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BACKSCATTERED ELECTRON IMAGING AND ENERGY-DISPERSIVE X-RAY MICROANALYSIS
STUDIES OF EVIDENCE FOR CALCIUM SALT HETEROGENEITY IN
FIFTEEN GALLSTONES FROM AN ELDERLY HUMAN
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Abstract
We examined 15 variably-sized gallstones, taken from an elderly male, by backscattered electron imaging and energy-dispersive X-ray microanalysis to learn the structural and distribution patterns of gallstone calcium (Ca-) salts. Of the 13 cholesterol-rich stones, nine stones had peripheral concentric layers of Ca-carbonate, whereas 2 stones had peripheral layers of Ca-phosphate. No Ca-salts were detected from 2 cholesterol-rich stones. The 2 stones containing Ca-phosphate had no Ca-salt cores, whereas the stones containing Ca-carbonate were separated into 3 different types: two stones with a Ca-carbonate core, four stones with several Ca-bilirubinate cores of glass-like structure, and 3 stones lacking Ca-salt cores. A closer view of the Ca-salt layers, which may be occasionally coexistent with Ca-bilirubinate, mainly showed either laminate deposits or numerous globules with a few laminae. Of the 2 cholesterol-poor stones, one had dispersed particles mainly of Ca-phosphate, and the other had loosely dispersed particles with small amounts of Ca-phosphate, bilirubinate, and/or palmitate. Some relationship between the size and Ca-salt species of these gallstones was suggested. Gallstones collected from the same individual showed a considerable heterogeneity of Ca-salts.

Key Words: Gallstones, scanning electron microscopy, backscattered electron imaging, energy-dispersive X-ray microanalysis, calcium salts, structure, distribution.

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Introduction
Human gallstones have been classified into many groups including the cholesterol [3, 6, 7, 8, 14, 16, 17, 18, 19, 22, 25, 26, 27, 28], the bilirubinate [18, 28], the pigment [24, 25, 26, 27], the brown pigment [6, 7, 8, 14, 15], the black pigment [6, 7, 8, 14, 15, 28, 30], various calcium (Ca-) salt stones [3, 22, 24, 27], and others [3, 18, 27]. Though cholesterol is a common constituent in the majority of gallstones [7, 8, 18, 19, 21, 26, 27, 28, 29], it is not found in all. In addition, gallstones share Ca-salts of various compositions; many gallstones differ from each other merely in the proportion of each Ca-salt they share [1, 2, 3, 6, 7, 8, 14, 16, 17, 18, 20, 22, 24, 25, 27, 28, 29, 30]. Thus, some of these stone groups may often overlap.

Inorganic and organic Ca-salts found in gallstones are mainly Ca-carbonate, phosphate, bilirubinate, and palmitate or fatty acid-Ca [1, 2, 3, 5, 6, 7, 8, 14, 17, 21, 22, 24, 27, 28, 29, 30]. These Ca-salts are concentrated in the cores [1, 2, 3, 5, 6, 7, 8, 16, 17, 18, 23, 27, 28] and in the peripheral concentric layers [1, 3, 8, 16, 23, 28]. In some other cases, they are dispersed diffusely throughout the entire stone [2, 3, 18, 22, 24, 28].

For identifying the composition of gallstone Ca-salts, chemical analysis [15, 18], microradiography [3, 27, 28], X-ray diffraction [1, 2, 3, 21, 22, 23, 27, 28, 29, 30], infrared spectroscopy [1, 2, 5, 14, 17, 24, 25, 27, 28, 29, 30], and atomic absorption spectroscopy [29, 30] have been applied. However, the precise structural and distribution patterns of gallstone Ca-salts could not be defined by these methods because the Ca-salts, except for Ca-salt gallstones, were very small in amount and size.

For the study of the distribution pattern and fine structure of Ca-salts in gallstones, scanning electron microscopy (SEM) [1, 3, 7, 8, 9, 15, 17, 27, 28], backscattered electron (BSE) imaging [7, 8], electron diffraction [9], and wavelength-dispersive X-ray [1, 2, 15, 16] and energy-dispersive X-ray (EDX) microanalysis [1, 2, 6, 7, 8, 9, 16, 17, 28] have been employed. These
studies have made a more detailed analysis possible. Furthermore, Kaufman et al. [7] established the EDX spectra of Ca-carbonate, phosphate, bilirubinate, and palmitate by using the respective standard samples.

In this study, the Ca-salts in 15 gallstones, collected from an elderly human, have been examined by conventional SEM, BSE, and EDX to learn the structural and distribution patterns of Ca-salts in the stones. The Ca-salts are identified on the basis of the EDX data of Kaufman et al. [7]. In addition, the relationships between the size and Ca-salt species of the stones are discussed.

Materials and Methods

Fifteen variably-sized gallstones were taken from the gallbladder of a 76-year-old male who died of lung cancer. His body had been soaked in a solution of 10% neutral formaldehyde for a post-mortem examination. All the stones were rinsed in running distilled water for a few minutes and air dried.

