Urol Res (1999) 27:409–416

RAPID COMMUNICATION

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The effect of pH changes on the crystallization of calcium salts in solutions with an ion composition corresponding to that in the distal tubule

Received: 8 July 1998 / Accepted: 12 March 1999

Abstract The effect of pH changes on the crystallization in solutions with an ion composition assumed to correspond to that of urine in the distal part of the distal tubule was examined by recording the number and volume of crystals with a Coulter Multisizer and by studying the crystal morphology with scanning electron microscopy at different degrees of volume reduction. The experiments were carried out with 100 ml samples at different starting pH values without and with 20% of dialysed urine (dU). The number of crystals increased in response to the volume reduction. In solutions without dU, 100 or more crystals with a diameter in the range 2.4–45 μm were observed already at a volume reduction of 40% when the initial pH was 7.28. For solutions with a pH of 5.80 and 6.45 the corresponding values were 60% and 80%, respectively. In the presence of dU, an appearance of crystals was recorded at volume reductions of less than 20%. In solutions with an initial pH of 5.80 and 6.45, the crystal number was greater with dU than without; such a difference was not recorded at pH 7.28. In samples containing dU, the mean crystal volume (MCV) varied very little when the sample volume was reduced. The same was found in solutions without dU when the initial pH was 5.80 and 7.28, whereas the MCV was greater in the samples with pH 6.45. Scanning electron microscopy of solutions reduced to 30–40% of the original volume showed that calcium phosphate had formed in solutions with a starting pH of 7.28 and 6.45. In solutions with pH 5.80 calcium oxalate crystals were observed with calcium phosphate.

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Key words Crystallization · Calcium phosphate · Calcium oxalate · Distal tubule · Macromolecules · Promotion · Inhibition · pH

Introduction

The development of urinary stones is the result of a crystallization process that involves nucleation, crystal growth and crystal aggregation. Chelators as well as inhibitors and promoters of crystallization therefore influence the crystallization; these may have either a low molecular weight (e.g. citrate, pyrophosphate and urate) or a high molecular weight (e.g. nephrocalcin, uropontin, Tamm-Horsfall protein and glycosaminoglycans).

In two recent studies we have shown that calcium phosphate (CaP) might be the type of crystal that most easily forms in the proximal and distal tubule of the nephron [14, 20]. In salt solutions with a pH of 6.45 and an ion composition assumed to correspond to that of urine in the distal tubule CaP nucleation was induced both by an increased calcium concentration and by changes in solution composition brought about by volume reduction. Urinary macromolecules thus appeared to have a promotive effect on the nucleation of CaP and an inhibitory effect on crystal growth, crystal aggregation or both, since they counteracted the development of large CaP crystals or crystal aggregates.

It is well known that urinary pH is of great importance for the crystallization of both calcium oxalate (CaOx) and CaP [4, 10–12, 24, 27, 28]. Most of these conclusions were based on experiments carried out in whole urine. It is also well known that the influence on the crystallization process of both low- and high-molecular-weight compounds depends on the pH [3, 5, 7, 13, 25, 26, 33].

Since it is reasonable to assume that the first step in the crystallization process leading to pure CaP and mixed CaOxCaP stones starts in the loop of Henle [2, 6, 16] or in the distal tubule [20, 30], we found it worthwhile to study the effects of pH on the crystallization of

calcium salts in solutions with a composition corresponding to that in the distal tubule.

Materials and methods

A salt solution with an ion composition assumed to correspond to that of urine in the distal part of the distal tubule (DTd) was prepared as previously described [20]. One litre of this solution was given the following ion composition: calcium 1.04 mmol, magnesium 0.41 mmol, phosphate 4.17 mmol, oxalate 0.04 mmol, citrate 0.35 mmol, sodium 96 mmol, potassium 22.5 mmol and sulphate 13.8 mmol. No ammonium was added to avoid the risk of precipitating ammonium salts.

The pH in the salt solution was adjusted to 6.45, 5.80 and 7.28, by addition of small amounts of sodium hydroxide or hydrochloric acid, in three different series. A pH of 6.45 was assumed to represent the average pH in the distal tubule under normal conditions [22]. The pH in the samples was measured with a pHM 84 pH meter (Radiometer, Copenhagen, Denmark) immediately before and after evaporation (see below).

We used pooled dialysed urine from normal male subjects as a source of macromolecules. This urine was collected between 2200 and 0600 hours in bottles containing 15 ml of 3 mmol/l sodium azide as a preservative. The urine was screened for bacteria, protein and glucose before being pooled. The preparation of dialysed urine (dU) was carried out as previously described [14]. According to the degree of dilution at different nephron levels the normal concentration of macromolecules in DTd was assumed to correspond approximately to a 20% concentration of dU. It needs to be emphasized, however, that the concentration of Tamm-Horsfall protein and probably also other macromolecules might be lower than that normally found in distal tubular urine as a result of the sample preparation technique [20].

