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THE EFFECT OF PH CHANGES ON CRYSTALLIZATION OF CALCIUM SALTS IN SOLUTIONS WITH AN ION-COMPOSITION CORRESPONDING TO THAT IN THE DISTAL TUBULE

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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 by scanning electron microscopy at different degrees of volume reduction. The experiments were carried out with 100 ml samples at different starting pH without and with 20% of dialyzed urine (dU).

The number of crystals increased in response to volume reduction. In solutions without dU, 100 or more crystals with diameters in between 2.4 and 45 µm were observed already at a volume reduction of 40% when the initial pH was 7.28. For solutions with 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; 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.

Key Words: Crystallization, calcium phosphate, calcium oxalate, distal tubule, macromolecules, promotion, inhibition, pH.

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Introduction

The development of urinary stones is the result of a crystallization process that involves nucleation, crystal growth and crystal aggregation. The crystallization is, therefore, influenced by chelators as well as inhibitors and promoters of crystallization either with a low molecular weight, such as citrate, pyrophosphate, and urate, or with a high molecular weight, such as 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 (Lupták *et al*., 1994; Højgaard *et al*., 1996). 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 crystallization of both calcium oxalate (CaOx) and CaP (Robertson *et al*., 1978; Tiselius, 1981, 1983; Berg and Tiselius, 1986; Hallson and Rose, 1989a, b; Grases *et al*., 1993). 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 (Smith, 1976; Wilson *et al*., 1985; Scurr and Robertson, 1986; Baumann *et al*., 1989; Hess *et al*., 1989; Coe *et al*., 1991; Boevé *et al*., 1994).

Since it is reasonable to assume that the first step in the crystallization process leading to pure CaP and mixed CaOx/CaP stones starts in the loop of Henle (Coe and Parks, 1990; Kok, 1995; Asplin *et al*., 1996) or in the distal tubule (Lupták *et al*., 1994; Tiselius *et al*., 1999), 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.

Material 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 (Lupták *et al*., 1994). One liter of this solution was given the following ion composition: 1.04 mmol calcium, 0.41 mmol magnesium, 4.17 mmol phosphate, 0.04 mmol oxalate, 0.35 mmol citrate, 96 mmol sodium, 22.5 mmol potassium and 13.8 mmol sulphate. 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 the 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 (Rector, 1983). 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 dialyzed urine from normal 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 dialyzed urine (dU) was carried out as previously described (Højgaard *et al*., 1996). According to the degree of dilution at different nephron levels, the normal concentration of macromolecules in DTd was assumed to approximately correspond to a 20% concentration of dU. However, it needs to be emphasized 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 (Lupták *et al*., 1994).

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 S.A., Molsheim, France), after which evaporation was carried out in a Büchi Rotavapor RE at 37°C (Büchi AG, Flawil, Switzerland).

Immediately after the evaporation, 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 (Coulter Electronic Ltd, Luton, U.K.). 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 counting non-crystalline material, as our method for particle detection did not allow 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 was obtained from solutions both with and without dU immediately after the crystal counting. The samples were prepared for scanning electron microscopy as previously described (Lupták *et al*., 1994).

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 (Werness *et al*., 1985) 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: $a_{Ca}2+ \cdot a_{PO4}3-$. Similar to our previous observations (Højgaard *et al*., 1996), 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.

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 ^{10}log crystal number and the degree of volume reduction in solutions with dU at all three pH levels, the coefficients of correlation 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 Figure 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 an 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 solutions