The dried stones were weighed and then embedded in epoxy resin (Epo-Mix Epoxide; Buehler, Evanston, IL, USA). Two large gallstones were cut into halves with a diamond wheel and all the stones were ground with grindstones. When the core structures were exposed, the ground surfaces were polished with 5 and 0.3 μm alumina on polishing cloths, and cleaned ultrasonically in distilled water. After the polished surfaces were photographed and coated with carbon in a high vacuum evaporator (HUS-5GB; Hitachi, Tokyo, Japan), BSE images were obtained with a Hitachi S-2500CX SEM operated at an accelerating voltage of 25 kV. Each area, showing a different BSE signal, was analyzed qualitatively and the areas containing Ca were analyzed quantitatively at 5 points with a S-2500CX SEM equipped with a Kevex (Foster City, CA, USA) Delta-4 EDX microanalysis system. The microanalysis conditions were an accelerating voltage of 15 kV, a specimen irradiation current of $1 \times 10^{-7}$ mA, and a counting time of 100 seconds. The standard mineral samples of fluorapatite, strontium sulfate, and magnesium oxide were prepared for the quantitative analysis of Ca, P, S, and Mg, and ZAF corrections were applied.

After the BSE and EDX investigations, all the stones were fractured across the core region into several pieces. One piece from each stone was soaked in ether for 5 to 15 minutes to dissolve cholesterol [19]. This treatment shows whether a gallstone is highly soluble in ether (cholesterol-rich) or poorly soluble in ether (cholesterol-poor). The remaining pieces without ether treatment were coated with an approximately 15 nm thick platinum-palladium layer in an ion sputtering apparatus (IB-5; Eiko, Tokyo, Japan). These samples were observed with a Hitachi S-430 SEM operated at 20 kV.

<table>
<thead>
<tr>
<th>Sample Weight (g)</th>
<th>Weight Ether Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number Dry Group¹</td>
<td>Solubility²</td>
</tr>
<tr>
<td>1</td>
<td>1.50 W¹</td>
</tr>
<tr>
<td>2</td>
<td>1.38 W¹</td>
</tr>
<tr>
<td>3</td>
<td>0.91 W¹</td>
</tr>
<tr>
<td>4</td>
<td>0.44 W²</td>
</tr>
<tr>
<td>5</td>
<td>0.43 W²</td>
</tr>
<tr>
<td>6</td>
<td>0.41 W²</td>
</tr>
<tr>
<td>7</td>
<td>0.36 W²</td>
</tr>
<tr>
<td>8</td>
<td>0.32 W²</td>
</tr>
<tr>
<td>9</td>
<td>0.29 W²</td>
</tr>
<tr>
<td>10</td>
<td>0.16 W³</td>
</tr>
<tr>
<td>11</td>
<td>0.15 W³</td>
</tr>
<tr>
<td>12</td>
<td>0.05 W⁴</td>
</tr>
<tr>
<td>13</td>
<td>0.05 W⁴</td>
</tr>
<tr>
<td>14</td>
<td>0.04 W⁵</td>
</tr>
<tr>
<td>15</td>
<td>0.01 W⁵</td>
</tr>
</tbody>
</table>

¹Weight groups: W¹: 1.50-0.91 g; W²: 0.44-0.29 g; W³: 0.16-0.15 g; W⁴: 0.05-0.04 g; and W⁵: 0.01 g.
²Ether solubility: +: Soluble (cholesterol-rich stone); -: Insoluble (cholesterol-poor stone).
Figure 2. Energy-dispersive X-ray spectrographs of 4 types of Ca-salts. (a) Ca-carbonate. (b) Ca-bilirubinate. (c) Ca-phosphate. (d) Unidentified Ca-salts. Ca-salts in the stones are identified by quantitative EDX on the basis of the data of Kaufman et al. [7]; see Table 2 also.

Table 2. Type of Ca-salts identified from the composition profiles of Ca, P, S, and Mg (percentage by weight) in 13 gallstones. Based on EDX as shown in Figure 2.

<table>
<thead>
<tr>
<th>Type of Calcium Salt</th>
<th>Carbonate</th>
<th>Bilirubinate</th>
<th>Phosphate</th>
<th>Unidentified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca</td>
<td>31.20 ± 2.94</td>
<td>2.18 ± 0.54</td>
<td>13.50 ± 7.20</td>
<td>1.43 ± 0.86</td>
</tr>
<tr>
<td>P</td>
<td>0.24 ± 0.12</td>
<td>±</td>
<td>7.07 ± 3.61</td>
<td>0.58 ± 0.40</td>
</tr>
<tr>
<td>S</td>
<td>0.04 ± 0.05</td>
<td>2.31 ± 1.93</td>
<td>0.10 ± 0.09</td>
<td>0.23 ± 0.12</td>
</tr>
<tr>
<td>Mg</td>
<td>0.05 ± 0.07</td>
<td>±</td>
<td>0.09 ± 0.18</td>
<td>±</td>
</tr>
<tr>
<td>n</td>
<td>90 (18 x 5)</td>
<td>25 (5 x 5)</td>
<td>25 (5 x 5)</td>
<td>5 (1 x 5)</td>
</tr>
</tbody>
</table>

Mean ± S.D. (n = The number of sample regions x 5, the number of points analyzed).

±: Trace *: Molar ratio of Ca/P = 1.61 ± 0.17 (Mean ± S.D.; n = 25).

