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BACKSCATTERED ELECTRON IMAGING AND WINDOWLESS ENERGY DISPERSIVE X-RAY MICROANALYSIS: A NEW TECHNIQUE FOR GALLSTONE ANALYSIS

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Abstract

Scanning electron microscopy with or without conventional energy dispersive x-ray microanalysis is currently used to identify gallstone microstructure and inorganic composition. Organic calcium salts are among many biliary constituents thought to have a role in gallstone nidation and growth. However, current analytical techniques which identify these salts are destructive and compromise gallstone microstructural data. We have developed a new technique for gallstone analysis which provides simultaneous structural and compositional identification of calcium salts within gallstones. Backscattered electron imaging is used to localize calcium within cholesterol at minimum concentrations of 0.01%. Windowless energy dispersive x-ray microanalysis produces elemental spectra of gallstone calcium salts which are qualitatively and quantitatively different. These combined techniques provide simultaneous structural and compositional information obtained from intact gallstone cross-sections and have been used to identify calcium salts in gallstones obtained at cholecystectomy from 106 patients.

Key Words: Gallstones, Calcium Salts, Backscattered Electron Imaging, Windowless Energy Dispersive X-ray Microanalysis

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Introduction

Gallstones are the product of years of biochemical and mechanical derangements occurring within the gallbladder (5-7, 11, 25). A standard method for studying gallstone pathogenesis has been the microscopic and biochemical analysis of bile (1, 5-7, 10, 11, 13, 26, 29, 37). However, bile obtained at cholecystectomy often represents the end stage of disease and may not reflect the biochemical state during the early stages of gallstone formation. Determination of gallstone composition and structure may thus provide the only available data reflecting the early events of gallstone nidation and growth.

Current techniques for gallstone analysis include scanning electron microscopy (SEM) with or without energy dispersive x-ray microanalysis (EDX) (2, 3, 8, 12, 14, 16, 17, 24, 31, 38), x-ray diffraction (4, 9, 32-34, 38), and infrared spectroscopy (15, 18, 27, 30, 35, 38). These physical and chemical methodologies have been utilized to define the microstructure and composition of intact, cross-sectioned, or pulverized gallstones. Conventional SEM/EDX has been valuable in demonstrating the microstructure of gallstones, but cannot differentiate the non-phosphate calcium salts (bilirubinate, palmitate and carbonate) that are known to play a key role in gallstone pathogenesis. Infrared spectroscopy and powder x-ray diffraction can quantitatively identify specific organic and inorganic calcium salts within gallstones but require destruction of the specimen and sacrifice structural data.

In order to overcome these limitations, we have developed a new technique of gallstone analysis using the backscattered electron imaging (BEI) technology of SEM coupled with windowless EDX. In the BEI mode, calcium salts appear bright within an organic (cholesterol) background. Windowless EDX generates elemental spectra which include carbon and oxygen peaks which cannot be detected with a conventional beryllium window x-ray detector. The relative intensities of these peaks and the calcium peak can then be used to differentiate the organic calcium salts of gall-

stones. These methods have been used to determine the prevalence of calcium salts in gallstones obtained from 106 consecutive patients undergoing cholecystectomy.

Methods

Windowless EDX Spectra and Backscattered Electron Imaging of Calcium Salts

Windowless EDX spectra of calcium salts known to be important in gallstone pathogenesis were determined from standards of calcium bilirubinate, calcium palmitate, calcium carbonate, and calcium phosphate. Unconjugated bilirubin, cholesterol, sodium palmitate, calcium carbonate, and calcium phosphate were purchased (Sigma Inc., St. Louis, MO) and were more than 99% pure. Calcium bilirubinate was prepared by the method of Edwards, et al. (9). Calcium palmitate was prepared as described by Wosiewitz, et al. (39). These known standards were then pulverized, mounted on aluminum SEM stubs with silver paint, and sputter-coated with 10-15 nm of silver (Polaron Instruments Inc., Doylestown, PA).

Standard EDX and SEM conditions were determined since EDX peak intensities are dependent upon the accelerating voltage of the primary electron beam as well as other microscope and sample parameters. Calcium salt standard samples were analyzed at accelerating voltages of 5, 7, 10, 15, and 20 kV. Take-off angle (at 51.7°), probe current (at 1.1 nA), and collection time (of 30 seconds) were held constant. A JEOL JSM 840 II SEM (Peabody, Mass.) with 4 nm secondary electron image (SEI) and 10 nm backscattered electron image (BEI) resolution was used for analysis. EDX spectra were generated and analyzed using a Princeton Gamma-Tech System IV EDX (Princeton, New Jersey) with a windowless, thin window, and beryllium window turret-type detector.

Spectral differences were determined by quantification of calcium salt spectra. The continuum x-ray spectra were subtracted from a series of calcium salt spectra. Integrated peak areas were determined, and peak area ratios of carbon to oxygen, calcium to oxygen, and carbon to calcium were calculated for the non-phosphate calcium salts. Energy windows for integration of the Kα x-ray peak of each element were defined at 212-340 eV for carbon, 458-590 eV for oxygen, and 3605-3775 eV for calcium. Phosphorus Kα x-rays were detected at 1936-2088 eV.

