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CRYSTAL ASSOCIATED DISEASES: ROLE OF SCANNING ELECTRON MICROSCOPY IN DIAGNOSIS

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Abstract

As crystals are important etiologic agents for disease, their accurate identification in tissues and body fluids is of utmost importance. This paper surveys the roles of crystals in disease process and outlines current analytical techniques for crystal detection and identification in bone tissues. The value of multiple correlated techniques is demonstrated including scanning electron microscopy, x-ray energy spectroscopy and powder diffraction analysis. The current feasibility of utilizing intermediate voltage scanning transmission analytical electron microscopy to integrate these analytical techniques on the same tissue sample is emphasized.

Key words: scanning electron microscopy, crystal associated diseases, apatite, calculi, analytical electron microscopy.

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Introduction

As etiologic agents of disease, crystals have several special characteristics which permit their detection and identification in tissues by scanning electron microscopy (SEM). These characteristics include insolubility, specific three dimensional structure (habit), and specific atomic and molecular composition (14, 25). In this paper, we will briefly review the disease processes associated with crystals, the classes of crystals associated with pathologic disorders, and then discuss the roles of SEM in the diagnosis and elaboration of pathogenesis of these diseases.

Crystals and Pathologic Processes

First, it should be noted that crystals may have physiological functions. As examples, calcium apatite present in an orderly manner on collagen fibrils, forms the mineral of bone (2, 12); calcium carbonate crystals as oticonia, act as part of the sensory mechanism for motion.

To an extent not yet completely defined, crystals of specific size and molecular composition are associated with specific disease processes as illustrated in Table 1. Crystals of similar size vary considerably in their pathogenicity. For example, carbon crystals, commonly inhaled as dust, are relatively inert provoking little reaction beyond phagocytosis (11). In contrast, following phagocytosis, urate, calcium pyrophosphate crystals and apatite amplify inflammation (19); silica crystals promote fibrosis (34); and asbestos crystals ultimately induce neoplasia (33).

Calcium apatite is a most useful example of a biological crystal which has both physiological and pathological roles. Although the atomic and molecular composition may be identical, as bone mineral, these crystals have very well defined low order crystallinity; i.e., extent of long range order, whereas in soft tissue pathologic apatite crystal deposition, this order may be decreased towards amorphous mineral or increased towards larger crystals (3, 10, 13, 21, 27). Moreover, physiologically these crystals form in an ordered array within the matrix, whereas pathologically apatite crystals tend to form in agglomerates. Presently, there is evidence that the determination of crystal formation and possibly other crystal properties, such as orientation and phase, are related to the matrix macromolecules and the ambient ion concentration (15). These deposits are usually of sufficient size to enable their detection by SEM.

TABLE 1: Crystals and Pathologic Processes

Pathologic Process	Crystals (Examples)
Duct Obstruction	Gall stones, eg., cholesterol Renal calculi, eg., oxalate
Acute inflammation	Monosodium urate Calcium pyrophosphate dihydrate Calcium apatite
Chronic inflammation	Cholesterol monohydrate
Fibrosis	Silica
Degeneration	Calcium pyrophosphate, apatite Cholesterol e.g., atherosclerosis
Neoplasia	Asbestos
Metabolic storage diseases	Cystine, calcium oxalate

TABLE 2: Pathologic Crystals

1. **Exogenous Crystals**
   - 1.1 Inhaled crystals
     - Carbon
     - Silica
     - Asbestos
   - 1.2 other routes
     - Intravenous - e.g., talc
     - Dermabrasion - e.g., cosmetics
     - Intraarticular - e.g., drugs-corticosteroids

2. **Endogenous Crystals**
   - 2.1 Calcium Salts
     - Phosphate
       - . Basic - apatite
       - . Neutral - Whitlockite
       - . Acidic - Brushite, Monetite
     - Pyrophosphate dihydrates (M) or (T)
     - Oxalates
     - Carbonates
     - Bilirubinate
     - Carboxylates
   - 2.2 Crystals without Calcium
     - Monosodium urate
     - Uric acid
     - Ammonium urate
     - Magnesium ammonium phosphate
     - Cholesterol
     - Cystine
     - Xanthine
     - Macromolecules, eg., immunoglobulin

Specific crystals may participate in multiple pathologic processes. For example, calcium pyrophosphate deposits in cartilage produce cartilage degeneration; when released in synovial fluid, these same crystals can induce acute inflammation in the synovial space (8, 26, 31).

For convenience, pathologic crystals can be grouped as exogenous and endogenous crystals. Because of their relative insolubility and ease of detection, it is useful to separate calcium containing crystals from those without this element (Table 2). It should be recognized that molecules as large as proteins can crystallize in tissues with pathologic effects dependent on site of deposition (22).

