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原　　著

Mechanism of the Formation of Bilirubin Stones II. Analysis of Conjugated and Unconjugated Bilirubin by Highperformance Liquid Chromatography and Measurements of Calcium Ion by Ion-selective Electrode in Bile of Patients with Gallstones

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Introduction

As gallstones are formed as a result of the alteration of bile composition, numerous studies on the analysis of bile components have been performed to clarify the mechanism of the formation of gallstones[25,71]. Among them, the work by Admirand and Small[1] succeeded in showing that the supersaturation of cholesterol in bile against micelles formed by bile acids and phospholipids possibly initiates the formation of cholesterol stones in the gallbladder. The results are now utilized widely in clinical fields for the treatment and the prevention of this type of gallstone[54].

On the other hand, the mechanism of the formation of bilirubin stones, which are one type of pigment gallstones, has not yet been elucidated and still remains under discussion[62,76,79]. This is mostly because the difficulty in the methods to measure correctly the concentrations of bilirubin and calcium in bile have prevented the physicochemical analysis of these two main components of bilirubin stones.

It has been clinically believed that bile stasis and infection were the initiating factors on the formation of bilirubin stones. Maki and his co-workers[35] found in their clinical studies that bilirubin stones were almost always associated with bile infection predominantly E. coli, and they proposed that beta-glucuronidase from E. coli infecting the bile causes hydrolysis of conjugated bilirubin to unconjugated bilirubin (UCB), which then precipitates as its insoluble calcium salts (calcium bilirubinate), forming the so-called "bilirubin calcium stones", which the author call bilirubin stones because bilirubin is the main component of this type of gallstone.

Key words: Bilirubin stone, Unconjugated bilirubin, high-performance liquid chromatography, Calcium ion, Bile acids.
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Caution should be taken to the fact that there are several assumptions behind this hypothesis. Firstly, the bilirubin secreted from the liver into bile is only of the conjugated type. Secondly, UCB is not soluble in bile. Thirdly, UCB should easily combine with calcium in bile, and fourthly the main component of bilirubin stones is calcium bilirubinate. However, all these assumptions have not yet been validated, and still several more studies pointed out the conflicting data against these assumptions. Thus, the proposed hypothesis regarding bile stasis and infection as the main factors in the pathogenesis of bilirubin stones is based more on speculation than data.

Recently developed thin-layer chromatographic (TLC) methods with diazo-reactions, largely owing to the works of Heirwegh and his colleagues[23], made possible the detailed analysis of bilirubin in bile. It was discovered by Boonyapisit et al.[7] using this TLC analysis of bilirubin that UCB was detected even in sterile human gallbladder bile from normal controls and that the concentrations of UCB in the bile far exceeded those resolved in buffer solutions. These results uncovered many problems about the etiology of bilirubin stones. That is, UCB should originate in the bile other than as a result of the hydrolysis of conjugated bilirubin by bacterial beta-glucuronidase; UCB dose not always react with calcium; some components of bile may contribute to the solubilization of UCB; as such.

However, as the analysis of bilirubin by TLC with diazo-reaction is very tedious and time-consuming, the possibility of inaccuracy[53] in the measurements can not be excluded because bilirubin is very labile under light and oxygen[2,42], thus, advanced analysis on bilirubin in bile has become difficult by using TLC methods.

Concerning calcium, although there are many reports about total calcium[63,64], few investigations were performed to determine the concentration of calcium ions in bile[57,66] which in practice is an active form. And little is known about the reactivity of calcium against bilirubin.

It is mandatory for resolving these problems to analyze bilirubin and calcium in bile more accurately.

The purposes of this study are following; firstly, to establish high-performance liquid chromatographic (HPLC) methods for the accurate analysis of the bilirubin in bile, secondly to determine the concentrations of unconjugated bilirubin in bile and to clarify its origin, thirdly to evaluate the reactivity of calcium ion in bile by measuring its concentrations, using a newly developed calcium ion analyzer equipped with an ion-selective electrode.

Materials and Methods

Drugs

Water was distilled and stored in glass. Tetrahydrofuran, KH_2PO_4, and phosphoric acid, obtained from Kanto Chemical Co. (Tokyo, Japan), and taurine from Tokyo Kasei (Tokyo, Japan), were all analytical reagent grade. Commercial bilirubin as a standard sample of unconjugated bilirubin IX-alpha was obtained from both British Drug House Co. (Poole, England, U.K.) and Sigma (St. Louis, Mo., U.S.A.) to examine their isomer composition[41]. Bilirubin

diglucuronide was purchased from Sigma. The $CHCl_3$ used contained 0.75% ethanol stabilizer (Wako HPLC grade, Wako, Japan). Methanol, ethanol, and dimethyl sulfoxide (DMSO) were also reagent grade solvents obtained from Kanto Kagaku (Tokyo, Japan). Beta-glucuronidase (type IX from E. coli) was obtained from Sigma (St. Louis, Mo., U.S.A.). All bile acids were also purchased from Sigma. Ascorbic acid and silicagel-plates (layer thickness 0.25 mm 20× 20 cm) were purchased from Merck A.–G. (Darmstadt, F.R.G.). Ethylanthranilate were obtained from Eastman Organic Chemicals (Rochester, NY, U.S.A.). All other chemicals were the best grade commercially obtainable and were used without further purification.

Apparatus

High-performance liquid chromatography described here employed a HPLC apparatus (Shimadzu LC-3A, Kyoto, Japan), equipped with a 200-μl injection loop (Shimadzu SIL-1A), connected to a Shimadzu variable-wavelength monitor (Model SPD-1A), set at 454 nm. The columns used were PCH packed columns (25 cm × 4.6 mm I.D., particle size 5 μm; Shimadzu. Kyoto, Japan) and were used in reversed-phase mode. A small pre-column packed with Hypersil-ODS was used to protect the analytical columns. Tetrahydrofuran-Water (46 · 54, v/v), containing 10 mM taurine and 5 mM KH_2PO_4, was adjusted to pH 3.0 with phosphoric acid and was used as the mobile phase. Taurine was used to eliminate the tailing of bilirubin IX-alpha peak and to obtain complete separation from bilirubin III-alpha peak. The mobile phases were thoroughly degassed by ultrasonification before use.

The quantitative determination of unconjugated bilirubin IX-alpha was performed on one PCH column with the solvent at a flow rate of 1.1 ml/min. The separation of both conjugated and unconjugated bilirubin was performed on two PCH columns connected together in series, eluted by the same solvent at a flow rate of 0.8 ml/min. Separations were achieved at 40°C for one column and at 60°C for two connected columns, respectively. The peak areas were calculated electronically by a Chromatopac C-R1A (Shimadzu, Kyoto. Japan). Peak identifications were made by the TLC analysis of p-iodoaniline azopigments as described by Heirwegh et al.[23] and by the comparison with the retention time of commercial unconjugated bilirubin solubilized in tetrahydrofuran. A standard curve for the calibration of UCB concentrations was made by using commercial bilirubin in tetrahydrofuran as a standard solution.

The calcium ion analyzer (Orion Model SS-20, Orion Biomedical, USA) was equipped with a calcium ion selective electrode. Standard solutions were prepared using calcium carbonate.

Bile pH was measured with a HITACHI-HORIBA pH meter Model M-7E (Hitachi-Horiba, Tokyo, Japan). Hitachi spectrophotometer Model 200–10 (Hitachi. Tokyo, Japan) was used for the measurements of total bilirubin, total bile acids, total cholesterol and total phospholipids. Total bilirubin was always measured in fresh sample by using the modified Michaelson method (Bilirubin Kit, Nippon Shoji Co., Osaka, Japan). Total bile acids (Sterognost-3 alpha, Nyegaard & Co. A/S, Oslo, Norway), total cholesterol (Boeringer Mannheim GmbH, Mannheim, F.R.G.), total phospholipids (PL Kit-K, Nippon Shoji Co., Osaka, Japan) were all determined by enzymatic assay[22,55].

Samples

Patients with cholecystitis or overt liver diseases, and white or greenish bile samples were excluded. Because preliminary experiments[69] revealed the rapid increase of unconjugated bilirubin in bile during storage at −20°C, and even at −80°C, the author examined only fresh bile samples aspirated at surgery in the operation rooms at Kyoto University Hospital. Human bile samples collected at laparotomy were immediately sent for analysis in a dark ice bottle at 4°C, and 10–100 μl of bile were injected on to HPLC, only with filtration through a 0.45μm milipore filter (Milipore Corp., Bedford, USA) without any other preparation. Thus, measurements of UCB were completed within 30 minutes after their collections, to minimize changes in bilirubin in samples during measurements. The concentrations of bilirubin IX-alpha were directly determined with reference to commercial bilirubin. Each bile sample was incubated under argon gas at 38°C in a dark sealed flask without buffer solution and UCB concentrations were measured every 30 minutes for 2 hrs to calculate the rates of hydrolysis of conjugated to unconjugated bilirubin. Because of the instability of bilirubin to oxygen and light, standard solution of bilirubin dissolved in tetrahydrofuran was freshly prepared at the time of calibration. Filtered samples were also used for the determination of concentrations of calcium ion, total bilirubin, total bile acids, total cholesterol and total phospholipids.