The types of Ca-salts are identified on the basis of Kaufman et al. EDX data [7].
Results

Table 1 shows the dry weight and the ether solubility of the 15 gallstones. The total combined weight of the stones was 6.5 g. The variety in weight ranged between 1.50 to 0.01 g (0.43 ± 0.47 g, mean ± standard deviation (S.D.)), and they were divided into 5 groups by weight. The ether treatment caused the pieces of 13 multifaceted or smaller granule-shaped stones (#2-#11 and #13-#15) to be sub-totally or completely dissolved; they were designated as cholesterol-rich stones. Figure 1 shows one example, sample #2, of the cholesterol-rich stones has an ether-insoluble layer between two ether-soluble regions. In the other 2 samples, a small black (#12) and a large brown stone (#1), both the fractured and polished surfaces showed no changes by SEM as well as by naked eye after ether treatment. They were recognized as cholesterol-poor stones.

Figure 3. The polished surface of sample #2, a W1, cholesterol-rich stone. (a) Optical photograph. (b) Backscattered electron (BSE) image. OP and IP1, IP2: Outer and inner thick layers formed by black-brown deposits (a) showing a high (OP, IP1) or a relatively high (IP2) BSE signal (b). The EDX of OP and IP1 yielded element contents consistent with Ca-carbonate, whereas the layer of IP2 was consistent with Ca-bilirubinate. The black core (C) shows a relatively low BSE signal.

The polished surfaces of the gallstones were roughly separated into 4 patterns according to the BSE signal strength: high, relatively high, relatively low, and low (see Figs. 3b to 9b). When analyzed quantitatively by EDX, only traces of Ca, if any, were detected from the regions of low and relatively low BSE signal in any stone, whereas a small amount of S was detected from some of them. From the regions of high and relatively high BSE signals, Ca was clearly detected together with other elements such as P and S; four types of characteristic element compositions were found (Fig. 2, Table 2). These 4 Ca-salt types were identified as Ca-carbonate with a small amount of S, Ca-bilirubinate, and Ca-phosphate with a small amount of S, while the remaining one
### Table 3. Elemental contents of 15 gallstones and type of Ca-salts.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Region</th>
<th>Ca</th>
<th>P</th>
<th>S</th>
<th>Mg</th>
<th>Ca-salt Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>W</td>
<td>1.43</td>
<td>0.58</td>
<td>0.23</td>
<td>±</td>
<td>Unidentified</td>
</tr>
<tr>
<td>2</td>
<td>IP1</td>
<td>29.34</td>
<td>0.30</td>
<td>±</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>IP2</td>
<td>1.77</td>
<td>±</td>
<td>5.70</td>
<td>±</td>
<td>Bilirubinate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>33.39</td>
<td>0.13</td>
<td>0.04</td>
<td>0.02</td>
<td>Carbonate</td>
</tr>
<tr>
<td>3</td>
<td>IP</td>
<td>33.60</td>
<td>0.04</td>
<td>±</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>34.60</td>
<td>0.27</td>
<td>±</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td>4</td>
<td>C</td>
<td>1.78</td>
<td>±</td>
<td>1.17</td>
<td>±</td>
<td>Bilirubinate</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>29.30</td>
<td>0.29</td>
<td>0.02</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>32.57</td>
<td>0.17</td>
<td>0.02</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td>5</td>
<td>C</td>
<td>3.02</td>
<td>0.02</td>
<td>2.04</td>
<td>0.16</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>32.86</td>
<td>0.37</td>
<td>0.05</td>
<td>0.25</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>29.90</td>
<td>0.25</td>
<td>±</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>1.89</td>
<td>±</td>
<td>1.13</td>
<td>±</td>
<td>Bilirubinate</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>29.04</td>
<td>0.41</td>
<td>0.10</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>31.87</td>
<td>0.20</td>
<td>0.05</td>
<td>0.03</td>
<td>Carbonate</td>
</tr>
<tr>
<td>7</td>
<td>C</td>
<td>33.74</td>
<td>0.22</td>
<td>0.20</td>
<td>0.05</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>23.82</td>
<td>0.10</td>
<td>0.03</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>33.20</td>
<td>0.20</td>
<td>0.04</td>
<td>0.02</td>
<td>Carbonate</td>
</tr>
<tr>
<td>8</td>
<td>P</td>
<td>29.25</td>
<td>0.23</td>
<td>0.05</td>
<td>0.07</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33.30</td>
<td>0.23</td>
<td>0.04</td>
<td>0.04</td>
<td>Carbonate</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>2.44</td>
<td>±</td>
<td>1.49</td>
<td>±</td>
<td>Bilirubinate</td>
</tr>
<tr>
<td></td>
<td>IP</td>
<td>29.63</td>
<td>0.27</td>
<td>0.09</td>
<td>0.06</td>
<td>Carbonate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>30.86</td>
<td>0.34</td>
<td>0.06</td>
<td>0.04</td>
<td>Carbonate</td>
</tr>
<tr>
<td>10</td>
<td>W</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>P</td>
<td>31.80</td>
<td>0.28</td>
<td>0.02</td>
<td>±</td>
<td>Carbonate</td>
</tr>
<tr>
<td>12</td>
<td>W</td>
<td>14.38</td>
<td>6.93</td>
<td>0.02</td>
<td>0.09</td>
<td>Phosphate</td>
</tr>
<tr>
<td>13</td>
<td>IP</td>
<td>4.90</td>
<td>2.36</td>
<td>±</td>
<td>±</td>
<td>Phosphate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>8.49</td>
<td>4.53</td>
<td>±</td>
<td>±</td>
<td>Phosphate</td>
</tr>
<tr>
<td>14</td>
<td>IP</td>
<td>17.03</td>
<td>7.38</td>
<td>0.11</td>
<td>0.03</td>
<td>Phosphate</td>
</tr>
<tr>
<td></td>
<td>OP</td>
<td>23.02</td>
<td>11.60</td>
<td>0.15</td>
<td>0.29</td>
<td>Phosphate</td>
</tr>
<tr>
<td>15</td>
<td>W</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>-</td>
</tr>
</tbody>
</table>

