Partial replacement of bile salts causes marked changes of cholesterol crystallization in supersaturated model bile systems

Tomoji NISHIOKA, Susumu TAZUMA¹, Gunji YAMASHITA and Goro KAJIYAMA

First Department of Internal Medicine, Hiroshima University School of Medicine, 1-2-3, Kasumi, Minami-ku, Hiroshima, Japan

Cholesterol crystallization is a key step in gallstone formation and is influenced by numerous factors. Human bile contains various bile salts having different hydrophobicity and micelle-forming capacities, but the importance of lipid composition to bile metastability remains unclear. This study investigated the effect of bile salts on cholesterol crystallization in model bile (MB) systems. Supersaturated MB systems were prepared with an identical composition on a molar basis (taurocholate/phosphatidylcholine/cholesterol, 152 mM:38 mM: 24 mM), except for partial replacement of taurocholate (10, 20, and 30 %) with various taurine-conjugated bile salts. Cholesterol crystallization was quantitatively estimated by spectrophotometrically measuring crystal-related turbidity and morphologically scanned by video-enhanced microscopy. After partial replacement of taurocholate with hydrophobic bile salts, cholesterol crystalliza-

tion increased dose-dependently without changing the size of vesicles or crystal morphology and the rank order of crystallization was deoxycholate > chenodeoxycholate > cholate (control MB). All of the hydrophilic bile salts (ursodeoxycholate, ursocholate and β -muricholate) inhibited cholesterol precipitation by forming a stable liquid-crystal phase, and there were no significant differences among the hydrophilic bile-salt species. Cholesterol crystallization was markedly altered by partial replacement of bile salts with a different hydrophobicity. Thus minimal changes in bile-salt composition may dramatically alter bile lipid metastability.

Key words: bile, bile metastability, bile salt species, cholesterol crystal, crystal habits, liquid crystal.

INTRODUCTION

Crystallization of cholesterol from supersaturated gall-bladder bile is a key step in the formation of gallstones. Such crystallization is believed to follow the aggregation and fusion of unilamellar vesicles rich in cholesterol [1]. Cholesterol is solubilized in phospholipid vesicles, as well as together with phospholipids and bile salts in mixed micelles [1–3]. Since the formation of mixed micelles requires more phospholipids than cholesterol, a larger amount of phospholipids is removed relative to cholesterol during the conversion of vesicles into mixed micelles. As a result, the residual vesicles become progressively richer in cholesterol and depleted of phospholipids, and the cholesterol/phospholipid (C/P) ratio steadily increases. These cholesterol-rich vesicles tend to aggregate, leading to the precipitation of cholesterol crystals [3,4]. Cholesterol/phosphatidylcholine vesicles are present in the gall-bladder bile of humans and play a key role in the nucleation of cholesterol crystals [3–5]. On the basis of video-enhanced microscopy of human bile, Halpern et al. [3] reported that vesicle aggregation and fusion precede cholesterol nucleation. Recently, Wang and Carey demonstrated that five crystallization pathways are present in pathophysiologically relevant model biles (MBs) [6]. The crystallization of cholesterol is affected by the presence of various factors in bile, such as biliary proteins [7–12], various phospholipid species [4,13–16], and various bile-salt species [17–21].

We have already compared the activity of these various promoting factors to determine their relative potency in causing cholesterol crystallization, and bile-salt species were found to be less effective than phospholipid species and biliary proteins [22].

Human and animal bile contain a complex and variable mixture of bile salts and phospholipids, the relative composition of which may vary in gallstone disease [23]. For instance, the highly hydrophobic bile salt deoxycholate has been associated with the pathogenesis of gallstones in humans [15,24]. Studies have shown a linear relationship between the percentage of biliary hydrophobic bile salts and the molar percentage of cholesterol in bile and the biliary cholesterol saturation index, indicating that hydrophobic bile salts directly and indirectly affect cholesterol nucleation [15]. Conversely, dissolution of cholesterol gallstones can be achieved through oral administration of ursodeoxycholate, a highly hydrophilic bile salt [25,26]. In the same fashion, prevention of cholesterol gallstones in animal models has been achieved by replacing part of the native biliary bile salts with more hydrophilic salts such as hyodeoxycholate [27], hyocholate [28] or β -muricholate [29]. It is noteworthy that recent data obtained for hamsters have suggested that the hydrophobic/hydrophilic balance of bile salts, calculated as the hydrophobic index, is the best predictor of cholesterol gallstones [30]. Thus it would appear that the composition of the bile-salt pool largely influences cholesterol crystal formation. We have already demonstrated that ursodeoxycholate prolongs the nucleation time in

