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原　　著

Mechanism of the Formation of Bilirubin Calcium Stones and Black Stones

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We previously studied the causes of gallstone formation, particularly, cholesterol stones. It is clear that after the 2nd World War, the Japanese diet shifted toward the European or American models with a change in 3 factors : increased sugar intake, increased animal fat intake, and reduced crude starch and fiber intake[4] These changes produced an increase in liver hydroxymethylglutaryl-CoA reductase activity, which determines the bio-synthesis of hepatic cholesterol. We have published information on this increased activity[6]. In this paper, we will discuss a different aspect of gallstone formation : namely, the formation of bilirubin stones and black stones.

First of all, we analysed the composition of various types of gallstones. Biochemical analysis, as shown in Fig. 1, was done systematically[8] We used computer processing for multivariant analysis of inorganic substances like, Cu, Mn, Mg, Zn, Ca and P and for the values of other components. Fig. 2 shows the results of this analysis. Most gallstones can be classified into the 3 groups: cholesterol stones; bilirubin stones such as bilirubin calcium stones or bilirubin-fatty acid-calcium stones; and black stones[10].

Using this classification Fig. 3 shows the distribution of cholestrol stones according to age. As shown, the peak distribution was found in people between the age of 40 and 50. The pattern of black stones is indicated by the broken line in Fig. 4. As shown, the occurrence of black stones increases with age. The frequency of bilirubin stones, as displayed in Fig. 5, increases abruptly after the age of 50[6].

In addition to the above epidemiological study, patients with gallstone disease in which the

Key words: Bilirubin-Calcium stone, Black stone, Bile acids, Unconjugated bilirubin, Ca-ion in bile.
索引語．ビリルビンカルシウム石，黒色石，胆汁酸，非抱合型ビリルビン，胆汁中 Ca イオン．
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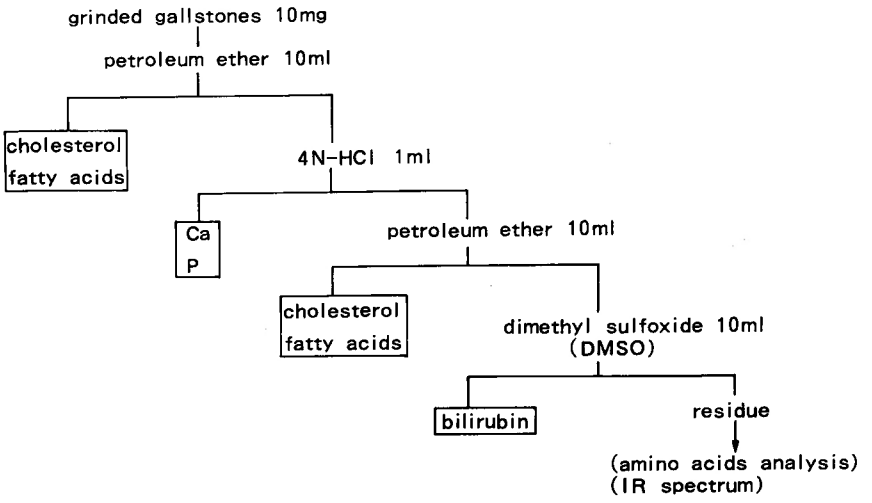


Fig. 1. Chemical analysis of gallstone

common bile duct had dilated more than 1 cm were analysed according to age and type of gallstone. The results are shown in Fig. 6. In the patients with cholesterol stones, the peak frequency appeared in the 40-50 year-old group ; however there were very few patients in this group with dilatation of the common bile duct. In the patients with black stones, the frequency was even less. However, 60-70% of the bilirubin stone patients in their 30's already demonstrated dilatation of the common bile duct, although the frequency of stone formation was still low. Such findings suggest the influence of a low protein and low fat diet over a long period which was historically characteristic of the Japanese diet[11]. These results support the classification of most gallstones into 3 types: cholesterol stones, bilirubin stones, and black stones. Of course, a good percentage of three all types of gallstone patients show dilatation of the common bile duct when they reach their 70's.

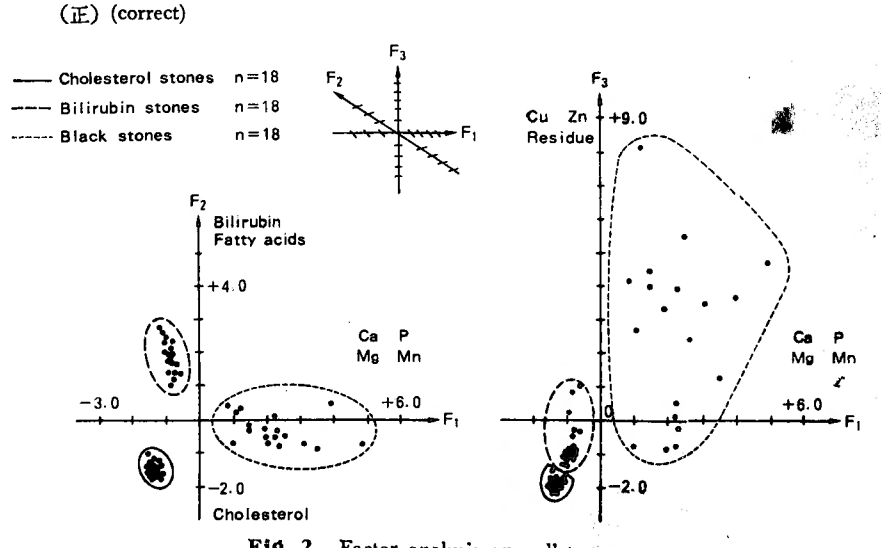


Fig. 2. Factor analysis on gallstones

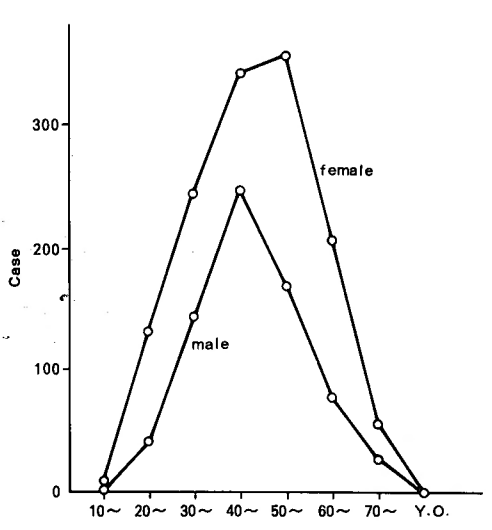


Fig. 3.

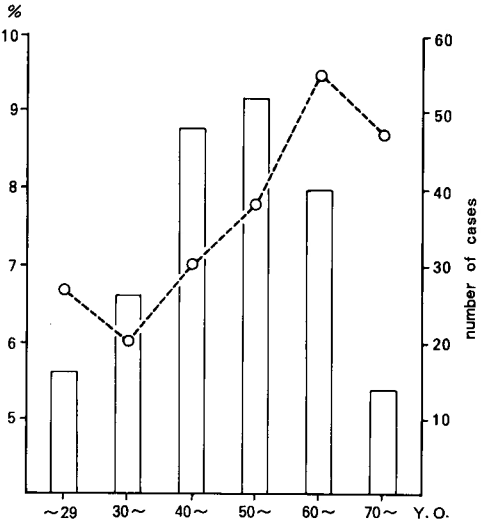


Fig. 4.

Fig. 3. Age distribution of patients with cholesterol stones in gallbladder
Fig. 4. Age distribution of patients with black stones

This can be explained as a common phenomenon of aging. Exactly the same results were obtained when a similar study was done on the three types of gallstones found in the gallbladder.

