Bile salt-induced cholesterol crystal formation from model bile vesicles: a time course study[1]

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Abstract Precipitation of cholesterol crystals from vesicles is an important step in the pathogenesis of cholesterol gallstones. Little is known, however, about the kinetics and the mechanisms involved in cholesterol crystallization. Therefore, the time course of cholesterol crystal precipitation and lipid exchange between vesicles and micelles were monitored in a model bile system. Vesicles obtained from supersaturated model bile (cholesterol saturation index (CSI) 1.4; 10 g/dl) by KBr density gradient ultracentrifugation, were incubated with various bile salts: deoxycholate (DC), chenodeoxycholate (CDC), cholate (C), ursodeoxycholate (UDC), and their respective taurine and glycine conjugates. Vesicle integrity was assessed in a leakage-assay of carboxyfluorescein-loaded vesicles (0–15 min) and by the change in optical absorbance at 340 nm of a vesicle solution (0–50 min). Fluorescence increased within 1 min after addition of bile salt, and was stable within 5–10 min. After addition of bile salt, absorbance fell immediately and stabilized within 30 min. Fluorescence and absorbance were dependent on bile salt hydrophobicity and concentration. At several time points after addition of bile salt to vesicles (from 1 to 72 h), the extent of cholesterol nucleation was determined semiquantitatively and incubation mixtures were again subjected to ultracentrifugation to assess the lipid distribution among residual vesicles, de novo formed mixed micelles, and cholesterol crystals. Nucleation occurred within 0.5 h after exposure of vesicles to the hydrophobic bile salts DC or CDC, and the cholesterol/phospholipid (c/p) ratio of the vesicles showed a transient rise from 1.45 to 3–4 (at t = 0.5 h) that coincided with the appearance of mixed micelles. Then the vesicular c/p ratio decreased to 0.6–0.8 (at t = 24 h) concomitantly with increasing precipitation of cholesterol crystals. In the case of UDC, the most hydrophilic bile salt used, < 5% micellization, no nucleation, and a constant vesicular c/p ratio were observed. We conclude that under the conditions used in the present model study, the kinetics of cholesterol crystallization are governed by the hydrophobicity of the added bile salts and their capacity to form mixed micelles. The results emphasize the pivotal role of time, and the dynamic aspects of the processes involved in cholesterol crystal formation.—**van de Heijning, B. J. M., M. F. J. Stolk, K. J. van Erpecum, W. Renooij, A. K. Groen, and G. B. vanBerge-Henegouwen.** Bile salt-induced cholesterol crystal formation from model bile vesicles: a time course study. *J. Lipid Res.* 1994. **35:** 1002–1011.

Supplementary key words bile stasis • cholesterol crystallization • cholesterol/phospholipid ratio • hydrophobicity index • mixed micelles • model bile • nucleation • ultracentrifugation • vesicle dissolution

In bile, cholesterol is solubilized in vesicles, together with phospholipids, and in mixed micelles, together with phospholipids and bile salts. Bile salts are amphipathic molecules with a planar hydrophobic moiety enabling them to penetrate into lipid bilayers. Owing to these physico-chemical features bile salts are detergents and form micelles in aqueous solutions in concentrations above their critical micellar concentrations (CMC).

Mixed micelles contain 3–5 times more phospholipid than cholesterol. This is in contrast to vesicles in supersaturated model bile in which the cholesterol/phospholipid (c/p) ratio ranges from 1 to 1.5. Cholesterol and phospholipid can interchange between vesicles and micelles dependent on bile salt hydrophobicity and concentration (1–5).

As formation of mixed micelles requires more phospholipid than cholesterol, a larger proportion of phospholipid will be removed relative to cholesterol in the conversion of vesicles into mixed micelles (1, 2, 6–8). As a result the residual vesicles become progressively enriched with cholesterol and depleted in phospholipid, and their c/p ratio will steadily increase (1, 2, 6, 8–10). It has been shown that cholesterol-rich vesicles tend to aggregate and that subsequently cholesterol crystals may precipitate from these aggregates (8, 11–13).

Biliary cholesterol supersaturation, bile stasis, and the subsequent precipitation of cholesterol crystals are essential in cholesterol gallstone formation. As bile becomes

Abbreviations: C, cholate; DC, deoxycholate; UDC, ursodeoxycholate; CDC, chenodeoxycholate; T-, tauro-; G-, glyco-; c/p ratio, cholesterol/phospholipid ratio; CSI, cholesterol saturation index; CMC, critical micellar concentration; CF, carboxyfluorescein.

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concentrated in the gallbladder and the bile salt concentration increases, the formation of mixed micelles and cholesterol-rich vesicles is favored. The time necessary for these dynamic processes to occur may be provided by gallbladder bile stasis. Impaired gallbladder motility may therefore be considered as a pivotal adjunct to cholesterol gallstone formation.

In addition, we previously showed that the relative hydrophobicity of added bile salts has a major impact on the amount and type of cholesterol crystals precipitated from supersaturated model bile vesicles (14, 15). In these experiments only one time point (5 days) was studied, most likely representing the end-stage equilibrium of the system. At equilibrium either two (crystals and micelles) or three phases (crystals, micelles and vesicles) are present, depending on the bile salt species and concentration (2). Little is known however about the early stages and the time scale of the process leading to cholesterol crystal precipitation.

We therefore investigated the time course of effects of bile salts added to model bile vesicles, and monitored vesicle integrity, the interconversion of vesicles into mixed micelles, and cholesterol crystal precipitation. We hypothesized that due to mixed micelle formation of bile salt and vesicle constituents, the c/p ratio of the remaining vesicles increases, which makes them more inclined to aggregate and to precipitate cholesterol into crystals.