The sensitivity of detecting a calcium salt in a gallstone cross-section by BEI/windowless EDX was approximated from standards of calcium salts embedded in an organic material. Known quantities of calcium carbonate and calcium bilirubinate were suspended in EPO-MIX epoxide resin (Buehler, Lake Bluff, IL) to final concentrations of 0.05% and 0.01% calcium salt by weight. The surface of each standard was polished using a Buehler Ecomet IV polisher. Finished surfaces were coated with 10-15 nm of sputtered silver and observed in the SEM in BEI and SEI modes at

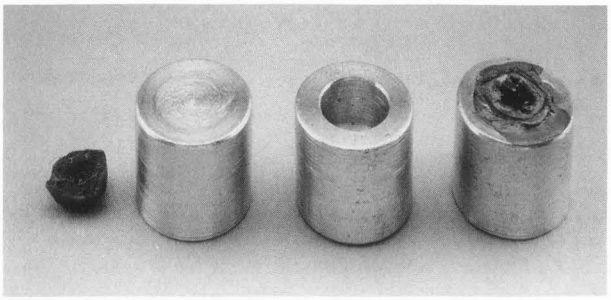


Figure 1. From left to right: fractured gallstone half, standard 10 mm aluminum SEM stub, SEM stub with drilled central depression, gallstone half mounted inside depression to keep fracture surface at level of stub surface (note silver paint placed along dried cement from stub to gallstone edge).

50-10,000x magnification. Working distance ranged from 14-39 nm, and accelerating voltage was 15-25 kV. The presence of calcium and the elemental composition of the backscattered structures were confirmed by windowless EDX.

Qualitative infrared spectroscopy was used to confirm the results of EDX. Purchased and synthesized calcium salts and cholesterol were analyzed by qualitative infrared spectroscopy using the potassium bromide disc technique (36) (Perkin Elmer 1310 Infrared Spectrophotometer, Perkin-Elmer, Norwalk, CT). In addition, selected areas of eight gallstone cores previously analyzed by SEM/windowless EDX were dissected, pulverized, and analyzed by infrared spectroscopy.

Gallstone Analysis

Gallstones were obtained fresh at cholecystectomy, washed briefly in deionized water, dried under vacuum, and classified as cholesterol or black or brown pigment stones by physical characteristics (12, 16, 37) and cholesterol content. Percent cholesterol of dry stone weight was determined by the method of Roschlau (28). Gallstones with more than 50% cholesterol by weight were classified as cholesterol stones whereas those with less than 20% cholesterol were considered to be pigment stones.

Dried gallstones were fractured to create a cross-section, mounted on aluminum SEM stubs, and coated with 10-15 nm of sputtered silver. Specialized stubs were prepared by drilling out part of the stub surface so that specimens could be sunk into the stub with the gallstone cross-section surface near the level of the remaining stub surface. Stone halves were mounted into the stub depression with Duco cement. A thin film of silver paint was then brought along the dried cement from the stub surface to the edge of the stone to maximize electrical conduction and minimize sputter coating requirements (Figure 1).

Entire cross-section surfaces were scanned in BEI at magnifications of 10-1000x to localize calcium salts. Random areas of each stone were further

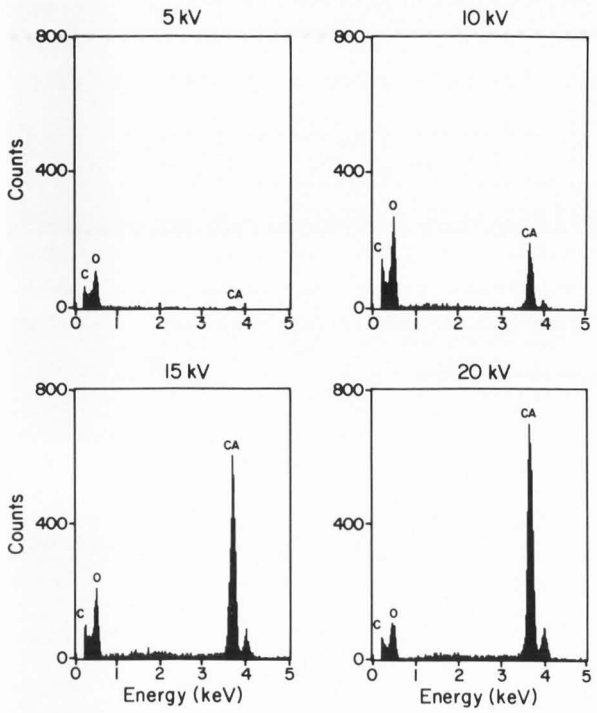


Figure 2. Windowless EDX spectra of calcium carbonate standard at varying electron beam accelerating voltages.

studied by BEI and SEI at magnifications of 1000-20,000x. BEI localized calcium salts and morphologically suspicious structures seen by SEI were then identified by windowless EDX. This technique has been used to analyze gallstones from 106 consecutive patients undergoing cholecystectomy to determine the prevalence of cholesterol and calcium salts (bilirubinate, palmitate, carbonate, and phosphate).

Statistical Analysis

Calcium salt peak ratios and cholesterol content are reported as mean ± standard error of the mean. Statistical differences were confirmed by analysis of variance. Prevalence of gallstone calcium salts are presented as the percent for each type of stone studied.