Abbreviations used: BA/PL ratio, bile acid/phospholipid ratio; CGR, crystal growth rate; CH, cholesterol; C/P ratio, cholesterol/phospholipid ratio; DCA, deoxycholic acid; PC, phosphatidylcholine; FCM, final crystal mass; IMC, intermixed micellar and vesicular bile-salt concentration; MB, model bile; PL, phospholipid; T β MC, tauro- β -muricholate; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TUC, tauroursocholate; TUDC, tauroursodeoxycholate; UDCA, ursodeoxycholic acid.

¹ To whom correspondence should be addressed (e-mail stazuma@mcai.med.hiroshima-u.ac.jp).

human gall-bladder bile without causing marked biliary cholesterol desaturation [26]. Ursodeoxycholate may stabilize non-micellar lipid particles where cholesterol nucleation occurs and thus retard the progression of nucleation, since the micellar solubility of cholesterol is considered poor [31].

Accordingly the present study was performed to evaluate the effect on cholesterol crystallization of partial replacement with several bile-salt species in supersaturated MB systems.

This work was presented in part at the 98th Annual Meeting of the American Gastroenterological Association, on 17 May 1998, in New Orleans,

MATERIALS AND METHODS

Materials

Egg-yolk phosphatidylcholine (PC), taurocholate (TC), and taurodeoxycholate (TDC) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Cholesterol was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Taurochenodeoxycholate (TCDC), tauroursodeoxycholate (TUDC), tauroursocholate (TUC), and tauro- β -muricholate (T β MC) were gifts from Tokyo Tanabe Co (Tokyo, Japan). All glassware was washed with 99% methanol and rinsed with distilled water before drying.

Preparation of MB solutions

MB solutions were prepared according to the previously described method [32]. Aliquots of stock solutions (TC in methanol, PC in chloroform/methanol, and cholesterol in chloroform/methanol) were first added to a volumetric flask to achieve the desired lipid levels. The organic solvent was then evaporated under a stream of nitrogen until the mixture condensed to a viscous paste. Next, the mixture was freeze-dried to achieve complete solvent removal and then stored at -80°C . Then, the required volumes of Tris/NaCl (25 mM/150 mM) buffer containing 0.02% NaN_3 , pH 7.45, were added to the freeze-dried lipids. These solutions were flushed with nitrogen, sealed, and incubated at 55°C for 12 h with shaking at 150 rev./min (Double Shaker NR-3; Taitec, Saitama, Japan) until the solutions became microscopically isotropic.

To compare the effects of various bile acids on cholesterol crystallization, we prepared MB solutions with an identical lipid composition under the following conditions. The control system had a total lipid concentration of 12 g/dl. It contained 38 mM PC, 152 mM TC and 24 mM cholesterol, with a final bile acid/phospholipid molar ratio (BA/PL) of 4.0. This control MB system was altered by replacing 10, 20 and 30% of TC with TDC, TCDC, TUDC, TUC or T β MC respectively. All MB systems had an equal total lipid concentration.

Conventional nucleation time study

Nucleation time was determined essentially as described by Holan et al [33]. Isotropic MB solutions were filtered through a sterile 0.20 μm -mesh filter (Advantec; Toyo Roshi Co., Tokyo, Japan) and aliquots of these solutions were mixed in 1 ml glass microvials. The vials were flushed with nitrogen, sealed, and incubated at 37°C with gentle shaking. The zero nucleation time was defined as 30 min after thermal equilibration. Samples were observed every 24 h by video-enhanced differential interference microscopy to detect cholesterol crystals as well as any morphological changes of the crystals.

Measurement of the intermixed micellar/vesicular bile-salt concentration (IMC)

The isotropic MB solutions were filtered through a sterile 0.20 μm filter and equilibrated for 1 h at 37°C . The IMC of each MB solution was determined by the method of Donovan and Jackson [34]. In brief, 500 μl of MB solution was placed in a Centricon 10^R (Amicon, Beverly, MA, U.S.A.) and centrifuged for 15 min at 1500 *g*. The bile-salt concentration of the filtrate was measured, then corrected by a factor derived from the Donnan equilibrium equation to obtain a cumulative IMC value.

