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ARCHITECTURE OF MIXED CALCIUM OXALATE DIHYDRATE
AND MONOHYDRATE STONES

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Abstract

Calcium oxalate dihydrate (COD) and monohydrate (COM) are the most frequent constituents of urinary stones, and there still exist some questions about the interrelation between the two hydrates. Architecture of mixed COD and COM stones was observed by electron microscopy to solve the questions.

The fractured surface of a stone is composed of the fractured face of the crystals. In this situation a morphological criterion of typical dipyrmaid shape is useless to identify COD. But we could identify COD using the partial dissolution method, which etched square pits on COD crystals.

COD and COM formed distinctly separate layers. COD was always found in the stone surface and COM in the center. The stone surface was covered by a thick layer of organic matrix, and the intercrystalline space was filled with matrix. The crystals were grown thrusting the matrix aside to minimize the space.

Although COD is more soluble than COM, the urine contains specific substances that favor the formation of COD. Supposing the stone matrix excludes these substances selectively, the gel-state matrix provides a preferable condition for COM formation. This hypothesis is suitable to explain the high incidence of COM stones. An abrupt change of the crystalline constituent can be explained by COD crystal deposition on COM stones. Frequent COD crystalluria can explain why COD is always found in the stone surface. Once the stone surface is covered with COD crystals, they continue to grow in the gel-state matrix or deposit further to form the bulk of the stone.

Key Words: Calcium oxalate stone, Calcium oxalate dihydrate, Calcium oxalate monohydrate, Crystal dissolution, Organic matrix.

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Introduction

Calcium oxalate dihydrate (COD) and monohydrate (COM) are the most frequent constituents of urinary stones. Therefore the effort of stone research is mainly focused on the pathogenesis of calcium oxalate stones. Nevertheless many questions remain unsolved. From the morphological point of view, the following points can be given.

1. The dihydrate crystals are more frequently observed in the urinary sediment, while in the stone both hydrates are equally often found (5, 7, 20).

2. The crystal shape of dihydrate (octahedral dipyrmaid) is essentially the same in the urinary sediment and in the stone, but that of monohydrate is quite different (15, 25).

3. When both hydrates exist in a stone, the dihydrate is always found in the stone surface and the monohydrate in the deeper layers (4, 18, 20).

Recently scanning electron microscopy (SEM) has been widely used in stone research. Compared to polarization microscopy, SEM has some disadvantages since it is difficult to observe the interior of the stones. In 1985 we reported the partial dissolution method which allows the observation of the crystal arrangements and crystal-matrix interrelations on the fractured surface of the stones (10). We later found that this method etches surface marks which are characteristic of each crystalline constituent (12). The following studies were performed to solve the questions described above using the partial dissolution method.

Materials and Methods

Calcium oxalate dihydrate (COD) crystals

COD crystals were obtained from the urine of a hypercalciuric patient. The crystal suspension was filtered through 0.22 μ m Millipore filter, which was settled on the suction funnel. The crystals remained on the filter paper were washed with pure water. Then several drops of 5%(wt/v) tetrasodium ethylenediaminetetraacetate (EDTA) solution, adjusted to pH 7.2 with HCl, were poured on the filter paper to start the dissolution of the crystals. The dissolution was

