Less hydrophobic phosphatidylcholine species simplify biliary vesicle morphology, but induce bile metastability with a broad spectrum of crystal forms

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Cholesterol crystallization in bile is affected by phosphatidylcholine (PtdCho) hydrophobicity. The aim of the present study was to determine whether PtdCho species modulate the metastable–labile limit and equilibrium solubility of cholesterol in the micellar phase of bile, thereby altering the distribution of cholesterol to biliary lipid carriers and thus influencing cholesterol crystallization. Supersaturated model bile (with a cholesterol saturation index of 2.0 and a total lipid concentration of 10 g/dl) was prepared with various PtdCho/(bile salt + PtdCho) ratios (0.1–0.5) using egg yolk or soya bean PtdCho. Subsequently, the following features were determined: metastable–labile limit, equilibrium solubility of cholesterol, metastable zone, and cholesterol crystallization process. Less hydrophobic PtdCho species destabilized bile cholesterol to induce rapid crystallization, because of a broad integrated metastable zone, whereas more hydrophobic species stabilized bile cholesterol

with a less integrated metastable zone and thus retarded cholesterol crystallization. Cholesterol crystallization was accelerated by a decrease in the PtdCho/(bile salt + PtdCho) ratio, whereas the final nucleated crystal mass was increased by an increase in this ratio. With decreasing hydrophobicity of the PtdCho species, the intermixed micellar/vesicular concentration of bile salts decreased in association with less formation of vesicles and increased formation of micelles, and a variety of crystal forms were detected. In conclusion, PtdCho species directly influenced the cholesterol crystallization process in model bile by remodelling the bile mesophase, and also had an indirect influence by altering the balance between bile salt micelles and vesicles.

Key words: cholesterol crystal growth assay, equilibrium solubility of cholesterol, metastable–labile limit, metastable zone.

INTRODUCTION

Crystallization of cholesterol from supersaturated bile is a key step in the formation of gallstones. Previous studies have indicated that changes in the biliary species of phosphatidylcholine (PtdCho) may have an important influence on the solubility of cholesterol and on the formation of cholesterol gallstones in humans [1,2]. We have previously shown that the degree of unsaturation of PtdCho in model bile systems affects bile metastability, which is reflected by cholesterol crystal nucleation, and that PtdCho hydrophobicity modulates biliary vesicle aggregation/fusion and cholesterol crystallization [3–6]. Cholesterol crystallization occurs in vesicles or micelles, or both, so the distribution of cholesterol among biliary lipid particulate species is one of the major factors affecting this process.

Cholesterol crystallization was believed to occur after aggregation and fusion of cholesterol-rich vesicles in bile [7,8]. However, Ahrendt et al. [9] reported that cholesterol is carried exclusively in, and nucleates rapidly from, mixed micelles without forming vesicles in the cholesterol-fed prairie dog. Also, Wang and Carey [10] showed that cholesterol crystallization in the left 2-phase zone (region A and B) on a ternary phase diagram occurs without the presence of liquid crystals. Cholesterol in bile is carried by particles, the formation of which is dependent upon the balance between bile salts and biliary lipids. Accordingly, the PtdCho species in bile may modulate the mesophase of a ternary phase diagram, and thereby alter the distribution of cholesterol to biliary lipid carriers. Such a process may be associated with the regulation of cholesterol crystallization.

In the present study, we analysed the effect of changes in the hydrophobicity of biliary PtdChos on the distribution of cholesterol between bile salt micelles and vesicles, focusing on the effect of PtdCho species on the metastable–labile limit and equilibrium solubility of cholesterol in the micellar phase.

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MATERIALS AND METHODS

Materials

Cholesterol was obtained from Wako Pure Chemical Industries (Osaka, Japan) and was stored under nitrogen at 4 °C. Egg yolk PtdCho, soya bean PtdCho, palmitoyl–linoleoyl PtdCho and sodium taurocholate were obtained from Sigma (St Louis, MO, U.S.A.) and were stored at –80 °C.