Mean (n = 5)

Regions: C: Core; P: Periphery (IP = Inner, OP = Outer); W: Whole

The type of Ca-salts is defined in Table 2 (-: No Ca-salts).
Table 4. Distribution pattern of Ca-salts in 15 gallstones.

<table>
<thead>
<tr>
<th>Cholesterol-rich stones</th>
<th>Periphery</th>
<th>Core</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-carbonate</td>
<td>-</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>(with Ca-bilirubinate)</td>
<td></td>
<td></td>
<td>(1)</td>
</tr>
<tr>
<td>Ca-carbonate</td>
<td>Ca-carbonate</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ca-carbonate</td>
<td>Ca-bilirubinate</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Ca-phosphate</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cholesterol-poor stones</th>
<th>Whole</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca-phosphate</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unidentified Ca-salts</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Total = 15

The type of Ca-salts is defined in Table 2.

No Ca-salts.

Table 5. Relationship between the weight and the stone type containing Ca-salts in 15 gallstones.

<table>
<thead>
<tr>
<th>Ca-salt group</th>
<th>Weight Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W1</td>
</tr>
<tr>
<td>Ca-carbonate</td>
<td>2</td>
</tr>
<tr>
<td>(with Ca-bilirubinate)</td>
<td>(1)</td>
</tr>
<tr>
<td>Ca-carbonate and Ca-bilirubinate cores</td>
<td>0</td>
</tr>
<tr>
<td>Ca-phosphate</td>
<td>0</td>
</tr>
<tr>
<td>Unidentified Ca-salts</td>
<td>1</td>
</tr>
<tr>
<td>No Ca-salts</td>
<td>0</td>
</tr>
</tbody>
</table>

n 3 6 2 3 1 15

The Ca-salt group is shown in Table 3.
The weight group, W1 to W5, is defined in Table 1.

----

appeared as a very small ring in the core region. Sample #11 had an outer concentric layer including Ca-carbonate, as did sample #2, but lacked the inner layers.

Sample #3, another large cholesterol-rich stone, had many dark-brown concentric rings (Fig. 4a), which corresponded with the thin layers of numerous particles showing a high BSE signal of Ca-carbonate (Fig. 4b). The black core showed a relatively low BSE signal with a small amount of S and a trace of Ca. Sample #7 had the black core (Fig. 5a), where numerous particles showing a high BSE signal of Ca-carbonate were concentrated (Fig. 5b). In the brown periphery, numerous particles formed thin concentric layers, mainly of Ca-carbonate around the core.

In sample #6, a brown concentric layer running along the stone surface (Fig. 6a) showed a high BSE signal of Ca-carbonate (Fig. 6b). In the inner region several black cores surrounded with dark-grey material were present (Fig. 6a). The cores and the scallop-shaped peripheries of the dark-grey material showed a relatively high and a high BSE signal (Fig. 6b), and Ca-bilirubinate and carbonate were detected, respectively. The dark-grey material showed a relatively low BSE signal with a small amount of S and a trace of Ca. The BSE image of sample #6 basically resembled those of the other 3 cholesterol-rich stones: #4, #5, and #9.

In sample #13, several dark-brown entire or partial concentric layers were present (Fig. 7a). The layers showed a relatively high BSE signal of Ca-phosphate, while the core showed a low BSE signal (Fig. 7b). The BSE image of sample #13 resembled that of #14.

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Sample #12 was a cholesterol-poor black stone. The polished stone surface displayed black-brown to dark-brown particles, which were dispersed coarsely throughout and separated by dark-grey trabeculae except for the core (Fig. 8a). The coarse particles showed a relatively high BSE signal of Ca-phosphate (Fig. 8b). Sample #1 was a cholesterol-poor brown stone, which had already been broken into one large and several smaller pieces at the time of collection. Numerous dark-brown to brown particles with dark-grey trabeculae were dispersed loosely throughout but no cores were found (Fig. 9a). The particles showed a relatively high BSE signal of an unidentified Ca-salt (Fig. 9b). Both the trabeculae of samples #1 and #12 showed a relatively low BSE signal with a small amount of S and a trace of Ca.

The BSE micrographs at higher magnifications of the 4 Ca-salt types are presented in Figures 10 to 13.
Figures 5 and 6. The polished surface of sample #7 (Fig. 5) and #6 (Fig. 6), W2, cholesterol-rich stones. (a) Optical photographs. (b) BSE images. Figure 5. Outer and inner layers (OP, IP) of deposits with a high BSE signal are seen. The black core (C) contains a dense aggregate of particles with a high BSE signal. These layers and core were consistent with Ca-carbonate. Figure 6. The black core (C) shows a relatively high BSE signal. The EDX of the core yielded element contents consistent with Ca-bilirubinate. The elemental contents of the scalloping border of the core (IP) and the outer layer (OP) were consistent with Ca-carbonate.

sample #12 and #1, which contained Ca-phosphate and unidentified Ca-salts respectively, the BSE images showed aggregated sand-grain deposits with irregular outlines but no laminae (Figs. 12d and 13).

