In Vitro Formation of "Urinary Stones": Generation of Spherulites of Calcium Phosphate in Gel and Overgrowth with Calcium Oxalate Using a New Flow Model of Crystallization

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IN VITRO FORMATION OF "URINARY STONES": GENERATION OF SPHERULITES OF CALCIUM PHOSPHATE IN GEL AND OVERGROWTH WITH CALCIUM OXALATE USING A NEW FLOW MODEL OF CRYSTALLIZATION

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Abstract

Calcium phosphate (CaP) has been detected in the majority of urinary stones containing predominantly calcium oxalate (CaOx). Therefore, crystal phases of CaP might play an important role with respect to the formation of urinary calcium stones in general. Very often, CaP found in stones or tissue of human kidney occurs in the shape of small spherulites. In this paper, we report on a new flow model of crystallization (FMCG), which has been used to generate spherulites of CaP in a gel matrix of 1% agar-agar at 37°C from a supersaturated, metastable solution continuously flowing over the gel surface. Scanning electron microscopy (SEM), X-ray diffraction and microscopic Fourier transformed infrared spectroscopy (FTIR) revealed that the particles formed (diameter: up to 200 μm) consisted of a poorly crystalline core of carbonatoapatite which was partly surrounded by a well-crystallized shell of octacalcium phosphate (OCP) showing radially oriented sheet-like structures. Subsequently, CaOx was grown on these spherulites from a flow of a correspondingly supersaturated solution conducted over the gel matrix. It could be shown by SEM that growth of calcium oxalate monohydrate (COM) was characteristically induced by the OCP shell. Radial sheet-like forms of OCP were directly continued by COM showing a certain radial orientation.

The model of crystallization in gel matrices applied here should be well-suited to simulate the process of urinary stone formation under in vitro conditions.

Key Words: Calcium oxalate, calcium phosphate, crystal growth, flow model of crystallization, gel matrices, nucleation, octacalcium phosphate, spherulites, urolithiasis.

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Introduction

Calcium phosphate (CaP) has been proven to be a component of most of the urinary stones which are mainly composed of calcium oxalate (CaOx) [6, 23, 24]. Furthermore, it is well-known that epitaxy occurs between different CaP phases and CaOx in in vitro growth experiments [10, 20, 25, 27]. These and other observations suggest that the primary formation of CaP in human kidneys, which is essential for subsequent nucleation of CaOx, is generally a key event in calcium urolithiasis, [4-7, 12, 23, 24, 28, 29, 34, 36, 38].

In vivo, CaP frequently forms small spherulitic particles of different crystalline structure. These spherulites have been found in the cores of calcium containing urinary stones [5-7, 18, 19, 23, 24, 35], in the tissue of human kidneys [28, 29], and in human urine [5, 6, 28, 37]. Spheroidal shapes of amorphous and crystalline CaP have also been produced in vitro under different conditions [8, 9, 14, 21, 22, 26, 32]. However, their formation has not yet been followed systematically in crystallization experiments aimed at simulating urinary stone formation. Particularly, studies about the influence of spherulitic superstructures on the induction and growth of CaOx crystals have been lacking, until now.

It has been the intention of this work to find suitable experimental conditions for the generation of CaP spherulites in a gel matrix from a flow of supersaturated solutions and to evaluate their ability to induce secondary growth of CaOx.

Since urinary stone formation, including nucleation, growth and agglomeration of crystals, takes place in human kidney in a fixed, gel-like state [7, 11, 16, 17] from a flow of supersaturated urine, we developed a crystallization model which simulates these physiological conditions as closely as possible.

