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Deconjugation of bilirubin accelerates coprecipitation of cholesterol, fatty acids, and mucin in human bile—In vitro study

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Abstract: To examine the initial step of brown pigment gallstone formation, sterile human gallbladder bile samples were incubated with or without β-glucuronidase in vitro. Enhanced bilirubin deconjugation achieved by adding β-glucuronidase significantly accelerated the formation of a precipitate that contained bilirubin (28.2 ± 3.8% of dry weight), cholesterol (14.3 ± 5.2%), free fatty acids (12.0 ± 1.3%), and glycoprotein (10.0 ± 6.7%). Both the composition and scanning electron microscopic appearance of the precipitate were similar to these features in brown pigment gallstones. The cholesterol saturation index and nucleation time in the supernatant did not change with various incubation periods. The weight ratios of bilirubin to cholesterol in the precipitates correlated with those in bile ($r = 0.76$; $P = 0.017$). Gel chromatography of the precipitate showed high molecular weight glycoprotein to be the major constituent. Bilirubin, cholesterol, fatty acids, and mucin were found to coprecipitate in accordance with bilirubin deconjugation, which process may play an important role in an early stage of the formation of brown pigment gallstones.

Key words: Brown pigment gallstone, calcium bilirubinate, bilirubin, cholesterol, free fatty acids, β-glucuronidase

Introduction

Brown pigment (calcium bilirubinate) gallstones are composed mainly of calcium salts of bilirubin, fatty acids, and cholesterol.[1,2] Bacterial infection and bile stasis have been considered to be the most important pathogenetic factors in the formation of this type of gallstone.[3,4] Maki[5] proposed that β-glucuronidase released from bacteria hydrolyzes bilirubin glucuronides into unconjugated bilirubin, leading to the sedimentation of calcium bilirubinate in the initial stage. Junipor et al.[6] observed calcium bilirubinate granules in the duodenal bile of patients with brown pigment gallstones.

The chemical analysis of brown pigment stones has revealed that these stones contain considerable amounts of bilirubin, mucin, and lipids, composed of cholesterol and free fatty acids. Gallbladder bile of patients with brown pigment stones is usually unsaturated with cholesterol and the cholesterol nucleation time is not significantly different from that of control subjects.[7] In infected bile, the concentration of biliary free fatty acids is usually very low, allowing crystallization of their calcium salts.[8] There is little information about the coprecipitation of biliary lipids and mucin with bilirubin in the process of brown pigment gallstone formation. We hypothesized that cholesterol, free fatty acids, and mucin coprecipitate together with unconjugated bilirubin. In the present study, we analyzed the chemical composition of the precipitate and supernatant of bile incubated with or without β-glucuronidase in vitro to examine whether deconjugated bilirubin coprecipitates with cholesterol and free fatty acids. The formed precipitate was compared with brown pigment gallstones by electron microscopy and the presence of glycoproteins in the precipitate was examined by gel chromatography to confirm the participation of biliary glycoprotein.

Materials and methods

Materials

All solvents used were of analytical grade or were distilled prior to use. Palmitic, heptadecanoic, oleic, li-

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noleic, and arachidonic acids; mucin; and β-glucuronidase (from *Escherichia coli*, optimal pH, 6.8; highly purified lyophilized powder, 9300 U/mg solid, Lot No, 92H 6825) were purchased from Sigma (St. Louis, MO, USA). Stearic acid was obtained from Wako Junyaku (Osaka, Japan). Blue Dextran 2000 was from Pharmacia (Uppsala, Sweden). Human gallbladder bile was obtained by needle aspiration at the time of surgery from six patients with gastric cancer without hepatobiliary disease. These patients underwent cholecystectomy to dissect lymph nodes in the hepatoduodenal ligament, and bacterial cultures of all bile samples were negative. The protocol was approved before the study by the Senior Staff Meeting at our institute and informed consent was obtained from all patients.