Aliquots of 100 ml of the salt solutions with and without dU were used in volume reduction experiments. These samples were passed through Millipore filters with a pore size of 0.22 μm (Millipore, Molsheim, France) after which evaporation was carried out in a Büchi Rotavapor RE at 37°C (Büchi, Flawil, Switzerland).

Immediately after the evaporation the number and volume of crystals in the size interval 2.4–45 μm were recorded in a Coulter Multisizer with a 100 μm capillary tube (Coulter Electronic, Luton, UK). The mean crystal volume (MCV) was the quotient between the total volume (μm^3) and the total number of crystals.

A significant formation of crystals was not considered to have occurred until the number of particles in 50 μl exceeded 100. This level was chosen to minimize the risk of drawing conclusions from the counting of non-crystalline material, as our method for particle detection did not enable a distinction between crystals and other particles. Based on previous microscopic observations a nucleation is conceivable when 100 crystals are recorded.

Aliquots for examination of the crystal morphology were obtained from solutions both with and without dU immediately after the crystal counting. The samples were prepared for scanning electron microscopy as previously described [20].

The ion-activity products of calcium oxalate (AP_{CaOx}), brushite (AP_{Bru}) and hydroxyapatite (AP_{HAP}) were calculated by means of computerized iterative approximation with the EQUIL2 program [32] at different degrees of evaporation. As the CaP crystal phase that forms is highly dependent on the pH, we also calculated the ion-activity product of CaP (AP_{CaP}) by means of the product of the activities of calcium and phosphate: $a_{Ca^{2+}} \times a_{PO_4^{3-}}$. Similar to our previous observation [14] the pH decreased following evaporation of solutions with a starting pH of 6.45 and 5.80, but not in solutions with a starting pH of 7.28 (Fig. 1). Therefore we used the pH value recorded at the end-point of the volume reduction for calculation of the AP_{Bru}, AP_{HAP} and AP_{CaP}. Figure 1 also shows the pH in solutions without calcium. The ion-activity products for the different salts during the crystallization process might have been overestimated because of a rapid nucleation and the fact that we did not take into account any complexation between ions and macromolecules.

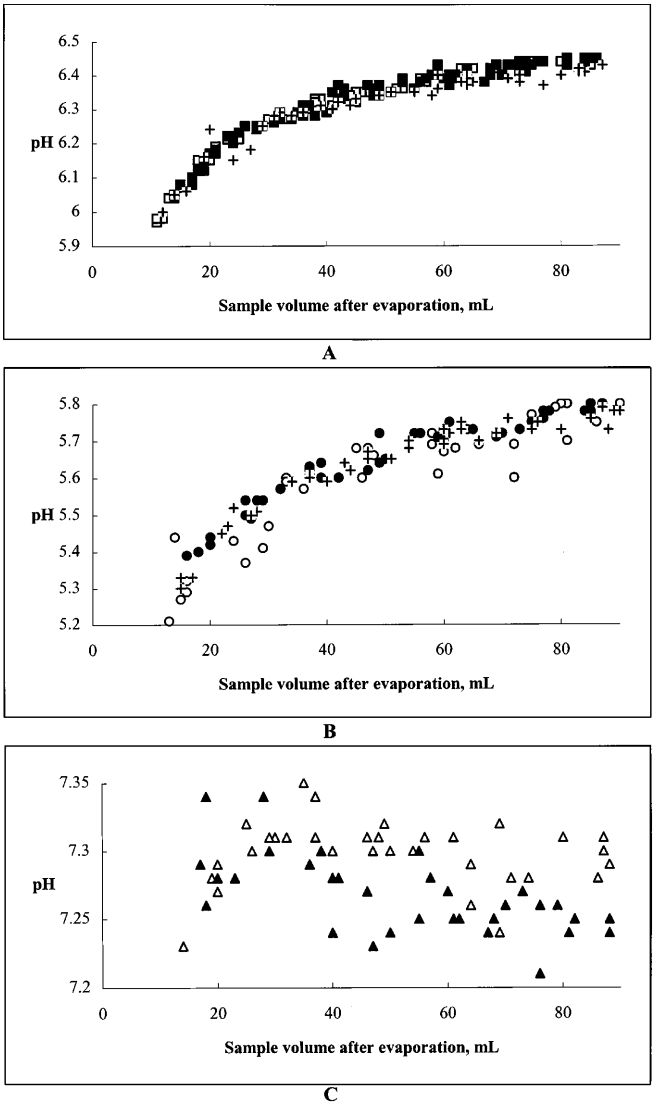

Fig. 1A–C Effects on pH of volume reduction of 100 ml solutions with an initial composition corresponding to that in the distal tubule. **A** Solutions with an initial pH of 6.45 without dialysed urine (*open squares*), with 20% of dialysed urine (*filled squares*) and without either dialysed urine or calcium (*crosses*). **B** An initial pH of 5.80 without dialysed urine (*open circles*), with 20% dialysed urine (*filled circles*) and without either dialysed urine or calcium (*crosses*). **C** An initial pH of 7.28 without dialysed urine (*open triangles*) and with 20% dialysed urine (*filled triangles*)

Statistical analysis

Regression analysis was used to record any association between different variables, and Student's *t*-test to decide on statistically significant differences.