As outlined in Table 3, the site specificity of crystal deposition is complex but always involves presentation or formation of insoluble particles with partial or total failure of clearance mechanisms. For exogenous crystals in airways, crystals must be in the narrow range of size to be suspended and not larger than 6-10 micrometers. Larger crystals tend to be precipitated higher in the airway and cleared by the ciliary action of endobronchial cells; smaller crystals remain suspended and are exhaled (11). For endogenous substances, the site of crystal formation relates closely to crystal phase detected. As most body fluids are supersaturated with respect to common solutes, e.g., calcium phosphate, oxalate, etc., crystal formation is dependent on nucleation phenomena which may involve exposure of surfaces, increased concentration of promoters, decreased concentration of inhibitors or local increased solute concentration either by phagocytosis, by altered local matrix structure or by fluid composition changes induced by stasis (20). Increasingly, it is recognized that crystal deposition is favored by the presence of organic surfaces such as cell membrane fragments (1, 4, 16), degraded collagen or degraded proteoglycan.

TABLE 3: Crystal Deposits: Site Specificity

1. Intracellular	
- Phagolysosome	- Phagocytosis
- Golgi vesicles	- Cell biosynthesis
- Membranes	- Nucleating molecules
2. Extracellular	
- Fluids and spaces	- Stasis, supersaturation, mechanical force - e.g., biliary, renal, and salivary calculi
	- Exogenous, e.g., inhalation
- Matrix	. Agglomerates - Restricted flow, increased binding of solutes, - e.g., CPPD in cartilage, e.g., Cholesterol in arteries
	. Ordered Deposition - Apatite crystals in bone, teeth

The pattern of crystal formation can provide an indication of the biological control under which the crystals form. With a high level of control, crystals that form will be highly regular and oriented. Tooth enamel is perhaps the best example of controlled crystal deposition (29). Pathologic deposits tend to be agglomerates, i.e., collections of randomly arranged crystals indicating that nucleation has proceeded rapidly, with control provided by solute availability and the size of the space that permits crystal

TABLE 4: Application of Crystal Structure Determination Techniques

Crystal Properties	SEM	TEM 100 kV	STEM 300 kV	X-Ray Diffraction	Polarized Light Microscopy
Minimum Crystal Deposit Size in micrometers	0.25	0.1	0.1	30	1
Crystal Surfaces	++++	--	++	--	+++
Birefringence	--	--	--	--	++
Powder Diffraction	-	+	+++	+++	--
Lattice Pattern	-	+	++++	++++	--
Elemental Composition (X-ray Energy Spectroscopy)	++++	++	+++	+	--

growth.

Crystal agglomerates are intrinsically unstable, which may lead to shedding of individual crystals from matrix deposits. This may have varying pathologic effects, depending on the nature of the tissue, e.g., acute inflammation from calcium pyrophosphate crystals shed from cartilage (8), ischemia resulting from cholesterol crystal emboli shed from artery walls (17), or from exogenous particulate emboli such as talc which has been injected into veins by drug abusers (9).

SEM for Analysis of Crystals in Tissues

SEM is only one of several complementary techniques utilized in crystal analysis. Other techniques commonly include compensated polarized light microscopy (32), X-ray diffraction, transmission electron microscopy (TEM) and a variety of bulk chemical analytical techniques such as infra-red spectroscopy (5, 6, 7, 23, 28). The selection of each of these tools is dependent on several factors including the nature of the crystals, the amount of crystals present, the degree of diagnostic precision required, as well as the cost, convenience, and accessibility of each technique for pathologists. As the initial screening technique, it is most convenient and effective to use compensated polarizing light microscopy on stained or unstained tissue sections, smears or fluid sediments (6). While this technique gives excellent information on the amount of crystals in tissues, and the sign and amplitude of birefringence if present, it is limited with respect to precise crystal identification by the small particle size of crystals usually seen in biological specimens. Biological crystals are usually less than 20 micrometers in length and may in fact be at or below the limits of light microscopy resolution of about 0.5 micrometers.

Thus, the higher resolving power of SEM enables the observer to determine the three dimensional shape (habit) of crystals, information which in the context of known tissues, does have considerable specificity. Second, SEM can determine the features of the crystal surface, whether it is smooth or is covered by amorphous material, whether the crystals show dislocations, epitaxial growth, etc. Third, using x-ray energy spectroscopy, the presence of elements with atomic number greater than 10 can be assessed and the elemental ratios, e.g., calcium : phosphorus, determined (30).

