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ORIGINAL PAPER

Studies on the role of calcium phosphate in the process of calcium oxalate crystal formation

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Abstract Crystals of calcium phosphate (CaP) added to solutions with a composition corresponding to that at different levels of the collecting duct (CD) and with different pH were rapidly dissolved at pH 5.0, 5.25 and 5.5. Only minor or no dissolution was observed at higher pH levels. Despite this effect, CaP crystals induced nucleation or heterogeneous crystallization of CaOx up to a pH of 6.1, whereas CaP was the type of crystalline material that precipitated at higher pH. Accordingly, small crystal volumes were recorded at pH 5.5 and great volumes at pH 6.7 4 h after the addition of CaP crystals to the solutions. Dialyzed urine appeared to counteract the dissolution of CaP and to reduce the rate of secondary crystallization. The CaP induced crystallization of CaOx was confirmed by a reduction of ^{14}C-labeled oxalate in solution. The AP_{CaOx} required for a nucleation or heterogeneous crystallization of CaOx in the presence of CaP was around 1.5×10^{-8} $(mol/l)^2$. For CaP crystal formation on CaP, an AP_{CaP} ($^aCa^{2+} \times {}^aPO_4^{3-}$) of approximately 50×10^{-14} $(mol/l)^2$ appeared to be necessary. The CaOx crystals formed were microscopically found in association with the CaP crystalline material and were most frequently of CaOx dihydrate type. Stepwise crystallization experiments comprising supersaturation with CaP (*Step A*), supersaturation with CaOx (*Step B*) and subsequently acidification (*Step C*) showed that CaOx crystal formation occurred when CaP crystals were dissolved and thereby served as a source of calcium. The ensuing formation of CaOx crystals is most likely the result from high local levels of supersaturation with CaOx caused by the increased concentration of calcium. These experimental studies give support to the hypothesis that crystallization of CaOx at lower nephron levels or in caliceal urine might be induced by dissolution of CaP formed at nephron levels above the CD, and that a low pH is prerequisite for the precipitation of CaOx. The observations accordingly provide additional evidence for the important role of calcium phosphate in the crystallization of calcium oxalate, that might occur both at the surface of Randall's plaques and intratubularly at the papillary tip.

Keywords Crystallization · Calcium oxalate · Calcium phosphate · Nephron · Collecting duct · Randall's plaque · Distal tubule · Urine

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Introduction

In approximately 80% of stones formed in the urinary tract calcium oxalate (CaOx) is the major constituent, and it also has been shown that a calcium phosphate crystal phase (CaP) commonly accompanies CaOx, albeit usually only in small amounts [1–3]. The high recurrence rate that is associated with calcium stone formation makes it desirable to provide medical preventive programs to these patients.

Such procedures have been variably successful, most certainly because of our incomplete understanding of the details of the mechanism of calcium stone formation. Recent observations, however, have shed new light on the possible role of CaP in the formation of calcium stones. Endoscopic as well as mineral analyses have demonstrated the papillary origin of CaOx stones and their close association with CaP [4–8]. Interest thereby has been redirected to the mechanism of stone initiation that was described by Randall almost 70 years ago [9–14]. In accordance with these observations it has been suggested that CaOx crystallization occurs at the surface of CaP deposits at the papillary level either by growth of CaOx on the surface of CaP or by co-precipitation [4].

There is indeed a bulk of information in support of CaP-induced CaOx-stone formation. The detection of CaP, in a majority of calcium stones is an important observation. Moreover, a sufficiently high supersaturation with CaOx is unlikely in urine at nephron levels above the distal part of the collecting duct (CD) [15–18]. The development of a renal stone requires retention of crystalline material and in this regard fixation of CaOx crystals to a preformed crystal phase of CaP appears logically attractive.

This article provides experimental evidence for a sequence of events that start, with CaP-precipitation at a high nephron level followed by CaP-dissolution and an ensuing CaOx precipitation in acid urine. Such a series of crystallization events accordingly seems commensurate both with the role of Randall's plaques and of intratubularly formation of CaOx crystal deposits [19–22].

Previous experimental work has shown that CaP was the favored type of crystal precipitated at nephron levels above the CDs [16, 17, 23–26]. When data from urine analyses were extrapolated to a situation assumed to exist in the distal part of the distal tubule (DTd), the ion activity product of calcium oxalate (AP_{CaOx}) was very low and most certainly below the risk of crystal formation [16]. In contrast the ion activity products of amorphous calcium phosphate (ACP), hydroxyapatite (HAP), octacalcium phosphate (OCP) and brushite (Bru) were well above the solubility products. The risk of forming urine supersaturated with CaP-salts in DTd was considered to be higher in stone formers that in normal subjects [22, 27, 28]. While the risk of CaP crystallization apparently is negligible at low pH levels, the risk rapidly increases with increasing alkalinity.

Although it is reasonable to assume that ACP is the initial CaP crystal phase [29, 30], this has been difficult to prove in our experiments, and, for sake of simplicity, we have chosen to express the supersaturation of calcium phosphate as AP_{CaP}, obtained from the product of ion activities of calcium and phosphate: $AP_{CaP} = {}^{a}Ca^{2+} \times {}^{a}PO_4^{3-}$. Preliminary data thus have shown that, following titration with calcium chloride, crystals first appeared at an AP_{CaP} of $123–131 \times 10^{-14}$ $(mol/l)^2$ [16]. In solutions left at constant supersaturation levels, crystals formed spontaneously when the AP_{CaP} was in the range $225–435 \times 10^{-14}$ $(mol/l)^2$ [28].

CaP crystals, thus, might develop in the nephron either in the loop of Henle [9, 12, 14, 25, 26, 31] or possibly in the DTd [16, 28] particularly following an alkaline load together with high concentrations of calcium and phosphate. The aim of the experiments presented in this article was to study the effects that CaP crystals formed in solutions with a composition approximately corresponding to that in DTd might have on CaOx crystal development in urine, with a composition roughly corresponding to that in the CD. The latter nephron level was chosen in order to reflect the crystallization properties at a high nephron level.

Materials and methods

Preparation of crystals

Crystals of CaP were prepared by adding 8 ml of 1.0 mol/l $CaCl_2$ to 200 ml of a solution with the following composition: calcium 1.04 mmol/l, magnesium 0.41 mmol/l, phosphate 4.17 mmol/l, sodium 96 mmol/l, potassium 22.5 mmol/l, citrate 0.35 mmol/l, sulfate 13.8 mmol/l, oxalate 0.04 mmol/l. The pH was adjusted to 6.75 to get a substantial amount of crystals. The solution also contained 20% of dialyzed urine. This urine was obtained from a normal subject and dialyzed according to principles previously described in detail [16] and subsequently passed through a Millipore filter with a pore size of 0.8 μm (Millipore, SA Molsheim, France).