Crystal-growth-rate (CGR) assay

To evaluate the comparative potencies of the various bile acids for influencing cholesterol nucleation in supersaturated MB systems, the appearance of cholesterol crystals and their growth were quantitatively assayed using a modification of the method described previously by Sömjen et al. [35,36]. Isotropic MB solutions were filtered through a sterile 0.20 μm filter (Advantec) and transferred into 1 ml glass microvials. The vials were flushed with nitrogen, sealed, and incubated at 37°C with gentle shaking. Zero time was defined as 30 min after thermal equilibration. A 30 μl aliquot of each MB solution was added to an ELISA plate (NUNC[®]; Nalge Nunc International, Roskilde, Denmark) and diluted with 100 μl of Tris/NaCl buffer containing TC at its 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 turbidity due to multilamellar vesicles, stacked lamellae, liquid crystals and cholesterol crystals. Then 100 μl of Tris/NaCl buffer containing 120 mM TDC was added to each MB solution to dissolve vesicles, and the absorbance was measured 30 min later at 620 nm to determine the turbidity of the cholesterol crystals alone. Measurements were performed every 24 h. The final crystal mass (FCM) was calculated at the plateau point of each nucleation curve.

Video-enhanced contrast microscopy

One drop of each MB solution was placed between two coverslips at frequent intervals during incubation and the samples were observed using video-enhanced contrast microscopy (Nikon, Tokyo, Japan). The images were monitored with a video-camera control system (C-2400; Hamamatsu Photonics, Hamamatsu, Japan) and a video monitor (PMV-1442Q; Sony, Tokyo, Japan). The number of different cholesterol crystals per microscopic field ($\times 400$ magnification) or per 1/40 microscopic field was reported on a semi-quantitative scale: 0, no crystals; 1, 1–5 crystals; 2, 6–10 crystals; 3, 11–15 crystals; 4, 16–20 crystals; 5, > 20 crystals. Observed values were smoothed manually.

Analytical procedures

PC concentrations were measured by the methods of Bartlett [37] and Fiske and SubbaRow [38], and cholesterol concentrations were determined enzymically with a commercially available assay kit that deleted cholesterol esterase and cholesterol oxidase (Nihon Shoji, Osaka, Japan). Bile acid concentrations were measured enzymically using 3 α -hydroxysteroid dehydrogenase (Daiichi Kagaku, Tokyo, Japan).

Statistical analysis

The statistical analyses of the CGR curves was performed using analysis of variance at each time point to determine whether differences existed among the groups.

RESULTS

Effect of each bile salt in the CGR Assay

The effect on cholesterol crystallization kinetics of replacement with each bile-salt species was assessed by the CGR assay using

Figure 1 Relative promotion of cholesterol crystallization by hydrophobic bile salts assessed with the CGR assay

(A) Effect of TDC on cholesterol crystallization. The MBs used in the CGR assay had the following compositions: ○, control MB; ●, 10% replacement of TC with TDC; □, 20% replacement with TDC; ■, 30% replacement with TDC. Each data point represents the mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$ compared with control. (B) Effect of TCDC on cholesterol crystallization. The MBs used in the CGR assay had the following compositions: ○, control MB; ●, 10% replacement of TC with TCDC; □, 20% replacement; ■, 30% replacement. Each data point represents the mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$ compared with control. Both hydrophobic bile salts promoted cholesterol nucleation dose-dependently.

supersaturated MB systems. Growth curves for each MB system are shown in Figures 1, 2 and 3. The CGR and FCM are compared in Tables 1 and 2.