We have also analysed bile acid from the bile of patients with each type of gallstone using high performance liquid chromatography. The results disclosed that the glycine/taurine ratio and the profile of the glycine conjugates were rather similar in both cholesterol and black stone

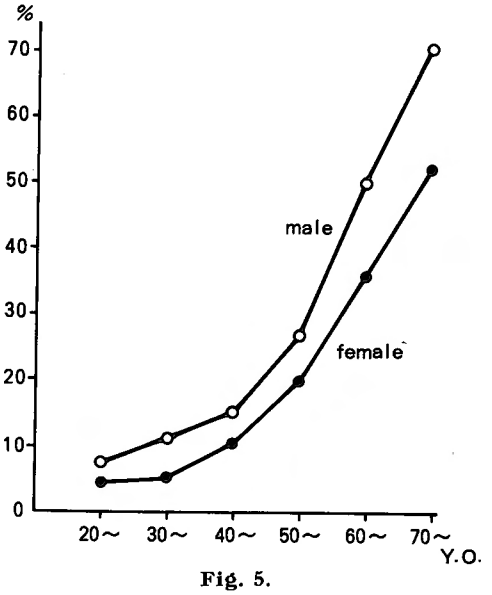


Fig. 5.

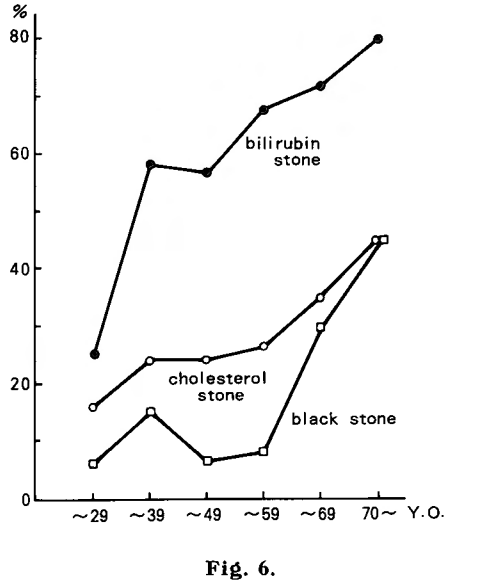


Fig. 6.

Fig. 5. Age distribution of patients with bilirubin stones
Fig. 6. Common bile duct dilatation and types of gallstones

Table 1. Comparison of cholesterol and black stones from the epidemiological survey

Similarity
(1) Both types are mainly observed in the gallbladder
(2) Both types have been increasingly observed more frequently among the Japanese
(3) Male : female ratio
(4) Bile acid composition and glycine／taurine ratio in conjugated bile acids, and the amount of total bile acids
(5) No abnormal dilatation of common bile duct before formation of gallstone
Difference
Age distribution

patients[7]. After all, both similarity and reciprocity are found between the two, as indicated in Table 1. We also found that only bile from patients with bilirubin stones always contained extraordinarily low levels of glycochenodeoxycholic acid. In normal bile, glycochenodeoxycholic acid is the largest constituent. This is the case even when there is a bile infection. This is shown in Figs. 7 and 8. Glycine conjugates have a particularly strong inhibitory effect on calcium ionization in bile as shown in Fig. 9; that is, it inhibits the formation of calcium ions which are able to combine with unconjugated bilirubin. Bile acids, forming micelles in bile, make compound substances with calcium ions. These compound substances inhibit the ionized state of calcium. The glycine conjugates show the highest inhibitory effect on the bile acids[14].

On the other hand, the dissolution of unconjugated bilirubin into bile largely depends on bile acids as shown in Fig. 10. Therefore, the reduction of glycine conjugates especially, glycochenodeoxycholic acid, in bile accelerates calcium ionization and decreases the dissolving capability of unconjugated bilirubin in bile. In this way, a favorable environment is created for the formation

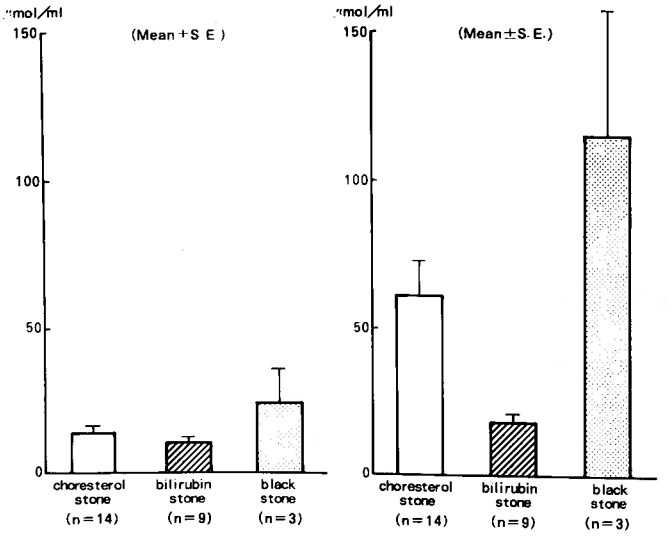


Fig. 7. Concentrations of taurince and glycine conjugated bile acids in gallbladder bile

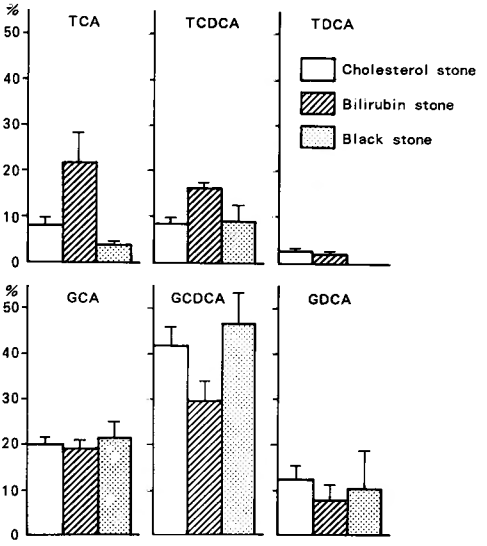


Fig. 8. Conjugated bile acid composition of gallbladder bile

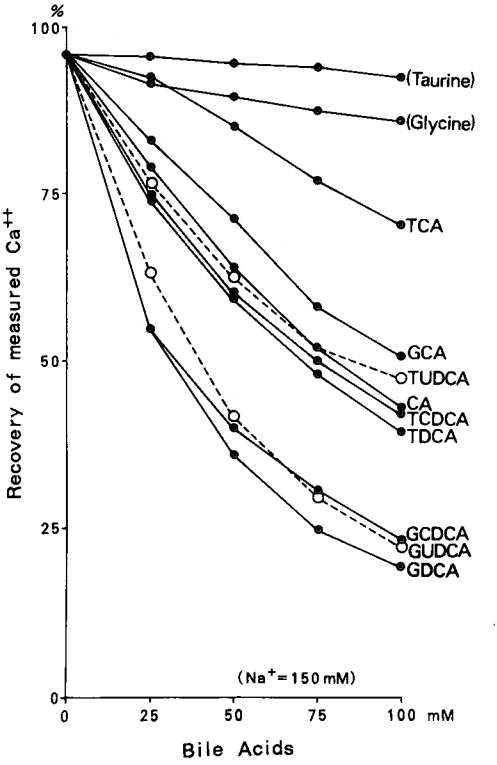


Fig. 9.

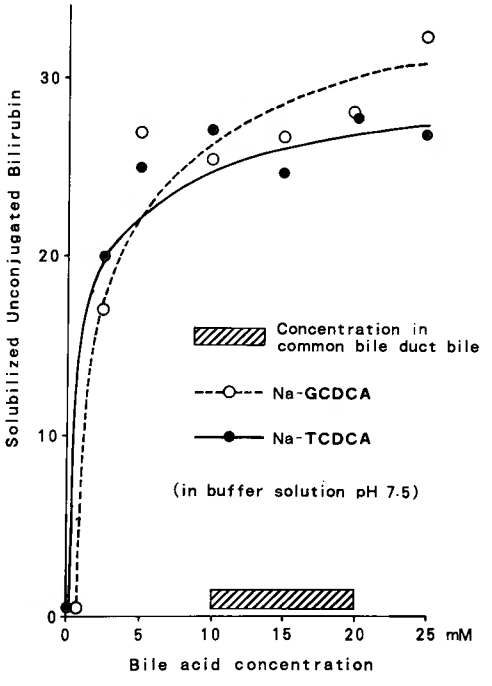


Fig. 10.