The Millipore-filtered $CaCl_2$ solution was added dropwise and pH maintained at 6.75 as long as the solution was macroscopically clear. When the first sign of crystal formation appeared, the remaining aliquot of $CaCl_2$ was added. The solution was left with magnetic stirring for 1 h, after which the solution was poured into a separation funnel. The crystals were allowed to sediment during 40 min. The sediment was subsequently isolated from the remaining solution, transferred to an empty and clean separation funnel, after which the precipitate was washed twice with 200 ml of a 0.15 mol/l sodium chloride solution saturated with Bru. The crystals were re-suspended in 20 ml of the same solution and used in the experiments after approximately 12 h. The crystal suspension had a concentration of 10–15 g/l. Microscopic examinations of the suspension disclosed an apatite-like morphology and although we were unable to define the crystal phase formed in this way, the precipitate undoubtedly was a CaP crystal phase. The crystal size distribution determined in a Coulter counter showed a mean crystal volume of 389–428 μm^3 with 92–93% of the crystals smaller than 15 μm.

Solutions with a composition corresponding to that in collecting duct urine

For determination of the formation product of CaOx crystals in the presence of a CaP precipitate, we prepared *Solution M*, which was given a composition corresponding to that in the middle part of the CD. *Solution M* had the following ion composition: calcium 1.6 mmol/l, citrate 1.21 mmol/l, phosphate 12.1 mmol/l, magnesium 1.45 mmol/l, sodium 94 mmol/l, potassium 53 mmol/l, sulfate 7.8 mmol/l and pH 6.0. The oxalate concentration was varied in the range 0.12–0.50 mmol/l. A solution with an oxalate concentration of 0.12 mmol/l but otherwise as described above, and a pH 5.00, 5.25, 5.50, 6.00 and 6.25 was used to assess the dissolution of added CaP crystals. Six analyses were carried out at each pH.

An aliquot of 0.2 ml of the CaP crystal suspension was added to 100 ml of *Solution M*. This low concentration of crystals was chosen to keep the crystal volume as small as possible, thereby avoiding a rapid supersaturation with calcium oxalate during the process of CaP dissolution.

For further studies on the crystallization process, a series of solutions with different salt compositions and with different pH were prepared. *CD-Solution A*, which was considered to correspond to an undiluted urine from the distal part of the CD was given the following final ion composition: calcium 4.5 mmol/l, sodium 109 mmol/l, potassium 63.7 mmol/l, phosphate 32.3 mmol/l, sulfate 20.8 mmol/l, citrate 3.21 mmol/l and oxalate 0.32 mmol/l. *CD-solutions B, C, D, E* and *F* were obtained by diluting *CD-solution A* with water to get 80, 60, 40, 20 and 10% of its concentration, respectively. *CD-solution A* was prepared both without and with dialyzed urine and the pH of all solutions was subsequently adjusted to 5.5, 5.8, 6.1, 6.4 and 6.7. Finally all solutions were passed through 0.22 µm Millipore filters before use in the crystallization experiments. All these experiments were carried out in triplicate.

The ion-concentrations and ion-activity products of different calcium salts are summarized in Table 1. In this set of samples, *CD-solution A* had a composition corresponding to that of final urine with a 24-h excretion of 6.75 mmol of calcium, 4.6 mmol of magnesium, 2.9 mmol of citrate and 0.3 mmol of oxalate in a volume of 1.5 l.

To 10 ml of each *CD-solution* were added 500 µl of the CaP crystal suspension. This concentration of crystalline material was considered necessary to discover a nucleation of CaOx (if that would occur) and to avoid a complete early dissolution of the crystals. The samples were subsequently placed on a shaking table and slowly moved during the following 20 h. Aliquots were drawn immediately after crystal addition and after 4, 8 and 20 h. At these occasions, the crystal size distribution was assessed in a Coulter Multisizer (Coulter Electronics Ltd., Luton, UK) with a 50 µm

Table 1 Ion composition and ion-activity products in the different experimental solutions

Solution	M	A	B	C	D	E
Calcium, mmol/l	1.6	4.5	3.6	2.7	1.8	0.9
Magnesium, mmol/l	1.45	3.85	3.08	2.31	1.54	0.77
Sodium, mmol/l	94	109	87	65	44	22
Potassium, mmol/l	53.0	63.7	51.0	38.2	25.5	12.7
Phosphate, mmol/l	12.1	32.3	25.8	19.4	12.9	6.5
Sulfate, mmol/l	7.8	20.8	16.6	12.5	8.3	4.2
Citrate, mmol/l	1.21	3.21	2.57	1.93	1.28	0.64
Oxalate, mmol/l	0.12	0.32	0.26	0.20	0.13	0.06
pH	5.00–6.25	5.5–6.7	5.5–6.7	5.5–6.7	5.5–6.7	5.5–6.7
dU%	0	0/100	0/80	0/60	0/40	0/20
AP_{CaOx}, $10^8 \times (mol/l)^2$	0.54–0.47	1.97–1.70	1.58–1.36	1.18–1.01	0.78–0.66	0.37–0.32
AP_{CaP}, $10^{14} \times (mol/l)^2$	0.11–20.1	4.85–410	3.53–312	2.35–218	1.32–131	0.48–53.5
AP_{HAP} $(mol/l)^2$	8.5×10^{-57}–5.2×10^{-49}	1.05×10^{-50}–61×10^{-45}	2×10^{-51}–13.6×10^{-45}	0.02×10^{-50}–1.76×10^{-45}	0.9×10^{-53}–9.4×10^{-47}	0.38×10^{-55}–5.4×10^{-49}
AP_{Bru}, $10^7 \times (mol/l)^2$	1.99–19.6	5.2–411	3.11–244	14.59–124	0.61–47.5	0.11–9.08
AP_{OCP}, $10^{43} \times (mol/l)^2$	0.05–6.7	5.2–411	3.11–244	1.59–124	0.61–47.5	0.11–9.08
Seed concentration, mg/l	20–30	500–750	500–750	500–750	500–750	500–750

tube. The number and volume of crystals in the size range 1.9–45 μm were recorded. Each experiment was repeated five times.

