

CRYSTALLIZATION DURING VOLUME REDUCTION OF SOLUTIONS WITH A COMPOSITION CORRESPONDING TO THAT IN THE COLLECTING DUCT. THE INFLUENCE OF HYDROXYAPATITE SEED CRYSTALS AND URINARY MACROMOLECULES

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Abstract

To examine the effect of hydroxyapatite (HAP) seed crystals and urinary macromolecules on the crystallization under conditions similar to those in the collecting duct (CD), we evaporated 100 ml samples of salt solutions with an ion-composition assumed to correspond to that in the CD without and with HAP seed crystals. The crystallization in seeded solutions was assessed both with and without dialyzed urine (dU). After evaporation, the number and volume of crystals were recorded and the crystal morphology was examined with scanning electron microscopy (SEM) and X-ray crystallography.

Addition of HAP crystals was apparently followed by an approximately 15-20% increase in heterogeneous nucleation of calcium oxalate (CaOx). In these experiments, SEM and X-ray crystallography showed a high percentage of CaOx in the precipitate.

In samples reduced to 40-69 ml, addition of dU to the CD solution containing HAP seed resulted in a greater mean (SD) number of crystals: 3895(1841) in samples with dU and 1785(583) in samples without. This was mainly explained by an increased mean (SD) number of small crystals. The mean crystal volume was 17.8(1.1) and 34.3(9.1) in samples reduced to 40-69 ml with and without dU, respectively. This might reflect the inhibitory effect of dU on the growth and/or aggregation of the CaOx-CaP precipitate or a promoted nucleation resulting in a great number of small crystals.

It is concluded that calcium phosphate (CaP) formed above the collecting duct might induce heterogeneous nucleation of CaOx at lower levels of the renal collecting system, and that urinary macromolecules are powerful modifiers of these processes.

Key Words: Crystallization, hydroxyapatite, calcium phosphate, calcium oxalate, collecting duct, macromolecules, promotion, inhibition.

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Introduction

The mechanisms of formation of urinary calcium oxalate containing concrements are incompletely understood, and the process can certainly not be explained only by a crystallization of calcium oxalate (CaOx). Urinary stones have an organic matrix accounting for approximately 2.5% of the weight of the stone (Boyce, 1956). It has also been shown that a considerable fraction of CaOx stones is composed of mixtures of CaOx and calcium phosphate (CaP) (Prien, 1955; Pak *et al.*, 1971, Peacock *et al.*, 1979; Larsson *et al.*, 1984a, b; Ohkawa *et al.*, 1992; Tiselius, 1996). Furthermore, Leusmann *et al.* (1984) showed that more than 70% of oxalate rich stones had CaP within or near the central core of the stone.

It is reasonable to assume that the crystallization is initiated in the nephron (Coe and Parks, 1990; DeGanello *et al.*, 1990; Lupták *et al.*, 1994; Kok, 1995; Asplin *et al.*, 1996; Højgaard *et al.*, 1996, 1997; Tiselius *et al.*, 1997). Finlayson and Reid (1978) showed that even with a maximal crystal growth of CaOx the renal transit time was too short to result in crystals with a size that would obstruct the collecting duct. Baumann *et al.* (1984) showed that after an oral oxalate load, the size of the crystals was generally smaller than 200 μm in a concentrated overnight urine. In a recent experimental study, Kok and Khan (1994), however, concluded that during the normal transit time for urine through the nephron, crystalline particles might form large enough to be retained, mainly due to the size-increasing effect of agglomeration. Thus, a pronounced agglomeration and crystal retention are factors that might be of importance for stone development. Randall's plaques seem to start from a CaP deposition in the papillary tissue (Prien, 1975) and it has been shown that crystals of hydroxyapatite (HAP) can induce a heterogeneous crystallization of CaOx (Smith and Werness, 1983; Baumann *et al.*, 1989; Berg and Tiselius, 1989; Hallson and Rose, 1989; Grases and Conte, 1992). Crystals of CaP that are retained in the nephron or in the renal collecting system are, therefore, highly interesting as possible promoters of CaOx nucleation.

In two recent works (Lupták *et al.*, 1994; Højgaard *et*

al., 1996), we have shown that there is a risk of CaP crystal formation in the proximal and distal tubule, whereas, CaOx is the primary type of crystal that forms in the collecting duct. In a recent study, Kok (1995) also found that crystallization of CaOx is most likely in the collecting duct, and it has been shown by several authors that there is a high risk of CaP crystallization at a nephron level above the collecting duct (Coe and Parks, 1990; Kok, 1995; Asplin *et al.*, 1996). These observations have raised the question on whether CaP formed at a high level in the nephron is a natural and common promoter of CaOx nucleation in the collecting duct. A heterogeneous crystallization of CaOx induced by CaP is a mechanism that previously has been proposed by several authors (Meyer *et al.*, 1975, 1977; Pak *et al.*, 1976; Koutskoukos and Nancollas, 1981; Pak, 1981; Baumann, 1985; Berg and Tiselius, 1989; Achilles *et al.*, 1994; Hufnagel *et al.*, 1995).

The aim of this study was to examine if CaP crystals can induce a heterogeneous nucleation of CaOx under conditions corresponding to those in the collecting duct and to study the possible influence of the urinary macromolecules on this process. Since HAP is thermodynamically the most stable form of CaP (Christoffersen *et al.*, 1990), HAP crystals were used in these experiments.

Materials and Methods

Salt solutions with an ion-composition assumed to correspond to that of urine in the distal part of the collecting duct were prepared by dissolving the following commercially available salts: calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$), magnesium chloride ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), sodium chloride (NaCl), potassium chloride (KCl), sodium sulphate (Na_2SO_4), sodium phosphate (Na_2HPO_4), sodium oxalate ($\text{C}_2\text{Na}_2\text{O}_4$) and sodium citrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$) in Millipore (Millipore S.A., Molsheim, France) filtered water as previously described (Lupták *et al.*, 1994). One liter of this solution was given the following ion-composition: calcium 4.50 mmol, magnesium 3.85 mmol, phosphate 32.3 mmol, oxalate 0.32 mmol, citrate 3.21 mmol, sodium 106 mmol, potassium 63.7 mmol, and sulphate 20.8 mmol. No ammonium was added to avoid the risk of precipitating ammonium salts. The pH was set to 5.80 which was considered the average pH in the collecting duct according to the results reported by Rector (1983).