The fine structures of the fractured surfaces of the 4 Ca-salt types were examined by SEM (Figs. 14, 15, 16, and 17). Figure 14 shows the concentric layers of Ca-carbonate. In sample #2, the inner layer between cholesterol crystal deposits had several laminae (Figs. 14a and 14b), which were occasionally contiguous with aster-shaped globules (Fig. 14c). In another cholesterol-rich stone, small globules of Ca-carbonate as shown in
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Figures 7 and 8. The polished surfaces of sample #13 (Fig. 7) and #12 (Fig. 8), W4, cholesterol-rich stones.
(a) Optical photographs. (b) BSE images. Figure 7. Dark-brown layers (OP, IP) show a relatively high BSE signal (b); EDX demonstrated that the layers were formed by Ca-phosphate. Figure 8. Diffuse deposits of black-brown to dark-brown, coarse particles (W) are separated by dark-grey, homogeneous trabeculae (Tr). The deposits of Ca-phosphate by EDX show a relatively high BSE signal. The homogeneous core (C) and trabeculae show a relatively low BSE signal.

Figure 10c were surrounded by cholesterol crystals (Fig. 14d). These Ca-carbonate deposits were composed of needle or sand-grain-shaped crystals. The cores of Ca-bilirubinate in sample #4-#6 and #9 showed a homogeneous, glass-like structure with cracks (Fig. 15). In sample #2, the fine structures of the discontinuous layer of Ca-bilirubinate (Fig. 3b), similar to that of the cores, were observed in close apposition to the layer of Ca-carbonate (Figs. 14a and 14b).

The coarse particles by BSE of Ca-phosphate in sample #12, divided by trabeculae, showed a fine sand-grain appearance on the fractured surface (Fig. 16). The loosely dispersed particles of unidentified Ca-salts in sample #1 (Fig. 9) were not further characterized by SEM (Fig. 17a). However, the particles showed a rougher surface as opposed to the smooth trabeculae. Figures 17b and 17c show the scanning electron micrograph and BSE image of the same region containing unidentified Ca-salts. Though the areas of relatively high and low BSE signals were noted, these structures were
Figure 9. The polished surface of sample #1, a W1, cholesterol-poor brown stone. The stone was fragmented at the time of its recovery. (a) Optical photograph. (b) BSE image. The diffusely dispersed, dark-brown to brown particles (a) show a relatively high BSE signal (b). The particles were unidentified by EDX. The dark-grey trabeculae (Tr) show a relatively low BSE signal.

not distinguishable by SEM. The morphology of the trabecular materials of the 2 cholesterol-poor stones (#1, #12) was somewhat similar to Ca-bilirubinate (Fig. 15); no cholesterol crystals were found in these stones.

Discussion

Thirteen gallstones with ether-soluble components had numerous cholesterol crystals by SEM [7, 8, 19, 28] as shown in Figures 14a and 14d, whereas 2 ether-insoluble gallstones showed no cholesterol crystals anywhere as shown in Figure 17. Therefore, in this study, these gallstones were designated cholesterol-rich and poor stones, respectively.

It has been reported in SEM studies that Ca-carbonate deposits in gallstones show a laminate [3, 26], a columnar [3], an aster-like [27], a spindlish [27], and a globular structure [7, 27, 28]. We observed the deposits of Ca-carbonate in 9 out of 13 cholesterol-rich stones, and demonstrated that most Ca-carbonate deposits were concentrically arranged, parallel to the stone surfaces, and assumed the appearance of parallel and semi-concentric laminae. In addition, an aster-like and a globular Ca-carbonate structure with a few laminae were also noted.

In crystallography of Ca-carbonate, calcite was reported to be present in gallstones [1, 2, 3, 5, 9, 21, 22, 27, 29]. However, the typical morphology of calcite in gallstones does not appear to have been clearly illustrated, and we could not identify calcite in this study. Aragonite [2, 3, 9, 21, 22, 27, 29] and vaterite [1-3, 9, 21, 22, 27, 29] have been reported to be present in the stones. Wosiewitz [27] reported that these crystals were detected as larger and smaller needle shapes. We observed small needle-shaped crystals similar to vaterite. The BSE image in Figure 10d might represent aragonite.

In 2 small cholesterol-rich stones, our BSE observations revealed that the structural pattern of Ca-phosphate deposits was similar to that of Ca-carbonate. On the other hand, the Ca-phosphate deposits in the cholesterol-poor, small black stone of sample #12 were dispersed coarsely throughout (Fig. 8). This small black stone may be classified as a Ca-phosphate stone [22] or might be a variety of the black pigment stones.

The species of Ca-phosphate crystals in gallstones have been identified as hydroxyapatite or apatite [1, 2, 21, 27, 29] and as whitlockite or Mg-containing whitlockite [2, 21, 29]. The Ca/P molar ratio of 1.61 of Ca-phosphate deposits (Table 2) is attributable to the presence of Ca-deficient and/or carbonate apatites [4, 10, 12, 13]. We could not find the characteristic structures of whitlockite, however, Mg, though in a very small amount, was detected; hence, the presence of Mg-whitlockite could not be ruled out.