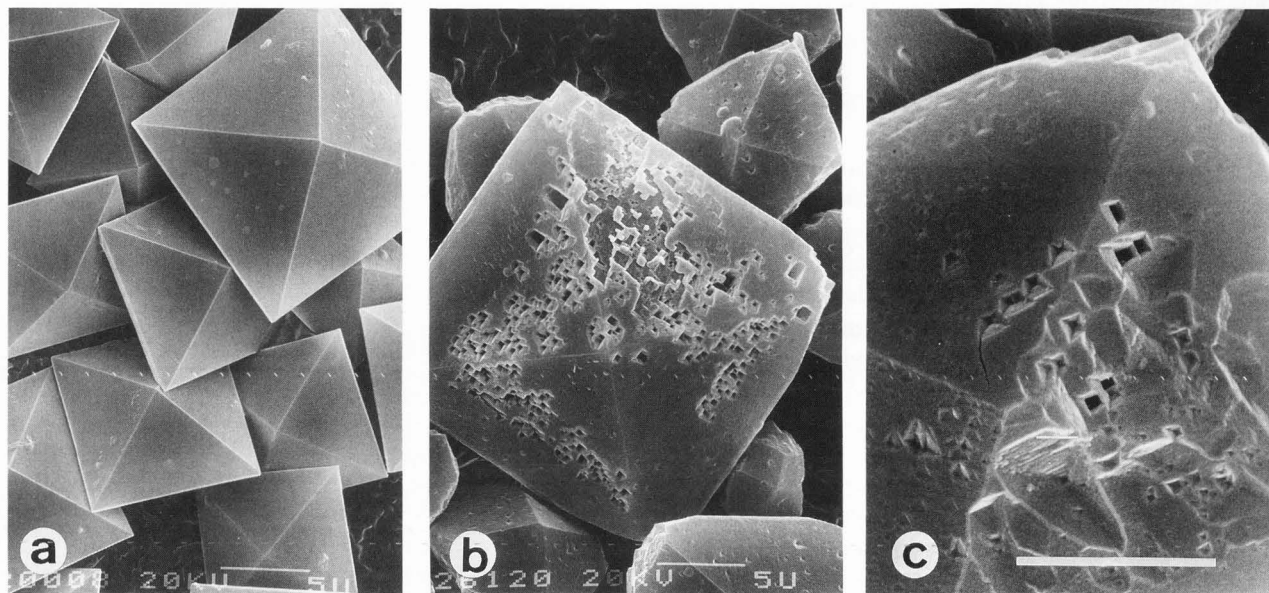


Figure 1. SEM of COD crystals from the urine of a hypercalciuric patient. (a) Octahedral dipyramid shape of undissolved crystals. (b) Five-minute dissolution with a EDTA solution etched square pits on the surface of the crystals. The side of the square is always parallel to the base of the crystal pyramid. (c) In the early phase of dissolution the bottom of the pits is made up of four triangular planes. Bar = 5 μ m.

carried out at room temperature, about 20°C. The dissolution was stopped after several minutes by washing with pure water. The filter papers were dried in a vacuum, mounted on aluminum stubs, coated with platinum and observed with a Hitachi S-500A scanning electron microscope.

Calcium oxalate stones

Calcium oxalate stones selected for this study were surgically removed from eight patients and were stored in our laboratory without any treatment for many years in the room atmosphere. Each of these patients had multiple renal stones less than 1 cm in diameter and either a spheroid or mulberry shape, which are typical of calcium oxalate monohydrate (COM) stones. Five patients had spheroid type stones and another three had mulberry type stones.

Partial dissolution method: Each stone was fractured through its center with a razor blade. One fragment of each stone was immersed in the EDTA solution and another fragment was left untreated. The temperature of the dissolution solution was maintained at 20°C and dissolution time ranged from 5 to 30 minutes. Then the fragments were rinsed with pure water and dried in a vacuum. Both fragments of a stone were mounted on aluminum stubs, and further treated for SEM as described above.

In order to observe the crystal-matrix interface, transmission electron microscopy (TEM) was used. Some of the stone fragments were fixed in a fixative mixture for about one week at room temperature. The fixative mixture contained 5%(v/v) formalin, 2%(v/v) glutaraldehyde and 1%(wt/v) cetylpyridinium chloride in 0.1M phosphate buffer, pH 7.2 (10, 11). Then the

fixative solution was exchanged for a dissolution solution, which was made by adding 5%(wt/v) EDTA to the fixative mixture without phosphate buffer and was adjusted to pH 7.2 with HCl. It took about two weeks for total dissolution of the crystals with a daily change of the dissolution solution.

The decrystallized specimens were further fixed in the fixative mixture for one day. The specimens were post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer, pH 7.2, for 4 hours. The post-fixed specimens were dehydrated with graded ethanol solutions and embedded in Epon-812. Thin sections were cut and were double stained with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-800 transmission electron microscope. Toluidine blue stained sections were examined by light microscopy (LM).

Results

Calcium oxalate dihydrate (COD) crystals

The COD crystals obtained from the urine of a hypercalciuric patient showed typical octahedral dipyramid of about 10-20 μ m in size (Fig. 1a). A five-minute treatment with the EDTA solution etched square pits on the surface of the crystals. The side of the square was always parallel to the base of the crystal pyramid (Fig. 1b). In the early phase of dissolution the bottom of the pits was made up of four triangular planes (Fig. 1c). These findings indicate that COD crystals have an octahedral or dodecahedral substructure. Thus the square etch pits can be used as another criterion to identify COD.