A suspension of hydroxyapatite (HAP) seed crystals with a concentration of 400 $\mu\text{g}/\text{ml}$ was prepared by adding the commercially available salt (Merck AG, Darmstadt, Germany) to Millipore filtered water. After ultrasonication for 30 minutes, the crystal size distribution was analysed in the Coulter Multisizer (Coulter Electronic Ltd., Luton, U.K.). No crystals were found with a size greater than 15.4 μm and the size distribution showed that 93% of the crystals had a

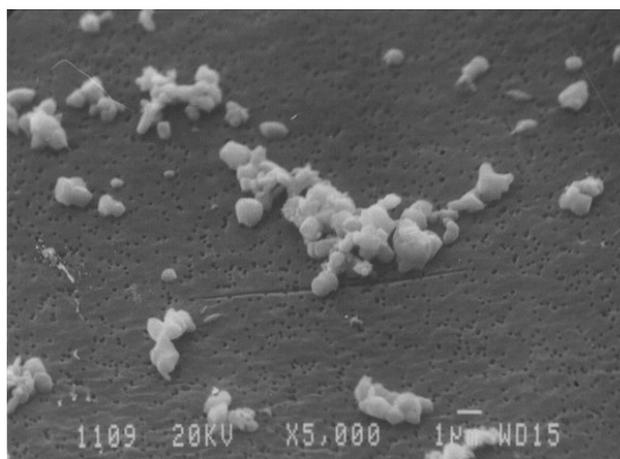


Figure 1. Scanning electron micrograph of HAP seed crystals at a concentration of 400 $\mu\text{g}/\text{ml}$. Bar = 1 μm .

diameter of 2.4 to 4.4 μm , 5% 4.5 to 5.4 μm , and 2% 5.5 to 15.4 μm . Scanning electron microscopy of suspensions showed that a major part of the crystals were smaller than 2.4 μm and that the fractions found in different size intervals were composed of HAP crystal aggregates (Fig. 1).

Pooled dialyzed bladder urine (dU) from 10 normal male subjects was used as a source of macromolecules, thereby, ignoring the fact that certain macromolecules might have been added to the urine as a result of its passage and storage in the lower parts of the urinary system, whereas, others might have been removed during the preparation of dU. This urine was collected between 2200 and 0600 hours, and screened to exclude bacteria, protein and glucose before being pooled. Urine was dialyzed as previously described (Lupták *et al.*, 1994). According to the concentration of urine in the distal part of the collecting duct, the samples with dU were prepared by dissolving the necessary salts in the dU.

Solution samples of 100 ml were passed through 0.22 μm pore size Millipore filters before evaporation in a Büchi Rotavapor RE (Büchi AG, Flawil, Switzerland) at 37°C. The filtration was carried out at room temperature at a rate of approximately 1 ml/sec. Evaporation was performed of salt solutions without both HAP and dU (CD-solution), of salt solutions with 1 ml of HAP suspension but without dU (CD-HAP-solution), and of salt solutions with 1 ml of HAP suspension and with dU (CD-HAPdU-solution). After evaporation to a predetermined volume, the number and volume of crystals in the size interval 2.4 to 45 μm were recorded in a Coulter Multisizer with a 100 μm capillary tube which we have found most appropriate for assessing crystals in this interval. Furthermore, the distribution of the crystals was recorded in the following size interval: 2.4 to 5.1 μm , 5.1

Table 1. The percentage of different calcium salts in the precipitate at different degrees of evaporation of 100 ml samples of CD, CD-HAP and CD-HAPdU-solutions analysed by X-ray crystallography.

| Sample volume after evaporation | Salt solution | | Per cent of calcium salt in the sample | | | | | total CaOx | total CaP |
|------------------------------------|------------------|--------------|--|-----------|-----------|-----------|-----------|---------------|--------------|
| | | | COD | COM | Bru | HAP | | | |
| 70-80 ml | CD | 0 min | 50 | 0 | 40 | 10 | 50 | 50 | |
| | | 60 min | 35 | 0 | 55 | 10 | 35 | 65 | |
| | HAP | 0 min | 45 | 20 | 5 | 30 | 65 | 35 | |
| | | 60 min | 15 | 45 | 5 | 35 | 60 | 40 | |
| | HAP-CDdU | 0 min | 35 | 0 | 5 | 60 | 35 | 65 | |
| | | 60 min | 20 | 0 | 5 | 75 | 20 | 80 | |
| 40-50 ml | CD | 0 min | 35 | 0 | 60 | 5 | 35 | 65 | |
| | | 60 min | 20 | 0 | 65 | 15 | 20 | 80 | |
| | HAP | 0 min | 50 | 5 | 10 | 35 | 55 | 45 | |
| | | 60 min | 40 | 0 | 50 | 10 | 40 | 60 | |
| | HAP-CDdU | 0 min | 60 | 0 | 5 | 35 | 60 | 40 | |
| | | 60 min | 35 | 0 | 5 | 60 | 35 | 65 | |
| 10-20 ml | CD | 0 min | 10 | 0 | 70 | 20 | 10 | 90 | |
| | | 60 min | 5 | 0 | 90 | 5 | 5 | 95 | |
| | HAP | 0 min | 30 | 0 | 40 | 30 | 30 | 70 | |
| | | 60 min | 20 | 0 | 70 | 10 | 20 | 80 | |
| | HAP-CDdU | 0 min | 20 | 0 | 75 | 5 | 20 | 80 | |
| | | 60 min | 5 | 5 | 85 | 5 | 10 | 90 | |

to 10.2 μm , 10.2 to 20.2 μm and 20.2 to 45 μm . Recordings were carried out both immediately after evaporation and after 60 minutes of continuous agitation in an incubator at 37°C. The mean crystal volume (MCV) was calculated as the ratio between the total volume (μm^3) and the total number of crystals. After evaporation, the mean and standard deviation (SD) were calculated for the following sample volume intervals: 70 to 95 ml (Sample A), 40 to 69 ml (Sample B), and 10 to 39 ml. No sample volume was reduced to more than 10% of its original volume.

The ion-activity products of CaOx (AP_{CaOx}), calcium hydrogen phosphate, brushite (AP_{Bru}), hydroxyapatite (AP_{HAP}), amorphous calcium phosphate (AP_{ACP}), and octacalcium phosphate (AP_{OCP}) at different degrees of evaporation, were calculated by means of computerized iterative approximation with the EQUIL2 program (Werness *et al.*, 1985). For ACP, we used the approximate formula $\text{Ca}(\text{PO}_4)_{0.74}(\text{H})_{0.22}$ (Christoffersen *et al.*, 1990).

