Mucins and calcium phosphate precipitates additively stimulate cholesterol crystallization

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Abstract **Human biliary mucin and calcium binding protein (CBP) influence formation of both calcium salt precipitates and cholesterol crystals and colocalize in the center of cholesterol gallstones. We investigated how physiological concentrations of these proteins regulate cholesterol crystallization in model biles, supersaturated with cholesterol and calcium salts, mimicking pathological human bile. Using polarizing light microscopy and nephelometry to assess cholesterol crystallization, the influence of calcium ions and calcium phosphate precipitates in the absence and presence of mucin, CBP, and human serum albumin was determined. Calcium phosphate precipitates stimulated cholesterol crystallization more strongly than soluble calcium. Mucin also stimulated, and with soluble calcium or calcium phosphate precipitates additively increased, the cholesterol crystal mass. In the absence of mucin, only human serum albumin plus CBP, not these proteins individually, decreased the stimulating effect of calcium phosphate precipitates but not of soluble calcium. However, seeding of calcium phosphate precipitates in biles with mucins resulted in near complete cholesterol crystallization within one day whether CBP and HSA were or were not also present. In conclusion, calcium salt precipitates plus human biliary mucins induce rapid and complete crystallization of cholesterol from model biles, little influenced by human biliary calcium binding proteins.—**van den Berg, A. A., J. D. van Buul, G. N. J. Tytgat, A. K. Groen, and J. D. Ostrow. **Mucins and calcium phosphate precipitates additively stimulate cholesterol crystallization. *J. Lipid Res.* 1998.** 39: **1744–1751.**

Supplementary key words cholesterol crystals • model bile • calcium phosphate • mucin • CBP

All cholesterol gallstones consist principally of cholesterol crystals. Virtually all cholesterol gallstones, however, also contain calcium salts (1–4) of "calcium sensitive anions" (5) derived from bile. Such salts are almost always present in the cores of stones, mainly as phosphates and bilirubinates. They are often also present in concentric rings in the periphery or in the shell of the stones, mainly as calcium carbonates and bilirubinates. Studies of stone microstructure by light microscopy and by immunolocalization of protein components (6) revealed that mucin forms the structural matrix of stones. The calcium salts in the core are deposited on this structural mucin matrix, apparently anchored by APF/CBP, a small amphipathic, acidic, biliary protein, the cholesterol binding protein (CBP) form of which binds calcium (7). Zones of cholesterol crystals in stones are bordered by mucin.

To explain this organized structure of gallstones, the biomineralization theory for gallstone formation was developed (6). It is hypothesized that small regulatory proteins, like CBP, when not bound to mucin inhibit calcium salt precipitation. After increase of biliary mucin or decrease in unbound CBP to the level at which all the regulatory protein is bound, calcium salts precipitate on the matrix of CBP bound to the mucin network. This complex is thought to be the stone nidus, and cholesterol crystals can subsequently bind to the unglycosylated hydrophobic domains of the mucins that cover the outer surface of the nidus (6).

Patients with and without cholesterol gallstones have similar degrees of supersaturation of bile with cholesterol, but the cholesterol characteristically crystallizes more rapidly from the biles of patients with stones (8). This indicates that the biles of patients contain additional factors that promote cholesterol nucleation and cholesterol crystal growth and/or that control biles contain factors that inhibit these processes. Calcium (9) and mucin (10) have been reported to act as potent stimulators of cholesterol crystallization from biliary vesicles. Calcium ions bind to bile salts, affect micellar and vesicular cholesterol content, and promote vesicle fusion (9), while mucin promotes vesicle aggregation and fusion (11, 12). In model systems containing calcium plus phosphate or carbonate but no lipids, bovine gallbladder mucin has been shown to affect precipitation of calcium salts depending on the amounts of CBP present (13).

Abbreviations: CBP, calcium binding protein; CCM, cholesterol crystal mass; CDT, crystal detection time; CSI, cholesterol saturation index; EYL, egg yolk lecithin; HSA, human serum albumin; TC, taurocholate; TDC, taurodeoxycholate; PIPES, piperazine-N,N′-bis[2-ethanesulfonic acid]; XOL, cholesterol.