In vitro incubation of bile with or without β-glucuronidase

The gallbladder bile samples, kept at 37°C, were immediately ultracentrifuged at 100000 g for 2 h at 37°C to obtain the isotropic bile. After the addition of 0.1 ml β-glucuronidase solution (4000 U/0.1 ml 50 mmol/l Tris HCl buffer, pH 7.30) or buffered solution without enzyme (control) to 3-ml aliquots of isotropic bile samples, incubation was carried out at 37°C under nitrogen, without shaking. The pH of the bile sample was 7.3 ± 0.3. After the addition of a pH 7.3-buffered solution with or without β-glucuronidase, the pH was essentially constant, ranging from pH 7.4 to 7.6, throughout the experiment. After various incubation periods (1.5, 3, 6, 9, and 12 h) the bile samples were centrifuged (1000 g, 5 min, 37°C) and a brown precipitate and the supernatant were obtained. The precipitate was washed with deionized water and dried in a desiccator for 3 days.

Analysis of supernatant

An aliquot of bile was used for measurement of the nucleation time.[9] Total cholesterol was measured by gas-liquid chromatography (GLC), as described previously.[10] Total bile acids were determined enzymatically[11] and phospholipid concentration was quantified by the method of Bartlett.[12] The cholesterol saturation index was calculated, using a computer program[13] based on Carey's critical tables.[14] Free fatty acids were analyzed as described previously.[8,15] Briefly, after 0.2 ml of bile was added to 2 ml Dole's reagent (isopropanol:heptane, 0.5 mol/l H_2SO_4 [40:10:1, v/v/v]) containing 50 μg of heptadecanoic acid as an internal standard, free fatty acids were extracted three times. The combined organic layer was evaporated to dryness under a stream of nitrogen, dissolved in 0.2 ml of chloroform, and then applied to a silica gel 60 column (500 mg). The eluate with 9 ml of diethylether was evaporated to dryness under reduced pressure and methylated with 1.0 ml of 50 g/l methanolic hydrogen chloride. Free fatty acid methyl ester was analyzed by gas-liquid chromatography (GLC) (GC-6AM; Shimadzu, Kyoto, Japan); the apparatus was equipped with a flame ionization detector, a van den Berg solventless injector, and a data processing system (Chromatopac C-R3A; Shimadzu). A fused-silica capillary column (15 m × 0.2 mm ID) coated with a non-polar stationary phase (HiCap CBP1; Shimadzu) was used. The column temperature was programmed to change from 185°C to 280°C at the rate of 5°C per min, and the temperature of the injection port was maintained at 305°C. Total bilirubin was determined by the method of Michaelsson[16] and analysis of subcomponents was performed by high-performance liquid chromatography (HPLC).[17] Ionized calcium was measured with a calcium ion meter (CAL-101; Shimadzu).

Analysis of precipitate

The precipitate was dissolved in dimethyl sulfoxide-acetone-1 mol/l HCl (90:9:1, v/v/v), followed by ultrasonification and centrifugation. The insoluble residue was not analyzed further. The supernatant obtained was used for the following determinations. Cholesterol and free fatty acids were analyzed as described above. Bilirubin content was determined by the sulfanilic acid diazo-reaction, according to Malloy and Evelyn.[18] The precipitate was dissolved in 5 ml of 12.5 mmol/l ethylene diammine tetraacetic acid (EDTA) in 0.1 mol/l NaOH, with shaking, followed by exposure to long-wave UV light for 24 h. The concentration of glycoprotein in the solution was measured according to the method of LaMont et al.,[19] using porcine gastric mucin as a standard.