In one series of *CD-solutions A* to *F*, 100 μl to [^{14}C]-oxalate, with a specific radioactivity of 109 μCi/μmol (Amersham, Buckinghamshire, UK), were added, and an aliquot filtered immediately and after 4, 8 and 20 h for assessing the isotope remaining in solution. The isotope was measured in a liquid scintillations spectrometer (model 1217 Wallac; LKB, Turku, Finland). Isotope also was added to *CD-solution M* and aliquots for isotope measurements drawn after 1, 2 and 4 h.

Step-wise crystallization experiments

In order to simulate the sequences of crystal formation assumed to take place at different levels of the nephron, the following experimental three-step crystallization system was designed.

The initial crystallization (*Step A*) was accomplished by mixing 200 μl of 5 mmol/l $CaCl_2$ and 100 μl of 100 mmol/l Na_2HPO_4 at pH 7.0. Addition of phosphate solution was made at $t = 0$. Each sample also contained either 50 μl of distilled water or 50 μl of dialyzed urine prepared as described above. These samples with a volume of 350 μl were placed in a 96-well microplates and the absorbance recorded at 655 nm for periods up to 90 min.

In crystallization *Step B*, 250 μl of the crystal suspension formed after the first 65 min in *Step A* were transferred to another well containing 100 μl of 3 mmol/l sodium oxalate. The absorbance was measured after between 10 s and 10–20 min.

After 20 min a small aliquot of Millipore-filtered hydrochloric acid (1 mol/l) was added to the 350 μl sample in *Step B* in order to get a pH in the solution of 5.75 (*Step C*). The absorbance was subsequently recorded at 655 nm for periods between 1 s and 240 min, slightly different in the various experiments.

All experiments in the step-wise crystallization system were carried out with six samples in 96-well Benchmark microplates (BioRad Laboratories, CA, USA) that were sealed and stored at 37°C during the 24 h following the last absorbance measurement in *Step C*. At that point of time aliquots of the sample were examined in a light microscope in order to roughly record presence of amorphous CaP as well as relative occurrence of COM and COD crystals and aggregates. All absorbance readings were made after 1 s of sample mixing.

Microscopic examinations

Crystals were examined either by light microscopy at a magnification of 400 × or in some experiments with a JSM 840 JEOL (JEOL Ltd., Tokyo, Japan) scanning electron microscope (SEM). For SEM examination, the solutions were passed through polycarbonate membrane filters with a pore size of 0.2 μm (Poretics Corp., Livermore, CA, USA). The filters were rinsed with air, dried at room temperature and mounted with double stick tape on metallic stubs. The crystals were covered by a 10 nm layer of metallic platinum in a twin electron beam gun sputter coating unit (Model 3AM; Edwards, Crawley, Sussex, UK).

Statistical analysis

Student's *t*-test, Wilcoxon's rank sign test and Lord's range test were used for group comparisons and statistical conclusions.

Results

Dissolution of CaP crystals

When CaP crystals were added to *Solution M* with different pH and an oxalate concentration of 0.12 mmol/l, a rapid and early dissolution of crystals was recorded at pH 5.0 and 5.25 (Fig. 1). Already in the analysis carried out immediately after addition of CaP crystals ($t = 0$) were the mean (SD) crystal numbers in these two samples 3417 (718) and 7730 (519), significantly smaller than that recorded with pH 5.5 and higher ($P < 0.01$ and $P < 0.05$, respectively). After 5 min smaller crystal numbers were observed in all samples

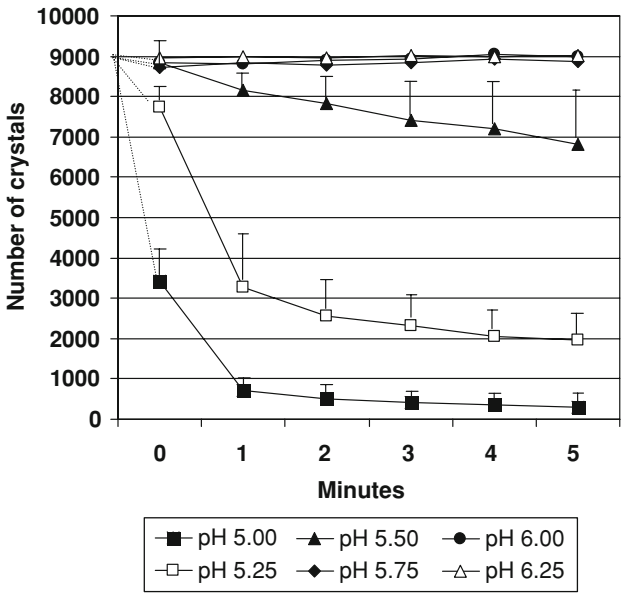

Fig. 1 Number of crystals recorded during the first 5 min after addition of CaP crystals to *Solution M* with different pH. The data are the mean values of five repeated measurements and the SD has been indicated for samples with pH 5.00, 5.25 and 5.50

with pH in the interval 5.00–5.50 compared with those seen in samples with pH 5.75–6.25 ($P < 0.01$). When the whole 5-min period was considered, the mean (SD) crystal numbers at pH 5.00, 5.25, 5.50, 5.75, 6.00, and 6.50 were 949 (1182), 3314 (2157), 7725 (1025), 8825 (170), 8921 (193) and 8981 (131). In comparison with samples that had a pH of 5.75 or higher the crystal numbers were significantly lower in the pH interval 5.0–5.50 ($P < 0.01$).

In terms of crystal volumes (Fig. 2) a similar pattern was obtained. After 5 min the mean (SD) crystal volumes in samples with pH 5.0, 5.25 and 5.75 were 6.5 (3.5), 28.2 (6.3) and 105 (18) $\times 10^3$ µm^3, all significantly smaller ($P < 0.01$) than the volumes of 182 (18), 184 (15) and 183 (13) $\times 10^3$ µm^3, respectively, in samples with pH 5.75, 6.00 and 6.50. For the whole observation period, the mean (SD) crystal volumes were 20 (23), 52 (39), 120 (30), 178 (11), 180 (11) and 186 (9) $\times 10^3$ µm^3, respectively, in samples with pH 5.00, 5.25, 5.50, 5.75, 6.00 and 6.50. While there were no differences in crystal volumes between samples with pH in the interval 5.75–6.50, significantly smaller crystal volumes were recorded in the pH range 5.00–5.50 ($P < 0.01$).