Methods and Materials

The principle of the dynamic Flow Model of Crystallization in Gels (FMCG) applied in this work is demonstrated in Figure 1 [2, 3]: a continuous flow of a solution S_{AB}, which is supersaturated but metastable with
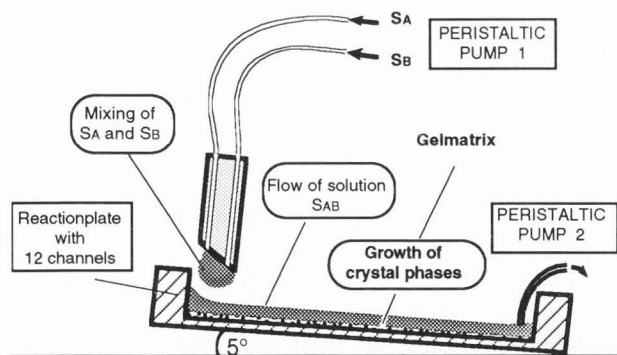
Figure 1. Schematic diagram of the Flow Model of Crystallization in Gel (FMCG).
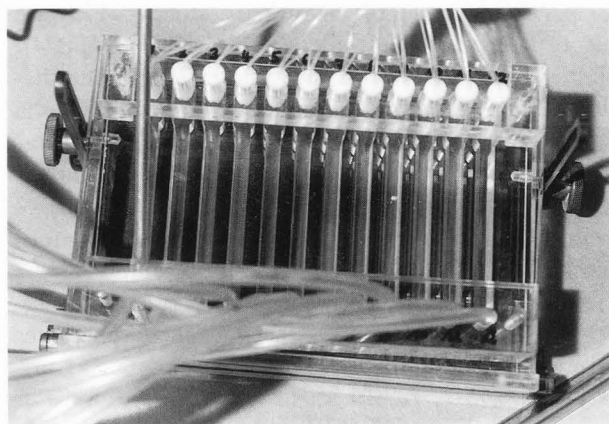

Figure 2. FMCG: reaction plate for the crystallization experiments described in this paper, with 12 channels containing gel, inlet tips and outlet tubes (size: 12.5 x 8.5 x 1.5 cm).

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respect to a sparingly soluble crystal phase AB, is generated by mixing two solutions, S_A and S_B, containing the phase components A and B, respectively. Mixture S_{AB} is conducted over a gel surface, resulting in crystal formation within the gel phase or on its surface. Solution flow is maintained at a constant rate by using multichannel peristaltic pumps.

The reaction plate used in this model is shown in Figure 2. It contains 12 channels with gel (750-1000 μl/channel). During experimentation, the channels are run simultaneously; the inlet tips are for the mixing process, while the outlet tubes remove solution S_{AB} at the lower end of the gel channel. The size of the reaction plate (125 x 85 x 15 mm) and the position of each channel (distance: 9 mm) within the plate are adapted to the dimensions of the 96-well microplate [1].

During crystallization, the reaction plate is kept at 37 ± 1° C in an incubator. The temperature is continuously monitored in the gel of the lower part of the first channel. The feeding solutions, S_A and S_B, are pre-

warmed in a thermostaticly controlled water bath. The constant flow rate of the final solution S_{AB} is 0.5 ml/min per channel, which is achieved by a 26-channel peristaltic pump (Type IPN 26; ISMATEC, Zuerich, Switzerland).

In this study, the gel used consisted of 1 wt% agar-agar (ROTH; no. 2266) containing 1 mM sodium azide, in order to prevent bacterial growth. All inorganic reagents used were of analytical grade (Merck, Darmstadt, Germany).

Crystallization of CaP phases was generated from a flow of solution I containing final concentrations of 200 mM NaCl, 20 mM NaH_2PO_4 / Na_2HPO_4, 1.7 mM, $CaCl_2$, and 1 mM NaN_3 (pH 6.6-6.7).

Growth of calcium oxalate monohydrate (COM) on CaP was achieved using two differently composed solutions: (1) Solution II contained 200 mM NaCl, 2 mM $CaCl_2$, 0.3 mM Na_2Ox, and 10 mM MES (morpholine ethane sulfonate; pH 6.0). (2) Artificial urine was composed as follows: (final total concentrations in parentheses given in mM) Na (131), K (40), NH_4 (25), Ca (4.0), Mg (3.0), phosphate (20), sulfate (15), citrate (2.0), chloride (149), urea (250), azide (1.0), oxalate (0.3), pH 6.0 [1].

Light microscopic observations of crystals within the gel matrix and documentation of crystal images were performed directly in the reaction plates using a computer controlled inverted microscope (IM 35) equipped with scanning-stage for 96-well microplates and a camera (ZEISS, Oberkochen, Germany) [1].

Specimens for the investigation by scanning electron microscopy (SEM) were mechanically prepared from the gel matrix after rinsing the gel with distilled water, followed by an air- or freeze-drying process (freeze-dryer Alpha I-5; Fa. Christ, Germany). Some of the samples were fractured into smaller pieces and coated with gold or carbon. SEM studies were performed using a CamScan4 microscope (Cambridge, UK) operated at 20 to 30 kV.