Gel chromatography

Gel chromatography of the precipitate was performed, by the method of LaMont et al.,[19] under dim light at 4°C. The precipitate was suspended in 20 ml of 12.5 mmol/l EDTA in 0.1 mol/l NaOH and stirred at 20°C for 5 min. The extract was then titrated to pH 8.0 with 0.1 mol/l HCl and centrifuged at 500 g for 10 min. The sediment was reextracted by the same procedure and the combined supernatants were dialyzed for 24 h against deionized water. The concentrated solution was subjected to gel chromatography (Sepharose 4B-Cl 0.8 × 65 cm), saturated with 0.2 mol/l NaCl, 0.01 mol/l Tris-HCl buffer and 0.4 g/l sodium azide pH 9.0, and eluted with 12.5 mmol/l EDTA in 0.1 mol/l NaOH. Fractions were collected every 10 min. Pigment and protein were monitored by spectrophotometer at wavelengths of 420 and

280 nm, respectively. and glycoprotein was determined by a periodic acid-Schiff (PAS) method.[20]

Electron microscopy of precipitate

Scanning electron microscopy (Hitachi S-430; Hitachi, Tokyo, Japan) was employed for observation of the precipitate and of brown pigment gallstones collected from humans.

Statistical analysis

Values are expressed as means ± SEM. Two-factor analysis of variance (ANOVA) was used for statistical comparisons. Probability values less than 0.05 were considered statistically significant.

Results

Analysis of supernatant

The concentrations of cholesterol, bile acids, phospholipids, and fatty acids in the supernatant did not change significantly during the incubation of the bile samples with or without β-glucuronidase (Table 1). The cholesterol saturation index (Fig. 1) and nucleation time (1 day; $n = 1$, no nucleation for 21 days; $n = 5$) of the supernatants were essentially constant throughout the 6 h incubation. Changes in total bilirubin and ionized calcium concentrations in the supernatant during the incubation period are shown in Table 1 and Fig. 2. Values for both decreased during the incubation period, even in the absence of β-glucuronidase. The ratio of unconjugated bilirubin to total bilirubin when the sample was incubated with β-glucuronidase increased rapidly up to 3 h of incubation, then reaching a plateau (Fig. 3). Without the enzyme, the ratio increased more slowly, reaching a value similar to that for the 3-h value with the enzyme at 12 h.

Analysis of precipitate

The light microscopic appearance of the precipitate is shown in Fig. 4. Cholesterol monohydrate crystals were not evident in the field. The dry weights of the precipitates, and total amounts of bilirubin, cholesterol, and free fatty acids in the precipitates obtained from the bile incubated with or without β-glucuronidase are shown in Fig. 5. The weight of the precipitate increased slowly until 3 h, and thereafter increased rapidly. β-Glucuronidase significantly accelerated precipitate formation. The precipitates ($n = 6$) at 6-h incubation with β-glucuronidase contained 28.2 ± 3.8% bilirubin, 14.3 ± 5.2% cholesterol, 12.0 ± 1.3% free fatty acids, and 10.0 ± 6.7% glycoprotein. The proportions of these constituents were essentially similar to the proportions in the pellets formed in the absence of β-glucuronidase. The weight ratios of bilirubin to cholesterol in the precipitates correlated significantly with those in the supernatants ($r = 0.76$; $P = 0.02$, $n = 8$). There was no significant difference between the weight ratios of bilirubin to cholesterol in the precipitates obtained from the bile samples incubated with β-glucuronidase and

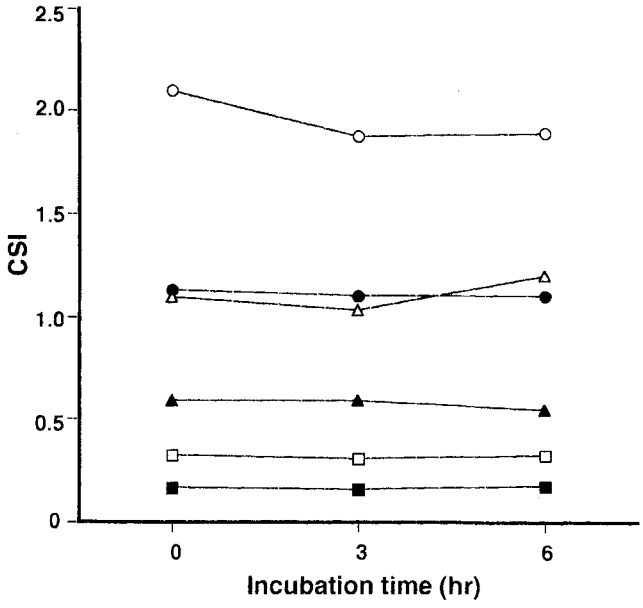

Fig. 1. Changes in cholesterol saturation index (*CSI*) during the incubation period. The CSI of the supernatant was essentially constant throughout the incubation period for each bile sample

