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ULTRASTRUCTURAL STUDIES OF CRYSTAL-ORGANIC MATRIX RELATIONS IN RENAL STONES

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Abstract

Biochemically the organic matrix of kidney stones contains mucoproteins, mucopolysaccharides, inorganic material and bound water. Morphologically, the organic matrix exists as either amorphous or fibrous forms. We have attempted to critically evaluate results from analytical and morphological studies on stone matrices using light microscopy, histochemistry, x-ray diffraction, scanning electron microscopy, x-ray energy dispersive spectrometry, transmission electron microscopy and selected area electron diffraction.

On the surface of calcium oxalate stones, there are usually large masses of randomly deposited calcium oxalate crystals each coated with organic matrix. Transmission electron microscopy shows these large surface crystals are composed of rows of smaller crystallites interleaved by organic matrix in a fairly orderly manner suggesting the crystallites are held together by organic matrix.

In the core of a calcium containing stone, the organic matrix frequently exists as concentric laminations alternating as calcium apatite covered fibrous matrix layers and amorphous matrix layers. Transmission electron microscopy suggests that the fibrous area is probably just an area heavily populated by calcium apatite crystallites which give the fibrous appearance while the amorphous area is sparsely populated.

Organic matrix richness in stones can be associated with infection and calcium apatite crystal deposition is favored in infection stones.

KEY WORDS: Scanning electron microscopy, x-ray diffraction, electron diffraction, transmission electron microscopy, renal stones, matrix stones, calcium oxalate, calcium apatite, organic matrix, proteus organisms.

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Introduction

The pathogenesis of stone formation in the urinary tract is not clearly understood. Although fluctuating ion supersaturation and decreased ambient inhibitors can account for the common event of crystal nucleation that results in crystalluria, these phenomena appear insufficient to explain the more sporadic agglomeration event that results in stone formation.

Stones are composed of agglomerates of one or more crystal phases and a variable amount of organic matrix. Whether the organic matrix is adsorbed passively to crystals or plays a more active role in facilitating lithogenesis by retarding ionic diffusion, catalyzing agglomeration or cementing agglomerates, continues to be a long standing unsettled controversy.

Extensive studies have shown that the concentrations of inhibitors for crystal growth and aggregation, e.g. pyrophosphate, citrate, glycosaminoglycans, Tamm-Horsfall mucoprotein and ribonucleic acid, in stone former urine are lower than in normal urine (Fleisch, 1980; Robertson et al., 1981). Yet no promoters have been identified in stone former urine. However, organic matrix is always present in urinary stones, albeit having only low contribution (mean 2.5% by weight) in most stones except in the rare radiolucent "matrix stones" (mean 65% by weight) (Boyce and King, 1959; Cheng et al., 1983b).

Biochemically the organic matrix of kidney stones contains mucoproteins (major component) mucopolysaccharides, residual inorganic material (calcium and phosphate bound to the matrix) and bound water (Boyce and Sulkin, 1956; Boyce and Garvey, 1956; King and Boyce, 1957 and 1959). The mucoprotein component does not contain collagen (no hydroxyproline) or elastin ($<$ 2% proline) (King and Boyce, 1957; Boyce, 1968) but contains substantial amounts of aspartic and glutamic acids and some γ-carboxyglutamic acid which facilitates calcium binding (Spector et al., 1976; Lian et al., 1977; Warpehoski et al., 1981). The mucopolysaccharide component does not contain hyaluronic acid, chondroitin sulphates but contains 'keratan sulphate like' material (Boyce and Sulkin, 1956; King and Boyce, 1957; Warpehoski et al., 1981). Sialic acid, though reported absent in earlier work, has been found to be present in

every stone, with a content close to that of the hexose (Melick et al., 1980). A detailed review on the biochemistry of renal stone matrix has been published recently (Malagodi and Moye, 1981).

The origin of the mucopolysaccharides is still unknown; the possible sources include glomerular ultrafiltrate, renal tubular cell brush border or basement membrane and bacterial cell wall.

Morphologically, the organic matrix exists as either amorphous or fibrous forms (Boyce and Sulkin, 1956; El-Sayed and Cosslett, 1977; Cheng et al., 1983b). However, the interrelationship between the crystal phase and the two morphological forms of organic matrix is still unclear. In order to better understand the roles of organic matrix in kidney stone pathogenesis, it is important to study in detail the crystal-matrix relationship in kidney stones.