MATERIALS AND METHODS

The solubilization of vesicles, isolated from supersaturated model bile by ultracentrifugation, into mixed micelles was monitored at regular time intervals up to 72 h after addition of bile salt. Vesicle integrity and conversion into mixed micelles was investigated by a leakage assay of carboxyfluorescein-loaded vesicles (time range 0 to 15 min), by the change in optical absorbance of a vesicle suspension (time range 0 to 50 min), and by determining the lipid distribution among vesicles, micelles, and crystals (up to 72 h). Experiments were carried out in duplicate or triplicate; representative experiments are shown in the Results section.

Bile salts

The sodium salts of the following bile acids and their respective taurine- and/or glycine-conjugates were used: deoxycholate (DC), chenodeoxycholate (CDC), ursodeoxycholate (UDC), and cholate (C). Taurocholate (TC) was purchased from Fluka Chemie (Buchs, Switzerland), and UDC and tauro-UDC (TUDC) were kindly donated by Tramedico (Weesp, The Netherlands) and by Dr. Falk (Freiburg, Germany). All other bile salts were obtained from Sigma Chemical Co. (St. Louis, MO). All bile salts were of analytical grade (> 98%) as confirmed by either thin-layer chromatography (16; unconjugated bile salts) or by high pressure liquid chromatography (17; conjugated species).

Vesicle leakage assay

Vesicle integrity was investigated by a leakage assay of carboxyfluorescein-loaded cholesterol/phospholipid vesicles. A solution of 13 mM cholesterol (Sigma) and 13 mM egg yolk lecithin (Sigma) in chloroform was evaporated into a dry film. Next, a buffer of 100 mM NaCl and 50 mM HEPES (pH 7.5), and the fluorophore carboxyfluorescein (CF; Kodak, Rochester, NY; 3 ml of a 175 mM solution in HEPES buffer) were added and the mixture was gently shaken, put on ice, and subsequently sonicated for 30 min at 20 W under a stream of nitrogen in a Vibra cell sonicator (Sonics & Materials, Danbury, CT). In this way CF-loaded cholesterol/phospholipid vesicles were prepared (c/p ratio = 0.95). The vesicles were centrifuged, resuspended in HEPES buffer, and loaded onto a G25 column (Pharmacia-LKB, Uppsala, Sweden) with 50 mM HEPES as eluting buffer. The eluted fractions were diluted in HEPES buffer (1000-fold) and fluorescence was measured before and after addition of a 0.1% Triton X-100 (Sigma) solution in HEPES buffer. The CF-loaded vesicles were excited at 492 nm and fluorescence was detected at 516 nm. Measurements were performed in a Perkin-Elmer 3000 fluorometer (Gouda, The Netherlands). The eluted fraction that gave the maximal fluorescence response upon addition of Triton was used for the experiments. The experiments involved exposure of the 10-fold diluted vesicle fraction (in HEPES buffer) to bile salts. At various time points a sample was taken from this incubation mixture (vesicles + bile salts) and the fluorescence of the sample was determined upon a 100-fold dilution in HEPES buffer (final vesicle dilution 1000-fold). Sodium salts of DC, UDC, and TC in final concentrations ranging from 0.3 to 22.5 mM were tested on the capacity to rupture the CF-loaded vesicles. Mixtures of DC and UDC with a constant final concentration of 1.25 mM, but with a varying hydrophobicity were also tested. The composition of the mixtures was 100 + 0, 75 + 25, 50 + 50, 25 + 75, and 0 + 100% of DC and UDC, respectively. According to Heuman (18) this yielded a hydrophobicity index ranging from −0.31 (UDC alone) up to +0.72 (DC alone) depending on the composition of the bile salt mixture.

The fluorescence due to CF leakage after addition of bile salts was determined up to 15 min. Then Triton was added to determine maximal fluorescence. Fluorescence after addition of bile salts was expressed as percentage of maximal fluorescence.

Model bile vesicle preparation

Supersaturated model bile with a CSI of 1.4 and a total lipid concentration of 10 g/dl, was prepared according to Kibe et al. (9) with slight modifications and consisted of 17

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mM cholesterol, 35 mM egg yolk lecithin, and 132 mM TC dissolved in 20 mM Tris/HCl buffer (pH 8.2). Model bile was incubated for 24 h at 56°C and was passed through a 0.8-μm filter (Millex-GS, Millipore, Molsheim, France).

The filtered model bile (2 ml/tube) was subjected to KBr density gradient ultracentrifugation for 20 h at 170,000 *g* at 20°C using an SW 40 Ti rotor (Beckman, Palo Alto, CA) and an L8-80 ultracentrifuge (Beckman), to separate vesicles and micelles (19).

After centrifugation, the vesicle fractions were pooled and cholesterol (Monotest®, Boehringer, Mannheim, Germany), phospholipid (SopaChem Phospholipids® Sopar Biochem, Brussels, Belgium), and bile salt (20) concentrations were determined. Vesicles prepared and isolated in this way were rather uniform and were ± 70 nm in diameter as previously shown by freeze-fracture transmission electron microscopy (21). The pooled vesicle solution was pH 7-8.

Vesicle dissolution measurement

Aliquots of pooled model bile vesicles were incubated at 37°C with different bile salt species mentioned above at varying final concentrations (0, 10, 30, 50, and 100 mM) in polystyrene multiwell plates (Greiner Labortechnik, Germany). The disappearance of the vesicles (i.e., the clearing of the opaque vesicle suspension) was monitored photometrically. Absorbance at 340 nm was measured at 1-min intervals using a thermostat multiwell plate reader (SLT 340 ATTC, SLT-LabInstruments, Salzburg, Austria).