Results

Formation of Spherulites of Calcium Phosphate

Spherulites of CaP could be generated in the unseeded gel matrix during a 4-8 hours flow of solution I over the gel surface. The size of the spherulites amounted up to about 200 μm in diameter. Typical light microscopic images of the spherulitic particles are shown in Figure 3. The characteristic Maltese cross pattern of the particles viewed between crossed polarizers evidenced their spherulitic nature (Fig. 3b).

Qualitative observation suggested a rather large variation in the number of particles formed per gel area/volume, as well as in their size distribution. This indicates
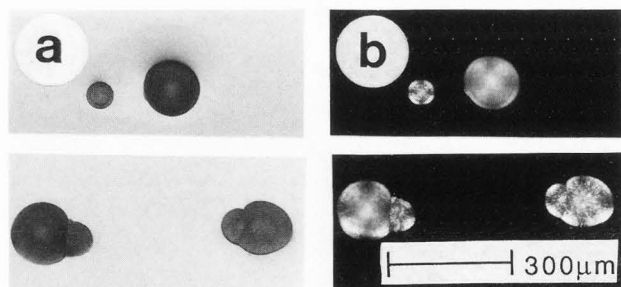
Figure 3. Typical light microscopic image of CaP spherulites formed in gel. Modes of observation: left = bright field; right = polarized light.

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the predominant role of nucleation factors.

Some special and unexpected features of the particles were revealed by SEM as shown in Figures 4a-f. A spherical or discoidal core (Figs. 4a-c), likely consisting of microcrystalline material (Fig. 4e), was partly covered by a well-crystallized shell of radially grown lamellated crystals (Figs. 4a-d and 4f).

The different crystal phases were analyzed by microscopic Fourier transformed infrared (FTIR)-spectroscopy (Dr. Daudon, Paris). Figure 5 shows examples of the resulting spectra. According to this result, the smallest and medium-sized spherulites were composed predominantly of octacalcium phosphate pentahydrate (OCP), some of them containing small amounts of poorly carbonated carbapatite. In the biggest spherulites shown in Figure 4, the unorganized core was a mixture of poorly carbonated and poorly crystallized carbapatite with minute amounts of OCP. The radially grown sheet-like shell contained mainly OCP with some portions of carbapatite.

These findings were confirmed by X-ray diffraction which showed that OCP was the predominant crystal phase with the corresponding d-values of 1.878, 0.2824 and 0.1997 nm (Dr. Schubert, Berlin).

Overgrowth of Calcium Phosphate Spherulites with Calcium Oxalate

In a second series of experiments, we investigated the potential role of CaP spherulites as substrates for the crystallization of CaOx. After rinsing with water, solution II (described in **Methods and Materials**), which was supersaturated but metastable with respect to CaOx, was conducted over the gel surface. After an 8 hour flow, the altered particles were observed by light microscopy and SEM.

The spherulites shown in Figure 6 correspond exactly to those demonstrated in Figure 3, however, only after subsequent overgrowth with CaOx. From this observation, preferential growth of monoclinic COM on

CaP spherulithes can be recognized.

More detailed information is gained from scanning electron micrographs, some of which are shown in Figure 7. Obviously, COM crystals are formed on the apatitic core as well as on the sheet-like outer OCP (Figs. 7a-c). However, while single COM crystals are irregularly arranged on the core surface (Fig. 7b), COM induced on OCP shows oriented, radially grown sheet-like crystals clearly resembling those of the preceding OCP phase. This may be seen especially from the detailed view on the outer shell as shown in Figures 7d-f. Here, COM crystals seem to directly continue the pre-formed OCP. A borderline, indicating a discrete growth of one crystal phase on the other, is not evident from the micrographs.

Qualitatively, quite similar features of COM grown on CaP spherulites were obtained when the artificial urine described above was used instead of solution II in overgrowth experiments.

Discussion

In this work, spherulites of CaP were generated in a new flow model of crystallization in gel matrices which has been aimed at simulating the formation of "urinary stones" under *in vitro* conditions.

CaP, particularly when it is in an apatitic crystal phase, seems to be a ubiquitous constituent of urinary stones. In his review on the "Organic Matrix of Human Urinary Concretions", Boyce [7] points out that, in each "oxalate calculus" investigated, a central or marginal spherule was found which invariably contained fibrous matrix and CaP, commonly as crystallized apatite. Even in stones consisting predominantly of uric acid, laminations were found containing fibrous matrix together with CaP. According to Boyce, the chemical analysis of stone matrix always revealed calcium and phosphate in the ash, even when all microscopically demonstrable crystals had been removed by dialysis against chelating agents [7].

