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CYCLIC DEPOSITION OF CALCIUM SALTS DURING GROWTH OF CHOLESTEROL GALLSTONES

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Abstract

Some cholesterol gallstones contain darkly pigmented centers or peripheral concentric pigmented bands. We examined the cross-sectional surface of three cholesterol gallstones which contained both central and peripheral pigmented areas with electron-probe microanalysis (EPM) and energy dispersive x-ray microanalysis (EDXA) to determine the elemental composition of the pigmented regions. Linear EPM across the cross-sectional surface of the stones demonstrated that most of the pigmented regions of all three stones had high Ca and P signals; the non-pigmented intervening areas had markedly lower or no detectable Ca and P signals. In two of the three stones, high O signals coincided with the high Ca and P signals suggesting that both calcium bilirubinate and calcium phosphate were present in these pigmented areas. EDXA of the central and peripheral pigmented areas of each stone confirmed the presence of a high Ca signal. Our results demonstrate that in some cholesterol gallstones there is cyclic deposition of calcium bilirubinate and other calcium salts.

Keywords: Cholesterol gallstones, calcium bilirubinate, calcium phosphate, scanning electron microscopy, electron probe microanalysis, energy dispersive X-ray analysis.

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Introduction

Human cholesterol gallstones are not homogeneous concretions in terms of either composition or structure (2,3,8,9). Cholesterol gallstones are comprised of 85% cholesterol on average (range 50–100%) with smaller amounts of pigment, calcium phosphate, palmitate and carbonate and protein (14). The average cholesterol stone contains less than 1–2% calcium bilirubinate pigment; the pigment can be distributed evenly throughout the stone or may be present in a central location and/or in peripheral rings. The purpose of this study was to examine the cross-sectional surface of cholesterol gallstones with pigmented areas to characterize the elemental composition of these areas, and of the intervening non-pigmented areas.

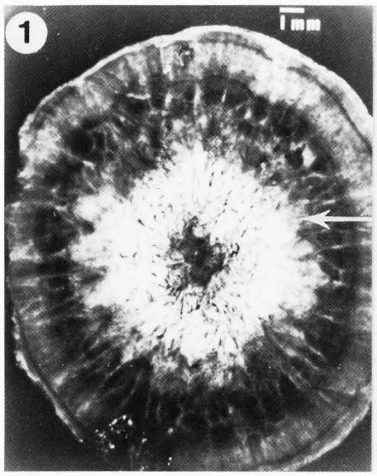
Materials and Methods

Selection and analysis of gallstones

Three sets of cholesterol gallstones with easily discernible pigmented rings and pigmented centers present on cross-section were selected. The stones' content of cholesterol and calcium bilirubinate was determined using quantitative infrared spectroscopy (12). Each stone contained at least 75% cholesterol (stone 1, near 100%, stone 2, 97% and stone 3, 75%) and less than 1% calcium bilirubinate (expressed as % of the total weight of the stone). Each stone contained a trace amount of calcium phosphate and no detectable calcium carbonate or palmitate.

Electron probe microanalysis (EPM)