Pilot experiments showed that, compared to vesicles, micelles were too small to cause significant absorbance changes at this wavelength. Mixed micelle solutions isolated from supersaturated model bile by ultracentrifugation (see above) gave an absorbance of about 0.20. Bile salt solutions up to 100 mM showed negligible absorbance (0.02-0.04). Thus, the time-dependent decrease in absorbance at 340 nm actually reflected reduction in size or number of the vesicles or their conversion into mixed micelles, and could hence serve to follow the kinetics of the vesicle solubilization process (3, 7, 22).

Vesicle disappearance was followed up to 50 min from the time of bile salt addition. A dose-response curve for each bile salt was established at t = 50 min. From these curves an ED_{50} value was derived expressing the concentration of bile salt where the absorbance of the vesicle-bile salt sample was 50% of the initial value. For UDC and TUDC the ED_{50} value was obtained by extrapolation.

In another series of experiments, bile salt mixtures of DC and UDC with a constant total bile salt concentration of 30 mM but with a distinct hydrophobicity were added to vesicles. The composition of these mixtures was 0 + 30, 10 + 20, 15 + 15, 20 + 10, and 30 + 0 mM of UDC and DC, respectively, yielding a hydrophobicity index ranging from −0.31 (UDC alone) up to +0.72 (DC alone) depending on the composition of the bile salt mixture (18).

Assessment of lipid distribution and cholesterol crystal formation

In this set of experiments DC, CDC, TC, C, and UDC were added to the pooled model bile vesicles in a final concentration of 30 mM. As shown previously, this concentration of bile salt induced considerable nucleation (14, 15). After an incubation time at 37°C of 0.5, 1, 6, 12, 24, 48, and 72 h, aliquots of 500 μl of each vesicle-bile salt sample and a control sample (saline added) were again subjected to KBr density ultracentrifugation to assess the actual lipid phase distribution. This technique gave excellent separation and did not require the addition of bile salts (unlike gel filtration chromatography) that might introduce artifacts and confound the phase distribution, whereas KBr is relatively inert (19, 23). This separation technique, however, required a 20-h run-time, and the incubation times mentioned are, therefore, relative and do not include ultracentrifugation time.

After centrifugation each tube was fractionated in 1-ml aliquots and part of each fraction was filtered (0.22 μm, Millex-GV4, Millipore, Bedford, MA). Cholesterol, phospholipids, and bile salts were determined, as mentioned above, in the unfiltered and in the filtered aliquots to assess their distribution between precipitate, vesicles, and micelles. Because the cholesterol concentration, and not the phospholipid concentration, decreased upon filtration, we assumed that only cholesterol crystals (> 0.22 μm) were removed and no vesicles were retained by the filter.

In addition, in 10 μl of each of the incubated vesicle-bile salt samples, the amount of cholesterol crystals was scored at all time points mentioned above. Polarized light microscopy and a semi-quantitative scale were used: 1 + = 1 crystal per field (100 × magnification), 2 + = 2-5 crystals, 3+ = 6-20 crystals, 4+ = 21-100 crystals, and 5 + = > 100 crystals in the microscopic field.

RESULTS

Vesicle leakage

Addition of bile salts to CF-loaded cholesterol/phospholipid vesicles (3.42 mM cholesterol; 3.59 mM phospholipid) induced leakage of CF as shown by a rapid increase in fluorescence (**Fig. 1**). Fluorescence stabilized at 5-10 min after addition of bile salts. Fluorescence was higher when more hydrophobic bile salt species (e.g., DC) were applied (Fig. 1). In the case of DC, fluorescence was maximal (100% of total) within 15 min even at a dose of 1.25 mM, whereas much higher concentrations of TC and UDC were needed to cause maximal vesicle leakage (Figs. 1 and 2B).

The effects on fluorescence of mixtures of DC and UDC with a constant total bile salt concentration of 1.25 mM were found to be intermediate of either pure DC or UDC (**Fig. 2A**). Fluorescence after addition of the various

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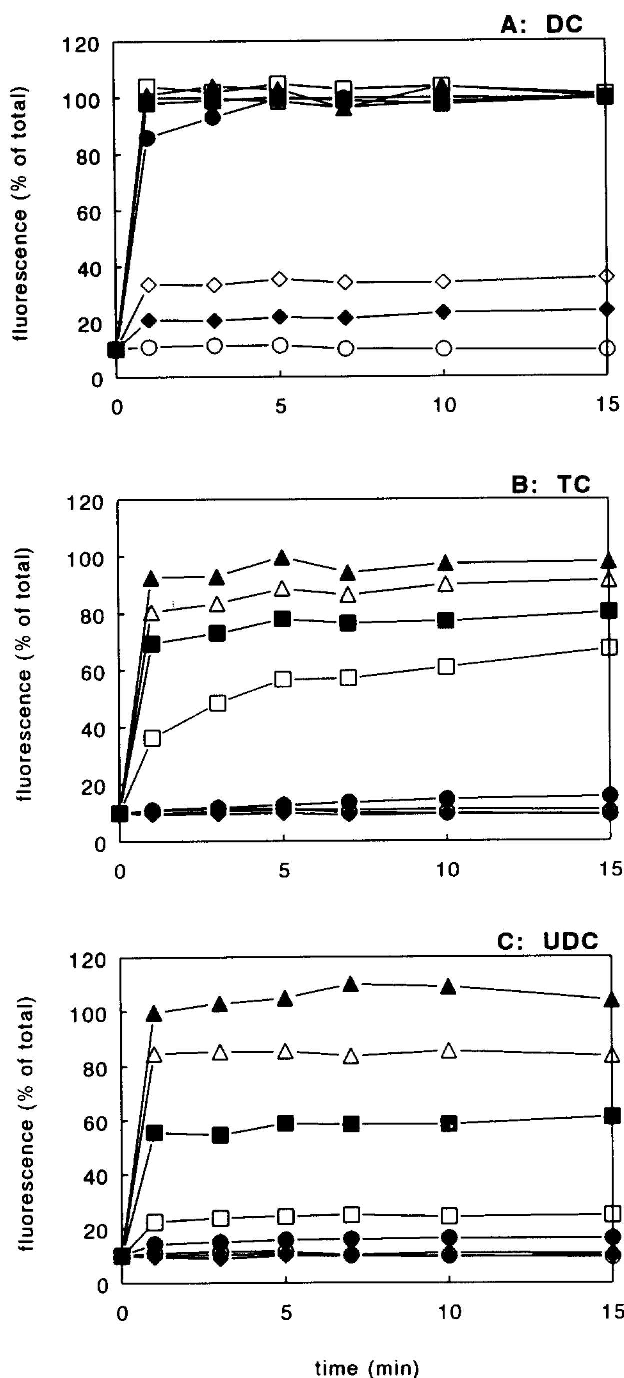