Architecture of Mixed Calcium Oxalate Stones

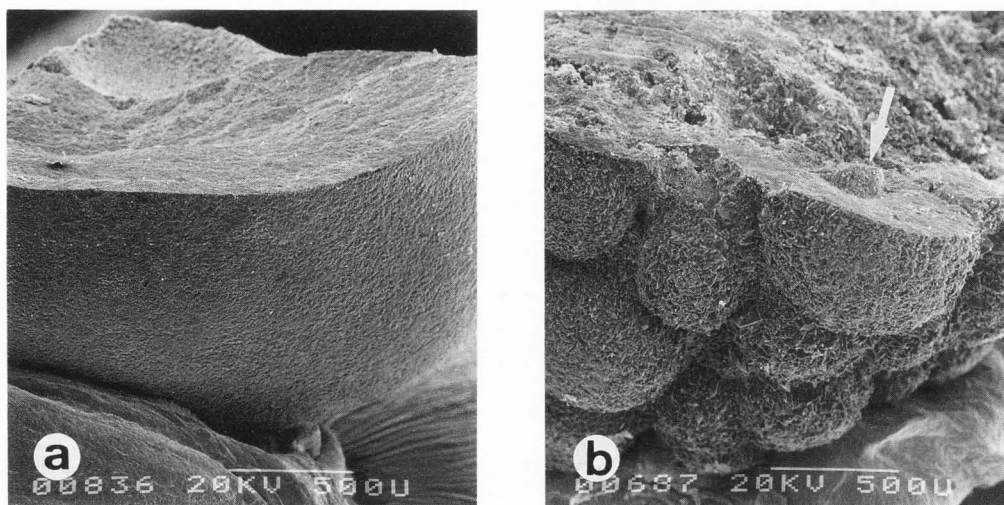


Figure 2. SEM of fractured (a) spheroid stone and (b) mulberry shaped stone composed of COD and COM. The surface of these stones is rough, because it is filled with dipyrnid COD crystals. A central core of one of the nodules is projecting on the fractured surface (white arrow). It is composed of a crystal aggregate. Bar = 500 μ m.

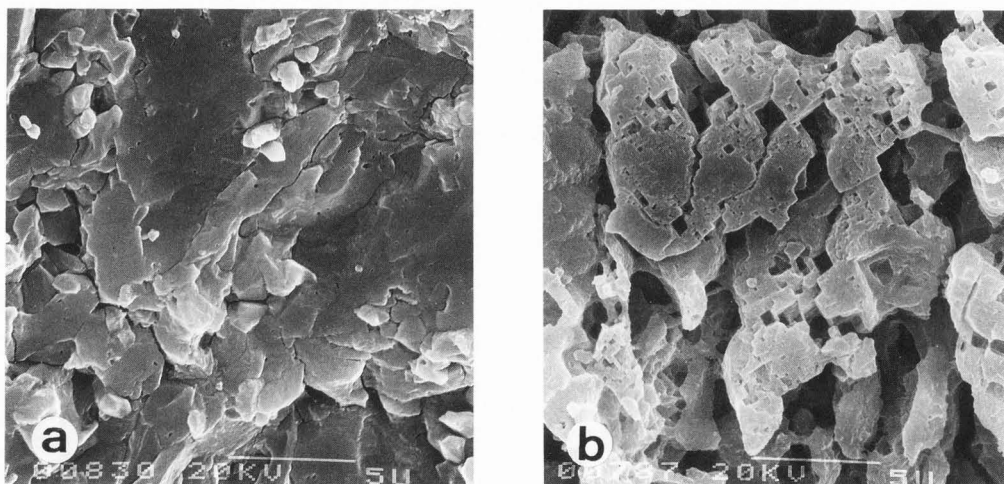


Figure 3. SEM of fractured surface of a COD stone. (a) Typical dipyrnid shape of COD is seldom observed on the fractured surface. (b) Five-minute dissolution etched square pits on the fractured face of COD crystals. Bar = 5 μ m.

