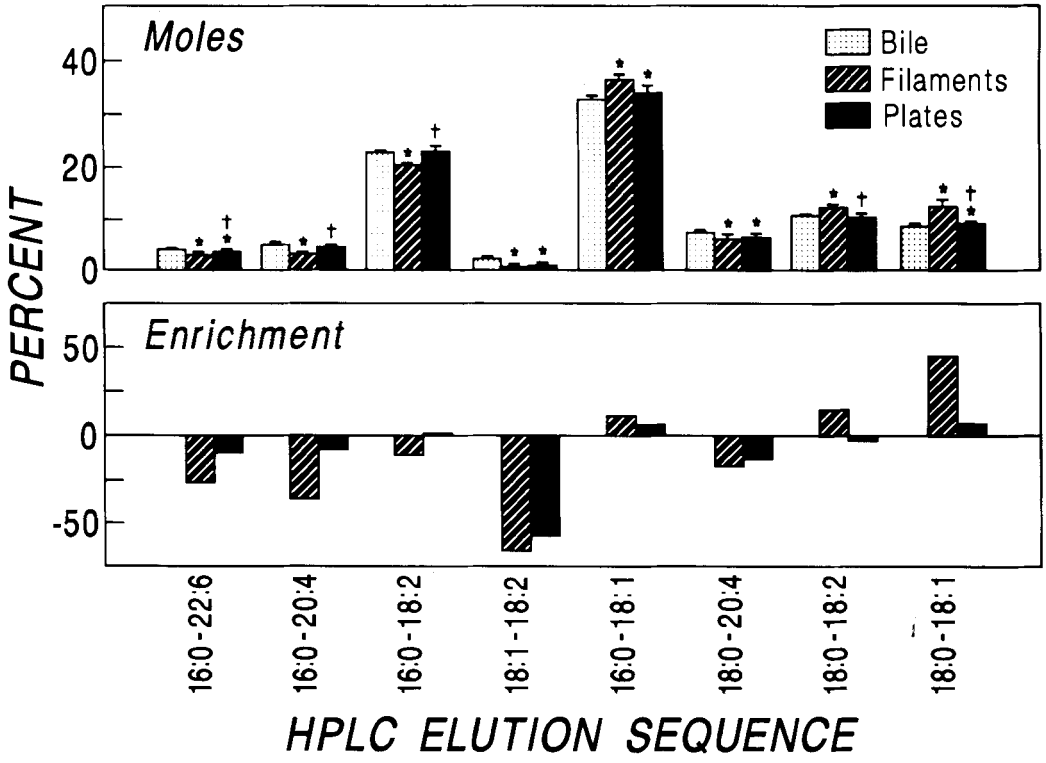


**Fig. 2.** Molar percentages + 1 SD (n = 3) (top panel) and relative enrichments (bottom panel) of the principal PC molecular species of egg yolk lecithin (denoted by carbon number and number of *cis* double bonds in *sn*-1-*sn*-2 acyl chains, respectively), isolated from the surfaces of early cholesterol filaments (hatched bars), and from plate-like crystals at equilibrium (filled bars), compared with the PC molecular species distribution in the original model bile (stippled bars). The PC species are depicted on the horizontal axis in elution order of their benzoyl diglyceride derivatives from the reverse-phase HPLC column, thus representing a relative estimate of their hydrophobicity (see Methods). Symbols over the bars indicate statistically significant differences ($P < 0.05$) from whole bile (*) and from filaments (†).

### Influence of phospholipids and related lipids on crystallization patterns

**Fig. 4** displays three distinct crystallization time-sequences with differing order of appearance of metastable intermediates and transitions between habits when nucleating biles were prepared with other phospholipids or related lipid classes and molecular species. With pure synthetic molecular species that constitute EYPC (16:0–18:1, 16:0–18:2, 16:0–20:4, 18:0–18:1, 18:0–18:3), as well as with other naturally occurring PC mixtures (SBPC, bovine heart or brain PC), or with related phospholipids (EYPE) the crystallization process remained the classic one (Fig. 1) as depicted in Fig. 4 (upper panel). However, when single molecular PC species contained either relatively saturated and/or long chains, or contained relatively unsaturated and/or short chains, the crystallization process was altered markedly (Fig. 4, middle and lower panels). Two distinct crystallization patterns were observed. First, disaturated lecithins with 6 to 12 carbon chains induced rapid precipitation of short filamentous crystals (Fig. 4, middle panel). The axial length of the filamentous crystals increased with increasing PC acyl chain length, and ranged from < 10 μm (6:0–6:0 PC) to > 30 μm (12:0–12:0 PC) (data not shown). These filaments transformed slowly into plate-like crystals via a metastable "arborization" pattern (Fig. 4b', middle panel) and lateral growth, a distinct and new crystalline transition pattern for this model bile system (14). Shown in **Fig. 5 (a–c)** are photomicrographs of the crystalline transitions with this unusual habit for a model bile composed with 18:3–18:3 PC. Polyunsaturated PCs derived from fish oils (16:0–20:5 and 16:0–22:6) resulted in a cholesterol crystallization sequence similar to that observed with EYPC (Fig. 4, upper panel), with the exception that transformation of filaments to plates involved some elements of the "arborization" pattern (Fig. 5). Second, bile composed of PCs with disaturated 16 to 18 carbon chains or sphingomyelins (also highly saturated C18 lipids) resulted in very slow and complex nucleation processes (Fig. 4, lower panel). Cholesterol monohydrate plates nucleated directly from the supersaturated model bile after 5 days and, in part, the plates evolved from filamentous crystals through metastable intermediates (helical and tube-like structures) that first appeared more



Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 2. Distribution and enrichment/depletion of soy bean phosphatidylcholine molecular species on nucleating cholesterol crystal surfaces compared with whole bile

| Species[a] | Whole Bile | Filaments[b] | | Plates[c] | |
|---|---|---|---|---|---|
| *sn*-1, *sn*-2 | Distr[d] (± SD) | Distr (± SD) | Enr[e]/Depl[f] +/− | Distr (± SD) | Enr/Depl +/− |
| 18:3<br>18:3 | 0.8 ± 0.2 | 0.6 ± 0.1 | −25.0 | 1.1 ± 0.2[g] | +37.5 |
| 18:2<br>18:3 | 10.5 ± 0.2 | 6.8 ± 0.4[h] | −35.2 | 8.1 ± 1.5[h] | −22.9 |
| 18:2<br>18:2 | 46.6 ± 0.4 | 35.6 ± 1.0[h] | −23.6 | 41.7 ± 5.3 | −10.5 |
| 16:0<br>18:2 | 21.4 ± 0.4 | 31.0 ± 1.1[h] | +44.9 | 24.9 ± 1.7[g,h] | +16.4 |
| 18:1<br>18:2 | 8.6 ± 0.4 | 7.1 ± 0.8[h] | −17.4 | 8.0 ± 1.6 | −7.0 |
| 16:0<br>18:1 | 1.8 ± 0.1 | 3.6 ± 0.1[h] | +100.0 | 1.7 ± 1.4[g] | −5.6 |
| 18:0<br>18:2 | 5.6 ± 0.0 | 10.5 ± 0.2[h] | +87.5 | 7.2 ± 0.8[g,h] | +28.6 |
| 18:0<br>18:1 | 0.3 ± 0.1 | 1.0 ± 0.0[h] | +233.3 | 0.7 ± 1.0 | +133.3 |

[a] Arabic numeral to left of colon represents number of carbons in respective acyl chain: numeral to right of colon represents number of *cis* double bonds.
[b] Filamentous crystals as in photomicrograph a of Fig. 1.
[c] Plate-like crystals as in photomicrograph c of Fig. 1.
[d] Distr, distribution (%).
[e] Enr enrichment (%).
[f] Depl depletion (%).
[g] Significantly different from filaments ($P < 0.05$).
[h] Significantly different from whole bile ($P < 0.05$).

than 15 days into the nucleation sequence (Fig. 4, lower panel). The sole exception to the behavior of disaturated lecithins was model bile composed of synthetic PC with medium length (14 carbon) chains, where the crystallization sequence was indistinguishable from that of the classic pattern (Fig. 4, upper panel).

In the case of model bile composed with cardiolipin, the earliest crystals (2–10 h) did not have a distinct morphology by light microscopy, but were certainly not filamentous (data not displayed). However, within 24 h the crystalline precipitates became typical plate-like cholesterol monohydrate crystals. By days 5–8 only a few needle-like crystals (< 1% of total) were observed and these disappeared completely by days 18–20. This crystallization sequence with cardiolipin resembled, but was not identical to, that observed with saturated long-chain lecithins and sphingomyelins (Fig. 4, lower panel).

In the total absence of phospholipids, cholesterol crystallization from simple bile salt micelles produced agglomerates of short (< 5 μm) needle-shaped crystals that remained stable except for some arborization growth (< 10%) and did not evolve into plate-like crystals (data not shown). An identical precipitation pattern was observed when an equimolar concentration of TC and monooleoyl-(lyso)-PC, monoolein, or oleoyl "acid soap" (pH 6.5) (25) were used as the model biles. When the molar concentration of oleoyl "acid soap" was doubled at a constant pH and bile salt concentration, the crystallization habit became filamentous (as with natural EYPC); however, these filaments remained needle-like throughout the entire incubation period, and even at 60 days had not evolved into typical plate-like cholesterol monohydrate crystals.