These results were confirmed by Leusmann [23, 24], who demonstrated in an extensive SEM study that more than 75% of stones consisting mainly of CaOx contained CaP in the core area. Zarembski and Grieve found by SEM that the nucleus of 15 "pure" COM stones consisted of apatite-type material. In each ring of the nucleus, the two components, CaOx and CaP, were detected in similar proportions [38].
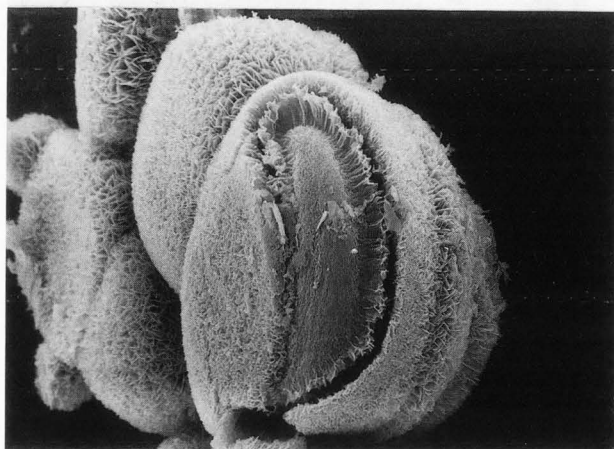
Other evidence of a potential causative relation between primarily formed CaP and the secondary growth of COM in urinary calculi comes from the classic observations of Randall [33] and later findings by Cifuentes [13], who studied Randall's plaques and the formation of papillary stones. Indirect arguments in
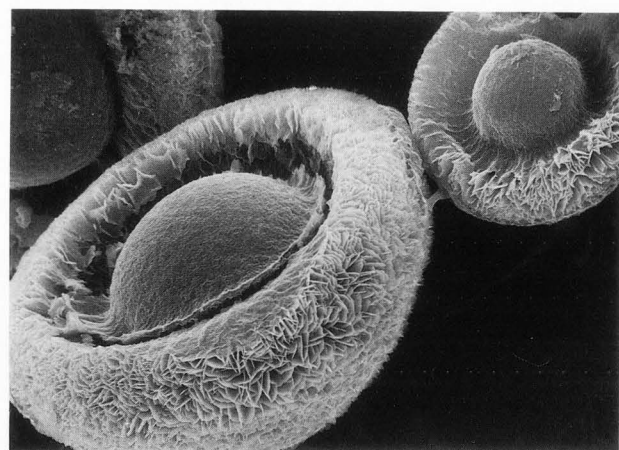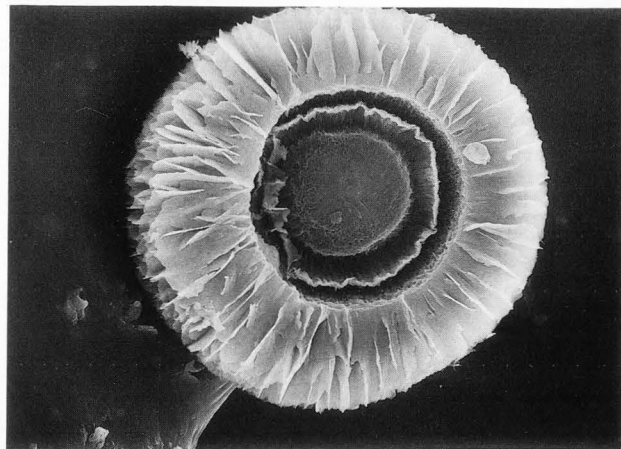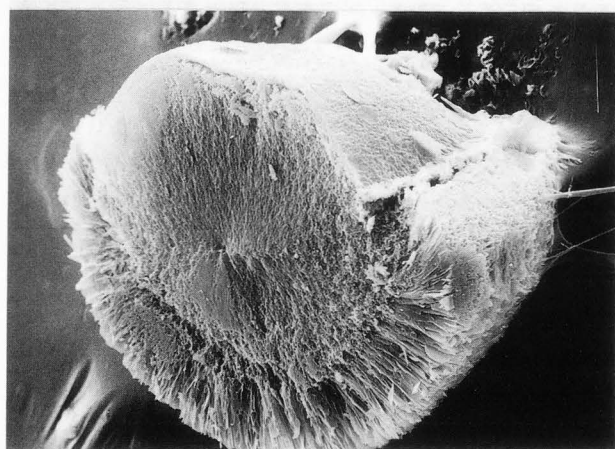
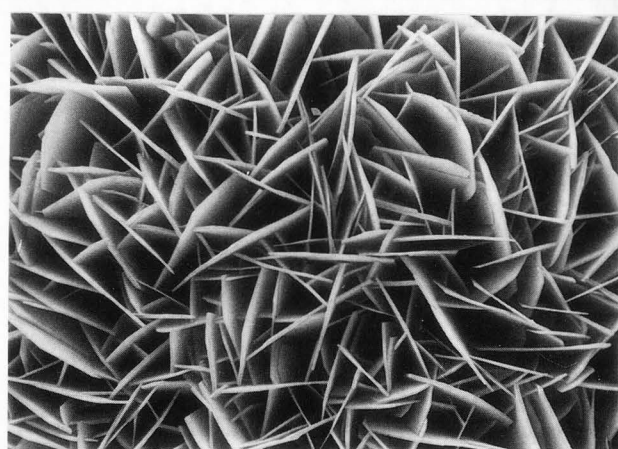**Figure 4.** Scanning electron micrographs of CaP spherulites: (**a, b**) Whole spherulitic particles are shown consisting of a microcrystalline core of carbonatoapatite and a partly formed shell of sheet-like OCP. (**c, d**) Fractured particles of the same type showing striated radial growth of OCP (the core in Figure 4d has been lost by mechanical manipulation). (**e**) Detailed view of the microcrystalline structure of core area, and (**f**) detailed view of the well-crystallized sheet-like outer OCP.