Results

1) HPLC analysis of Bilirubin in Human Bile

The separation of conjugated and unconjugated bilirubin in bile by the two-column HPLC system are shown in Fig. 1. Peaks 1 and 2 were identified as bilirubin diglucuronides (Peak 1) and bilirubin monoglucuronides (Peak 2) respectively by the analysis of TLC. Peak 3 was identified as unconjugated bilirubin IX-alpha both by the analysis of TLC and by comparing its retention time (38.0 min) with that of commercial unconjugated bilirubin. The retention time of commercial bilirubin diglucuronide (4.00 min) was not consistent with that of diglucuronide (6.00 min) detected in human and rat bile. By TLC analysis, commercial bilirubin diglucuronide separated into alpha azopigment, and beta- or gamma-azopigment. This suggested that the commercial bilirubin diglucuronide might include the isomer of bilirubin acylglucuronide other than bilirubin 1-O-acylglucuronide[3,16,17] which is a dominant form in human and rat bile, possibly because commercial bilirubin diglucuronide is extracted from bovine gallstones.

Figure 2 shows the changes of bilirubin in human gallbladder bile during incubation at 37°C in the dark, added with beta-glucuronidase of E. coli. Bilirubin diglucuronides (Peak 1) and monoglucuronides (Peak 2) rapidly decreased and unconjugated bilirubin (Peak 3) markedly increased with its two isomers. Peak 4 was thought to be bilirubin monoconjugates other than monoglucuronides[20], which might have resulted from the deconjugation of bilirubin monoglucuronide-monoconjugate (glucoside or xyloside) molecules which were probably included in Peak 1. The similar hydrolysis of bilirubin glucuronides was also found in bile after superfiltration by centrifugation (1,000 G×5 min) with a cut off of 25,000 daltons (Centriflo cones CF25A: Amicon Corp., Lexington, Mass., USA), though during a longer incubation time under

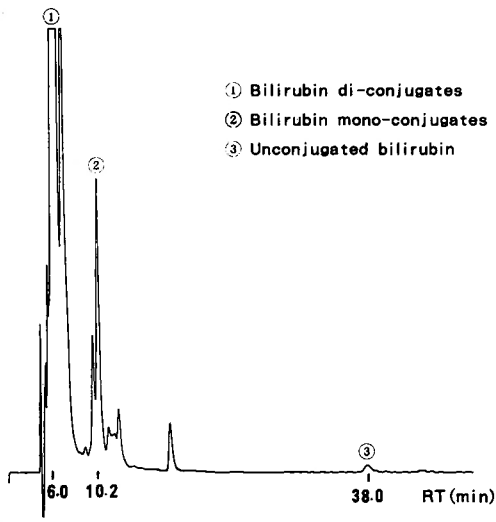


Fig. 1. Bilirubin in human gallbladder bile separated by HPLC. RT—retention time.

the same condition, as shown in Fig. 3. These results indicated that, although bacterial beta-glucuronidase can certainly hydrolyze bilirubin diglucuronide and monoglucuronide in human bile even under alkaline pH, bilirubin glucuronides are also hydrolyzed non-enzymatically[38,60]

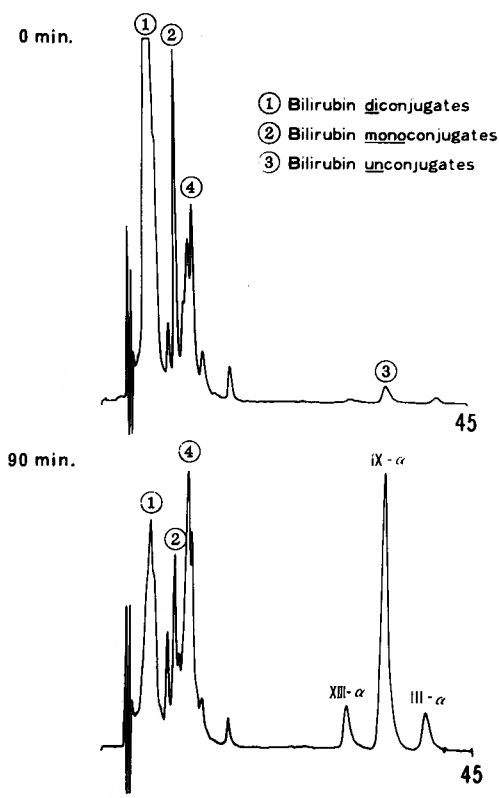


Fig. 2. HPLC analysis of hydrolysis of bilirubin conjugates in human gallbladder bile during incubation with beta-glucuronidase from E. coli. Peak 4 was considered to be bilirubin monoconjugates other than monoglucuronides.

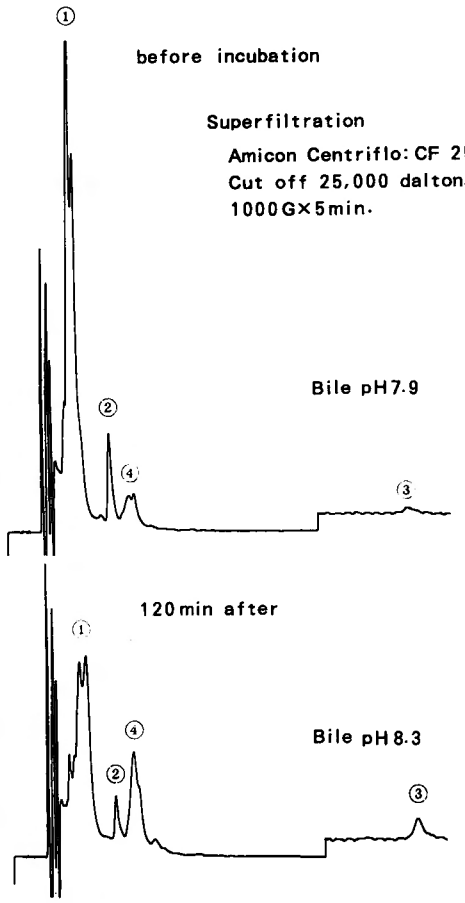


Fig. 3. HPLC analysis of hydrolysis of bilirubin conjugates in human gallbladder bile during incubation after superfiltration through Amicon Centriflo CF 25A with a cut off of 25,000 daltons. Bile pH increased from 7.9 to 8.3 during incubation.

under the same condition though at a slightly slower speed, and that bacterial infection is not always needed for the hydrolysis of bilirubin glucuronides in bile.

Figure 4 shows the changes in the bilirubin in common bile duct bile, aspirated from the same patient at surgery and through T tube after surgery. Although T tube bile was carefully aspirated to obtain bile as fresh as possible, the decrease in bilirubin diconjugates and the increases in monoconjugates and unconjugated bilirubin in T tube bile are clearly demonstrated in the chromatogram of T tube bile aspirated 7 days after surgery compare to that of common bile duct bile at surgery. These changes in bilirubin in T tube bile may be brought about by altered metabolism of bile acids, continuing cholangitis, bile infection and/or changes in bile pH, those caused by the insertion of the T tube, or by the physicochemical deconjugation occuring in stagnant bile around the T tube. In any case, the difference of bilirubin pattern between the two bile samples indicated that, to investigate the mechanism of the formation of bilirubin stones, the bilirubin in common bile duct bile must be analyzed using fresh bile samples aspirated only at surgery.

2) HPLC analysis of unconjugated bilirubin in human bile

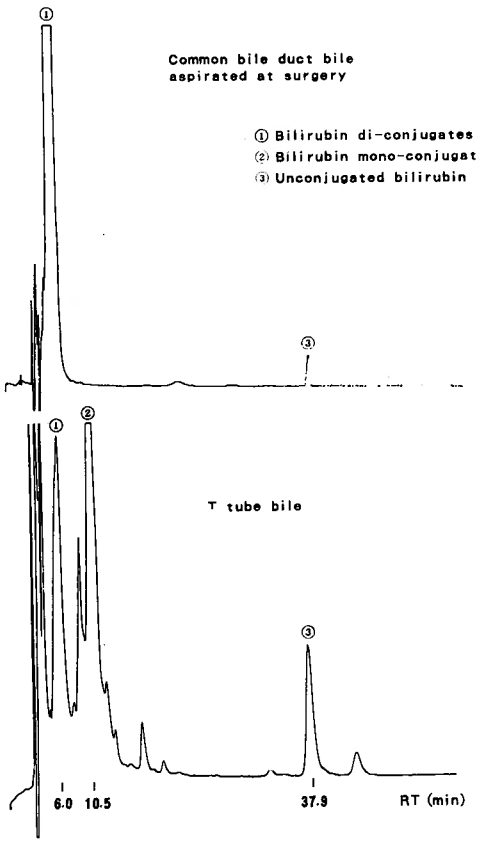
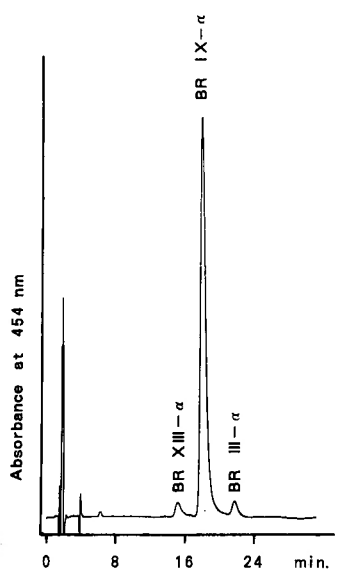


Fig. 4. Changes in bilirubin in human common bile duct bile aspirated at surgery and after surgery through a T tube (lower panel) in the same patients.