Fig. 9. Effects of bile acids on measured Ca^{++} in the standard solution (20 mEq/L).
Fig. 10. Solubilization of unconjugated bilirubin sodium salt by bile acids measured by HPLC.

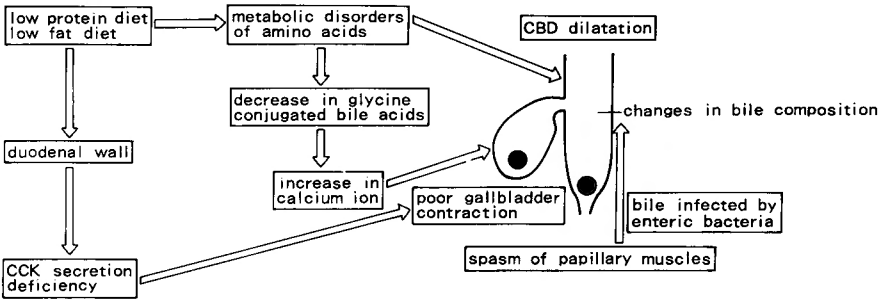


Fig. 11. Hypothesis of bilirubin stone formation

of bilirubin calcium stones in bile.

Given this process of bilirubin stone formation, we might consider the following hypothesis. For many years in the past the characteristic Japanese diet was low in both protein and fat. Insufficient protein intake might cause a deficiency affecting common bile duct development and also decrease digestive hormone secretion. Subsequently, a decline in the excretion of cholecystokinin (CCK), a type of gut hormone, may result in improper function of the biliary tree. The release of CCK from the pancreas is triggered only when enough protein and fat are taken in. When protein and fat intake is low, bile tends to be stagnant, and the common bile duct progressively dilates creating an environment for bilirubin stone formation. Unfortunately, the stagnation of bile allows enough time for the reaction between unconjugated billirubin and ionized calcium in the manner

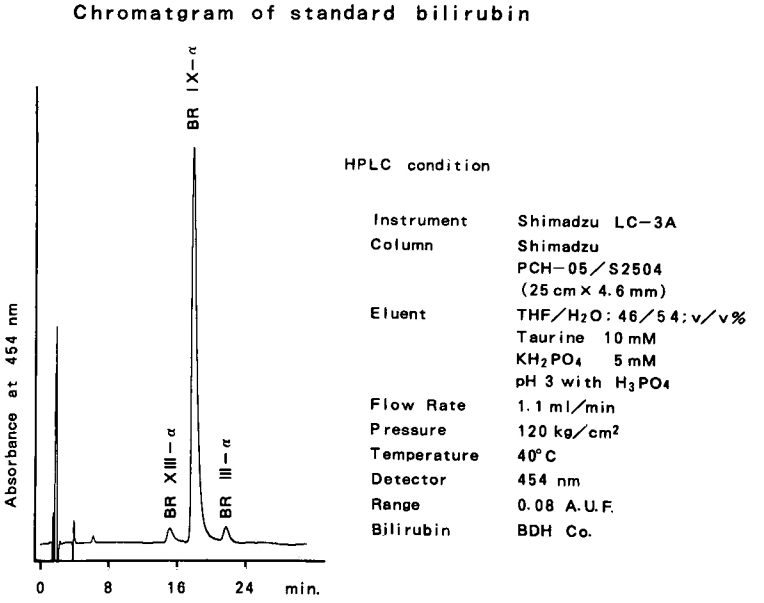


Fig. 12. Chromatogram of standard solution of commercial bilirubin and HPLC conditions for the quantitative analysis of unconjugated bilirubin in human bile. Complete separation of three bilirubin isomers was obtained by using taurine in the eluent.

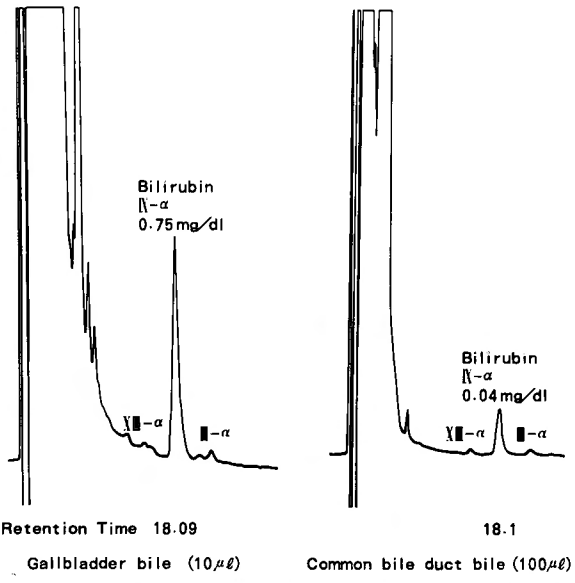


Fig. 13. Chromatograms of human bile. Concentration of bilirubin IX-alpha were calculated with reference to the calibration curves

just explained. A decrease in glycine conjugates in bile, which contributes to a favorable environment for stone formation, appears to be due to a shortage of glycine-rich animal protein, as was the case with the Japanese diet in the past. If we follow this concept, the reason for the very frequent development of bilirubin calcium stones among Japanese in the past may be explained by the process shown in Fig. 11. Therefore, we suggest that the initiating factor for bilirubin calcium stone formation is the diet, rather than a local factor such as biliary infection.

In addition to factors in the diet, we should also examine the biochemistry of bilirubin as another component contributing to bilirubin calcium stone formation. We used high performance liquid chromatography to examine whether unconjugated bilirubin could be found in human bile

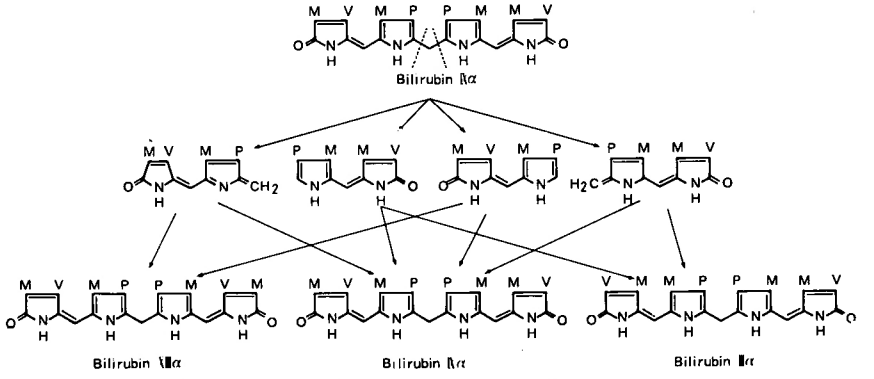


Fig. 14. Isomerization of bilirubin IX-α

Biochem. J. 170: 297-311, 1978
Brown SB et al.

and, if so, what type there was. The results are shown in Figs. 12 and 13[14]. Mainly unconjugated bilirubin of the IX-α type was found in the bile of patients with bilirubin calcium stones. The isomers of free III-α type and XIII-α type (Fig. 14)[2] were also present, though only in trace amounts. As for IX-α type free bilirubin, either the cis or trans type is formed, depending on the direction of the double bond between the two sets of pyrrole nuclei. After all, as shown in Fig. 15, 4 kinds of optical isomers, i. e., ZZ, ZE, EZ and EE, can be formed[12]. It was said that free bilirubin of the IX-α type in human bile is the ZZ isomer (Fig. 16)[1]. The concentration of unconjugated bilirubin of IX-α type was significantly increased in the gallbadder bile of patients with bilirubin calcium stones. In the duct bile, however, there was no increase, even if the bile was infected. These are shown in Figs. 17 and 18.