Results

Crystal number

There was a linear relationship between the $\log_{10}$ crystal number and the degree of volume reduction in solutions

with dU at all three pH levels; the correlation coefficients were statistically significant ($P < 0.001$). This was not observed for solutions without dU and an initial pH of 5.80 and 6.45 (Fig. 2). As shown in Fig. 2A, the number of crystals in solutions without dU and an initial pH of 6.45 did not exceed 100 until the volume had been reduced to 20 ml. In the presence of dU, this crystal number was exceeded already after evaporation to 85 ml. In solutions with a starting pH of 5.80, 100 crystals were recorded after a volume reduction to approximately 40 ml without dU and 90 ml with dU (Fig. 2B). At a starting pH of 7.28 the number of crystals exceeded 100 after a volume reduction to 60 ml without dU and 85 ml in the presence of dU (Fig. 2C). The number of crystals in solutions without dU and a starting pH of 5.80 or 6.45 was much smaller than that in solutions with dU. Such a difference was not recorded when the starting pH was 7.28.

Mean crystal volume

The MCV was fairly constant for solutions both with and without dU at all three pH levels (Fig. 3). Only for solutions without dU and an initial pH of 5.80 and so-

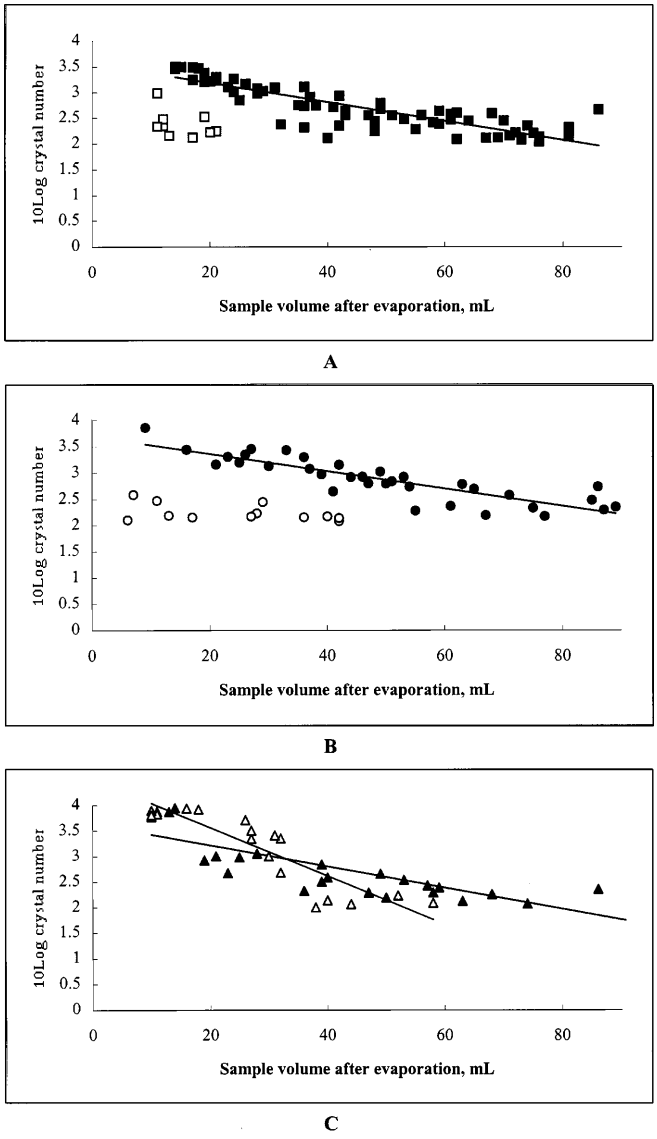

Fig. 2A–C Relationship between the sample volume and the number of crystals after evaporation of 100 ml samples with an initial composition corresponding to that in the distal tubule. **A** Solutions with an initial pH of 6.45 without dialysed urine (*open squares*) and with 20% of dialysed urine (*filled squares*). **B** An initial pH of 5.80 without dialysed urine (*open circles*) and with 20% of dialysed urine (*filled circles*). **C** An initial pH of 7.28 without dialysed urine (*open triangles*) and with 20% of dialysed urine (*filled triangles*)

