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CRYSTALLIZATION DURING VOLUME REDUCTION OF SOLUTIONS WITH AN ION-COMPOSITION CORRESPONDING TO THAT IN THE DISTAL TUBULI

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Abstract

The effect of macromolecules on the crystallization in solutions with an ion-composition and a pH corresponding to that of urine in the distal part of the distal tubuli was examined by recording the number and volume of crystals in a Coulter Multisizer and by studying the crystal morphology with scanning electron microscopy at different degrees of evaporation. The experiments were carried out with 100 ml samples of salt solutions with and without different concentrations of dialysed urine (dU) from normal subjects. Addition of dU resulted in a greater number of crystals and a reduction in the mean crystal volume (MCV). Under the experimental conditions, the maximal effect of the macromolecules appeared to be accomplished in solutions with an initial dU concentration of 10%.

The precipitate was strongly suggestive of calcium phosphate (CaP) as shown by scanning electron microscopy and Raman spectroscopy. This conclusion was further supported by the ion-activity products of calcium oxalate (CaOx) and different CaP salts in those samples in which crystal formation was recorded. The obtained results give support to the view that macromolecules might exert a promotive effect on the nucleation of CaP. The macromolecules also appear to counteract the development of large CaP crystals, but whether this is due to an inhibition of crystal growth, an inhibition of crystal aggregation or both could not be concluded from these experiments. The way in which CaP crystals initially form in the nephron might be of importance for the subsequent crystallization of CaOx and the formation of CaOx containing stones.

Key Words: Crystallization, calcium phosphate, distal tubule of the nephron, promotion, inhibition, urinary macromolecules.

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Introduction

Although calcium stones formed in the upper urinary tract have calcium oxalate (CaOx) as the dominant constituent, the majority of such stones are mixtures of CaOx and CaP (Pak *et al.*, 1971; Chambers *et al.*, 1972; Prien, 1975; Leusmann *et al.*, 1990). It has furthermore been shown that patients who developed stones with a high fraction of CaP had more frequent stone recurrences than those with a low content of CaP (Larsson *et al.*, 1984a,b; Tiselius and Larsson, 1993). In a recent experimental study, we have shown that CaP was the type of crystal that most easily formed in the proximal and distal tubuli when the concentration of calcium was increased (Lupták *et al.*, 1994).

This brings into focus the important question: whether mixed calcium stones might be a result of a primary nucleation of CaP followed by a heterogeneous deposition of CaOx crystals. Although such a model previously has been proposed by several authors (Pak, 1969; Pak *et al.*, 1971; Nancollas, 1983), the role of CaP to a great extent has been ignored in favour of CaOx, attributable to the pronounced influence that oxalate has in changing the supersaturation of urine.

It is generally accepted that low molecular weight molecules such as citrate, magnesium and pyrophosphate have inhibitory influences on the crystallization of both CaOx and CaP, but the macromolecules might be of even greater importance for the crystallization process (Coe *et al.*, 1980; Resnick *et al.*, 1982; Nakagawa *et al.*, 1983; Rose, 1984; Nakagawa *et al.*, 1985; Ryall *et al.*, 1985; Tiselius *et al.*, 1987; Hess *et al.*, 1989)

Most studies on the crystallization of CaOx and CaP have been carried out in solutions with a composition similar to that in urine from normal subjects or stone formers. This makes the interpretation difficult as it is reasonable to assume that the important pathophysiologic processes occur at a higher level of the nephron where the urine composition is different from that in final urine.

In an attempt to increase our understanding of the initial steps of the crystallization during formation of

mixed calcium stones, we have studied the influence of different concentrations of macromolecules on the crystallization process in solutions with an ion-composition corresponding to that in the distal tubuli.

Material and Methods

A salt solution with an ion-composition assumed to correspond to that of urine in the distal part of the distal tubuli (DTd) was prepared as previously described (Lupták *et al.*, 1994). In this solution the ion-composition was as follows: 1.04 mM calcium, 0.41 mM magnesium, 4.17 mM phosphate, 0.04 mM oxalate, 0.35 mM citrate, 96.0 mM sodium, 22.5 mM potassium and 13.8 mM sulfate. No ammonium was added to avoid the risk of precipitation of ammonium salts.