Preparation of model bile solutions

Model bile solutions were prepared according to a previously described method [11,12]. Aliquots of stock solutions [sodium taurocholate in methanol, PtdCho in chloroform/methanol (2:1, v/v), and cholesterol in chloroform/methanol (2:1, v/v)] were mixed in a volumetric flask and shaken overnight at 37 °C. The resulting mixture was then evaporated under a stream of nitrogen, freeze-dried and stored at –80 °C. Next, the required volumes of 10 mM Hepes/150 mM NaCl buffer containing 4 mM EDTA and 0.02 % NaN₃ (pH 7.5) were added to the freeze-dried lipids. These solutions were flushed with nitrogen, sealed and incubated

Abbreviations used: CCG, cholesterol crystal growth; CGR, crystal growth rate; FCM, final crystal mass; IMC, intermixed micellar/vesicular concentration; PtdCho, phosphatidylcholine; VCC, vesicle and cholesterol crystal.

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Table 1 Composition of the model bile used

CSI, cholesterol saturation index.

PtdCho (mM/mol%)	Bile salt (mM/mol%)	Cholesterol (mM/mol%)	PtdCho/ (bile salt + PtdCho)	Bile salt/ PtdCho	CSI	Total lipid concentration (g/dl)
0.00/0.00	180.71/96.18	7.18/3.82	0.0	—	2.0	10
16.80/9.18	151.19/82.62	15.00/8.20	0.1	9.0	2.0	10
31.22/17.42	124.88/69.70	23.07/12.88	0.2	4.0	2.0	10
44.08/25.16	102.85/58.70	28.28/16.14	0.3	2.3	2.0	10
56.71/33.39	85.06/50.09	28.04/16.51	0.4	1.5	2.0	10
68.92/41.97	68.92/41.97	26.36/16.05	0.5	1.0	2.0	10

at 55 °C for 12 h with shaking at 150 rev./min (Double Shaker NR-3; Taitec, Saitama, Japan) until microscopically isotropic. To determine the metastable–labile limit, the equilibrium solubility of cholesterol, and the metastable zone for each model bile, we prepared model bile solutions (cholesterol saturation index of 2.0, and a total lipid concentration of 10 g/dl) with various PtdCho/(bile salt + PtdCho) ratios (0.1–0.5) using egg yolk PtdCho and soya bean PtdCho (Table 1). The cholesterol saturation index was determined from the critical table of Carey [13]. All of the model bile systems had an equal total lipid concentration.

Vesicle and cholesterol crystal (VCC) and cholesterol crystal growth (CCG) assays

To evaluate the relative influence of each PtdCho species and PtdCho/(bile salt + PtdCho) ratio on the distribution of cholesterol between bile salt micelles and vesicles, and on nucleation of cholesterol in the model bile systems, the appearance of vesicles or cholesterol crystals and their growth were assayed quantitatively using a modification of the method described previously by Yamashita et al. [14–16]. Isotropic model bile solutions were filtered through a sterile 0.20 µm filter (Advantec) and were transferred into 1 ml glass microvials. The vials were flushed with nitrogen, sealed and incubated at 37 °C with gentle shaking (Bio-shaker BR-15; Taitec). Zero time for the VCC and CCG assays was defined as 60 min after thermal equilibration. A 30 µl aliquot of each model bile solution was added to an ELISA plate (NUNC™; Nalge Nunc International, Roskilde, Denmark) and diluted with 100 µl of buffer [25 mM Tris, 150 mM NaCl and 0.02 % Na₂S₂O₃ (pH 7.4)] containing sodium taurocholate at its intermixed micellar/vesicular concentration (IMC). After shaking, the absorbance was measured at a wavelength of 620 nm using a spectrophotometer (ImmunoMini NJ-2300; System Instruments, Tokyo, Japan). This absorbance represented the turbidity caused by a combination of multilamellar vesicles, stacked lamellae, liquid crystals and cholesterol crystals. Subsequently, 100 µl of buffer [25 mM Tris, 150 mM NaCl and 0.02 % Na₂S₂O₃ (pH 7.4)] containing 120 mM sodium taurodeoxycholate was added to each model bile solution to dissolve the vesicles, and the absorbance was measured 30 min later at 620 nm to determine the turbidity of the cholesterol crystals alone. Subsequently, the vesicle-related turbidity was estimated by subtraction of the absorbance measured in the presence of sodium taurodeoxycholate at the concentration required to completely dissolve the vesicles from the absorbance in the absence of sodium taurodeoxycholate. Measurements were performed every 24–48 h. The calculation of the crystal growth rate (CGR) was performed by adjusting data plotted to the sigmoidal curve tangent as previously described [6,14,17]. The slope of this

linear line was used as the CGR. The final crystal mass (FCM) was calculated at the plateau point of each nucleation curve.