Figure 1 shows the effect of replacing TC with hydrophobic bile salts. In Figure 1(A), curves show the effect of 10, 20 and 30% replacement of TC with TDC MB and control MB respectively. After 30% replacement of TC with TDC MB, there were significant increases of CGR and FCM compared with the control bile system (CGR: 6.973 ± 0.622 versus 4.406 ± 0.463 ; FCM: 0.316 ± 0.038 versus 0.182 ± 0.030) (Table 1). In contrast, 10% replacement of TC with TDC MB did not significantly change CGR or FCM. After 20% replacement, FCM was not significantly different from the control values, but CGR was increased. Figure 1(B) shows 10, 20 and 30% replacement of TC with TCDC MB. After 30% replacement there was a significant increase of CGR and FCM compared with the control (CGR: 7.871 ± 0.545 versus 4.406 ± 0.463 ; FCM: 0.285 ± 0.010 versus 0.182 ± 0.030). After 10 and 20% replacement, CGR and FCM were not significantly different from the control values.

Figure 2 shows the effect of replacing TC with hydrophilic bile salts. After 30% replacement of TC with any hydrophilic bile-salt, CGR and FCM were significantly decreased compared with control values (Table 2). After 10% and 20% replacement of TC with T β MC MB, there were no significant changes in CGR and FCM (Figure 2A). TUDC and TUC MB inhibited cholesterol crystallization dose-dependently (Figures 2B and 2C).

Figure 3 shows the relative potencies of bile salts in the CGR assay after 30% replacement. In the hydrophobic bile salts, the ranking order of the CGR values was TDC > TCDC > control (TC). All of the hydrophilic bile-salts (TUDC, TUC and T β MC) inhibited cholesterol precipitation and there were no significant differences among the hydrophilic bile-salt species.

Morphological changes of vesicles and cholesterol crystals

Vesicle formation and the morphological changes of cholesterol crystals were observed by video microscopy. Figure 4 shows the time course of cholesterol crystal formation in control (Figure 4A) and TDC bile systems (Figure 4B). Vesicular aggregates were observed immediately, the aggregates increased to a maximum 40 hours later, and then gradually decreased and disappeared completely after about 160 h. Accompanying the decrease of vesicles, intermediate forms appeared. Then mature plate-like crystals appeared and eventually became predominant. The key factor in the amount of crystallization was the appearance and increase of intermediate crystal forms, especially arches. Representative patterns of crystallization in control and 30% TDC systems are shown in Figures 5 and 6. In the control study, multilamellar vesicle formation and aggregation were rapid (Figure 5A, 24 h), filamentous crystals appeared (Figure

Table 1 Effect of hydrophobic bile salts on cholesterol crystal growth and vesicle aggregates in the CGR assay

CGR is the maximal growth rate (maximum slope of the experimental curve); $n = 4$. Statistical significance: * $P < 0.05$; ** $P < 0.01$ versus control.

	Control	TDC			TCDC		
		10%	20%	30%	10%	20%	30%
FCM	0.182 ± 0.030	0.168 ± 0.026	0.163 ± 0.022 $P = 0.0079$	$0.316 \pm 0.038^{**}$	0.158 ± 0.025	0.180 ± 0.071 $P = 0.0021$	$0.285 \pm 0.010^{**}$
CGR	4.406 ± 0.463	5.129 ± 0.557 $P = 0.0449$	$5.904 \pm 0.261^*$ $P = 0.012$	$6.973 \pm 0.622^*$	3.890 ± 0.182	5.108 ± 0.791 $P < 0.0001$	$7.871 \pm 0.545^{**}$

Figure 2 Relative inhibition of cholesterol crystallization by hydrophilic bile salts assessed with the CGR assay

(A) Effect of T β MC on cholesterol crystallization. The MBs used in the CGR assay had the following compositions: $\circ$, control MB; $\bullet$, 10% replacement of TC with T β MC; $\square$, 20% replacement; $\blacksquare$, 30% replacement. Each data point represents the mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$ compared with control. (B) Effect of TUDC on cholesterol crystallization. The MBs used in the CGR assay had the following compositions: $\circ$, control MB; $\bullet$, 10% replacement of TC with TUDC; $\square$, 20% replacement; $\blacksquare$, 30% replacement. Each data point represents the mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$ compared with control. (C) Effect of TUC on cholesterol crystallization. The MBs used in the CGR assay had the following compositions: $\circ$, control MB; $\bullet$, 10% replacement of TC with TUC; $\square$, 20% replacement; $\blacksquare$, 30% replacement. Each data point represents the mean value $\pm$ S.D. ($n = 4$). * $P < 0.05$ compared with control. All hydrophilic bile-salt species inhibited cholesterol crystallization dose-dependently.