The pH was measured with a glass electrode (pHM 84; Radiometer, Copenhagen, Denmark) and adjusted to 6.45 (Rector, 1983) immediately before and after evaporation of 100 ml samples with and without dialysed urine (dU). Because pH declined with increasing degree of evaporation, we also measured the pH in salt solutions without calcium but otherwise with the same concentrations of ions as in the standard DTd solution. These measurements were undertaken in order to record pH changes that occurred as a result of volume reduction but not associated to the precipitation of calcium salts.

Pooled dU from normal subjects was used as a source of macromolecules. This urine was collected between 2200 and 0600 hours. The urine was screened for the presence of bacteria, protein and glucose before being included in the pool. The pooled urine was subsequently centrifuged at 2800 rpm for 30 minutes after pH adjustment to 5.8. The supernatants were decanted and saved, and the sediments were combined in one tube. Following repeated centrifugation of the sediment fraction, this supernatant was combined with the previously collected supernatants. The crystals in the sediment were dissolved by addition of 1 mM HCl. After centrifugation and neutralisation, this supernatant was combined with the other urine. The undissolved material in the tube was discarded. The pH of the urine was then readjusted to 5.8. One hundred milliliter aliquots of the pooled urine were then transferred to Spectrapore No. 3 dialysis tubings with an exclusion limit of 3500 Daltons (D) (Spectrum Medical Industries, Inc., Houston, TX). The aim was to get a source of macromolecules with a wide range of molecular weights. Each tubing was placed in 1000 ml of deionized water with magnetic stirring at room temperature. The water was exchanged eight times, once every hour. During the following 15 hours, still under continuous stirring, the samples were kept in deionized water at 4°C, after which the deionized water was replaced by Milli-Q filtered water (Millipore, S.A., Molsheim, France), exchanged six times, once every hour. The samples were subsequently filled up to the original volume with water and stored at -20°C until used in the experiments. Samples of salt solutions with different concentrations of macromolecules were subsequently used in the experiments. According to the concentration of urine in the distal tubuli, the normal concentration of macromolecules at that level was assumed to approximately correspond to that in a 20% solution of dU.

Samples of 100 ml salt solutions without and with different concentrations of dU were passed through Millipore filters with a pore size of 0.22 μm (Millipore S.A.) before evaporation in a Büchi Rotavapor RE (Flawil, Switzerland) at 37°C.

After evaporation to a predetermined solution volume, the number and volume of crystals in the size interval 2.4 to 45 μm were recorded in a Coulter Multisizer with a 100 μm capillary tube (Coulter Electronic Ltd., Luton, U.K.). Recordings were carried out both immediately after the evaporation and after 60 minutes of continuous agitation in an incubator at 37°C. The mean crystal volume (MCV) was calculated as the quotient between the total volume (μm^3) and the total number of crystals.

For studies of crystal morphology, aliquots of 0.5 ml obtained immediately after the evaporation had been completed were passed through polycarbonate membrane filters with a pore size of 0.2 μm (Poretic Corp., Livermore, CA). The filters were rinsed with air, mounted with double stick tape on metallic stubs and dried at room temperature. The crystals were subsequently covered by a 10 nm layer of metallic platinum in an Edwards (Edwards High Vacuum, Crawley, Sussex, U.K.) model 3AM twin electron beam gun sputter coating unit. The crystals were examined in a JEOL (Tokyo, Japan) JSM-840 scanning electron microscope.

Crystal material formed in solutions with final volumes around 35, 15 and less than 10 ml was centrifuged at 1500 rpm for 15 minutes and the sediments were dried at room temperature. Raman spectroscopy, using a IFS 106 (Brucker Instruments, Billerica, MA) unit connected to a Brucker 66V fourier transform spectrometer, was performed on this material to qualitatively examine if it contained CaOx or CaP. The Raman effect is based on the inelastic scattering of photons when a monochromatic light interacts with the matter.