## DISCUSSION

This study demonstrates that during cholesterol crystallization from a dilute bile salt-rich model bile, the lecithin molecular layer covering filamentous cholesterol crystals (14) is preferentially enriched with PC molecular species containing saturated acyl chains. This preferential enrichment with certain PC molecular species occurred only when cholesterol crystals nucleated and grew in model bile, and not when pre-formed cholesterol crystals were added to cholesterol-free biliary lipid solutions. We eschewed the use of cholesterol for this experiment as we



Downloaded from www.jlr.org by guest, on June 18, 2012

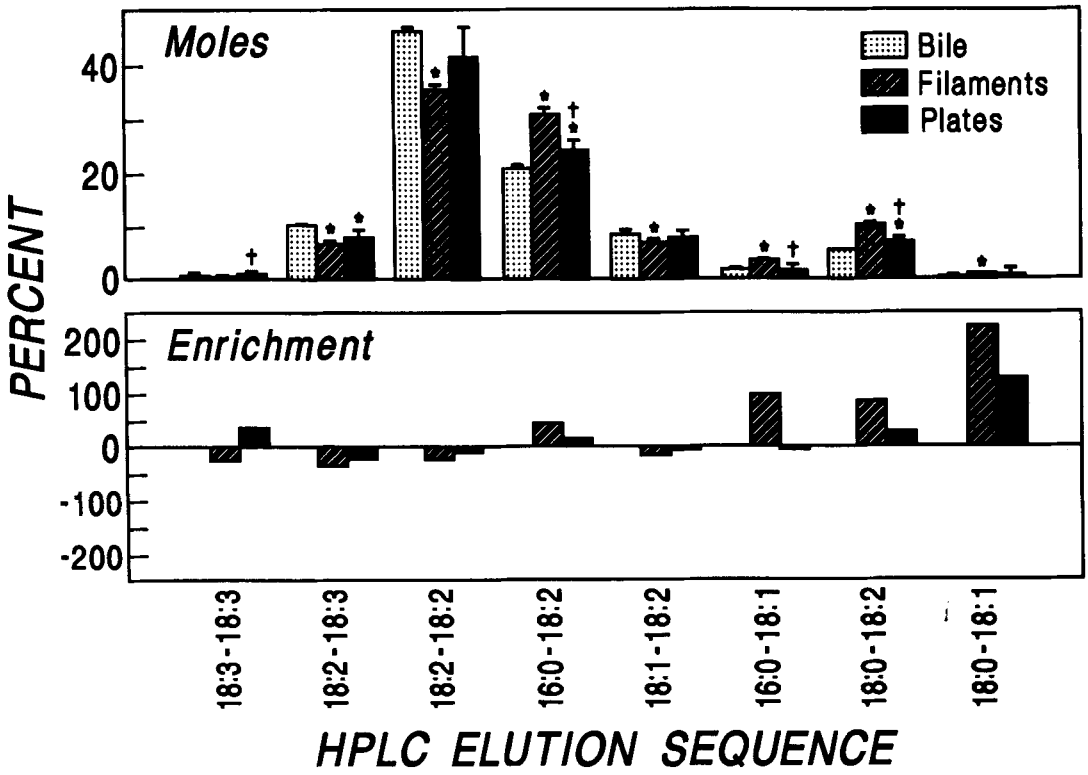


**Fig. 3.** Molar percentages + 1 SD (n = 3) (top panel) and relative enrichments (bottom panel) of the principal molecular species of soy bean lecithin (in increasing order of HPLC elution sequence of their benzoyl diglyceride derivatives, with respect to *sn*-1–*sn*-2 acyl chains), isolated from the surfaces of early cholesterol filaments (hatched bars) and from plate-like crystals at equilibrium (filled bars), compared with PC molecular species distribution of the original bile (stippled bars). HPLC elution sequence provides an estimate of increasing hydrophobicity of the PC molecular species. Symbols over the bars indicate statistically significant differences ($P < 0.05$) from whole bile (*) and filaments (†).

wished to avoid its preferential ordering of the more unsaturated PC molecular species in EYPC. Moreover, we show here that crystallization time-sequences and transitions between metastable intermediates and equilibrium cholesterol crystals can be altered by substituting certain synthetic lecithin species for the natural lecithins, as well as by other phospholipid classes and related lipids. Lecithins with short or polyunsaturated acyl chains resulted in rapid initial precipitation of short filamentous crystals with subsequent metastable "arborization" of filaments to form plate-like cholesterol monohydrate crystals, whereas long-chain saturated lecithins and other "stiff" membrane phospholipids retarded the cholesterol crystallization process and usually produced plate-like crystals directly and later, via metastable intermediates. These findings indicate that the earliest phases of biliary cholesterol crystallization can be highly heterogeneous and are influenced and possibly controlled in part by a layer of lecithin molecules (14) covering the crystal surfaces.