5B, 70 h) and transformed into spiral shapes and arches (Figure 5C, 96 h). Finally, the classical monohydrate crystal became the predominant form in the MB solution (Figure 5D, 144 h). Although neither the morphology of the vesicles nor the formation of the crystals in these MBs was altered, the appearance time and the quantity of arch crystal were different. In 30% TDC MB, arch crystals were already appeared at 50 h (Figure 6B). In the hydrophilic-bile-salt systems, liquid crystals were observed throughout the study (Figure 7, 144 h). After 30%

Table 2 Effect of hydrophilic bile salts on cholesterol crystal growth and vesicle aggregates in the CGR assay

CGR is maximal growth rate (maximum slope of the experimental curve); $n = 4$. Statistical significance: $P < 0.05$; ** $P < 0.01$ versus control.

	Control	T β MC			TUDC			TUC		
		10%	20%	30%	10%	20%	30%	10%	20%	30%
FCM	0.182 $\pm$ 0.03	0.167 $\pm$ 0.066	0.132 $\pm$ 0.02	0.031 $\pm$ 0.019**	0.156 $\pm$ 0.065	0.066 $\pm$ 0.015**	0.015 $\pm$ 0.01**	0.147 $\pm$ 0.03*	0.058 $\pm$ 0.008**	0.018 $\pm$ 0.01**
				$P = 0.0045$		$P = 0.0054$	$P = 0.0057$	$P = 0.0167$	$P = 0.0057$	$P = 0.0015$
CGR	4.406 $\pm$ 0.463	4.412 $\pm$ 0.786	3.821 $\pm$ 0.499	0.542 $\pm$ 0.276**	4.414 $\pm$ 0.985	3.069 $\pm$ 0.698	0.254 $\pm$ 0.214*	4.267 $\pm$ 1.658	1.927 $\pm$ 0.433*	0.525 $\pm$ 0.330**
				$P = 0.0014$			$P = 0.005$		$P = 0.0344$	$P = 0.0061^*$

Figure 3 Relative effect on cholesterol crystallization of replacement with 30% of various bile salts assessed with the CGR assay

In the hydrophobic bile salts, the ranking order of the CGR values was TDC > TCDC > control (TC). All of the hydrophilic bile salts [TUDC, TUC and TbMC ('TbMC' on the Figure symbol key)] inhibited cholesterol precipitation, and there were no significant differences among the hydrophilic bile salts.

Figure 4 Time-course of the different cholesterol crystal forms in control and TDC (30%) MB systems

The semi-quantitative scale represents the number of structures per microscopic field as explained in the Materials and methods section. Observed values (not plotted) were manually smoothed: ·····, liquid particles; ----, filamentous structures; —, spirals and helices; —, typical cholesterol monohydrate plates. (A) control MB; (B) 30% TDC MB.

replacement with hydrophilic bile salts, the liquid crystals were stable, there were few needle-like or spiral crystals, and there were no monohydrate crystals.

Figure 5 Particles in control MB solutions

The Figure shows a representative pattern of crystallization in control MB observed by video microscopy ($\times 195$). (A) Earliest vesicle aggregation at 24 h; (B) filamentous crystals have appeared at 70 h; (C) arched crystals at 96 h; (D) monohydrate plate-like crystals at 144 h.

Figure 6 Particles in 30% TDC MB solutions

The Figure shows a representative pattern of crystallization in 30% TDC MB observed by video microscopy ($\times 192$). (A) Earliest vesicle aggregation at 24 h; (B) arched crystals have appeared at 50 h; (C) arched and plate-like crystals at 70 h; (D) monohydrate plate-like crystals at 144 h.

DISCUSSION

Cholesterol, a highly insoluble molecule, is transported in bile by specialized lipid aggregates. The number and type of cholesterol-containing aggregates in bile are still controversial. It has become generally accepted that biliary cholesterol is solubilized by simple and mixed micelles, single-bilayered (unilamellar) vesicles and, under certain conditions, multilamellar vesicles (liposomes or liquid crystals) all composed of bile salts, phospholipids and cholesterol in different proportions. Sömjen et al. have reported that structures named 'lamellae' represent the major cholesterol carriers in human bile [39]. Current concepts suggest that, in lithogenic biles, multilamellar vesicles result from aggregation and fusion of unilamellar vesicles and are a principal source from