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Despite the presence of precipitates from both calcium and cholesterol crystallization systems in biles and stones, the influences of regulatory components have not been systematically studied in complex model biles containing factors from both systems. Moreover, the effect of insoluble calcium salts on cholesterol crystallization has not been studied. Therefore we have now studied the influence of soluble and precipitated calcium salts, human biliary mucin, CBP, and human serum albumin on cholesterol crystallization from two model bile systems. In the first model system we investigated the influence of soluble calcium ions. In the second system, rapid seeding of the phosphate salt of calcium was induced as a model to study the effects of insoluble calcium salts (13). In both systems we studied the influence of CBP, and of human serum albumin (HSA) on the effects of calcium and calcium salt precipitates on cholesterol crystallization. These studies, using a system simulating physiological concentrations of biliary lipids and bile salts, were done in the presence and absence of human biliary mucin. Mucin, calcium, CBP, and HSA were all studied at their physiological concentrations in human gallbladder bile at the pH of 7.0, typical of human gallbladder bile. Furthermore, we tested the biomineralization hypothesis for gallstone formation by also determining whether microstone formation occurred within 3 months in model bile solutions containing cholesterol crystals, precipitated calcium phosphates, biliary calcium-binding proteins, and biliary mucin.

MATERIALS AND METHODS

Chemicals

Egg yolk lecithin (EYL), sodium taurocholate (TC), sodium taurodeoxycholate (TDC), cholesterol (XOL), piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES), and human serum albumin, essentially fatty acid free (HSA), were obtained from Sigma (St. Louis, MO). CsCl and Sepharose CL-4B were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). All regular chemicals, reagent grade, were obtained from Merck (Darmstadt, Germany). Unconjugated bilirubin (UCB) was purified from commercial UCB (Calbiochem, La Jolla, CA) according to the method of McDonagh and Assisi (14) and recrystallized twice from chloroform–methanol.

Isolation of mucin from human hepatic bile

Mucin was isolated from a pool of human hepatic biles collected by T-tube drainage using Sepharose CL-4B gel permeation chromatography and CsCl zonal ultracentrifugation as described by Pearson et al. (15). After extensive dialysis, lyophilization, and redissolution in demineralized water, the concentration of mucin in stock solutions was determined by measurements of fluorescence of cyano–acetamid-derivatized human mucin isolates using similarly derivatized bovine submaxillary gland mucin as a standard (16). Purity of the isolated mucins was assessed by SDS-polyacrylamide gel electrophoresis (17) with separate bands stained with alcian blue or the periodic acid-Schiff reagents. The mucin stocks used in model bile studies contained less than 10% breakdown products (<1000 kD) entering the 7% gel. The human hepatic bile mucins were the only mucins used in this study and shall be further referred to as mucin.

Isolation of calcium binding protein (CBP)

Calcium binding protein (CBP) was isolated from the same pool of human hepatic biles as the mucins, using the method described by Lafont et al. (18), but without the Laemmli SDS-PAGE purification. One-tenth volume of 0.1 m Tris-HCl, pH 8.0, was added to the pool of human hepatic biles. While stirring, small quantities of $CaCl_2$ were added to a final concentration of 0.25 m and after 4 days the mature $CaCO_3$ precipitates were centrifuged at 1000 *g* for 10 min. Pellets were washed with 0.25 m $CaCl_2$ in 0.01 m Tris-HCl and redissolved over 4 days in an alkaline solution of 0.5 m EDTA. Contaminating proteins of molecular mass over 30 kD were removed by serial ultrafiltrations through 100 kD and 30 kD cut-off membranes (Amicon, Danvers, MA). The retained material was washed with ten volumes of distilled water while concentrating via 3 kD ultrafiltration. The protein concentrate was subjected to Sephadex G-25 gel filtration with phosphate-buffered saline, pH 7.0, as eluant. Eluted fractions containing protein were collected and lyophilized. Based on the dry weight, the protein was dissolved to a concentration of 40 mg/mL in distilled water. One important functional characteristic of the CBP isolate, its ability to retard the formation of calcium phosphate precipitates from aqueous solutions of 10 mm HPO_4 plus 4 mm calcium at pH 6.8, was tested in the rapid seeding assay system (13). Purity and molecular weight of the isolated CBP was assessed by SDS-PAGE in the Schägger von Jagow system (19) with silver staining of protein bands using the Bio-Rad kit. The CBP used in the model bile studies showed the characteristic single broad band at apparent molecular mass of less than 10 kDa. The protein isolated this way is the calcium binding protein component, CBP, of the APF/CBP fraction of bile (18).