The crystal morphology was studied in a JEOL JSM-840 scanning electron microscope (JEOL Ltd., Tokyo, Japan). Preparation for scanning electron microscopy (SEM) was performed as described in detail previously (Lupták *et al.*, 1994).

Solutions CD, CD-HAP and CD-HAPdU were evap-

orated down to a volume of 70-80 ml, 40-50 ml and 10-20 ml. Half of the crystalline material recovered from these samples was pooled before being centrifuged at 1500 rpm for 15 minutes. The other half was left for 60 minutes of continuous agitation in an incubator at 37°C before being pooled and centrifuged at 1500 rpm for 15 minutes. The sediment was dried in air and then subjected to X-ray crystallographic examination at L.C. Herring and Company (Orlando, FL, USA) to quantitatively assess the content of CaOx and CaP.

Statistical analysis

Wilcoxon's rank sum test (Swinscow, 1976) was used for comparing samples with and without HAP, and with and without dU. Wilcoxon's signed rank test was used to compare samples immediately after evaporation with those obtained after 60 minutes of continuous agitation in the incubator.

Results

Scanning electron microscopy showed that volume reduction of the CD-solution resulted in formation of CaOx and CaP crystals. CaOx dihydrate (COD) was the predominant crystal phase in the least concentrated samples immediately after the evaporation (Fig. 2A), whereas, CaP

Table 2. Mean (SD) number, total crystal volume and mean crystal volume (MCV) at different sample volumes immediately and 60 minutes after evaporation of 100 ml samples of CD, CD-HAP and CD-HAPdU-solutions.

| Crystal size 2.4 to 45 µm. | Sample A (70-95 ml) | | | Sample B (40-69 ml) | | |
|---|-------------------------|-------------------------|---------------------------|----------------------------|--------------------------|----------------------------|
| | CD | CD-HAP | CD-HAPdU | CD | CD-HAP | CD-HAPdU |
| The number of crystals | | | | | | |
| 0 min | 131 (133) ^{††} | 1372 (378) [°] | 1479 (588) | 347 (315) ^{††°} | 1785 (533) [°] | 3895 (1841) ^{**°} |
| 60 min | 127 (142) | 926 (232) [°] | 1414 (755) | 1999 (1476) ^{††°} | 2659 (1089) [°] | 5398 (2254) [°] |
| The total crystal volume (1000 x µm³) | | | | | | |
| 0 min | 30.4 (41.0) | 54.3 (26.6) | 61.6 (42.3) | 30.2 (31.5) ^{†°} | 59.9 (19.3) [°] | 134 (50.9) ^{**°} |
| 60 min | 27.0 (34.7) | 44.7 (13.5) | 88.5 (81.8) | 405 (508) [°] | 396 (474) [°] | 482 (325) [°] |
| MCV | | | | | | |
| 0 min | 178 (133) [†] | 38.9 (9.7) | 16.7 (1.1) ^{**°} | 101 (57.7) ^{††} | 34.3 (9.1) [°] | 17.8 (1.1) ^{**°} |
| 60 min | 234 (120) | 49.7 (12.9) | 55.2 (34.2) [°] | 164 (150) | 122 (110) [°] | 82.3 (32.0) [°] |
| number of experiments | | | | | | |
| | 5 | 5 | 6 | 6 | 8 | 6 |

Comparison of CD and CD-HAP samples [†]p < 0.05; ^{††}p < 0.01.

Comparison of CD-HAP and CD-HAPdU samples *p < 0.05; **p < 0.01.

Comparison of samples immediately and 60 minutes after evaporation [°]p < 0.05.

became more common in samples subjected to a more excessive volume reduction (Fig. 2E). CaP predominated in samples drawn after 60 minutes irrespective of the degree of volume reduction (Figs. 2B, 2D and 2F). These findings were supported by X-ray crystallographic analysis (Table 1) showing that the precipitate obtained from the CD-solution immediately after evaporation contained 50% COD, 40% Bru and 10% HAP following a volume reduction to 70-80 ml, and 35% COD, 60% Bru and 5% HAP when the volume was reduced to 40-50 ml. The dominance of CaP, mainly as Bru, was even more pronounced with further volume reduction.

During the 60 minutes following evaporation of the CD-solutions, the crystal number and volume in Sample A did not change (Table 2). In contrast, these variables increased significantly with the more extensive volume reduction in Sample B.

As a result of the presence of seed crystals, a larger number of crystals as well as greater crystal volumes were recorded in the CD-HAP-solutions in comparison with the CD-solutions immediately after evaporation in both Samples A and B (Table 2). As expected, this difference was mainly seen in the size interval of 2.4-5.1 µm.

In comparison with the CD-solution, Samples A and

B from the CD-HAP-solutions, had a lower MCV both immediately after evaporation and 60 minutes later (Table 2).

In addition to the added HAP-seed in the CD-HAP-solutions, the larger number of crystals in Samples A and B could probably be explained by the development of CaOx crystals as shown by X-ray crystallographic analysis of the precipitate. As illustrated in Table 1, a volume reduction to 70-80 ml and 40-50 ml increased the CaOx content in comparison with that recorded in the precipitate retrieved from the CD-solution. Crystals of calcium oxalate monohydrate (COM) were almost entirely found in these samples, whereas, in the absence of HAP crystals, only COD was found. A more common occurrence of CaOx crystals in the CD-HAP-solution compared to the CD-solution was also demonstrated with SEM (see, Figs. 2C and 3C).

In Sample B, the number of crystals was about twice as high in the CD-HAPdU-solution as in the CD-HAP-solution, both immediately after evaporation and after 60 minutes (p < 0.01; Table 2). A much smaller and statistically insignificant difference between the two solutions was recorded in sample A. The total stone volume was significantly higher in Sample B from the CD-HAPdU-