Ca-bilirubinate deposits in gallstones have been reported as small granules to homogeneous, glassy masses by SEM [7, 8, 15, 17, 28]. The glass-like structures shown in Figures 11 and 15 resembled those of Ca-bilirubinate [8], of the pigment core containing 26.8% Ca-bilirubinate in a cholesterol stone [17], and of a black bilirubinate stone containing Ca and S [15]. The black pigment core of a cholesterol stone and the periphery of a small black pigment stone containing Ca and S [28]
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Figure 10. BSE images of Ca-carbonate. (a) An area of sample #2 (OP in Fig. 3b). (b) An area of sample #3 (IP in Fig. 4b). (c) An area of sample #4 (OP in Fig. 4b). (d) An area of sample #8. Ca-carbonates from parallel, semi-concentric laminae, partially aggregated globules with a few laminae, and aggregations of needle or sand-grain-shaped particles. Arrows indicate the stone surfaces.

also showed a similar glass-like structure. We found such glass-like deposits of Ca-bilirubinate in the black core regions of the 4 cholesterol-rich stones. In each stone, the deposits formed several cores, that appeared to be packed together by Ca-bilirubinate with a scalloped periphery of Ca-carbonate (Fig. 6b). Thus, such a stone may have been formed by the aggregation of several small black pigment stones followed by the deposition of a layer of Ca-carbonate [28].

The deposits of Ca-bilirubinate were also observed in close apposition to the concentric laminate layer of Ca-carbonate in sample #2 (Table 3, Figs. 3b, 14a, and 14b). In other stones, a small amount of S was detected from black to brown deposits containing Ca-carbonate or phosphate (Table 3), therefore, these Ca-salt deposits may occasionally be mixed with some amounts of Ca-
Figure 11. BSE image of one of the several Ca-bilirubinate cores in sample #6 (C in Fig. 6b). The deposit shows a homogeneous, glass-like structure.

bilepigment and/or bilepigment complex [18, 20, 26] or bilepigment [14, 15, 28, 29, 30]. On the other hand, no Ca-bilirubinate deposits, but only a small amount of S could be detected in the black cores of several cholesterol-rich stones and in the dark-grey trabeculae of the 2 cholesterol-poor stones (#1, #12). The deposits of the cores and the trabeculae showed a smooth, glass-like structure, similar to that of Ca-bilirubinate deposits, as well (Figs. 14, 16, and 17a). Therefore, these deposits, without EDX pattern of Ca-bilirubinate, probably contain bilepigment complex or bilepigment as previously reported [14, 15, 18, 20, 26, 28, 29, 30].

The nature of Ca-salt deposits in sample #1, which were loosely dispersed throughout (Fig. 9), was not identified. Nevertheless, the EDX data (Table 3) and the BSE images and scanning electron micrographs (Figs. 13 and 17) indicate that they might contain small amounts of Ca-phosphate, bilirubinate, and/or palmitate [7, 8, 28]. This cholesterol-poor, large brown stone may belong to the bilirubinate stones [18, 28] or might be a variety of the brown pigment stones.

In the Ca-salt core of cholesterol gallstones, Ca-carbonate [1, 2, 3, 8, 16, 27, 28], phosphate [1, 2, 8, 16, 27, 28], bilirubinate [2, 8, 16, 17, 28], and palmitate [8, 27] have been reported. Our BSE and EDX data (Table 4) concurred with the presence of Ca-carbonate (2 stones) and bilirubinate (4 stones) in the core. The periphery of cholesterol stones frequently contains Ca-carbonate [1, 3, 8, 16], phosphate [8], bilirubinate [8, 16], and palmitate [8]. As shown in Table 4, Ca-carbonate (9 stones) and phosphate (2 stones) were detected as concentric layers in the periphery. In addition, Ca-bilirubinate might also co-exist with these Ca-salts as shown in sample #2 (Table 3, Figs. 3b, 14a, and 14b). Ca-palmitate could not be identified with certainty in this study.

Little is known about the relations between the size and Ca-salt species of gallstones, although Kaufman et al. [6] reported that Ca-salt composition differed among variably-sized gallstones in 90% of the patients. The findings in this study demonstrate some relationship between the size (weight) and composition of the stones (Table 5). Most cholesterol-rich stones, ranging from 1.38 to 0.15 g, had the concentric layers of Ca-carbonate in the periphery, whereas cholesterol-rich stones between 0.44 to 0.29 g had several Ca-bilirubinate cores in addition to the peripheral concentric layers of Ca-carbonate. Ca-phosphate deposits were seen in the cholesterol-rich and poor stones of 0.05 and 0.04 g only. However, further research using stones from many patients will be necessary to establish this relationship.

Gallstone Ca-salts may be intermittently and concentrically deposited after cholesterol crystal deposition, first around the core formed by either Ca-salts or other materials, while cholesterol stones are growing [1, 3, 8, 16, 18, 23, 28]. Occasionally, several small black pigment stones may be packed together with Ca-carbonate deposits and become a larger cholesterol stone [28]. In some cholesterol-poor stones, the mechanism of Ca-salt deposition appears to be different: Ca-salt particles may aggregate with certain organic materials in an early stage of the stone formation.