Figure 6. Typical light microscopic image of CaP spherulites in gel, after overgrowth with calcium oxalate. Modes of observation: left = bright field; right = polarized light.

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factor in the genesis of calcium urolithiasis.

Apart from their larger size, the particles formed in the gel matrix of our flow model bear a strong resemblance to native spherulites found in urinary stones and in sections of human kidneys [5, 6, 13, 18, 23, 24, 28, 35]. This demonstrates the physiological relevance of the crystallization model used. Scanning electron micrographs, together with physicochemical analysis, revealed that the spherulites are composed of a disordered, poorly crystallized core of carbapatite and a shell made of sheet-like radially grown OCP.

Spherulitic amorphous calcium phosphate (ACP) has been prepared in aqueous solution and investigated by several authors [8, 9, 14, 26, 32]. From these results, it may be concluded, by analogy, that ACP should also have originated in the gel matrix and later been transformed into the microcrystalline apatite of the core. Brecevic and Füredi-Milhofer [8] demonstrated in their experiments that, after a period of metastability, formation of crystalline material occurred within or upon the amorphous matter. The authors could show that the metastability of ACP ended with the secondary precipitation of OCP. The observations indicate a definite connection between the precursor and the secondary precipitate, making ACP appear as a template for OCP formation. These results are in many respects comparable to the findings in our work. However, the sheet-like radially oriented growth of OCP, which is clearly shown in our experiments, seems to be a consequence of the slow diffusion process in the gel matrix. This observation is in agreement with the results of LeGeros, who grew needle- and plate-like spherules of OCP by a double diffusion process in silica gel [21, 22]. Iijima and Moriwaki [15] reported about lengthwise and oriented growth of plate-like OCP on a cation selective membrane. It may be concluded from their results that the control of ionic diffusion and the undisturbed conditions of crystal growth contribute clearly to the orientation of crystals.
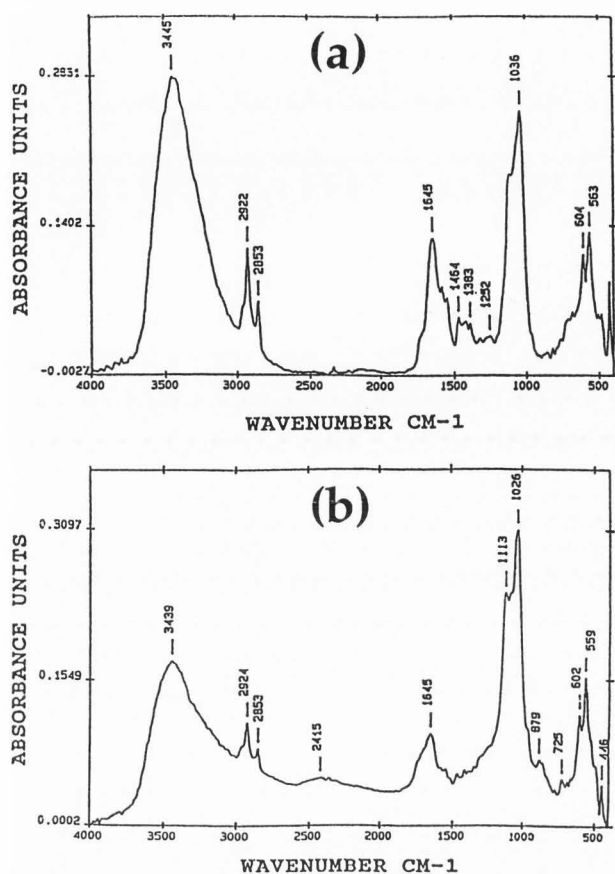