Table 1. Sequential changes in pH, bilirubin, bile acids, cholesterol, lecithin, and free fatty acids in supernatant

Incubation time	Solution	pH	Bilirubin (mg/ml)	Bile acid (mM)	Cholesterol (mM)	Lecithin (mM)	FFA (mg/ml)
0 h	With β-g	7.47 ± 0.14	3.27 ± 0.44	105.97 ± 34.14	17.02 ± 6.54	40.75 ± 7.02	0.72 ± 0.37
3 h	With β-g	7.50 ± 0.14	3.03 ± 0.41	103.79 ± 33.52	15.77 ± 6.25	41.70 ± 7.30	0.94 ± 0.55
6 h	With β-g	7.57 ± 0.12	2.70 ± 0.35	106.03 ± 34.38	15.51 ± 6.05	40.04 ± 7.14	0.99 ± 0.37
	Control	7.50 ± 0.14	3.0 ± 0.42	105.36 ± 54.29	15.11 ± 5.25	41.12 ± 7.42	0.72 ± 0.27

Values are expressed as means ± SEM ($n = 6$)
β-g; β-glucuronidase, FFA; free fatty acid

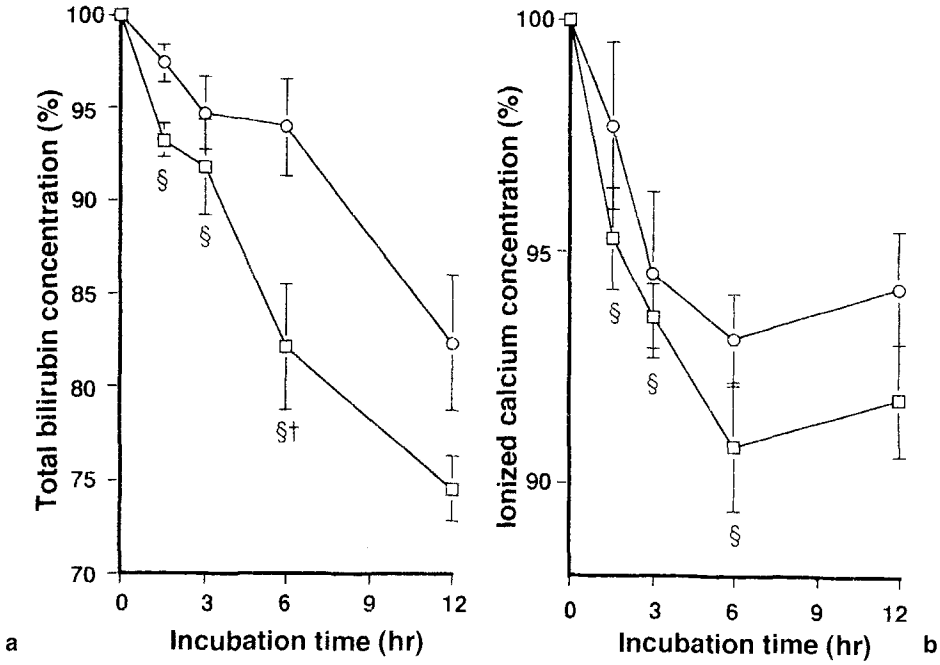

Fig. 2a,b. Changes in **a** total bilirubin and **b** ionized calcium concentrations of bile samples incubated with β-glucuronidase (*squares*) or without the enzyme (control; *circles*). Significant decreases ($P < 0.05$) in total bilirubin levels were seen in the supernatants of bile incubated with β-glucuronidase, compared with the control, at 6h. Values are percentages of the initial concentrations of bilirubin and ionized calcium in the bile supernatant and are expressed as means ± SEM ($n = 6$).[§] Compared with 0h;[<+>] compared with control. ($P < 0.05$)