The concentration of ions in solutions with volumes of 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10 ml, corresponding to the volume reduction brought about by evaporation, were used for calculating the ion-activity products of calcium oxalate (AP_{CaOx}), calcium hydrogen phosphate, brushite (AP_{BRU}), hydroxyapatite (AP_{HAP}), amorphous calcium phosphate (AP_{ACP}), and octacalcium

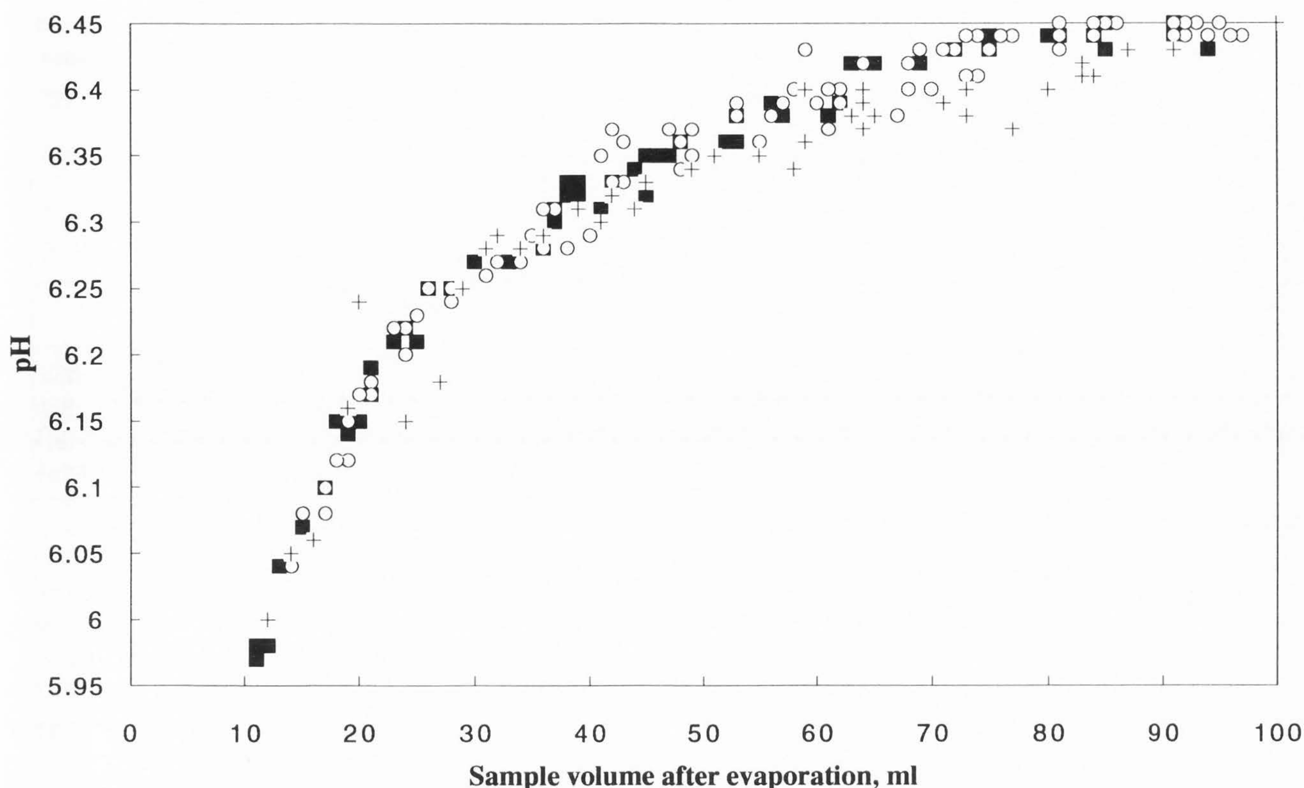

Figure 1. Relationship between the pH and the sample volume after evaporation of 100 ml solutions without dialysed urine (■), with 20% of dialysed urine (○), and without both dialysed urine and calcium (+).