Secondary crystallization in CD-solutions

As shown in Fig. 3, precipitation of [^{14}C]-oxalate during the first 20 h following addition of CaP crystals was recorded in *CD-solutions A and B*. In samples without dialyzed urine the percentage of isotope remaining in solution was significantly lower in sample *A* at 5.5–6.1 ($P < 0.01$)

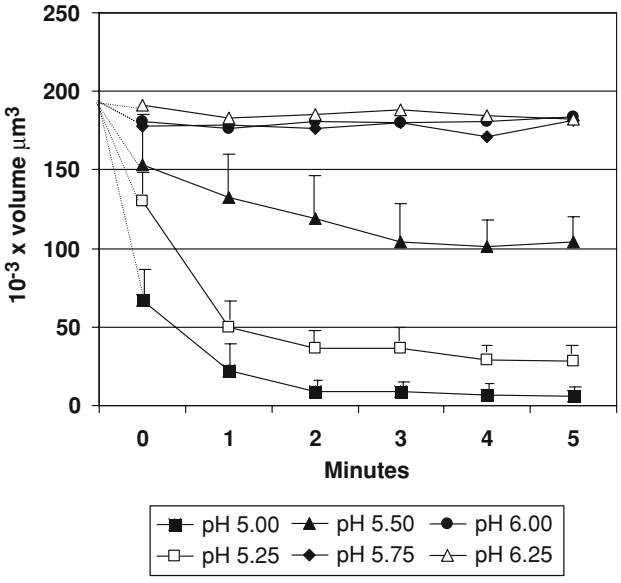

Fig. 2 Volume of crystals recorded during the first 5 min after addition of CaP crystals to *Solution M* with different pH. The data are the mean values of five repeated measurements and the SD has been indicated for samples with pH 5.00, 5.25 and 5.50

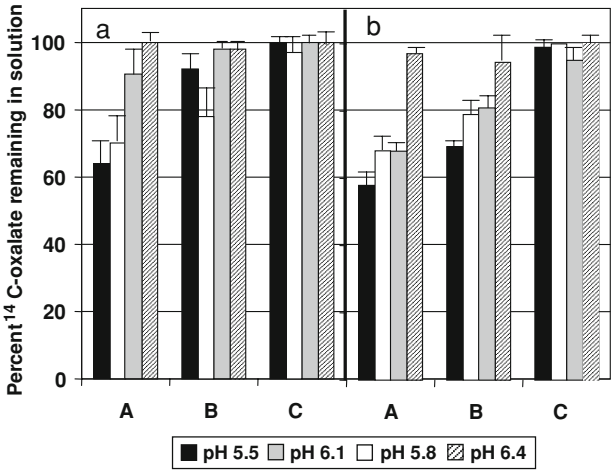

Fig. 3 Percent [^{14}C]oxalate remaining in solution 20 h after the addition of CaP crystals to solutions with different salt concentrations and different pH. The measurements were carried out in salt solutions without (**a**) and with (**b**) dialyzed urine

and in sample B at pH 5.5 and 5.8 ($P < 0.01$). No precipitation of CaOx was noted in samples with pH 6.4 and 6.7 and neither was there any precipitation in samples *D, E* and *F*.

At pH 5.5, 5.8 and 6.1, crystallization of CaOx was recorded at an AP_{CaOx} level between 1.4 and 1.5 $\times 10^{-8}$ (mol/l)^2. Although the secondary crystallization of CaOx in solutions containing CaP apparently was slow (Fig. 4), it is noteworthy that under these experimental conditions, crystal formation nevertheless occurred already at an AP_{CaOx} around 1.5 $\times 10^{-8}$ (mol/l)^2. It also should be noted that the crystallization was recorded earlier in *Solution M* than in *Solutions A, B* and *C*, despite the lower CaP crystal concentration in the former solution. Only at pH 5.5, 5.75 and 6.1 was there a detectable precipitation of CaOx.

There was a significant difference in isotope remaining in solution between samples with and without dialyzed urine at pH 6.1 ($P < 0.01$), but not at the other pH levels.

The number and volume of crystals 4 h after the addition of a CaP suspension are shown in Fig. 5. In the combined measurements in solutions *A* to *E*, the mean (SD) crystal number was 7.76 (1.01) $\times 10^3$ in samples without dialyzed urine; a value that was significantly lower than the 8.31 (0.41) $\times 10^3$ crystals in samples with dialyzed urine ($P < 0.05$). It was, moreover, observed that this effect mainly was explained by the lower crystal numbers at 5.5: 6.33 (0.44) $\times 10^3$ in samples without and 8.38 (0.44) $\times 10^3$ in samples with dialyzed urine. In the pH range 5.8–6.7 the crystal number remained at a stable level and did not differ significantly ($P > 0.10$).

In terms of crystal volumes the mean (SD) at pH 5.5 did not differ significantly between samples without and with dialyzed urine; 0.92 (0.69) $\times 10^6$ µm^3 and 1.42 (0.35) $\times 10^6$ µm^3, respectively ($P > 0.05$). The mean (SD) crystal