In this review, we will attempt to critically evaluate results from analytical and morphological studies using light microscopy, histochemistry, x-ray diffraction, scanning electron microscopy, x-ray energy dispersive spectrometry, transmission electron microscopy and selected area electron diffraction.

Methods

Polarized Light Microscopy and Histochemistry

Large undecalcified kidney stones are difficult to cut into sections. Thick sections (100-300 μm) were employed to study macroscopic distributions of crystalline and amorphous materials by polarized light microscopy, microradiography, x-ray microdiffractometry, scanning electron microscopy and x-ray energy dispersive spectrometry (Lagergren, 1956; Cheng et al., 1981). However, these thick sections are not suitable for studying crystal-matrix relationship. Semi-thin sections (1-10 μm) of small undecalcified and decalcified stones or fragments of large stones are more suitable for studying organic matrix by polarized light microscopy and histochemistry. These sections can be stained with hematoxylin and eosin for cellular elements, bacteria and structural characteristics of the matrix (the last stained only faintly), periodic acid Schiff for mucoproteins (brilliant purplish-red) and toluidine blue for sulfated mucopolysaccharides (blue and purple metachromatasia) (Boyce and Sulkin, 1956; Cheng et al., 1984). For polarized light microscopy, both stained and unstained sections can be examined in a polarizing microscope equipped with a polarizer, a first-order retardation plate and an analyzer. However, while the microscopic relationship between crystal and organic matrix phases can be studied histochemically, crystals, except for calcium oxalate, are difficult to detect in stained sections either because the crystals dissolve during the preparation or because the stain masks the birefringent properties of the crystals (Cheng et al., 1983a). Although unstained sections retain most crystals, some mounting media (e.g. Flo-texx, Lerner Laboratories; Permount, Fisher) used in securing cover slips on slides dissolve crystalline materials or quench birefringence.

X-ray Diffraction

X-ray powder diffraction can be used to positively identify the various crystal phases of the stones (Sutor and Scheidt, 1968). However it is only useful for studying large crystalline deposits in relatively thick sections (Cheng et al., 1981 and 1983a). Amorphous inorganic or organic materials do not give x-ray diffraction patterns (Cheng and Pritzker, 1983). However the organic matrix in "matrix stones" does give a very broad band (0.28-0.48nm) (Cheng et al., 1983b).

Analytical Scanning Electron Microscopy

Scanning electron microscopy has been useful in studying the surface morphology of kidney stones. A comprehensive review on identification of stones by surface morphology has been published (Kim, 1982). Organic matrix coatings of crystals have been reported in several studies of well-mineralized human and experimental animal stones (Malek and Boyce, 1977; Khan and Hackett, 1980; Rushton et al., 1980; Warpehoski et al., 1981; Kim and Johnson, 1981). Scanning electron microscopy and x-ray energy dispersive spectrometry have been used to study poorly mineralized "matrix stone" surfaces (Cheng et al., 1983b).

Analytical Transmission Electron Microscopy

The crystal-matrix relationship can best be studied by transmission electron microscopy. However stones are difficult to cut into thin sections (50 nm). Stones first embedded in agar then demineralized in 0.25 N EDTA can be cut easily and have been shown to preserve the information needed to study the crystal-matrix interface (Khan et al., 1983). However, in order to study the fine details of calcium phosphate crystallites and organic matrix interaction, it is highly desirable to study undecalcified stone sections. This has been shown to be a rewarding approach in the "matrix stones" study (Cheng et al., 1983b and 1984). Further examples on transmission electron microscopic studies of undecalcified mineralized kidney stones will be demonstrated below.

Recently it has been shown that fixation by aqueous fixatives including Karnovsky's leads to considerable loss of calcium deposits (Landis, 1979). As a consequence we have adopted the procedure of direct embedding of air dried stones in plastic without fixation. 50 nm thin sections can be cut with a diamond knife on a microtome, but large crystals have to be collected separately because they fall out from stone sections during cutting. Crystal deposits remaining in the sections can be identified by selected area electron diffraction (Cheng et al., 1983b).