TEM for Analysis of Crystals in Tissues

Until recently, TEM techniques have been less useful than SEM because of the necessity to cut sections, and the relative paucity of secondary x-rays generated for elemental analysis. However, TEM is useful in obtaining selected area electron diffraction and microdiffraction from crystal deposits in sections. Further, powder electron diffraction patterns can be obtained, which when compared to standards can be used to identify crystals precisely. With respect to diffraction techniques, x-ray diffraction, although more cumbersome and requiring greater amounts of material, still remains the standard technique as the library of known patterns is so extensive. Moreover, although micro x-ray diffraction can be performed, it is limited to deposits of greater than 30 micrometers in size and is much more tedious and less informative than electron diffraction.

While not yet generally available to diagnostic laboratories, the difficulties of thin sectioning and x-ray paucity have been largely obviated by the intermediate voltage scanning transmission electron microscopes (STEM) such as the 300 kV Philips EM 430. Instruments of this type can provide bright field imaging, back scatter imaging, x-ray energy spectroscopy and electron diffraction for crystal specimens more than 1 micrometer thick with superior resolution and less damage than conventional 100 kV TEM. With the currently availabile STEM, traditional SEM, i.e., scanning reflection electron microscopy, can be reserved for analysis of large specimens or analysis of surface details.

Over the past 15 years, as instrumentation has advanced, there has been rapid and continual evolution of crystal identification techniques with steady progress towards decreased sample size, increased sensitivity of x-ray energy spectroscopy analysis and ease of production and interpretation of electron diffraction patterns. The relative advantages of the various current crystallographic techniques in tissues are summarized in Table 4.

Practical Examples of Crystal Analysis

As stated above, the mixture of techniques for crystal analysis and the order of their application is highly dependent on the nature of the crystal deposit in tissues. In this section, to illustrate current techniques, three different clinical applications are described.

1. Crystal Mixtures in Calculi

Common stone deposits such as biliary and renal calculi are seldom composed of a solitary crystal phase. More commonly, biologic stones are composed

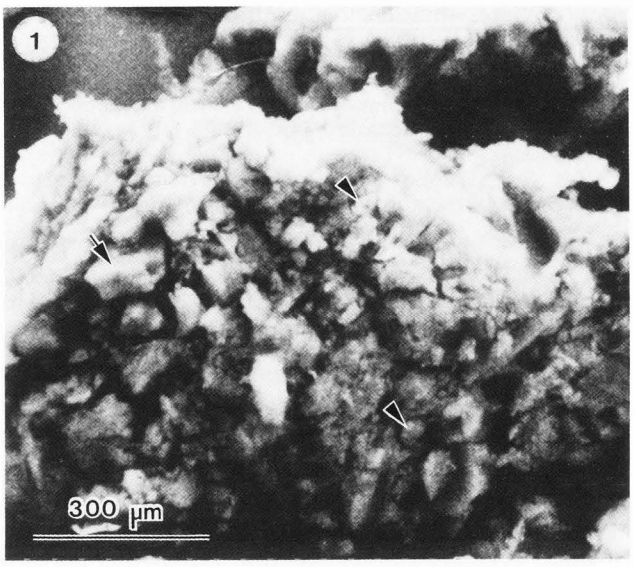

Figure 1 (on the left). Cut surface of a renal calculus demonstrating mixture of large plate-like crystals (arrow) and smaller spherical aggregates (arrowheads). Scanning electron photomicrograph.

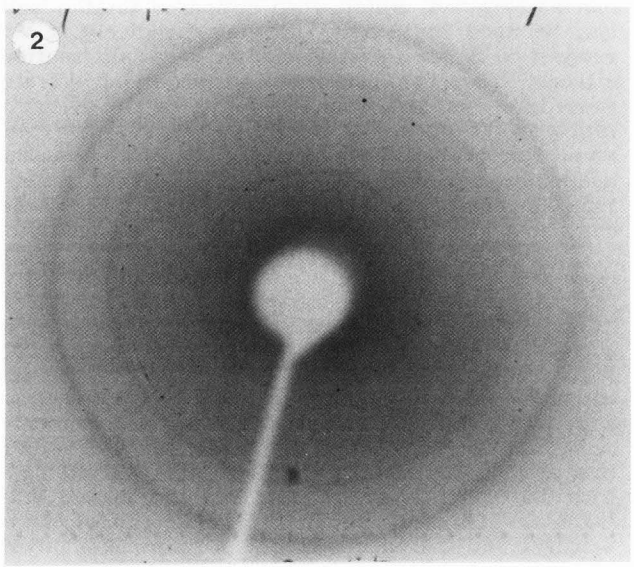

Figure 2 (on the left). Powder pattern x-ray diffraction of section of calculus in Figure 1. The 0.281, 0.344, 0.184 nm lines indicate apatite, the 0.618, 0.278, 0.442 nm, calcium oxalate dihydrate.