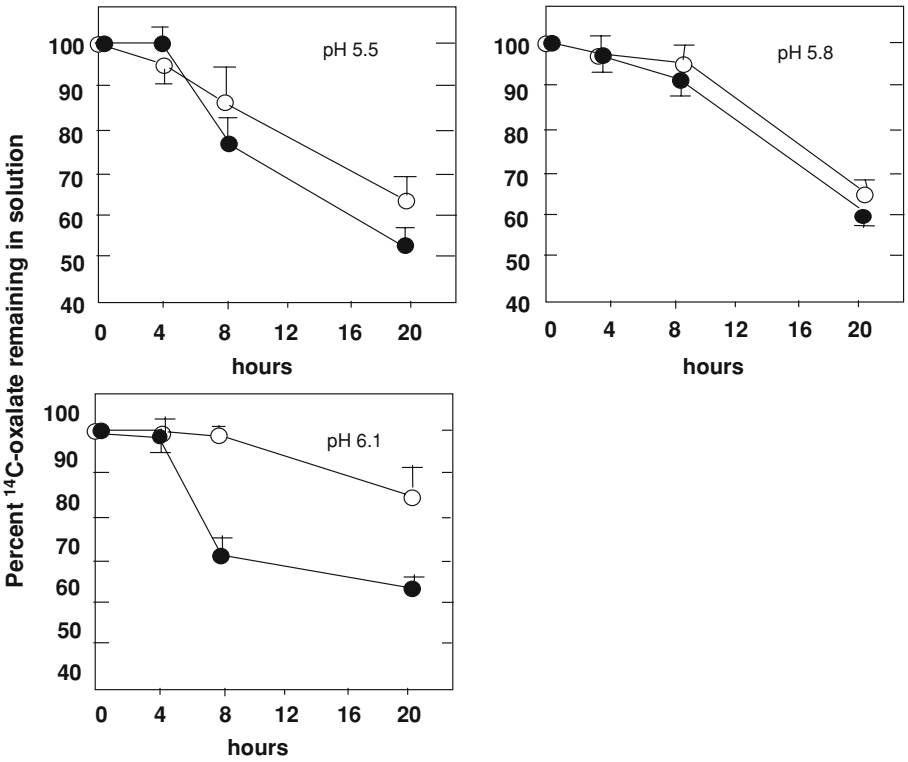

Fig. 4 Percent [^{14}C]oxalate remaining in solution in samples with pH 5.5, 5.8 and 6.1 during the first 20 h following the addition of CaP crystals to solutions with a composition corresponding to that of collecting duct urine (CDm) at different pH. The measurements were carried out in solutions without (*open circles*) and with (*filled circles*) dialyzed urine

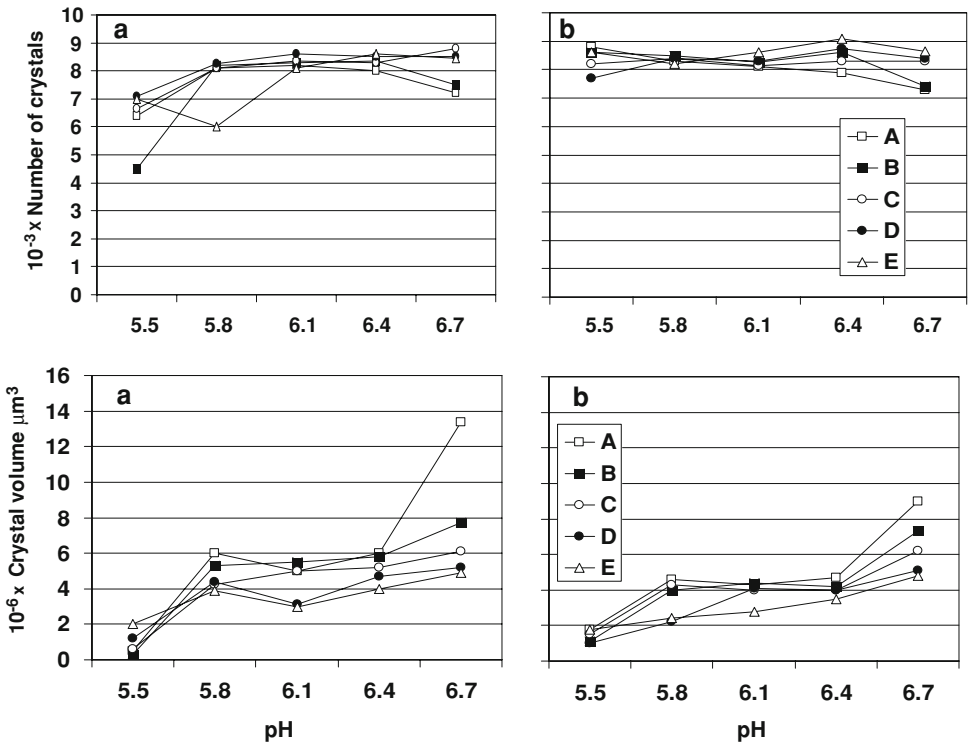

Fig. 5 Number (*above*) and volume (*below*) of crystals in *Solutions A, B, C, D* and *E,* without (**a**) and with (**b**) dialyzed urine 4 h after addition of CaP crystals

volume in samples with pH 5.5 was, however, much lower than the crystal volume of 5.4 (2.17) × 10^6 µm^3 recorded in the combined samples with pH 5.8–6.7 that did not contain dialyzed urine ($P < 0.01$). The corresponding volumes in samples that contained dialyzed urine were 1.42 (0.35) and 4.50 (1.56) × 10^6 µm^3, respectively ($P < 0.01$). The crystal volumes in samples both with and without dialyzed urine and a pH of 6.7 was numerically higher in samples with a high (solutions *A* and *B*) than in those with low (solutions C, D and E) concentrations of solutes, whereas the opposite

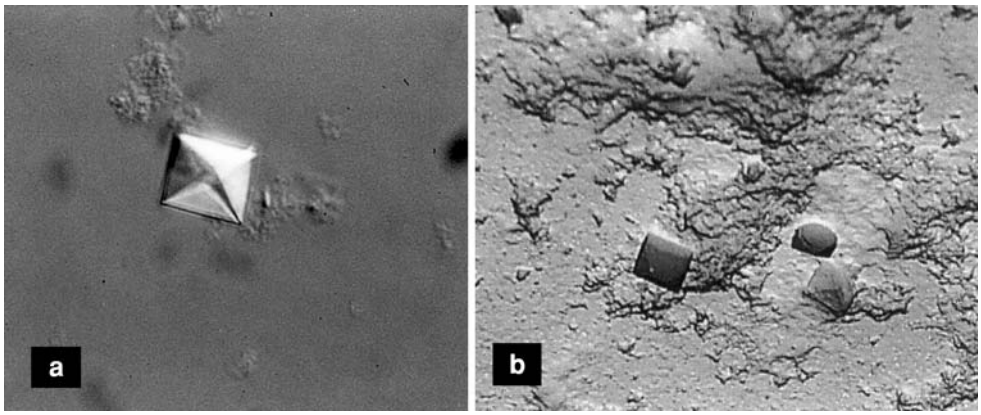

Fig. 6 A typical appearance of CaOx crystals observed in association with CaP crystalline material. Light microscopy (**a**) and scanning electron microscopy (**b**)

situation seemed to be the case in terms of crystal number. The mean (SD) crystal volumes in the *A* and *B* samples without dialyzed urine were 9.35 (2.38) $\times$ 10^6 μm^3 and in the *C, D* and *E* samples 5.38 (0.61) $\times$ 10^6 μm^3. The corresponding values for crystal number were 7.35 (0.13) $\times$ 10^3 and 8.52 (0.18) $\times$ 10^3, respectively ($P < 0.05$).