Results

Windowless EDX Identification of Calcium Salts

The dependence of peak intensity on electron beam accelerating voltage is shown in Figure 2 for a calcium carbonate standard. These spectra demonstrate the effect of absorption on the ratio of calcium to carbon and oxygen. With increasing accelerating voltage, the mean primary electron penetration depth and depth of x-ray generation increase. However, this results in increased absorption of lower energy (light element) x-rays, and the EDX spectra show a decrease in the peak intensity of the light elements. Fifteen kV was arbitrarily chosen as a standard accelerating voltage for calcium salt identification.

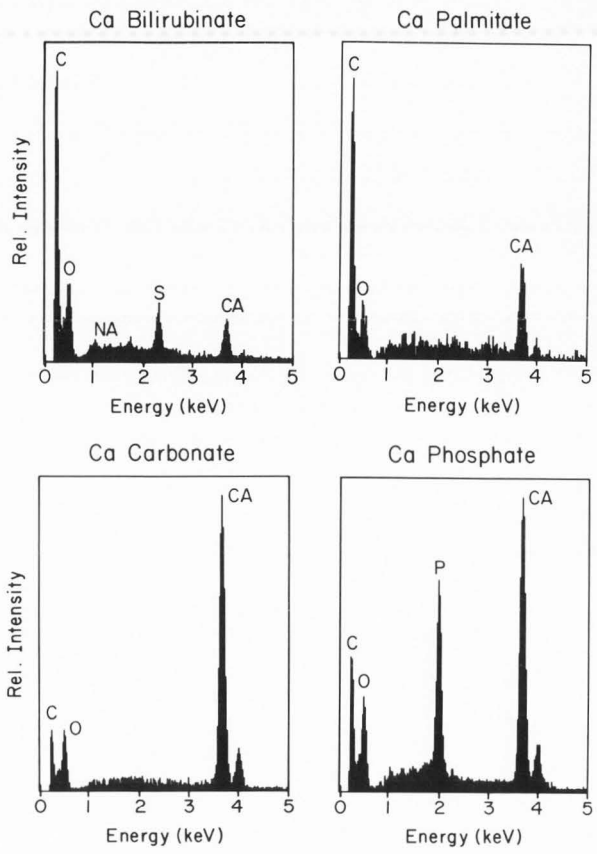


Figure 3. Windowless EDX spectra of gallstone calcium salts.

Windowless EDX spectra obtained from samples of calcium bilirubinate, palmitate, carbonate, and phosphate are displayed in Figure 3. A conventional beryllium window EDX spectrum of calcium palmitate is compared to the same spectrum generated without the window in place (Figure 4). Without the addition of the carbon and oxygen peaks, the beryllium window spectrum would be similar for all of the non-phosphate calcium salts. However, the windowless EDX spectra of these three calcium salts appear different.

Quantitative differences were determined from a series of spectra of calcium bilirubinate (n = 6), calcium palmitate (n = 4), and calcium carbonate (n = 4) analyzed through a background subtraction program by the PGT System IV. Ratios of integrated background subtracted peak areas for carbon to oxygen, calcium to oxygen, and carbon to calcium for each non-phosphate calcium salt spectrum are displayed in Table 1. Each of these three calcium salts provides distinct ratios which differentiates it from the other salts. Moreover, calcium bilirubinate often contains a sulfur peak, and calcium phosphate contains a phosphorus peak (Figure 3).

Backscattered Electron Imaging

Calcium carbonate and calcium bilirubinate were detected by BEI at concentrations of 0.05% and

Table 1. Integrated background subtracted peak ratios of gallstone non-phosphate calcium salts.

Calcium salt	n	Carbon/Oxygen	Calcium/Oxygen	Carbon/Calcium
Calcium bilirubinate	6	3.13 ± 0.60[a]	0.31 ± 0.10[a]	13.90 ± 2.70[a]
Calcium palmitate	4	5.34 ± 0.36[b]	2.52 ± 0.43[b]	2.30 ± 0.38[b]
Calcium carbonate	4	0.68 ± 0.08	5.21 ± 0.89	0.14 ± 0.02

a $p<.05$ vs calcium palmitate, and vs calcium carbonate
b $p<.05$ vs calcium carbonate

Table 2. Prevalence of calcium salts and cholesterol in 106 gallstones.

	Cholesterol Stones (n=75) %	Black Pigment Stones (n=26) %	Brown Pigment Stones (n=5) %
Cholesterol	100	4	80
Any calcium salt	89	100	100
Calcium bilirubinate	52	100	100
Calcium palmitate	41	27	100
Calcium carbonate	21	27	0
Calcium phosphate	31	27	20

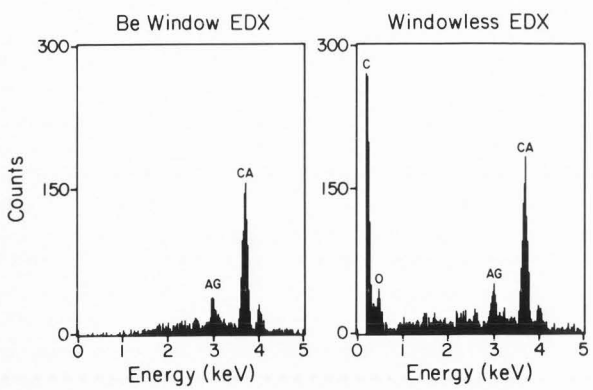


Figure 4. Conventional beryllium window EDX spectrum compared to windowless EDX spectrum of calcium palmitate. Silver peak generated from sputtered coating.