Video-enhanced contrast microscopy

One drop (approx. 10 µl) of model bile solution was taken at frequent intervals during incubation and was placed between two cover slips. The samples were observed at 37 °C using a video-enhanced contrast microscope (LABOPHOTO-2; Nikon, Tokyo, Japan). Images were monitored using a video camera control system (C-2400; Hamamatsu Photonics, Hamamatsu, Japan) with a video monitor (PMV-1442Q; Sony, Tokyo, Japan) and were recorded on videotape using a video recorder (BR-S811; Victor, Yokohama, Japan). Observations of each model bile were performed every 24–48 h.

Measurement of the IMC

Isotropic model bile solutions were filtered through a sterile 0.20 µm filter and equilibrated for 1 h at 37 °C. The IMC of each model bile solution was then determined by the method of Donovan and Jackson [18]. In brief, 500 µl of model bile solution was placed in a Centricon 10R (Amicon, Beverly, MA, U.S.A.) and centrifuged for 15 min at 1500 g. The bile salt concentration of the filtrate was measured, and then was corrected by a factor derived from the Donnan equilibrium equation to obtain a cumulative IMC value. The IMC assay was performed at the start of incubation and again after equilibrium was reached.

Determination of the metastable zone and the metastable–labile limit

The metastable–labile limit was determined using a modification of the method described previously by Carey and Small [19]. In brief, model bile solutions were equilibrated at 55 °C and equilibration was confirmed microscopically, after which the solutions were rapidly cooled to 37 °C. For each bile salt PtdCho series, the cholesterol content of total lipids (in mols/100 mols) was plotted against the time required for precipitation. Cholesterol concentrations were measured at 60 min after rapid cooling. Mixtures that precipitated rapidly (i.e. after seconds or minutes) contained a larger excess of cholesterol compared with those that precipitated slowly (i.e. after hours or days). The rapidly precipitating mixtures were defined as falling within a subzone of labile supersaturation, whereas the latter mixtures showed metastable supersaturation. The interpolated cholesterol concentration at the boundary was defined as the metastable–labile limit. The metastable zone was therefore bounded by the metastable–labile limit and the equilibrium solubility of cholesterol.

Separation of lipid particles

Separation of lipid particles was performed using an FPLC system for each model bile solution. After incubation for 1 h at 37 °C, 500 µl of model bile solution was applied on to a Superose-6® column, which had been equilibrated with buffer [25 mM Tris, 150 mM NaCl and 0.02 % Na₂S₂O₃ (pH 7.4)] that contained sodium taurocholate at its IMC, and was eluted with the same buffer at a flow rate of 0.3 ml/min. Thirty fractions of 1 ml each were collected and were assayed for cholesterol and PtdCho to identify the various particulate fractions.

Analytical procedures

PtdCho concentrations were determined using a commercial kit (Nippon Shoji, Osaka, Japan), and cholesterol concentrations

were determined enzymically with a commercially available assay kit that employed cholesterol esterase and cholesterol oxidase (Nihon Shoji, Osaka, Japan). Bile salt concentrations were measured enzymically using 3-hydroxysteroid dehydrogenase (Daiichi Kagaku, Tokyo, Japan). The cholesterol saturation index of each sample was determined from the table of Carey [13].

HPLC analysis of PtdCho species

PtdCho hydrophobicity was determined by the retention times on reverse-phase HPLC (Shimadzu LC-6A system; Shimadzu Instruments, Tokyo, Japan) as previously described [5,6,20]. PtdCho species were chromatographed on a 4.5 mm $\times$ 250 mm Ultrasphere 5 μ m octadecyl silane column (Shimadzu) equipped with a 4.5 mm $\times$ 50 mm pre-column of the same material (Shimadzu Techno-Research, Tokyo, Japan) and eluted with methanol/water/acetonitrile solvent (90.5:7:2.5, by vol.) containing 20 mM choline chloride. Solvents were of HPLC-grade and were filtered (0.45 μ m; Corning, Tokyo, Japan) prior to use. The flow rate was 2 ml/min and absorbance was monitored at

205 nm. The hydrophobic index of each PtdCho was calculated from the relative retention factors determined by retention times.