Calcium oxalate stones

Under the naked eye some of the stones were lustered and others were lusterless. Under the scanning electron microscope the surface of the former stones was smooth, and no crystal figure was found. These stones were found to be composed of COM only. But the surface of the latter stones was rough because it was filled with the dipyrnid crystals of COD (Fig. 2). Therefore, spheroid and mulberry shapes were not proper to pure COM stones. It was easy to identify COD on the stone surface. But on the fractured surface of the stones it was not easy because the surface was composed of the fractured

face of the crystals. Consequently, the typical dipyrnid shape was seldom seen (Fig. 3a). In this situation the partial dissolution method was very useful. The dissolution solution etched square pits on the fractured face of the crystals, and we could identify such crystals to be COD (Fig. 3b). We found that the stones obtained from four patients contained both COM and COD. The stones from another four patients contained only COM. The architecture of the stones from the same patient was essentially same.

The mixed stones from two patients were spheroid. COD crystals were observed only as a surface deposit in one patient (Fig. 4a). In

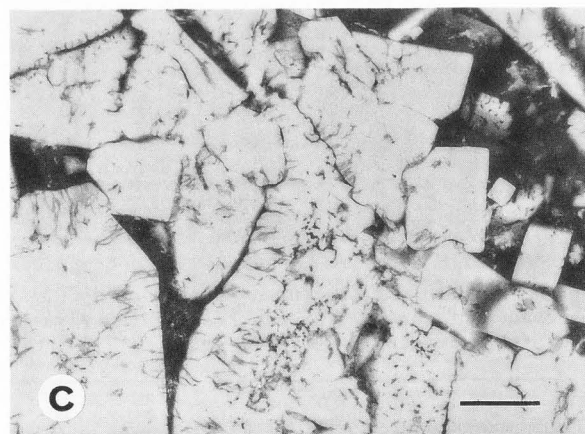
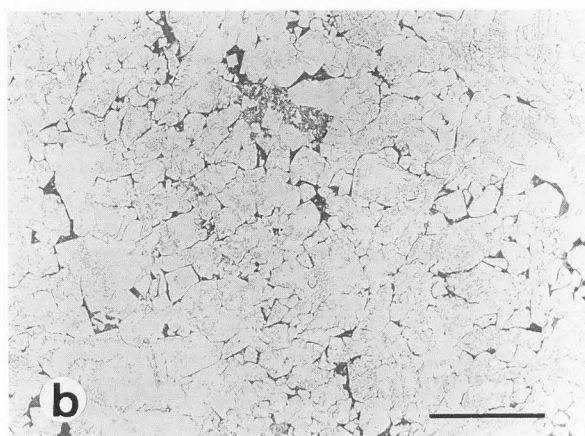
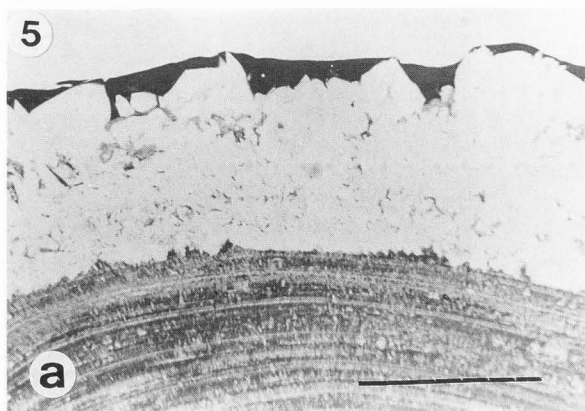
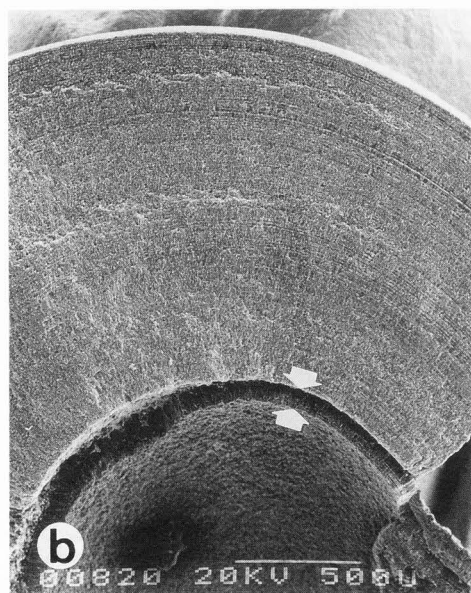
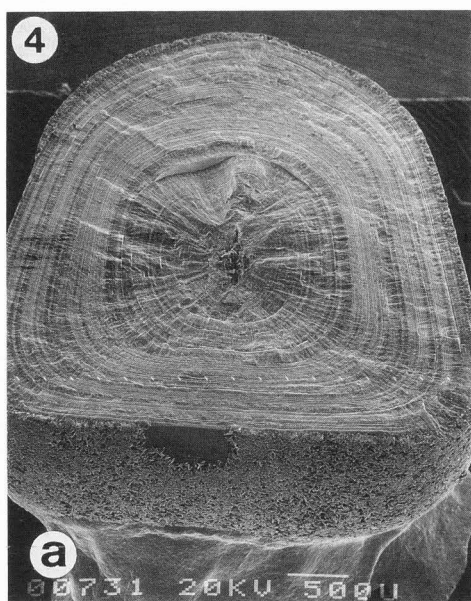