Figure 5. FTIR-spectra of material from different parts of CaP spherulites like those shown in Figure 4. (a) Substance from core area indicating poorly crystallized carbonatoapatite mixture with small proportions of OCP and (b) substance from the shell area demonstrating mainly OCP.

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favour of a causative role of CaP in stone formation were given by Smith *et al*. [34], Baumann [4], and Tiselius and Larsson [36]. Furthermore, epitactic growth relations between CaP and CaOx have been demonstrated by several authors [10, 20, 22, 25, 27].

In vivo, CaP very often has the shape of small spherulites or spherules. Referring again to Boyce, "the existence of spherules is a striking characteristic of all urinary calculi of whatever crystalline composition, size or shape" [7]. The occurrence of CaP spherulites and spherulitic aggregates in human urinary stones has been proven by Spector *et al*. [35], Blaschke *et al*. and Leusmann [5, 6, 23, 24] using SEM. Meyer-Jürgens *et al*., and other authors, have shown that spherulitic particles composed of CaP occur in the tissue of human kidney [28, 29], as well as in human urine [5, 6, 28, 37].

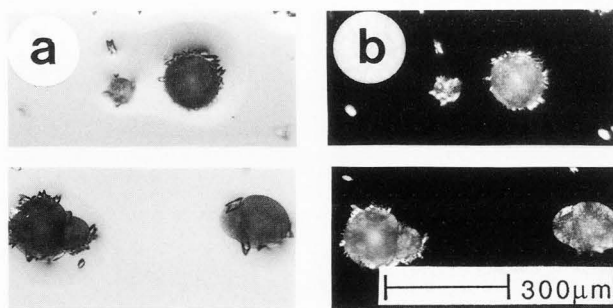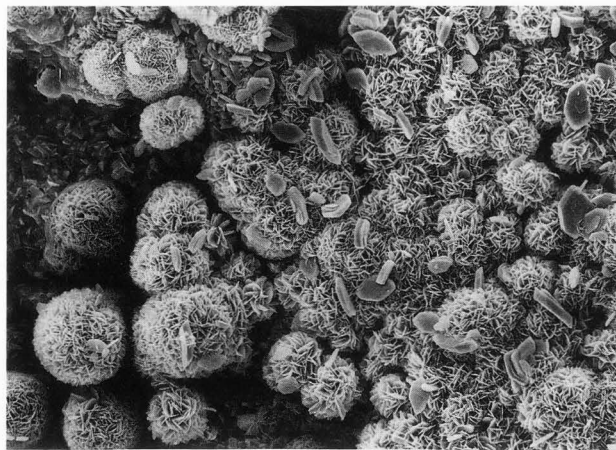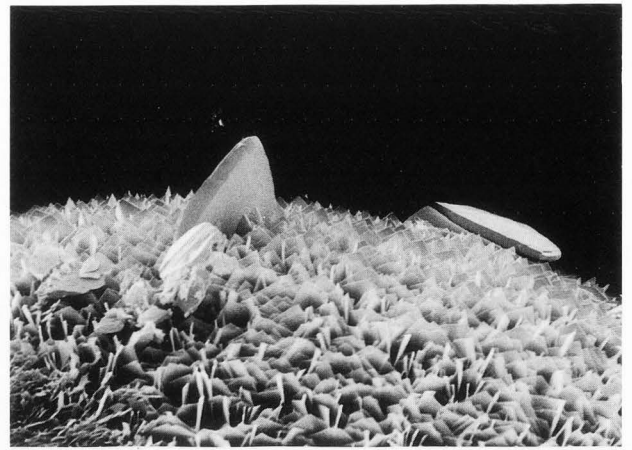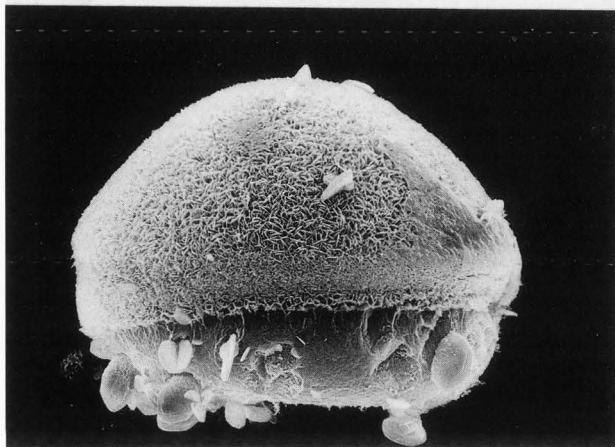Altogether, the formation of CaP spherulites in human kidneys seems to be a very likely etiological