Crystallization in the collecting duct

Table 3. Mean (SD) number and total crystal volume at different sample volumes immediately and 60 minutes after evaporation of 100 ml samples of CD, CD-HAP and CD-HAPdU-solutions

| crystal size interval μm | Sample A (70-95 ml) | | | Sample B (40-69 ml) | | |
|---|-------------------------|-------------------------|--------------------------|--------------------------|-------------------------|----------------------------|
| | CD | CD-HAP | CD-HAPdU | CD | CD-HAP | CD-HAPdU |
| Number of crystals | | | | | | |
| <i>2.4 to 5.1</i> | | | | | | |
| 0 min | 88 (92) ^{††} | 1279 (371) [°] | 1328 (444) | 294 (289) ^{††°} | 1670 (504) | 3592 (1755)* |
| 60 min | 94 (94) | 840 (206) [°] | 1198 (569) | 1484 (1109) [°] | 1833 (658) | 3979 (1359)** |
| <i>5.1 to 10.2</i> | | | | | | |
| 0 min | 35 (32) [†] | 79 (19) | 142 (147) [°] | 42 (45) ^{†°} | 107 (47) [°] | 290 (126)** [°] |
| 60 min | 24 (36) | 77 (34) | 196 (190) [°] | 340 (345) [°] | 596 (523) [°] | 1291 (981) [°] |
| <i>10.2 to 20.2</i> | | | | | | |
| 0 min | 7 (12) | 5 (3.2) | 8.2 (6.0) | 11 (19) [°] | 6.8 (5.0) [°] | 12.3 (1.2) [°] |
| 60 min | 7 (13) | 7.8 (5.5) | 20 (28) | 164 (227) [°] | 172 (288) [°] | 125 (104) [°] |
| <i>> 20.2</i> | | | | | | |
| 0 min | 1 (1.4) | 1 (0.7) | 0.8 (1.0) | 0.83 (0.9) | 0.75 (0.9) | 0.8 (0.8) |
| 60 min | 2 (1.3) | 0.6 (0.6) | 0.5 (0.9) | 85 (195) | 3.3 (5.1) | 3 (4.0) |
| Total crystal volume (1000 x μm^3) | | | | | | |
| <i>2.4 to 5.1</i> | | | | | | |
| 0 min | 2.2 (1.7) ^{††} | 22.6 (7.8) [°] | 24.8 (10.3) | 5.5 (4.7) ^{††°} | 29.9 (9.5) | 70.4 (35.9)* [°] |
| 60 min | 6.1 (2.8) ^{††} | 15.9 (4.4) [°] | 23.4 (12.3) | 31.5 (24.6) [°] | 41.6 (15.1) | 99.4 (47.4) [°] |
| <i>5.1 to 10.2</i> | | | | | | |
| 0 min | 6.6 (6.5) | 11.6 (3.1) | 21.6 (22.9) [°] | 8.2 (9.3) ^{†°} | 17.1 (8.4) [°] | 41.7 (18.1)** [°] |
| 60 min | 6.3 (5.8) | 12.3 (5.4) | 35.1 (37.9) [°] | 69.6 (75.1) [°] | 219 (330) [°] | 23.0 (17.1) [°] |
| <i>10.2 to 20.2</i> | | | | | | |
| 0 min | 8.3 (15.2) | 6.3 (5.1) | 9.8 (7.3) | 12.7 (21.9) [°] | 7.7 (5.4) [°] | 14.8 (4.1) [°] |
| 60 min | 9.6 (17.5) | 10.2 (9.7) | 22.1 (29.2) | 233 (336) [°] | 189 (335) [°] | 133 (135) [°] |
| <i>> 20.2</i> | | | | | | |
| 0 min | 17.1 (20.7) | 13.3 (13.8) | 5.3 (6.0) | 4.4 (5.5) [°] | 11.1 (16.8) | 6.9 (7.8) |
| 60 min | 11.3 (10.0) | 8.5 (9.3) | 7.9 (16.9) | 190 (377) [°] | 23.1 (39.3) | 20.8 (23.5) |
| number of experiments | | | | | | |
| | 5 | 5 | 6 | 6 | 8 | 6 |

Comparison of CD and CD-HAP samples [†]p < 0.05; ^{††}p < 0.01.

Comparison of CD-HAP and CD-HAPdU samples *p < 0.05; **p < 0.01.

Comparison of samples immediately and 60 minutes after evaporation [°]p < 0.05.