Fig. 1. The time course of fluorescence of CF-loaded vesicles after addition of increasing concentrations of A: deoxycholate (DC), B: taurocholate (TC), and C: ursodeoxycholate (UDC); (○) blank; (◆) 0.3; (◇) 0.6; (●) 1.25; (□) 2.5; (■) 5; (△) 10; (▲) 22.5 mM.

DC/UDC mixtures was related to the proportion of DC in the mixture, and hence to the hydrophobicity index of the bile salt mixture. Fig. 2B depicts the fluorescence at 15 min and clearly shows that fluorescence increased with the concentration of either bile salt and that fluorescence is related to the hydrophobicity of the bile salt used.

Vesicle dissolution

The opaque pooled vesicle suspension (4.42 ± 0.46 mM cholesterol; 3.01 ± 0.32 mM phospholipid; c/p ratio 1.48 ± 0.04 [n = 14]) isolated from model bile, cleared upon addition of bile salts. Clearing was studied quantitatively by determining the change in absorbance of a vesicle suspension in time at 340 nm. **Fig. 3** summarizes the results obtained after addition of DC, TC, and UDC. The clearing was instantaneous, dose- and bile salt species-dependent, and stabilized rapidly. From Fig. 3A it can be derived that addition of DC to vesicles up to a final concentration of 50 or 100 mM induces all vesicles to solubilize within 1 min. After addition of 10 mM DC, an initial increase preceded the decline in absorbance (Fig. 3A), as also observed with TDC and GDC at this concentration (results not shown). Conjugation of DC, C, and UDC, either by taurine or by glycine, had little or no effect on their vesicle dissolution capacity (results not shown).

Fig. 4 shows the effects of mixtures of DC and UDC on vesicle dissolution. The total bile salt concentration was

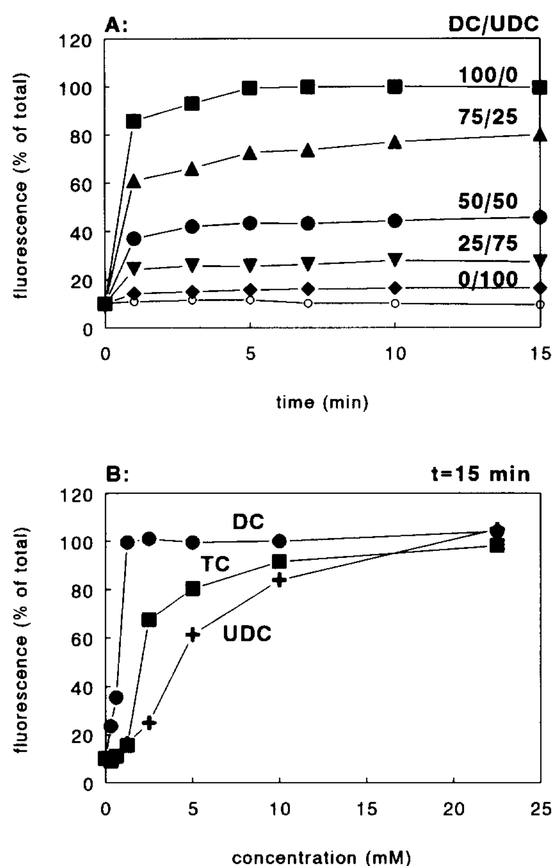

Fig. 2. A: The time course of fluorescence of CF-loaded vesicles after addition of DC/UDC mixtures with a total concentration of 1.25 mM. The percentile composition of each mixture is mentioned in the figure. B: Effect of addition of increasing concentrations of deoxycholate (DC), taurocholate (TC), and ursodeoxycholate (UDC) on the fluorescence of CF-loaded vesicles at t = *15 min.*

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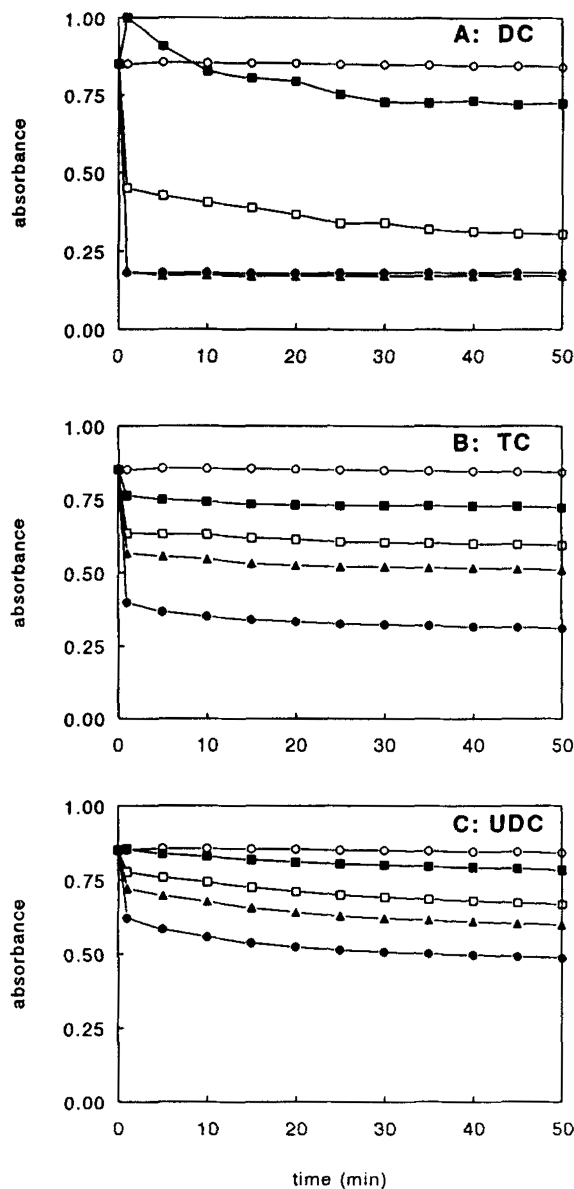