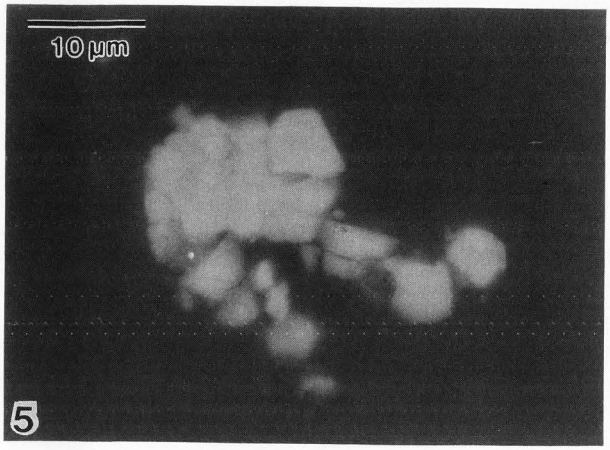
0.01% by weight in epoxide (Figure 5). Backscattering calcium salt particle sizes ranged from 1.0 - 20.0 μm. The windowless EDX spectrum of epoxide identified carbon and oxygen, although the oxygen peak was more intense than that obtained by cholesterol (carbon and oxygen only in a ratio greater than 20/1 for both organic compounds). In a control preparation of 1% cholesterol embedded in epoxide, cholesterol crystals could not be detected by BEI and were poorly imaged by SEI as defects in the polished epoxide surface. Cholesterol crystals could be seen suspended in epoxide by observation in the dissection light microscope.

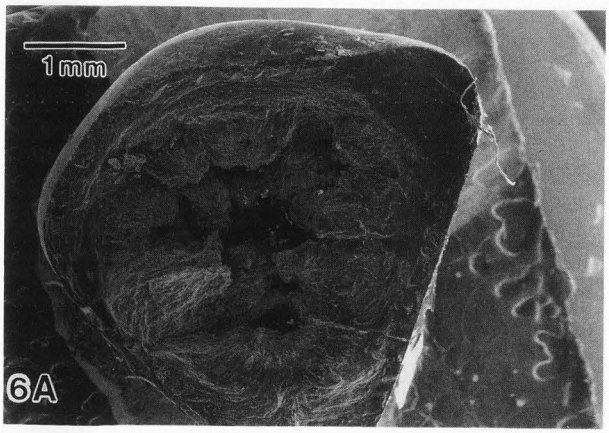
Calcium standards and pulverized gallstone samples (n = 8) containing EDX identified calcium salts were analyzed by qualitative infrared spectroscopy (IR). Qualitative IR confirmed the identity of each synthesized or purchased standards and identified calcium salts suspected by BEI/windowless EDX analysis in six cholesterol and two black pigment gallstones.

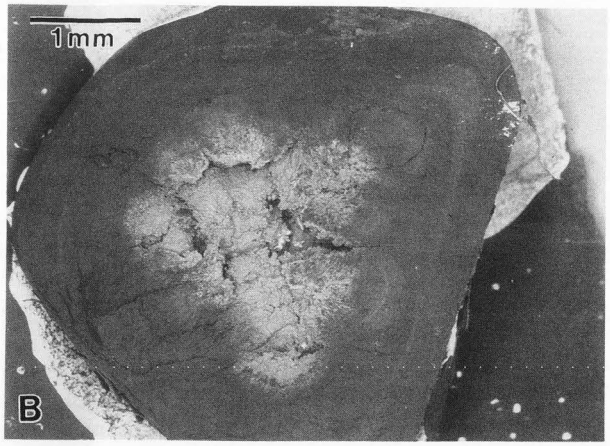
Gallstones

A cholesterol gallstone cross-section is shown in Figure 6 at low magnification as imaged by secondary and backscattered electrons. At slightly higher magnification (Figure 7), a calcium salt is imaged in BEI interacting with cholesterol crystals at the interface of the calcium salt nidus and cholesterol periphery of this gallstone.

Calcium bilirubinate granules ranged from clusters of 0.5 μm to amorphous glassy masses > 100







μm in diameter (Figure 8). Calcium phosphate particles were often < 1.0 μm and reached maximum diameters of 10-20 μm when agglomerated (Figure 9). Whereas macroscopic palmitate rosettes were often > 100 μm, partial rosettes and individual leaves were seen < 10 μm (Figure 10). Calcium carbonate microspheroliths ranged from 0.5 μm to 10 - 20 μm in diameter (Figure 11).

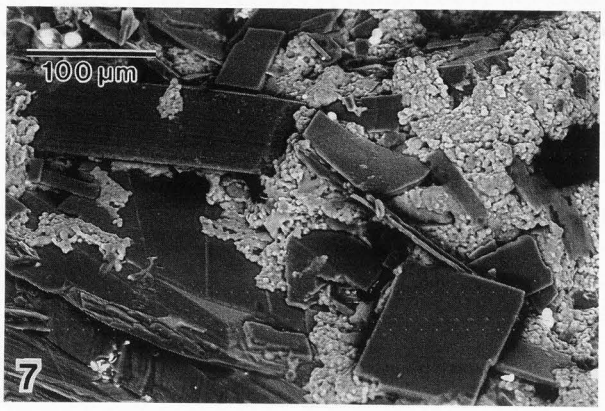


Figure 5. BEI of calcium carbonate standard embedded in epoxide resin and fine polished.