Filamentous, helical, and tubular cholesterol crystals ("metastable intermediates") have also been observed by us during cholesterol crystallization in model biles with physiological lipid compositions and concentrations as well as in native human gallbladder biles (preliminary report in ref. 14). A systematic study is now under way in our laboratories to explore the time-sequences and transformations between these crystal forms as functions of several physical-chemical conditions of pathophysiological relevance. Preliminary data suggest that filamentous crystallization occurs preferentially in the 2 phase zone of the relevant phase diagram (22), whereas a liquid crystal (multilamellar vesicle) to plate-like crystal pathway is favored in the 3 phase zone (D. Q-H. Wang, F. M. Konikoff, M. C. Carey, unpublished observations). The dilute, bile salt-rich model system used in the present study highlights the filamentous pathway and enables systematic investigation of a sequence of events occurring during cholesterol crystallization. Hence, these results should be viewed as representing only part of a heterogeneous spectrum that comprises highly complex processes during cholesterol crystallization from bile.

Preferential surface-adsorption of saturated lecithin species on cholesterol crystals found in our work suggests that lecithins on the crystals' surfaces are selected and stabilized by hydrophobic forces. The observation that this enrichment is more pronounced on early filamentous



Downloaded from www.jlr.org by guest, on June 18, 2012

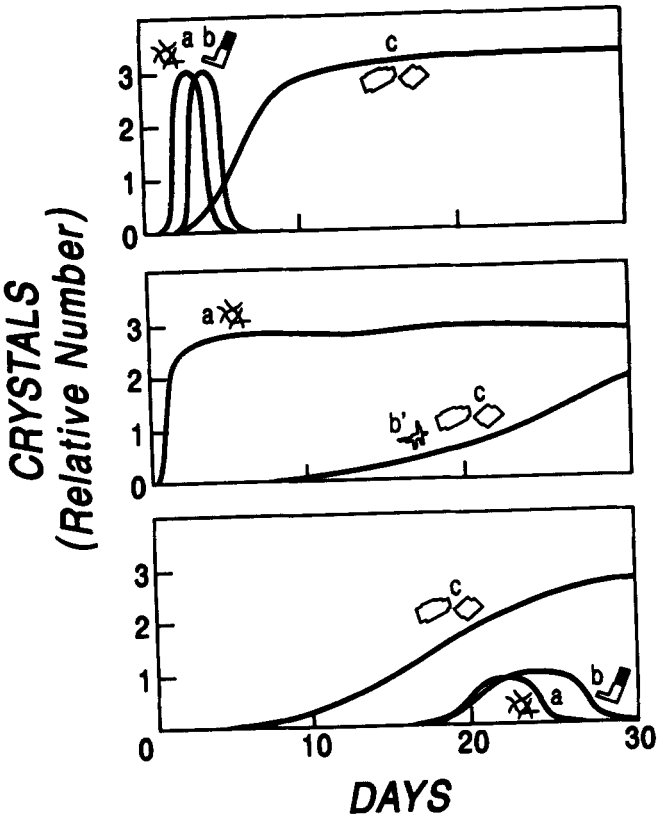


**Fig. 4.** Schematic diagrams of cholesterol crystallization sequences in model biles composed of different phospholipid species. Upper panel: natural PC mixtures (EYPC, SBPC, and EYPE) and pure 16:0–18:1, 16:0–18:2, 18:0–18:3, 18:1–18:1, 16:0–20:4, and 14:0–14:0 PC species. Middle panel: synthetic 6:0–6:0, 7:0–7:0, 9:0–9:0, 12:0–12:0, and 18:3–18:3, PC species. Lower panel: disaturated long chain PC species 16:0–16:0, 18:0–18:0, and sphingomyelin (see Materials and Discussion). Symbols a, b, and c correspond to the crystal habits in Fig. 1; b' denotes an "arborization" pattern described in the text (see Fig. 5).

crystals rather than plate-like cholesterol monohydrate crystals could imply that these crystals are more hydrophobic, possibly reflecting their anhydrous nature (13). This conclusion is supported by synchrotron X-ray diffraction (14) and density gradient ultracentrifugation data (26) that suggest that earliest cholesterol filaments are not fully hydrated. It is also likely that the lecithin species distribution may reflect a vesicular origin of both filamentous crystalline cholesterol and the associated lecithin molecules. Earlier work on model systems from our laboratory (23), and verified recently in human bile (27), has shown that biliary vesicles display a similar preferential distribution of lecithin molecular species as we have now found on the earliest cholesterol filaments. In fact, **Fig. 6** demonstrates that the enrichment of lecithin molecular species on filamentous crystals is positively correlated with enrichment of the same species in vesicles when separated from micelles by chromatography (data from ref. 23) yielding slopes (±SE) for EYPC (1.23 ± 0.16) and SBPC (1.26 ± 0.12) that are not significantly different from the line of identity (displayed). It has been proposed that adsorption of lecithin molecules onto cholesterol crystals represents an interfacial barrier that controls cholesterol dissolution in bile salt solutions (28). Likewise, it is possible that this interfacial barrier also affects the crystallization process and maturation of cholesterol crystals in bile.