Despite these findings, clinical and epidemiological studies showed that the frequency of bilirubin calcium stones in the gallbladder, and in the common bile duct, is roughly even in Japan. There is even some literatures suggesting that stone formation of this type in the common bile duct is more frequent (Table 2). This fact suggests that, while the existence of unconjugated bilirubin in bile is surely necessary for the formation of bilirubin calcium stones, a remarkable increase in it is not absolutely needed. Indeed, bilirubin calcium stones are formed in bile which has a low concentration of unconjugated bilirubin. It is unlikely that an increased concentration of unconjugated bilirubin in bile plays a key role in forming bilirubin calcium stones.

The conventional theory on the cause of bilirubin calcium stones states that unconjugated bilirubin in infected bile is increased, and it plays a leading role in the formation of bilirubin

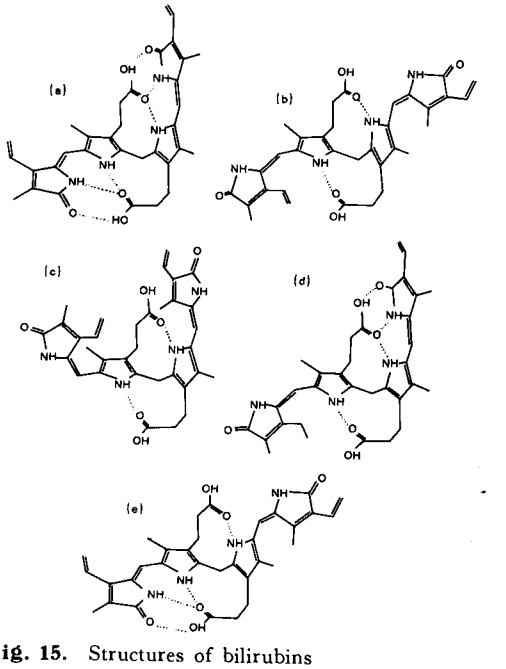
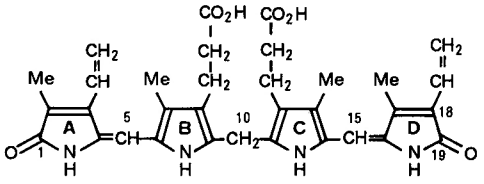


Fig. 15. Structures of bilirubins
Biochem. J. 183: 139-146, 1979
Stoll, MS et al.



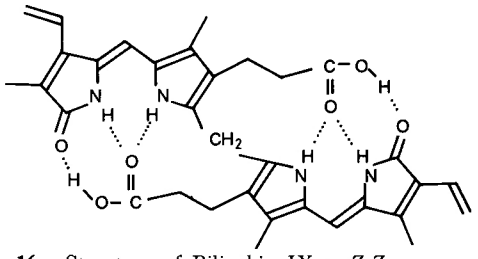
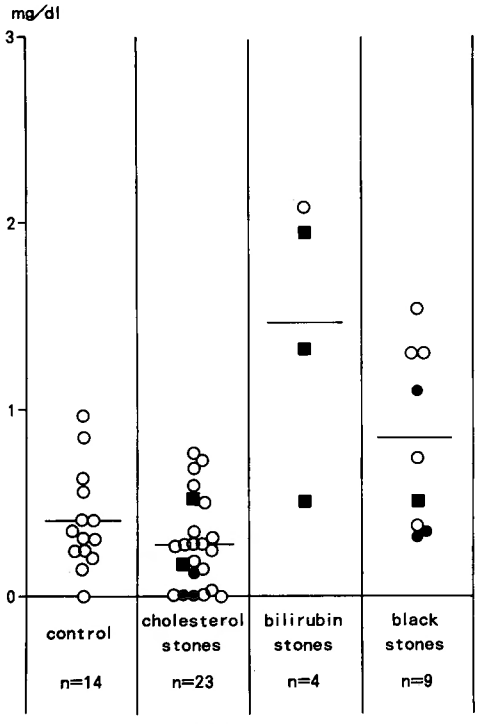


Fig. 16. Structure of Bilirubin IX-α, Z-Z
Nature 262: 326-328, 1976
Bonnett R et al



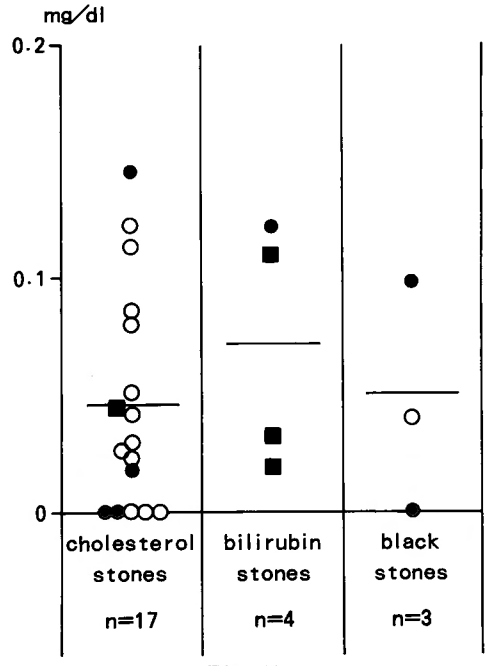


Fig. 17. **Fig. 18.**

Fig. 17. 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. 18. 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.

Table 2. Type of gallstones and localization in the biliary tract

	GB	GB + CBD	CBD	IHD	other: cystic duct remnant of small intestine	total
cholesterol stones	2,617	384	102	24	0	3,127
bilirubin stones	395	297	383	164	(3 2	1,244
black stones	274	27	1	3	0	305
total	3,286	708	486	191	5	4,676

GB: gallbladder, CBD: common bile duct, IHD: intrahepatic bile duct

calcium stones. On the contrary, the results we have found suggest the significant factor in bilirubin calcium stone formation lies in the ionization of calcium in bile. Also, the conventional theory of bilirubin calcium stone formation cannot explain the reason for the recent drastic reduction in bilirubin calcium stones in Japan.

Results on analysis of bilirubin in bilirubin calcium stones using high performance liquid chromatography (HPLC) are shown in Fig. 19. The stones are dissolved with dimethyl sulfoxide and show a peak with retention time of 38.5 minutes. Such a significant peak suggests the existence of unconjugated bilirubin of the IX-α type. As shown in Fig. 20, its retention time was same as that of the standard synthetic bilirubin calcium. Accordingly, it was verified that the form of bilirubin contained in bilirubin calcium stones is unconjugated, free bilirubin of the IX-α type.

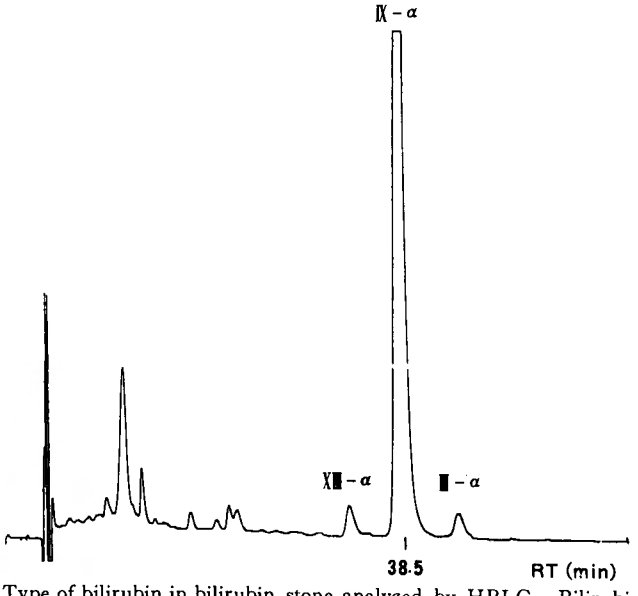


Fig. 19. Type of bilirubin in bilirubin stone analyzed by HPLC. Bilirubin stone was dissolved with dimethyl sulfoxide. As seen in the chromatogram, unconjugated bilirubin IX-alpha was the predominant form in bilirubin stone.