phosphate (AP_{OCP}). For ACP, we used the approximate formula $Ca(PO_4)_{0.74}(H)_{0.22}$ (Christoffersen *et al.*, 1990). The ion activity products were calculated by means of computerized iterative approximation with the EQUIL2 program (Werness *et al.*, 1985). The pH values used in the calculations were those measured at the different degrees of evaporation because there was no difference in pH between solutions with and without calcium (Fig. 1). The concentration of carbon dioxide in our samples was not measured despite the fact that it certainly was reduced as a result of the evaporation. Under the compositional conditions in our system, the effects of carbon dioxide on the ion-activities of calcium, phosphate and oxalate appeared to be of minor importance. For this reason, we set this concentration to zero in all EQUIL2 computations. Complex formation between ions and urinary macromolecules were not accounted for in our calculations. Although macromolecules form complexes with ions in urine (Sheinfeld *et al.*, 1978; Morse and Resnick, 1989; Nishio *et al.*, 1990), their effects in our experimental system are difficult to predict. Sheinfeld *et al.* (1978) showed that the concentration of calcium in urine was reduced by approximately 9% following ultrafiltration, that of phosphate by 12% and that of oxalate by 13%. In addition, there might be a reduced ion strength brought about by a binding also of sodium and other ions. Attributable to the low concentration of macromolecules, the ion-activities in the solutions at the start of the evaporation were probably not more than a few percent lower than those prospected from our calculations. During the evaporation, the effect of macromolecules might be more important as their relative concentrations increase. We have, therefore, most certainly overestimated the different ion-activity products at the end-point of the evaporation. Although it is possible to measure the ion-concentration of calcium directly in the samples and in this way get information on the complexation with macromolecules, such measurements are not useful after the evaporation when the ion-concentration is affected not only by the complexation with macromolecules but also by the precipitation. We believe, however, that the simplification introduced by ignoring the effects of macromolecules in no way has influenced our conclusions.

Formation of crystals was not considered to have occurred until the number of particles in a 100 μl aliquot exceeded 100. This level was chosen to minimise the risk of counting non-crystal material, since a distinction between crystals and other particles was not possible in the Coulter equipment. Based on previous microscopic