The slower initial crystallization that occurs in the presence of more saturated (i.e., hydrophobic) lecithins is likely a result of high affinity binding of these lecithins to crystals as well as to each other, which presumably interferes with critical crystal nucleation and growth. Conversely, more hydrophilic (i.e., very short or unsaturated) lecithins probably bind less avidly to crystal surfaces, and thus may permit more rapid initial crystal formation as well as growth. Alternatively, or in addition, these effects might be attributed to a shift in the phase equilibria of bile above the micellar zone induced by changing the phospholipid species in the system; this subject has yet to be explored experimentally. The nonspecific adsorption of lecithin species onto surfaces of pre-formed cholesterol crystals suggests that the specific selection is not a secondary phenomenon but is a primary process that occurs during crystal formation and growth apparently from biliary vesicles (14).

Lecithin is known to play an important role in solubilization of cholesterol in bile, both in mixed micelles and in vesicles (2). Preliminary data, published in abstract form (18, 29, 30) suggest that dipalmitoyl and distearoyl PCs added to bile prolong nucleation times. These observations confirm the present work and demonstrate further that this antinucleating effect is likely related to the suppression of early crystal formation, as we have demonstrated systematically here (Fig. 4, lower panel). Our data indicate that hydrophilic lecithins have an opposing effect in enhancing early filamentous crystal formation and altering their subsequent crystal transformations (Fig. 4, middle panel). However, in contrast to recent claims (31), we found no evidence in the present work that biles enriched with fish oil lecithins exhibit by themselves an altered crystallization pattern. This supports observations of Berr et al. (32) that when the diet of gallstone patients is supplemented with fish oil, cholesterol nucleation from bile is slowed, principally due to decreases in biliary cholesterol saturation.

Lecithin is the main (> 95%) phospholipid in human bile (33), in which 16:0–18:2, 16:0–18:1, and 16:0–20:4 are the predominant (> 80%) molecular species (24). Although several groups (34–39) have suggested that the profile of lecithin molecular species in gallstone patients differs from controls, data are conflicting and recent results from our laboratory (24) and another (40) have found no statistically significant differences. The reasons



Downloaded from www.jlr.org by guest, on June 18, 2012

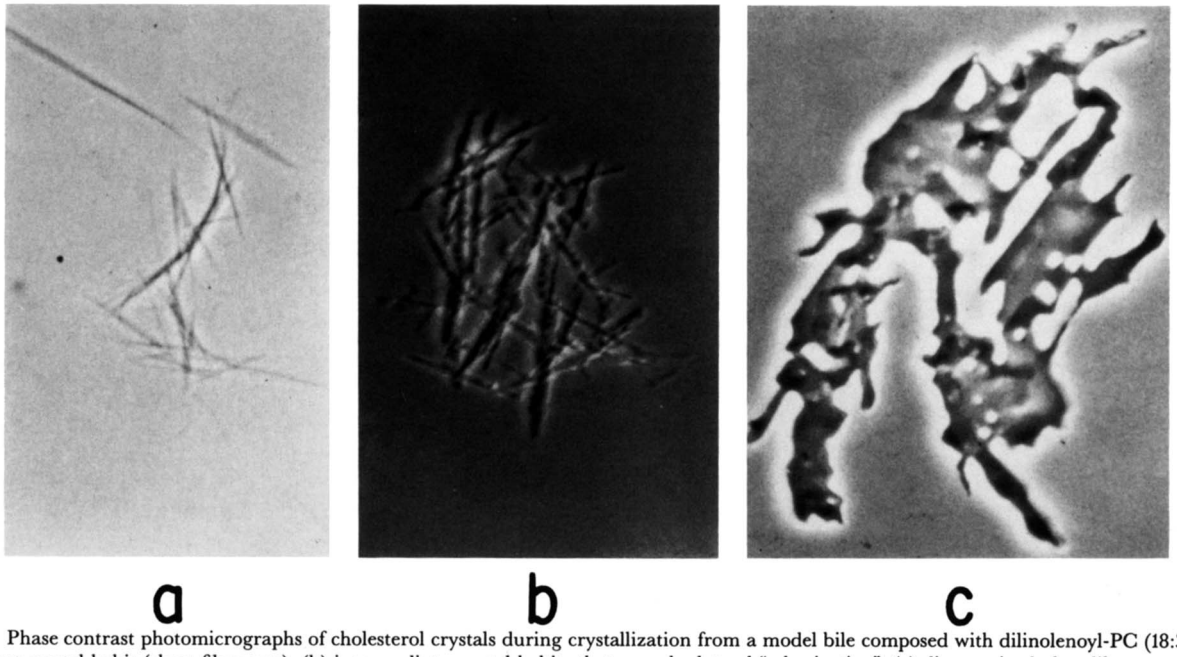


**Fig. 5.** Phase contrast photomicrographs of cholesterol crystals during crystallization from a model bile composed with dilinolenoyl-PC (18:3-18:3). (a) Earliest crystal habit (short filaments); (b) intermediate crystal habits that grow by lateral "arborization"; (c) disorganized plate-like crystals. The arborization growth pattern was observed also with other hydrophilic PC species, e.g., fish oil PCs (see text) and its time-sequence during crystallization is depicted in Fig. 4 (middle panel).