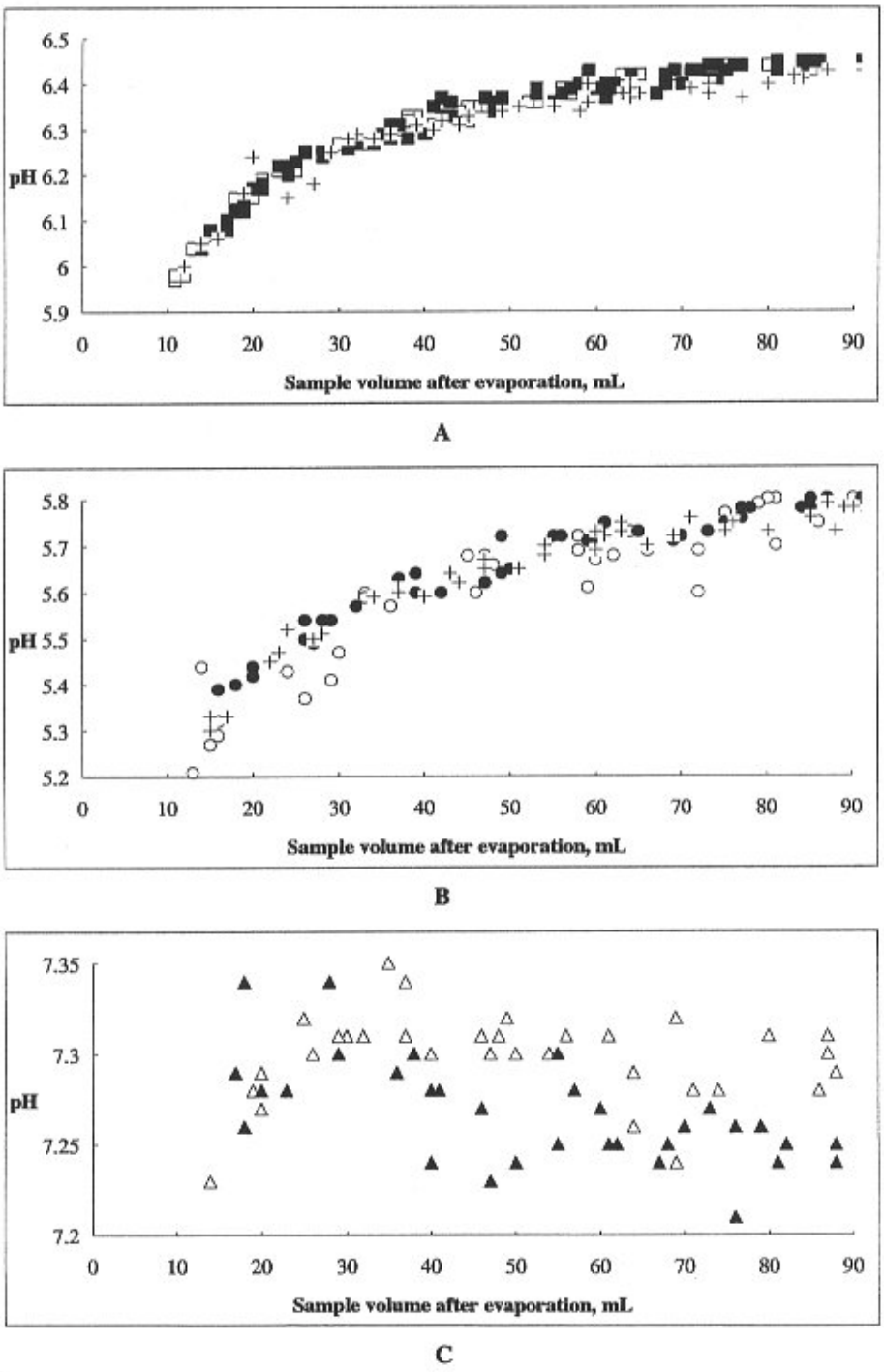

Figure 1. Effects on pH of volume reduction of 100 ml solutions with an initial composition corresponding to that in the distal tubule. (**A**) Solutions with an initial pH of 6.45 without dialyzed urine (hollow squares), with 20% of dialyzed urine (solid squares) and without both dialyzed urine and calcium (+); (**B**) an initial pH of 5.80 without dialyzed urine (hollow circle), with 20% dialyzed urine (solid circles) and without both dialyzed urine and calcium (+); (**C**) an initial pH of 7.28 without dialyzed urine (hollow triangle) and with 20% dialyzed urine (solid triangle).