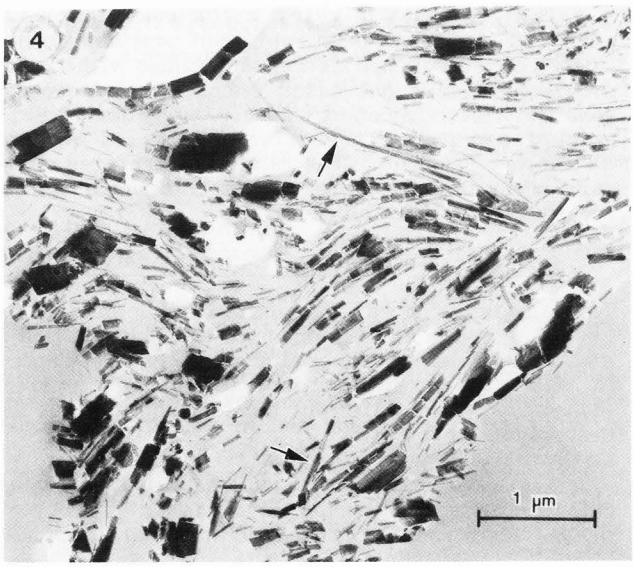

Figure 3 (on the facing page). 3A. Cut surface of gallstone of mixed composition. Reflected light microscopy. 3B. Scanning electron photomicrograph of surface from 100 micrometer of stone demonstrating areas of heterogeneous composition. 3C. Back scattered electron image of section, demonstrating two domains containing elements of higher atomic number (arrows). 3D. X-ray energy spectroscopy maps demonstrating that these areas contain calcium. 3E. Micro x-ray powder diffraction pattern of calcium containing domains. The lines at 0.33, 0.273, 0.182 nm identify the Vaterite phase of calcium carbonate. 3F. Micro powder x-ray diffraction pattern of non calcium containing areas. The lines at 0.599, 0.381, 0.476 nm identify cholesterol monohydrate.

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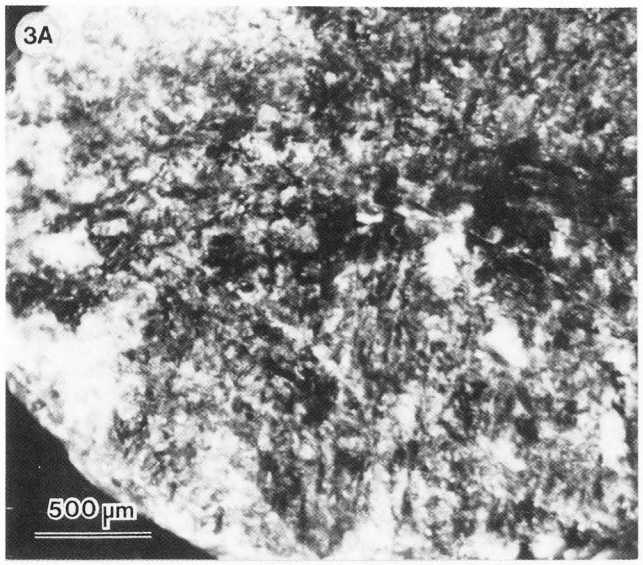
of a mixture of amorphous matrix and one or more crystal phases (24). A major advantage of SEM is that morphologic analysis and elemental composition can be determined on the bulk sample without the technical problems of interpretation that may be induced by elemental translocation and phase change inherent in fixation and embedding (18). The renal stone in Fig. 1 contains both calcium apatite and calcium oxalate as shown by the mixed diffraction pattern in Fig. 2. Energy dispersive analysis could localize the different crystals within the stone.

However, for analysis of specific areas, it is still useful to perform analysis on sectioned materials. This is shown in a gallstone in Fig. 3A. With SEM, utilizing back scatter imaging, areas of different elemental composition can be assessed (Figs. 3B and 3C). The distribution of elements such as calcium can then be determined by x-ray energy spectroscopy after localizing areas of different composition (Fig. 3D). Further, these studies permitted micro x-ray diffraction of crystal domains demonstrating the mixture of Vaterite, a phase of calcium carbonate, and cholesterol monohydrate (Figs. 3E & 3F).