for these discrepancies are not clear, but may reflect different populations, diets, stages of gallstone disease, as well as methodologies used in separation and quantitation of individual lecithin molecular species (cf. 24). Based on available human data, therefore, the direct relevance of our findings with respect to gallstone pathophysiology is not predictable. Nonetheless, although filamentous lecithin-covered cholesterol crystals appear in human bile (14), their frequency, fate, and pathophysiological roles remain to be determined. Notwithstanding these deficiencies in our knowledge, the basic paradigm of the nucleation sequence and metastable intermediates in the prototype model bile of this work has been confirmed by us (D. Q-H. Wang, F. M. Konikoff, and D. C. Carey, unpublished results) in more concentrated and pathophysiologically relevant model bile systems. Therefore, because the contents and molecular species of biliary lecithins are subject to dietary manipulations (41-46), our findings might provide a scientific basis for novel strategies that delay or prevent the earliest events in cholesterol gallstone formation.

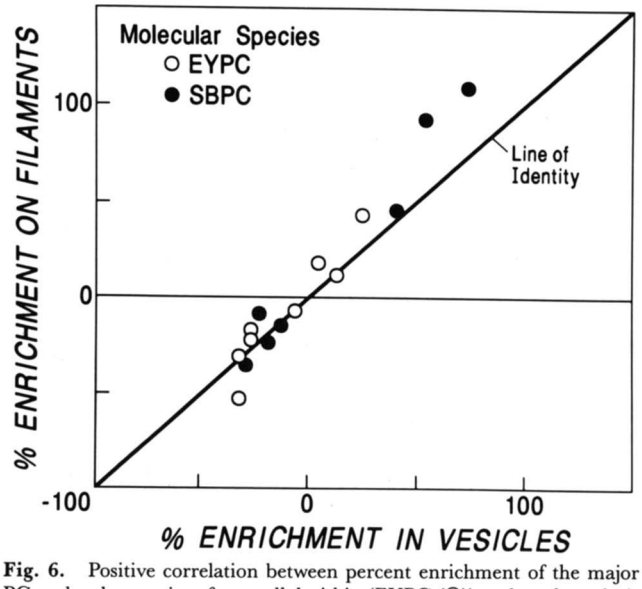


**Fig. 6.** Positive correlation between percent enrichment of the major PC molecular species of egg yolk lecithin (EYPC (○)) and soy bean lecithin (SBPC (●)) on the surfaces of filamentous cholesterol crystals and in the biliary vesicles of nucleating bile (from ref. 23). Each data point represents a single PC molecular species. The slopes of the regressions (not displayed) for each mixed lecithin species (EYPC or SBPC) were not significantly different from each other or from the line of identity (see discussion).

This work was supported by grants DK36588 and DK34854 from the National Institutes of Health NIDDK (US Public Health Service). Dr. Konikoff was supported in part by a fund from The American Physicians Fellowship Inc. Dr. Cohen was supported by grants from The Medical Foundation (Boston) and the American Liver Foundation.

*Manuscript received 22 April 1993 and in revised form 21 July 1993.*

## REFERENCES

1. Cabral, D. J., and D. M. Small. 1989. Physical chemistry of bile. *In* Handbook of Physiology—The Gastrointestinal System III. Section 6. S. G. Schultz, J. G. Forte, and B. B. Rauner, editors. American Physiological Society, Waverly Press, Baltimore, MD. 621-662.
2. Cohen, D. E., and M. C. Carey. 1990. Physical chemistry