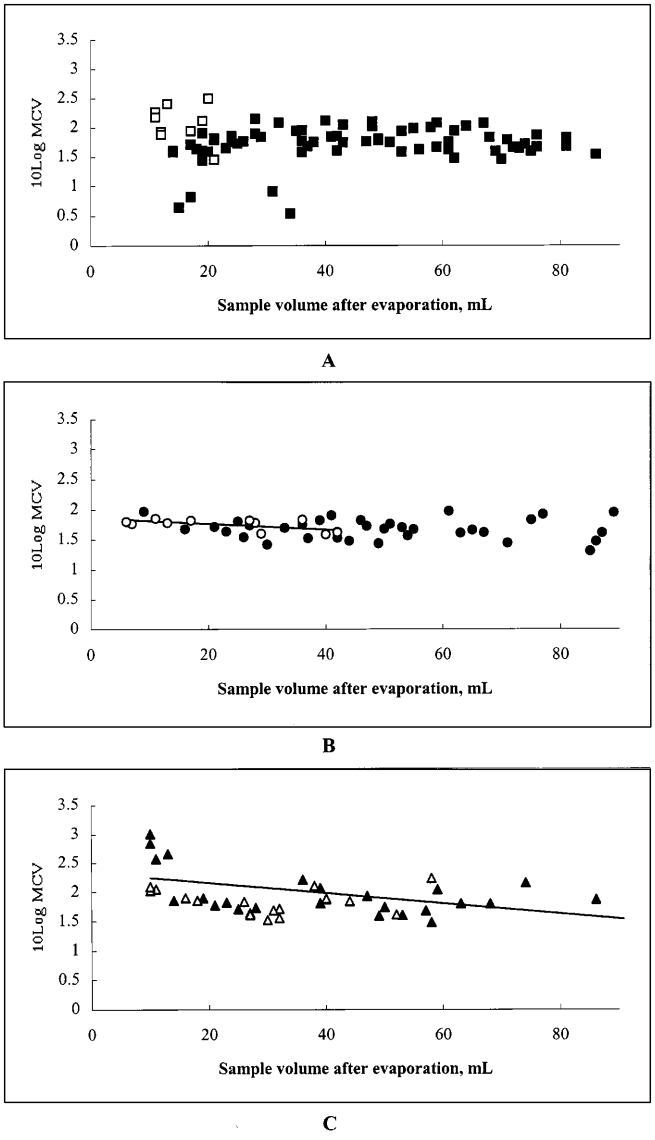

Fig. 3A–C Relationship between the sample volume and the mean crystal volume (MCV) after evaporation of 100 ml samples with an initial composition corresponding to that in the distal tubule. Evaporation was carried out of the following samples: **A** Solutions with an initial pH of 6.45 without dialysed urine (*open squares*) and with 20% of dialysed urine (*filled squares*). **B** An initial pH of 5.80 without dialysed urine (*open circles*) and with 20% of dialysed urine (*filled circles*). **C** An initial pH of 7.28 without dialysed urine (*open triangles*) and with 20% of dialysed urine (*filled triangles*)

lutions with dU and an initial pH of 7.28 was the relationship between the degree of volume reduction and the $\log_{10}$ MCV statistically significant; however, the slopes were weak. From Fig. 3A it is evident that the MCV in the most concentrated samples was slightly greater without than with dU when the initial pH was 6.45. In solutions with an initial pH of 7.28, the MCV remained at a constant level in both the presence and the absence of dU, except for the most concentrated samples (Fig. 3C). A fairly constant MCV was also recorded in dU-containing samples with a starting pH of 6.45 and 5.80. The MCVs recorded at each pH level are summarized in Table 1. The greatest MCV was found in solutions without dU and an initial pH of 6.45, where the mean MCV was 2.07 compared with 1.74 in solutions without dU and an initial pH of 5.80, and 1.84 in solutions without dU and an initial pH of 7.28. The difference between the MCV in solutions without dU and an initial pH of 5.80 and 6.45 was statistically significant ($P < 0.01$), whereas there was no significant difference between the MCV in solutions without dU and an initial pH of 5.80 and 7.28 ($P > 0.05$) or an initial pH of 6.45 and 7.28 ($P > 0.05$). At a starting pH of 5.80 and 6.45 the presence of dU resulted in a smaller MCV than when the starting pH was 7.28. When the dU-containing solutions with different pH were compared, it was obvious that the MCV was greatest at a high pH and smallest at a low pH. This difference was statistically significant ($P < 0.02$).

Table 1 Mean (SD) mean crystal volume (*MCV*) in solutions with and without dialysed urine (*dU*) at different sarting pH

pH	Solution	MCV	*P* value
5.80	Without dU	1.74 (0.10)	>0.05
5.80	With 20% dU	1.68 (0.17)	
6.45	Without dU	2.07 (0.32)	<0.05
6.45	With 20% dU	1.71 (0.30)	
7.28	Without dU	1.84 (0.22)	>0.05
7.28	With 20% dU	1.98 (0.39)	

Crystal morphology

Scanning electron microscopy showed a crystal morphology in conformity with that of CaP crystals in solutions with an initial pH of 6.45 and 7.28, both in the presence and in the absence of dU (Figs. 4, 6). The samples were all taken from solutions in which the volume had been reduced to 30–40 ml, except for Fig. 6E, F which represents samples evaporated to a final volume around 20 ml. In the solutions with an initial pH of 5.80, however, the samples both with and without dU and reduced to a volume between 30 and 40 ml contained a precipitate suggestive of both CaP and CaOx (Fig. 5); the latter crystal phase was predominantly calcium oxalate dihydrate (COD) (Fig. 5E, F). The precipitate in solutions with an initial pH of 7.28 was strongly suggestive of hydroxyapatite, particularly when the volume had been reduced to around 20 ml (Fig. 6E, F).