Preparation of model biles

Model biles were prepared according to the method of Kibe et al. (20) with slight modifications described by De Bruijn et al. (21) and were composed of TC/TDC (50/50), EYL and XOL (5:2:1 mole percentages), with a total lipid content of 10–12 g/dL in solutions containing 25 mm PIPES and 125 mm NaCl buffered to pH 7.0. This resulted in model biles with a cholesterol saturation index CSI of 1.6–1.8 according to the critical tables of Carey (22). The final concentrations of the bile lipids were measured by standard enzymatic methods. To maintain constant ionic strength, NaCl concentrations were decreased by 30 mm whenever 10 mm CaCl was added. All solutions used were filtered through a sterile MILLEX-GP 0.22 μm pore filter (Millipore, Bedford, MA) before use.

Incubation of biles and measurements of cholesterol crystallization

Bile incubations at 37°C, were performed in a 24-well cell culture cluster (Costar, Cambridge, MA) and were started by adding 1.5 or 2 ml of sterile-filtered warm model bile to a well in which the protein components had been placed previously to yield the specified final concentrations: mucins, 1 mg/mL; human serum albumin (HSA), 1 mg/mL; calcium binding protein CBP, 0.25 mg/mL; and $CaCl_2$, 10 mm. Calcium chloride was added last after preincubating all the other model bile constituents for 30 min. In the experiments studying the influence of soluble ionized calcium, calcium carbonate precipitation was prevented by both an argon atmosphere which excluded CO_2, as well as the neutral pH, minimizing formation of supersaturating carbonate anion concentrations.

The time to initial detection of cholesterol crystals (CDT) was established by inspection of 10-μl bile aliquots under polarizing light microscopy at 100 times magnification. Cholesterol crystal masses (CCM) formed in the biles were determined by double light-scattering measurements as described by De Bruijn et al.

(21), using a BN 100 nephelometer (Behring, Germany). To establish whether the same light scattering was obtained with various samples containing different crystal morphs, the mass was also measured colorimetrically in duplicate, after redissolution of cholesterol crystals in isopropanol at 37°C for 30 min, using cholesterol oxidase according to the method of Deeg and Ziegenhorn (Boehringer Mannheim) (23).

Statistics

Statistical analyses were performed using the Student's *t*-test. Data are expressed as mean ± SD of 3–5 separate experiments. *P* values <0.05 were judged as indicating significant differences.

RESULTS

Effects of soluble calcium ions and human hepatic bile mucin on cholesterol crystallization from model biles (Table 1 and Fig. 1)

In supersaturated model biles containing only biliary lipids, cholesterol crystals were first detected after 2 or 3 days. The average increase in the cholesterol crystal mass then accelerated between 5 and 9 days (from 50 μg/ml to 150 μg/ml per day) and subsequently declined to achieve a near constant plateau for the cholesterol crystal mass (CCM) of approximately 1 mg/mL after the twelfth day of incubation. Addition of 1 mg/mL mucin accelerated the crystallization of cholesterol, reducing the CDT to 2–8 h, enhancing the increases in CCM to 200 μg/ml per day between days 5 and 7 and also increasing the final CCM ($P < 0.05$), which was attained within 9 days. Addition of 10 mm calcium chloride also stimulated the crystallization, though less than did 1 mg/mL mucin; the CDT was shortened one day, the stable CCM attained at 7 days was greater ($P < 0.05$). Thereafter however, the CCM did not differ from model biles without calcium. Addition of both mucin and calcium shortened the CDT to 1 h and additively increased the CCM formed at all time points ($P < 0.01$). The increase in CCM between days 5 and 7 was even higher (250 μg/ml per day) compared to addition of mucin only and the plateau was achieved at 9 days. Thus, physiological concentrations of both mucin and calcium ions individually stimulated cholesterol crystal formation from supersaturated model biles, and together additively increased this crystallization.