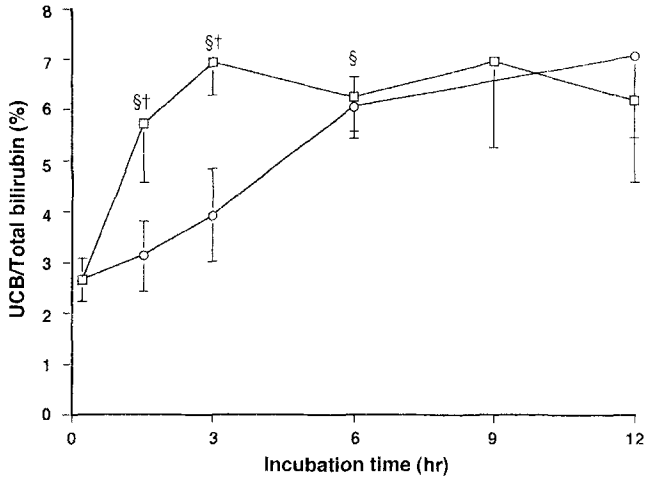

Fig. 3. Changes in the ratio of unconjugated bilirubin (*UCB*) to total bilirubin of bile supernatant incubated with β-glucuronidase (*squares*) or without the enzyme (control; *circles*). Values are expressed as means ± SEM ($n = 6$).[§] Compared with 0h;[<+>] compared with control. ($P < 0.05$)

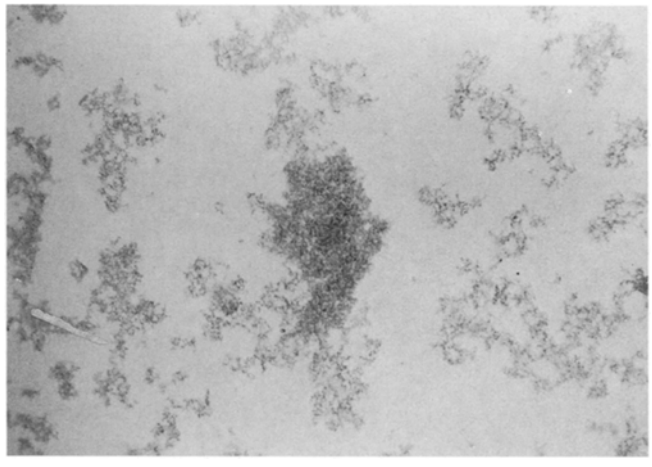

Fig. 4. Microphotograph of precipitate incubated with β-glucuronidase for 12h. ×400

those incubated without the enzyme ($P = 0.46$). The composition of fatty acids in the precipitates is shown in Table 2. Palmitic acid was the major component, in the precipitates, representing 50% of free fatty acids.

Gel chromatographic analysis of precipitate

Sepharose 4B-Cl chromatography of the precipitate revealed two major peaks: a high molecular weight peak in the void volume and a lower molecular weight peak in the included volume (Fig. 6). The first peak contained bile pigment and glycoprotein mucin, as confirmed by absorbance at 420nm and the PAS method, respec-