Results and Discussion

Crystal-Organic Matrix Relationship at the Stone Surface

The organic matrix content in kidney stones may be low by weight but it occupies much more space than is suggested by its proportional weight (Khan et al., 1983). Even though it is present throughout the stone, it is not evenly distributed, its content being higher at the stone surface (5.7%) than at the core (2.7%) (Warpehoski et al., 1981). Surface matrix content is greater than amounts predicted by physical adsorption suggest-

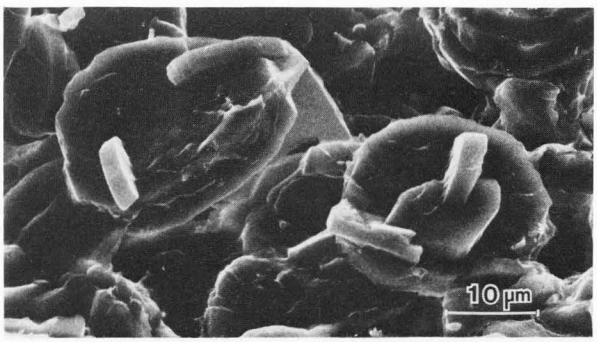

Fig. 1: Scanning electron micrograph of a stone surface full of large matrix coated crystals.

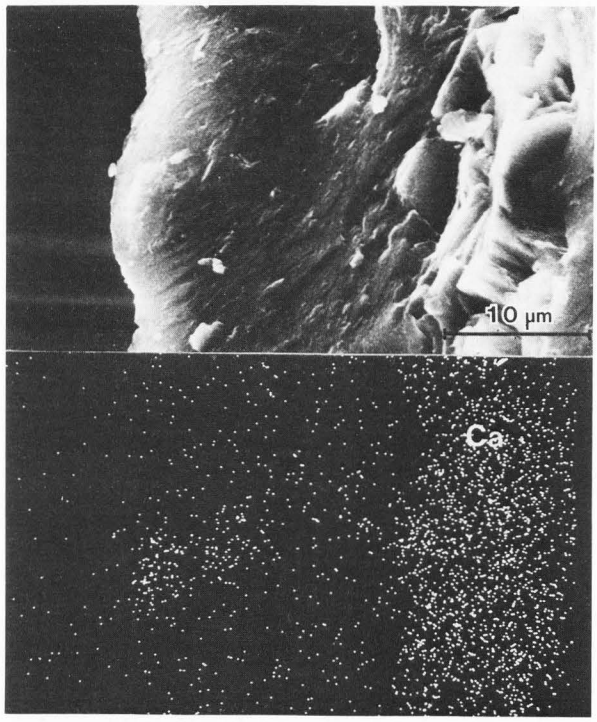

Fig. 2: a) Scanning electron micrograph of a matrix rich area of the same stone from Fig. 1. b) The corresponding Ca x-ray dot map.

Fig. 3: Scanning electron micrograph of a calcium oxalate-matrix rich stone.

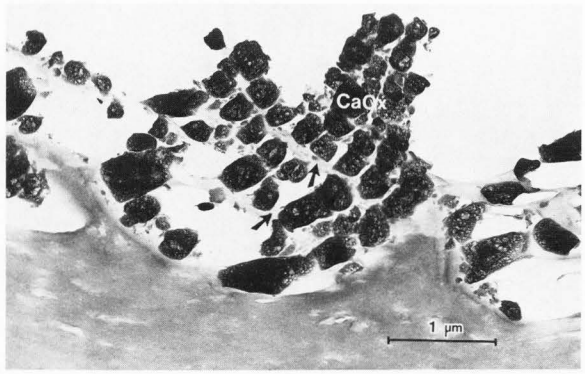

Fig. 4: Transmission electron micrograph of a 50 nm section of a surface area from the stone as shown in Fig. 1 showing rows of small calcium oxalate crystallites (CaOx) interleaved by organic matrix (arrows).

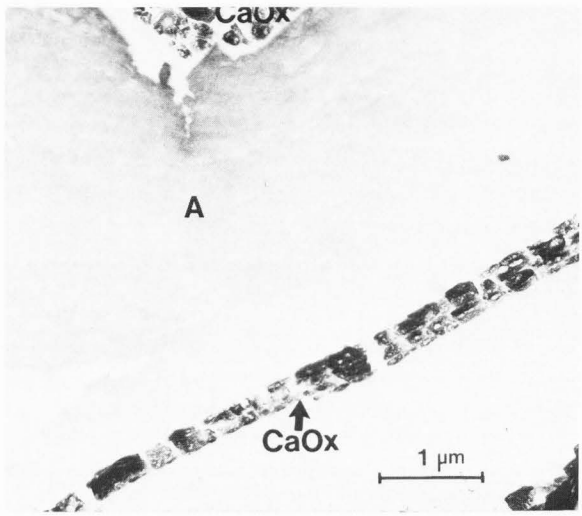

Fig. 5: Transmission electron micrograph of a 50 nm section of an area just beneath the surface from the stone as shown in Fig. 1 showing calcium oxalate crystallites (CaOx) embedded in substantial amount of amorphous organic matrix (A).