Figure 6. A. Low magnification SEI micrograph of cholesterol gallstone cross-section. **B.** Same section in BEI mode displaying obvious calcium salt core.

Figure 7. BEI micrograph of calcium salt intimately related to cholesterol crystals within cholesterol gallstone.

The prevalence of calcium salts and cholesterol found anywhere within the gallstone cross-section of 106 gallstones obtained at cholecystectomy is presented in Table 2. Details of this patient population and data on the prevalence of individual calcium salts within the center, periphery, and shell of these gallstones will be the subject of a separate report.

Discussion

Approximately 29 million Americans have gallstones and nearly 500,000 cholecystectomies are performed annually in the United States (20). Gallstone disease therefore places a large financial burden on the U.S. health care system. Calcium salts are one of the many biliary components thought to play a role in gallstone nidation and cholesterol crystal agglomeration (18, 19, 24, 38). The simultaneous study of gallstone structure and composition with respect to calcium salts thus may provide clues to the initiating and promoting events of gallstone formation and lead to pharmacologic and dietary manipulations targeted at primary prevention of gallstones.

Previous methods available to identify organic calcium salts in gallstones have required destruction of the stone and loss of structural data. Infrared spectroscopy has been used both quantitatively and qualitatively with sensitivities reported from 0.1% to 0.5% (15, 36). Powder x-ray diffraction is able to determine the crystalline form of each calcium salt and has a detection limit on the order of 1% (4, 32). The radial distribution of carbon, oxygen, calcium and other elements in gallstone cross-sections has been determined by wavelength dispersive electron probe microanalysis

(17). However, with this technique each element is analyzed individually, and this information has not been used to differentiate non-phosphate calcium salts. Finally, gallstones have been studied by conventional scanning electron microscopy to determine cholesterol crystal growth patterns in gallstone pathogenesis (3, 21-23), the role of bacteria in pigment gallstone formation (8, 12, 14, 31), and the morphology of calcium salts detected by other methodologies (16, 18, 38). EDX has previously been used to demonstrate calcium, sulfur, and trace elements present in gallstones (2, 16, 38), but the application of windowless EDX is new to gallstone research.

Quanta generated from the fractured surface of a gallstone by an incident electron beam simultaneously provide both structural and compositional data. Of interest in this application are secondary electrons, backscattered electrons, and x-rays. Since backscattered electrons from calcium containing structures produce a stronger signal than the surrounding cholesterol crystals, calcium salts are easily localized within cholesterol gallstones. Once localized, the electron beam can be focused on the salt of interest and a windowless EDX spectrum generated. Calcium bilirubinate, palmitate, carbonate and phosphate produce windowless EDX spectra that are quantitatively different. Therefore, with preservation of structure, specific calcium salts can be identified within gallstones.

The detection of calcium in organic matrix by backscattered electron imaging is dependent upon both microscope and its operating conditions, and sample structure and composition. Adjustable microscope parameters include accelerating voltage, probe size, and distance from sample to detector (in this case approximated by working distance to pole piece). These can be optimized for calcium salt localization by BEI and windowless EDX analysis. However, for best results, working distance and accelerating voltage are different in each application.

Gallstone sample characteristics can be modified but would require polishing and therefore changing the natural fracture surface, an effect purposely avoided in this technique. An attempt was therefore made in sample mounting to prepare a semi-flat surface with good conductive properties in order to minimize charging phenomena.

Both size of calcium salt structures and salt density also affect backscattered imaging intensity. Since backscattered electrons are generated from depths on the order of microns beneath the sample surface, the detectability of these salts is both size and depth dependent. Regarding density, calcium carbonate provides the strongest signals independent of particle size followed by calcium phosphate, palmitate, and bilirubinate.

The variables listed above, as well as variation in amount of surface area examined, make it difficult to assign a sensitivity to this method. Pulverized calcium salts were suspended in epoxide and a smooth surface prepared in order to experimentally approximate this sensitivity. Calcium carbonate and bilirubinate, at opposite ends of signal intensity in BEI, were easily localized in an organic matrix at 0.01%. The smooth surface represents an ideal condition for backscattered electron imaging and may not be applicable to fractured gallstone cross-sections. However, 0.01% calcium salt is easily detected in epoxide. Since theoretically lower concentrations could be detected by searching a larger surface area, we estimate 0.01% as an approximate sensitivity for the BEI sensitivity of localization of calcium salts within gallstones. In addition, the particle sizes detected, 1.0 - 20.0 μm, are similar to those of calcium salts found in actual gallstones (38).

The specific identification of calcium salts by windowless EDX is also dependent upon microscope and x-ray analyzer operating conditions. The accelerating voltage of 15 kV was chosen since it was well in excess of the critical excitation energy of calcium but did not overly "dampen" the light elements of interest, carbon and oxygen by absorption effects. This accelerating voltage is not optimal for detection of trace elements such as iron and copper; however, these elements were occasionally found when the accelerating voltage was increased to 25 kV.