Fig. 3. The time course of the absorbance at 340 nm of a vesicle solution after addition of increasing concentrations of A: deoxycholate (DC), B: taurocholate (TC), and C: ursodeoxycholate (UDC); (○) blank; (■) 10; (□) 30; (▲) 50; (●) 100 mM.

kept constant at 30 mM, but the proportion of DC was varied. The absorption values at t = 50 min were clearly related to the proportion of DC in the mixture, and hence to the hydrophobicity of the bile salt mixture. Increasing the mol% of DC in the mixtures decreased absorbance progressively.

The dose-response curves (at t = 50 min) for the unconjugated bile salts UDC, C, CDC, and DC are shown in **Fig. 5A.** The ED_{50} value of each bile salt species is plotted against its hydrophobicity index. From Fig. 5B it is apparent that the ED_{50} values of the individual bile salts are strongly related to their hydrophobicity index.

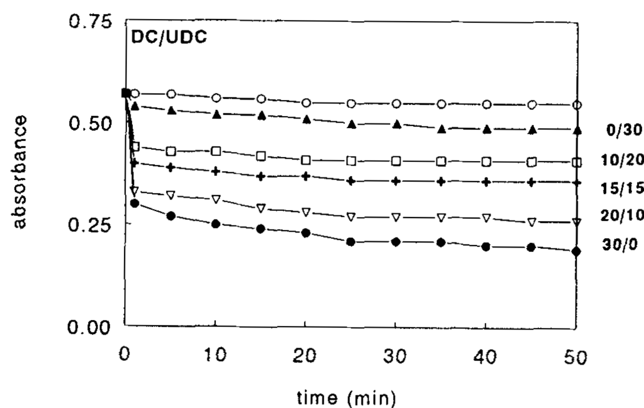

Fig. 4. Effect of DC/UDC mixtures with a total concentration of 30 mM on the absorbance at 340 nm of a vesicle solution. Hydrophobicity index in parentheses; (○) blank; (▲) 0/30 (−0.31); (□) 10/20 (0.03); (+) 15/15 (0.21); (▽) 20/10 (0.38); (●) 30/0 (0.72) mM DC/UDC.

Lipid distribution and cholesterol crystal formation

The phase distribution of cholesterol in the suspension which initially (at t = 0) consisted only of vesicles (5.92 ± 0.06 mM cholesterol; 4.08 ± 0.03 mM phos-

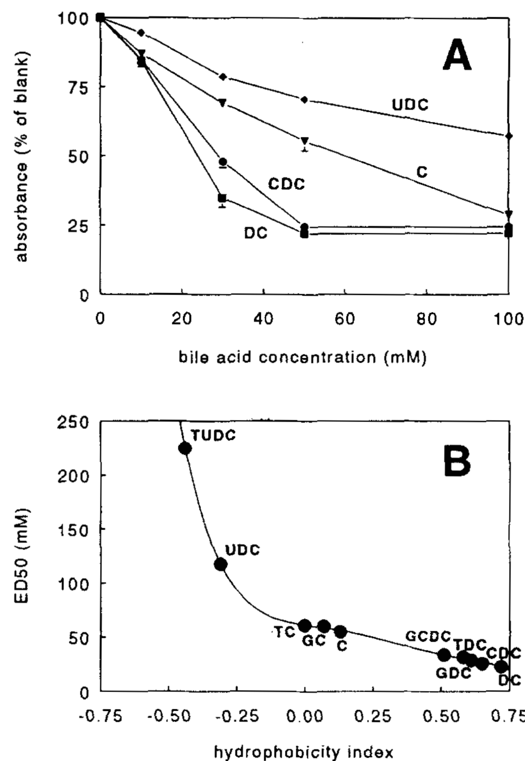

Fig. 5. A: Dose-response curves of the effect of bile salts on the change in the absorbance at 340 nm of a vesicle solution (mean ± SEM; n = 3). DC, deoxycholate; CDC, chenodeoxycholate; UDC, ursodeoxycholate; C, cholate. B: The ED_{50} value of individual bile salts for the change in absorbance at 340 nm of a vesicle solution versus their hydrophobicity index.

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pholipid; no detectable bile salts [< 0.3 mM]), was monitored for 72 h at regular time intervals after addition of bile salts (final concentration of 30 mM).

The cholesterol distribution between the vesicular, micellar, and crystalline phase is shown in **Fig. 6** for the samples incubated with DC, CDC, TC, or UDC. From Fig. 6 is it clear that the addition of the hydrophobic bile salt species DC and CDC to vesicles induced mixed micelle formation at 0.5 h after incubation. Also cholesterol crystals were observed as early as 30 min after addition of DC or CDC. In contrast, the hydrophilic species UDC induced almost no mixed micelles and no cholesterol precipitation. The effects of TC were intermediate to the aforementioned. TC induced some mixed micelle formation and cholesterol precipitation although much less and much later than DC and CDC did (Figs. 6 and 7).

