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Composition of Intrahepatic Calculi
Etiological Significance

NORIO YAMASHITA, MD, JIRO YANAGISAWA, MD, and FUMIO NAKAYAMA, MD, FACS

Gallstones in intrahepatic (N = 42) and extrahepatic (N = 22) bile ducts and gallbladder (N = 23) were subjected to chemical analysis modified to suit the analysis of brown pigment stones with the aim of determining if stone location at surgery influenced stone composition. Dimethylsulfoxide–acetone–1 N HCl (90:9:1, v/v/v) was used to dissolve gallstone specimens. Intrahepatic calculi were divided into two groups, ie, nine cholesterol stones and 33 brown pigment stones. Cholesterol stones in the intrahepatic bile ducts had a similar composition to those in the gallbladder and extrahepatic bile ducts, suggesting a similar pathogenesis wherever formed throughout the biliary tract. Intrahepatic brown pigment stones contained significantly less bilirubin ($P < 0.001$) and more cholesterol ($P < 0.05$) by chi-square analysis than brown pigment stones found in the extrahepatic bile ducts, suggesting that the site of formation affects stone composition and modifies stone pathogenesis.

KEY WORDS: intrahepatic calculi; gallstone analysis; brown pigment stone; dimethylsulfoxide.

Intrahepatic calculi or hepatolithiasis is prevalent in East Asia including Japan, China, Korea, Taiwan, Philippines, Vietnam, Thailand, Malaysia, Singapore, and Indonesia (1, 2). The distinctive feature is an intractable course requiring multiple operative interventions with frequent recurrence. This is in distinct contrast to cholesterol or black pigment stones which originate in the gallbladder and, when present in the extrahepatic bile ducts, are removed with an uneventful recovery after common bile duct exploration. In contrast, intrahepatic calculi are mostly brown pigment stones (calcium bilirubinate stones) and are prone to recur whenever bile stasis and bacterial infection remain in the biliary tract.

In the present communication, the composition of intrahepatic calculi has been chemically analyzed and compared with that of stones in either the gallbladder or extrahepatic bile ducts with the aim of determining whether the site of formation affected stone composition and therefore modified stone pathogenesis. The method of analysis had to be modified to suit brown pigment stones, the predominant stone type of intrahepatic calculus, since the extracting solvent used in the previous analytical procedure was found to be unsatisfactory, leaving considerable unextractable residue.

MATERIALS AND METHODS

The patients studied were admitted to Kyushu University Faculty of Medicine, Department of Surgery I, from 1973 to 1983. Gallstones from the intrahepatic bile ducts of 42 cases were analyzed, representing 70% of all patients operated upon for hepatolithiasis at our institution during 11 years. Thirty-four had gallstones also in the extrahepatic bile duct and/or gallbladder. For comparison, gallstones in extrahepatic bile ducts from another 22

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From Kyushu University Faculty of Medicine, Department of Surgery I, Fukuoka, Japan.

Address for reprint requests: Prof. Fumio Nakayama, Kyushu University Faculty of Medicine, Department of Surgery I, Fukuoka 812, Japan.