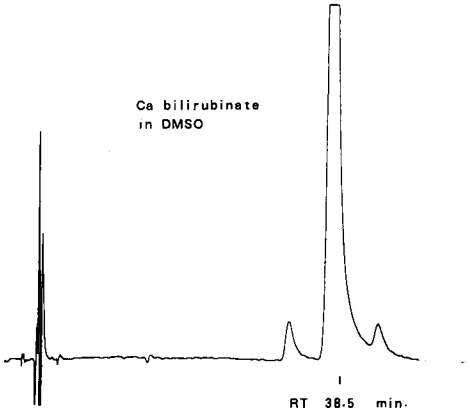


Fig. 20. Chromatogram of synthesized calcium bilirubinate dissolved in dimethyl sulfoxide. The retention time of bilirubin was the same as that of unconjugated control.

It can be said that bilirubin calcium stones are formed in rather dilute bile, as shown in Figs. 21 and 22. As mentioned already, hepatic bile shows a low concentration of unconjugated bilirubin in patients with bilirubin calcium stones, but significantly high concentration is observed

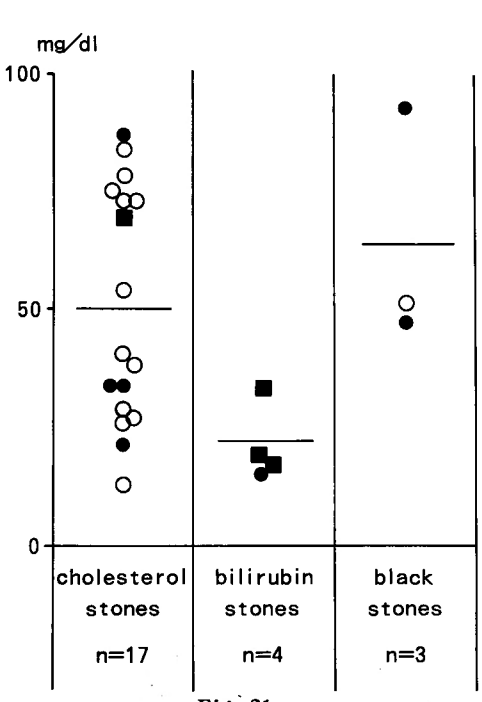


Fig. 21.

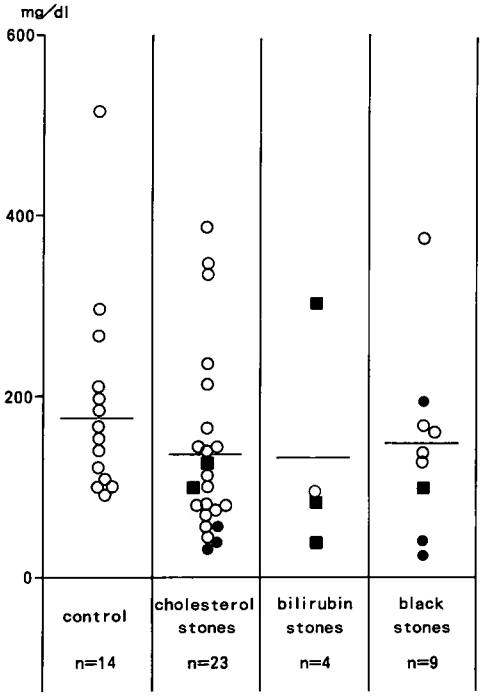


Fig. 22.

Fig. 21. Total bilirubin concentration in human common bile duct bile. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

Fig. 22. Total bilirubin concentration in human gallbladder bile. ○ no growth, ■ E. coli, ● other bacteria, by bile culture.

in gallbladder bile. We must consider the possibility that de-conjugation may occur in conjugated bilirubin, even after bile is excreted from the liver. Fig. 23 shows the results of analysis of human bile which was collected and immediately incubated at 38°C, under argon gas, and analysed by HPLC to determine the ratio of deconjugated bilirubin. The percent of the di-conjugated bilirubin is reduced, and the percent of both mono-conjugated and unconjugated bilirubin is increased. The peak with the 4 mark in this figure might show the mono-conjugated bilirubin obtained by the de-conjugation of glucuronic acid from a special di-conjugated bilirubin having a glucuronic conjugation at one end, and material other than glucuronic acid, such as xyloside or glycoside at another end.

When the de-conjugation ratio of conjugated bilirubin was measured, as shown in Fig. 24, it was independent from bacterial infection in bile, and the degradation reaction was higher in bile of black stone patients than of bilirubin calcium stone patients. These results suggest that the concentration of unconjugated bilirubin in gallbladder bile should be higher in patients with black stones than in patients with bilirubin calcium stones. Actually, the reverse relation prevails.

This can be explained as follows : in patients with black stones, glycochenodeoxycholic acid is not reduced. Accordingly, the ionization of calcium does not take place in the bile and

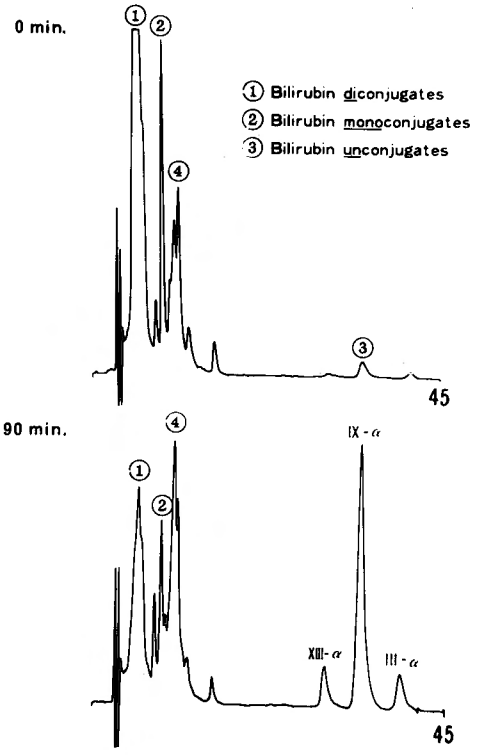


Fig. 23. HPLC analysis of hydroysis of bilirubin conjugates in human bile during incubation with beta-glucuronidase from E. coli. Peak 4 was considered to be bilirubin monoconjugates other than monoglucuronides.

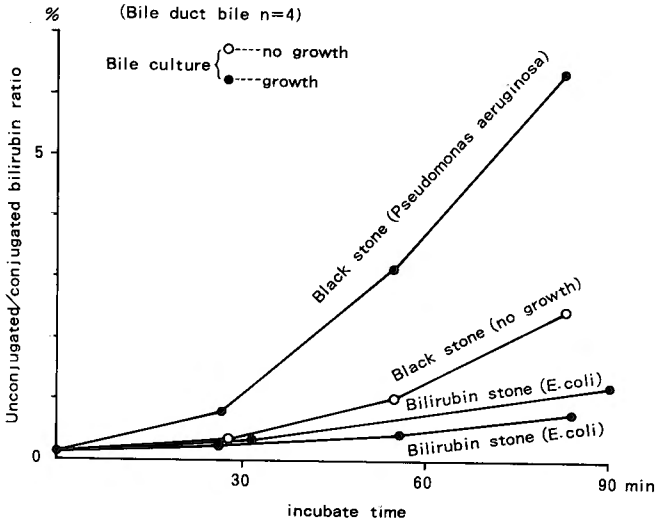


Fig. 24. Changes of unconjugated/conjugated bilirubin ratio during incubation

bilirubin calcium stone formation is not possible. As clarified already by Carey et al., the unconjugated bilirubin in bile begins a self-aggregation reaction above a certain concentration, and the dimer → multimer → polymer shift will take place[3]. Because of this, quantitative analysis of free bilirubin is not possible in bile of patients with black stones. From the HPLC qualitative viewpoint, the properties of the dimer, multimer and polymer are entirely different. Because of