2. Crystal Size and Shape in Crystal Deposits

Calcium pyrophosphate dihydrate (CPPD) crystals form in human cartilage under a restricted range of biological conditions (26). A major question related to the deposition of these crystals is their localization as agglomerates related to the territorial matrix of cartilage. To date, conventional SEM has been inadequate to show relationships between crystals and individual matrix components. Similarly, conventional TEM has been unsatisfactory because of the considerable crystal loss with preparation and beam induced artefact during electron microscopic examination. Recently, intermediate voltage STEM

Figure 4 (to the left). Agglomerate of calcium pyrophosphate dihydrate crystals in cartilage. Note the presence of greatly elongated crystals (arrows), and absence of "bubble" artefact related to damage of crystals by electron beam. TEM photomicrograph, of unstained 1 micrometer section, 300 kV.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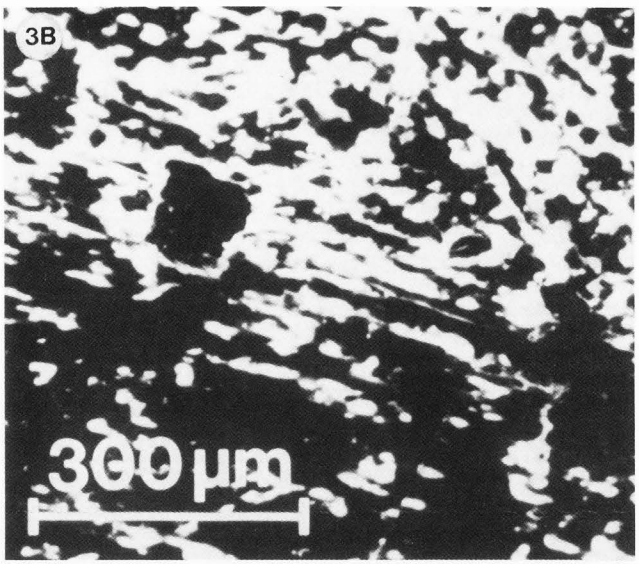
500 µm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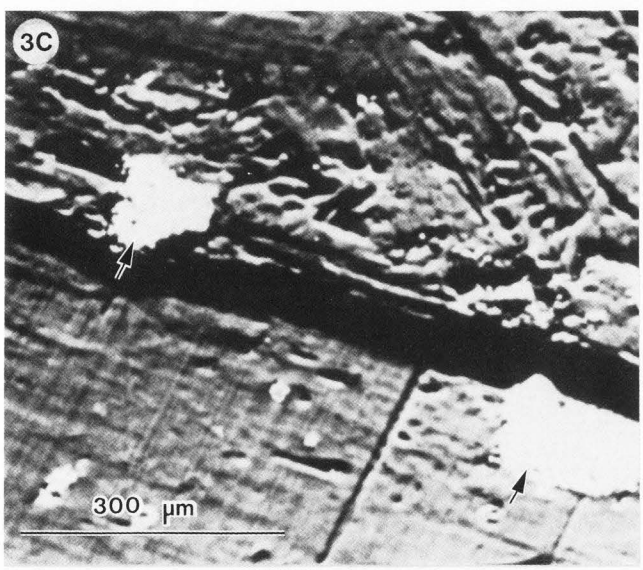
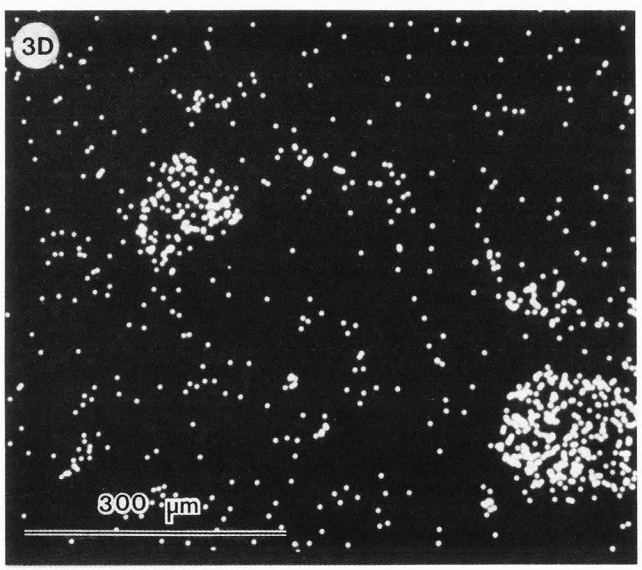
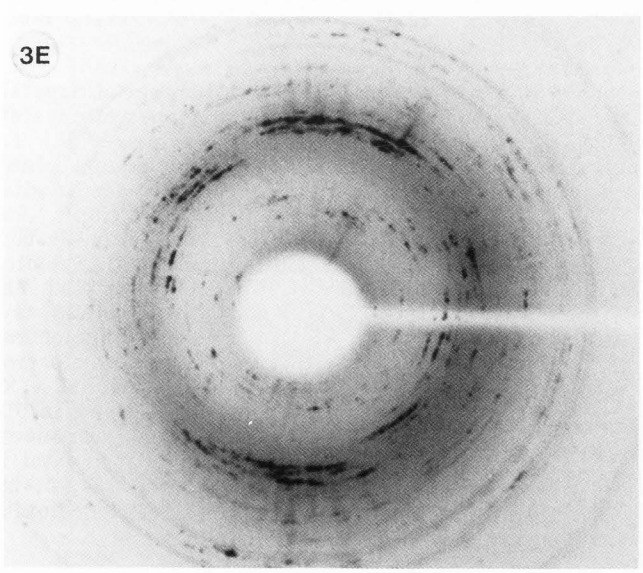
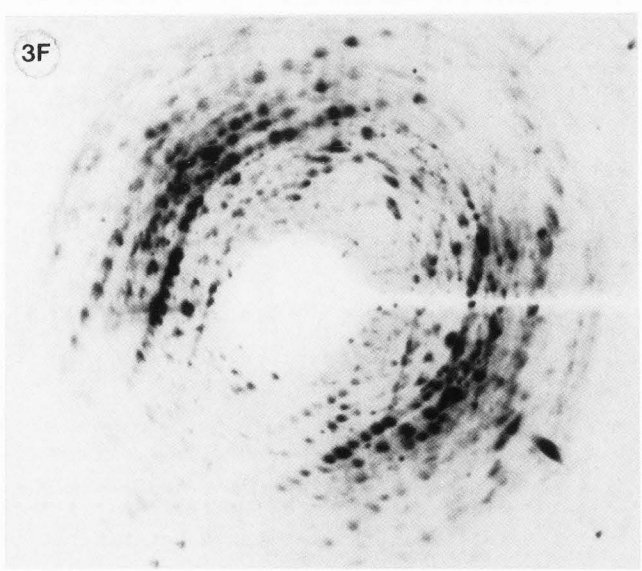
300 µm