In conclusion, we studied the structural and distribution patterns of several Ca-salts in human gallstones, and showed that the gallstones from the same individual differed in their patterns of Ca-salt distribution. We also suggest that there is some relationship between the size and the Ca-salt composition in gallstones.

Acknowledgments

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References

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Figure 12. BSE images of Ca-phosphate. (a, b, and c) An area of sample #14 similar to #13 (Fig. 7b). (d) An area of sample #12 (W in Fig. 8b).

Figure 13. BSE image of unidentified Ca-salts in sample #1 (W in Fig. 9b). The deposits form irregular outlines but no laminae.


Discussion with Reviewers

H.S. Kaufman and K.D. Lillenoe: Calcium (Ca-) salts are known to re-precipitate in aqueous solutions. In our studies of gallstones, we have taken extensive measures to ensure that the stones were dried, fractured,
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Figure 14. Scanning electron micrographs of Ca-carbonate. (a and b) Areas of Figure 3b (IP1, 2) show the Ca-carbonate layer of a sand-grain pattern in close apposition of the glass-like Ca-bilirubinate layer (*) with cholesterol crystals (Ch). (c) An area of sample #2 showing an aster-shaped formation. (d) An area of Figure 10b showing Ca-carbonate globules surrounded by cholesterol crystals. Arrows indicate the stone surfaces.

and not exposed to an aqueous environment prior to study. During our preliminary studies, we also embedded and polished stones but found that this changed the Ca-salt morphology. Did the authors study any of the stones on a purely fractured surface before any manipulation, and did this change the Ca-salt structure? Why were the stones fixed in glutaraldehyde?

R.D. Soloway: All stones were fixed with 10% neutral formaldehyde. Most other studies are on unfixed stones obtained at the time of surgery. Although formalin may not structurally affect Ca-salts, I wonder if any component of gallstones are dissolved in formalin? I also wonder if any structural changes occur due to the denaturation of stone proteins by formalin? Others have fixed stones in glutaraldehyde and have had better protein preservation. Did the authors try this alternative method?

Reviewer IV: What is the effect of immersing the stones in formaldehyde prior to analysis?
Figure 15. Scanning electron micrographs of Ca-bilirubinate in an area of sample #6 (Fig. 11). The deposit is homogeneous and glass-like.

Figure 16. Scanning electron micrograph of Ca-phosphate in sample #12 (Fig. 12d). The fractured surface shows a sand-grain pattern. Tr: Trabecula.

Figure 17 (a and b). Scanning electron micrographs of unidentified Ca-salts in sample #1 (Fig. 13). The deposits appear to be formed by irregularly shaped plates and blocks with a smooth trabecula (Tr). (c) BSE image of (b).
U. Wosiewitz: Are you sure that the way of sample preparation you chose is adequate? Considerations concerning possible dislocations of microstructures by the grinding procedure are missed. What reasons speak against investigating native fractured surfaces even in BSE mode?

Authors: Fixation: As discussed in Materials and Methods, the gallstones were collected from the body of an elderly male, preserved in 10% neutral formaldehyde for a post-mortem examination. Certainly, formalin fixation is an old-fashioned method compared with that of glutaraldehyde. However, some investigators [5, 33] used formalin fixation for gallstones; in addition, we used it for dental calculus [10, 11], several human calculi [31], and human pineal bodies with calcareous concretions [32]. Henichart et al. [5] detected Ca-carbonate and bilirubinate from Ca-palmitate gallstones by infrared spectroscopy. Ogata and Murata [34] observed crystals of cholesterol stones by SEM. Previously, we observed Ca-phosphate crystals including biological apatites, octacalcium phosphate, and Mg-containing whitlockite (β-tricalcium phosphate) in several calculi [10, 11, 31] and apatite in pineal concretions [32] by SEM, BSE, and EDX. In addition, there were no differences in the morphology of whitlockite crystals between formalin fixation and no fixation [11]. Although some minor dissolution of the deposits might occur in an aqueous solution at the molecular level, formalin fixation did not appear to grossly alter these crystals. We believe that neutral formalin causes little effect on Ca-salts at the magnifications used in this study. On the other hand, we cannot clearly answer the questions regarding the effect of fixation on organic material. In general, organic material will gradually degrade when they are left unfixed.

Exposure to aqueous solutions: Biological specimens, fixed in a chemical solution, are usually rinsed with an aqueous solution: In our previous studies [10, 11, 31], several calculi containing Ca-phosphate crystals, fixed in formalin, were observed by SEM after water rinsing. Several investigators [5, 15, 25, 30] washed fixed or unfixed (?) gallstones with distilled water. Malet et al. [15], Trotman et al. [25], and Wosiewitz and Schroebler [30] reported various components including minerals in gallstones. We believe that re-precipitation of Ca-salts would be minimal, if any, when rinsed in distilled water for a few minutes after fixation.

Grinding procedure: The grinding and polishing procedures may rarely cause gallstone microstructures to be removed or dislocated. However, the BSE images, at less than 1,000x magnifications, showed clear laminate structures (Figs. 10a, 10b, 10c, 12a, and 12b). We believe that such micrographs are highly unlikely to be artifacts. Any loose particles were removed by sonication. The clear structures by BSE cannot be obtained under the fractured surfaces, because of their roughness. Our previous studies with the BSE images, using the polished stone surfaces, did not encounter notable dislocation of crystals [31, 32]. Scanning electron micrographs of the fractured surfaces of each stone have been carefully compared with their BSE images in this study.