Fig. 25. Intramural black stones in the gallbladder

the role of unconjugated bilirubin in stone formation, bilirubin calcium stones and black stones should be clearly classified in different categories according to their pathological processes. But when the self-aggregation reaction proceeds, it does not mean that the black stones are formed immediately. Co-existence of self-aggregated bilirubin and a high molecular protein excreted into the bile favors black stone formation. In view of this, the initial reaction in black stone formation may occur in Rokitansky-Aschoff sinus, since all the calculi in the gallbladder wall of patients with cholecystitis showed only black stone properties, as shown in Fig. 25. After this initial reaction in the gallbladder wall, the growth can be either in Rokitansky-Aschoff sinus or in the gallbladder. In regard to the shift of free bilirubin to the dimer, multimer or polymer type, the catalytic reaction of Cu cannot be completely dismissed. As we will explain later, Cu is always present in black stones and, as shown in Fig. 26, the movement of Cu into bile always paralleled bilirubin excretion.

The facts we have just mentioned seem to include some problems. If bile of patients with bilirubin calcium stones is a residual bile left after bilirubin calcium stones have formed, the bile does not fully reflect what was present the time of the actual bilirubin calcium stone formation. The best evidence, however, was obtained from a patient with primary common bile duct stones. In the initial operation we performed a cholecystectomy and choledocholithotomy, but one year later the patient developed recurrent stones in the common bile duct. Studies have shown that the outer layer of bilirubin calcium stones removed at the initial operation had exactly the same composition, as shown in Fig. 27, as that of the inner layer of recurrent stones, as indicated in Fig. 28. The outer layer of initial stone and inner layer of recurrent stone were almost identical. In this case, it can be assumed that the bile composition in the common bile duct is always

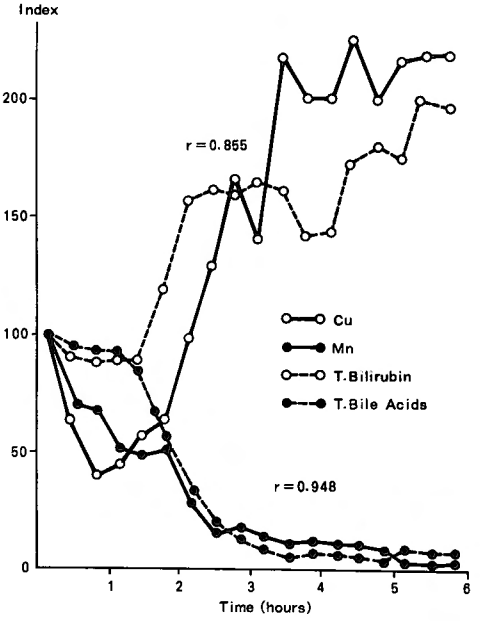
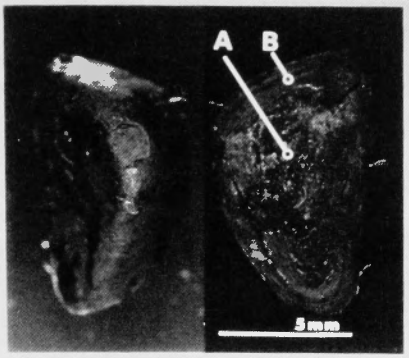


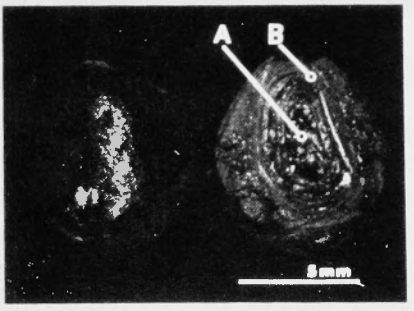
Fig. 26. Excretion of bile components



CHEMICAL COMPOSITION

	A	B
Cholesterol	4.5 %	7.0 %
Bilirubin	29.4	39.3
Fatty Acids	22.1	13.1
Ca	2.1	1.7
PO_4	0.2	0.3
CO_3	0.0	0.0
Protein	—	—
Residue	0.9+α	4.2+α

Fig. 27.



CHEMICAL COMPOSITION

	A	B
Cholesterol	6.2 %	6.0 %
Bilirubin	38.7	43.6
Fatty Acids	13.8	6.4
Ca	1.6	1.2
PO_4	0.1	0.2
CO_3	0.0	0.0
Protein	—	—
Residue	1.3+α	2.1+α

Fig. 28.

Fig. 27. Common bile duct stone (bilirubin stone) at the initial operation
Fig. 28. Recurrent common bile duct stone (bilirubin stone) at the second operation

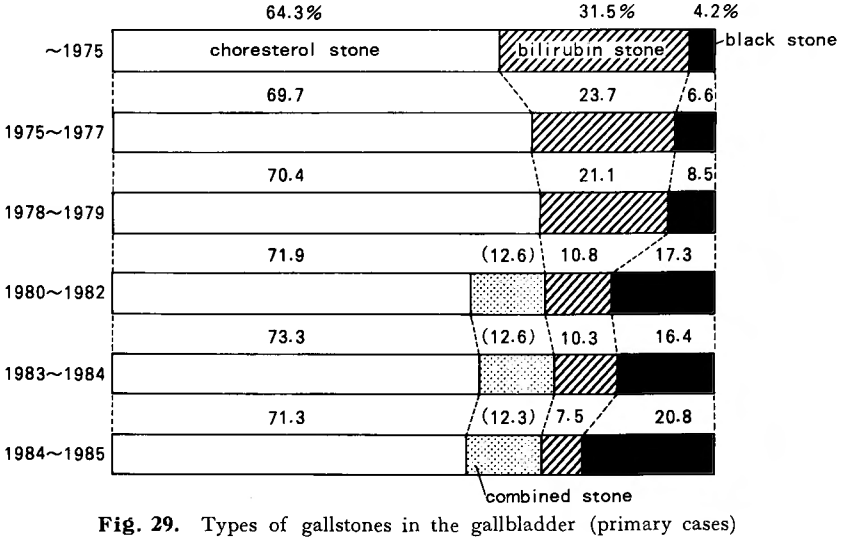


Fig. 29. Types of gallstones in the gallbladder (primary cases)

Table 3. Classification of black stones

components \ type	I (n=23)	II (n=31)	III (n=4)	IV (n=24)
	%	%	%	%
Ca	2.7±0.3	12.3±1.0	23.6±2.0	27.0±1.6
PO_4	1.4±0.3	16.7±1.6	17.0±3.6	1.4±0.3
CO_3	1.1±0.3	1.2±0.3	18.6±5.6	38.5±2.6
bilirubin	17.2±1.7	11.7±1.0	5.5±0.9	5.2±0.8
cholesterol	0.7±0.2	0.4±0.1	0.2±0.1	0.8±0.4
fatty acids	0.9±0.2	1.3±0.3	1.3±0.4	1.3±0.3
residue	36.7±3.0	21.2±3.0	6.2±3.5	8.9±2.0
protein	8.5 (n=9)	2.3 (n=7)	2.6 (n=1)	3.2 (n=5)
unknown	41.0±2.0	35.3±1.4	25.6±3.9	17.5±1.9

mean±S.E.

maintaied in the same condition, and results in repeated formation of bilirubin calcium stones[15].