Statistical analysis

Statistical analysis of the CCG, VCC and IMC data was performed by analysis of variance to determine whether differences existed among the groups at each specified time of examination. Statistical analysis of data on the lipid distribution in the model bile systems was performed using the Mann-Whitney U test.

RESULTS

Effect of the PtdCho species and the PtdCho/(bile salt + PtdCho) ratio on cholesterol crystallization

The effect of the PtdCho species and the PtdCho/(bile salt + PtdCho) ratio on cholesterol crystallization kinetics was assessed using various supersaturated model bile systems. The CCG curves for each model bile system are shown in Figure 1 and the

Figure 1 Effect of the PtdCho/(bile salt + PtdCho) ratio on cholesterol crystallization

(A) Model bile containing egg yolk PtdCho. (B) Model bile containing soya bean PtdCho. The model bile system used in the CCG assay had the following PtdCho/(bile salt + PtdCho) ratios: $\circ$, 0; $\bullet$, 0.1; $\triangle$, 0.2; $\blacktriangle$, 0.3; $\diamond$, 0.4; and $\blacklozenge$, 0.5. (A) When the PtdCho/(bile salt + PtdCho) ratio was 0.1, 0.2 or 0.3, there was a significant increase in CGR and FCM compared with the values at ratios of 0, 0.4 and 0.5. (B) CGR and FCM showed similar trends in model bile containing soya bean PtdCho, but the absolute increases were far greater than those shown in (A). Data are presented as means $\pm$ S.D. ($n = 3$).

Figure 2 Effect of the PtdCho/(bile salt + PtdCho) ratio on vesicle formation

(A) Model bile containing egg yolk PtdCho. (B) Model bile containing soya bean PtdCho. The PtdCho/(bile salt + PtdCho) ratios are as follows: $\circ$, 0; $\bullet$, 0.1; $\triangle$, 0.2; $\blacktriangle$, 0.3; $\diamond$, 0.4; and $\blacklozenge$, 0.5. (A) When the PtdCho/(bile salt + PtdCho) ratio was 0 or 0.1, vesicles were not detected. When the PtdCho/(bile salt + PtdCho) ratio was 0.3, 0.4 or 0.5, vesicles were prominent and did not change much over time. When the PtdCho/(bile salt + PtdCho) ratio was 0.2, vesicles increased initially, and then diminished. (B) When the PtdCho/(bile salt + PtdCho) ratio was 0, 0.1 or 0.2, vesicles were not detected. When the PtdCho/(bile salt + PtdCho) ratio was 0.4 or 0.5, vesicles were prominent and did not change much over time. When the PtdCho/(bile salt + PtdCho) ratio was 0.3, vesicles grew initially, and then diminished. Data are presented as means $\pm$ S.D. ($n = 3$).

Table 2 Effect of PtdCho hydrophobicity on cholesterol growth and vesicle aggregates in the CGR assay

Values are presented as means $\pm$ S.D. ($n = 3$). * $P < 0.05$, compared with egg yolk PtdCho with the same PtdCho/(bile salt + PtdCho) ratio.

Model bile	PtdCho/(bile salt + PtdCho) ...	FCM (absorbance)					Maximal CGR (Δ absorbance $\times 10^{-3}$ /h)		
		0.1	0.2	0.3	0.4	0.5	0.1	0.2	0.3
Egg yolk PtdCho		0.170 $\pm$ 0.002	0.175 $\pm$ 0.005	0.049 $\pm$ 0.003	0.009 $\pm$ 0.002	0.007 $\pm$ 0.0005	8.33	0.70	0.167
Soya bean PtdCho		0.217 $\pm$ 0.011*	0.527 $\pm$ 0.009*	0.932 $\pm$ 0.013*	0.006 $\pm$ 0.002	0.001 $\pm$ 0.004	40*	16.7*	6.67*

Table 3 Percentage molecular composition and hydrophobic index of PtdCho determined by HPLC