Figure 4. SEM of fractured surface of spheroid stones after five-minute dissolution. (a) The bulk of this stone is composed of COM crystals, which display radial orientation and concentric laminations. COD is observed only as a surface deposit. (b) The bulk of this stone is composed of COD crystals. COM crystals are found in a deeper layer (sandwiched between white arrows). The central core of this stone was lost by the cutting force. This stone and that shown in figure 2a were obtained from the same patient. Bar = 500 μ m.

Figure 5. LM and TEM of the organic matrix of a mixed stone. (a) A surface layer (upper half) of the stone was composed of COD, and COM occupied deeper layers (lower half). The stone surface

is covered with a thick layer of the organic matrix. Toluidine blue stain. Bar = 100 μ m. (b) The matrix fills the space among COD crystals. Therefore, COD crystals can be recognized as ghosts. Bar = 10 μ m. (c) The matrix is also observed in the crystalline boundaries. COD crystals are interlocked with each other, but a thin matrix film remains between the crystals. The crystal shape of COD is distorted because the crystals grew thrusting the matrix aside to minimize the intercrystalline space. Bar = 1 μ m.

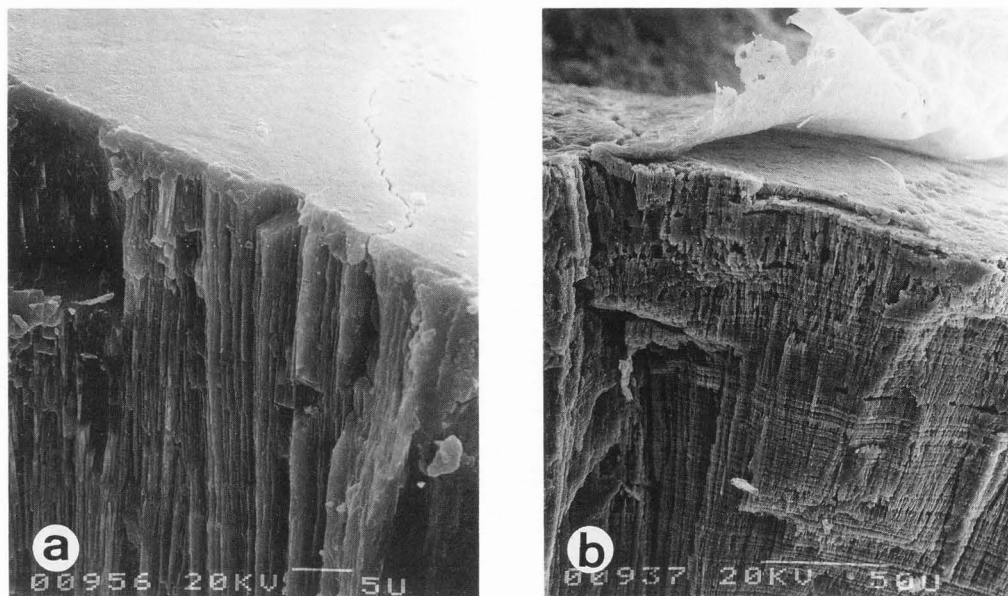


Figure 6. SEM of a fractured COM stone. (a) The surface of COM stone (upper part) is smooth and no crystal figure is seen. Bar = 5 μ m. (b) A flake of surface coat is observed after the partial dissolution. Bar = 50 μ m.

this patient the bulk of the stones was composed of COM crystals, which displayed radial orientation and concentric laminations. In the stones from the other patient, COD occupied the bulk of the stones and COM crystals were found in a deeper layer (Fig. 4b). The COD layer also showed concentric laminations which became apparent after the partial dissolution.