Black stones have a black colour, and show no definite structure when observed under the scanning electron microscope[16]. They are a type of gallstone produced mainly in the gallbladder. Our epidemiological survey in Japan indicated that the frequency of black stones has recently increased. This is clearly shown in Fig. 29. These results suggest that diet is involved in this type of stone formation. In light of this, the recent trend in Japan toward the European or American diet needs attention.

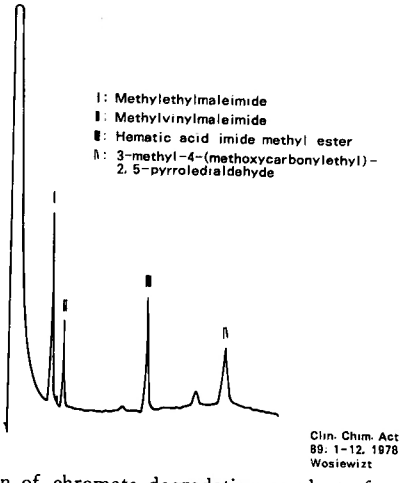


Fig. 30. GLC-separation of chromate degradation products from "black" pigment in black stone

Generally speaking, black stones can be classified into two major groups according to appearance. In one group, the colour is balck and the shape is of a coke or coal form. When the composition of this group is analysed biochemically, there is a large black residual component, the content of Cu is large, and S is also present. In the other group, the main components are calcium carbonate, calcium phosphate, Mn and Mg. The black residue and bilirubin contents are relatively low, and the ranges from shape spherical to a ragged ball form. Subsequent studies show that each group can be further divided into 2 subgroups. In total, as shown in Table 3, black stones can be classified into 4 types[9]. As can be seen from the biochemical analysis of black stones, the collapse of body protein, even membrane protein, appears closely related to black stone formation.

In the past, there was a great deal of discussion about the black residue contained in black stone. Wosiewitz et al. reported it was a degenerated tetrapyrrolic substance from bile pigment as indicated in Fig. 30[18]. Prof. N. Suzuki of Tohoku University thought it consisted of a metal complex of bilirubin and a polymer of a bilirubin derivatives[13]. To clarify this, we performed further studies on the chemical composition of the black residue. The residual components of black stones were dissolved as much as possible, and 5% SDS-polyacryl gel electrophoresis and amino acid analysis were done. From this we obtained the amino acid composition shown in Table 4. In addition, a high polymer protein having a molecular weight of at least one million was found, as shown in Fig. 31. When hydrolysis was done prior to amino acid analysis, black-coloured, non-hydrolyzed fumin was always produced. Therefore, it appears the stones also contained a sugar substance. This high molecular weight polymer protein with sugar appropriately called a "proteoglycan", always existed in the crude black residue component. Consequently, S was naturally found there, as shown in Fig. 32. Therefore, when hydrolysis by caustic soda and neutralization followed by processing and refining are performed on the above crude residues, the final, black-coloured residual components are obtained. Analysis of this material shows the black

Table 4. Composition of amino acids in black stone

Lys	5.9%
His	2.1
Arg	5.3
Asp	9.6
Thr	4.9
Ser	5.4
Glu	12.4
Pro	5.3
Gly	8.8
Ala	5.9
Cys	8.6
Val	6.6
Met	1.4
Ile	2.1
Leu	6.6
Tyr	3.3
Phe	5.8

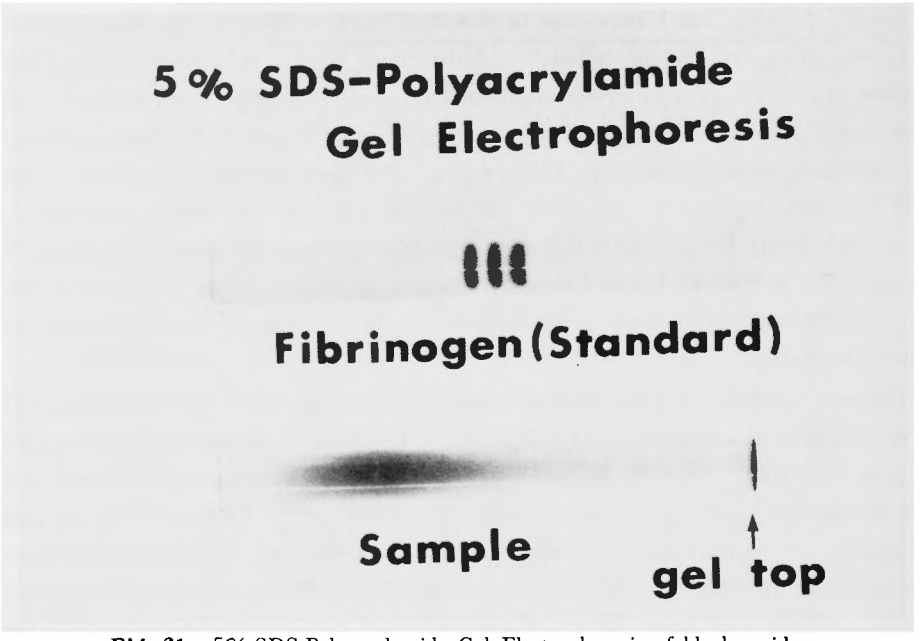


Fig. 31. 5% SDS-Polyacrylamide Gel Electrophoresis of black residue

components are made only of carbon, hydrogen, nitrogen and oxygen, and no metal element, except for Cu. The percentage of Cu by weight is nearly constant in all types of black stones, as shown in Table 5[10] After gel filtration analysis and each fraction is analysed by spectrophotometry, the spectral absorbance of Cu always maintained a parallel retention. This suggests that the black

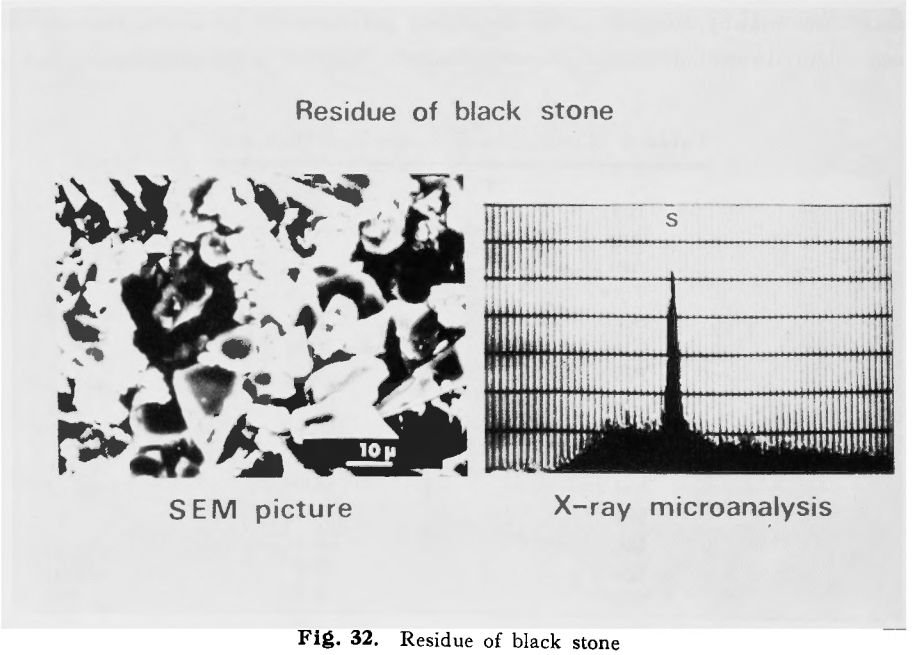


Fig. 32. Residue of black stone

Table 5. Cu concentration in extraced fraction from black stones

black stone	Ⅱ) 1N+HCl	Ⅳ) DMSO	Ⅴ) residue	Ⅳ) final residue
	ng/ml	ng/ml	μg/g	μg/g
No. 1	2830	2430	2090	177
2	8750	2360	10600	177
3	3770	1740	1870	134
4	1970	645	1220	190
5	9320	2240	2250	98
6	1090	777	1890	170

final residue: black coloured precipitates after alkaline hydrolysis followed by HCl neutralization

residual component in black stones was made of a copper binding substance. To examine the properties of the material binding Cu, gel filtration analysis was carried out. The results are shown in Fig. 33 and 34. The material in question has a maximum molecular weight of about 5 million. During gel filtration, we found that the pitch black colour of the final residue changed to yellow during the filtration step. Therefore, we concluded that the final black residue was a bilirubin polymer.