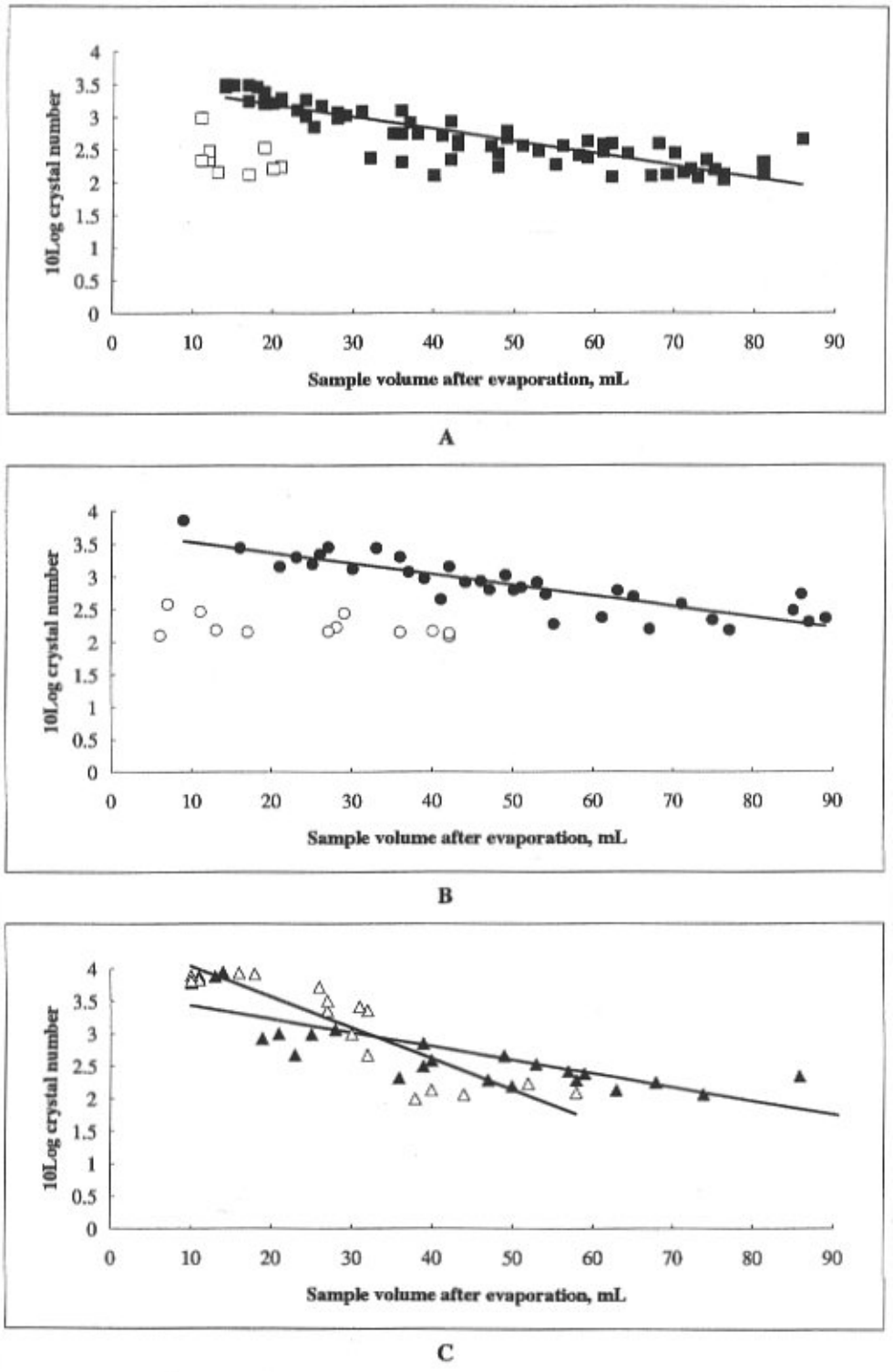

Figure 2. Relationship between the sample volume and the number of crystals after evaporation of 100 ml samples with an initial composition corresponding to that in the distal tubule. (**A**) Solutions with an initial pH of 6.45 without dialyzed urine (hollow squares) and with 20% of dialyzed urine (solid squares); (**B**) an initial pH of 5.80 without dialyzed urine (hollow circle) and with 20% of dialyzed urine (solid circles); (**C**) an initial pH of 7.28 without dialyzed urine (hollow triangle) and with 20% dialyzed urine (solid triangle).