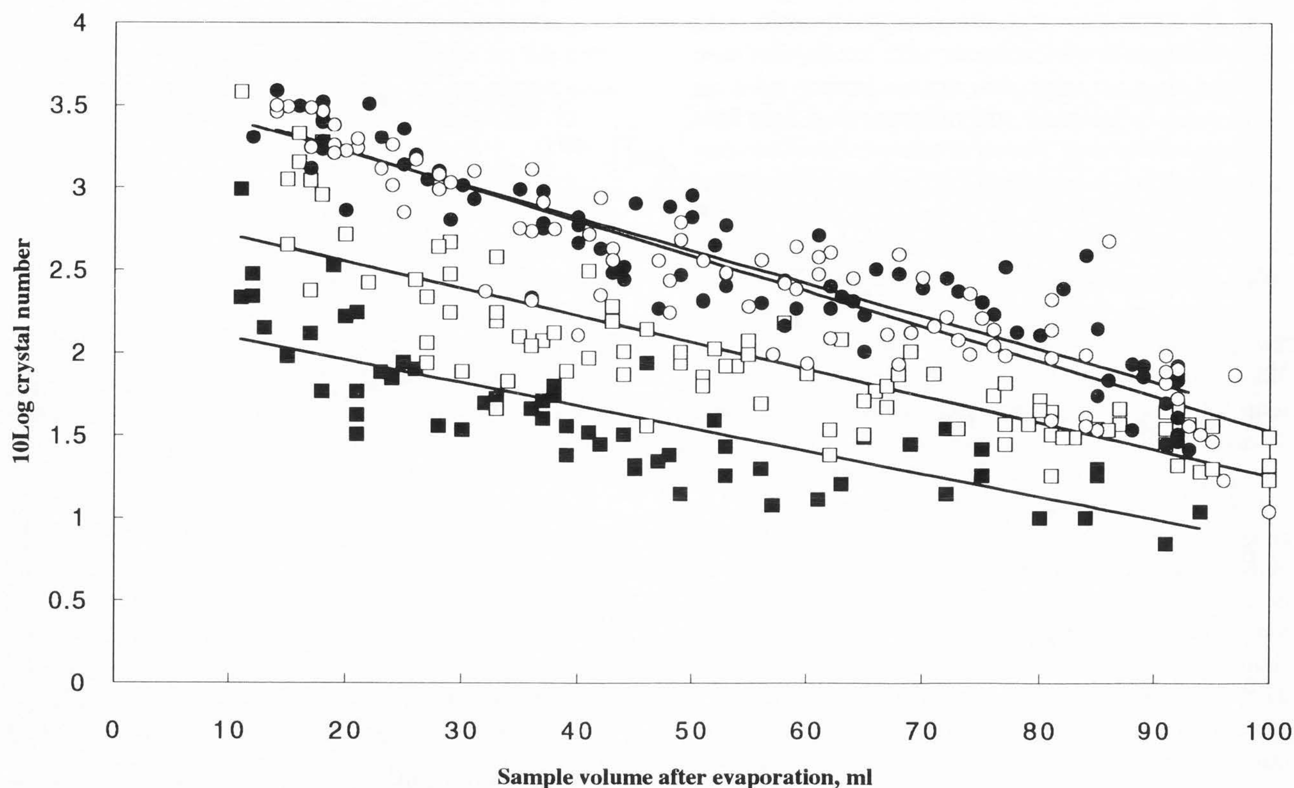

Figure 2. Relationship between the number of crystals and the sample volume after evaporation of 100 ml solutions with an initial composition corresponding to that in the distal tubuli. The evaporation was carried out of samples without dialysed urine (■) or with dialysed urine in concentrations of 5% (□), 10% (●) and 20% (○).

observations, a nucleation is conceivable when 100 crystals has formed.

Statistical analysis

Regression analysis was carried out to record any association between different variables and Student's t-test was used to decide on statistical significance.

Results

Crystal number

There was a linear relationship between the ^{10}log crystal number and the degree of evaporation of solutions without and with dU in concentrations of 5%, 10% and 20% (Fig. 2). The coefficients of correlation were all statistically significant ($p < 0.001$), both for samples analysed immediately after evaporation and for those analysed after 60 minutes (Table 1). As shown in Figure 1, the slopes of the different regression lines were almost parallel in the four series of experiments, and it is evident that in the salt solution without dU, the number of crystals was below the critical limit of 100 until the solution volume had been reduced to 15 ml. With 5% of dU, this limit was already reached at a volume of 55 ml and at a volume of 80 ml in the presence of 10% of dU. There were no major differences between the samples containing 10% and 20% of dU in terms of the number of crystals formed during evaporation.

There were no obvious differences in the relationship between the ^{10}log crystal number and the degree of evaporation between the crystal counts after 0 and 60 minutes, but there was a tendency towards a smaller number of crystals in the 60 minute samples containing 10% and 20% of dU.

Mean crystal volume

Although less pronounced than the relationship between the volume reduction and the crystal number, there was also a significant correlation ($p < 0.025$) between the degree of evaporation and the MCV in samples analysed immediately after the evaporation (Table 1), and the relationship between the degree of evaporation and the ^{10}log MCV is shown in Figure 3. It is evident that, whereas the MCV became greater as the volume of solutions without dU was decreased, the opposite was found for solutions with dU. This effect was most pronounced at the highest concentrations of dU. Similar to the recorded crystal number, there were no major differences in MCV between samples containing 10% and 20% of dU.

Table 1. The number of crystals and the mean crystal volume (MCV) in solutions without and with different concentrations of dialysed urine (dU).