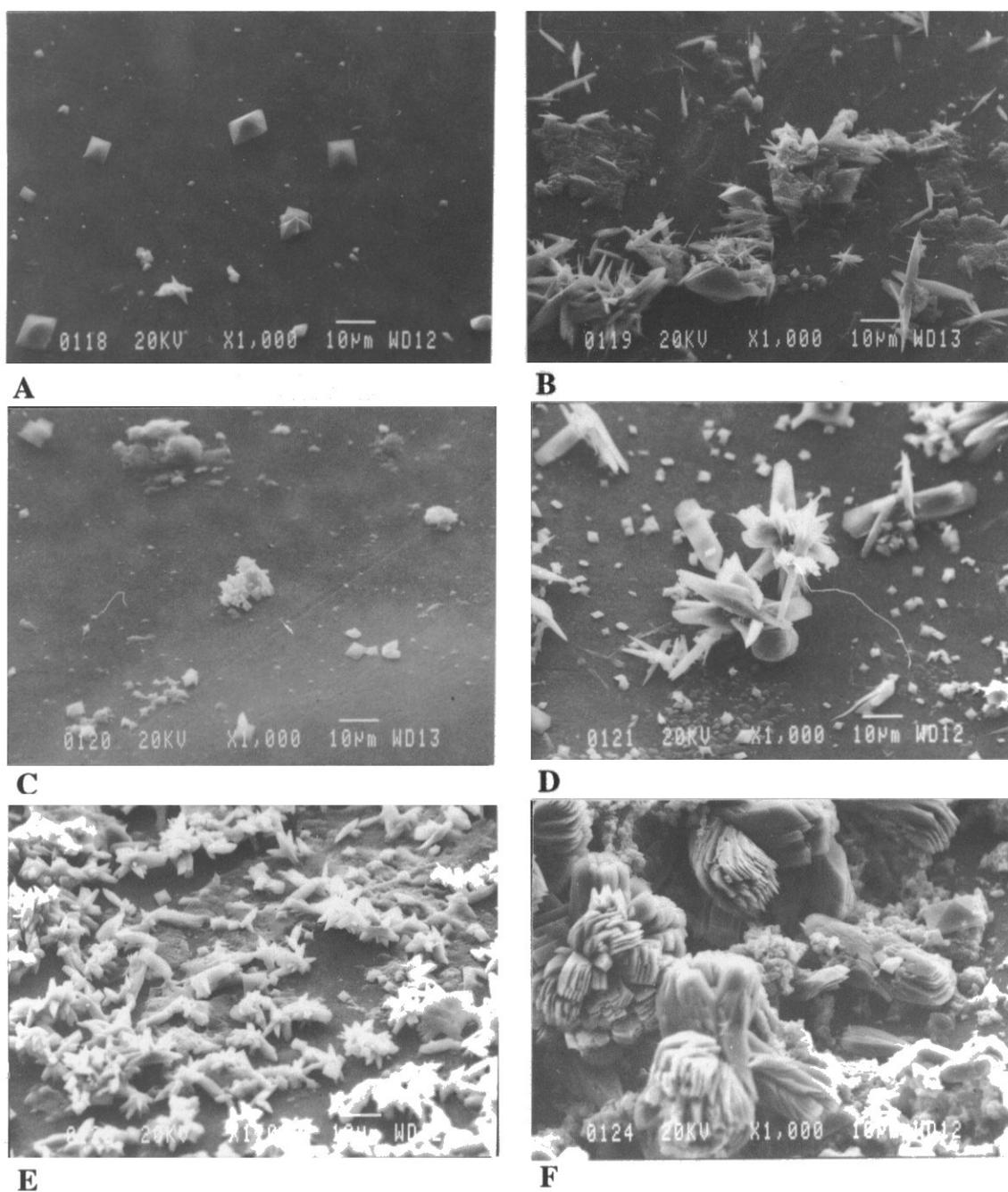


Figure 2. Scanning electron micrographs of the precipitate after evaporation of 100 ml samples of CD-solution. (A) and (B) evaporated down to a final volume of 70-80 ml; (C) and (D) evaporated down to a final volume of 40-50 ml; (E) and (F) evaporated down to a final volume of 10-20 ml: (A, C, and E) immediately after evaporation and (B, D, and F) 60 minutes after evaporation. Bars = 10 µm.

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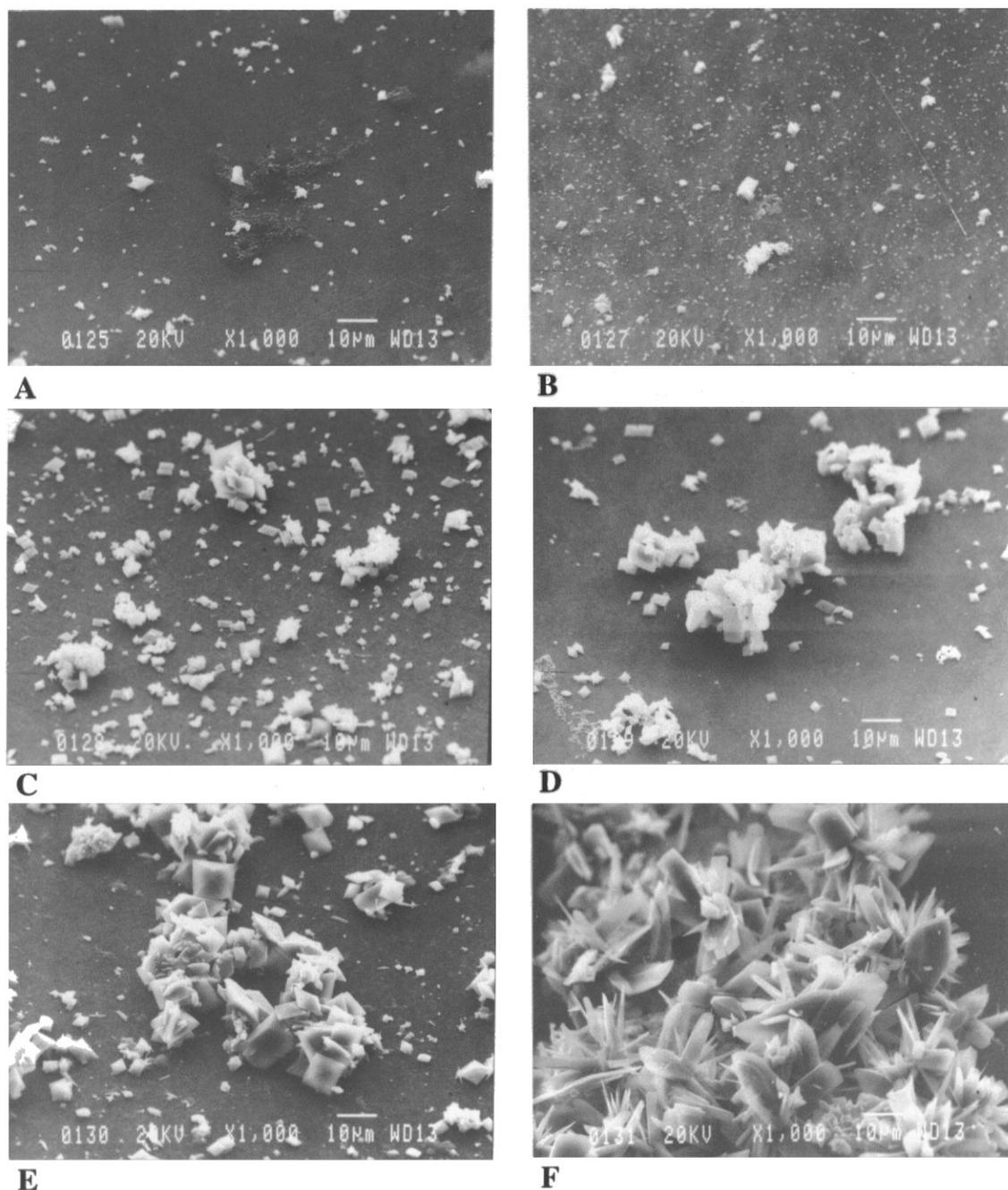


Figure 3. Scanning electron micrographs of the precipitate after evaporation of 100 ml samples of CD-HAP-solution. (A) and (B) evaporated down to a final volume of 70-80 ml; (C) and (D) evaporated down to a final volume of 40-50 ml; (E) and (F) evaporated down to a final volume of 10-20 ml: (A, C, and E) immediately after evaporation and (B, D, and F) 60 minutes after evaporation. Bars = 10 µm.