In conclusion, a non-destructive technique for the simultaneous study of gallstone microstructure and composition is presented. Backscattered electron imaging easily localizes organic and inorganic calcium salts within gallstones with a detection limit more sensitive than for previously available methods. These calcium salts can then be specifically identified by windowless energy dispersive x-ray microanalysis which provides elemental spectra that keep quantitatively distinct for calcium bilirubinate, palmitate, carbonate and phosphate. These combined methodologies, therefore, allow the sensitive and specific detection of organic calcium salts within gallstones and should be useful in further investigations of gallstone pathogenesis.

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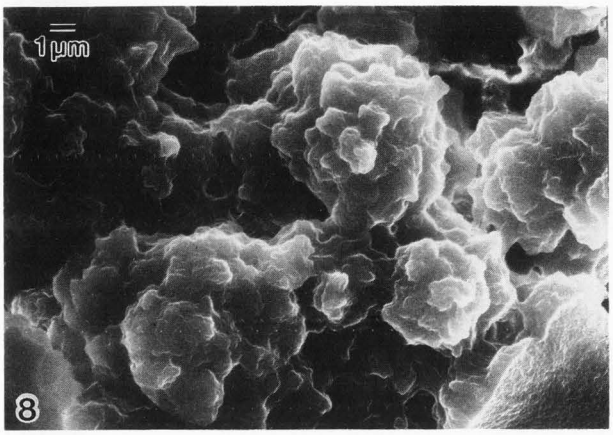

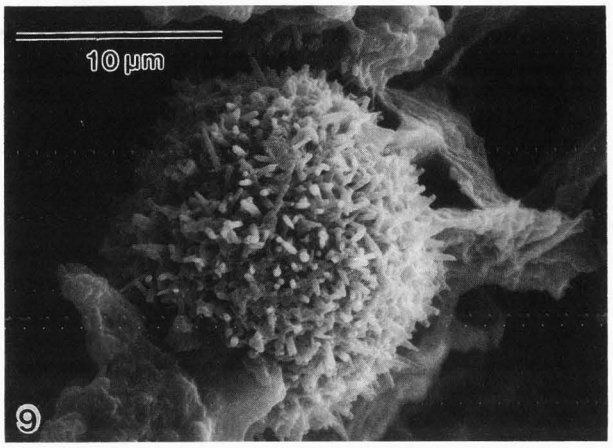

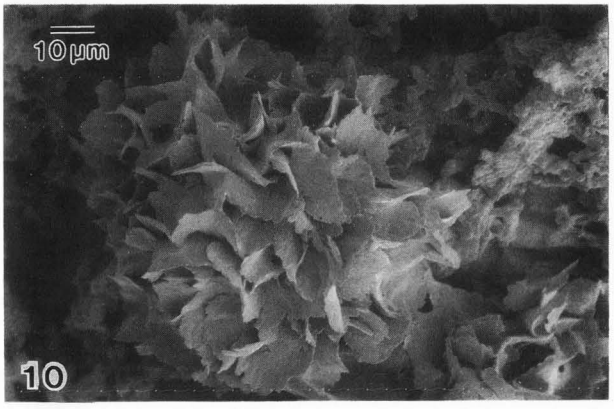

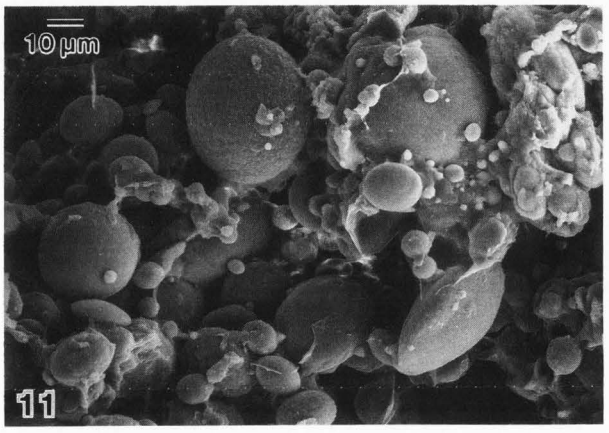

Figure 8. Secondary electron image of calcium bilirubinate clusters found within pigment gallstone core.

Figure 9. Calcium phosphate structure seen within cholesterol gallstone core (SEI).

Figure 10. Calcium palmitate rosette from cholesterol gallstone core (SEI).

Figure 11. SEI of calcium carbonate microspheroliths detected within pigment gallstone core.

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Discussion with Reviewers

S.R. Kahn: Why the elaborate preparation, drilling a hole in the stubs, etc? Did you try to use colloidal graphite to mount the specimen?

Authors: Since the gallstone cross-sections are similar to hemispheres, the use of a standard flat stub would give a small point of contact from stone-to-stub. The amount of stone surface in contact with a conductive surface was increased by the sample preparation method described above. This allowed for increased conductivity and permitted a decrease in the amount of sputtered silver necessary to eliminate charging phenomena. We did not attempt to use colloidal graphite to mount the specimens.