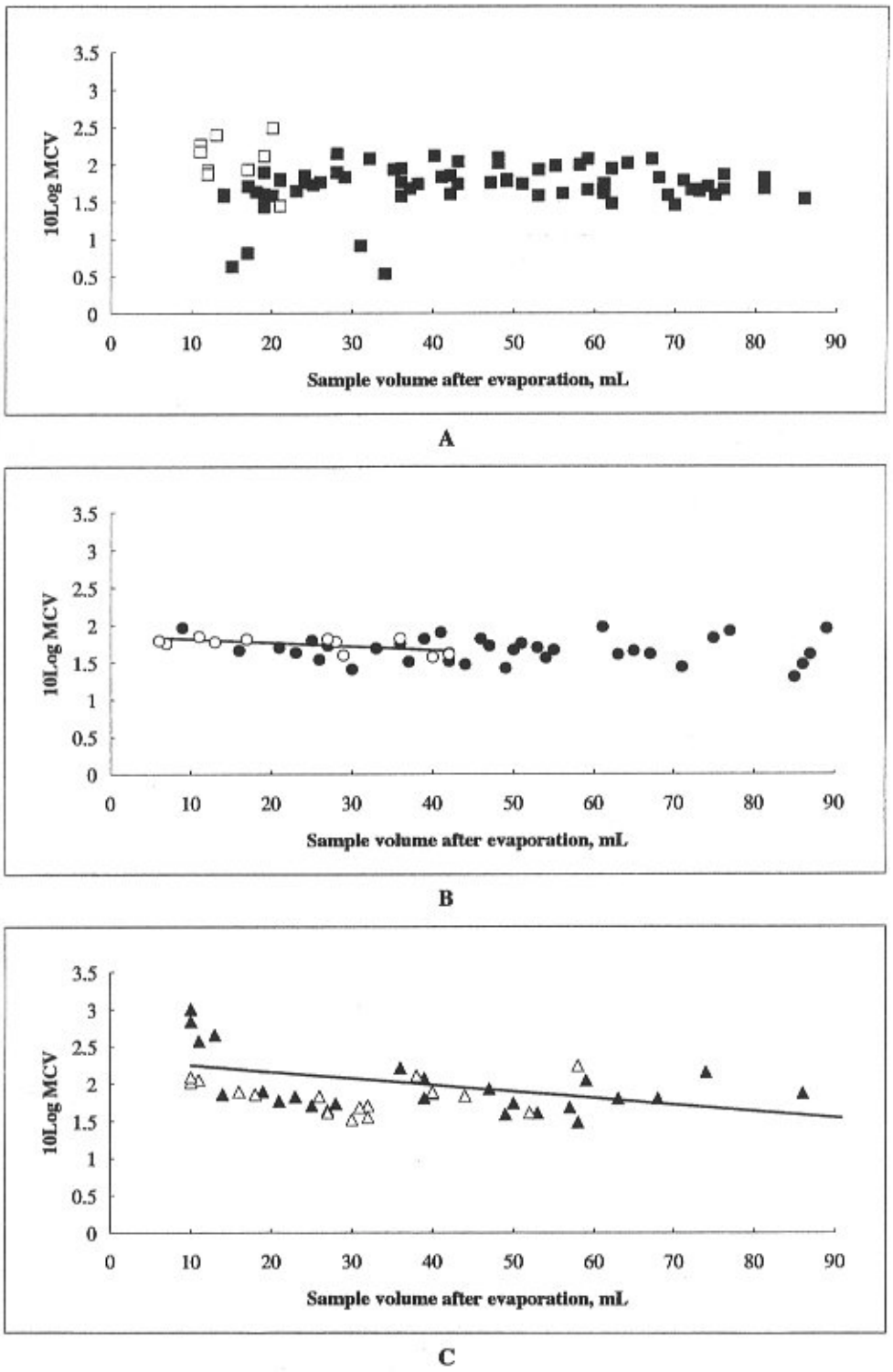

Figure 3. Relationship between the sample volume and the MCV after evaporation of 100 ml samples with an initial composition corresponding to that in the distal tubuli. Evaporation was carried out of samples with (**A**) solutions with an initial pH of 6.45 without dialyzed urine (hollow squares) and with 20% of dialyzed urine (solid squares); (**B**) an initial pH of 5.80 without dialyzed urine (hollow circle) and with 20% of dialyzed urine (solid circles); (**C**) an initial pH of 7.28 without dialyzed urine (hollow triangle) and with 20% dialyzed urine (solid triangle).

Table 1. Mean (standard deviation in parentheses) crystal volume (MCV) in solutions with and without dialyzed urine (dU) at different starting pH.

pH	Solution	MCV
p > 0.05		
5.80	without dU	1.74 (0.10)
5.80	with 20% dU	1.68 (0.17)
p < 0.05		
6.45	without dU	2.07 (0.32)
6.45	with 20% dU	1.71 (0.30)
p > 0.05		
7.28	without dU	1.84 (0.22)
7.28	with 20% dU	1.98 (0.39)

with dU and an initial pH of 7.28 was the relationship between the degree of volume reduction and the ^{10}log MCV statistically significant; however, the slopes were weak.