The mixed stones from another two patients were of the mulberry type. Each nodule had a laminated structure as in the case of the spheroid stone and had a central core, which was composed of a crystal aggregate (Fig. 2b). In both patients COD crystals occupied the bulk of the nodules, and COM crystals were found in the deeper layers. The layers of COM and COD continued beyond the border of the neighboring nodules. In all the mixed stones observed, COD was always observed in the stone surface and COM deeper into the stone. The layers of COM and COD were distinctly separated.

Thin sections of the organic matrix of mixed stones revealed that the stone surface was covered with a thick layer of the organic matrix (Fig. 5a). Furthermore the organic matrix filled the space among the crystals. Therefore, COD crystals were recognized as ghosts (Fig. 5b). The matrix was also observed within the crystalline boundaries. The COD crystals were interlocked with each other, but a thin matrix film remained between the crystals (Fig. 5c). These findings indicate that the crystals were grown thrusting the matrix aside to minimize the intercrystalline space. Consequently the dipyrmaid crystal shape was distorted. This was the second reason that we had difficulty in identifying COD on the fractured surface of the stone.

As already mentioned, the surfaces of pure

COM stones were smooth even at a higher magnification. In most cases the bulk of the stones was composed of the plate-like COM crystals oriented perpendicular to the stone surface (Fig. 6a). When these stones were treated with the partial dissolution method, a flake of surface coat was often recognized (Fig. 6b). These findings indicate that the organic matrix of calcium oxalate stones arose from a mucinous surface coat, and the bulk of the stones was formed by the crystal growth limited in this matrix.

Discussion

The square etch pits on the surface of COD crystals were occasionally observed by SEM (1, 14). These pits were thought to be a result of *in vivo* dissolution by the tide of undersaturated urine. The partial dissolution method created these pits effectively. Our observations showed that the octahedral or dodecahedral substructure of COD crystals is due to the formation of the square pits. Therefore, these pits were useful for identifying the COD crystals especially on the fractured surface of the stones, where the COD crystals seldom showed typical dipyrmaid shape.

When sodium oxalate is added to calcium chloride solution, only COM crystals are formed (8). This result is reasonable because COD is reported to be more soluble than COM (24). But when sodium oxalate is added to the urine, COD crystals are formed exclusively (8). These observations suggest that urine contains specific substances that favor the formation of thermodynamically unstable COD crystals (6, 9, 17). Murphy and Pyrah (18) proposed the idea that the growth of COM is inhibited by some substances in

urine, and COM growth can only proceed within the stone matrix, from which the substances would be excluded by a process of selective diffusion. Although we think the word "growth" in their statement should be replaced by "formation" or "nucleation", this matrix theory is very interesting.

As we have shown in Fig. 5 and 6, the crystal growth in the stone proceeded in the organic matrix, which must have been a gel when the stone was *in vivo* (11, 12). The crystal growth in a gel is reported to be different from that in a solution (2, 16). Slow diffusion of ions may allow the growth of large crystals and gel composition could play a role in crystal orientation. Supposing the formation (nucleation) and subsequent growth of crystals in the gel-state matrix is one of the main mechanisms of stone growth, the first two questions can be solved.

From the findings of polarization microscopy, transformation of COD to COM is reported by many authors (1, 3, 18, 21). This phenomenon is convenient to explain the high incidence of COM stones and the existence of COM in the deeper layers. But these authors also found that the transformation is limited in the crystalline boundaries of COD and the dipyrmaid shape is maintained even after the complete transformation. Therefore, the transformation cannot explain the radially oriented structure of COM layers. Furthermore the layers of COM and COD are distinctly separated. This means that an abrupt change happened at this point. The abrupt change of the crystalline composition can most easily be explained by crystal deposition on a growing stone surface, which is composed of other crystalline components (Fig. 4a). Deposition of COD crystals on the stone surface is very likely to occur because COD crystalluria is often observed in stone former's urine (25). Once the stone surface is covered with COD crystals, they continue to grow or further deposit to form the bulk of the stone (Fig. 7). On the contrary COM crystalluria is less likely (5). This explanation is sufficient to solve the third question, that COD is always observed on the stone surface.