PtdCho species	Retention time (min)	Relative retention time	Soya bean PtdCho (%)	Palmitoyl-linoleoyl PtdCho (%)	Egg yolk PtdCho (%)
18:2-18:3	12.6	0.55	10	—	—
18:2-18:2	17.1	0.75	42	—	—
16:0-20:4	22.3	0.97	—	—	6
16:0-18:2	22.9	1	23	100	19
18:1-18:2	25.3	1.11	9	—	—
16:0-18:1	30.8	1.35	2	—	38
18:0-20:4	36.2	1.58	—	—	7
18:0-18:2	37.8	1.65	6	—	8
16:0-16:0	40.8	1.78	—	—	—
18:0-18:1	52.1	2.27	—	—	9
Hydrophobic index			0.9	1	1.4

VCC curves for each system are shown in Figure 2. CGR and FCM values are compared in Table 2. Figure 1(A) shows the effect of various PtdCho/(bile salt + PtdCho) ratios (0, 0.1, 0.2, 0.3, 0.4 and 0.5) on supersaturated model bile containing egg yolk PtdCho. The model bile systems with ratios of 0.1, 0.2 and 0.3 showed significant increases in CGR and FCM compared with the model bile systems having ratios of 0, 0.4 and 0.5 (Table 3). Figure 1(B) shows the effect of various PtdCho/(bile salt + PtdCho) ratios (0, 0.1, 0.2, 0.3, 0.4 and 0.5) on supersaturated model bile containing soya bean PtdCho. The CGR and FCM values showed the same trends as in model bile systems containing egg yolk PtdCho, but the absolute extent of the changes was much greater. Figure 2(A) shows the results of the vesicle assay at various PtdCho/(bile salt + PtdCho) ratios (0, 0.1, 0.2, 0.3, 0.4 and 0.5) using supersaturated model bile containing egg yolk PtdCho. When the PtdCho/(bile salt + PtdCho) ratio was 0 or 0.1, vesicles were not detected by the optical assay or by microscopy. When the ratio was 0.3, 0.4 or 0.5, numerous vesicles were detected, and they did not change much throughout the observation period. When the ratio was 0.2, vesicles grew initially and then diminished as cholesterol crystals appeared. These changes were also recognized microscopically. Figure 2(B) shows the results of the vesicle assay at various PtdCho/(bile salt + PtdCho) ratios, (0, 0.1, 0.2, 0.3, 0.4 and 0.5) using supersaturated model bile containing soya bean PtdCho. When the PtdCho/(bile salt + PtdCho) ratio was 0, 0.1 or 0.2, vesicles were not detected by the optical assay or by microscopy. When the ratio was either 0.4 or 0.5 there were numerous vesicles that did not change much throughout observation. When the ratio was 0.3 vesicles grew initially, and then diminished as cholesterol crystals appeared. These changes were also recognized microscopically.

Morphological changes of vesicles and cholesterol crystals

Vesicle formation and morphological changes of cholesterol crystals were observed by video-enhanced microscopy every

24–48 h. Vesicle formation was rapid in the model bile solutions containing both egg yolk PtdCho and soya bean PtdCho with higher PtdCho/(bile salt + PtdCho) ratios (0.4 and 0.5). In model bile solutions with middling PtdCho/(bile salt + PtdCho) ratios (0.2 and 0.3) crystals could also be observed in the experimental period. When the PtdCho/(bile salt + PtdCho) ratio was 0.1 cholesterol crystallization was accomplished rapidly in model bile solutions containing both egg yolk PtdCho and soya bean PtdCho. In model bile solutions containing both EYPtdCho and SBPtdCho with lower PtdCho/(bile salt + PtdCho) ratios (0 and 0.1) no vesicles were observed. In contrast, when the PtdCho/(bile salt + PtdCho) ratio was 0.2, vesicles were observed in model bile containing egg yolk PtdCho, whereas no vesicles were found in model bile containing soya bean PtdCho. Some crystals were transformed into arc-like crystals, spiral structures and then plate-like crystals. As the final step, classical monohydrate crystals became the predominant form in the model bile solutions. Representative patterns of crystallization in each model bile are shown in Figure 3.