Reviewer IV: Did the authors use the same technique as Kaufman et al. [7]? If not, how did their technique differ?

Authors: Kaufman et al. [7] used "windowless" EDX, whereas, we detected quantitatively inorganic elements by ordinary EDX using the standard mineral samples and ZAF corrections (Tables 2 and 3).

U. Wosiewitz: Certainly, an about 10 µm diameter microspherolith is shown in Figure 14d. This is a typical microstructure of biliary Ca-carbonate. Did you observe similar structures composed of Ca-phosphate?

Authors: No, we did not. However, globular deposits of Ca-phosphate observed by BSE are similar to those of Ca-carbonate (Figs. 10c and 12b). These results suggest that some Ca-phosphate deposits may assume similar appearances.

U. Wosiewitz: Are the microstructures shown in Figures 15 and 16 are typical for Ca-bilirubinate and phosphate, respectively?

Authors: We cannot conclude that these deposits, observed in this study, are typical, since we have only examined a limited number of the stones.

R.D. Soloway: When the protein components are preserved, small pore-like spaces are seen, especially in the pigmented layers of stones. These pores are now thought to be the spaces which were filled by bacteria. Is there any evidence of these spaces in the stones examined in this study?

Authors: We observed no such pores either by SEM or by BSE imaging. However, no special attempts were made to search for these pores.

U. Wosiewitz: Can you please make your own suggestion on what biliary material sulfur could be derived from, and on the close topical relation of sulfur and pigment?

R.D. Soloway: Sulfur is described as present in the stone. This has been mentioned by several groups, including our own. It has repeatedly been identified as a sulfhydryl S rather than a sulfate S, indicating the presence of proteins. If that is the case in these stones, it should be so indicated.

Authors: EDX analysis detects elements only; therefore, we cannot comment on the chemical component
present. However, sulfur may be derived from such organic substances as cysteine and taurine, since they contain sulfur and are present in bile acid with bilirubin (biliary pigment).

H.S. Kaufman and K.D. Lillemoe: What were sizes of the precipitates of the unidentified Ca-salts? Could the P and S be coming from below that of the unidentified Ca-salt precipitated surface, suggesting co-precipitated bilirubinate and/or phosphate?
Authors: We could not measure the sizes because the calcified material was not distinguishable from non-calcified material by SEM and BSE (Figs. 17b and 17c). In the BSE image (Fig. 13), the deposits ranged from less than 3 µm in diameter to about 90 µm in length, although fine sand-grain structures were observed in the particles. It is likely that small amounts of unidentified Ca-salts might be co-precipitated with other Ca-salt species.

Reviewer IV: What is your hypothesis for the differing arrangement of Ca-salts in these stones? What is the hypothesis for the possible association between stone weight and Ca-salt content in these stones?
Authors: One explanation would be that a certain group of cholesterol stones showing a deposition of similar Ca-salts is formed during approximately the same time period, whereas the other groups are formed during another period. These differences may be caused by changes in dietary habits and the variability of biliary components during aging. It appears that the stones containing Ca-carbonate are formed at an early time, while the stones containing Ca-phosphate are formed later in life. Another explanation would be that cholesterol stones containing Ca-carbonate grow more rapidly and become large-sized regardless of their formation periods, whereas gallstones containing Ca-phosphate remain smaller.

Reviewer IV: Currently, ether solubility is not used by investigators in the field to separate types of stones. Can the authors justify classifying the stones on basis of ether solubility?
Authors: As mentioned in Materials and Methods, we used ether following the method of Phillips et al. [19] for determination of the presence of cholesterol in gallstones. However, we agree that this method may not be totally specific for identification of cholesterol. However, the ether-soluble stones contained numerous cholesterol crystals identified by SEM (Figs. 14a and 14d), whereas in the ether-insoluble stones, no cholesterol crystals were observed by SEM on the fractured surfaces.

Reviewer IV: Could the stone you term cholesterol-poor actually be cholesterol stones with an unusually high Ca-salt content? How would the authors respond to the following hypothesis? All the stones from this patient were in fact cholesterol (which are generally agreed to being defined as containing ≥ 60% cholesterol) and that the differences the authors observed were due to the percentage and type of Ca-salts and other non-cholesterol material such as mucin glycoproteins?
Authors: Cholesterol crystals have been shown to have characteristic morphology. The two cholesterol-poor stones did not show a high Ca-salt content (Table 3). We cannot support your hypothesis, because these stones were quite insoluble in ether and no cholesterol crystals could be observed by SEM in these stones (Fig. 17). In addition, both stones did not show a radial or concentric growth pattern which is one of the characteristics of cholesterol stones [18, 19, 33].

Reviewer IV: Since mucin glycoproteins are known to comprise approximately 10% or so of cholesterol stones’ weight, could these have influenced the authors’ results?
Authors: We did not attempt the detection of mucin. However, we think that the proteins, although they are likely to be present in these stones, have not influenced the SEM and BSE observations of gallstone Ca-salts except for the detection of sulfur by EDX.

O. Johari: Can you please provide a reference for the ZAF correction?
Authors: A standard reference for conventional ZAF is Martin and Poole [33].

Additional References