Each gallstone was bisected using a scalpel and the cross-sectional surface was then polished with very fine abrasive paper. The surface of each stone was then vacuum-coated with carbon and mounted on a carbon stub. Linear electron probe microanalysis using a Cameca model MBX electron probe microanalyzer was then performed on the cross-sectional surface of each stone from the edge of the stone to the center along the locus as indicated in the results section. The microanalysis was performed at 20 kV with the electron beam at 90° to the sample. A stepwise analysis was performed with a 200 μm wide beam to average out concentrations transversely; 50 μm steps were used for stone 1, 25 μm steps for stone 2 and 10 μm steps for stone 3. Dwell time was 10 seconds and the working distance 9 mm. Each stone was examined for Ca, P, O, Cu and S.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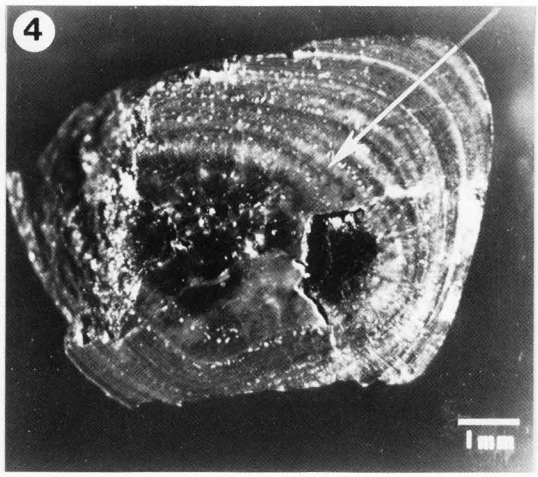
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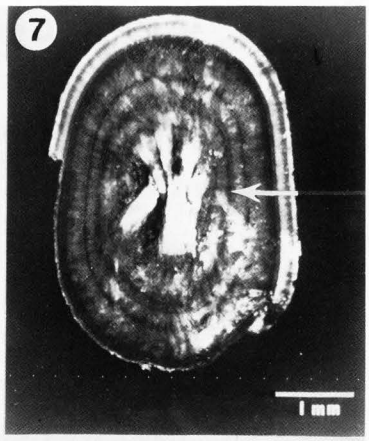
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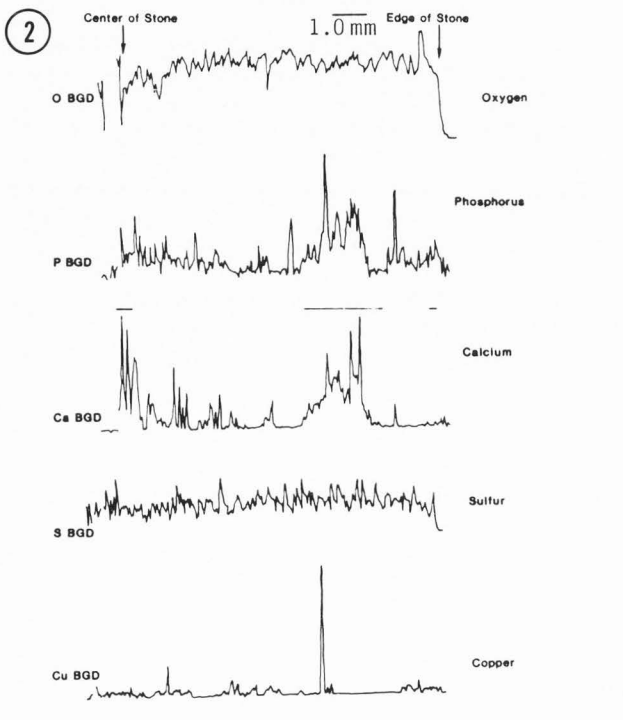
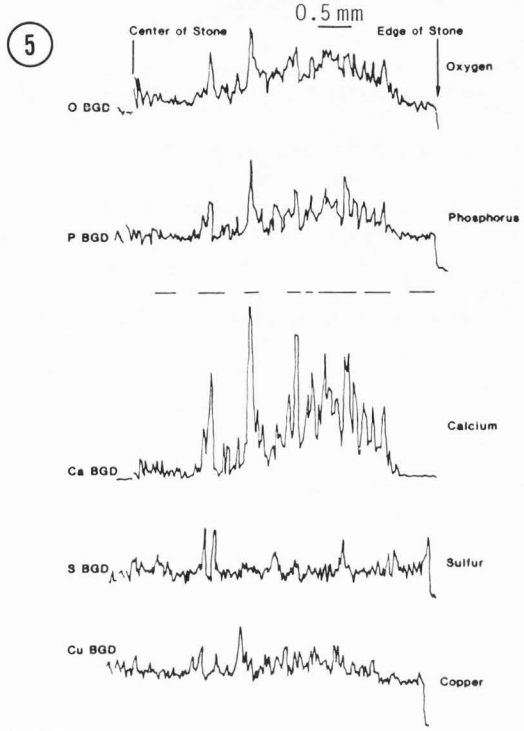