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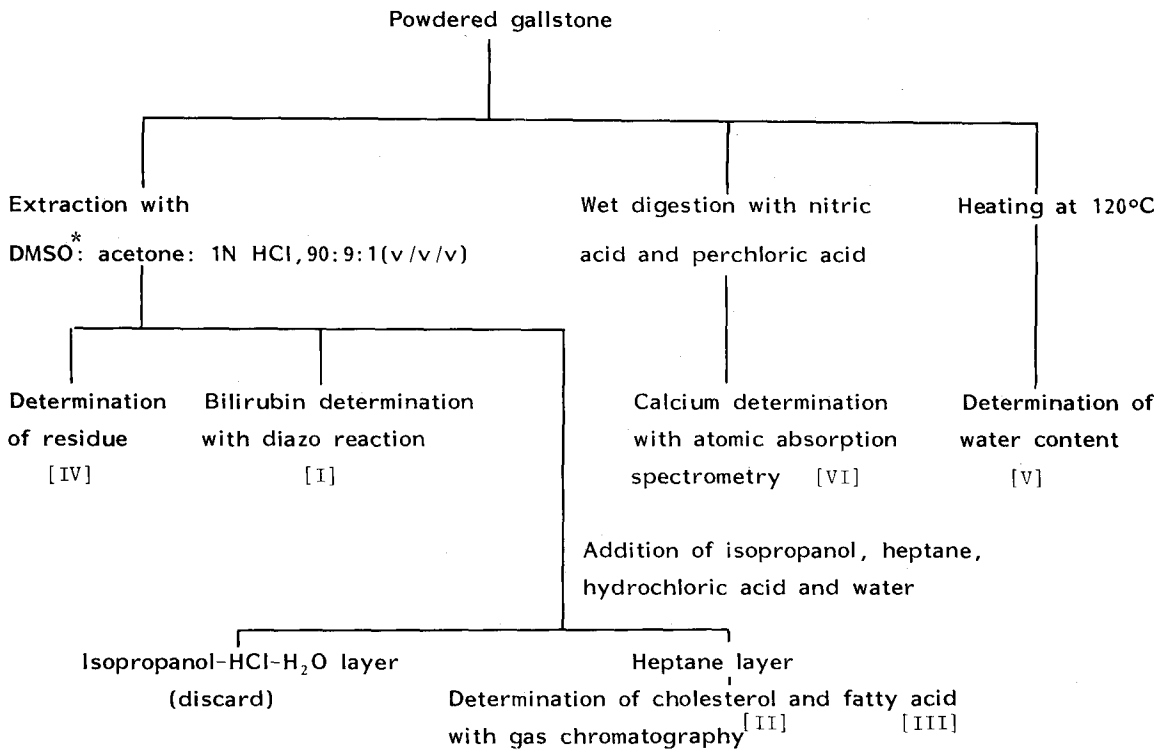

Fig 1. Chemical analysis of gallstone. *Dimethylsulfoxide; Roman numerals correspond to those in the text.

patients were analyzed, which were not accompanied by intrahepatic and gallbladder stones—so-called primary common bile duct stones. Gallstones in gallbladder from another 23 patients without intra- or extrahepatic bile duct stones were also analyzed.

Cholesterol, coprostanol, bilirubin, heptadecanoic acid, palmitic acid, and other fatty acids were purchased from Sigma Chemical Co., St. Louis, Missouri. The fatty acids were checked for their purity by gas–liquid chromatography and used as standards. Prior to chemical analysis, gallstones were classified by visual inspection as cholesterol, brown pigment, or black pigment stones. Since the acidified chloroform–methanol previously used (3) for dissolving gallstones was found to be a rather poor solubilizer of calcium bilirubinate, a new solvent system was developed. Dimethylsulfoxide (DMSO) seems to be a good solvent for bilirubin (4). Several solvent mixtures were tested. Of these, two solvent systems, ie, DMSO–acetone–1 N HCl (90:9:1, v/v/v) and acidified chloroform–methanol (2:1, v/v) were found to dissolve powdered cholesterol and brown pigment stones well. Dissolution of brown pigment stones with these solvent mixtures was compared for five gallstone specimens by determining the weight of residue remaining after extraction. The former solvent gave significantly less residue according to paired t test ($P < 0.05$). The effect of hydrochloric acid and acetone on determination of bilirubin by sulfanilic acid diazo reaction (5) was tested and showed negligible effect when the determination was made immediately following the extraction. Based on these preliminary experiments, DMSO–acetone–1 N HCl (90:9:1, v/v/v) was used as the dissolving agent for further analysis.