HPLC condition

Instrument	Shimadzu LC-3A
Column	Shimadzu PCH-05/S2504 (25 cm × 4.6 mm)
Eluent	THF/H_2O: 46/54; v/v% Taurine 10 mM KH_2PO_4 5 mM pH 3 with H_3PO_4
Flow Rate	1.1 ml/min
Pressure	120 kg/cm²
Temperature	40°C
Detector	454 nm
Range	0.08 A.U.F.
Bilirubin	BDH Co.

Fig. 5. Chromatogram of standard solution of commercial bilirubin and HPLC condition for the quantitative analysis of unconjugated bilirubin in human bile. Complete separation of three bilirubin isomers was obtained by using taurine in the eluent.

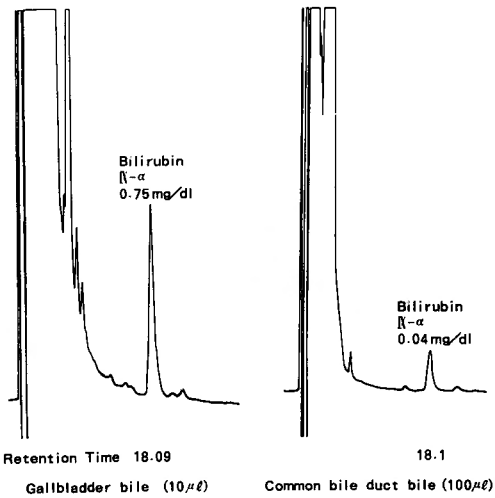


Fig. 6. Chromatograms of human gallbladder and common bile duct bile, injected with 10μl and 100 μl respectively. Concentrations of bilirubin IX-alpha were calculated with reference to the calibration curves.

The chromatogram of a standard sample of unconjugated bilirubin dissolved in tetrahydrofuran is shown in Fig. 5, with the condition of the chromatography. Three bilirubin isomers, bilirubin XIII-alpha, IX-alpha and III-alpha, which were present in commercial bilirubin[41], were completely separated, by using taurine 10 mM in the eluent. The retention time of bilirubin IX-alpha isomer, which is a predominant form in vivo[11,24], was 18.1 minutes. The chromatograms of human gallbladder bile and common bile duct bile for the quantitative analysis of UCB are shown in Fig. 6. The standard curve of bilirubin IX-alpha and its recovery test in a gallbladder bile for the calibration are demonstrated in Figs. 7 and 8. Regression coefficients of these curves were both 0.999 in the range of 0.01 to 16 mg/dl. Because the main forms of bilirubin in bilirubin stones may be free and metal chelates (such as calcium bilirubinate) of unconjugated bilirubin, it was considered that the most important factor in determining the mechanism of the formation of bilirubin stones is whether the concentration of unconjugated

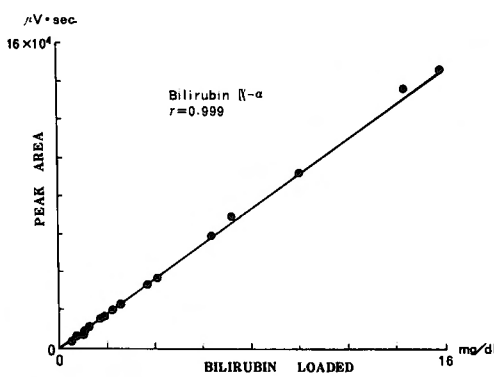


Fig. 7. Standard curve for calibration of unconjugated bilirubin IX-alpha. Regression coefficient was 0.999 from 0.01 to 16 mg/dl.

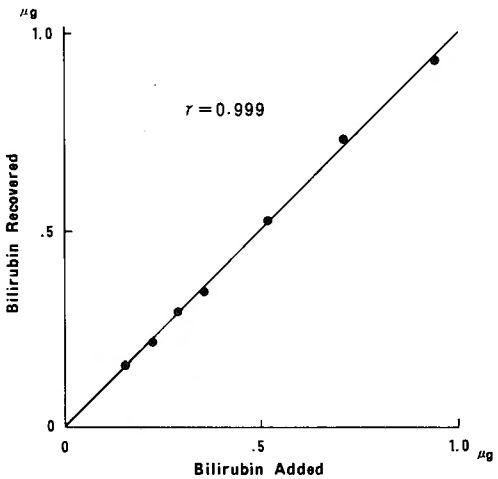


Fig. 8. Recovery curve of commercial unconjugated biliruin in human bile.

bilirubin and the rate of hydrolysis of conjugated bilirubin increase in human bile with bilirubin stones. This HPLC method with one PCH column was more suitable than that with two columns above mentioned for rapid and accurate measurements of unconjugated bilirubin in human bile. For this reason, it was decided that the present study should measure the concentrations of unconjugated bilirubin in bile from patients with and without gallstones, using the one-column HPLC method described here.

3) Solubilization of unconjugated bilirubin by bile acids

The solubility of unconjugated bilirubin into bile acid solutions was investigated[85]. Commercial bilirubin was dissolved in 0.2 M NaOH solution and the final concentration of unconjugated bilirubin of this suspension was about 1,000 mg/dl. Then, 0.02ml of the supernatant of this suspension was added to 2 ml of KH_2PO_4 buffer solutions pH 7.5 with and without bile acids. These buffer solutions with 0.02 ml bilirubin suspension were centrifuged and the supernatant were injected into HPLC. The results in Fig. 9 indicated that bile acids contribute to the solubilization of unconjugated bilirubin and that even low concentrations of about 5 to 10mM of bile acids were sufficient for the effects. Furthermore, the supersaturation of unconjugated bilirubin against bile acids might not readily occur, because the concentrations of bile acids usually exceed 5 mM even in common bile duct bile from patients with stones.

4) Bilirubin in the gallbladder bile

The presence of unconjugated bilirubin in bile from normal controls that has been reported by TLC with diazo-reaction[7] was confirmed by this study because the HPLC method minimized the possibility of the changes in bilirubin in samples during measurements and eliminated the false detection of diazo-positive pigments as UCB. The results of the analysis of bilirubin in human gallbladder bile are summarized in Table 1.

UCB concentrations (Fig. 10) and UCB/total bilirubin ratios (Fig. 11) of bile from bilirubin stone patients were 1.46 ± 0.35 mg/dl; $1.44\pm0.34\%$, and from black stone patients were $0.82\pm$

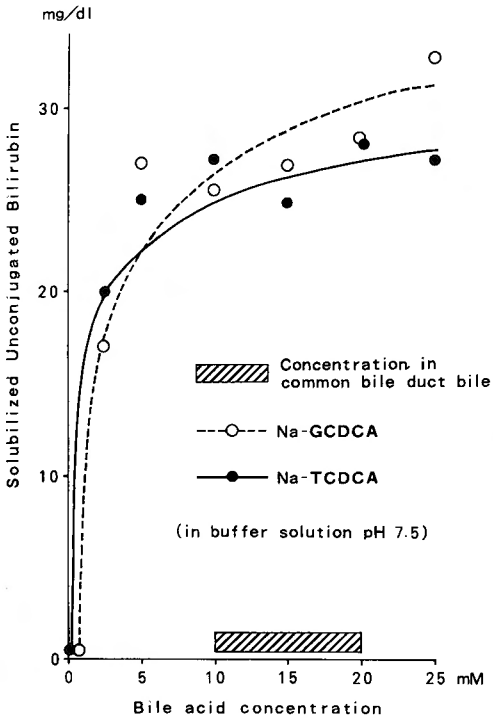


Fig. 9. Solubilization of unconjugated bilirubin sodium salt by bile acids measured by HPLC.

0.16 mg/dl; 0.67±0.11%, respectively. These were significantly ($p<0.05$) higher than those from cholesterol stone patients which were 0.28±0.05 mg/dl; 0.23±0.04% and showed the minimum values among the four groups. UCB in control bile without stones averaged 0.39± 0.07 mg/dl and were lower than bile from bilirubin and black stone patients, though not statisti cally significant. All four groups showed the values of UCB concentrations 2 to 5 times lower than those of previous reports using thin-layer chromatography and the diazo-method[7,39]. This suggested that TLC and the diazo-method probably overestimated the concentrations of UCB in bile.

Table 1. Summary of HPLC measurements of bilirubin in fresh human gallbladder bile from 50 patients with and without gallstones.

Stone groups (n)	Controls (no stone) (14)	Cholesterol stones (23)	Bilirubin stones (4)	Black stones (9)
UCB (mg/dl)	0.39± 0.07	0.28± 0.05	1.46± 0.35*	0.82± 0.16*
Total BR (mg/dl)	176.7±31.3	135.6±21.1	130.0±59.6	148.5±34.0
UCB/Total BR (%)	0.25± 0.05	0.23± 0.04	1.44± 0.34*	0.67±0.11*
Hyd. rates (%/hr) (n)	1.50± 0.33 (7)	1.42± 0.25 (17)	1.98± 0.28 (4)	0.80± 0.51 (4)

BR-Bilirubin, Hyd.-Hydrolysis *$p<0.05$ Mean±S.E.