2
Center of Stone
1.0 mm
Edge of Stone
O BGD
Oxygen
P BGD
Phosphorus
Ca BGD
Calcium
S BGD
Sulfur
Cu BGD
Copper


5
0.5 mm
Center of Stone
Edge of Stone
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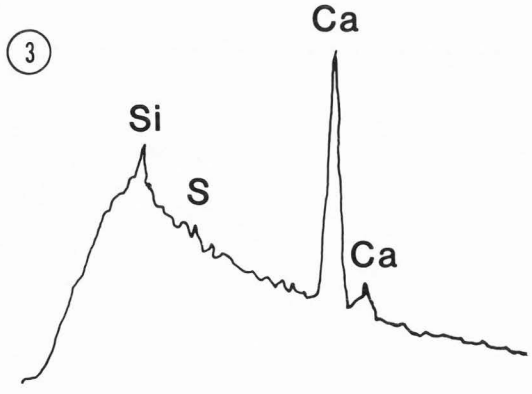
Oxygen
P BGD
Phosphorus
Ca BGD
Calcium
S BGD
Sulfur
Cu BGD
Copper


3
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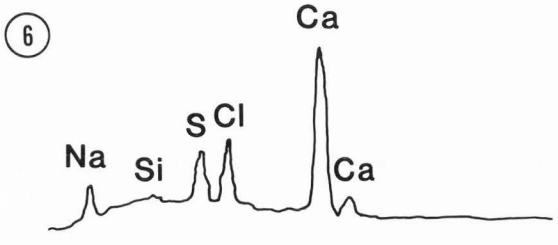
Si
S
Ca


6
Ca
Na
Si
S
Cl
Ca

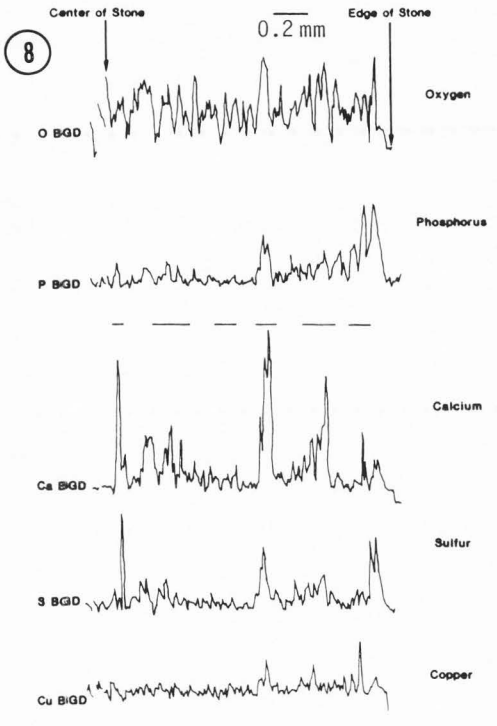
Energy dispersive x-ray microanalysis (EDXA)

EDXA was performed on the central pigmented area and on a peripheral pigmented band of each stone using a Tracor Northern model 2000 system at 20 kV.

Results

Gallstone 1 had a thin pigmented band in the distal periphery, a wide pigmented band in the mid-section and central pigmentation (Fig. 1). Electron probe microanalysis (Fig. 2) demonstrated that broad, high Ca and P signals corresponded spatially with the wide mid-sectional pigmented band: there was also a high narrow peak of Cu in this ring. In the outer pigmented band, narrow lower signals for Ca and P were seen. In the center of the stone, there was a high Ca signal and a smaller P signal. There were no O or S peaks along the cross-sectional surface. The rim of the stone, which was nonpigmented, as well as the nonpigmented areas between the pigmented bands had generally low or no detectable signals for Ca, P, O and Cu.

EDXA also demonstrated that the center of the stone had a high Ca signal (Fig. 3). EDXA of a peripheral pigmented band demonstrated signals for Ca and S.