P.T. Cheng: What are the actual values of carbon/oxygen, calcium/oxygen, and carbon/calcium ratios (ranges, means and standard deviations) as observed on the unpolished fracture surfaces on which the identities of the calcium salts had been confimed by

infrared spectroscopic data?
Authors: The ranges for the ratios in Table 1 are: calcium bilirubinate - C/O: 1.77-5.47, Ca/O: 0.08-0.76, C/Ca: 4.87-21.9; calcium palmitate - C/O: 4.75-6.39, Ca/O: 2.09-3.81, C/Ca: 1.25-3.04; calcium carbonate - C/O: 0.56-0.91, Ca/O:3.40-7.69 C/Ca: 0.08-0.18. The mean and standard error of the mean for each ratio are presented in Table 1.

G.M. Roomans: How did you produce the background spectrum that you subtracted from your salt spectra?
Authors: The Bremsstrahlung selected for background subtraction was generated from cholesterol crystals found near calcium salts in a cholesterol gallstones. This background spectrum was stored in the PGT System IV memory and subtracted point by point from each calcium salt spectrum. The remaining background subtracted carbon, oxygen, and calcium peaks were then integrated at full-width, half-height for calculation of elemental ratios.

G.M. Roomans: Semi-quantitative analysis of light elements (in this case C and O) in bulk specimens is notoriously difficult because of x-ray absorption. The absorption is highly dependent on the geometrical conditions and hence on the surface structure of the specimen. The local surface structure determines the pathlength of the x-rays in the specimen, and consequently, absorption. Errors will occur with unpolished specimens. Have the authors considered these problems?
Authors: You are correct in stating that a polished surface would have diminished x-ray absorption. However, we were concerned that polishing would change the calcium salt and cholesterol morphology, an effect purposely avoided with our fracture technique. Even without polishing, the ratios of C/O, Ca/O, and C/Ca were all statistically different among the calcium bilirubinate, palmitate and carbonate salts.

G.M. Roomans: Table 1 raises a number of questions. Given that in carbonate the molar ratio of C:O is 1:3, is this compatible with an intensity ratio of 2:3? Are you, maybe, losing oxygen under the beam? Similarly, if we accept the measured C/O ratio for carbonate, does this fit with the measured ratio vs the known C/O ratio for calcium bilirubinate and calcium palmitate?
Authors: We also initially questioned the experimentally observed C/O ratio for calcium carbonate. However, when these same standards or gallstone samples of calcium carbonate were analyzed using a second system (JEOL JEM 1200 with a Kevex Quantum detector at 40 kV), a similarly low oxygen peak was generated with respect to calcium. The background subtracted peak ratios obtained with our semi-quantitative analysis were not expected to equal the stoichiometric ratios of carbon to oxygen, calcium to oxygen or carbon to calcium for these salts. Corrections for absorbance and fluorescence were not made either by ZAF correction or peak to background ratios.

R.D. Soloway: Windowless EDX of mixtures of standards should be reported. Can they be identified in the presence of cholesterol? What is the diameter of the field of interest that can be independently analyzed? What is the reproducibility of the methods?
Authors: We have not performed windowless EDX on mixtures of standards or in the presence of cholesterol because at magnifications high enough to visualize individual calcium salts, specific spectra are obtained. The purpose of this technique is to be able to determine simultaneously the structure and composition of calcium salts in and cholesterol in gallstones. A structure must first be identified in SEI or BEI and then analyzed for composition by EDX. This method is not designed to determine an amount of a calcium salt within a mixture. Relative proportions of salts can be estimated morphologically, but not determined from peak heights or ratios.

Although carbon, oxygen and calcium peaks can be generated from submicron sized particles, x-ray signal is generated from a teardrop shaped region (whose size is a function of the accelerating voltage and density of the specimen) extending micrometers below the sample surface. At 15 kV the field size that is possible to analyze without changing calcium salt peak ratios due to sub-surface x-ray signal is approximately 10-20 μm^2. This field size applies in bulk samples and assumes that the calcium salt of interest is approximately spherical. Thus, calcium containing structures with diameters less than 1 μm cannot be accurately identified as a specific calcium salt by windowless EDX. As stated in the text, the windowless EDX observations were reproduced by IR.

R.D. Soloway: Calcium bilirubinate often contains a sulfur peak. Is there also associated copper? Several previous studies have noted copper and sulfur in association, suggesting that a copper protein is present. Does this sulfur peak suggest associated protein? Is the sulfur a thiol or sulfate in frequency? These two forms have differing peaks in the EDX spectrum.
Authors: Calcium bilirubinate does often contain a sulfur peak. A copper peak was identified often when the accelerating voltage was increased to 25 kV. This was not the usual operating accelerating voltage used in this study. This sulfur peak may suggest an associated protein and has been shown by other investigators (16) to be in a low valance state, suggestive of a sulfide as opposed to a sulfate moiety. The valance state cannot be determined by conventional or windowless EDX and has been shown before using electron spectroscopy for chemical analysis (ESCA).

R.D. Soloway: In Figure 7, can the individual spectra of the cholesterol crystals and the calcium phosphate be determined? Is calcium bilirubinate ever intermixed with calcium phosphate?
Authors: Yes, individual windowless EDX spectra of cholesterol crystals (Figure 12) and the calcium salts in Figure 7 can be determined. The calcium salt shown in Figure 7 was identified as calcium bilirubinate by windowless EDX analysis. Calcium bilirubinate and calcium phosphate were identified together

in 10% of the gallstones studied.