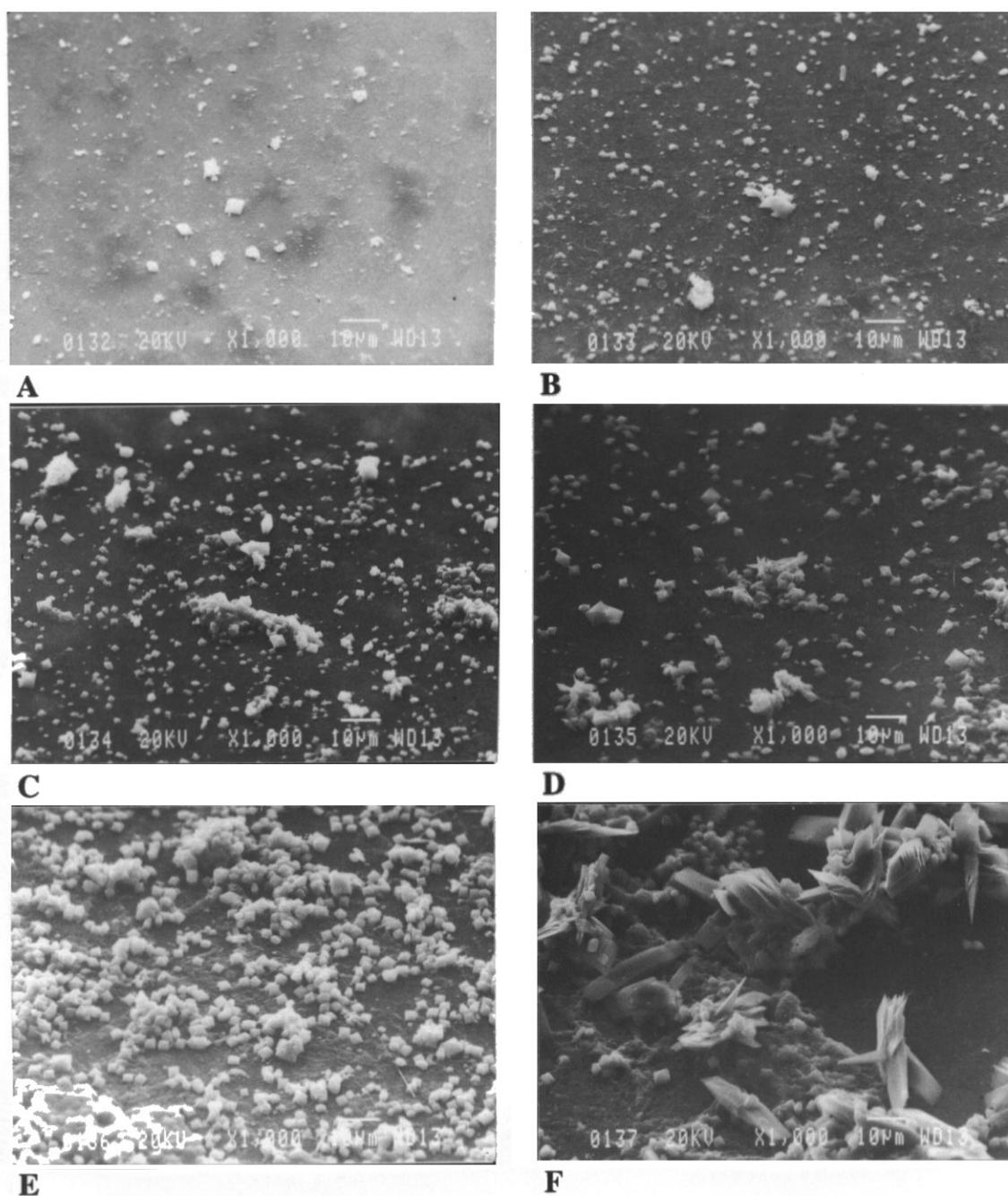


Figure 4. Scanning electron micrographs of the precipitate after evaporation of 100 ml samples of CD-HAPdU-solution. (A) and (B) evaporated down to a final volume of 70-80 ml; (C) and (D) evaporated down to a final volume of 40-50 ml; (E) and (F) evaporated down to a final volume of 10-20 ml; (A, C, and E) immediately after evaporation, and (B, D, and F) 60 minutes after evaporation. Bars = 10 µm.

Table 4. Ion-activity products for CaOx and different CaP salts at different volumes after evaporation of 100 ml of the CD-solution.

| volume after evaporation (ml) | $10^8 \cdot AP_{CaOx}$ (mol/l) ² | $10^{12} \cdot AP_{ACP}$ (mol/l) ² | $10^{25} \cdot AP_{OCP}$ (mol/l) ⁸ | $10^{48} \cdot AP_{HAP}$ (mol/l) ⁹ | $10^7 \cdot AP_{Bru}$ (mol/l) ² |
|-------------------------------|--|--|--|--|---|
| 100 | 1.94 | 1.82 | 1.60 | 0.74 | 4.59 |
| 90 | 2.18 | 1.83 | 2.03 | 1.56 | 5.32 |
| 80 | 2.43 | 1.86 | 2.67 | 3.54 | 6.28 |
| 70 | 2.78 | 1.90 | 3.59 | 8.51 | 7.56 |
| 60 | 3.20 | 1.98 | 5.08 | 22.5 | 9.38 |
| 50 | 3.86 | 2.13 | 7.57 | 65.0 | 12.1 |
| 40 | 4.82 | 2.43 | 12.2 | 204 | 16.7 |
| 30 | 6.43 | 3.15 | 21.9 | 645 | 25.1 |
| 20 | 9.55 | 5.59 | 45.7 | 1454 | 45.0 |
| 10 | 17.1 | 9.36 | 183 | 23000 | 108 |

solution. These effects could be explained by a larger number of small crystals (Table 3). A significantly lower MCV in the CD-HAPdU-solution was observed in both Samples A and B immediately after evaporation ($p < 0.01$).

The scanning electron micrographs in Figures 3C, 3D, 4C and 4D show that the crystals were smaller and apparently less aggregated in the CD-HAPdU-solutions than in the CD-HAP-solutions.

For the most extensively evaporated samples which were reduced to a volume of 10-39 ml, there was no evident difference in terms of number of crystals, crystal volume or MCV compared with the CD-HAP-solution. In the CD-HAPdU solution, however, there was a larger number of small crystals than in the CD-HAP-solution. By SEM examination of the precipitate reduced to a volume of 10-20 ml, it was also obvious that the crystals from the CD-HAPdU-solutions were smaller than those from the CD-HAP-solutions (Figs. 3E and 4E), while no important difference was recorded between samples from CD-solutions and CD-HAP-solutions (Figs. 2E and 3E).