From Figure 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 both in the presence and 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 MCV recorded at each pH level is 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 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 ($p < 0.02$).

Crystal morphology

Scanning electron microscopy showed a 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 absence of dU (Figs. 4 and 6). The samples were all taken from solutions in which the volume had been reduced to 30-40 ml, except for Figures 6E and 6F, which represent 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) (Figs. 5E and 5F). 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 (Figs. 6E and 6F).

Ion-activity products

At a volume reduction to 40-30 ml, the AP_{CaOx} was 0.41-0.56 x 10^{-8} M^2, which only is about twice the solubility product of 0.23-0.25 x 10^{-8} M^2 (Pak *et al.*, 1975; Tomazic and Nancollas, 1979). Even following the pronounced volume reduction to 10 ml, the AP_{CaOx} was not higher than 1.9 x 10^{-8} M^2. The AP_{CaP} at volume reductions resulting in a significant crystallization ($\geq$ 100 crystals) for solutions with and without dU was 1.01 x 10^{-14} M^2 and 1.78 x 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 x 10^{-14} M^2 and 44.3 x 10^{-14} M^2; and for samples with an initial pH of 7.28, they were 189 x 10^{-14} M^2 and 288 x 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 x 10^{-8} M^2 and 9.6 x 10^{-8} M^2, respectively. This should be compared with a solubility product of 1.9 x 10^{-7} M^2 (Koutsoukos and Nancollas, 1981). The AP_{HAP} at volume reductions resulting in a significant crystallization were all much above the solubility product of 1.87 x 10^{-59} M^9 (Koutsoukos and Nancollas, 1981), (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 compositions assumed to correspond to that in the distal part of the distal tubule (Lupták *et al.*, 1994) were subjected to a volume reduction similar to that of urine during its passage from the distal tubule to the calyx. Urine will be concentrated on average six 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 reabsorption which 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

Table 2. The ion-activity products of calcium oxalate, calcium phosphate, hydroxyapatite and brushite 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	3800000	2.61
7.28	with dU	85	0.18	189	387000	1.72