The c/p ratio of the vesicles isolated from supersaturated model bile was 1.45 ± 0.03. At 0.5 h after incubation with 30 mM DC or CDC, a sharp increase of the vesicular c/p ratio up to 4 was observed (**Fig. 7**). Within 24 h, however, the c/p ratio gradually declined and stabilized at 0.6–0.8. The sharp initial increase of the vesicular c/p ratio coincided with the appearance of mixed micelles, whereas precipitation of cholesterol occurred concomitantly with the fall in the vesicular c/p ratio (Figs. 6 and 7). Only a minor increase of the vesicular c/p ratio was noted after 30 min incubation with TC (Fig. 7), and a small decline in this ratio was seen concomitant with cholesterol nucleation at 24 h after incubation. No apparent changes in vesicular c/p ratio were found upon addition of UDC to vesicles (Fig. 7). In all samples the c/p ratio of the de novo formed mixed micelles was 0.3–0.45.

The extent of cholesterol precipitation was also monitored semiquantitatively by microscopic examination of the samples (Fig. 7; hatched bars). Whereas DC and CDC induced abundant nucleation (> 100 crystals per microscopic field) at 6 and 12 h of incubation, respectively, TC caused microscopically detectable nucleation only after 12 h. In contrast, vesicles exposed to UDC never did precipitate cholesterol into crystals (Fig. 7).

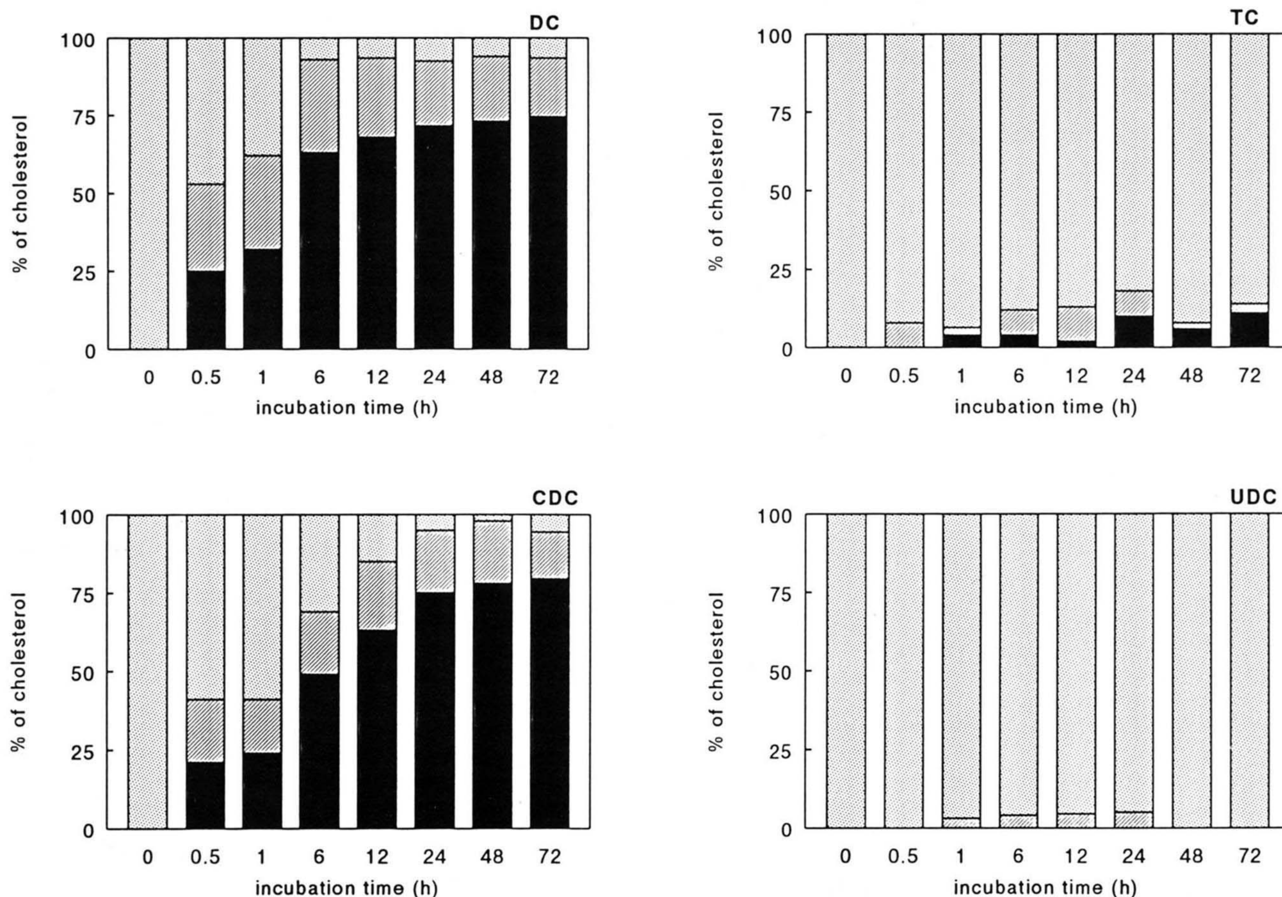

Fig. 6. Cholesterol distribution (% of total) between the vesicular, micellar, and crystalline phase after various incubation times of vesicles with 30 mM of either deoxycholate (DC), chenodeoxycholate (CDC), taurocholate (TC), or ursodeoxycholate (UDC). Solid bars: crystals; hatched bars: micelles; stippled bars: vesicles.