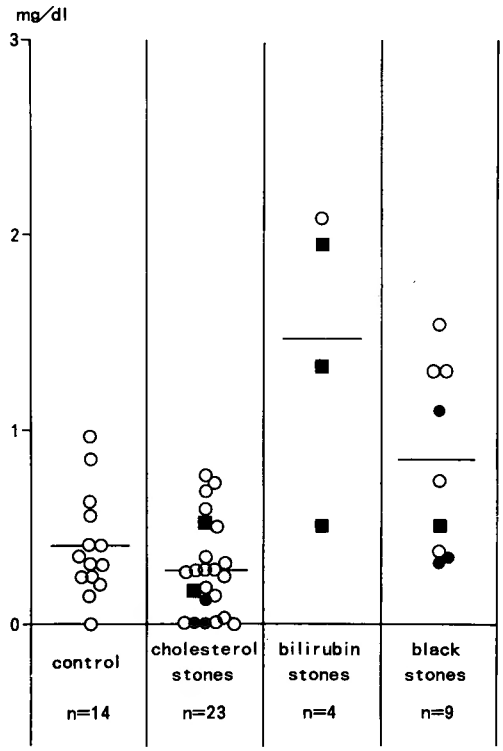


Fig. 10. Absolute concentrations of unconjugated bilirubin IX-alpha in human gallbladder bile measured by HPLC. ○ no growth, ■ E. coli, ● other bacteria, by bile culture. Each bar shows the mean value of the group.

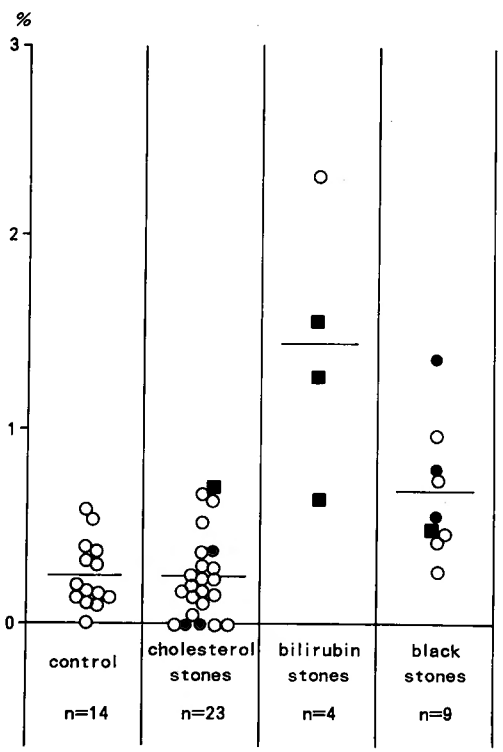


Fig. 11. Proportion of unconjugated bilirubin to total bilirubin in human gallbladder bile. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

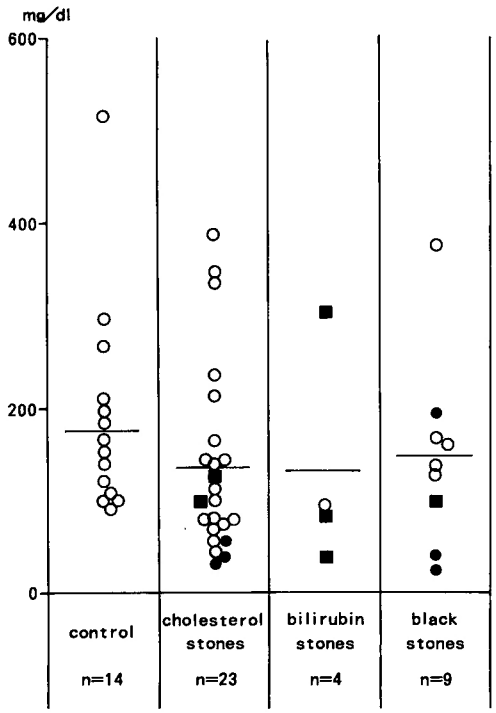


Fig. 12. Total bilirubin concentrations. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

Total bilirubin concentrations (Fig. 12) according to stone groups were 135.6±21.1mg/dl with cholesterol stones, 130.0±59.6mg/dl with bilirubin stones, and 148.5±34.0mg/dl with black stones. The values were lower than that of controls (176.7±31.3mg/dl). These findings were

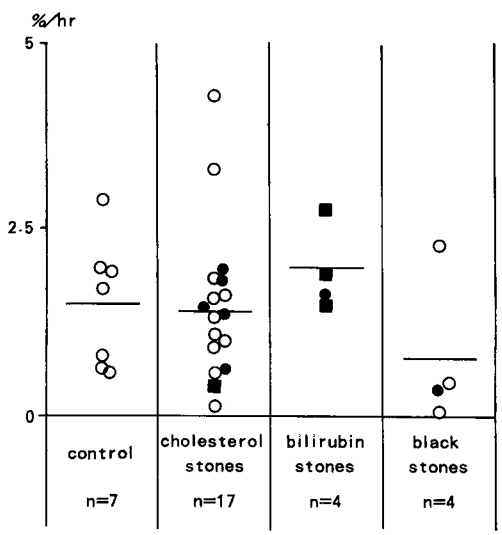


Fig. 13. Rates of hydrolysis of conjugated to unconjugated bilirubin in human gallbladder bile during incubation at 38°C in the dark under argon gas. Rates are expressed as % per hour to total bilirubin concentrations. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

in agreement with those of previous reports[49].

Hydrolysis of conjugated to unconjugated bilirubin was found in all bile samples and the rates of hydrolysis (Fig. 13) were 1.50±0.33%/hr in control, 1.42±0.25%/hr in cholesterol stone group, 1.98±0.28%/hr in bilirubin stone group and 0.80±0.51%/hr in black stone group, respectively; no significant differences were seen among the groups. The present findings conflict with those of Boonyapisit et al.[8]. The discrepancy is probably due to the fact that the incubation of bile in this study was carried out without buffer solutions, because it was found that bile pH increased about 1.0 during incubation and the hydrolysis was inhibited by adding phosphate buffer pH 5.0. Bacterial cultures were performed both aerobically and anaerobically with all bile samples to determine the effect of bile infection on biliary bilirubin concentrations. The isolated bacteria are summarized in Table 3 and the correlation of positive or negative bile culture with bilirubin concentrations in each case is illustrated in Figs. 10 to 13. The results indicated that bile infections with either E. coli or others, does not affect any one of absolute UCB concentrations, UCB/total bilirubin ratios, total bilirubin concentrations or rates of hydrolysis in bile.

5) Bilirubin in the common bile duct bile

Using the TLC method with diazo-reaction, measurement of the concentrations of unconjugated bilirubin in common bile duct bile was difficult and inaccurate because its concentration in bile duct bile is very low. In this study by using the HPLC method, unconjugated bilirubin was also detected in common bile duct bile and even in fresh bile samples aspirated directly from the intrahepatic duct.

The results of the analysis of bilirubin in common bile duct bile are summarized in Table 2. The mean unconjugated bilirubin concentrations in common bile duct bile were 0.05±0.05 mg/dl from cholesterol stone group, 0.07±0.03mg/dl from bilirubin stone group and 0.05±0.03 mg/dl from black stone group respectively and were about ten times lower than in the gallbladder bile. Although UCB/total bilirubin ratio (Fig. 15) was higher in bilirubin stone group (0.36±0.16%) than in cholesterol (0.11±0.01%) and black stone groups (0.06±0.03%), the absolute UCB concentrations (Fig. 14) were almost the same among these groups. This was because the total bilirubin concentration (Fig. 16) of the bilirubin stone group (21.2±4.0 mg/dl) was significantly ($p<0.05$) lower than those of the other two groups, that is, 50.3±6.1 mg/dl from cholesterol

Table 2. Summary of HPLC measurements of bilirubin in fresh human bile duct bile from 24 patients with gallstones.

Stone groups (n)	Cholesterol stones (17)	Bilirubin stones (4)	Black stones (3)
UCB (mg/dl)	0.05±0.05	0.07±0.03	0.05± 0.03
Total BR (mg/dl)	50.3 ±6.1	21.2 ±4.0*	63.6 ±14.6
UCB/Total BR (%)	0.11±0.01	0.36±0.16	0.06± 0.03
Hyd. Rate (%/hr)	—	(0.49, 2.2)	(4.6, 1.67)

BR-Bilirubin, Hyd.-Hydrolysis *$p<0.05$ Mean±S.E.

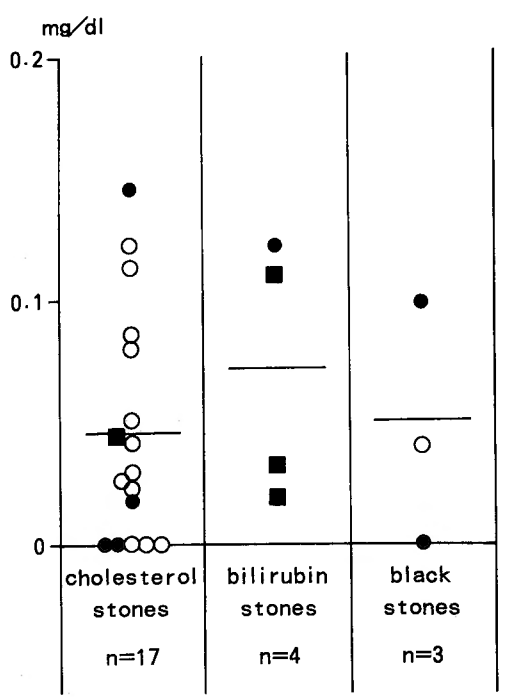


Fig. 14.