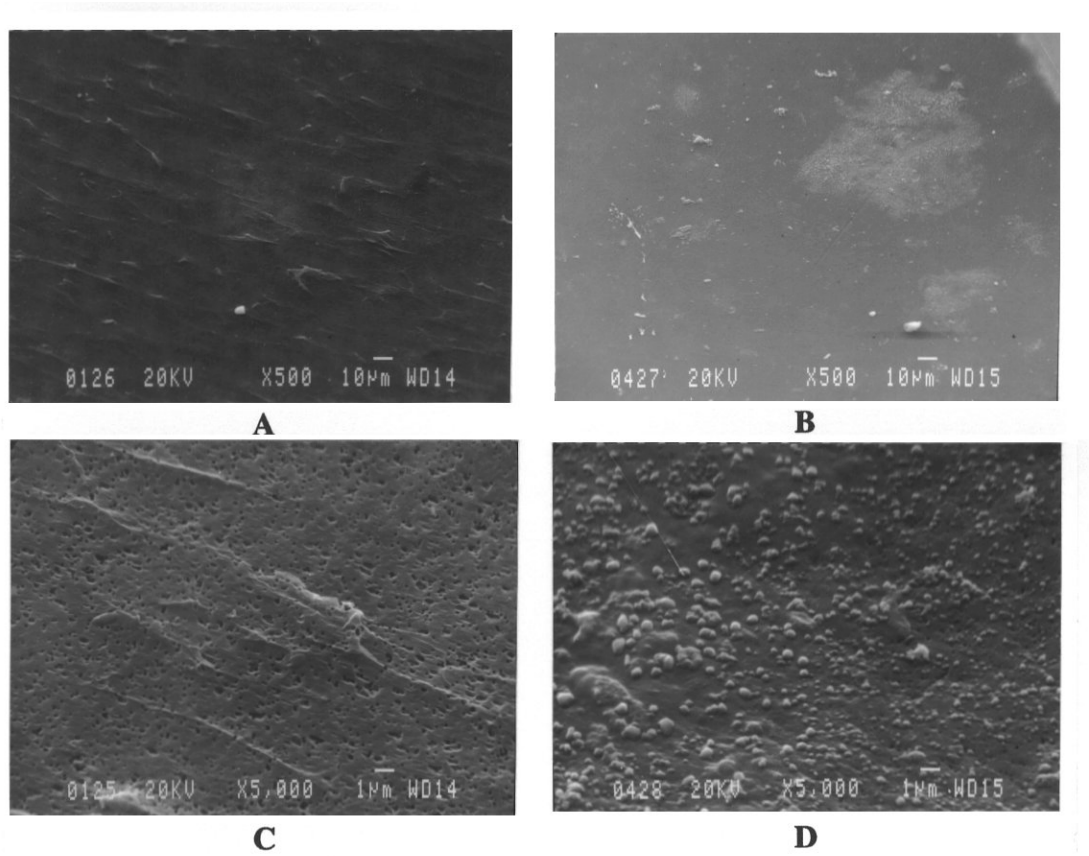

Figure 4. Scanning electron micrographs of the precipitate after evaporation of 100 ml salt solutions with an initial pH of 6.45 to a final volume between 30-40 ml: without (**A** and **C**) and with 20% of dialyzed urine (**B** and **D**) at low (**A** and **B**, bars = 10 µm) and high magnifications (**C** and **D**, bars = 1 µm).

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 holding 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 larger number of crystal nuclei, a higher rate of crystal growth or