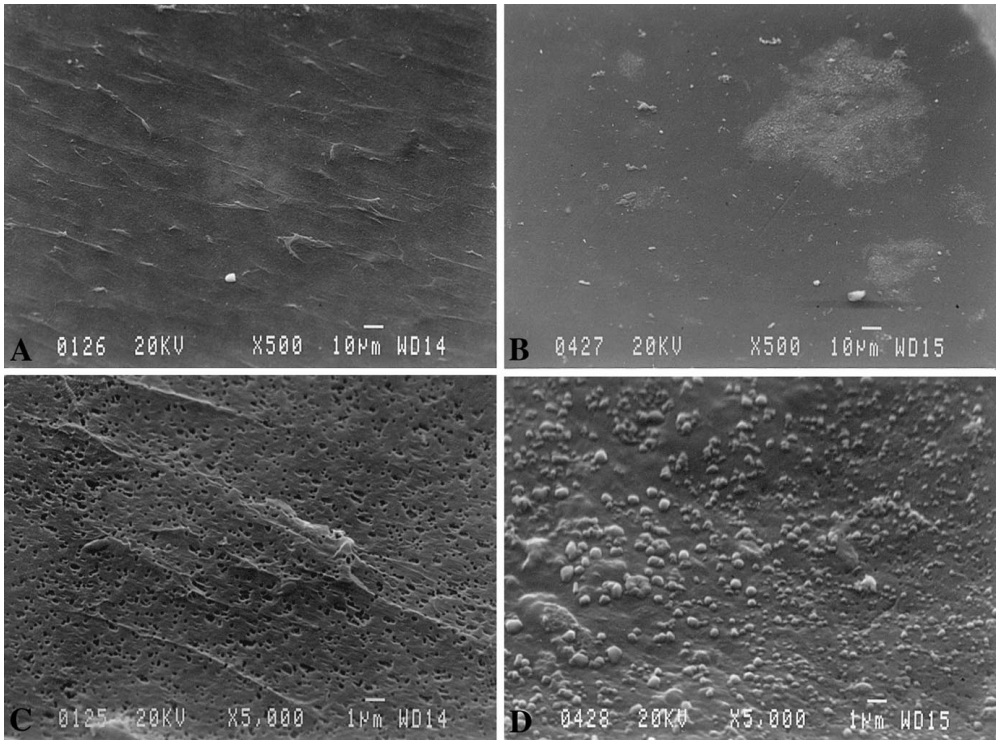

Fig. 4A–D Scanning electron microscopic examination of the precipitate after evaporation of 100 ml salt solutions with an initial pH of 6.45 to a final volume between 30 and 40 ml: without dialysed urine (**A**) at a magnification of ×500 and (**C**) at a magnification of 5000×; with 20% of dialysed urine (**B**) at a magnification of ×500 and (**D**) at a magnification of ×5000