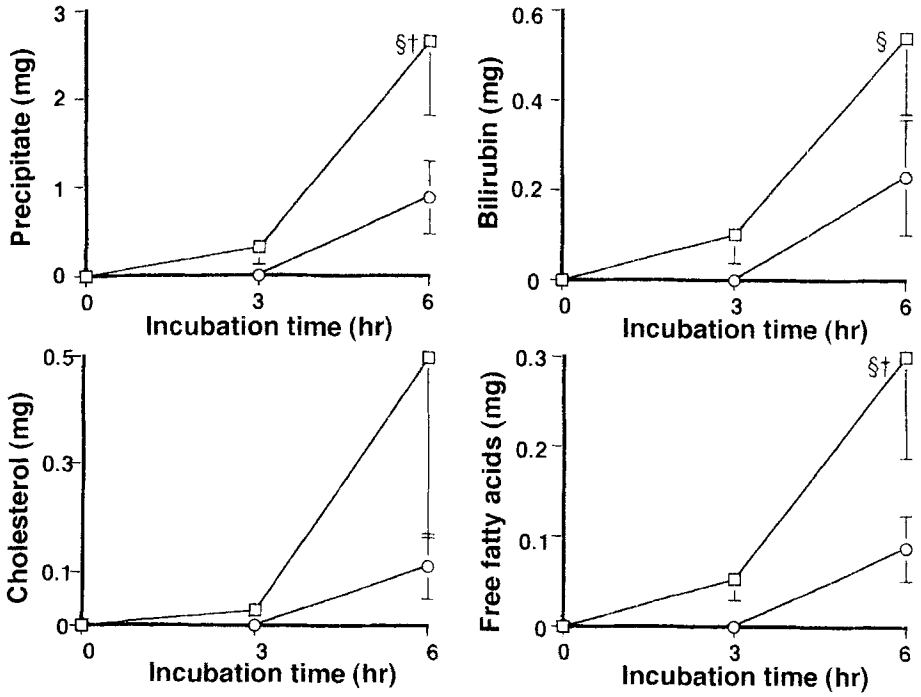

Fig. 5. Changes in dry weights of the precipitate, and in amounts of total bilirubin, cholesterol, and free fatty acids in the precipitate during incubation with β-glucuronidase (*squares*) or without the enzyme (control; *circles*). After 3-h incubation. The total weight of the precipitate increased rapidly in accordance with increases in bilirubin, cholesterol, and free fatty acids. Values are expressed as means ± SEM (n = 6).[§] Compared with 0 h;[<+>] compared with control. ($P < 0.05$)

Table 2. Free fatty acid composition of precipitate

	16:0	18:2	18:1	18:0	20:4
With β-glucuronidase (n = 6)	49.2 ± 3.9	7.8 ± 2.9	18.3 ± 4.2	17.8 ± 4.4	7.0 ± 2.3
Control (n = 4)	53.5 ± 3.4	8.4 ± 3.5	18.2 ± 2.3	13.1 ± 4.3	4.0 ± 1.4

Values are expressed as percent weight of total free fatty acids (mean ± SEM)
16:0, Palmitic acid; 18:2, Iinoleic acid; 18:1, oleic acid; 18:0, stearic acid; 20:4, arachidonic acid

tively. The other components present in the two peaks and proteins in the second peak were not further characterized.

Electron microscopy of precipitate and human brown pigment stone

The dried precipitate consisted of particles with a diameter of about 100 μm. Each particle looked like a coral or tree branches with many crevices (Fig. 7a). A similar structure was observed in a human brown pigment stone (Fig. 7b).

Discussion

Incubation of bile samples with β-glucuronidase may represent pigment gallstone formation with bacterial infection and incubation without the enzyme may be a model of bile stasis for biliary sludge and pigment gallstone formation.[21] Lee and Nicholls[22] reported on the nature and composition of biliary sludge, finding the biliary sludge to contain cholesterol monohydrate crystals and a higher ratio of cholesterol to bilirubin than that in precipitate, as found in the present study. Our study demonstrated that the brown precipitate formed by incubating bile samples with β-glucuronidase contained cholesterol, free fatty acids, unconjugated bilirubin, and glycoprotein, at ratios similar to those in brown pigment gallstones[19,23] and the microscopic features of the samples resembled those of the pigmented gallstones. The observed result is considered to represent an initial step of brown pigment gallstone formation rather than the formation of biliary sludge. Although all bile samples used in the present study were sterile, bile incubated without β-glucuronidase formed a precipitate