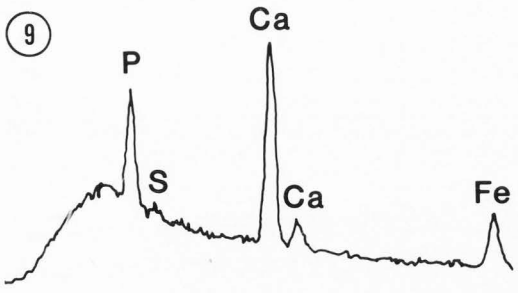


FIGURE LEGENDS:

Fig. 1. Optical photograph of the cross-sectional surface of the first cholesterol gallstone. This gallstone has a pigmented central area, a wide mid-peripheral pigmented band and a thin pigmented band near the edge. The arrow in this figure and in Figs. 4 and 7 indicates the locus along which electron probe microanalysis was performed to produce a line-profile.

Fig. 2. Electron probe microanalysis performed on the cross-sectional surface of stone 1 along the locus indicated by the arrow in Fig. 1. The line-profiles for O, P, Ca, S and Cu are shown from the edge of the stone to the center. In this figure and in the other electron probe microanalysis figures, BGD indicates the background and the shaded areas above the calcium line-profile indicate the location of the most visually prominent pigmented areas along the stone's cross-section. The relative scales of the EPM line-profiles compared to that of S at 1 are: O at 5:1, P at 8:1, Ca at 10:1 and Cu at 10:1.

Fig. 3. EDXA spectrum from the central pigmented area of stone 1. In this and in the following figures of EDXA spectra, the ordinate is relative intensity and the abscissa is energy in kV.

Fig. 4. Optical photograph of the cross-sectional surface of the second cholesterol gallstone. This gallstone has a pigmented central area and numerous concentric pigmented peripheral rings.

Fig. 5. Electron probe microanalysis performed on the cross-sectional surface of stone 2 along the locus indicated by the arrow in Fig. 4. The line-profiles for O, P, Ca, S and Cu are shown from the edge of the stone to the center. The relative scales of the EPM line-profiles compared to that of S at 1 are: O at 7:1, P at 7:1, Ca at 10:1 and Cu at 4:1.

Fig. 6. EDXA spectrum from the central pigmented area of stone 2.

Fig. 7. Optical photograph of the cross-sectional surface of the third cholesterol gallstone. This gallstone has a slightly eccentric pigmented center and several concentric pigmented peripheral rings.

Fig. 8. Electron probe microanalysis performed on the cross-sectional surface of stone 3 along the locus indicated by the arrow in Fig. 7. The line-profiles for O, P, Ca, S and Cu are shown from the edge of the stone to the center. The relative scales of the EPM line-profiles compared to S at 1 are: O at 6:1, P at 1:1, Ca at 5:1 and Cu at 4:1.

Fig. 9. EDXA spectrum from the central pigmented area of stone 3.

Stone 2 had numerous thin concentric pigmented bands and central pigmentation (Fig. 4). Linear EPM demonstrated that high signals for Ca, P and O coincided with the pigmented areas in the periphery and center of the stone (Fig. 5). The center also had Cu and S peaks as did several of the peripheral rings.

EDXA confirmed that Ca and S were present in the central area of the stone (Fig. 6) as well as in the peripheral pigmented band examined.

Stone 3 had several peripheral pigmented bands and a pigmented center (Fig. 7). EPM demonstrated Ca, P and O peaks corresponding with several of its peripheral pigmented bands; high Cu and S signals also coincided with the peripheral pigmented bands (Fig. 8). The center of stone 3 had high Ca and S signals but no discernible P, O, or Cu peaks. A high narrow S peak was located in the center of the stone. The nonpigmented areas of the stone had low or no detectable signals for Ca, P, Cu and S.

EDXA performed in the center of the stone in a different area from the locus along which electron probe microanalaysis was performed, confirmed the presence of Ca and also demonstrated the presence of P (Fig. 9). EDXA of a peripheral pigmented band demonstrated the presence of Ca and S.