Figure 7. Scanning electron micrographs of CaP spherulites overgrown with calcium oxalate: **(a-c)** Whole and fractured spherulitic CaP particles with COM crystals grown on the core surface and on the sheet-like OCP shell. **(d-f)** Detailed views on the crystal shape of CaOx grown on OCP: oriented radial growth of COM seems to be induced by the similarly shaped OCP.

An interesting reference with respect to our results is the paper of Carr [11] on the phenomenon of crystalline radial striation, which is one of the most characteristic features found in the majority of urinary stones [7, 16, 30]. Carr supposes spherulites, which are growing in a gelatinous medium, to be the substrates of radially oriented crystal growth in stones. The gel medium, where stone growth takes place, is formed by the macromolecular mucinous layer which always covers a calculus. Accordingly, this mucinous layer provides the necessary gel-like framework in which undisturbed diffusion can occur. During the further crystal growth, the mucinous layer is incorporated in the crystalline material, thus becoming the organic matrix of the calculus. The Carr's suggestion on the development of stones in a gel-like state has been supported by results of Iwata *et al.* [16, 17].

Referring to our investigations, the above findings and views are of considerable importance. They strongly suggest that crystallization studies with respect to urolithiasis should be carried out in a gel medium [1]. Furthermore, spherulites may be considered physiologically relevant crystalline substrates which promise successful imitation of "urinary stone" formation *in vitro*.

Our experiments on the overgrowth of the CaP spherulites with CaOx reveal that OCP is most probably a primary template or, preferably, substrate, for the "heterogeneous" nucleation of COM crystals. Since OCP is a metastable crystal phase which is easily transformed into hydroxyapatite [31], it may not be detected in calculi by later analysis. From the radial orientation of COM crystals, which may be observed on the micrographs in Figures 7d-f, one may hypothesize that OCP is also responsible for the induction of the phenomenon of radial striation typical for COM stones. However, such a suggestion has to be proven by further experiments.

Lonsdale [25] suggested plausible crystallographic preconditions for epitaxial growth of many crystal phases relevant in stone formation. She proposed to attempt the growth of artificial stones on suitable crystal substrates in order to find out potential growth effectors. The flow model of crystallization from the gel state used in this study seems to be a preferable tool in order to carry out this kind of investigation.

In conclusion, the results of the present work support the previously suspected crucial role of CaP, especially in its spherulitic shape, in calcium stone formation.

Acknowledgements

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lytes and proteins as mineralization promotors and inhibitors of calcium oxalate monohydrate. Calcif. Tissue Int. **45**, 122-128.

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Discussion with Reviewers

S.R. Khan: To interpret the results of their experiment, the authors should take into account a couple of things: 1. How common is OCP in the human urinary stones, particularly in association with COM? 2. Mandel and Mandel [39] have provided evidence that epitaxy can not play a major role in urinary stone formation. 3. My personal work with experimentally induced foreign body stone formation *in vivo* [40] has clearly demonstrated formation of radially arranged crystals of COM in discrete layers on stone surfaces, in the total absence of any type of calcium phosphate. Could you comment please?