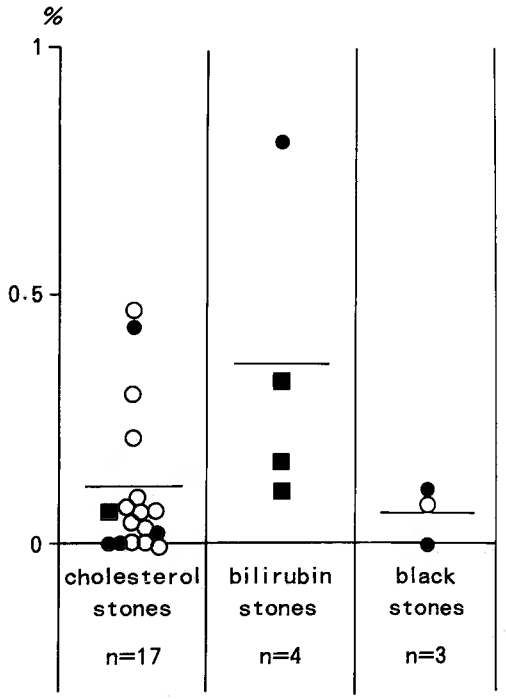


Fig. 15.

Fig. 14. Absolute concentrations of unconjugated bilirubin in human common bile duct bile measured by HPLC. Each bar shows the mean value of the group. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

Fig. 15. Proportions of unconjugated bilirubin to total concentrations in human common bile duct bile. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

and 63.6±14.6 mg/dl from black stone patients.

Rates of hydrolysis (Table 2) were measured in four cases (bilirubin 2, black 2), and the bile with black stones (4.6, 1.67%/hr) showed rates about 2 times higher than that with bilirubin stones (0.49, 2.2%/hr). Considering these rates and that the total bilirubin concentration with black stones was three times higher than that with bilirubin stones, it was calculated that the amount

Table 3. Results of bile cultures of all samples. The relationship between bile infection and bilirubin concentrations of each case is illustrated in Fig. 10 to Fig. 16.

Gallbladder bile		Common bile duct bile	
no growth	38	no growth	13
infected	12	infected	11
E. coli	6	E. coli	4
Klebsiella	2	Klebsiella	2
Enterobacter cloaca	2	Enterococcus	2
Pseudomonas aeruginosa	1	Pseudomonas aeruginosa	1
Morganella	1	Citrobacter freundii	1*
		Gram negative rods	1
		Candida	1
total	50	total	24

*Mixed infection with Enterococcus

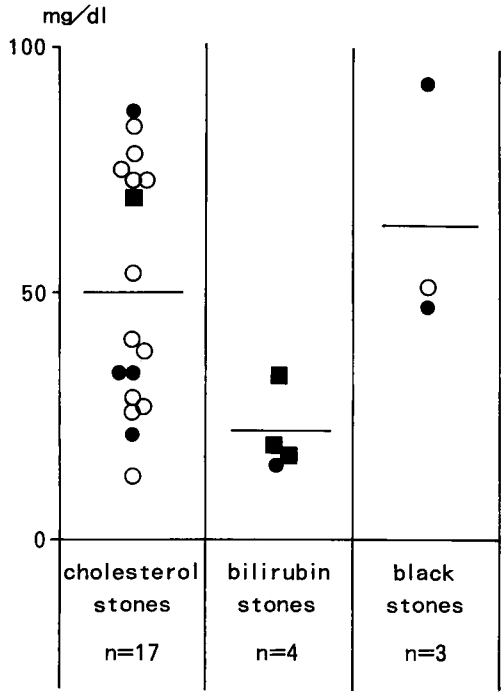


Fig. 16. Total bilirubin concentrations in human common bile duct bile. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

of UCB produced by every unit of time by hydrolysis in common bile duct may be six times higher in the black stone group than in the bilirubin stone group.

The UCB concentration of bile from a patient with hemolytic anemia was 0.26 mg/dl and

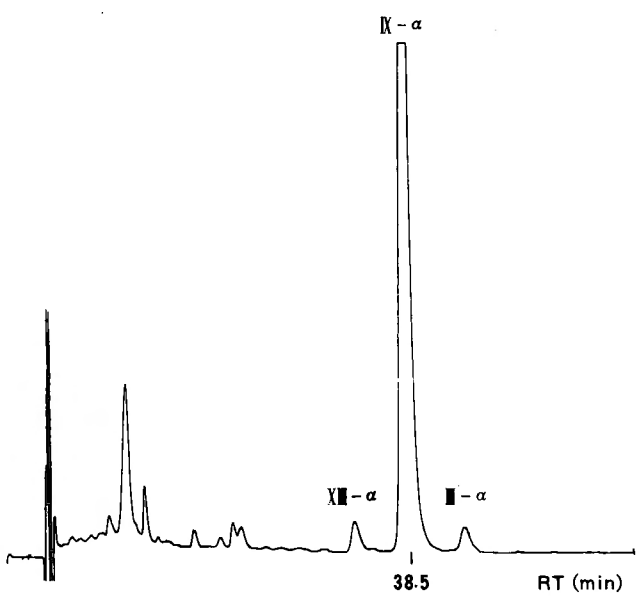


Fig. 17. Bilirubin in bilirubin stone analyzed by HPLC. Bilirubin stone was dissolved in dimethyl sulfoxide. As seen in the chromatogram, unconjugated bilirubin IX-alpha was the predominant form of bilirubin in bilirubin stone.

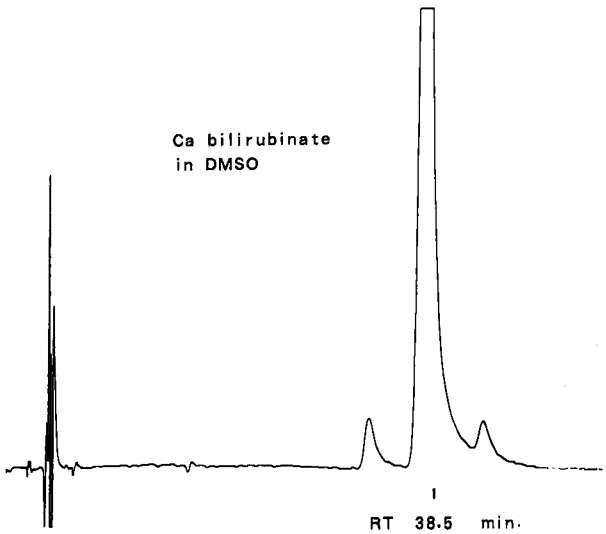


Fig. 18. Chromatogram of synthesized calcium bilirubinate dissolved in dimethyl sulfoxide. The retention time was the same as that of commercial unconjugated bilirubin.

this was the maximum value in common bile duct bile. The patient also showed a high total bilirubin concentration (107.3 mg/dl). However the UCB/total bilirubin ratio (0.24%) was still lower than the average of the bilirubin stone group.

Bacterial cultures were also carried out on all bile samples and the results are demonstrated in Table 3 and Figs. 14 to 16. As with the gallbladder bile, there was no correlation between the results of bile cultures and the results of the analysis of bilirubin in the common bile duct bile.

6) Bilirubin in bilirubin stones

Figure 17 shows the chromatogram of the bilirubin in bilirubin stone. A bilirubin stone was pulverized and dissolved in dimethylsulfoxide. The solution was centrifuged and the supernatant was injected into HPLC. The bilirubin in bilirubin stone is predominantly un-

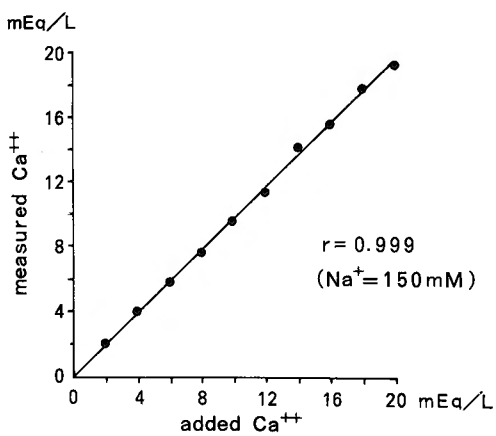


Fig. 19. Standard curve of Ca^{++} for calibration. The regression coefficient was 0.999 in the range of 2 to 20 mEq/L.