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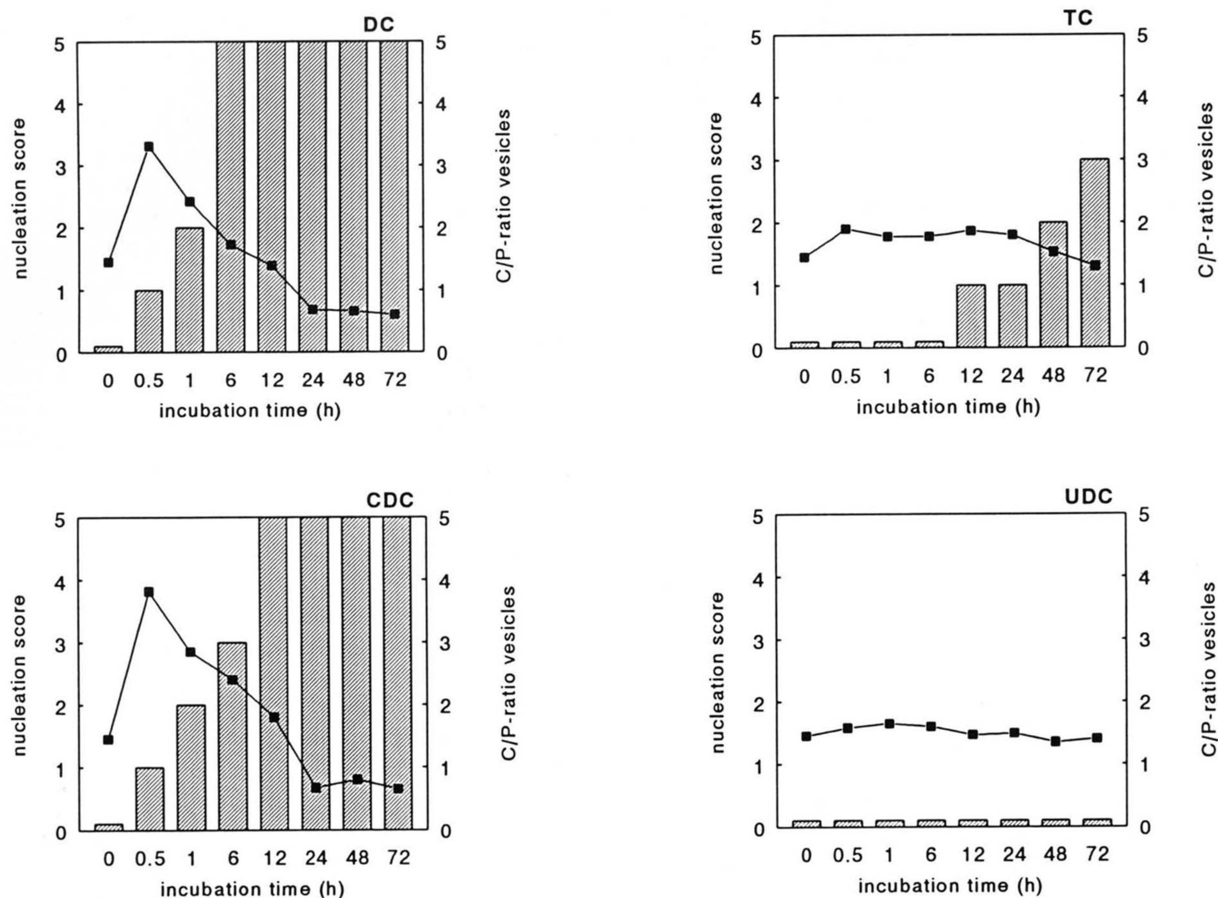

Fig. 7. Semiquantitative nucleation score (hatched bars, left ordinate) and the cholesterol/phospholipid (c/p) ratio of the remaining vesicles (-■-, right ordinate) at various incubation times of vesicles with 30 mM of either deoxycholate (DC), chenodeoxycholate (CDC), taurocholate (TC), or ursodeoxycholate (UDC).

DISCUSSION

Using an in vitro system of cholesterol-phospholipid vesicles to model the vesicular phase of gallbladder bile, we evaluated the time course of effects of progressive increments in bile salt concentration on the interconversion of vesicles into mixed micelles and the phase distribution of cholesterol.

Bile salts are detergents that form micelles when the bile salt concentration exceeds the CMC. Bile salts are thought to break down vesicles by solubilizing the lipid bilayer and to convert them into mixed micelles. The solubilizing capacity is strongly dependent on the relative hydrophobicity of the bile salt species, and hence on its CMC. This is clearly demonstrated by the effects of bile salts on increased fluorescence and decreased absorbance (Figs. 1 and 3). The capacity to dissolve vesicles is: DC > CDC > C > UDC, which is their rank on the hydrophobicity scale (Fig. 5B). The ranking order in membranolytic effects of the glyco- and tauro-conjugates is found to be similar, as might be expected because their hydrophobicity differs only slightly from their unconjugated congeners (Fig. 5B, (18)). The relation between lytic capacity and detergent hydrophobicity is also demonstrated by the effects of the DC/UDC mixtures (Figs. 2A and 4).

Many reports exist on the cytotoxic and detergent effects of bile salts on various cell types (24–28) or lecithin dispersions (22). In these studies it was also observed that the membrane-damaging effect strongly depends on the hydrophobicity of the bile salt (mixture).

The present study shows lytic effects of bile salts (Fig. 1A) and hence, presumably, formation of micelles at concentrations just below their individual CMC in pure water (29, 30). This is confirmed by previous reports (27, 31), and might be explained by the fact that sufficient amounts of counterions were available in the solution (100 mM NaCl and 50 mM HEPES) to decrease the CMC considerably (22, 30, 32). This explains the rapidity of vesicle solubilization and the effectiveness of the submicellar amounts of bile salts added. The low effectiveness of UDC in solubilizing vesicles is in accordance with its relative hydrophilicity, its low affinity for counterions, and its

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low affinity for lecithin (22, 33). As a result, the micellization capacity is poor and vesicle dissolution proceeds slowly.