Figure 7 Photograph of the stable liquid-crystal phase observed by video microscopy in hydrophilic bile-salt solutions at 144 h ($\times 250$)

which cholesterol precipitates to form gallstones. The cholesterol-solubilizing capacity of a bile is a critical function of the hydrophobic-hydrophilic balance of the principal bile salt [6]. The effect of bile-salt species on cholesterol nucleation is correlated with the micellization ability of each bile salt, and bile salts regulate cholesterol crystallization by the stabilization of vesicles. Crystallization of cholesterol is affected by the presence of various factors in bile, such as biliary proteins [7–12], various phospholipids [4,13–16] and various bile salts [17–21]. We recently compared the activity of these promoting factors to determine the relative potency in causing crystallization of cholesterol, and showed that replacement of bile salts was less effective than changing the phospholipid species and specific biliary protein [22]. These findings suggest that changes in the composition of bile salts must be large to promote cholesterol nucleation. Few investigations have compared the potencies of different bile salts. Oral administration of ursodeoxycholic acid (UDCA) leads to 20–30% replacement of the total bile-salt pool, retarding cholesterol nucleation [26]. This finding led us to use supersaturated MB solutions to clarify the effects of partial replacement with various bile-salt species (10–30%) and to rank them in order of activity.

First, we confirmed the effect of changes in the bile-salt composition on the CGR assay. Replacing 10 and 20% of TC with TDC did not significantly alter the CGR assay results (Figure 1 and Table 1), whereas a 30% replacement promoted crystallization. Replacement with TCDC showed the same effect as TDC (Figures 1 and 3). This indicated that, to cause a cholesterol pro-nucleating effect by changing the composition of bile-salts, large amounts of hydrophobic bile-salts, at least 30%, are required, and that the potency of these salts was correlated with their hydrophobicity. In a morphological study we found that the key factor in the amount of crystallization was the appearance and increase of intermediate crystal forms, especially arches (Figures 4–6).

The level of deoxycholic acid (DCA) in bile has been implicated in the development of cholesterol-rich gallstones [17,24]. Hussaini et al. [17] studied native bile and found that a relative increase in the percentage of DCA favoured the partitioning of cholesterol into vesicles and led to an increase in the vesicular C/P ratio and thus to a decrease in the cholesterol microcrystal nucleation time. van de Heijning et al. [20,21] studied isolated vesicles and reported that cholesterol crystallization from vesicles depended on the hydrophobicity of the bile salts added and apparently

occurred from fused or aggregated vesicles of extended magnitude and with a multilamellar constitution. These studies have shown a linear relationship between the percentage of biliary DCA and the molar percentage of cholesterol in bile and the biliary cholesterol saturation index. DCA directly and indirectly affects cholesterol nucleation [17]. The hydrophobic detergent effect of DCA on the canalicular membrane may lead to hypersecretion of cholesterol. If an increased percentage of biliary DCA induces an increase in the biliary cholesterol concentration that exceeds the capacity of the micelles to solubilize, this would increase in turn the molar concentration of cholesterol in the vesicles as well as the vesicular C/P ratio. DCA is also more hydrophobic than many other bile acids [17]. Thus, if micelles are rich in DCA, they will draw in lipids from the vesicles. Since bile acids solubilize relatively more phospholipid than cholesterol does, the preferential transfer of phospholipid from the vesicles to DCA-rich micelles will produce vesicles with a high cholesterol concentration and a high C/P ratio. The indirect action of DCA on cholesterol nucleation occurs via the excretion of mucin. The concentration of DCA in bile is correlated positively with both the molar percentage of cholesterol and the amount of arachidonic acid-rich PC in bile [15]. This finding suggests that the secretion of mucin and its release by the gall-bladder mucosa, which is presumably regulated by the prostanoid pathway, may be stimulated via arachidonic acid (a precursor of prostanoids), although we did not investigate this indirect action of DCA in this *in vitro* study.

We confirmed a dramatic alteration of the cholesterol crystallization process after partial replacement with hydrophobic bile salts. Although there was no change of cholesterol crystal habits, the key factor in the amount of crystallization was the appearance and increase of intermediate crystal forms, especially arches.