Chemical analysis of gallstones, thus revised, is outlined in Figure 1. Briefly stated, gallstone powder (1–2 mg) was dissolved in the solvent followed by ultrasonification and centrifugation. The supernatant was obtained by two more repeated extraction procedures and used for the subsequent determination of bilirubin, cholesterol, and fatty acid. Bilirubin concentration ([I] in Figure 1) was determined by the sulfanilic acid diazo reaction according to Malloy and Evelyn (5) modified for DMSO. Cholesterol [II] and fatty acid [III] in gallstones were extracted by the method modified from that of Dole and Meinertz (6). To an aliquot of supernatant with internal standards, ie, coprostanol for cholesterol and heptadecanoic acid for fatty acid, heptane and isopropanol were added. Partition was obtained by addition of water and 0.1 N hydrochloric acid. The organic layer was transferred, evaporated, methylated with 5% (w/v) methanolic hydrogen chloride, and acetylated with acetic anhydride and pyridine (7). Quantitative analyses were carried out by gas–liquid chromatography using a Shimadzu GC-6AM (Kyoto, Japan) equipped with a glass capillary column (SE 30, 25 m × 0.37 mm, id). The residue [IV] remaining after the extraction was transferred to a preweighed aluminum planchet and dried in an oven kept at 120° C. After cooling, the planchet was weighed and the residue was determined. For determination of water content [V], another 5–10 mg of powdered gallstone was placed in a preweighed aluminum planchet and dried in an oven at 120° C to a constant weight. The

TABLE 1. COMPOSITION OF GALLSTONES FROM INTRAHEPATIC BILE DUCTS*

	Cholesterol stone (N = 9)		*Brown pigment stone (N = 33)*	
	Mean ± SE	*Range*	*Mean ± SE*	*Range*
Bilirubin	1.7 ± 2.0	6.3– 0.3	25.7 ± 11.1	48.5–0.3
Cholesterol	82.3 ± 15.6	97.0–50.5	15.5 ± 13.0	48.4–2.9
Fatty acid	5.9 ± 4.4	16.5– 0.3	13.8 ± 7.7	23.5–5.1
Calcium	1.2 ± 2.1	6.3– 0	1.8 ± 0.6	4.1–0.7
Residue	1.8 ± 3.8	11.3– 0	7.3 ± 7.5	20.6–0

*Expressed in weight percent. Total mass determined as bilirubin, cholesterol, fatty acid, calcium, and residue accounted for 92.9 ± 6.9% of dry weight in cholesterol stone and 64.1 ± 13.5% in brown pigment stone.

difference in weight before and after heating was considered as water content. For determination of calcium content [VI], another 1–2 mg of powdered gallstone was digested with concentrated nitric acid and perchloric acid at 150° C (8, 9). Calcium in the digest was measured by atomic absorption spectrometry on a Shimadzu AA 630-01.

RESULTS

Reproducibility of the present method was checked by a triplicate run of one sample. The coefficients of variation were very small; 0.9% for bilirubin, 1.0% for cholesterol, and 2.6% for fatty acid. A recovery experiment was carried out by adding various amounts of compounds to gallstone powder of known composition. The recovery was satisfactory, ie, 98.0–100% for bilirubin, 98.0–103.0% for cholesterol, and 98.6–102.5% for fatty acid.

The composition of intrahepatic stones is shown in Table 1. The composition of stones in gallbladder and extrahepatic bile duct from patients without intrahepatic calculi is shown in Table 2. The classification of gallstones used in these tables was based mostly on visual inspection and, in doubtful cases, on chemical analysis. The majority of intrahepatic calculi were brown pigment stones as previously described (10). In nine intrahepatic calculi identified as cholesterol stones, cholesterol averaged 80% of total dry weight, which was similar to cholesterol gallstones from the extrahepatic bile duct and gallbladder (Table 2). In contrast, the composition of 33 brown pigment stones in intrahepatic bile duct varied greatly. The mean percentages of major constituents were: 25.7% for bilirubin, 15.5% for cholesterol, and 13.8% for fatty acid. However, the macroscopic appearance of intrahepatic stones was not distinguishable from stones in other locations.

Fatty acid in gallstones was found to be almost exclusively palmitic acid, in agreement with an earlier report (11). Other fatty acids detected in trace amounts were stearic, oleic, palmitoleic, and linoleic acids.