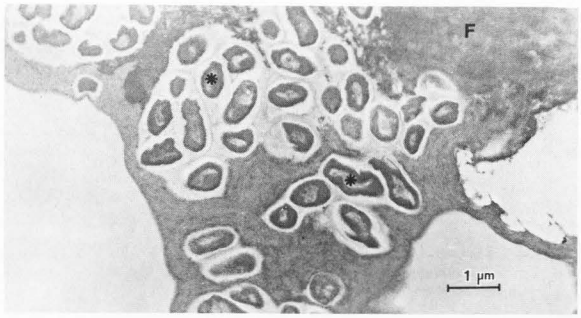

Fig. 6: Transmission electron micrograph of a 50 nm section of another area near the surface from the stone as shown in Fig. 1 showing casts of microorganisms (*) in a fibrous area (F).

ing mature stones grow by organic matrix facilitated microcrystal aggregation on stone surfaces.

On the surfaces of calcium oxalate stones, there are usually large masses of randomly deposited calcium oxalate crystals (Malek and Boyce, 1977) in both dihydrate (COD) (predominant) and monohydrate (COM) forms. Organic matrix coating of surface COD crystal ghosts has been observed in decalcified agar embedded stones (Khan et al., 1983).

An example of an organic matrix rich COM stone (not a "matrix stone") is given here. No other kinds of crystals have been detected by x-ray diffraction and the matrix richness has been confirmed by transmission electron microscopy. Fig. 1 shows the stone surface full of large matrix coated crystals. Only the crystals but not the matrix contain calcium (Fig. 2). For comparison, a "matrix stone" surface containing small COM (identified by x-ray diffraction) crystals embedded in organic matrix is shown in Fig. 3.

A 50 nm thin section of the same organic matrix rich COM stone shows the large surface COM crystals are composed of rows of smaller crystallites interleaved by organic matrix in a fairly orderly manner (Fig. 4) suggesting the crystallites are held together by organic matrix. The thickness of the matrix layers ranges 50-200 nm substantially thicker than the 1 nm gaps observed between COM crystals in sterile stones (Ogbuji and Finlayson, 1981). Similar results have been reported on COM containing matrix rich stones recovered from a cystic kidney (Cheng et al., 1984). Just beneath the surface, plate-like crystals can be seen embedded in substantial amorphous organic matrix (Fig. 5). Also near the surface, casts of micro-organisms can be seen in a more fibrous matrix area showing double membranes and haloes around them (Fig. 6). Their size (approximately 0.5 μm in cross sectional diameter) agrees well with the family of Enterobacteriaceae (0.4-0.6 x 2-3 μm) which includes E. coli and other Proteus organisms frequently responsible for urinary tract infection (Davies et al., 1980). Although bacterial infection is not related to calcium oxalate stones, the matrix richness may be related to the infection, either as a bacterial cell wall degradation product or an inflammatory reaction product.

Crystal-Organic Matrix Relationship at the Stone Core

In the core of a calcium containing stone, the organic matrix frequently exists as concentric laminations alternating as calcium apatite covered fibrous matrix layers and amorphous matrix layers (Boyce and Sulkin, 1956; Malek and Boyce, 1977; El-Sayed and Cosslett, 1977; Khan et al., 1983). While large crystals e.g., COM, COD, struvite ($MgNH_4PO_4.6H_2O$) have been found mainly in the amorphous matrix layers, the small calcium phosphate crystallites are associated mainly with the dense fibrous layers (Boyce and Garvey, 1956; Malek and Boyce, 1977; Cheng et al., 1981). Since fibrous areas have also been observed in decalcified stones which should not contain hydroxyapatite crystals if complete decalcification is assumed, then fibrous areas do not necessarily