The instantaneous increase in CF leakage (Fig. 1) shows that the vesicles are ruptured extremely fast by bile salts. However, the stable level of fluorescence within a few minutes indicates that the membrane-damaging effects of the bile salts are limited. As suggested by Schubert and co-workers (34, 35) the addition of bile salts to vesicles induces membrane disturbances, during which intramembrane pores are transiently formed and CF can be released from the intravesicular space. The formation and resealing of pores are probably related to bile salt species and concentration.

It remains unclear from the present study whether all vesicles are temporarily leaking CF or whether only a few vesicles are immediately ruptured while the others remain unaffected (22). Once a vesicle-mixed micelle equilibrium in the solution is reached, the vesicular bilayer might reseal or fuse with another vesicle, and CF leakage and vesicle dissolution will stop (34). In the case of 1.25 mM DC, the most hydrophobic species, fluorescence is maximal (100%) within 1 min (Fig. 1A). Instead, membrane damage caused by TC and UDC, or by 0.6 or 0.3 mM DC, apparently is restricted to a discrete amount of vesicles or to pores in vesicles that reseal before all CF is released. Dependent on the bile salt concentration, a discrete submaximal level of fluorescence is observed (Fig. 1).

Looking on a larger time scale (up to 50 min), bile salt-induced vesicle-to-micelle conversion was monitored photometrically. Pilot experiments showed that the decrease in absorbance probably reflected mixed micelle formation as a solution of mixed micelles gave a stable level of absorbance of 0.20 (cf. Fig. 3A: addition of 50 or 100 mM DC to vesicles). This enabled us to follow the formation of mixed micelles beyond the point of membrane rupture (within 1 min). In essence, the results were similar to those obtained with the vesicle leakage assay. Like fluorescence, absorbance also stabilized in time, indicating that vesicle dissolution is limited and dependent on the hydrophobicity and the concentration of the bile salt species applied (Figs. 3 and 5A). Probably, bile salts lose their lytic effects once the simple micelles are converted into mixed micelles by incorporation of phospholipids (and to a far lesser extent of cholesterol).

After addition of 10 mM of DC (or TDC or GDC; results not shown) to vesicles, an initial increase in absorbance was observed (Fig. 3). This might be attributed to initial fusion of vesicles into hexagonal rods, followed by dissolution into particles of micellar size (6, 7). Upon addition of higher concentrations of bile salts, dissolution into mixed micelles prevails. Rapid cleavage of vesicles into smaller membrane pieces (budding) causing a transient increase in the light-scattering activity has also been reported (25, 35).

Our results clearly show that lipids can interchange between vesicles and micelles as a function of bile salt concentration. As phospholipid is removed more easily from vesicular membranes than cholesterol, cholesterol-rich vesicles will arise (1, 6, 8). Cholesterol-rich vesicles aggregate and undergo phase transitions into large lipid aggregates with liquid crystalline properties from which solid cholesterol crystals may ultimately precipitate (8, 9, 11, 12).

The present study shows for the first time that the vesicular c/p ratio is only transiently increased after addition of bile salts. This might be attributed to the progressive and preferential solubilization of phospholipids into mixed micelles. When no micelle formation occurs, as in the case of UDC, the c/p ratio does not change (Fig. 7). Although we did not measure vesicle size and the transition into aggregates as shown by others (9, 11, 12), we monitored nucleation and determined the exact amount of cholesterol precipitated from the vesicle solution. As can be seen in Fig. 7, the increase of vesicular c/p ratio preceded nucleation. This is in accordance with the hypothesis that cholesterol-rich vesicles tend to remodel into large liquid crystalline aggregates from which cholesterol crystals precipitate (8, 9, 11, 12). Nucleation of cholesterol crystals caused the c/p ratio to decrease to a low, stable value (at t = 24 h), which indicates that the remnant vesicles discarded excess cholesterol and hence stabilized. These changes of c/p ratio reflect the dynamic nature of the processes involved in cholesterol crystal nucleation.

Several reports support our observations, although most papers only describe part of the processes involved. Harvey and co-workers (1) performed gel-filtration chromatography of gallbladder bile with increasing concentrations of sodium cholate in the eluting buffer. This resulted in increasing vesicular c/p ratios with decreasing nucleation times. This is in accordance with our observations of increasing c/p ratio and nucleation.

Studying the stability of cholesterol-rich codispersions of cholesterol and phosphatidylcholine, Collins and Phillips (36) observed a spontaneous decline to a low, stable c/p ratio concomitant with aggregation, liquid crystal formation, and cholesterol monohydrate crystal formation. This study confirms our observations of declining vesicular c/p ratio upon cholesterol precipitation. And more recently, Jüngst et al. (37) showed a decrease in vesicular cholesterol and c/p ratio to coincide with a prolongation of nucleation time upon addition of phospholipids but not bile salts to whole gallbladder bile.

In conclusion, the present in vitro study showed that the formation of mixed micelles by bile salts is an immediate process (< 1 min) resulting in vesicle disruption. Mixed micelle formation renders the remaining vesicles cholesterol-rich as shown by an increase of their c/p ratio. The increase of the vesicular c/p ratio is transient, and it

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decreases concomitantly with precipitation of cholesterol crystals. In this way the remaining vesicles discard excess cholesterol and stabilize at a c/p ratio below 1.

The present study shows that bile salt hydrophobicity plays a critical role in the rate of cholesterol crystal formation. Moreover, it emphasizes the dynamic nature of the nucleation process and the pivotal role of time in the formation of cholesterol crystals.

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