The water content of intrahepatic cholesterol and brown pigment stones was 1.3 ± 1.1% and 4.0 ± 2.7%, respectively. The water content in the latter varied considerably because of the pastelike nature of intrahepatic calculi. Mean recovery which comprised the sum of the weight percentages of bilirubin, cholesterol, fatty acid, calcium, and residue was about 92% for intrahepatic cholesterol stones and 64% for intrahepatic brown pigment stones. The latter were found to be similar to those of brown pigment stones from the extrahepatic bile duct and gallbladder.

Since the major gallstone constituents were bilirubin, cholesterol, and fatty acids, the composition of the stones was plotted on a triangular coordinate diagram. Brown pigment stones in the extrahepatic bile ducts were present exclusively in the upper corner enclosed in a dashed line (Figure 2). When the same line was arbitrarily drawn on the diagram of intrahepatic calculi (Figure 3), 21 of 33 brown pigment stones were found outside the area. Comparison of the composition between the intra- and extrahepatic brown pigment stones, using the chi-square test, revealed the former were composed of significantly less bilirubin ($P < 0.001$) and more cholesterol ($P < 0.05$) than the latter.

DISCUSSION

Gallstone analysis has presented problems because of the presence of various constituents such as cholesterol, bilirubin and its polymers (12), fatty acid, and calcium and its salts, with widely different solubility characteristics. In order for the gallstone to become amenable to chemical analysis, such constituents should preferably be brought into solution. However, no single solvent serves this purpose. Dimethylsulfoxide (DMSO) was found to dissolve bilirubin efficiently by interfering with hydrogen-bond formation in the bilirubin molecule (13). Substitution of methanol in the acidified chloroform–methanol mixture previously used (3) with DMSO in the present study greatly facilitated extraction of gallstones, especially of brown pigment stones. Acetone was added to enhance disso-

TABLE 2. COMPOSITION OF GALLSTONES FROM GALLBLADDER AND EXTRAHEPATIC BILE DUCT*

	Cholesterol stone (N = 21)		*Black pigment stone (N = 9)*		*Brown pigment stone (N = 15)*	
	Mean ± SE	*Range*	*Mean ± SE*	*Range*	*Mean ± SE*	*Range*
Bilirubin	2.3 ± 4.5	20.0–0	9.4 ± 3.9	14.0–4.7	33.4 ± 7.5	46.4–21.9
Cholesterol	79.5 ± 20.3	101.5–31.7	1.5 ± 2.0	5.9–0	5.0 ± 4.0	15.6–0.3
Fatty acid	2.9 ± 2.2	9.3–0.7	4.0 ± 4.5	14.7–0.3	9.0 ± 6.2	21.4–1.4
Calcium	2.0 ± 4.1	18.9–0	12.2 ± 4.4	16.8–1.8	2.1 ± 0.6	2.4–1.3
Residue	1.5 ± 4.1	19.0–0	14.0 ± 10.2	26.5–1.7	2.4 ± 3.2	10.0–0

*Expressed in weight percent. Total mass determined as bilirubin, cholesterol, fatty acid, calcium, and residue accounted for 89.1 ± 16.8% of dry weight in cholesterol stone, 41.1 ± 7.9% in black pigment stone, and 51.1 ± 9.8% in brown pigment stone.

lution of cholesterol. Subsequently DMSO–acetone–1 N HCl (90:9:1, v/v/v) was chosen for the analysis of gallstones.

The classification of pigment stones according to the proceedings of the first National Institutes of Health—International Workshops on Pigment Gallstone Disease (14) was adapted in the present study. Gallstones from the gallbladder and extrahepatic bile duct could be divided into cholesterol, brown pigment, and black pigment stones. In contrast to the general belief concerning hepatolithiasis, not only brown pigment stones but also cholesterol stones were present, although the former predominate (79%). Some of the latter may have migrated from the gallbladder passing through the cystic duct and the common bile duct. However, review of the operative findings revealed that in some cases the stones were present in the intrahepatic bile ducts with stricture at the liver hilum which precluded the upward migration of stones originating from the gallbladder into the intrahepatic bile duct. In other cases, the stones filled the entire bile duct from the common bile duct and common hepatic duct upward toward the periphery of the intrahepatic bile duct. In these cases, it seems most unlikely that all stones present originated in the gallbladder, filling out the entire bile duct. Therefore, these cholesterol stones must have been formed *in situ* in the intra- and extrahepatic bile ducts.