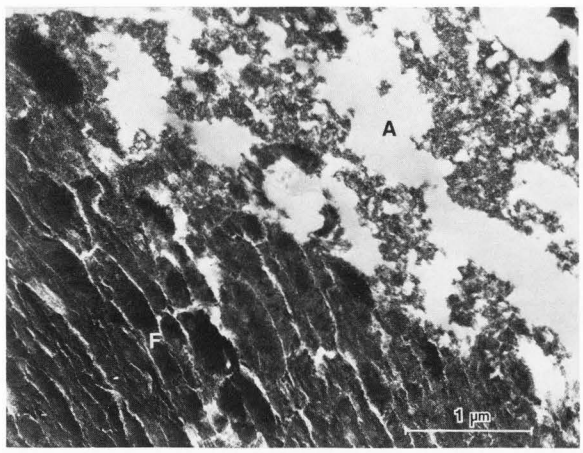

Fig. 7: Transmission electron micrograph of a 50 nm section of a calcium apatite containing stone matrix showing the interface of a fibrous area (F) and an amorphous area (A).

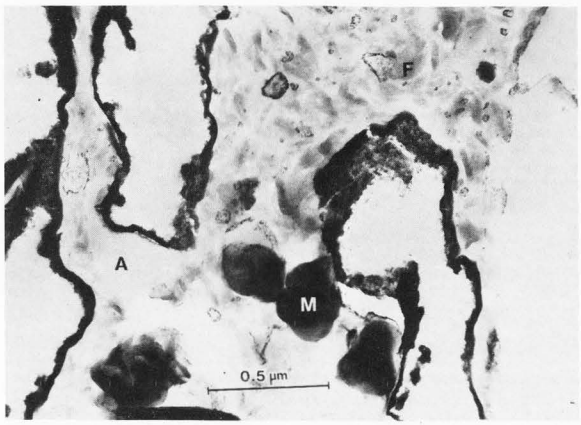

Fig. 8: Transmission electron micrograph of a 50 nm section of a calcium oxalate-matrix stone showing areas of amorphous material (A), fibrous matrix (F) and mineral deposits (M).

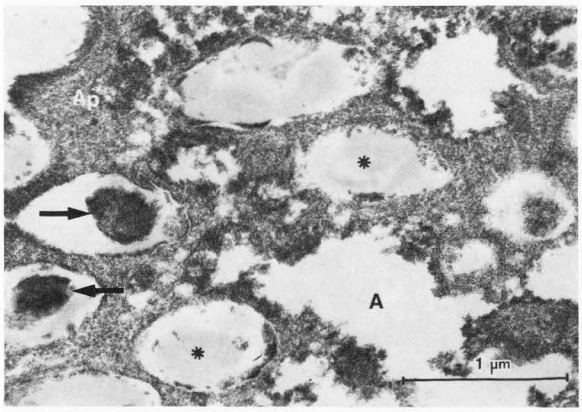

Fig. 9: Transmission electron micrograph of a 50 nm section of another area of the stone as shown in Fig. 7 showing bacterial casts (*) at various stages of calcification (arrows) surrounded by apatite crystallites (Ap).

imply the presence of hydroxyapatite crystals.

The relationship between dense fibrous and amorphous matrix areas is not clear. However, in a 50 nm thin section (Fig. 7) it can be seen that the dense fibrous area (F) is probably just an area heavily populated by calcium apatite crystallites which give the fibrous appearance while the amorphous area (A) is sparsely populated. This would agree with the indistinguishability of the two areas by toluidine blue and periodic acid Schiff stains (Boyce and Sulkin, 1956). However the diameters (50 - 200 nm) of the calcium apatite "fibres" shown in Fig. 7 are somewhat larger than those (13 - 54 nm) of "ribbon-like fibrils" published by Malek and Boyce (1977). In our "matrix stone" study, both COM and calcium apatite crystallites have been found embedded in amorphous matrix areas. However, the fibrous areas in "matrix stones" are populated by "finger print like" paracrystalline structures with approximately 4.5 nm spacings between lattice rows (Cheng et al., 1983b) (Fig. 8).

In another area of the same section as shown in Fig. 7, bacterial casts at various stages of calcification can be seen surrounded by very fine apatite crystallites (approximately 2-5 x 10-20 nm)(Fig. 9). Once again, organic matrix can be associated with infection and this example confirms that calcium apatite crystal deposition is favored in infection stones (Wickham, 1976).