Effect of PtdCho species and PtdCho/(bile salt + PtdCho) ratio on the metastable zone

Figure 4 shows triangular co-ordinate plots of the metastable–labile limit and the equilibrium solubility of cholesterol in different model bile systems. The area enclosed by the two curves represents the metastable zone. Figure 4(A) shows the metastable zone for model bile systems containing egg yolk PtdCho. The upper boundary (metastable–labile limit) was not as high as that of model bile containing soya bean PtdCho (Figure 4B), but the lower boundary (the equilibrium solubility of cholesterol) was not much different. As a result, the metastable zone of model bile containing egg yolk PtdCho was smaller than that of model bile containing soya bean PtdCho. The width of the metastable

Figure 3 Particles detected in model bile systems by video-enhanced contrast microscopy

(A–C) Model bile containing egg yolk PtdCho. (D–F) Model bile containing soya bean PtdCho. (A1, A2 and A3) Model bile at 26, 77 and 282 h respectively [PtdCho/(bile salt + PtdCho) ratio = 0.1]. (B1, B2 and B3) Model bile at 26, 219 and 425 h respectively [PtdCho/(bile salt + PtdCho) ratio = 0.2]. (C1, C2 and C3) Model bile at 26, 219 and 425 h respectively [PtdCho/(bile salt + PtdCho) ratio = 0.4]. (D1, D2 and D3) Model bile at 26, 77 and 282 h respectively [PtdCho/(bile salt + PtdCho) ratio = 0.1]. (E1, E2 and E3) Model bile 26, 219 and 425 h respectively [PtdCho/(bile salt + PtdCho) ratio = 0.3]. (F1, F2 and F3) Model bile at 26, 219 and 425 h respectively [PtdCho/(bile salt + PtdCho) ratio = 0.4]. Vesicle formation was rapid in both model bile solutions with higher PtdCho/(bile salt + PtdCho) ratios (over 0.4). However, few vesicles were observed in those with a lower PtdCho/(bile salt + PtdCho) ratio (0.1). In model bile containing egg yolk PtdCho with a PtdCho/(bile salt + PtdCho) ratio of 0.2 and in model bile containing soya bean PtdCho with a ratio of 0.3, after vesicles decreased, needle-like crystals appeared and some crystals were transformed into spiral structures and then plate-like crystals appeared. Finally, monohydrate crystals became the predominant form. Scale bars = 10 μ m.

zone at each PtdCho/(bile salt + PtdCho) ratio almost corresponded to the FCM for both egg yolk PtdCho- and soya bean PtdCho-containing model bile systems. In brief, as the PtdCho/

(bile salt + PtdCho) ratio increased to 0.3, the metastable zone became wider, but it subsequently showed a rapid decrease in width.

Figure 4 Triangular coordinate plot of the metastable-labile limit (●-●) and the maximum cholesterol solubility (○-○) in model bile systems

The hatched area represents the metastable region. (A) Metastable zone of egg yolk PtdCho-containing model bile solutions. (B) Metastable zone of soya bean PtdCho-containing model bile solutions. The upper boundary (metastable-labile limit) of egg yolk PtdCho-containing model bile is not as high as that of soya bean PtdCho-containing model bile, but the lower boundary (the equilibrium solubility of cholesterol) is not much different. Therefore the metastable zone of egg yolk PtdCho-containing model bile solutions is smaller than that of soya bean PtdCho-containing model bile solutions. Abbreviations used: BA, bile salt; CH, cholesterol; PC, PtdCho.

Effect of PtdCho species on the IMC

Figure 5 shows the IMC values of each model bile solution. At the start of the incubation, the IMC values of model bile containing soya bean PtdCho were lower than those of model bile containing egg yolk PtdCho (Figure 5A). In contrast, the IMC values of model bile containing soya bean PtdCho increased at equilibrium, whereas they hardly changed in model bile containing egg yolk PtdCho. Consequently, the difference between the two model biles became smaller compared with that observed at the start of the incubation (Figure 5B).

Effect of PtdCho species on the distribution of cholesterol among lipid particles

The effect of the PtdCho species on the pattern of cholesterol and PtdCho distribution among lipid particles was evaluated (Figure

Figure 5 IMC values of model bile solutions

(A) At the start of incubation, the IMCs of model bile solutions containing soya bean PtdCho (●) were lower than those of solutions containing egg yolk PtdCho (○). (B) The IMC values of soya bean PtdCho-containing model bile (●) were increased at equilibrium, whereas they were unchanged in egg yolk PtdCho-containing model bile (○), and thereby the difference between those two became smaller when compared with that observed at the start of incubation. Abbreviations used: BA, bile salt; PC, PtdCho.