Downloaded from www.jlr.org by guest, on June 18, 2012

of biliary lipids during bile formation. *Hepatology.* **12:** 143S–148S.
3. Holzbach, R. T. 1990. Nucleation of cholesterol crystals in native bile. *Hepatology.* **12:** 155S–161S.
4. LaMont, J. T., and M. C. Carey. 1992. Cholesterol gallstone formation. 2. Pathobiology and pathomechanics. *Prog. Liver Dis.* **10:** 165–191.
5. Halpern, Z., M. A. Dudley, A. Kibe, M. P. Lynn, A. C. Breuer, and R. T. Holzbach. 1986. Rapid vesicle formation and aggregation in abnormal human biles. A time-lapse video-enhanced contrast microscopy study. *Gastroenterology.* **90:** 875–885.
6. MacDonald, R. C. 1990. Surface chemistry of phospholipid vesicles relevant to their aggregation and fusion. *Hepatology.* **12:** 56S–60S.
7. Womack, N. A., R. Zeppa, and G. L. Irvin III. 1963. The anatomy of gallstones. *Ann. Surg.* **157:** 670–686.
8. Broomfield, P. H., R. Chopra, R. C. Sheinbaum, G. G. Bonorris, A. Silverman, L. J. Schoenfield, and J. W. Marks. 1988. Effects of ursodeoxycholic acid and aspirin on the formation of lithogenic bile and gallstones during loss of weight. *N. Engl. J. Med.* **39:** 1567–1572.
9. Sedaghat, A., and S. M. Grundy. 1980. Cholesterol crystals and the formation of cholesterol gallstones. *N. Engl. J. Med.* **302:** 1274–1277.
10. Juniper, K., Jr., and E. N. Burson, Jr. 1957. Biliary tract studies. II. The significance of biliary crystals. *Gastroenterology.* **32:** 175–208.
11. Loomis, C. R., G. G. Shipley, and D. M. Small. 1979. The phase behavior of hydrated cholesterol. *J. Lipid Res.* **20:** 525–535.
12. Craven, B. M. 1976. Crystal structure of cholesterol monohydrate. *Nature.* **260:** 727–729.
13. Shieh, H. S., L. G. Hoard, and C. E. Nordman. 1977. Crystal structure of anhydrous cholesterol. *Nature.* **267:** 287–289.
14. Konikoff, F. M., D. S. Chung, J. M. Donovan, D. M. Small, and M. C. Carey. 1992. Filamentous, helical and tubular microstructures during cholesterol crystallization from bile: evidence that biliary cholesterol does not nucleate classic monohydrate plates. *J. Clin. Invest.* **90:** 1155–1160.
15. Holan, K. R., R. T. Holzbach, R. E. Hermann, A. M. Cooperman, and W. J. Claffey. 1979. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology.* **77:** 611–617.
16. Holzbach, R. T., A. Kibe, E. Thiel, J. H. Howell, M. Marsh, and R. E. Hermann. 1984. Biliary proteins. Unique inhibitors of cholesterol crystal nucleation in human gallbladder bile. *J. Clin. Invest.* **73:** 35–45.
17. Groen, A. K., J. P. J. Stout, J. A. G. Drapers, F. J. Hoek, R. Grijm, and G. N. J. Tytgat. 1988. Cholesterol nucleation-influencing activity in T-tube bile. *Hepatology.* **8:** 347–352.
18. Jüngst, D., T. Lang, and G. Paumgartner. 1989. Phosphatidylcholines normalize cholesterol nucleation time in gallbladder bile of patients with cholesterol gallstones. *Hepatology.* **10:** 599.
19. Kibe, A., M. A. Dudley, Z. Halpern, M. P. Lynn, A. C. Breuer, and R. T. Holzbach. 1985. Factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile. *J. Lipid Res.* **26:** 1102–1111.
20. Konikoff, F., G. Goldman, Z. Halpern, G. J. Sömjen, and T. Gilat. 1990. Polyamines—potential nucleating factors in bile. *Liver.* **10:** 173–176.
21. Pope, J. L. 1967. Crystallization of sodium taurocholate. *J. Lipid Res.* **8:** 146–147.
22. Carey, M. C., and D. M. Small. 1978. The physical chemistry of cholesterol solubility in bile. Relationship to gallstone formation and dissolution in man. *J. Clin. Invest.* **61:** 998–1026.
23. Cohen, D. E., and M. C. Carey. 1991. Acyl chain unsaturation modulates distribution of lecithin molecular species between mixed micelles and vesicles in model bile. Implications for particle structure and metastable cholesterol solubilities. *J. Lipid Res.* **32:** 1291–1302.
24. Hay, D. W., M. J. Cahalane, N. Timofeyeva, and M. C. Carey. 1993. Molecular species of lecithins in human gallbladder bile. *J. Lipid Res.* **34:** 759–768.
25. Staggers, J. E., O. Hernell, R. J. Stafford, and M. C. Carey. 1990. Physical-chemical behavior of dietary and biliary lipids during intestinal digestion and absorption. 1. Phase behavior and aggregation states of model lipid systems patterned after aqueous duodenal contents of healthy adult human beings. *Biochemistry.* **29:** 2028–2040.
26. Konikoff, F. M., and M. C. Carey. 1992. Monitoring biliary cholesterol crystallization, transformation and growth: density gradient evidence that initial crystals are anhydrous and hydrate slowly. *Hepatology.* **16:** 124A.
27. Booker, M. L., W. W. LaMorte, S. A. Ahrendt, K. D. Lillemoe, and H. A. Pitt. 1992. Distribution of phosphatidylcholine molecular species between mixed micelles and phospholipid-cholesterol vesicles in human gallbladder bile: dependence on acyl chain length and unsaturation. *J. Lipid Res.* **33:** 1485–1492.
28. Hoelgaard, A., and S. Frokjaer. 1980. Adsorption of lecithin by cholesterol. *J. Pharm. Sci.* **69:** 413–415.
29. Jüngst, D., P. Huber, and G. Paumgartner. 1990. Phospholipids but not bile acids normalize cholesterol nucleation time in gallbladder bile of patients with cholesterol stones. *Gastroenterology.* **98:** A251.
30. Tao, S., S. Tazuma, T. Ohya, H. Sagawa, S. Hatsushika, G. Yamashita, N. Aihara, M. Sasaki, I. Horiuchi, and G. Kajiyama. 1992. Different processes in cholesterol crystal nucleation: importance of fatty acid composition in biliary lecithin. *Gastroenterology.* **102:** A899.
31. Janowitz, P., W. Swobodnik, J. G. Wechsler, A. Janowitz, D. Saal, and H. Ditschuneit. 1991. Fish oil enriched with polyunsaturated fatty acids of the omega-3-type accelerates the nucleation time in healthy subjects. *Klin. Wochenschr.* **69:** 289–293.
32. Berr, F., J. Holl, D. Jüngst, S. Fischer, W. O. Richter, B. Seifferth, and G. Paumgartner. 1992. Dietary n-3 polyunsaturated fatty acids decrease biliary cholesterol saturation in gallstone disease. *Hepatology.* **16:** 960–967.
33. Hay, D. W., and M. C. Carey. 1990. Chemical species of lipids in bile. *Hepatology.* **12:** 6S–16S.
34. Ahlberg, J., T. Curstedt, K. Einarson, and J. Sjövall. 1981. Molecular species of biliary phosphatidylcholines in gallstone patients: the influence of treatment with cholic acid and chenodeoxycholic acid. *J. Lipid Res.* **22:** 404–409.
35. Marks, J. W., G. G. Bonorris, G. Albers, and L. J. Schoenfield. 1992. The sequence of biliary events preceding the formation of gallstones in humans. *Gastroenterology.* **103:** 566–570.
36. Dam, H., I. Kruse, I. Prange, H. E. Kallehauge, J. Fenger, and M. Krogh-Jensen. 1971. Studies on human bile. III. Composition of duodenal bile from healthy young volunteers compared with composition of bladder bile from surgical patients with and without uncomplicated gallstone disease. *Z. Ernährungswiss.* **10:** 160–171.
37. Cantafora, A., A. Di Biase, D. Alvaro, M. Angelico, M. Marin, and A. F. Attili. 1983. High performance liquid