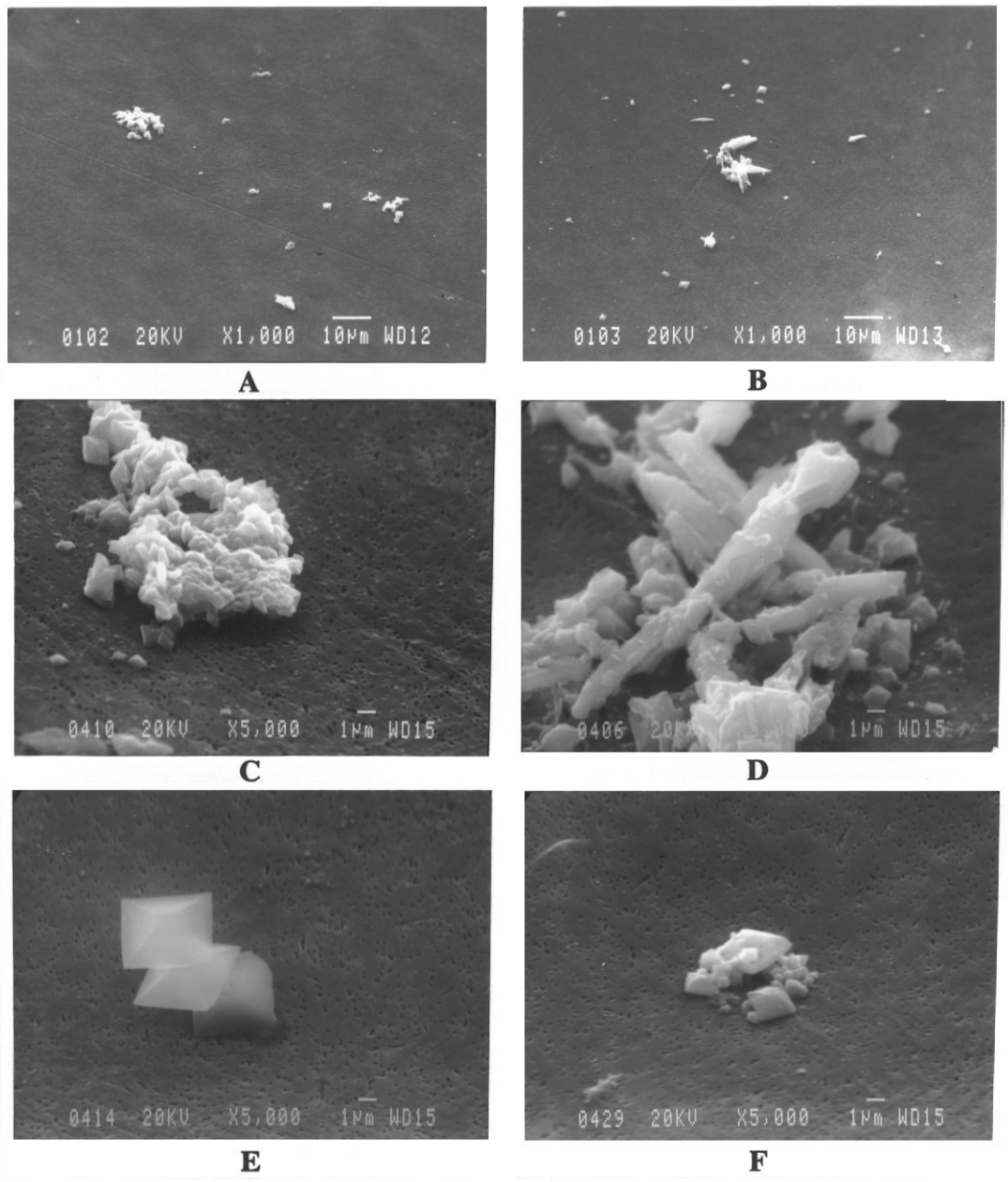

Figure 5. Scanning electron micrographs of the precipitate after evaporation of 100 ml salt solutions with an initial pH of 5.80. Samples evaporated to a final volume between 30 to 40 ml without dialyzed urine (**A**, **C** and **E**), and with 20% of dialyzed urine (**B**, **D** and **F**) at low (**A** and **B**, bars = 10 µm) and high magnifications (**C**, **D**, **E**, and **F**, bars = 1 µm). Calcium oxalate dihydrate crystals are clearly seen at high magnification (**E** and **F**).

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. Another possibility, that deserves some attention, is that CaP crystals that might have formed, dissolve when the pH is reduced and that such an effect is counteracted by protection of CaP crystals with urinary macromolecules.

Recent experimental results from our laboratory have shown that CaP crystals formed at a high pH might induce CaOx crystallization when exposed to urine with a lower pH (Tiselius *et al*., 1999; Højgaard *et al*., unpublished). In samples with an initial pH of 6.45 which is considered as an