Ion-activity products

The ion-activity product of AP_{CaOx} exceeded the thermodynamic solubility product for CaOx (SP_{CaOx}) of 0.23 to $0.25 \times 10^{-8} M^2$ (Pak *et al.*, 1975; Tomazic and Nancollas, 1979) in the samples already before evaporation. A formation product of CaOx (FP_{CaOx}) around $2 \times 10^{-8} M^2$ was achieved already at a volume reduction to only 90 ml (Table 4). The solutions were supersaturated with respect to CaP, thus, the AP_{OCP} and AP_{HAP} much exceeded the solubility products of $8.3 \times 10^{-48} M^8$ for SP_{OCP} and of $2.35 \times 10^{-59} M^9$ for SP_{HAP} (Koutsoukos and Nancollas, 1981) (Table 4). The AP_{Bru} exceeded the SP_{Bru} of $1.87 \times 10^{-7} M^2$ in the non-evaporated solutions but the FP_{Bru} of $2 \times 10^{-6} M^2$ (Koutsoukos and Nancollas, 1981; Nancollas, 1982) was reached only

after a volume reduction to 30 ml. It has to be emphasized, however, that when the ion-activity products were calculated, the decrease in the pH during volume reduction and complex binding between ions and urinary macromolecules were not accounted for. The ion-activity products, particularly of different calcium phosphate salts might, therefore, be overestimated.

Discussion

As shown in these experiments, addition of HAP crystals to a CD solution resulted in deposition of CaOx already at a volume reduction of only 20-30%. This finding gives support to the hypothesis that CaP crystals formed at a nephron level above the collecting ducts (Coe and Parks, 1990; DeGanello *et al.*, 1990; Lupták *et al.*, 1994; Kok, 1995; Asplin *et al.*, 1996; Højgaard *et al.*, 1996; Tiselius *et al.*, 1997) might promote the formation of a CaOx precipitate when these crystals encounter the urine environment in the collecting duct at which level of the nephron both pH and urine volume are reduced.

Under normal conditions, the pH will be around 6.75 in the proximal tubule and 6.45 in the distal tubule (Rector, 1983) and it has previously been shown in our laboratory that CaP is the type of crystal that most easily forms at these levels, whereas CaOx is the most likely product of crystallization in the collecting duct (Lupták *et al.*, 1994; Højgaard *et al.*, 1996). According to measurements carried out by Asplin *et al.* (1996), the pH and the risk of CaP precipitation might also be high in the loop of Henle. Irrespective of the exact level of CaP precipitation in the nephron, the crystals material will probably move downwards in the nephron. Although small and unretained crystals will be eliminated with urine, this will not be the

case with crystals that are retained either because of particle size or because of morphological abnormalities in the tubular layer of the collecting duct. Khan (1995), thus, has shown that the crystals easily can get lodged in the distal part of the collecting duct where the channels bend. The interaction between crystals and cells that has been demonstrated by several authors (Khan and Hackett, 1991; Boevé *et al.*, 1993, 1994; Lieske and Toback, 1993; de Bruijn *et al.*, 1994; Verkoelen *et al.*, 1996), might be extremely important for the development of crystals formed in the nephron.

When retained CaP crystals are exposed to low pH, they might dissolve either partially or completely. Although this dissolution obviously is counteracted by urinary macromolecules (Højgaard *et al.*, 1997, unpublished; Tiselius *et al.*, 1997), it can nevertheless be sufficient to cause a local increment in the supersaturation with CaOx and hence a CaOx nucleation (Højgaard *et al.*, 1997).

The effect of HAP was studied both in the presence and absence of dU. The presence of macromolecules resulted in a larger number of crystals and a reduced MCV. This is most probably caused by the inhibition of growth and aggregation of the CaP crystals (Højgaard and Tiselius, unpublished). Urinary macromolecules can also be expected to inhibit the heterogeneous nucleation of CaOx. In comparison with solutions without dU, the content of CaOx that precipitated following a small volume reduction was lower in solutions with dU. With the higher CaOx supersaturation following a more extensive evaporation, this difference essentially disappeared. The exact mechanism behind this inhibition has not been elucidated, but it is reasonable to assume that liberated Ca²⁺-ions bind to γ -carboxyglutamic acid groups in the macromolecules (Lian *et al.*, 1977; Nishio *et al.*, 1990). Such a binding would, thus, counteract the development of a local critical supersaturation when the solution supersaturation remains low, but not when it becomes high as a result of a pronounced volume reduction.

The increased number of small crystals that appeared following a limited volume reduction of CD-HAP solutions was most certainly the result of CaOx nucleation on CaP crystals previously below the detection limit in the Coulter counter. The increased fraction of CaP associated with extensive volume reductions in the form of brushite was an interesting and unexpected finding. It is reasonable to assume that this is explained by a transformation of HAP to Bru in the acid environment (Abbona and Franchini-Angela, 1990; Lundager-Madsen and Christensson, 1991) and as a result of the increased calcium/magnesium ratio (Abbona and Franchini-Angela, 1990). Obviously, brushite is most easily formed in the absence of both HAP seed and urinary macromolecules. The very high content of brushite, that was recorded following volume reduction to 12-20 ml,

is unlikely to occur under clinical conditions and probably a volume reduction to 70-80 ml best reflects what happens in stone-formers' urine.

The presence of small HAP seed apparently resulted in a controlled secondary nucleation since both CD-HAP and CD-HAPdU solutions had an MCV smaller than that in the CD-solutions, at least with a size distribution of the kind used in these experiments. A more heterogeneous size distribution with large CaP crystals or crystal aggregates would, in contrast, provide a basis for the formation of easily retained CaP/CaOx crystal masses.

In conclusion, we are able to demonstrate that volume reduction of solutions with a composition corresponding to that in the collecting duct resulted in precipitation of CaOx and that such a process might be augmented by HAP crystals. The precipitation of CaOx was most obvious when limited volume reduction was carried out, according to that expected under clinical conditions.

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Discussion with Reviewers

B. Hess: What is the authors' explanation for the relatively high percentage of COM crystals in CD-HAP samples, especially after 60 minutes in the sample evaporated to 70-80 ml?

Authors: We are not able to definitely explain this finding, but it is reasonable to assume that transformation occurs from COD to COM. This process, which is time dependent, might be counteracted by the higher magnesium and citrate concentrations in samples subjected to the most extensive volume reduction.

J.M. Baumann: Ion activity of brushite compared to the activity product of calcium oxalate showed a disproportionately high increase with the increasing volume-depletion by evaporation. Can this phenomena be attributed to the relatively low degree of phosphate chelation (about 5%) compared to the much higher degree of oxalate chelation (about 30%) and can it explain the predominance of brushite precipitation in the highly concentrated samples?