Downloaded from www.jlr.org by guest, on June 18, 2012

chromatographic analysis of molecular species of phosphatidylcholine—development of quantitative assay and its application to human bile. *Clin. Chim. Acta.* **134:** 281–295.
38. Cantafora, A., M. Angelico, A. DiBiase, U. Pièche, F. Bracci, A. F. Attilli, and L. Capocaccia. 1981. Structure of biliary phosphatidylcholine in cholesterol gallstone patients. *Lipids.* **16:** 589–592.
39. Berr, F., E. Schreiber, and C. V. Frick. 1992. Interrelationships of bile acid and phospholipid fatty acid species with cholesterol saturation of duodenal bile in health and gallstone disease. *Hepatology.* **16:** 71–81.
40. Angelico, M., S. Ginanni-Corradini, R. Masella, D. Alvaro, A. Cantafora, and L. Capocaccia. 1992. Molecular composition of biliary phosphatidylcholines, as related to cholesterol saturation, transport and nucleation in human gallbladder bile. *J. Hepatol.* **15:** 59–66.
41. Nervi, F., C. Covarrubias, P. Bravo, N. Velasco, N. Ulloa, F. Cruz, M. Fava, C. Severin, R. Del Pozo, C. Antezana, V. Valdivieso, and A. Arteaga. 1989. Influence of legume intake on biliary lipids and cholesterol saturation in young Chilean men. Identification of a dietary risk factor for cholesterol gallstone formation in a highly prevalent area. *Gastroenterology.* **96:** 825–830.
42. Booker, M. L., T. E. Scott, and W. W. LaMorte. 1989. Effect of dietary cholesterol on phosphatidylcholines and phosphatidylethanolamines in bile and gallbladder mucosa in the prairie dog. *Gastroenterology.* **97:** 1261–1267.
43. Dam, H., I. Kruse, M. Krogh-Jensen, and H. E. Kallehauge. 1967. Studies on human bile. II. Influence of two different fats on the compositions of human bile. *Scand. J. Clin. Invest.* **19**: 367–376.
44. Scobey, M. W., F. L. Johnson, J. S. Parks, and L. L. Rudel. 1991. Dietary fish oil effects on biliary lipid secretion and cholesterol gallstone formation in the African green monkey. *Hepatology.* **14:** 679–684.
45. Booker, M. L., T. E. Scott, and W. W. LaMorte. 1990. Effects of dietary fish oil on biliary phospholipids and prostaglandin synthesis in the cholesterol-fed prairie dog. *Lipids.* **25:** 27–32.
46. Cohen, B. I., E. H. Mosbach, N. Ayyed, S. Miki, and C. K. McSherry. 1992. Dietary fat and fatty acids modulate cholesterol cholelithiasis in the hamster. *Lipids.* **27:** 526–532.

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on June 18, 2012