In an epidemiological study by Soloway and his group, pigment stones were not as rare in the U.S.A. as they were supposed to be in Fig. 35[17] In Japan, studies indicate that pigment stones are characteristic of aged patients. After, Soloway spent some time in our laboratory, he was convinced that the pigment stones were not bilirubin calcium stones, but the black stones we refer to.

Considering the above facts, we undertook further studies to assess whether or not aging

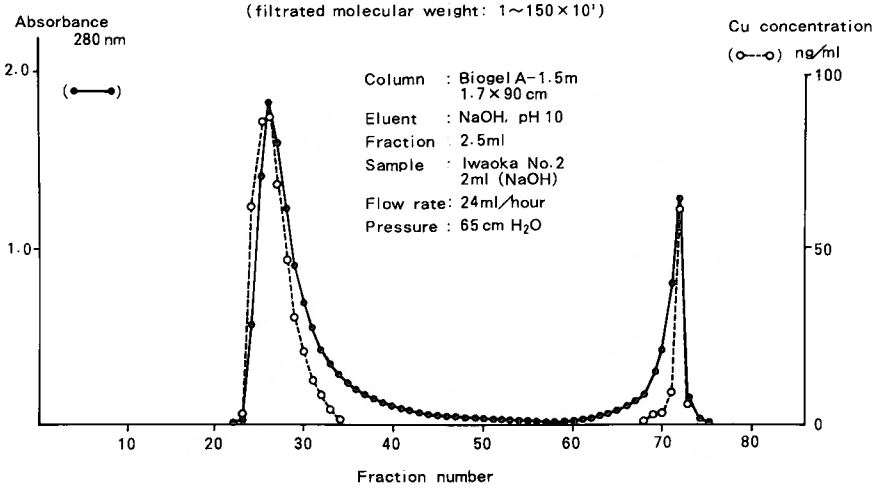


Fig. 33. Gel filtration of black components
(filtrated molecular weight: 1~150×10⁴)

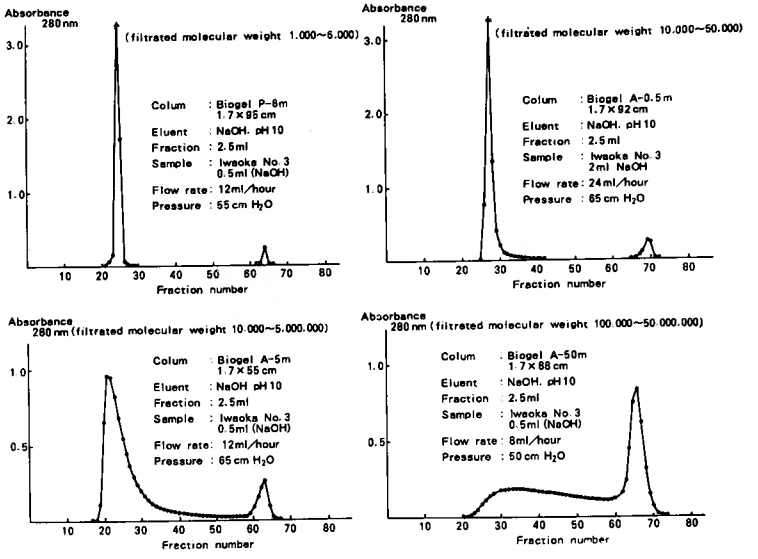


Fig. 34. Gel filtration of black components

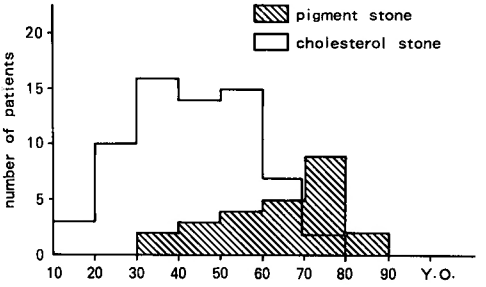


Fig. 35. Age distribution of types of gallstones
(B.W. Trotman et al. 1975, Philadelphia)

results in black stone formation. We selected aged hamsters in which the collapse of body protein is apt to have a physiological cause. The hamsters were given an experimental diet corresponding to the western diet (lithogenic diet) which produces pure cholesterol stones as previously reported. The precise composition of the diet is shown in Table 6. We fed this experimental diet to aged hamsters. They did not produce pure cholesterol stones at a rate of 100%, but 20-30% of them formed the experimental black stones shown in Fig. 36[5].

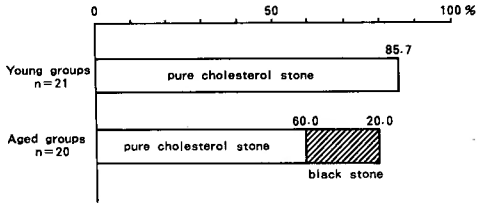


Fig. 36. Experimental gallstone formation in hamsters

Table 6. Composition of experimental diet for induction of pure cholesterol stone (Hamster)

Glucose	60.0%
Casein	20.0
Butter	10.0
Vitamins	1.5
Minerals	5.0
Cellulose	3.5

Summary

For our basic analysis of bile components we used high performance liquid chromatography which promptly and accurately separates and quantitatively analyses bilirubin without using thin layer chromatography with diazo-reaction. This is important because bilirubin is especially unstable in light and oxygen. We also found a method to determine the concentration of calcium ions in bile using a calcium ion analyser with an ion selective electrode. Our purpose in undertaking this research was to search for a better explanation for the formation of bilirubin calcium stones and black stones, as the conventional explanation failed to fully satisfy us.

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和文抄録

ビリルビンカルシウム石および黒色石の形成機序

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コレステロール石の成因は，食餌性因子による肝コレステロール生合成の律速酵素活性亢進に起因することを証明した．一方，ビリルビン石や黒色石と呼称されるいわゆる色素胆石の成因は，疫学的考察や臨床的経験から幾つかの仮説が提唱されているものの，その具体的根拠は曖昧な状態であった．我々が色素胆石の成因について今日までに得た成果を中心としてその成因を検討した．

従来から肉眼的外観で分類されてきた各種胆石について，その構成成分の詳細な系統的化学的分析結果と原子吸光分析で得られた無機物 (Cu, Mn, Mg, Zn) の含有値についてコンピューターによる多変量解析（因子分析）を行なった結果，ヒト胆石はコレステロール石，ビリルビン石，黒色石に分類されることを示し，これに基いて色素胆石の研究を開始した．

ビリルビン石の成因は，低蛋白・低脂肪食により胆汁中総胆汁酸濃度なかでもグリシン抱合型胆汁酸が減少し，Ca がイオン化しやすくなるとともに，疎水性の非抱合型ビリルビンの溶解度が下がり，Ca イオンとビリルビンが反応，析出しやすい環境になることを，HPLC を用いた胆汁酸とビリルビンの分析および Ca イオン濃度の測定から明らかにした．

他方，黒色石については，黒色成分の原子吸光分析やゲル濾過，ゲル電気泳動，HPLC によるビリルビンやアミノ酸分析まで徹底した分析を行ない，この黒色成分がビリルビンの Polymer と Cu との結合物であると考えられ，その生成には胆汁中で十分な胆汁酸と高分子蛋白および Cu の存在下に，非抱合型ビリルビンが増加することが必要であることが示唆された．