The volume increment thus was most pronounced in the most supersaturated solutions. The change in crystal volume had a similar pattern in solutions with and without dialyzed urine, albeit, the greatest volumes were recorded in the urine-free samples. When samples with and without dialyzed urine, as a source of urinary macromolecules, were compared (Figs. 3, 4, 5), the presence of dialyzed urine apparently was associated with slightly more pronounced precipitation of oxalate.

An AP_{CaP} level of at least 50×10^{-14} $(mol/l)^2$ was apparently required for growth of CaP crystals, but a more detailed analysis of the AP_{CaP} level necessary for CaP crystal growth was not carried out. CaOx crystals were usually of dihydrate type and associated with CaP crystalline material (Fig. 6) and this reflects that a high calcium concentration was an important determinant for the precipitation CaOx.

Step-wise crystallization experiments

In crystallization *Step A*, Fig. 7 shows the increased levels of absorbance during the first 60 min following addition of calcium chloride to a solution of sodium phosphate at pH 7.0. A continuous rise in absorbance was noted until 40 min after the supersaturation with calcium phosphate. A significant increase in absorbance was observed between 10 s and 40 min after the addition of calcium chloride ($P < 0.002$). The slight reduction in absorbance observed after 50 and 60 min most likely reflects aggregation of CaP crystals.

When an aliquot of the crystal suspension drawn from the solution in *Step A* after 65 min, was mixed with a solution containing sodium oxalate in *Step B* (Fig. 8), the level of absorbance remained stable during the 10-min observation period, despite the high ion-activity product of CaOx and there were no significant differences between the absorbance values recorded ($P > 0.10$). No pH adjustment was carried out during this period.

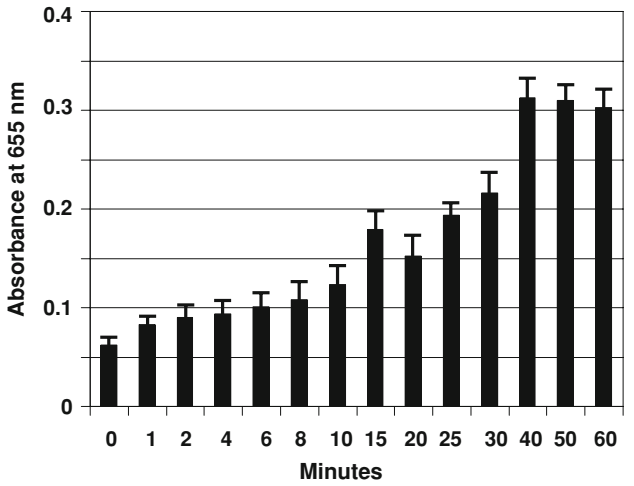

Fig. 7 Mean (+SD) absorbance in six samples during the first 60 min in crystallization *Step A*. The absorbance was measured at 655 nm in a microplate spectrophotometer

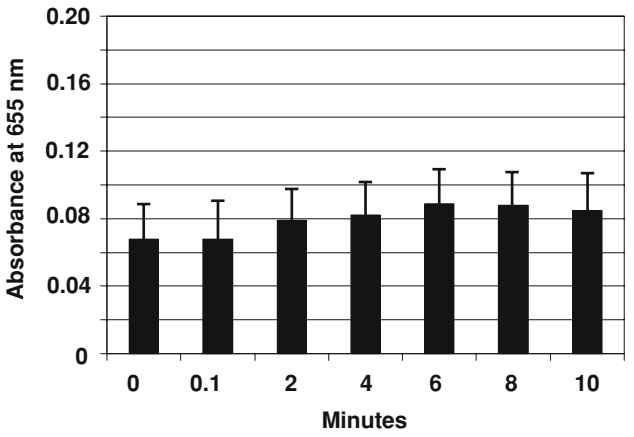

Fig. 8 Mean (+SD) absorbance in six samples during the 10 min in crystallization *Step B*. The absorbance was measured at 655 nm in a microplate spectrophotometer

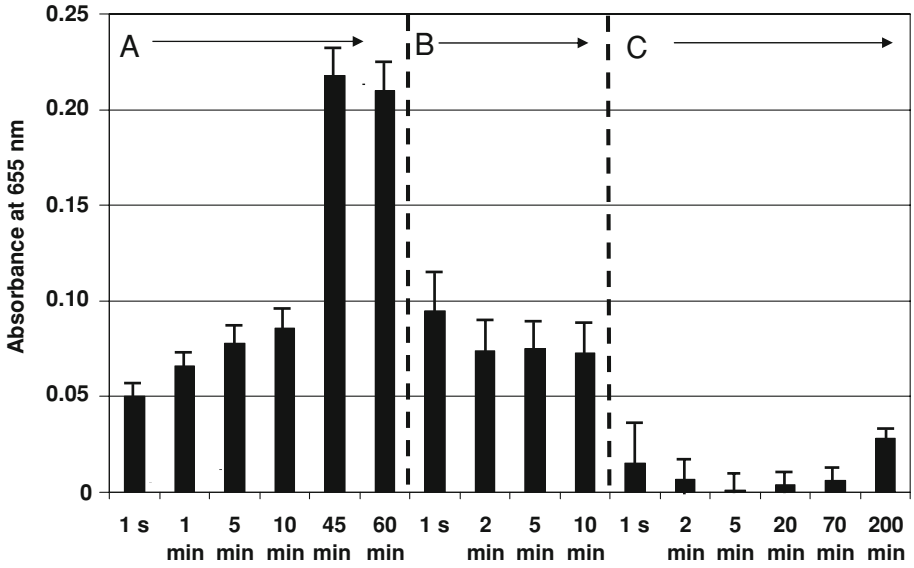

Fig. 9 Mean (+SD) absorbance in six samples recorded in crystallization *Steps A*, *B* and *C*

Figure 9 shows the typical mean (SD) absorbance in six experiments with urine-free solutions. The pronounced precipitation of CaP in *Step A* was followed by a stable level of absorbance during 10 min in *Step B*. The reduced absorbance compared with that in *Step A* is explained by dilution. Following acidification in *Step C*, a rapid marked reduction in absorbance with a lowest value after 5 min (occurred when the CaP crystals were dissolved. The subsequently increased absorbance ($P < 0.002$) reflected the formation of CaOx crystals as verified by light microscopy.