Thirty-four of 42 cases in the present study with stones in the intrahepatic bile ducts had stones also in the extrahepatic bile duct and/or gallbladder. In these cases the chemical composition of the paired

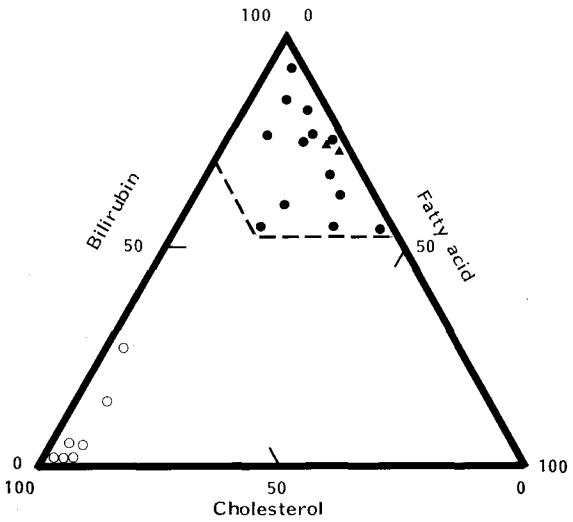

Fig 2. Composition of stones in the extraheptic bile duct. Cholesterol, bilirubin, and fatty acid were expressed as weight percent, which was calculated using the assumption that gallstones were composed entirely of these three constituents. Gallstone classification depended on visual inspection: (●) brown pigment stone; (▲) black pigment stone; (○) cholesterol stone. Dashed line indicates the upper limit of cholesterol content or the lower limit of bilirubin content in the extrahepatic brown pigment stones.

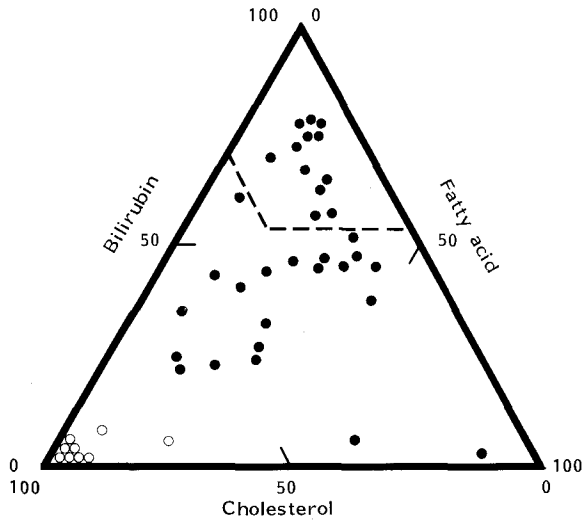

Fig 3. Composition of intrahepatic calculi expressed in weight percent: (●) brown pigment stone; (○) cholesterol stone.

gallstone specimens from intrahepatic and extrahepatic bile duct were quite similar, suggesting that the stones in the extrahepatic bile duct had descended from the intrahepatic bile ducts.

This study demonstrated that, compared to extrahepatic brown pigment stones, intrahepatic brown pigment stones contained significantly less bilirubin and more cholesterol, in agreement with an earlier report (15). Other investigators have distinguished two types of brown pigment stones in intrahepatic ducts, ie, the stratified type with low and amorphous type with high cholesterol content (16). Thus, the present study suggested the site of formation affected stone composition. The presence of brown pigment as well as cholesterol stones among intrahepatic calculi suggests the complex nature of the problem, ie, not only the formation and precipitation of calcium bilirubinate but also the solubility of cholesterol and formation of calcium salts of fatty acids should be considered when dealing with intrahepatic calculi.

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