Discussion

Our findings demonstrate the cyclic deposition of calcium bilirubinate pigment and other calcium salts within these cholesterol gallstones. Other calcium salts known to be present in small quantities in some cholesterol gallstones are calcium phosphate (12,15), calcium carbonate (3,8,15) and calcium palmitate (5,15). It has previously been demonstrated (3,15,16) that calcium carbonate is present in the center or in concentric layers in some cholesterol gallstones. It is important to emphasize that although the pigmented bands are easily discernible visually and the calcium signals relatively high in the pigmented areas, the amount of pigment present in each stone is quite small quantitatively (less than 1%). The microstructure of the pigmented areas basically consists of flat stacked cholesterol crystals (6) and, if the pigment content is particularly high, some features of pigment gallstones (6,7,15) may be seen admixed with the cholesterol crystals.

It is interesting that different types of calcium salts, for example, calcium bilirubinate and calcium phosphate, seem to be deposited together alternating with layers containing a paucity of calcium salts. The cyclical deposition of calcium salts in gallstones has been noted previously (11) and has been postulated to be a reflection of changes in bile composition or changes in other aspects of the environment within the gallbladder lumen. However, no specific factors or conditions in bile have been clearly shown to promote the precipitation of calcium salts during cholesterol gallstone formation.

Cu and S were also co-distributed with the calcium salts in some of the pigmented areas; this was particularly prominent in stone 3. Cholesterol gallstones are known to contain mucin glycoproteins (10,14). Been et al. (1) and Wosiewitz and Juling (16) have also demonstrated that S is found in pigmented regions of cholesterol gallstones and exists in the sulfide or disulfide state, consistent with its presence in protein molecules. It is possible the cyclic hypersecretion of Cu and Ca-binding proteins, such as mucin glycoproteins, into gallbladder bile during cholesterol gallstone formation may be responsible for the intermittent deposition of calcium salts within the stones.

The presence of pigmented bands in cholesterol gallstones may have clinical relevance regarding the efficacy of medical dissolution with chenodeoxycholate. The small amounts of calcium bilirubinate and other calcium salts in some cholesterol gallstones, when present in high concentration bands, may interfere with attempts at medical dissolution (4,13).

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Discussion with Reviewers

U. Wosiewitz: What was the roughness of the surface used for EPM? Were there larger holes or pinnacles?

Authors: The cross-sectional surface of the stone was polished with very fine abrasive paper. Naturally, when examined with the electron microscope, there were some small peaks and valleys on the surface. The probe diameter was set at 200 μm in order to average out concentrations.

U. Wosiewitz: As the background of EPM depends on the inclination and roughness of the surface: which of the peaks of the line profiles have to be considered significant?
Authors: The background readings are indicated in the appropriate figures for the EPM line profiles. We would draw the reader's attention mainly to the very high peaks that clearly stand out from the rest. The line profiles for the five different elements are presented on somewhat different scales from one another so as to make the absolute size of the recordings similar to one another.

Reviewer IV: In Figure 2 the first small Ca peak from the edge of the stone corresponds to a high P peak whilst half way along the trace another P peak has no corresponding Ca peak; could another P-containing compound such as a phospholipid be present?
Authors: It is entirely possible that high P peaks with no corresponding Ca peaks in the same area could represent other P-containing compounds such as phospholipids.

K.M. Kim: Proteins and mucoproteins are said to be responsible for the calcium containing deposits. What is the mechanism?
Authors: Evidence from various sources, including animal models of gallstone formation, supports the concept that mucin glycoproteins play a role in both cholesterol and pigment gallstone formation. One postulated mechanism is that mucin binds and/or complexes with cholesterol or calcium bilirubinate. For example, gallbladder mucin-bilirubinate complexes have been demonstrated in cholesterol gallstones (text ref. 10). In addition, gallbladder mucin has been shown to bind bilirubin *in vitro* (Smith BF, LaMont JT. (1983). Bovine gallbladder mucin binds calcium bilirubinate *in-vitro.* Gastroenterology 85:707–712.) Gallbladder mucin has also been shown to accelerate greatly the nucleation of cholesterol crystals from bile supersaturated with cholesterol (Lee SP, LaMont JT, Carey MC (1981). Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. J Clin Invest 67:1712–1723.)