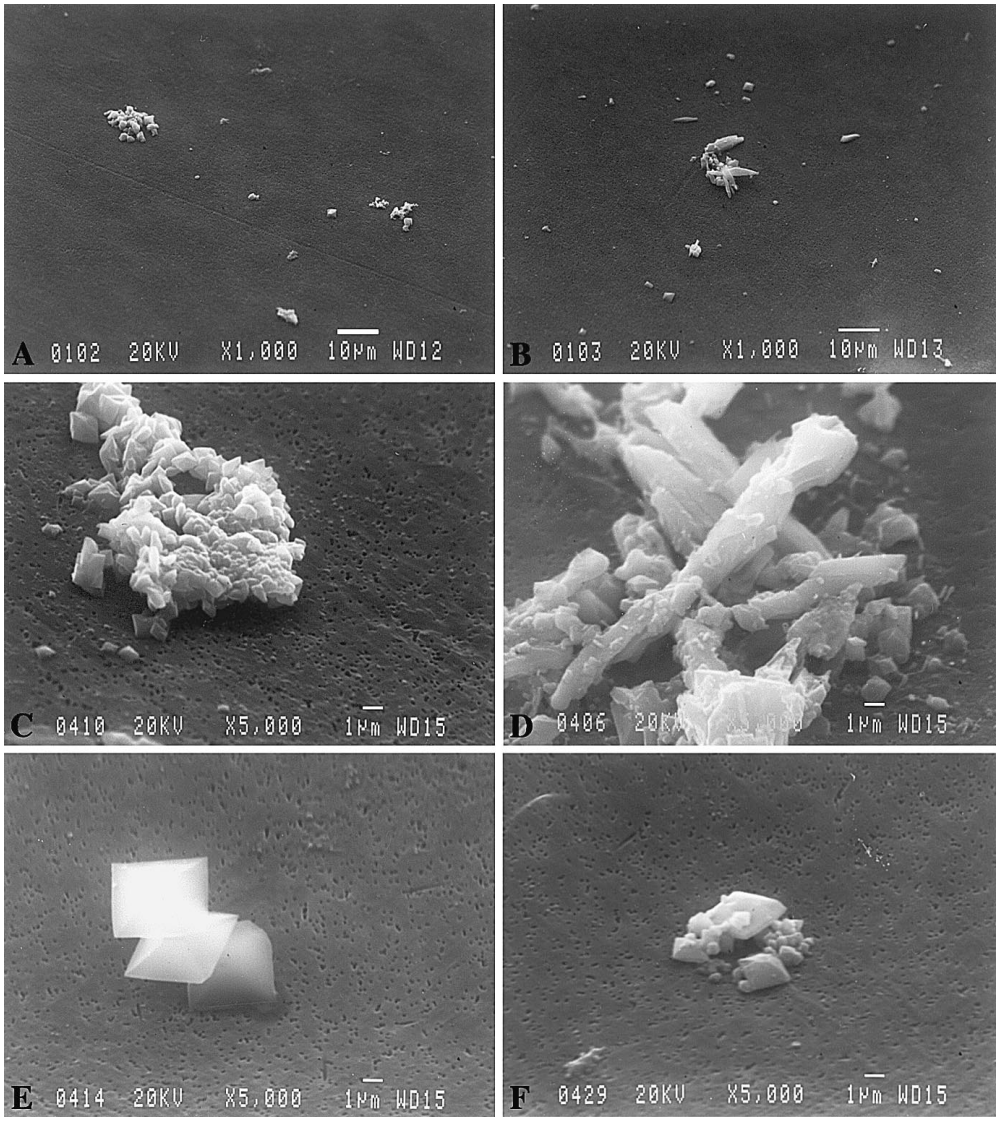

Fig. 5A–F Scanning electron microscopic examination of the precipitate after evaporation of 100 ml salt solutions with an initial pH of 5.80. Samples were evaporated to a final volume between 30 and 40 ml without dialysed urine (**A**) at a magnification of ×1000 and (**C**, **E**) at a magnification of ×5000, where **E** clearly shows calcium oxalate dihydrate crystals; and with 20% of dialysed urine (**B**) at a magnification of ×1000 and (**D**, **F**) at a magnification of ×5000, where **F** clearly shows calcium oxalate dihydrate crystals

Ion-activity products

At a volume reduction to 40–30 ml, the AP_{CaOx} was 0.41–0.56 × 10^{-8} M^2, which only is about twice the solubility product of 0.23–0.25 × 10^{-8} M^2 [21, 31]. Even following the pronounced volume reduction to 10 ml, the AP_{CaOx} was not higher than 1.9 × 10^{-8} M^2. The AP_{CaP} at volume reductions resulting in a significant crystallization (>100 crystals) for solutions with and without dU was 1.01 × 10^{-14} M^2 and 1.78 × 10^{-14} M^2, respectively, for samples with an initial pH of 5.80 (Table 2). The corresponding values for samples with an initial pH of 6.45 were 14.1 × 10^{-14} M^2 and 44.3 × 10^{-14} M^2; and for samples with an initial pH of 7.28, they were 189 × 10^{-14} M^2 and 288 × 10^{-14} M^2 (Table 2). The AP_{Bru} following evaporation of the solution with an initial pH of 5.80 to a volume of 40–30 ml were 7.4 × 10^{-8} M^2 and 9.6 × 10^{-8} M^2, respectively. This should be compared with a solubility product of 1.9 × 10^{-7} M^2 [18]. The AP_{HAP} at volume reductions resulting in a significant crystallization were all much above the solubility product of 1.87 × 10^{-59} M^9 [18] (Table 2), irrespective of the pH level.

Discussion

The objective of these experiments was to study the crystallization under conditions similar to those in the nephron. For this reason, solutions with a composition assumed to correspond to that in the distal part of the distal tubule [20] was subjected to a volume reduction similar to that of urine during its passage from the distal tubule to the calyx. Urine will be concentrated an average of 6 times as a result of water absorption in the collecting duct. It should be emphasized that the experimental model reflects effects brought about by changes in pH and supersaturation caused by this volume reduction. Other alterations such as a reduced concentration of calcium or other ions as a result of