Salt solution	Coefficient of correlation		Slope		p value		Number of experiments
	Immediately after evaporation	After 60 minutes	Immediately after evaporation	After 60 minutes	Immediately after evaporation	After 60 minutes	
Number of crystals							
without dU	0.78	0.64	0.0138	0.0105	< 0.001	< 0.001	60
with 5% dU	0.85	0.82	0.0163	0.0166	< 0.001	< 0.001	93
with 10% dU	0.92	0.86	0.0199	0.0159	< 0.001	< 0.001	85
with 20% dU	0.89	0.91	0.0212	0.0164	< 0.001	< 0.001	90
MCV							
without dU	0.310	0.150	0.0046	0.0021	< 0.025	> 0.05	60
with 5% dU	0.280	0.040	-0.0044	-0.0007	< 0.01	> 0.05	93
with 10% dU	0.470	0.360	-0.0060	-0.0053	< 0.001	< 0.001	85
with 20% dU	0.510	0.180	-0.0075	-0.0024	< 0.001	> 0.05	90

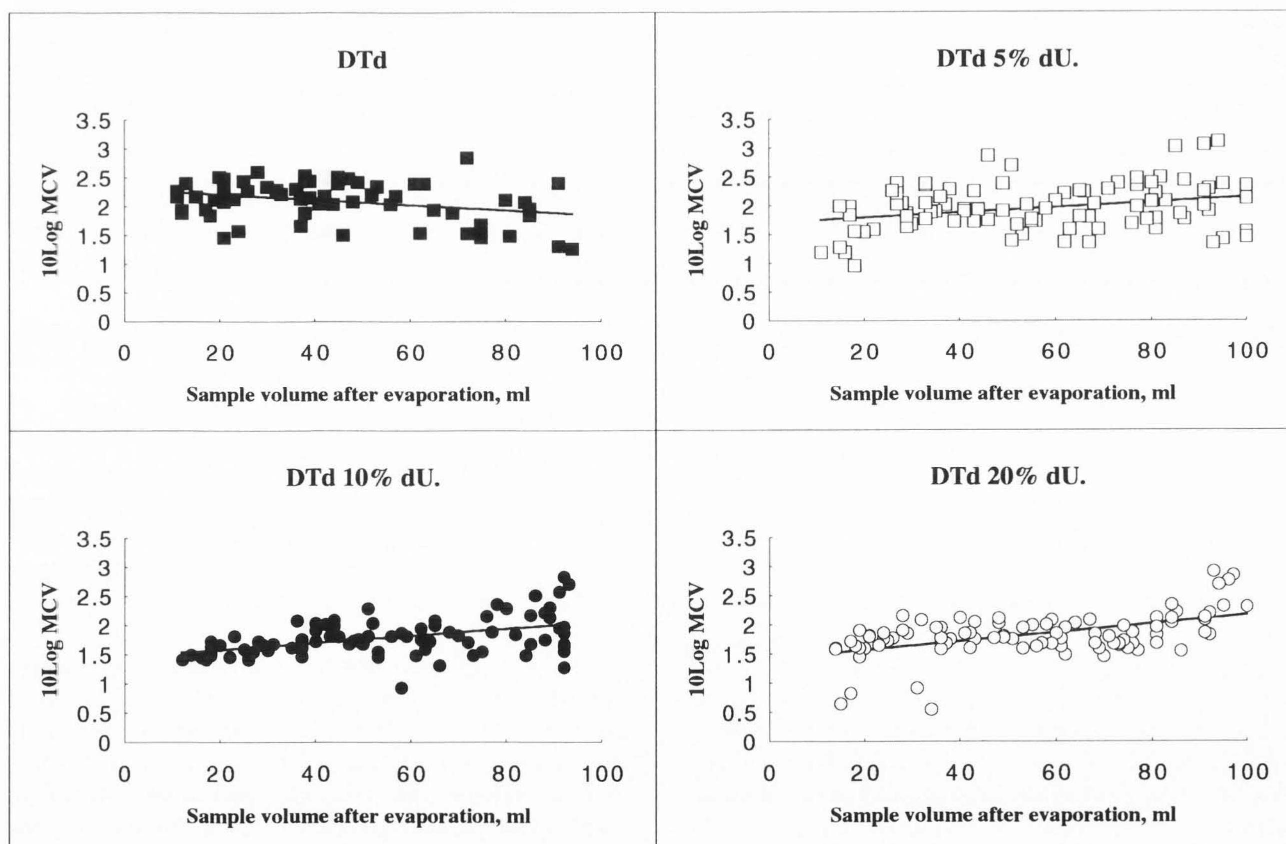

Figure 3. Relationship between the MCV and the sample volume after evaporation of 100 ml solutions with an initial composition corresponding to that in the distal tubuli. The evaporation was carried out of samples without dialysed urine (DTd) or with dialysed urine in concentrations of 5% (DTd 5% dU), 10% (DTd 10% dU) and 20% (DTd 20% dU) of dialysed urine.

Table 2. The ion-activity products at different volume after evaporation of 100 ml samples of salt solutions without dialysed urine (dU) and with a starting pH of 6.45.

volume after evaporation	pH	AP_{CaOx} 10^8 x M^2	AP_{ACP} 10^{12} x M^2	AP_{OCP} 10^{25} x M^8	AP_{HAP} 10^{48} x M^9	AP_{BRU} 10^7 x M^2	ion-strength
100 ml	6.45	0.15	1.79	0.63	0.08	0.74	0.15
90 ml	6.44	0.17	1.77	0.80	0.17	0.85	0.16
80 ml	6.42	0.19	1.75	1.01	0.31	0.97	0.18
70 ml	6.40	0.22	1.74	1.33	0.65	1.13	0.21
60 ml	6.37	0.26	1.73	1.79	1.36	1.35	0.24
50 ml	6.35	0.31	1.78	2.74	3.80	1.71	0.29
40 ml	6.32	0.41	1.89	4.52	11.3	2.27	0.35
30 ml	6.27	0.56	1.94	5.01	17.9	2.90	0.46
20 ml	6.16	0.90	3.09	16.9	54.6	5.30	0.68
10 ml	5.98	1.96	7.05	59.8	64.9	12.1	1.00

Crystal morphology