Conclusion

Organic matrix content is very high in "matrix stones", relatively high in "infection stones", but is low in "metabolically induced" calcium oxalate and uric acid stones. It has been suggested that organic matrix is not a major factor in urolithiasis, but deposited in stones by adsorption on crystal surfaces (Finlayson, 1982). This is probably true for the "metabolically induced" sterile stones, but unlikely for the matrix-rich "infection stones" and "matrix stones". In the last two cases, crystallites may coprecipitate with matrix (Finlayson et al., 1961) and then become a stone when the "matrix bond" is strong enough to hold the crystallites together. The time needed for the stone to grow (Finlayson et al., 1961) may be provided by virtue of partial or complete stasis (Cheng et al., 1984).

The chemical composition of the organic matrix is still uncertain although it is probably uromucoid in origin. However, whether uromucoid is a promoter of crystal aggregation is also controversial. Contradictory in vitro results have been published suggesting uromucoid recovered from urine to be a weak inhibitor (Robertson et al., 1981) or a promoter (Hallson and Rose, 1979). Hence it is obvious that many more morphological and biochemical studies on the crystal-organic matrix interaction in kidney stones are needed to advance our understanding of kidney stone pathogenesis.

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Discussion with Reviewers

W.G. Robertson: Is there any satisfactory technique which will distinguish between the possibility that the organic matrix is a fortuitous inclusion in a stone (arising from adsorption on to growing crystals) and that it is an essential "glue" necessary to hold the crystals together to form an entity which is not easily passed from the urinary tract? Another possibility is that it merely aggravates an already abnormal situation by loosely holding crystals in close proximity until such time as they have been joined by crystal bridging.

Authors: The role of organic matrix may be assessed by studying the size distribution of growing crystal clusters in synthetic urine with or without stone matrix extract. With respect to the "net theory" that matrix retains crystallites until the latter join together, this could be tested by studying the properties of whole and de-matrixed stones.

W.G. Robertson: Would the authors like to comment on the possibility that the main role of matrix in stones (particularly phosphatic stones) is to inhibit re-dissolution of the stone during periods of undersaturation? This would allow the stone to survive until the next period of urinary supersaturation when it would continue to grow by crystal accretion and growth.

Authors: It is possible that stone surface matrix forms a physical barrier inhibiting stone dissolution at the end of each growth period.

G. Faure: Two kinds of material can be studied: decalcified and undecalcified stone sections; what are the advantages and limits of these two techniques of preparation in the various methods?

Authors: Decalcification enables us to visualize the fibrous and amorphous components of the organic matrix which are hidden by the crystalline phases. However, even partial decalcification or mere immersion in an aqueous medium other than the original urine, may result in loss or translocation of crystal phases. These two methods provide complementary information - decalcified sections provide access to matrix where as undecalcified sections provide more information on crystalmatrix interaction.

G. Faure: Do you think that immunohistological or immunochemical techniques can be applied to this material? In other words, would it be possible to detect for example immunoglobulins or complement factors in the matrix or around the microorganisms that can be seen in some kidney stones?

Authors: Immunohistological techniques would be very useful to detect not only immunoglobulin components on microorganisms but also fragments of connective tissue molecules. The limitations of this technique of course relate to the availability and specificity of antibodies.

K.M. Kim: On what basis do you consider the organic matrix to cement whewellite crystals? A layer of the matrix on the crystal surface may interfere with the ion exchange across the solid-liquid interface and disturb the crystal growth. In addition to the cementing effect, does the organic matrix have any other function(s) in stone formation?

Authors: We think the organic matrix cements crystallites together in matrix-rich stones because in more than one study we observed interleaving layers of crystallites and organic matrix. The thickness of the matrix layers is substantially

thicker than would have resulted from surface adsorption. Crystallites are not expected to grow once they have been incorporated into a stone which grows by crystallite accretion. Also, organic matrix on the surface of a growing stone would inhibit stone dissolution at the end of each growth period.

K.M. Kim: Does the organic matrix play similar role in other types of stones?
Authors: Perhaps.

K.M. Kim: Crystals are frequently anhedral (non-facetted). How do you distinguish anhedral crystals from the organic matrix?
Authors: X-ray or electron diffractions should be able to differentiate between anhedral crystals and organic matrix.