Influence of human calcium-binding biliary proteins on calcium ion-enhanced crystallization

Addition of 0.25 mg/mL CBP, 1 mg/mL HSA, or both did not decrease the stimulating effects that 10 mm calcium chloride exerted on cholesterol crystallization. CBP and/or HSA added to model biles without calcium did not significantly stimulate or inhibit cholesterol crystallization (not shown).

Effects of calcium phosphate precipitates on the crystallization of cholesterol and the influences of biliary proteins

The CBP dose-dependently inhibited calcium phosphate precipitation in a supersaturated aqueous system (13), with complete inhibition for 30 min, at a concentration of 50 μg/ml.

In the system with 50 mM phosphates, we observed formation of precipitates immediately after addition of calcium chloride, in both the absence and presence of added proteins. In the model biles with calcium phosphate precipitates but no proteins, we observed an increase in cholesterol crystallization in comparison with model biles with only lipids ($P < 0.01$) (Table 1 and **Fig. 2**). This enhancement was greater than in model biles with the same concentration of total calcium but with no phosphates ($P < 0.05$) (Table 1 and cf. Figs. 1 and 2). The calcium phosphate precipitates did not artifactually contribute to the light scattering used to assess cholesterol crystal mass (**Fig. 3**).

Addition of either 1 mg/mL HSA or 0.25 mg/mL CBP alone did not alter the effects of calcium phosphate precipitates. The simultaneous addition of these proteins did not affect the initial CCM at day 2 but thereafter decreased the CCM ($P < 0.001$) to the level observed in the control model biles without calcium or phosphates. One mg/mL mucin more potently stimulated cholesterol crystallization in the presence of calcium phosphate salts com-

TABLE 1. The effects of calcium ions, calcium phosphate precipitates, and the biliary proteins mucin, CBP and HSA on the crystallization of cholesterol from model biles

	No Mucin						1 mg/mL Mucin[c]					
				$CaHPO_4$[d]						$CaHPO_4$		
	No Ca	10 mm Ca		CBP	HSA	Both	No Ca	10 mM Ca		CBP	HSA	Both
	mg/mL						*mg/mL*					
CCM 2[a]	0.01	0.2	0.2	0.6	0.5	0.3	0.5	1.1	3.0	2.6	3.2	2.4
CCM 14[b]	1.1	1.1	2.2	1.8	2.2	1.0[e]	1.8	2.7	3.7	3.5	3.6	3.4

Data are presented as the mean of three to five independent experiments.

[a] CCM 2 is the cholesterol crystal mass in mg/mL model bile formed within 2 days representing the initial crystal growth.

[b] CCM 14 is the cholesterol crystal mass in mg/mL model bile after 14 days of incubation, representing the stable phase. CBP and HSA were present at 0.25 mg/mL and 1 mg/mL respectively.

[c] $P < 0.05$ for mucin versus no mucin in the absence of calcium, with soluble calcium or calcium phosphate precipitates.

[d] $P < 0.05$ for calcium phosphate precipitates versus no calcium or soluble calcium.

[e] $P < 0.001$ for CBP plus HSA versus HSA or CBP or none.