We have already reported that the nucleation time was significantly prolonged by UDCA therapy [26]. There are several possible explanations to account for the stabilization of bile by UDCA. First, biliary cholesterol in the metastable phase could be shifted to the stable phase by UDCA, which somehow shifts non-micellar cholesterol from the vesicles to the phospholipid lamellae along with a decrease in the C/P ratio, resulting in the stabilization of non-micellar cholesterol. In the present study we confirmed that partial replacement with hydrophilic bile-salt species (TUDC, TUC and T β MC) inhibited cholesterol precipitation through the formation of a stable liquid-crystal phase or stacked lamellae, and found similar inhibition with all three bile salts (Figures 2, 4 and 5, and Table 2). Secondly, it is also possible that UDCA enhances the activity of antinucleating factors, decreases the activity of pronucleating factors, or does both. Our previous observation that serum levels of apolipoprotein A-1 and A-2 were raised by UDCA administration supports this hypothesis [31]. Since the biliary apolipoproteins are transported from the plasma into the liver [40], an increase of the plasma level may be reflected by an increase in the bile. An increase of apolipoproteins A-1 and A-2 in bile as a result of UDCA treatment might play an important role in the stabilization of biliary lipid particles, resulting in prolongation of the nucleation time. Thirdly, UDCA decreased the biliary PC level of arachidonic acid, which is a substrate for prostaglandins, and this may have led to decreased mucin secretion and/or synthesis by the gall-bladder, since the prostanoid pathway is involved in these processes [41].

In conclusion, a marked change in the cholesterol crystallization process was evident after partial replacement with bile salts having a different hydrophobicity, and the acceleration of crystallization correlated with their micelle-forming capacity. Hydrophilic bile-salts (TUDC, TUC and T β MC) inhibited

cholesterol precipitation through the formation of a stable liquid-crystal phase, whereas hydrophobic bile salts (TDC and TCDC) shifted vesicular lipids into micelles, resulting in destabilization of the vesicles. Thus minimal changes in bile-salt composition may lead to marked alterations in bile lipid metastability.

This study was supported, in part, by the Ministry of Education of the Japanese Government (grant no. 10670477 awarded to S.T.).