6). At the same PtdCho/(bile salt + PtdCho) ratio, numerous vesicles were observed in model bile containing egg yolk PtdCho, but few vesicles were found in model bile containing soya bean PtdCho. There were more bile salt micelles in model bile containing soya bean PtdCho than in model bile containing egg yolk PtdCho. In the model biles using an identical PtdCho, the higher the PtdCho/(bile salt + PtdCho) ratio the more vesicles that were observed.

Degree of hydrophobicity of each PtdCho species determined by HPLC

The molar percentages of PtdCho molecular species in each PtdCho used in the present study are reported in Table 3. The retention time of palmitoyl-linoleoyl PtdCho was used as a reference, and the retention time of the other species was divided by that of palmitoyl-linoleoyl PtdCho. Based on the weighted average of relative retention times of PtdCho species, the overall hydrophobic index of each PtdCho was estimated. The rank order of hydrophobic indexes of PtdCho species used in the present study was egg yolk PtdCho (1.4) > palmitoyl-linoleoyl PtdCho (1.0) > soya bean PtdCho (0.90).

Figure 6 Cholesterol and PtdCho distribution among lipid particles using Superose-6® column chromatography after 1 h

(●) Vesicles of model bile containing egg yolk PtdCho. (■) Micelles of model bile containing egg yolk PtdCho. (▲) Vesicles of model bile containing soya bean PtdCho. (×) Micelles of model bile containing soya bean PtdCho. At the same PtdCho/(bile salt + PtdCho) ratio, numerous vesicles were observed in model bile containing egg yolk PtdCho, but few vesicles were found in model bile containing soya bean PtdCho. There were more bile salt micelles in model bile containing soya bean PtdCho than in model bile containing egg yolk PtdCho. In the model biles using an identical PtdCho, the higher the PtdCho/(bile salt + PtdCho) ratio the more vesicles that were observed. Data are presented as means ($n = 3$). Abbreviations used: BA, bile salt; CH, cholesterol; PC, PtdCho.

DISCUSSION

Previous findings have suggested that biliary PtdCho species play a crucial role in the solubilization of cholesterol in bile. Jüngst et al. [21] have shown that an increased PtdCho concentration in bile has a greater effect on cholesterol nucleation than an increased bile salt concentration. Halpern et al. [22] have demonstrated that disaturated synthetic PtdCho species prolong the nucleation time in comparison with egg yolk PtdCho or mono-unsaturated PtdCho species. The PtdCho species are asymmetrically distributed between vesicles and micelles in human bile and model bile systems [23,24]. We recently demonstrated that changes in PtdCho hydrophobicity within the physiological range could have a marked influence on bile metastability as well as on the cholesterol crystal nucleation process [6]. In this context, the present study demonstrated that less hydrophobic PtdCho species destabilized cholesterol in bile (as indicated by a broad integrated metastable zone) and thus induced rapid cholesterol crystallization, whereas more hydrophobic PtdCho species stabilized cholesterol (decreasing the integrated metastable zone) and thus retarded cholesterol crystal-

lization. Therefore the changes in cholesterol crystallization associated with differences in fatty acyl chain saturation can be explained by modulation of the mesophase in bile. Several investigators have reported that nucleation of cholesterol crystals commences from vesicles in bile [7,25]. However, Ahrendt et al. [9] reported that cholesterol is exclusively carried by, and nucleates rapidly from, mixed micelles without forming vesicles in cholesterol-fed prairie dogs. Similarly, Wang and Carey [10] showed that cholesterol crystallization in the left 2-phase zone on a ternary phase diagram occurs without the presence of liquid crystals.

Recently we also demonstrated that cholesterol crystals originate predominantly from mixed micelles in model bile containing soya bean PtdCho and exclusively from these micelles in model bile containing dilinoleoyl PtdCho [6]. These results suggest that vesicles containing cholesterol and PtdCho are not always required for nucleation. In the present study, decreasing the hydrophobicity of the PtdCho species in model bile resulted in less vesicle formation, although a variety of crystal forms developed (arc-like crystals, spiral structures and plate-like crystals as well as classical monohydrate crystals). Wang and Carey [10] showed that decreases in temperature, total lipid concentration and bile salt hydrophobicity progressively shifted all crystallization pathways to lower PtdCho contents, retarded crystallization and decreased micellar cholesterol solubilities. van Erpecum and Carey [26] showed that ternary phase diagrams of sphingomyelin (from egg yolk, buttermilk and brain) and dipalmitoyl PtdCho with cholesterol and taurocholate are identical, and, compared with systems containing egg yolk PtdCho, the 1-phase micellar zone and 2- and 3-phase solid cholesterol crystal-containing zones are reduced markedly while the 2-phase zone with stable cholesterol- sphingomyelin liquid crystals is greatly expanded.