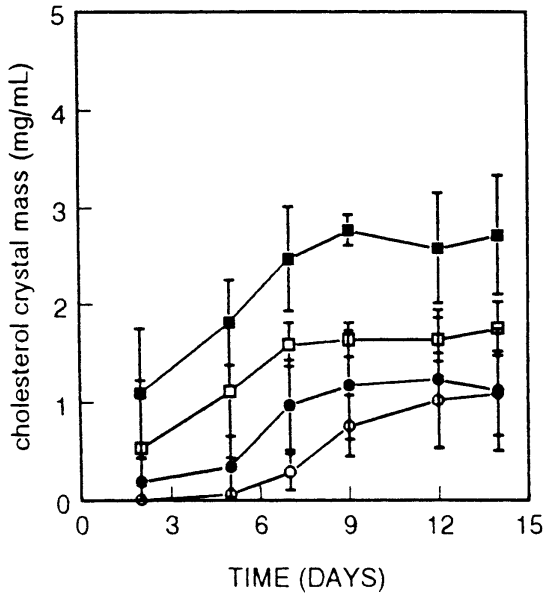

Fig. 1. Crystallization of cholesterol from supersaturated model bile solutions (circles) and model biles after addition of 10 mm $CaCl_2$ (solid circles), 1 mg/mL human hepatic bile mucins (open squares), or both (solid squares). Crystal masses were calculated from nephelometric measurements of bile samples according to the method of De Bruin et al. (21). Means of three independent experiments with standard deviations.

pared to the system without phosphates ($P < 0.01$) (compare Fig. 1 and **Fig. 4**). Simultaneous addition of both 0.25 mg/mL CBP and 1 mg/mL HSA along with the mucin did not alter the enhancing effect of mucin alone. Thus, mucin in model bile with calcium phosphate precipitates strongly stimulated cholesterol crystallization, irrespective of the presence of the biliary calcium binding proteins.

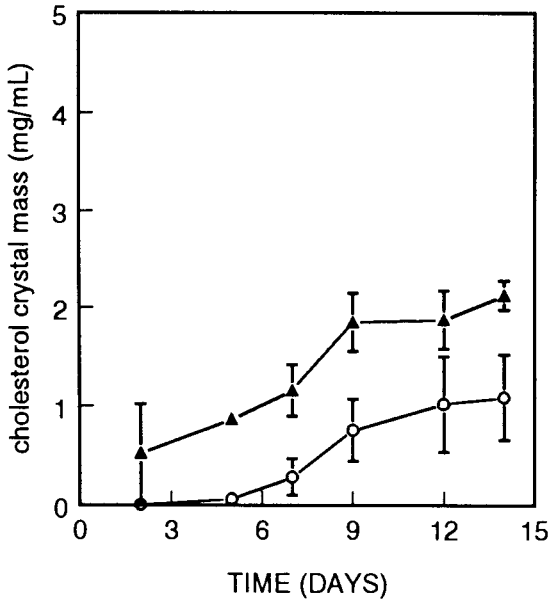

Fig. 2. Comparison of the formation of cholesterol crystals in supersaturated model bile solutions without (circles) or with (solid triangles) induced rapid seeding of calcium phosphate salts. Seeding was accomplished via injection of calcium to 10 mm final concentration in model bile buffered with 50 mm phosphate. Means of three independent experiments with standard deviations.

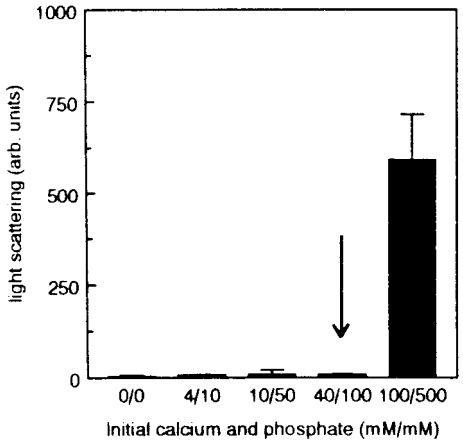

Fig. 3. The light-scattering by calcium phosphate precipitates from aqueous solutions. Model biles containing 50 mm phosphates plus 10 mm calcium salts give minimal light-scattering in the nephelometer (arrow); nb: model bile aliquots are diluted ten times before measurements. Thus, the maximal amounts of calcium phosphate salts present in the nephelometer during measurements of light scattering by cholesterol crystals are probably about 5 mm phosphate plus 1 mm calcium. Means of three duplicate experiments with standard deviations.