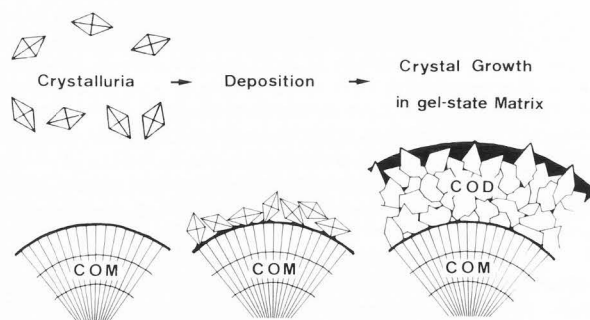


Figure 7. Schematic drawing of the formation of mixed COD and COM stone. COD crystalluria is common, and the crystals deposit on the surface of COM stone. Once the stone surface is covered with COD crystals, they continue to grow in the gel-state matrix or further deposit to form the bulk of the stone.

Although the hypothesis that "the formation of COM is inhibited by some substances in urine, and COM formation can only proceed in the stone matrix, from which the substances would be excluded" has not yet been proven, this matrix theory is very attractive. Analytical studies revealed that the stone matrix contains some urinary macromolecules on a selective basis (19, 22, 23). For example, heparan sulfate and hyaluronic acid were found in the matrix, but the most prominent urinary glycosaminoglycan, chondroitin sulfate, was excluded from it. Doubtlessly, the organic matrix provides a milieu, which is different from that of the ambient urine. We believe that the crystal deposition on the stone surface and the crystal growth within the gel-state matrix are the two mechanisms of stone growth (13). The architecture of a stone is determined by the combination of these two mechanisms.

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Discussion with Reviewers

M.I. Resnick: Calcium oxalate crystals obtained from only one patient were studied. It is suggested that crystals from other patients be studied as well to determine and particularly document the reproducibility of this finding.

Authors: The square etch pits were also observed on the COD crystals from another normocalciuric patients. But in this communication COD crystals from one hypercalciuric patient were used because his crystals were always large enough and he

voided large amount of crystals at every visit. Using this patient's crystals, experiments were repeated several times to take good micrographs. We believe the square etch pits of COD are highly reproducible.

M.I. Resnick: The calcium oxalate stones studied were obviously quite old and had been in storage for variable periods of time. The method of storage certainly could affect the composition of the stone and the subsequent observations by the investigators. It is suggested that fresh stones be used so that the investigators can compare these findings with those of their initial work on old stones.

Authors: The stones used in this study were selected from our collections from more than one thousand patients. Although these stones were stored for many years in the room atmosphere, the crystalline components were newly analyzed shortly before this morphological study using infrared spectroscopy. We found that the storage in the above mentioned condition affected little on the mineral component. Furthermore, one of the conclusion of this communication is that the COD-COM transformation does not affect the stone architecture. It is not exactly essential to use fresh stones, which are not available now by the change of the stone treatment.

S.R. Khan: What was the matrix percentage in your stones? It is interesting that you could maintain the architectural integrity of the crystal-matrix association during total dissolution for TEM without providing any support like agar bedding.

Authors: The matrix occupies about 2% of the stone weight (text reference 19). We were able to maintain the integrity by adding cetylpyridinium chloride (CPC) in the fixative mixture. When CPC was not added, the architectural integrity of the organic matrix was lost during the dehydration procedure (text reference 10). CPC is a long-chain quaternary ammonium salt. It forms insoluble complexes between water-soluble acid glycosaminoglycans. We reported that the stone matrix contains acidic glycosaminoglycans (text reference 19).

S.R. Khan: Does COD-COM transformation play any role during stone growth and architectural design of urinary stones?

A. Hesse: In your opinion, can the transformation of COD on the surface of urinary stones lead to radial arrangement of COM?

Authors: No. The transformation of COD to COM does not affect the stone architecture. It takes place only in the crystalline boundaries of COD, consequently the original dipyrmaid shape remains even after the complete transformation. We believe that the radial arrangement is a result of synchronous growth of COM crystal piles, which nucleated within the gel-state organic matrix.

A. Hesse: Does the dissolution of COD with EDTA also initiate a transformation of the adjacent layers into COM?

Authors: We didn't find any sign which indicated the transformation initiated by EDTA.

A. Hesse: Can you confirm that in most cases COD crystals grow directly on the surface of COM?

Authors: Our opinion is that COD crystals nucleate apart from the surface of COM stone and deposit on it subsequently. The existence of COM is not necessary for the growth of COD.