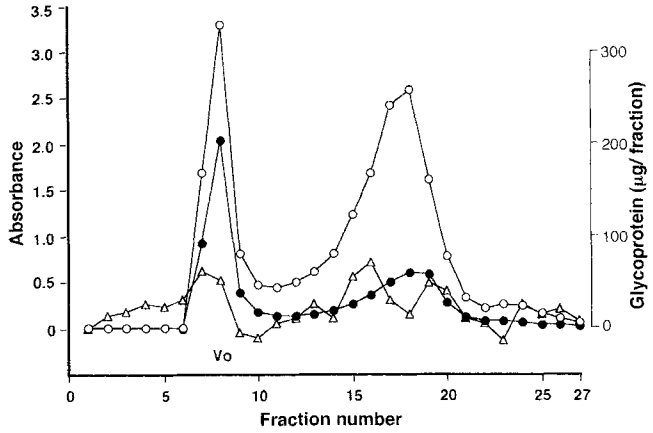

Fig. 6. Sepharose 4B-Cl gel chromatography of the precipitate. The precipitate was dissolved in EDTA/NaOH, dialyzed, concentrated, and loaded onto a column, as described in Materials and Methods. Each fraction was analyzed for bile pigment (*circles*; OD 420), and protein (*dots*; OD 280) by spectrophotometer and for glycoprotein (*triangles*; μg/fraction) by periodic acid-Schiff assay

similar to bile that in incubated with the enzyme. This result was probably due to the non-enzymatic hydrolysis of conjugated bilirubin[24] and to nonbacterial β-glucuronidase activity in bile.[25] The weight of free fatty acids in the precipitate increased during the incubation period; this we ascribed to a non-enzymatic reaction and nonbacterial phospholipase activity in bile.[26] The idea of the non-specific hydrolysis of lecithin[26] was supported by our finding of increased free fatty acid concentration in the supernatant during the incubation.

Smith and LaMont[27,28] reported that mucin binds bilirubin and biliary lipids, resulting in the formation of a mucin-bilirubin complex and a mucin-lipid complex in brown pigment and cholesterol stones. In the present study, the precipitate contained a considerable amount of glycoprotein, a finding consistent with the results of LaMont et al.[19] The electron microscopic appearance of the precipitate suggested that glycoprotein was the framework of the precipitate. Large molecular weight glycoprotein was eluted together with bilirubin, suggesting that glycoprotein in the precipitate binds to bilirubin. Although mucin has been suggested to play a role as a mediator between unconjugated bilirubin and biliary lipids, the mode of binding is still unclear. Gong et al.[29] estimated 60 affinity binding sites for cholesterol and 260 sites for free fatty acids on human gastric mucin. Whether these binding sites are responsible for the binding between mucin and biliary lipids is not clear at present; however, it is likely that mucin plays an important role in the coprecipitation of cholesterol and free fatty acids.

In the formation of brown pigment gallstones, calcium salts of fatty acids are reported to coprecipitate with calcium bilirubinate.[4] However, there is little information about the incorporation of cholesterol into brown pigment gallstones. Carey and Small[30] suggested that cholesterol supersaturation plays a role in the formation of brown pigment stones. In the present study, however, the brown precipitate contained a considerable amount of cholesterol, despite the relatively low cholesterol saturation index of the bile samples. Cholesterol crystals and liquid crystals were not observed by polarizing microscopy in the supernatant throughout the incubation period. Further, the ratio of cholesterol