I.A.D. Bouchier: It is sometimes claimed that *all* cholesterol stones have a pigment center. Is this so?
Authors: Previous reports have shown that most cholesterol stones do have pigmented centers. In our own recent studies of nearly 100 cholesterol gallstones, we found that over 80% of them had visually pigmented centers. This should not be taken to imply that the pigmented centers of cholesterol stones are true pigment stones. Quantitative analysis of 12 visually pigmented centers of cholesterol stones in our lab has revealed that in only 3 of the 12 stone centers did the actual amount of pigment exceed 1% (as a percent of sample weight). This indicates that although the centers of most cholesterol stones may appear pigmented, sometimes strikingly so, the actual quantity of pigment present is usually very small.

I.A.D. Bouchier: Do the authors have any views on why the pigment bands vary in number and thickness? Could the factor determining the precipitation of calcium be related to change in the quantity or characteristics of bilirubin in bile?
Authors: As yet there is no good explanation of why some cholesterol stones contain pigmented areas and others do not or why the number and thickness of the pigmented bands vary from stone to stone. It would seem reasonable to hypothesize that there is an intermittent change in some factor(s) in bile that affects the solubility of either calcium or bilirubin, resulting in the intermittent precipitation of calcium bilirubinate. An analogous situation is known to exist regarding cholesterol supersaturation of bile in that bile in many patients is only intermittently supersaturated (Smallwood RA, Jablonski P, Watts JMcK (1972). Intermittent secretion of abnormal bile in patients with cholesterol gall stones. Brit Med J 4:263–266.). It may be, for example, that there is intermittent hypersecretion of gallbladder mucin which then binds calcium salts causing them to precipitate on growing gallstones. Similarly, other factors in bile known to influence the solubility of bilirubin, such as bile salts or pH, could change and result in a decrease in bilirubin's solubility. It is also entirely possible that a slight increase in the concentration of biliary bilirubin or an increase in the proportion of biliary unconjugated bilirubin could result in concentrations of calcium bilirubinate that exceed its solubility product.

I.A.D. Bouchier: Could the availability of calcium in bile for precipitation be related to the bound and unbound (ionized) fractions attached to mixed micelles and that variation in micellar characteristics might influence the availability of calcium for precipitation?
Authors: Definitely. The ionized calcium concentration of bile is the fraction which is available for precipitation with a variety of anions including bilirubinate, phosphate, carbonate and palmitate. Normally, this ionized fraction is kept below the solubility product for the various calcium salts by "buffering" by micelles and several other moieties in bile. (Williamson BWA, Percy-Robb IW (1980). Contribution of biliary lipids to calcium binding in bile. Gastroenterology 78:696–702.). Variations in micellar characteristics, particularly involving the bile salt fraction, could definitely affect (either increase or decrease) the amount of ionized calcium in bile available to precipitate with the anions in bile. (Moore EW, Celic L, Ostrow JD (1982). Interactions between ionized calcium and sodium taurocholate: bile salts are important buffers for prevention of calcium-containing gallstones. Gastroenterology 83: 1079–1089.).

U. Wosiewitz: The P signal by EPM from the third stone center is different (lower) than the P signal detected by EDXA of the stone center. Is that because the stone center is inhomogeneous?
Authors: The EDXA was performed in a slightly different area of the stone center than was the EPM. This is good evidence that the center of the stone is inhomogeneous in composition.

U. Wosiewitz: Similarly, the EPM at the indicated center of stone 2 does not show any increase in Ca, P or S while the EDXA of this stone's center does. Why?
Authors: EDXA of the central area of stone 2 was not done in the precise center but rather in the same area as the Ca peak seen on the EPM line-profile which is within 100 μm of the indicated center.