When two urine samples were mixed with calcium chloride but without addition of phosphate (Fig. 10), no precipitation of CaP was recorded in *Step A*. A slight precipitation of CaOx obviously took place in *Step B* inasmuch as the absorbance was significantly increased during the 30-min observation period ($P < 0.002$) as a result of the supersaturation that was established when calcium from *Step A* increased the supersaturation with CaOx. No further crystal precipitation was recorded ($P > 0.10$) when the sample from *Step B* was acidified (*Step C*).

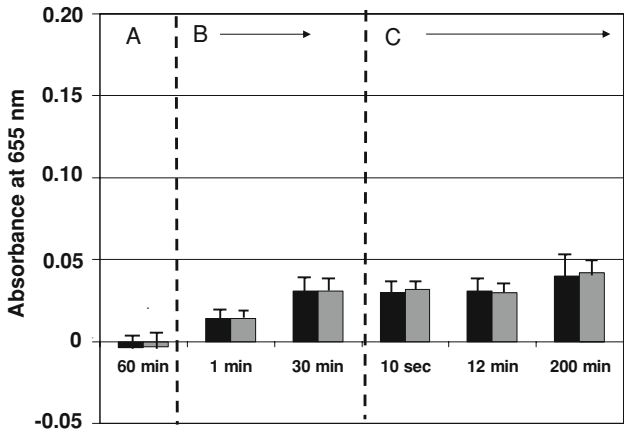

Fig. 10 Mean (+SD) absorbance in two urine samples to which was added $CaCl_2$ but no phosphate in *Step A*. The samples were further processed as described in Sect. "Materials and methods". The calcium concentration in *Step B* obviously resulted in a slight precipitation of CaOx. No further CaOx precipitated during *Step C*

Figure 11 summarizes the mean (SD) absorbance recorded from six experiments with samples containing pooled urine from stone-forming as well as non-stone forming subjects. There was a pronounced precipitation of CaP during the first 45–90 min after the supersaturation with CaP in crystallization *Step A*. As expected the absorbance in *Step B* was reduced, but remained stable during the 10 min. The reduced absorption during the first part of *Step C* most certainly was explained by dissolution of CaP, whereas the subsequent increment was caused by CaOx precipitation. A significantly increased absorbance was observed in both stone forming and non-stone forming patients ($P < 0.02$). The precipitation of CaOx was verified by light-microscopy with demonstration of both COD and COM crystals. The precipitation of CaP obviously was slower in non-stone forming urine than that in urine from stone forming patients.

Discussion

The possible importance of CaP during development of calcium oxalate containing stones has been emphasized by several authors [2, 18, 22, 25, 28, 32–40]. It is well recognized that CaP crystal phases, such as, HAP, OCP and Bru are capable of inducing heterogeneous growth with CaOx [38, 41–43]. Previously reported observations on CaP in gel systems, overgrown with CaOx, gave convincing support for an interaction between CaP and CaOx in the stone forming process [32]. Furthermore, CaP is a common constituent in a great proportion of CaOx containing stones

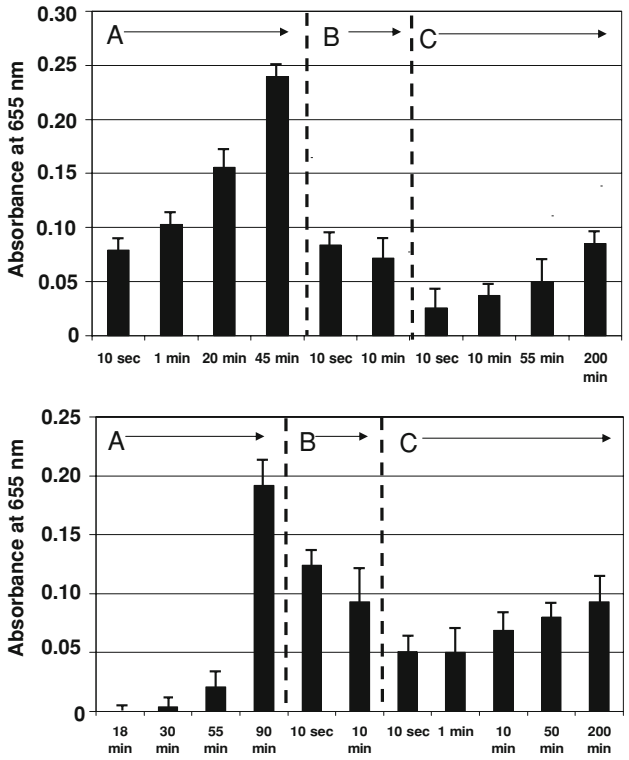

Fig. 11 Mean (+SD) absorbance in a sample of urine from stone forming patients (*above*) and from non-stone forming subjects (*below*). In crystallization *Step A* the phosphate solution was replaced by 100 µl of water. Otherwise the samples were treated as described

[2, 3, 44] and CaP crystals are frequently observed in urine from stone formers [45].

Although the details of the initiation of calcium stone formation are incompletely understood, it appears reasonable to assume that the first steps in this process take place in the nephron. At levels above the CD, CaP or calcium carbonate are the most likely products of crystallization [9, 15–18, 24, 25, 34]. Irrespective of whether this process occurs in the loop of Henle or in the distal tubule, and whether this crystallization requires a promoter or not, it appears reasonable to assume that CaP initiates a subsequent CaOx crystallization. Calculations on urine composition in different parts of the nephron have indicated that the risk of CaOx precipitation is most pronounced in the distal CD [20].

The importance of precipitation of CaP in the loop of Henle has been emphasized previously [15, 25, 26]. In the calculations by Asplin et al. [25], urine in the loop of Henle was supersaturated with respect to Bru and apatite and that in the CD with Bru and calcium oxalate. Although, these authors also found that the distal tubular urine was undersaturated with Bru, these data did not refer to urine in the late portion of the distal tubule and inasmuch as the urine in the early CD commonly was supersaturated with Bru, this would probably be the case also for urine in DTd.

The results in our experiments clearly showed that the CaP crystals, produced by increased supersaturation with CaP in a DTd-like solution, when added to CD-solutions, were capable of inducing CaOx crystallization. There was also microscopic evidence that the precipitated CaOx crystals were associated with the CaP material (Fig. 6). Although the CaOx crystallization, under these circumstances, was a slow process, both in the presence and absence of dialyzed urine, it is noteworthy that CaOx crystal formation was observed at AP_{CaOx} levels as low as 1.5×10^{-8} $(mol/l)^2$. This should be compared with an AP_{CaOx} in the range of $2.6–4.3 \times 10^{-8}$ $(mol/l)^2$ necessary for precipitation of CaOx in similar systems without seed crystals [46] and a formation product of 2×10^{-8} $(mol/l)^2$ as reported in the literature [1]. The importance of oxalate is emphasized by the much faster CaOx crystallization recorded in *Solution M* which had a higher concentration of oxalate at corresponding levels of supersaturation than *Solutions A, B* and *C*.