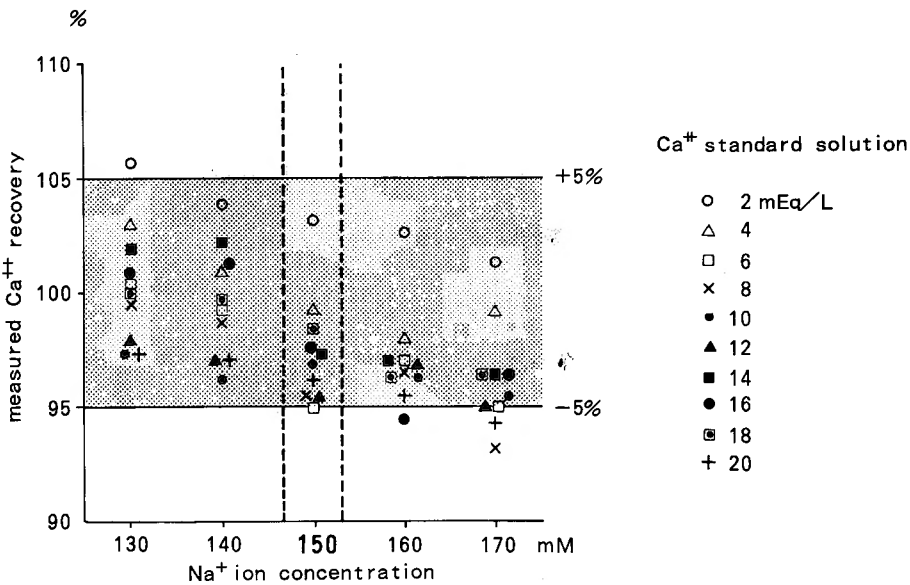


Fig. 20. Effects of Na^+ ion on measured Ca^{++}.

conjugated bilirubin (retention time 38.5 min). In addition, the retention time of calcium bilirubinate synthesized from Na-bilirubinate and $CaCl_2$[19,65], as described Edwards or Sutor, and dissolved in dimethylsulfoxide was also 38.5 min, as shown in Fig. 18.

7) The analysis of Ca ion

Standard curve (Fig. 19) showed high linearity and the regression coefficient was 0.999 from 2 to 20 mEq/L. The effect of Na ion on the measurements of Ca ion[43] was assessed in standard solutions. The results, as shown in Fig. 20, indicated that the variations remained within ±5.2% and is negligible in the physiological range of Na ion from 140 to 160 mM. The interassay and intra-assay variation coefficients for the measurements of standard solutions were 0.75% and 0.05% respectively, indicating high reproducibility of this measurements.

Measured Ca ion concentrations of diluted bile samples were almost always higher than those of the original samples. On the contrary, measured Ca ion recovery in the bile was always lower

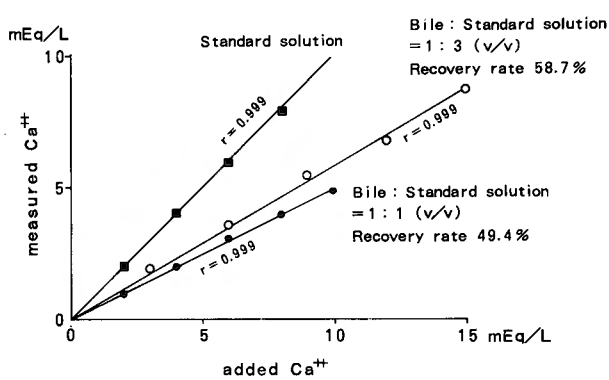


Fig. 21. Recovery test of calcium ion in human gallbladder bile.

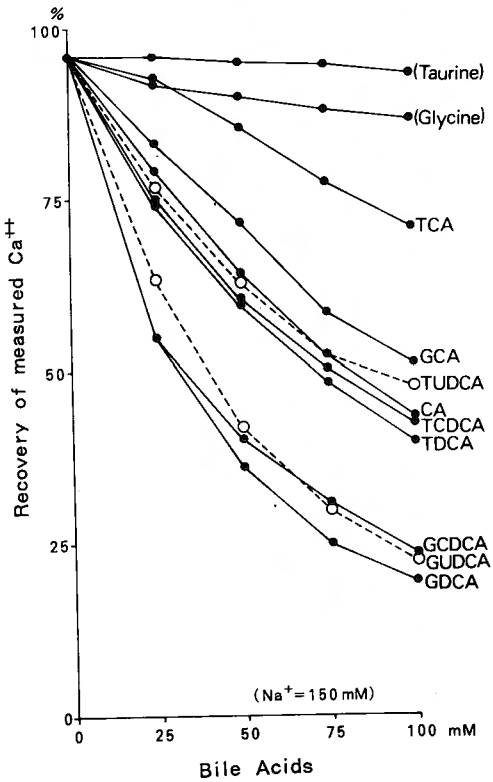


Fig. 22. Effects of bile acids on measured Ca^{++} in the standard solution (20 mEq/L).

than the theoretically calculated recovery, though at the constant ratio with each dilution of bile, as illustrated in Fig. 21. From these results of bile dilution and recovery tests, it was considered that some inhibitors against the ionization of Ca should be present in bile. Further experiments about this problem revealed that bile acids were one of the expected inhibitors in bile. Then, 9 types of free and conjugated bile acids were tested to evaluate their inhibitory effects on calcium ionization in bile[56,81]. Figure 22 illustrates the results. In summary, the effects of deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) were stronger than that of cholic acid (CA), and glycine conjugates were more potent than taurine conjugates (Table 4). Adding to the effects of bile acids, the effects of phospholipids (lecithine) with bile acids were tested[82]. As

Table 4. Summary of effects of bile acids on measured Ca^{++} in standard solution. Free forms of 100 mM DCA and CDCA did not dissolve in water.

Bile acids	Free form	Glycine conjugates	Taurine conjugates
CA	57.3%	50.0%	29.6%
DCA	turbid	80.5	60.5
CDCA	turbid	76.5	58.0
UDCA	—	77.6	54.3

(Bile acids 100 mM; Ca ion 20 mEq/L; Na ion 150 mEq/L)

Table 5. Total bile acids, total cholesterol, and total phospholipids in the human gallbladder bile from patients with and without gallstones.

Stone groups	Control (no stone)	Cholesterol stones	Bilirubin stonse	Black stones
Bile acids μmol/ml	163.6±21.7	137.4±23.4	57.8±34.4*	168.7± 36.6
Cholesterol mg/dl	277.7±42.5	247.6±43.1	116.1±34.0*	410.7±168.6
Phospholipids mg/dl	2671±337	1563±179	1222±262*	2754±252

*$p<0.05$ Mean±S.E.

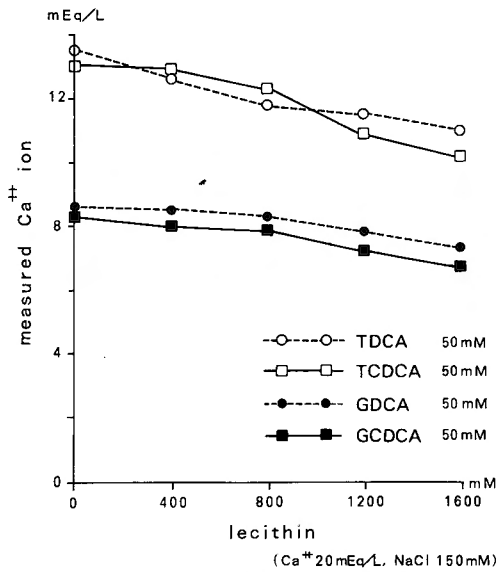


Fig. 23. Effects of phospholipids with bile acids (50 mM) on measured Ca^{++}.

shown in Fig. 23, lecithine slightly increased the effects of bile acids.

8) Total bile acids, total cholesterol and total phospholipids in the gallbladder bile

Table 5 summarizes the results. The gallbladder bile from patients with bilirubin stones showed total bile acids of 57.8±34.4 μmol/ml, total cholesterol 116.1±34.0 mg/dl, and total phospholipids 1222±262 mg/dl and all these data were significantly ($p<0.05$) lower than the other three groups. These results indicated that bile with bilirubin stones exhibits a low concentration of bile components.

Discussion

Although several methods to analyze bilirubin by HPLC were recently reported[4,5,15,28,30,32,34,51,52,83,86], none of them are sufficient in several respects. That is, the time for the analysis is too long; the separation is not complete; TLC or diazo-reaction is needed; or pretreatment to bilirubin is done before HPLC analysis. Thus these methods are not suitable for

determining the concentrations of unconjugated bilirubin in bile both accurately and rapidly. Thus, it is first necessary to establish the HPLC methods.

To establish the best condition of HPLC for the analysis of bilirubin in bile, many compositions of eluents were tested on several columns. Chloroform, acetonitrile, methyl- and ethyl-alcohol and tetrahydrofuran (THF) were tested with water, buffer solution (pH 4, 7, 8) at several mixing ratios. Dodecan-, pentan-, heptan-sulfonic acid sodium salts and tetra-n-butyl ammonium bromide were also tested as a counter ion in each eluent. After many trials, the combination with a Shimadzu PCH column and THF/water eluent was regarded as the best. Next, the optimal THF/water mixing ratio was investigated on a PCH column, by testing the ratio from 20/80 to 80/20 %/% at 5% increments. Further experiments such as these revealed that the mixing ratio of THF/water : 46/54 was most suitable, judging from the resolution factor and the separation time of bilirubin on a PCH column. However, in the chromatogram of standard solution of UCB under this ratio of eluents, the main peak of bilirubin IX-alpha still showed tailing and did not completely separate from its isomer, bilirubin III-alpha. Because it was considered that this tailing of the main peak is caused by the effects of silica-gel carriers, the effects of the pH of the eluent and/or KH_2PO_4 addition into it were tested to eliminate the tailing. KH_2PO_4 improved and the acidification of eluents with H_3PO_4 also decreased the tailing but not completely. Especially concerning acidification, pH 3 was the lower limit from the tolerance of the PCH column. Under the condition that bilirubin IX-alpha in standard solution still had the tailing, several bile samples were tentatively injected into HPLC and the chromatograms of complete separation of three bilirubin isomers were unexpectedly obtained. Furthermore, rather large amount of standard bilirubin added to bile was also completely separated into the isomers. These results suggested that, because bilirubin in bile samples apparently make ion-pairs with a certain component of bile and lose adherence to the silica-gel carrier of the column, the tailing of bilirubin IX-alpha disappears completely. As bile acids was regarded as the most probable counter ion in bile, glycocholic acid was added to standard solution, and the chromatogram of complete separation of bilirubin isomers in it were obtained as was expected. From these findings, the tailing of the peak of bilirubin IX-alpha was finally eliminated by the addition of taurine or glycine to the eluents. Thus, the best HPLC conditions for the analysis of bilirubin in bile were established.