The difference in vesicle appearance patterns between model biles containing egg yolk PtdCho and soya bean PtdCho in the present study can be explained by these phenomena. In fact, model bile containing soya bean PtdCho with a PtdCho/(bile salt + PtdCho) ratio of 0.3 is plotted in the 3-phase zone. However, if the ratio is 0.1 or 0.2, the model bile is plotted in the left 2-phase zone, and when the ratio is over 0.4, the model bile is plotted in the right 2-phase zone. In contrast, in hydrophobic egg yolk PtdCho-containing model biles, all crystallization pathways are shifted to the left (with lower PtdCho contents). As a result, when the PtdCho/(bile salt + PtdCho) ratio is 0.2 the model bile containing egg yolk is plotted in the 3-phase zone, whereas a ratio of 0.1 results in a model bile that is plotted in the left 2-phase zone, and a ratio of 0.3 results in a model bile that is plotted in the right 2-phase zone.

In addition, the present study provided the interesting findings that micellar cholesterol solubility at the equilibrium state was unchanged, whereas the metastable-labile limit of each model bile containing egg yolk PtdCho and soya bean PtdCho differed drastically. As a result, the metastable zone of model bile containing egg yolk PtdCho was smaller than that of model bile containing soya bean PtdCho.

In the present study, a decrease in the PtdCho/(bile salt + PtdCho) ratio accelerated cholesterol crystallization, whereas the FCM was increased with an increase in the ratio to 0.1–0.3. However, this change was not evident at higher ratios, of 0.4 and 0.5. This interesting crystal behaviour was more marked in model bile containing soya bean PtdCho, although model bile containing egg yolk PtdCho showed similar findings. Such changes could be explained by the function of PtdCho molecules as cholesterol carriers. PtdCho molecules might form metastable cholesterol-carrying particulate species at a specific range of the

PtdCho/(bile salt+PtdCho) ratio, thus increasing the total release of cholesterol from such metastable carrier particles. However, once PtdCho molecules reach a high enough concentration to stabilize the bile, the above phenomenon is no longer evident. Thus the total amount of PtdCho in bile is another important factor affecting the FCM.

Another interesting finding in the present study was the fact that the IMC was lower in model bile containing soya bean PtdCho compared with model bile containing egg yolk PtdCho. The FPLC elution suggested that model bile containing soya bean PtdCho formed significantly more micelles than model bile containing egg yolk PtdCho at the same lipid composition, and when they form some vesicles, their masses are much less than in model bile containing egg yolk PtdCho. Thus the amount of bile salt associated with PtdCho molecules may have been lower in model bile containing egg yolk PtdCho than in model bile containing soya bean PtdCho. This might be associated with the broad mesophase of model bile containing soya bean PtdCho, although further investigation is needed for clarification. Donovan et al. [27] investigated the relationship between IMC and total lipid concentration of model bile with various bile salt/(bile salt+PtdCho) ratios (0.5–1.0) using a modified equilibrium dialysis method [27], and suggested that above a bile salt concentration of 30 mM in the model bile system, the IMC value must be underestimated [18]. Thus data using an IMC of over 30 mM would be controversial, although such data fit well in a series of experiments regarding the effect of PtdCho molecules on micelle-forming capacity in the present study.

At any event, PtdCho species can influence the cholesterol crystallization process directly by remodelling the mesophase of model bile, and indirectly by altering the balance between bile salt micelles and vesicles in association with a change of IMC.

Since all experiments in the present study were performed using model bile solutions with a single total lipid concentration of 10 g/dl, the relevance to events in native bile might be limited. Nevertheless, the finding that PtdCho hydrophobicity affects bile metastability as well as the process of cholesterol crystal nucleation and growth in supersaturated model bile systems provides a new insight into our understanding of cholesterol gallstone formation.

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