REFERENCES

- Holzbach, R. T. (1990) *Hepatology* **12**, 155S–161S
- Harvey, P. R. C., Upadhyay, G. A. and Strasberg, S. M. (1991) *J. Biol. Chem.* **266**, 1–8
- Halpern, Z., Dudley, M. A., Kibe, A., Lynn, M. P., Breuer, A. C. and Holzbach, R. T. (1986) *Gastroenterology* **90**, 875–885
- Tazuma, S., Ochi, H., Teramen, K., Yamashita, Y., Horikawa, K., Miura, H., Hirano, N., Sasaki, M., Aihara, N., Natsushika, S. et al. (1994) *Biochim. Biophys. Acta* **1215**, 74–78
- Aldhal, N. H., Niu, N., Nunes, D. P., Bansil, R., Cao, X. X., Gantz, D., Small, D. M. and Offner, G. D. (1995) *Hepatology* **22**, 856–865
- Wang, D.Q.-H. and Carey, M. C. (1996) *J. Lipid Res.* **37**, 606–630
- de Bruijn, M. A. C., Goldhoorn, B. G., Zijlstra, A. I. M., Tygat, G. N. J. and Groen, A. K. (1995) *Biochem. J.* **305**, 93–96
- Yamashita, G., Ginanni, C. S., Secknus, R., Takabayashi, A., Williams, C., Hays, L., Chernosky, A. and Holzbach, R. T. (1995) *J. Lipid Res.* **36**, 1325–1333
- Offner, G. D., Gong, D. and Aldhal, N. H. (1994) *Gastroenterology* **106**, 755–762
- Abei, M., Nuutinen, H., Kawczak, P., Schwarzendrube, J., Pillay, S. P. and Holzbach, R. T. (1994) *Gastroenterology* **106**, 231–238
- Groen, A. K., Noordam, C., Drapers, J. A. G., Egbers, P., Jansen, P. L. M. and Tygat, G. N. J. (1990) *Hepatology* **11**, 525–533
- Groen, A. K. (1990) *Hepatology* **11**, 189S–194S
- Jüngst, D., Lang, T., Huber, P., Lange, V. and Paumgartner, G. (1993) *J. Lipid Res.* **34**, 1457–1464
- Ochi, H., Tazuma, S. and Kajiyama, G. (1996) *Biochem. J.* **318**, 139–144
- van Berge-Henegouwen, G. P., van der Werf, S. D. J. and Ruben, A. T. (1987) *Clinica. Chimica. Acta* **165**, 27–37
- Ringel, Y., Sömjén, G. J., Konikoff, F. M., Rosenberg, R., Michowitz, M. and Gilat, T. (1998) *J. Hepatol.* **28**, 1008–1014
- Hussaini, S. H., Pereira, S. P., Murphy, G. M. and Dowling, R. H. (1995) *Hepatology* **22**, 1735–1744
- Noshiro, H., Chijiwa, K., Makino, I., Nakano, K. and Hirota, I. (1995) *Gut* **36**, 121–125
- Juste, C., Catala, I., Henry, R., Chabanet, C., Gueugneau, A. M., Beguet, F., Lyan, B. and Corring, T. (1995) *Biochim. Biophys. Acta* **1254**, 89–97
- van de Heijning, B. J. M., Stolk, M. F. J., van Erpecum, K. J., Renooij, W., Groen, A. K. and van Berge-Henegouwen, G. P. (1994) *J. Lipid Res.* **35**, 1002–1011
- van de Heijning, B. J. M., Stolk, M. F. J., van Erpecum, K. J., Renooij, W. and van Berge-Henegouwen, G. P. (1994) *Biochim. Biophys. Acta* **1212**, 203–210
- Nishioka, T., Tazuma, S., Yamashita, G. and Kajiyama, G. (1998) *Biochem. J.* **332**, 343–350
- Marcus, S. N. and Heaton, K. W. (1988) *Gut* **29**, 522–533
- Berr, F., Mayer, M., Sackmann, M. F., Sauerbruch, T., Holl, J. and Paumgartner, G. (1994) *Gastroenterology* **106**, 215–224
- Corrigan, O. I., Su, C. C., Higuchi, W. I. and Hofman, A. F. (1980) *J. Pharm. Sci.* **69**, 869–871
- Tazuma, S., Sasaki, H., Mizuno, S., Sagawa, H., Hashiba, S., Horiuchi, I. and Kajiyama, G. (1989) *Gastroenterology* **97**, 173–178
- Singhal, A. K., Cohen, B. I., Finver-Sadowsky, J., McSherry, C. K. and Mosbach, E. H. (1984) *J. Lipid Res.* **25**, 564–570
- Dusserre, J. P., Montet, A. M. and Montet, J. C. (1988) *Can. J. Physiol. Pharmacol.* **66**, 1028–1034
- Montet, J. C., Parquet, M., Sacquet, E., Montet, A. M., Infante, R. and Amic, J. (1987) *Biochim. Biophys. Acta* **918**, 1–10
- Heuman, D. M. (1989) *J. Lipid Res.* **30**, 719–730
- Mizuno, S., Tazuma, S. and Kajiyama, G. (1993) *Dig. Dis. Sci.* **38**, 684–693
- Kibe, A., Holzbach, R. T., LaRusso, N. F. and Mao, S. J. T. (1984) *Science* **225**, 514–516
- Holan, K. R., Holzbach, R. T., Hermann, R. E., Cooperman, A. M. and Claffey, W. Y. (1979) *Gastroenterology* **77**, 611–617
- Donovan, J. M. and Jackson, A. A. (1993) *J. Lipid Res.* **34**, 1121–1129
- Busch, N., Tokumo, H. and Holzbach, R. T. (1990) *J. Lipid Res.* **31**, 1903–1909
- Sömjén, G. J., Ringel, Y., Konikoff, F. M., Rosenberg, R. and Gilat, T. (1997) *J. Lipid Res.* **38**, 165–169
- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Fiske, C. H. and Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- Gilat, T. and Sömjén, G. (1996) *Biochim. Biophys. Acta* **1286**, 95–115
- Sewell, R. B., Mao, S. T., Kawamoto, T. and LaRusso, N. F. (1983) *J. Lipid Res.* **42**, 391–401
- Lamorte, W. W., Booker, M. L., Scott, T. E. and Williams, L. F. (1985) *Surgery* **85**, 445–451

Received 2 July 1998/3 February 1999; accepted 11 March 1999