With 0.5 ml of the crystal suspension added to *Solutions A* to *E*, the concentration of CaP seed in the samples was of 500–750 mg/l. For HAP, OCP and ACP, this amount corresponds to urine calcium concentrations of 5–7.5, 4.5–6.8 and 4.1–6.1 mmol/l, respectively. Precipitations of these amounts of calcium can be encountered in vivo.

It is noteworthy that the amount of CaP seed in the experimental system with *Solution M* was only 20–30 mg/l. It is, thus theoretically possible that the faster CaOx crystallization in *Solution M*, apart from the effect brought about by the higher oxalate concentration, also can be explained by the smaller crystal surface area available in these samples. Therefore, a localized release of calcium ions from the precipitate during dissolution might attract more oxalate per crystal surface area and, thus result in a higher local supersaturation with CaOx than was the case in the presence of a greater amount of seed crystals.

The absence of CaOx crystal formation at pH 6.4 and 6.7 might be explained by a higher concentration of dissociated citrate [47] and by the fact that CaP is the favored crystal product when pH is increased [1, 48]. Such an effect is also suggested by the marked increase in crystal volume at pH 6.7 particularly in *Solutions A* and *B*. It needs to be emphasized, however, that due to the crystal morphology, the Coulter measurement of precipitated CaP is less accurate than that of CaOx, particularly, when the CaP precipitate has a high concentration. Nevertheless, we believe that this method is sufficiently accurate for the conclusions we have drawn.

The results of our experiments thus give support to the previously suggested crucial role of CaP for formation of calcium oxalate stones [10, 26, 49] and are in accordance with the assumption that CaP is the preferred crystal phase at high nephron levels [15, 16, 22, 24–26]. Theoretical

conclusions as well as experimental findings have indicated that the most likely place for CaP nucleation is the thin segment of the loop of Henle, because in this part of the nephron the concentrations of calcium and phosphate as well the pH are sufficiently high to result in an ion-activity product of CaP—probably ACP—that exceeds the formation product. There is, moreover, recent evidence that CaP accumulates in the basement membrane and that this precipitate eventually gives rise to the interstitial calcifications of Randall's plaques [10]. The alternative fate of CaP formed in the loop of Henle might be the intra-luminal transportation to low nephron levels or out of the nephron into the caliceal system [50]. The latter event probably represents the normal condition, whereas an abnormal quantity of CaP might result in tubular retention of crystalline material. Large amounts of crystals thus can be retained in the CD, probably at its distal part (duct of Bellini). CaP both in Randall's plaques and in intra-tubularly retained crystal deposits with or without protrusion into the caliceal cavity might subsequently be dissolved during periods with low urine pH. This dissolution releases calcium ions.

For Randall's plaques it was recently shown that a contact is established between caliceal urine and the CaP crystal phase by disruption of the papillary epithelium and that CaOx crystals form in an organic matrix associated with the crystalline CaP component [49]. A similar course of events might occur on the surface of CaP crystals retained at the papillary tip or otherwise retained in the CD [50]. The acidification with ensuing dissolution of CaP seems to be a necessary component in order to get CaOx, and it is tempting to speculate that this crystal formation is the result of nucleation rather than heterogeneous growth. From the step-wise crystallization experiments it was observed that only supersaturation with CaOx in *Step B* (Figs. 8, 9, 11) was insufficient to result in a significant formation of CaOx. Moreover, a similar outcome was noted in the absence of a sufficient supply of CaP from *Step A* (Fig. 10).

From Fig. 3, and 5 it appears that dissolution of CaP occurs only at pH levels below 6.1–6.4 and apparently attributable to the fact that CaP crystals at least at pH 5.5 were subject to dissolution.

The reduced crystal number observed at pH 5.5, in the absence of urine, was not recorded in solutions containing urine and the presence of urine seemed to counteract or reduce the rate of CaP dissolution. Although urinary macromolecules attached to the CaP crystal phase can act as inhibitors of CaP crystal dissolution as well as of crystal growth and crystal aggregation [21, 22, 51], they might also in the course of time provide a template or compartment for binding and accumulation of calcium ions from the dissolved CaP [51]. When this amount of calcium ions is mixed with peak concentrations of calcium and oxalate in urine, very high local levels of CaOx supersaturation can be expected. The results shown in Fig. 4 indicate that urinary macromolecules at least after 8 and 20 h had a crystallization promoting effect. The constant shaking of the experimental vessels partially might have counteracted this process and it is possible that a less agitated solution had resulted in a more substantial precipitation of CaOx.

The role of pH as a modifier of the crystallization process has been clearly demonstrated in our experiments. At pH-levels below 6.1–6.4, retained CaP crystals might induce CaOx nucleation, at least if the AP_{CaOx} exceeds 1.5×10^{-8} $(mol/l)^2$. It is logically attractive to assume that after inducing CaOx crystallization, a complete dissolution of the CaP crystal phase might result in pure CaOx stones. Consequently incomplete dissolution of CaP will result in stones containing both CaOx and CaP. At higher pH levels, the CaP crystallization will dominate, and at pH levels above 6.1–6.4 the CaP crystal phase remains or increases and in this regard it is of note that patients with renal tubular acidosis or primary hyperparathyroidism, who are unable to sufficiently acidify their urine form stones that are composed either of pure CaP or at least dominated by that crystal phase [52, 53].

High urinary concentrations of calcium and oxalate as well as low concentrations of citrate coinciding with periods of low pH and accordingly dissolution of CaP, provide an accumulation of risk factors for crystallization of CaOx. Such risk periods very high local supersaturation levels of CaOx can be expected particularly in association of an excessive intake of animal protein [54].

Further studies are necessary to disentangle the complexity of the events leading to CaOx stone formation, but the results obtained in this limited study provide further interesting evidence for a crucial role of CaP in CaOx stone formation. From the results reported here and by others it seems reasonable to assume that one of the major areas of interest with the aim of achieving a rational CaOx stone prevention should be the development of tools that effectively counteract the nucleation of CaP at high nephron levels.

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