The merits of this method are followings; 1) complete separation of three alpha isomers of unconjugated bilirubin, 2) short time for the measurements without gradient, 3) direct injection of bile into HPLC without extraction from bile, 4) direct measurement of unconjugated bilirubin in its tetrapyrolic form without using the diazo-reaction.

It has been considered that the liver normally secretes only conjugated bilirubin into bile and no unconjugated bilirubin is present in bile. In contrast to this assumption, the bilirubin in bilirubin stones is exclusively of the unconjugated type, as shown in Fig. 17. To explain this difference of bilirubin forms between in bile and in stones, hydrolysis of conjugated bilirubin into unconjugated bilirubin was thought to be theoretically needed for the initial step of the formation of bilirubin stones. It has been proposed that beta-glucuronidase from E. coli infecting

the bile may cause massive hydrolysis of conjugated to unconjugated bilirubin and that bile stasis and infection may lead to the formation of bilirubin stones[35].

Many studies in agreement with this proposal have been reported. Because E. coli accounts for only 25% of bacteria recently isolated in bile and is not the most frequently one, the activity of betaglucuronidase from other bacteria is additionally reported[68]. The reason why the number of patients with bilirubin stones have decreased rapidly in Japan associated with the Westernization of diet[26,46,47,48] is explained by the facts that the inhibitors against beta-glucuronidase, for example, glycine-conjugated bile acids[27] and/or glucaro-1,4-lactone[37,40], increase in bile from patients who take high-protein diet.

In these studies concerning the activity of beta-glucuronidase, however, the utilized substrate for the assay of beta-glucuronidase activity was phenolphthalein glucuronide (an ethereal glucuronide), which is different from bilirubin glucuronide (acyl glucuronide). The activity of beta-glucuronidase against ethereal and acyl glucuronide may differ greatly[73]. Moreover, the optimal pH of bacterial beta-glucuronidase (pH 5.5) is lower than the measured pH in bile (7.5–8.5) with stones[31]. From these findings, it is not clear whether the demonstrated increased or decreased values of the activity of beta-glucuronidase in vitro are also manifested in vivo. Basically, if bile infection is the initiating factor in the formation of bilirubin stones, determining the route is problematic[29]. It was postulated that oditis found in bilirubin stone patients caused the infection of bile. However, oditis too may be one of the results of stone formation.

Earlier studies have shown that conjugated bilirubin is unstable in alkaline solution[59] and is easily hydrolyzed non-enzymatically in it. The data of the present study demonstrated that the hydrolysis of conjugated bilirubin was also detected even in superfiltrated bile with a cut off of 25,000 daltons. The results of bile cultures indicated that bile infections are not correlated with the total bilirubin concentrations, unconjugated bilirubin concentrations, rates of hydrolysis or the type of gallstones. These findings indicated that the increase of beta-glucuronidase activity is not always necessary for the hydrolysis of conjugated bilirubin, and that bile infection may be the result of stone formation rather than the cause of it.

Recently developed thin-layer chromatographic methods have made possible the detailed analysis of bilirubin in bile and demonstrated that unconjugated bilirubin is present even in normal sterile gallbladder bile. The present study confirmed this and further the presence of unconjugated bilirubin in fresh hepatic bile.

The origin of unconjugated bilirubin found in bile is still controversial[38]. There are three possible origins; 1) hydrolysis of conjugated bilirubin in bile which is caused by enzymatic (bacterial or endogenous) or non-enzymatic (alkaline pH or the alteration of pH), 2) the direct secretion of unconjugated bilirubin from the liver[74], and 3) the spontaneous deconjugation during developments by TLC or the false detection of diazo-positive pigments other than unconjugated bilirubin. Because bilirubin is very labile under light and oxygen and TLC methods is time-consuming, the possibility of changes in bilirubin during measurements can not be excluded. The data described here eliminated those error during measurements by using HPLC system and that was one of the purposes of this study. In the gallbladder bile, the measurements by HPLC

directly showed the presence of unconjugated bilirubin even in sterile bile.

Although significantly higher concentrations of UCB were observed in bile of patients with bilirubin and black stones, the rates of hydrolysis of unconjugated and conjugated bilirubin during incubation did not differ among the four groups and was not correlated to bile infection. These facts suggested that the difference in UCB concentrations in the gallbladder bile depends on the difference in the amounts of UCB which would flow into the gallbladder with common bile duct bile.

In common bile duct bile, UCB concentrations were slightly higher in patients with bilirubin stones than with cholesterol and black stones. Unconjugated bilirubin was also detected in the hepatic bile aspirated directly from the intrahepatic duct. The rate of hydrolysis in bile duct bile from bilirubin stone patients were similar to those in the gallbladder bile, but the rate from black stone patients were higher than that from bilirubin stone patients. Bile infections did not affect the UCB concentrations in bile duct bile. It seems probable from these findings that the liver may secrete unconjugated bilirubin directly into bile and that the increase in total amounts of this hepatic secretion of UCB may contribute the increase in unconjugated bilirubin in the gallbladder bile found in bilirubin stone patients. The increased hydrolysis in common bile duct bile, probably caused by the alteration of bile pH, may contribute the increase in UCB in black stone patients.

The studies on biliary bilirubin performed until now have been based on a vague prediction that the increase of unconjugated bilirubin in bile results in the formation of bilirubin stones, whether the increase may depend on the hydrolysis of conjugated bilirubin in bile or the hepatic direct secretion of unconjugated bilirubin. However, patients and animal models of hereditary hemolytic anemia, show the high incidence of black stones[13,77,78] but not bilirubin stones, though it is demonstrated that the concentrations of unconjugated bilirubin in bile from these subjects are higher than those from normal subjects. Trotman demonstrated the high incidence of "bilirubin calcium stones", which were black stones judging from his explanation, in WBB6F mice[78] in which the increased concentrations of unconjugated bilirubin in hepatic bile were demonstrated.

The present study showed that the total bilirubin concentrations in bile from patients with bilirubin stones are lower than in bile from normal subjects and from patients with cholesterol or black stones. Clinically, bilirubin stones are the most frequent stone type found in the common bile duct, in which the concentration of the bilirubin is about ten-times lower than in the gallbladder.

The recent reports indicated that the bilirubin polymer may be the main component of black stones[12,67,84]. Other reports noted that bilirubin shows self-aggregation forming multimer above a certain concentration[14]. In common bile duct bile from black stone patients, the increase in unconjugated bilirubin at every unit of time was calculated to be about 6 times higher than in bilirubin stone patients from the data of the present study. This fact may suggest the tendency of making bilirubin multimers in the bile duct in black stone patients.

In the light of these facts and the data of this study, it appears that the increase in the bili-

rubin in bile including unconjugated bilirubin causes the polymerization of bilirubin, resulting in the formation of black stones, and bilirubin stones may be paradoxically formed in bile with low bilirubin concentrations[70].

Unconjugated bilirubin is poorly soluble in water[10], whereas the concentrations of unconjugated bilirubin in bile far exceeded its calculated concentrations in buffer solution. This indicates that some components of bile solubilizes unconjugated bilirubin. The solubilization of unconjugated bilirubin by bile salts[85] showed that the solubilization effects of bile acids depend on their concentrations in bile. From the results, it is speculated that the decrease in micelles may cause the supersaturation of unconjugated bilirubin and the formation of bilirubin stones. However, the required concentrations of bile acids to solubilize unconjugated bilirubin sufficiently are very low (5–10 mM) as shown in Fig. 9. In terms of clinical practice, patients with liver cirrhosis[50,75], in whom bile acids output from the liver decrease, have a higher incidence of black stones but not bilirubin stones. Therefore, it is difficult to explain the mechanism of the formation of bilirubin stones only with the increase and the supersaturation of unconjugated bilirubin against the micelles formed by bile acids.

The average concentration of unconjugated bilirubin in bile duct bile was 0.07 mg/dl and the daily output of bile from the liver is about 1,200 ml/day. By multiplying these values, 0.84 mg/day of unconjugated bilirubin enters into bile duct. The average bilirubin content of bilirubin stones is 30% ($34.7 \pm 2.7\%$, w/w) from the data in our laboratory[44]. The required bilirubin weight for the formation of a bilirubin stone in size of 1 cm in diameter is calculated as 150 mg. And this amount of bilirubin can be supplied in about 6 months ($150/0.84 = 179$ days). This period is consistent with that for the formation of a reccurent bile duct stone of the same size. These calculations mean that the massive increase in unconjugated bilirubin is not always needed for the formation of bilirubin stones[72].