3C
300 µm


3D
300 µm


3E


3F

has enabled the visualization of crystals in thick sections. This technique has shown that CPPD crystals may grow initially as very long thin structures, possibly providing an extensive nucleating surface for agglomeration (Fig. 4).

3. Identification of Small Quantities of Crystals in Biological Fluids

The recognition by clinicians that crystals can be associated with disease processes has led to requests to identify crystals in tissues and fluids which may be present in quantities barely detectable by light microscopy.

Typically, fluids are prepared for this type of analysis by cytocentrifugation at 5,000 rpm for 10-30 minutes. The sediment is either placed on a Formvar coated grid or embedded in Epon for preparation of 70 nm or thicker unstained sections. With the analytical STEM, it is possible to identify crystals in mixtures in which the area of crystals is circa $10nm^2$. This is illustrated by the exogenous material present in saliva from a patient who had the sensation of "grittiness" in her saliva, but for whom conventional analysis failed to reveal crystals.

Analysis of salivary fluid sediment revealed crystals of two types, elongate needles 0.5 x 0.03 micrometers and large flat crystals 0.3 micrometers^2 (Figs. 5A and 5D). X-ray energy spectroscopy demonstrated that the crystals were quite different in elemental composition (Figs. 5B and 5E).

Selective area electron diffraction analysis identified the crystals as titanium oxide (TiO) and silica (SiO_2) (Fig. 5C and 5F). This led to investigation of the erosion from faulty dentures as the source of the crystals.

Conclusions

The recognition of crystal associated diseases and the role of crystals in the pathogenesis of disease is limited only by the clinical awareness that crystals may be present and by the utilization of simple preparation techniques which preserve crystals in tissues. Scanning electron microscopy techniques are extremely useful for the precise identification of crystals and in elaborating the relationships of crystals to their tissue environment. Precise crystal identification requires analysis of diffraction patterns and x-ray energy spectroscopic analysis, techniques which are continually improving with the development of scanning microscopy instrumentation. Finally, the increasing accessibility of instrumentation and the increasing ease of crystal analysis make it now possible to identify precisely most unknown crystals detected in pathologic tissues.