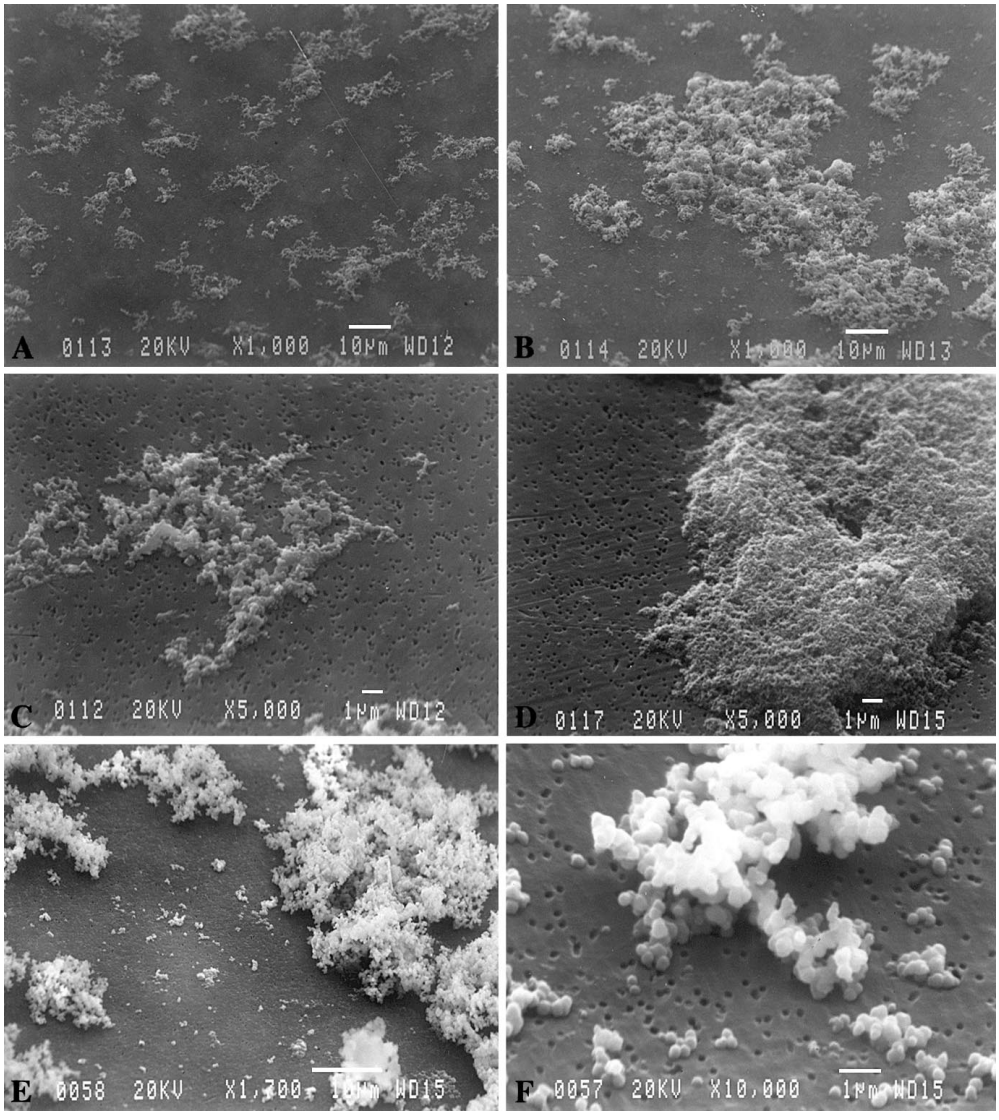

Fig. 6A–F Scanning electron microscopic examination of the precipitate after evaporation of 100 ml salt solutions with an initial pH of 7.28 to a final volume between 30 and 40 ml: without dialysed urine (**A**) at a magnification of ×1000 and (**C**) at a magnification of ×5000; with 20% of dialysed urine (**B**) at a magnification of ×1000 and (**D**) at a magnification of ×5000. **E** and **F** show the precipitate after evaporation to a final volume around 20 ml of samples without dialysed urine, **E** at a magnification of ×1700 and **F** at a magnification of ×10000

Table 2 The ion-activity products of calcium oxalate (*CaOx*), calcium phosphate (*CaP*), hydroxyapatite (*HAP*) and brushite (*Bru*) in samples with and without dU, following evaporation to a volume resulting in a significant crystallization (≥100 crystals)

pH	Solution	Volume (ml)	AP_{CaOx} ($10^8 \times M^2$)	AP_{CaP} ($10^{14} \times M^2$)	AP_{HAP} ($10^{50} \times M^9$)	AP_{Bru} ($10^7 \times M^2$)
5.62	Without dU	40	0.41	1.78	0.07	0.74
5.78	With dU	90	0.17	1.01	0.002	0.29
6.16	Without dU	20	0.9	44.3	5460	5.3
6.43	With dU	85	0.18	14.1	24.1	0.91
7.28	Without dU	60	0.26	288	3 800 000	2.61
7.28	With dU	85	0.18	189	387 000	1.72

reabsorption that normally takes place in the collecting duct were not accounted for.