Experiments to generate micro-choleliths

In both model bile systems, larger particles or microliths, i.e., aggregates with a mean diameter over 1 mm, did not form even after prolonged (3 months) incubation of the model biles. Small stacks of cholesterol crystal plates were sometimes observed, but their presence was not confined to a certain type of model bile. Incubating the tubes in a rocking waterbath to mimic the mixing of bile that occurs in vivo due to gallbladder contraction likewise did not result in aggregation to form nidi or microliths. Addition of a range of concentrations (1 μm, 5 μm, 10 μm) of unconjugated bilirubin to the model biles containing calcium phosphate salts, 1 mg/mL mucin, 0.25 mg/mL CBP, 1 mg/mL HSA resulted neither in changes in the amounts of cholesterol crystals nor in the size of the aggregates. Restricting the amounts of CBP added to the model biles with calcium phosphate salts, to reduce the weight ratio of CBP to mucin from 1:4 to 1:1000 and create a molar excess of mucin, also did not result in stone formation.

Finally, calcium hydroxyapatite particles of approximately 0.5 mm diameter were tested as nidi. These were sequentially bathed in aqueous stock solutions of both 40 mg/mL CBP and/or 20 mg/mL mucins to generate active surface, prior to their addition to model biles containing calcium binding proteins, mucin, and calcium phosphates. This also did not result in the formation of cholesterol microliths.

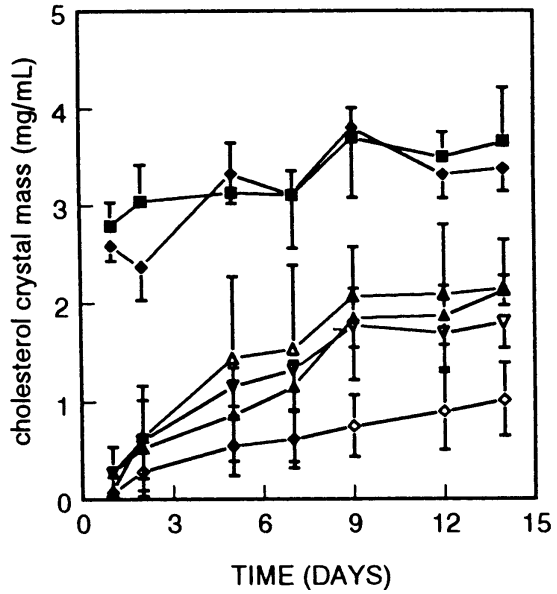

Fig. 4. Crystallization of cholesterol from supersaturated model bile solutions after rapid seeding of calcium phosphates (solid triangles). Effects of addition of 1 mg/mL human hepatic bile mucins (solid squares), 0.25 mg/mL CBP (open upright triangles), 1 mg/mL HSA (open downward triangles), 0.25 mg/mL CBP plus 1 mg/mL HSA (open diamonds), 0.25 mg/mL CBP plus 1 mg/mL HSA plus 1 mg/mL human hepatic bile mucins (solid diamonds). Crystal masses were calculated from nephelometric measurements of bile samples according to the method of De Bruin et al. (21). Means of three independent experiments with standard deviations.

DISCUSSION

Calcium ions and mucin additively stimulate the crystallization of cholesterol

Calcium ions and mucin have been regarded as potent promotors of cholesterol crystallization in human biles (9, 10). In prior studies with calcium, the stimulating effect was observed with addition of supraphysiological concentrations (75 mm) (24), or with more dilute model bile systems (2–8 g/dl total lipids) of low CSI (20). The physiological concentration of total calcium in gallbladder biles obtained from cholesterol gallstone patients ranges from 1.5 to 16 mM (25, 26). Our present studies show that 10 mm total calcium (with 9 mm bound to bile acids (cf. 27)) stimulates cholesterol crystallization in a model bile system with 10 g/dl total lipids, CSI 1.7. Gallinger et al. (28) questioned the relevance of calcium to cholesterol crystallization in vivo, as depletion of calcium from native human biles with EDTA did not change the CDT. In that study, however, cholesterol micro crystals, which are known to accelerate the CDT, were not first removed by filtration (21). Therefore, our findings of the stimulatory effects of calcium may well be relevant for human gallbladder biles.