It is reasonable from above mentioned reasons that, in the process of stone formation caused by changes in bile components[33] other than binding between bilirubin and calcium as calcium bilirubinate[9,45,58], unconjugated bilirubin which is possibly secreted directly from the liver and already present in bile, might only be deposit into the gallstones. The low concentration of bilirubin in bile might be the important condition for the deposition of bilirubin as it is, not altered into bilirubin multimers or polymers which may cause the formation of black stones.

It is reported that the increased proportion of bilirubin monoglucuronide in bile, which is associated with defective conjugation in the liver, could act as a trigger for gallstone initiation, regardless of the final composition of the stone[18]. It is interesting because some reports demonstrated the increase in bilirubin monoconjugates in bile of patients with bilirubin stones[39]. In terms of the bilirubin structure[6], monoconjugates may be more reactive than unconjugated bilirubin because monoconjugates have a free group of –COOH which may be covered by the intramolecular hydrogen bonding in unconjugated bilirubin in bile.

However, there are few reports that the increased incidence of gallstones especially bilirubin stones is found in patients with congenital non-hemolytic familiar jaundice, who have the increased concentrations of bilirubin monoconjugates in bile[21].

It was found in this study that calcium ionization was inhibited by bile acids and the degree of the effects depends upon the type of bile acid. The most effective bile acids were glycine-conjugated deoxycholic and chenodeoxycholic acid. On the other hand, Maruyama reported[36] that total bile acids and especially glycine-conjugated bile acids decreases in bile from patients with bilirubin stones. This study demonstrated that bile acids also contribute to the solubilization of unconjugated bilirubin. It was reported that biliary lipids decrease the activity of calcium in bile. Another report[61] demonstrated that UCB output is related to bile salt output. Considering these results in addition to the fact that the bile from patients with bilirubin stones had low concentrations of total bile acids and total phospholipids also measured in this study (Table 5), it seems likely that the bile of patients with bilirubin stones is a very suitable condition for bilirubin and calcium to react each other.

From these results mentioned above, it was concluded that the primary determinants of the formation of bilirubin stones are low concentrations of bilirubin, and calcium ion reactivity which depend on bile acids concentrations and their G/T ratios[80].

Conclusion

The HPLC method without TLC and diazo-reaction is suitable for the analysis of bilirubin and the HPLC conditions established in this study, which characteristically used taurine as a counter ion in the eluent, were very useful for rapid and accurate measurements of bilirubin in bile.

The following findings were obtained by using the methods;

1) The concentrations of unconjugated bilirubin in the gallbladder bile from patients with bilirubin and black stones were significantly higher than those from patients with cholesterol stones or normal subjects without stones.

2) There was no correlation between bile infection and any of the types of gallstones, the absolute concentrations of UCB, the UCB/total bilirubin ratios, or the rates of hydrolysis of conjugated to unconjugated bilirubin in the gallbladder bile.

3) Beta-glucuronidase from E. coli hydrolized the conjugated bilirubin in the incubated gallbladder bile at 37°C even under alkaline pH. However the same hydrolysis was observed in the gallbladder bile after the superfiltration with a cut off of 25,000 daltons.

4) The concentrations of UCB in common bile duct bile were relatively constant among cholesterol, bilirubin and black stone groups.

5) The rate of hydrolysis in common bile duct bile were higher from black stone patients than from bilirubin stone patients.

6) Total bilirubin concentrations in the gallbladder and the common bile duct bile from patients with bilirubin stones were lower than the other groups and is significant ($p<0.05$) in the common bile duct bile.

7) UCB was detected in fresh bile directly aspirated from a hepatic duct.

8) Bile acids contributed to the solubilization of UCB in bile.

9) Bile acids also had an inhibitory effect on calcium ionization and this effect was highest

with glycine-conjugated deoxycholic and chenodeoxycholic acid.

It is concluded from these findings that

I) Bilirubin stones are formed in bile with paradoxically lower concentrations of bilirubin and the increase in unconjugated bilirubin in bile may not cause their formation.

II) The origin of unconjugated bilirubin in bilirubin stones may be the direct hepatic secretion of it into bile.

III) Unconjugated bilirubin and calcium may easily react with each other in bile of patients with bilirubin stones in which the concentrations of both total bile acids and glycine-conjugated CDCA decrease.

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和文抄録

ビリルビン結石の成因に関する実験的・臨床的研究
Ⅱ．高速液体クロマトグラフィーによる胆石症例の胆汁中ビリルビン分析およびイオン電極法を用いたカルシウムイオン測定

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胆汁中のビリルビンとカルシウムの分析を行い，従来，ビリルビンカルシウム石と呼称されてきた，わが国に多い色素石の一つであるビリルビン石の成因に関する臨床的研究を行った．光と酸素に不安定なビリルビンに対しては，薄層クロマトグラフィーおよびジアゾ化法を必要とせず，迅速正確な分離定量が可能な高速液体クロマトグラフィーによる方法を開発し，カルシウムは，実際に反応性を有するカルシウムイオンを，カルシウムイオン選択的電極を使った血清用自動分析器を胆汁に応用可能として用いた．これらの方法による，種々の条件下の測定から，ビリルビンに関しては，以下の結果を得た．

1) 薄層クロマトグラフィーとジアゾ化法では否定できない測定誤差を，HPLC 法を応用して開発した独自の直接測定法により排除して，胆嚢胆汁中に，水に不溶の非抱合型ビリルビンが溶存していることを確実に立証した．かつ，その非抱合型ビリルビンの溶解には，胆汁酸が大きく関与していることも明らかにした．

2) 胆汁中の非抱合型ビリルビン測定値に及ぼす因子として，胆汁試料の −20°C 保存を検討し，24時間で50%の増加を示す例を認めた．この増加は抱合型ビリルビンの脱抱合によるものであり，pH 5.0 の緩衝液を加えると阻止されることを見い出した．ただし，緩衝液を添加するとビリルビンが極めて酸化されやすい状態となり，この方法では保存法を解決できないことを指摘した．

3) 抱合型ビリルビンの脱抱合は，胆汁を分子量25,000以下の超限外濾過処理後に incubate しても認められ，抱合型ビリルビンは，pH 7.0 以上では容易に非酵素的に脱抱合されることが判明した．従って，非抱合型ビリルビン測定を行う胆汁は，手術時に得られる新鮮な胆汁試料に限定し，各試料の採取から30分以内に定量を完了させた．

4) このようにして測定した胆石症例の胆嚢胆汁（50例）中非抱合型ビリルビン濃度は，ビリルビン石および黒色石群において，コレステロール石および無石の対照群よりも有意（$p<0.05$）に高かった．しかし，胆汁を incubate（遮光，アルゴンガス充塡下，38°C）して測定した抱合型ビリルビンの脱抱合速度は各胆石症群間に有意の差を認めず，特に，胆汁細菌感染の有無との関連性は認められなかった．

5) そこで，胆嚢胆汁中の非抱合型ビリルビンの由来を明らかにするため，胆管胆汁中の非抱合型ビリルビン濃度を測定した．その測定に際し，T tube 胆汁のビリルビン分画は，術中採取胆汁に比較して大きな相違が見られたので，胆汁試料は，術中採取の新鮮胆管胆汁に限定した．その結果，胆管胆汁中にも非抱合型ビリルビンが存在し，肝内胆管より直接採取した胆汁試料中にも検出されることを明らかにした．その濃度は，各胆石症3群ともに比較的一定していた．しかし，抱合型ビリルビンの脱抱合速度は，胆管胆汁でも細菌感染とは無関係に認められ，その速度は同じ色素石でも黒色石群がビリルビン石群より高い傾向を示した．

以上の成績から，非抱合型ビリルビンは肝臓から一定量ずつ直接排泄されており，その一日分泌総量の差が，胆嚢胆汁における非抱合型ビリルビン濃度の有意の差となるものと考えられた．さらに，臨床的には，胆管胆石は殆んどビリルビン石であるにも拘わらず，その濃度は胆嚢胆汁より約10倍稀薄であることから，むしろ逆説的に，ビリルビン石はビリルビン濃度の低い胆

汁中で発生するものと言え，この肝臓からの非抱合型ビリルビンの微量ではあるが連続的な直接分泌が，その成因に大きく関与しているものと考えられた．

最近，黒色石の成分としてビリルビンの polymer が推定され，ビリルビンは一定濃度以上では selfaggregation を来し multimer になることが報告されていることを考え合わせると，総ビリルビン濃度が高く，かつ，脱抱合速度が速いと非抱合型ビリルビン濃度が急激に上昇することによって，黒色石を生ずるものと思われた．

一方，胆汁中のカルシウムイオン測定から，胆汁酸がそのイオン化を抑制することを解明し，その抑制効果は，コール酸よりもデオキシコール酸，ケノデオキシコール酸のほうが，また，タウリン抱合型よりもグリシン抱合型の胆汁酸のほうが強力であった．

胆汁酸が，非抱合型ビリルビンの胆汁中への溶解に関与していること，カルシウムのイオン化を抑制していること，および，ビリルビン石症例の胆汁中では，総胆汁酸およびグリシン抱合型胆汁酸が有意に低下していることの3つの実験結果を合わせて考察すると，ビリルビン石症例の胆汁中では，ビリルビンとカルシウムが反応しやすい環境になっていると言え，ビリルビン石の重要な成因の1つであると考えられた．