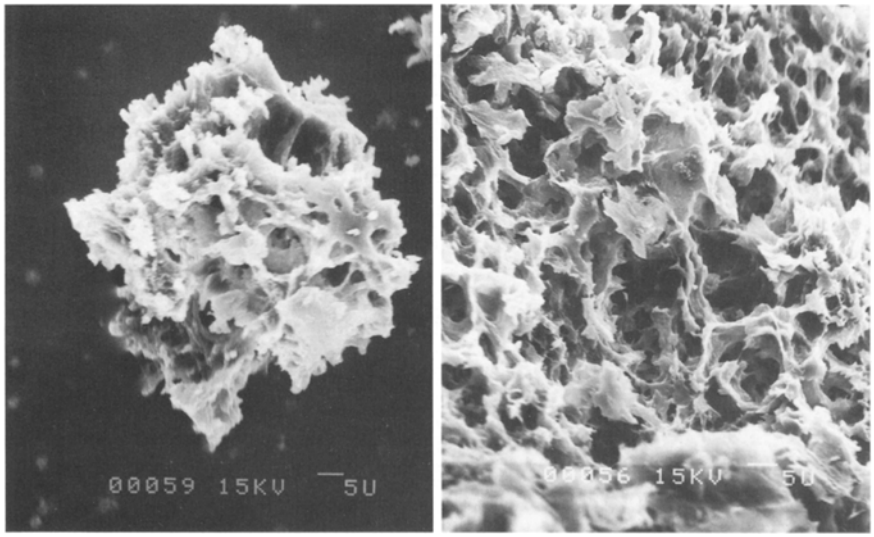

Fig. 7a,b. Scanning electron microscopy of dried precipitate; **a** It consisted of particles with a diameter 100 μm and looked like a coral with many crevices. The appearance was similar in a human brown pigment gallstone. (**a**,**b**. ×1200)

to bilirubin in the precipitate was similar to that in the bile. These observations indicated that: (i) supersaturation of cholesterol in bile is not always necessary for the incorporation of cholesterol into brown pigment stones and (ii) the cholesterol content of the stones may depend on the relative ratio of cholesterol to total bilirubin in bile. It has been thought that calcium bilirubinate, cholesterol, and free fatty acids are incorporated in brown pigment gallstones as independent components at different stages in the formation of these stones.[31] Our study demonstrated that these components may coprecipitate from the initial stage and that the coprecipitation of the lipids is accelerated by the deconjugation of bilirubin.

Cahalane et al.[32] reported that decreased capacity of bile salt micelles may promote cholesterol saturation, resulting in the incorporation of cholesterol crystals into brown pigment stones. In our study, the cholesterol saturation index and nucleation time remained constant during the incubation period. Since the amount of cholesterol in the precipitate, even at 12-h incubation, represented as little as 2% of total cholesterol in the bile sample, it appeared that the precipitation of cholesterol did not affect the cholesterol saturation index.

The concentration of ionized calcium in the supernatant decreased during the incubation period due to consumption of the ion as calcium salts of bilirubin and fatty acids. Total bilirubin and free calcium concentrations in the supernatant decreased slowly until 3 h, and thereafter decreased rapidly during the incubation period, corresponding to a rapid increase in weight of the precipitate after 3-h incubation. The weight of the precipitate also increased in a similar way. As shown in Fig. 5, the formation of unconjugated bilirubin seemed to precede the coprecipitation with biliary lipids, suggesting that the deconjugation of conjugated to unconjugated bilirubin plays a central role in the formation of pigmented gallstones. This study showed that the precipitate formed by either the enzymatic or non-enzymatic incubation of isotropic human bile samples contained considerable proportions of cholesterol, free fatty acid, and glycoprotein, as well as deconjugated free bilirubin. The composition and electron microscopic appearance of the precipitate resembled these features in brown pigment stones and suggested the importance of glycoprotein as the frame-work of the precipitate. We speculate that: (i) insoluble deconjugated bilirubin, which is formed either enzymatically or non-enzymatically, binds to glycoproteins, which also have binding sites for free fatty acids and cholesterol, and (ii) the bilirubin-glycoprotein-biliary hydrophobic lipid complex precipitates and aggregates to form brown pigment stones.

The present in vitro study suggests that the precipitation of cholesterol, free fatty acids, and bilirubin is accelerated by the presence of β-glucuronidase. The binding of unconjugated bilirubin to glycoprotein mucin may play an important role in the coprecipitation of these biliary lipids.

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