Authors: In these solutions, differences in the free concentrations of HPO_4^{2-} and Ox^{2-} , respectively, are highly important for whether CaOx or CaHPO_4 will be the favored product of precipitation. At the pH used, volume reduction caused an increase in HPO_4^{2-} that was more pronounced than for Ox^{2-} , a fact that might have been of great importance for brushite precipitation.

J.M. Baumann: Blocking growth sites on crystals and crystal nidus is a generally accepted mechanism to explain the effect of crystallization inhibitors. The authors support the interesting theory that calcium phosphate formed in renal tubules may dissolve in the CD due to a lower pH, produce local zones of high calcium concentration or calcium oxalate supersaturation, respectively, and thus, induce calcium oxalate nucleation. Macromolecular inhibitors are thought to bind calcium ions liberated by calcium phosphate dissolution and, thus, counteract nucleation of calcium oxalate. However, we wonder how the authors can explain

this effect knowing that calcium concentrations are in the millimolar range, whereas, macromolecular inhibitors, like Tamm Horsfall protein (THP), are only present in nanomolar concentrations. THP has a molecular weight of about 80 KD and is excreted in amounts of 40 to 50 mg per day.

Authors: The exact role of macromolecules in this respect is abstrusive. Macromolecules will certainly bind Ca^+ -ions to numerous specific binding sites. This complex formation can slightly affect the ion-activity products of CaOx as well as CaP , but the inhibition of CaOx crystal growth is counteracted by binding of macromolecules to the crystal surface. When CaP crystals occur in solutions containing macromolecules, they dissolve more slowly than without macromolecules. This is probably an effect of a slow diffusion of ions through the macromolecular layer surrounding the crystals. The establishment of a high concentration at these sites might induce nucleation, but, when the CaOx crystals have formed, normal macromolecules will protect them from growing. The macromolecules, thus, might act through the accumulation of Ca^{2+} -ions by forming areas with high levels of supersaturation and by providing a gel environment that facilitates nucleation but counteracts dissolution. In such a system, it is difficult to draw conclusions from purely stoichiometrical calculations.

J.M. Baumann: A further question is how the authors, by their theory, explain the finding of an increased crystal number and a decreased mean crystal volume in dU?

Authors: The increased crystal number and decreased mean crystal volume in the presence of dU might be an effect of aggregation inhibition brought about by the macromolecules.

J.M. Baumann: In most experiments, heterogeneous nucleation has been studied in solutions being saturated with respect to the nucleator (e.g., HAP), where neither a dilution nor a precipitation of the nucleator occurs. Obviously, the authors tried to simulate conditions as found in the CD also with respect to phosphate. Why did they omit uric acid?

Authors: Our aim was to make observations of CaOx and CaP precipitation in solutions that were supersaturated with these salts to approximately the same extent as tubular urine. Urate has a small influence on the ion-activity products of both CaOx and CaP , but this effect is only marginal. It is also possible, however, that urate promotes precipitation of CaOx . This has been a matter of debate for more than a decade. During the volume reduction, sodium urate as well as uric acid might, under certain circumstances, precipitate and although this might be of great importance for the crystallization, we thought that the presence of urate would make conclusions more difficult as our intention was to study what happened to CaOx and CaP . Urate, therefore, was excluded on the same grounds as ammonium: to assure

a pure calcium salt precipitation. This does not mean that we consider urate unimportant and future studies of the crystallization in solutions with and without urate might shed light on the role of urate in the calcium crystallization process.

L.-C. Cao: Why did the authors select to measure crystal nucleation in the size range 2.4-45 μm when a considerable proportion might be found between 2 and 2.4 μm ?

Authors: To assess the nucleation is indeed an extremely difficult task and ideally, a method should be used, which allows detection of particles as small as 1-2 nm. Besides the lack of technical equipment for such measurements, most solutions contain impurities giving a considerable background noise, which increases with decreasing size of the particles we want to measure. Although, the Coulter counter with a 100 μm tube allows for particle size analysis down to 2 μm , we found that the reproducibility for this type of experiments was much better with the threshold set at 2.4 μm .

F. Grases: Do you think that, in any case, the crystals formed in the distal tubules can obstruct them, and, if this would occur, what type of renal calculi would be formed?

Authors: Whether crystals formed in the tubular system might be sufficiently large to obstruct them is a matter of debate. In case of CaP nucleation in the distal part of the distal tubule, we believe that the risk of tubular obstruction is negligible. The crystals might, however, be retained at this level and, as long as they remain there, CaP will be the type of crystals encountered.

F. Grases: If crystals formed in the distal tubules are not retained there or within the collecting ducts, can they induce renal calculus formation? Why and where?

Authors: In our hypothetical model, the initial CaP precipitate induces CaOx precipitation in the lower part of the collecting duct, where adherence to the tubular cells might cause retention and further crystal growth. The final steps in this process are less well understood, but the experimental results reported by Khan (1995, **Additional References**) have shown an important interaction between the crystal material and the tissues at that level.

L.-C. Cao: Regarding your theory of intratubular crystallization, do you have any ideas on how to improve the treatment of patients with stone disease?

Authors: From a therapeutic point of view, it would be desirable to prevent CaP crystallization in the nephron. A reduced excretion of calcium and a strict control of intratubular pH might be of crucial importance to achieve this goal. Correction of an abnormally low citrate excretion, thus, has to be undertaken with the risk of intratubular CaP

crystallization in mind. We cannot say, however, whether an excessive alkalinization increases the risk of stone formation or not. The reason for this is that whereas CaP more easily forms at high levels of the nephron, the dissolution at CD-levels is counteracted. Further experimental and clinical studies will be necessary to answer this question.

L.-C. Cao: Will 24-hour urinary parameters be useless for evaluating metabolic disorders in a given stone patient?

Authors: The usefulness of 24-hour urine samples or any samples collected during periods of various duration for evaluation of the risk of stone-formation is an interesting and important issue to consider. Whatever the urine composition might be at the nephron level where we assume that the initial steps of the crystallization take place, this might at least, to some extent, be reflected in the composition of final urine. Recent calculations of the ion-activity product of CaP in the distal part of the distal tubule, by extrapolation from 16-hour urine, indicated a good relationship between AP_{CaP} index in voided urine and AP_{CaP} in tubular urine (Tiselius *et al.*, 1997, **Additional References**). In addition, final urine more closely reflects the composition below the CD-level where CaOx crystallization predominates. In our view, urine collections, thus, give valuable information on the risk situation in the renal collecting system, but the interpretation has to be made in view of the variations in urine composition that occur when urine passes through the nephron.

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