R.D. Soloway: What is the compositional identity of the other structures in the Figure 9?
Authors: The composition of the background structure in Figure 9 is calcium bilirubinate.

G.M. Roomans: The morphology of the calcium crystals appears rather distinct. Do you actually need windowless EDX to identify them?
P. Malet: How variable is the SEM appearance of calcium phosphate, carbonate and bilirubinate crystals?
Authors: Calcium phosphates varied from microspheroliths with granular surfaces to larger spherical structures with spiculated crystalline surfaces. Calcium carbonate was observed in three morphologies: rod-like structures in the shells and rings of cholesterol stones, singular ovoid or spherical structures found within both cholesterol and pigment stones, and an amorphous material seen mostly in pigment stones. The calcium bilirubinate precipitates were seen in clusters or amorphous glassy masses.

While the morphologic appearances of some crystal forms of the calcium salts are unique, others overlap considerably. Calcium bilirubinate clusters are often morphologically similar to some calcium phosphates. The EDX phosphorous peak easily differentiates these crystals. Calcium bilirubinate glassy masses can be morphologically confused with amorphous calcium carbonate as it appeared in pigment but not cholesterol stones. Sulphur was not uniformly present in the bilirubinate spectrum, and windowless EDX was necessary to discriminate between bilirubinate and carbonate. Amorphous palmitate was quite sensitive to higher beam kV, and this characteristic could help differentiate it from bilirubinate or carbonate. However, we often worked at lower accelerating voltages, and again, windowless EDX was necessary for salt identification.

P. Malet: Can the authors speculate as to why the calcium salts are deposited within the cholesterol gallstones?
Authors: While a considerable literature exists on calcium salts in the cores of gallstones and their potential role in stone nucleation (2, 16, 18, 19, 24, 38), fewer studies focus on the distribution of calcium in the periphery of the gallstone. Sutor and Wooley (34), characterized the deposition of crystalline material in gallstones from 578 patients by XRD and postulated that changes in bile composition were reflected in the variation of calcium salts and cholesterol in gallstones. Malet et al. (17), described the cyclic deposition of calcium salts in cholesterol gallstones and showed that S and Cu were present in the calcium containing bands. This observation was suggested to represent cyclic hypersecretion of Cu and Ca binding proteins.

Recently a low molecular weight acidic glycoprotein has been isolated from 13 cholesterol gallstones [Shimizu S, Sabsay B, Veis A, Ostrow JD, Rege RV, Dawes LG (1989) Isolation of an acidic protein from cholesterol gallstones, which inhibits the precipitation of calcium carbonate in vitro. J. Clin. Invest. 84, 1990-1996]. This protein was shown to bind pigment and inhibit precipitation of calcium carbonate in vitro. Indeed, cyclic secretion of this protein into bile may be responsible for the cyclic deposition of calcium bilirubinate, while a relative decrease in its concentration in bile could result in the cyclic calcium carbonate precipitates found in cholesterol gallstones. The calcium salt present in most radio-opaque gallstones is calcium carbonate. While precipitation of this salt onto a cholesterol stone appears to arrest further precipitation of cholesterol crystals in some stones, others incorporate the carbonate into an inner ring and continue with cholesterol stone growth. This finding is further indirect evidence for changing bile composition reflected in gallstone composition. However, whether this phenomenon represents a change in the saturation indices for cholesterol or calcium salts, or the presence of a pro-nucleating or anti-nucleating protein for cholesterol and/or calcium salt precipitation, remains to be elucidated.

P.T. Cheng: What tentative conclusions can you draw on the differences in the pathogenesis of cholesterol and black and brown pigment stones from your observed prevalence of calcium salts and cholesterol in the 106 gallstones studied.
Authors: Calcium salts were identified in the 89% of cholesterol gallstones and in 100% of pigment gallstones suggesting a role for calcium in the initiation of most cholesterol gallstones and all pigment gallstones. However, since the gallstones from any human patient are of only one stone type, the pathogenesis of these stones must be different.

Using the techniques described in this paper, we have confirmed previous observations that black and brown pigment stones differ with respect to the presence of cholesterol, calcium phosphate and calcium palmitate (12). Further observations with respect to the distribution of calcium salts within the different stone types and their implications regarding pathogenesis will be the subject of a separate paper.

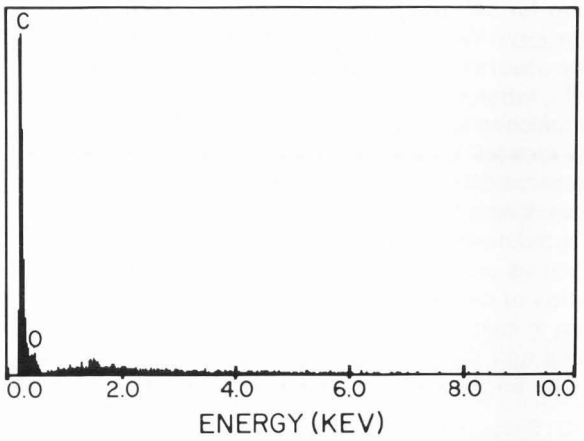

Figure 12. EDX spectra for cholesterol crystals.