According to the definition given above, crystal appearance was recorded earlier in all samples containing dU than in those without dU, irrespective of pH. A more pronounced crystallization in terms of crystal number was most clearly shown when the initial pH was 5.80 and 6.45. At pH 7.28, no such difference was recorded. An outcome like this can be explained by nucleation of different crystal phases in the presence and absence of urinary macromolecules, but the similarity in crystal morphology between samples of the same pH makes it reasonable to assume a modifying role of the macromolecules in dU. An increased number of crystals in the measurable size interval can be explained by the formation of a greater number of crystal nuclei, a higher rate of crystal growth or agglomeration of initially small crystals, or by the nucleation of a smaller number of

crystals with a larger size. The present experiment does not allow definite conclusions in this respect, but the small effects on MCV during evaporation indicate that an effect on nucleation is most probable. In this respect a pH-dependent crystallization-promoting effect has previously been reported [8, 9]. Another possibility that deserves some attention is that CaP crystals that might have formed will dissolve when the pH is reduced, and that such an effect is counteracted by protection of CaP crystals with urinary macromolecules.

A high risk of CaP nucleation above the collecting duct, especially during periods resulting in peaks of CaP supersaturation, has recently been proposed by several authors [2, 16, 17, 20, 29]. When these CaP crystals formed at higher nephron levels are exposed to a lower pH in the collecting duct they might partly or completely dissolve resulting in an increased local concentration of calcium and hence an increased supersaturation of CaOx [15, 30].

In samples with an initial pH of 6.45, which is considered an average normal pH in the distal tubule, volume reduction resulted in a nucleation of CaP [14]. At a volume reduction to 30–40 ml the pH was reduced to 6.2–6.3. This did not result in the appearance of CaOx, which is in agreement with observations that CaP crystals do not dissolve unless the pH is decreased to levels below 5.70–5.80 and that CaOx nucleation was not induced at pH levels above 6.10 [30].

In contrast, samples with a starting pH of 5.80 had a pH around 5.6 at a volume reduction to 40 ml. In these samples crystals of CaOx were formed. With an initial pH of 7.28 the numbers of crystals were similar with and without dU, at least with a volume reduction of less than 90%. It can therefore be assumed that at this high pH the driving force of CaP supersaturation is sufficient for a homogeneous nucleation, only marginally influenced by promoters.

Although dU obviously promoted the crystallization while maintaining the MCV at a fairly constant level, it is noteworthy that in the presence of dU, MCV was greatest at pH 7.28 and 6.45.

It should be noted the crystal counting of CaP is less accurate than that of CaOx, particularly in the presence of great crystal masses. We believe, however, that the accuracy was sufficient for the conclusions drawn.

Crystals of CaOx were observed in samples with an ion-activity product of $0.41–0.56 \times 10^{-8}$ M^2. This level was derived from the urine composition in those samples where CaOx was first detected, but without attention to increments in the calcium concentration due to a possible dissolution of CaP. Although previous studies have suggested a formation product of CaOx around 2×10^{-8} M^2 [23], a much lower saturation might be sufficient for inducing secondary CaOx nucleation, particularly in the presence of urinary macromolecules.

One important question is the level of supersaturation at which CaP nucleation occurs. This is, of course, highly dependent on the crystal phase that forms, but without exact information in this respect we have found it of value to use the product of $a_{Ca^{2+}} \times a_{PO_4^{3-}}$ to express the ion-activity product of CaP. From the data in Table 2, the lowest AP_{CaP} at which crystals were observed was in samples with an initial pH of 5.80, whereas the corresponding values were much higher in samples with an initial pH of 7.28. It has previously been shown that brushite can precipitate in poorly supersaturated solutions with a low pH, while other crystal phases of CaP form in solutions with higher a pH level and a higher CaP supersaturation [1, 19]. A direct comparison between the crystallization in solutions with different pH is hampered by the fact that different crystal phases probably are precipitated, but it is reasonable to assume that crystal phases other than brushite would predominate in solutions with an initial pH of 6.45 and 7.28, as suggested by the scanning electron microscopy in our experiments. Scanning electron microscopic analysis of samples containing dU with an initial pH of 5.80, reduced to a volume between 30 and 40 ml, showed a precipitate suggestive of both CaOx and CaP. The ion-activity product of brushite in these solutions varied between 7.4×10^{-8} M^2 and 9.6×10^{-8} M^2, which is below the solubility product of 1.9×10^{-7} M^2 [18]. For this reason it is very unlikely that the CaP phase constitutes brushite.

The most important findings recorded in this series of experiments were that crystals occurred earlier in the presence of dU, that the MCV was highest in samples with pH 7.28 and that CaOx crystals were only observed following volume reduction of the samples with a starting pH of 5.80. For the subsequent development of CaOx crystal masses and stones, the dissolution of previously formed CaP crystals might be an important factor.

Acknowledgements We are very grateful to Ms. Iréne Eriksson for assistance with the EQUIL2 calculations, and to Mr. Bengt-Arne Fredriksson for his unfailing help in the performance of scanning electron microscopy. The study was supported by grants from the Foundation of Maud and Birger Gustavsson.

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