Earlier studies of the stimulating effects of mucins were performed with non-human mucins (29) or high concentrations (2–8 mg/mL) of human mucins in model biles (10, 30). It was also shown that increases in mucin content of gallbladder biles preceded stone formation in laboratory animals on cholelithogenic diets (31). Increased production of mucins might contribute to cholesterol crystallization and cholesterol gallstone formation in humans (32). The physiological concentrations of soluble mucin in human gallbladder biles from patients and controls were reported in various studies to range from 0.1 to 5 mg/mL (15, 33–35). In our study, in both systems, addition of only 1 mg/mL human hepatic bile mucins produced similar additional increases in the CCM. Addition of 1 mg/ml mucin furthermore resulted in far shorter CDT, compared to prior studies, in which even higher concentrations of mucin were added. However, our model biles were of higher CSI with a high total lipid content. Other studies showed that increases in the total lipid concentrations and increasing the CSI resulted in higher cholesterol to phospholipid ratios in vesicles and in faster nucleation of cholesterol (36, 37). Apparently, pronucleating factors that act on vesicles, such as mucin and calcium, can further enhance crystal formation in such biles.

The mucins used in our present study were isolated from a pool of human hepatic biles. The relative potencies of mucins from lithic versus control hepatic biles in nucleation has not been established, although this might make a difference in their capacity to contribute to stone formation. However, previous studies have shown that mucins isolated from gallbladder biles of gallstone patients and controls did not differ in their enhancement of crystallization of cholesterol in patients biles (33, 38).

In our systems the effects of 1 mg/mL mucin and 10 mm calcium were additive. Recently, soluble calcium was reported to accelerate crystallization and also enhance the accelerating and crystal growth-promoting activities of a Con A-binding bile glycoprotein fraction (39). For mucin and calcium, partially overlapping mechanisms have been described, comprising stimulation of vesicle aggregation and fusion (12, 20, 40). Here we have observed that calcium accelerates the nucleation but did not affect amounts of crystals precipitated at the plateau phase (cf. 39) whereas mucin also increases the amounts of crystals at plateau. Nevertheless, because the stimulating effect of mucin depends on the hydrophobic domains of the molecule (11) and because calcium can increase the binding capacity of these hydrophobic domains (12, 41) the additivity may be based on the enhancement of the mucin effect by calcium, rather than on the sum of two separate direct influences on crystallization.

Compared to the model biles containing only lipids, addition of either mucin or mucin plus calcium led not only to acceleration of cholesterol crystallization but also to greater quantities of cholesterol crystals at the plateau. On a theoretical basis, the maximum quantity of cholesterol crystals that could be formed, however, i.e., when our model biles reach a CSI of 1.0, was 3.5–4 mg/mL. This is the maximum amount we observed in the biles containing calcium phosphate precipitates plus mucin. Thus, the biles with a lower crystal mass probably are in a metastable phase with high vesicular cholesterol content and

possibly also containing liquid crystals (undetectable by nephelometry).

Calcium phosphate precipitates enhance crystallization of cholesterol from model bile

As compared to the model biles with soluble calcium, crystallization of cholesterol was even more strikingly accelerated and increased in the model biles with calcium phosphate precipitates. The effect is probably not related to binding of bile salts, for it has been reported that glycine conjugated bile acids do, but taurine conjugated bile acids do not, bind to calcium phosphate precipitates (42). The effect of calcium phosphate precipitates in native human biles in which more than 70% of the bile salt pool is composed of glycine conjugates requires separate evaluation (43). The greater total mass of cholesterol crystals formed indicates that the calcium phosphate precipitates apparently also have an increased ability to destabilize biliary vesicles and/or promote crystal formation. The accelerating effect of calcium salt precipitates likely derives mainly from their effect as nidi catalyzing the initial formation of cholesterol crystals, as was originally hypothesized by Craven (44).