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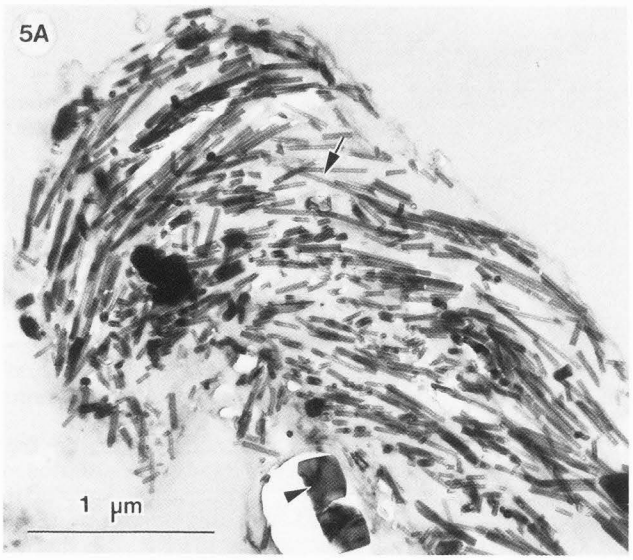
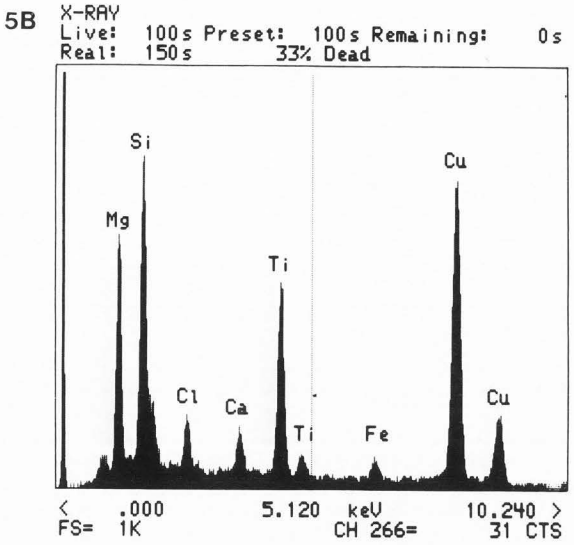
Figure 5 (on the facing page): Analysis of crystals present in salivary fluid. 5A. Two types of crystals are observed, elongated crystals (arrow) 0.2 - 1.0 micrometers in length, and electron opaque plates (arrowhead) approximately 0.3 micrometers in diameter. Transmission electron photomicrograph. 5B. X-ray energy spectroscopy of mixture demonstrating presence of both titanium and silicon. 5C. Selected area electron diffraction of elongated crystals. The lines at 0.210, 0.146 nm identify the crystals as a Titanium oxide (TiO). 5D. Transmission electron photomicrograph of plate-like crystals. Note electron opacity and absence of internal structure. 5E. X-ray energy spectroscopy of crystal observed in 5D. Silicon is detected as the major elemental component. 5F. Selected area electron diffraction of crystal in 5D. Lattice pattern with spacing at 0.334, 0.426, 0.182 nm identifies the crystal as silicon dioxide (SiO_2).


5A
1 μm


5B
X-RAY
Live: 100s Preset: 100s Remaining: 0s
Real: 150s 33% Dead
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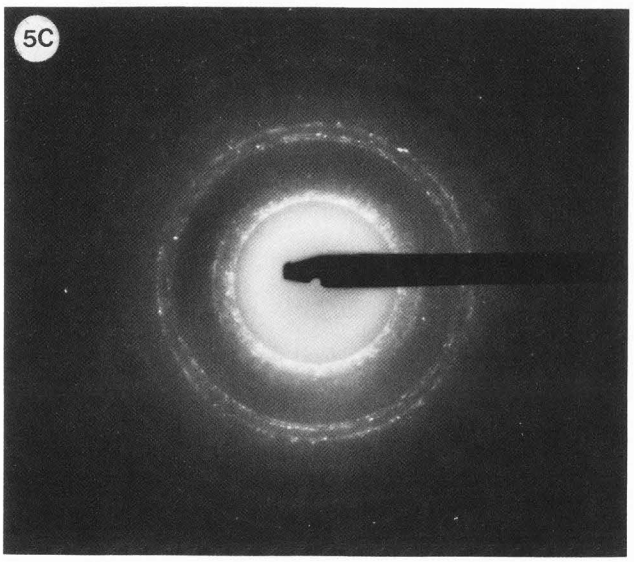
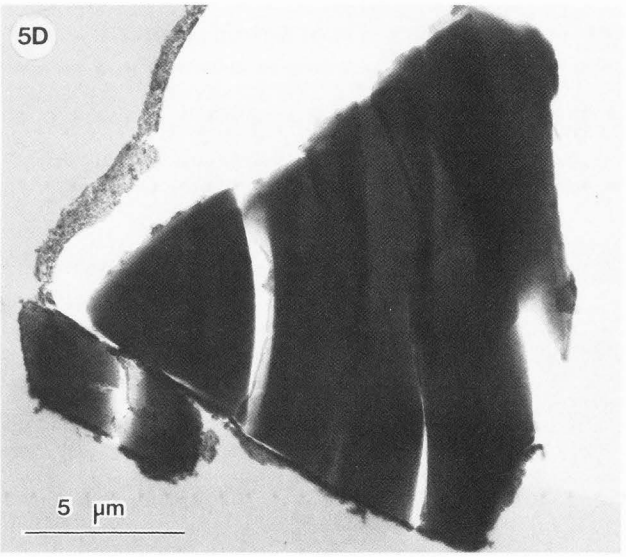
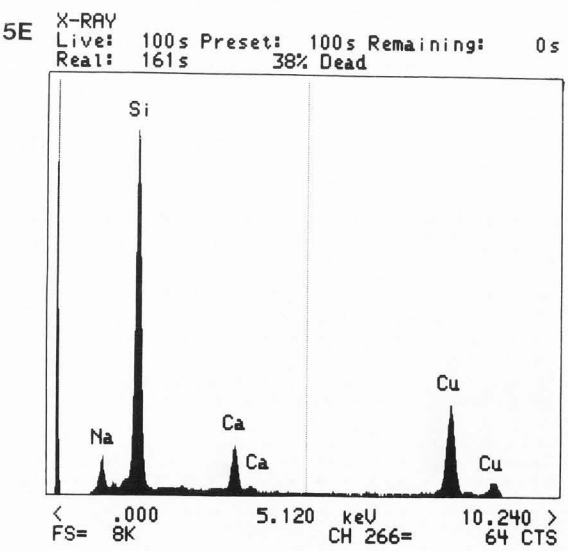
FS= 1K CH 266= 31 CTS