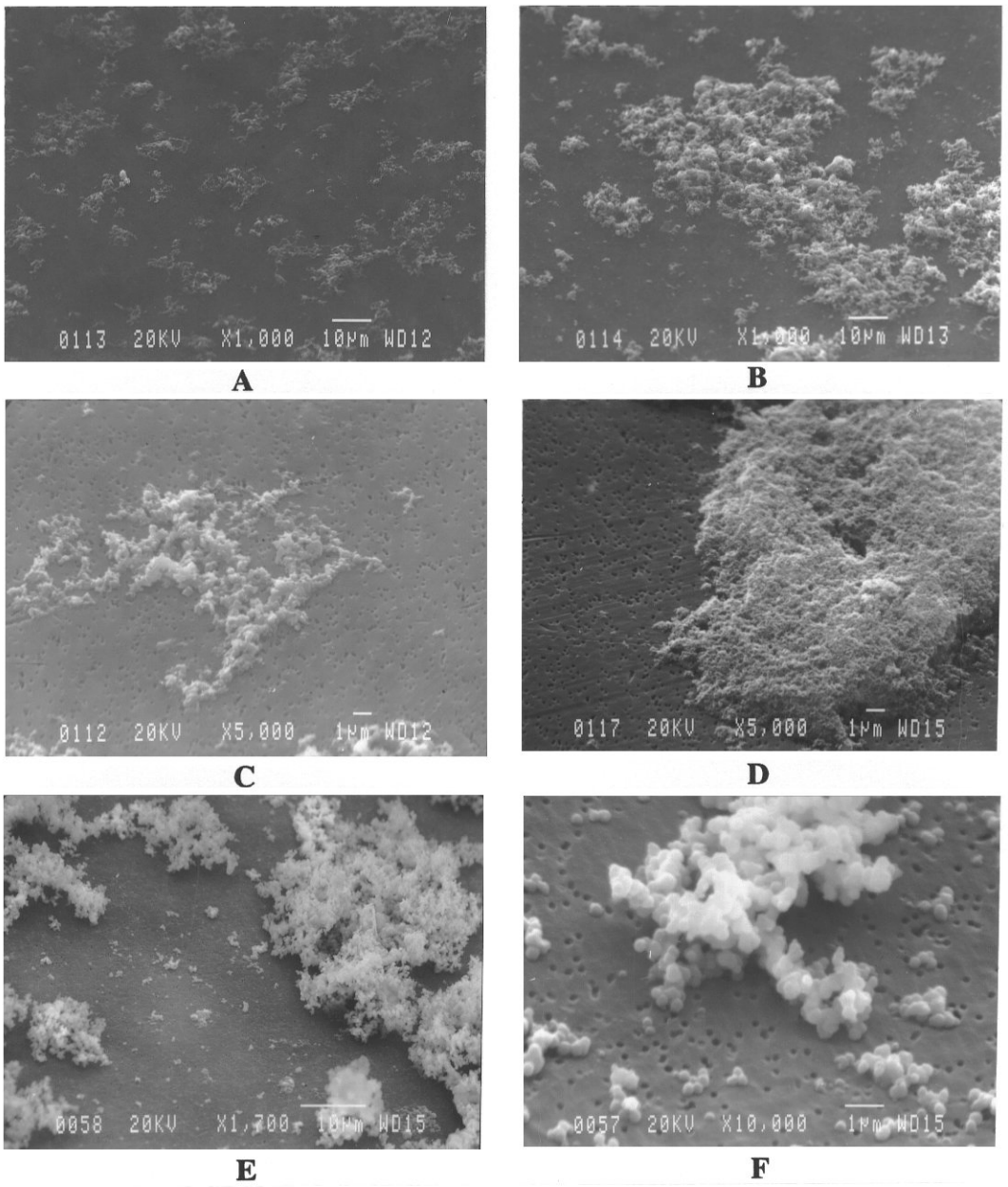

Figure 6. Scanning electron micrographs of the precipitate after evaporation of 100 ml salt solutions with an initial pH of 7.28 to a final volume between 30-40 ml: without dialyzed urine (**A** and **C**) and with 20% of dialyzed urine (**B** and **D**) at low (**A** and **B**, bars = 10 µm) and high magnifications (**C** and **D**, bars = 1 µm). Precipitate after evaporation to a final volume around 20 ml of samples without dU are also shown at low (**E**, bar = 10 µm) and high magnifications (**F**, bar = 1 µm).

average normal pH in the distal tubule, volume reduction resulted in a nucleation of CaP (Højgaard *et al*., 1996). 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 crystal 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 (Tiselius *et al*., 1999).

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 number of crystals were similar with and without dU, at least with a volume reduction of less than 90%. Therefore, it can 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, in the presence of dU, MCV was greatest at pH 7.28 and 6.45.

It should be noted that 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 x 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 CaP dissolution. Although previous studies have suggested a formation product of CaOx around 2 x 10^{-8} M^2 (Robertson *et al.*, 1968), a much lower saturation might be sufficient for inducing secondary CaOx nucleation, particularly in the presence of urinary macromolecules.

One important question is at which level of supersaturation CaP nucleation occurs. This is, of course, highly dependent on which crystal phase that forms, but without exact information in this respect we have found it of value to use the product of $a_{Ca}2+ \cdot a_{PO4}3-$ 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 (Abbona and Franchini-Angela, 1990; Lundager-Madsen and Christensson, 1991). 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 other crystals phases 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 x 10^{-8} M^2 and 9.6 x 10^{-8} M^2, which is below the solubility product of 1.9 x 10^{-7} M^2 (Koutsoukos and Nancollas, 1981). For this reason, it is 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 only were observed following volume reduction of the samples with a starting pH of 5.80. For the subsequent development of CaOx crystal masses and stones, a dissolution of previously formed CaP crystals might be an important factor.

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Discussion with Reviewers

L.C. Cao: Is it possible to really control CaP crystal formation in the upper nephron to prevent CaOx stone formation?
Authors: It probably is very difficult to change urine composition and CaP supersaturation in the upper parts of the nephron. We believe, however, that steps can be taken to avoid periods of excessive alkalinization in the distal part of the distal tubule as well as in the upper part of the collecting duct. Although a high fluid intake results in reduced calcium and phosphate concentrations, this effect undoubtedly will be most pronounced in the collecting duct. To our knowledge, there are no experimental studies from which conclusions in this respect can be drawn and it remains to be shown whether a reduced pH or a reduced concentration of calcium and phosphate in the distal part of the distal tubule or in the proximal part of the collecting duct can reduce the risk of CaOx crystal formation.

F. Grases: Do you think that the crystals formed in the distal tubules can obstruct them? If this would be the case, what type of renal calculi would be formed? 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: Our reply to this question can only be speculative, but it seems unlikely that CaP crystals formed in the distal tubule will obstruct the lumen at this level. Such a mechanism would result in intrarenal CaP concrement formation and this is not a common clinical finding. We rather believe that large CaP aggregates adhere to the tubular wall and, thus, move slowly down the nephron. By such a mechanism, the CaP crystal material can be retained so that it occasionally will be exposed to a urine with a low pH. Dissolution of CaP crystals can, in that way, bring about a high local concentration of calcium in the macromolecular layer surrounding the crystals and provide the necessary prerequisites for CaOx nucleation.