Only the combination of CBP and HSA decreases the effect of calcium phosphate precipitates

In the present study, in the absence of mucin, the combination of CBP and HSA reversed the increases in CCM produced by calcium salt precipitates, whereas either protein by itself did not. The mechanism of this effect of the combination of CBP and HSA remains unknown. CBP retards calcium phosphate salt precipitation from aqueous solutions supersaturated with calcium phosphate probably by adhering to the surface of newly formed submicroscopic crystals, thus inhibiting their further growth (13). HSA does not have this effect but can bind soluble calcium. Possibly the combination of HSA and CBP is able to decrease the concentration of calcium ions and/or retard the formation of calcium phosphate precipitates. In support, Fig. 4 suggests that in all model biles, except those with both HSA and CBP, the cholesterol crystal mass had reached a stable level within 14 days.

Mucin with calcium phosphate precipitates is the most potent combination stimulating cholesterol crystallization

Binding of CBP to mucin abrogates the inhibitory effects of the individual proteins on calcium salt precipitation from an aqueous solution (13); instead the combination actually stimulates calcium phosphate precipitation. Thus, the binding of CBP to the mucin probably explains why the combination of CBP and HSA could not inhibit calcium phosphate-induced increases in cholesterol crystallization in the presence of mucin. The mucin itself did not alter the effects of calcium phosphate precipitates. This is in agreement with the study of Qiu and coworkers (45) who observed a decrease in the size but not in the kinetics of calcium hydroxyapatite precipitation in the presence of 1 mg/mL human gallbladder mucin in vitro.

The stimulatory effect of the calcium salt precipitates towards the crystallization of cholesterol has not been described before. It has been hypothesized that, under certain circumstances, the ion products of calcium and phosphates exceed the solubility products of their salts, for example after increases in biliary inorganic phosphate due to hydrolysis of phospholipids by phospholipase C. This could explain the formation of calcium phosphate precipitates in bile and their presence in the core of cholesterol gallstones (5). Assuming the present findings apply also to native biles, calcium salts can be hypothesized to contribute to gallstone formation both as a constituent of the stone nidus and by enhancing formation of cholesterol crystals in bile. Interestingly, the pathological state of native biles preceding stone formation, called sludge, is composed of calcium salts, pigments, and cholesterol crystals in a matrix of mucin; this indicates that calcium salts might serve as promotors of cholesterol crystallization in vivo as well, especially in conjunction with mucin. Regarding the relatively brief residence time of bile in human gallbladder, it is of great interest that calcium phosphate precipitates and mucin together resulted in nearly complete crystallization of all cholesterol in a single day in our model bile system.

Stone formation in vitro

Based on the presence of calcium salts, pigments, mucin, and cholesterol crystals (35) and possibly of CBP (46) in sludge and in the centers of cholesterol gallstones (1, 6), it has been hypothesized that calcium salts precipitate on a template formed by CBP bound to mucin, thus forming a matrix for subsequent cholesterol crystal adherance (6). As described earlier, pigmented centers are present in a large proportion of human cholesterol gallstones. Moreover, it was recently reported that deconjugation of bilirubin resulted in enhanced precipitation from native human biles of solids containing cholesterol crystals and mucin (47). We hypothesized that addition of unconjugated bilirubin to our model biles might result in the formation of stone nidi, i.e., larger aggregates of calcium salts including bilirubinates, calcium binding proteins, and mucin, to which cholesterol crystals could adhere. However, our study showed that none of the combinations of stone core components used in our model biles resulted in aggregation to form a stone nidus, suggesting that other, minor stone core constituents might be needed to accomplish aggregation. Alternatively, the external influences of the gallbladder, such as contraction, or cyclic changes in pH or bile lipid concentrations and composition, may be required for the compaction of core components into a stone. A more fruitful approach to defining the factors that contribute to stone formation might be to use small gallstones isolated from human gallbladders and investigate the accretion of various bile constituents to such nidi. Such studies, focussing on growth rather than on nucleation, nidation, and aggregation, are in progress in our laboratory.

We conclude that in complex model biles, supersaturated with calcium salts and cholesterol, the formation of

calcium salt precipitates in the presence of human biliary mucins leads to very rapid and complete crystallization of cholesterol, little modified by the human biliary calcium binding proteins, CBP and albumin.

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