5C


5D
5 μm


5E
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Live: 100s Preset: 100s Remaining: 0s
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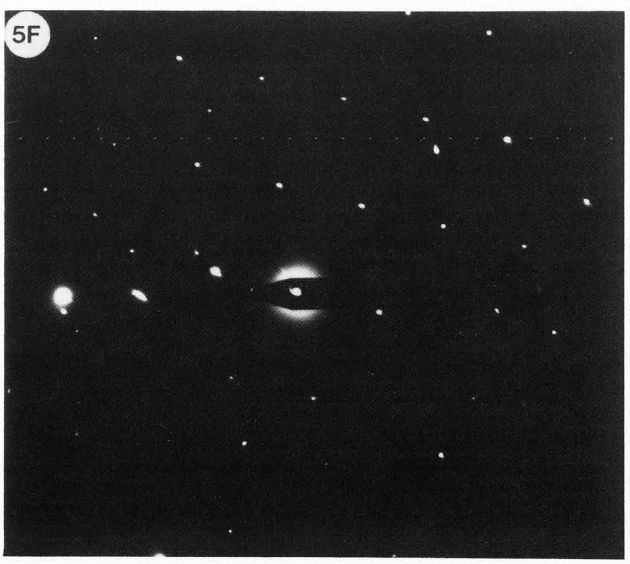
5F

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Discussion with Reviewers

E. Bonucci: From the properties and structure of the associated organic phase, can the crystal phase be identified and the crystal organization predicted?
Authors: Both crystal nucleation and growth of crystal deposits can be determined by the nature of the organic matrix. Mann [Mann S (1988). Molecular Recognition in Biomineralization, Nature 332, 119-124] has reviewed the evidence of the effects of organic phase on nucleation of specific crystal phases. Briefly, organic surfaces facilitate specific nucleation by lowering interfacial energies. This requires matrix preorganization with respect to ion chemistry (electrostatic accumulation), structural correspondence (epitaxy), and topological correspondence (stereochemistry).

The pattern of crystal deposition can be frequently identified with specificity by examination of crystal ghosts (dehydrated matrix within the calcification) using post embedding decalcification and staining techniques [Bonucci E (1987). Is there a calcification factor common to all calcifying matrices? Scanning Microscopy 1, 1089-1102].

E. Bonucci: Can crystal properties be changed by association of inorganic substances with organic material?
Authors: Crystal surfaces selectively adsorb ambient molecules. In general these molecules neutralize charges that may be present on crystal surfaces, thus altering the crystal properties. An example of the change in crystal properties by specific substances is the adsorption of complement or immunoglobulins on to crystals. These substances can act as opsonins to facilitate the phlogistic properties of crystals.

Reviewer 3: In recent years, several major contributions to urinary stone and crystal analysis, not cited in the text have appeared in Scanning Electron Microscopy. Please comment.
Authors: This paper presented a general review of crystal associated diseases. For more extensive discussion of scanning electron microscopy and urinary crystal analysis, the reader is referred particularly to:

Khan SR (1987). Role of Scanning Electron Microscopy and X-ray Microanalysis in the Identification of Urinary Crystals. Scanning Microscopy 1, 1405-1411.

Rogers AL (1985). Application of Physical-Chemical Procedures in the Analysis of Urinary Calculi. Scanning Electron Microsc. 1985;II: 745-758.

Leusmann DB (1984). Scanning Electron Microscopy of Urinary Calculi Scanning Electron Microsc. 1984;III: 1427-1432.