Authors: 1. OCP is a very rare crystal phase in urinary stones. However, it is metastable, can be easily transformed into hydroxyapatite and might, therefore, not be detectable. 2. Referring, for instance, to references [20], [25] and [27], this seems to be contradictory. 3. We have some doubt about your statement of "the total absence of any type of calcium phosphate" in the *in vivo* model to which you have referred. The experimental conditions you have described were quite favourable also for the formation of CaP crystal phases. It seems to be likely that traces of CaP at the struvite surface were present in your experiments but had not been detected.

In general, also referring to the remarks of Dr. Eanes, we would see the link between radially grown OCP and striated growth of COM as a hypothesis. Experiments of longer duration of COM growth on OCP, also in the presence of matrix forming macromolecules, are necessary for further proof.

S.R. Khan: Is it possible that slow and continuous movement of metastable solution through and over a gel results in this morphology? Did the authors study the formation of calcium oxalate only without prior formation of calcium phosphate?

Authors: It is without any doubt that the diffusion process slowed down by the gel medium favours the formation of spherulitic structures [11]. Up to now, we have not been able to demonstrate radial growth of COM without prior CaP formation. COM growth in the absence of CaP has always led to monoclinic crystals without preferred orientation.

H.-G. Tiselius: Are there any special structural or morphologic characteristics of the gel that explain how the nucleation of calcium phosphate is promoted?

Authors: One can not conclude from these experiments that the nucleation of calcium phosphate is promoted by the gel. The gel clearly has an impact on the morphology of the crystallites, which is mainly due to the altered diffusion process. We have no suggestion for the specific mechanism of nucleation within the gel.

H.-G. Tiselius: Is there any precipitation of CaOx in the gel if a solution, metastably supersaturated with CaOx, is conducted over the gel in the absence of previously precipitated CaP?

Authors: Yes. Under the conditions employed here we could observe monoclinic COM crystals, also in the absence of CaP.

H.-G. Tiselius: A period of 4-8 hours appears to be long compared to the time required for physiological crystallization. This is probably explained by the slow diffusion of solutes into the gel. Were there any CaOx crystals observed earlier than that, and if so, were these crystals also COM? Can you speculate on which CaOx crystal phase precipitates first on an apatite or OCP surface?

Authors: From our point of view, a duration of 4-8 hours for the overgrowth experiments is too short, rather than too long. Stone growth may occur within weeks or months. We have not registered the course of the crystallization at a different time, so we are not able to answer your questions. However, following the process as a function of time should provide interesting information.

E.D. Eanes: What is meant by "mechanically prepared"? Were the spherulites physically removed from the gel matrix before scanning or were they examined *in situ*? By what means were the spherulites fractured?

Authors: The particles were removed before SEM from the lyophilized or air-dried gel material using tweezers or a pointed knife. They were fractured using a scalpel.

E.D. Eanes: What accounts for the OCP shell not completely covering the core volume in the whole spherulitic particles? Was the exposed core area the part of the core that was in contact with the gel matrix?

Authors: Spherulites formed predominantly just under the gel surface. There were spherulites completely covered by an OCP shell and hemispherulites in which plate-like OCP crystals were directed only to the top of the gel. The gradient of supersaturation for OCP from the top to the bottom of the gel canals could be seen as a cause of the hemispherulitic morphology.

E.D. Eanes: The FTIR evidence for OCP is weak. The unique 917 cm^{-1} band for OCP appears to be absent

from spectrum b of Figure 5. This suggests that OCP possibly had undergone some hydrolysis to apatite before the spectrum was recorded. The finding of an 1.878 nm peak in the X-ray diffraction pattern of the shell material is more definitive evidence for this material being OCP.
Authors: Thank you for this remark.

E.D. Eanes: Are the terms "CaOx" and "COM" being used interchangeably, i.e., are all the CaOx crystals COM?
Authors: As a rule, the term "COM" is used in this paper when this crystal phase had been identified by its typical morphology.

E.D. Eanes: Was there any evidence of COM crystals forming directly on the gel matrix or were all the COM crystals found on the surface of the CaP spherulites?
Authors: Under our experimental conditions employed, we could observe single monoclinic COM crystals, also directly in the gel.

Additional References

[39] Mandel NS, Mandel GS (1981). Epitaxis between stone-forming crystals at the atomic level. In: Urolithiasis - Clinical and Basic Research. Smith LH, Robertson WG, Finlayson B (eds.). Plenum Press. pp. 469-480.
[40] Khan SR, Hacket RL (1987). Urolithogenesis of mixed foreign body stones. J. Urol. **138**, 1321-1328.