Examination of the crystals with scanning electron microscopy showed a morphology strongly suggestive of CaP (Fig. 4). When samples with different concentrations of dU were compared, it was evident that the number of crystals was greater in solutions containing 20% of dU than in the solutions without dU.

Raman spectroscopy

Raman spectroscopy of the crystalline material from solutions reduced to volumes of 30-40 ml, 10-20 ml and less than 10 ml showed that it was consistent with calcium phosphate. No calcium oxalate was observed.

Ion-activity products

The saturation with CaOx remained very low until the solution volume had been reduced to below 20 ml (Table 2). With a thermodynamic solubility product for CaOx (SP_{CaOx}) of 0.23 to 0.25 x 10^{-8} M^2 (Pak *et al.*, 1975; Tomazic and Nancollas, 1979), it is evident that the AP_{CaOx} also was below this level until the volume had been reduced to 60 ml, in those samples the AP_{CaOx} was 0.26 x 10^{-8} M^2. A formation product of CaOx (FP_{CaOx}) around 2 x 10^{-8} M^2 was reached only at the most extreme degree of evaporation to 10 ml. The AP_{CaOx} in all other solutions most certainly had a driving force that was too low to be associated with a nucleation of CaOx, even in the presence of macromolecular promoters.

As could be expected, AP_{ACP}, AP_{OCP}, AP_{HAP} and AP_{BRU} increased as a result of the volume reduction. With an SP_{OCP} of 8.3 x 10^{-48} M^8 (Koutsoukos and Nancollas, 1981) and an FP_{OCP} of 2.5 x 10^{-45} M^8 (Robertson *et al.*, 1968), it is evident that the solutions were highly supersaturated with respect to OCP (Table 2). The SP_{HAP} has been reported to be approximately 2.35 x 10^{-59} M^9 (Koutsoukos and Nancollas, 1981) and the AP_{HAP} were in all samples above this level. No information is available on FP_{HAP}. With an SP_{BRU} of 1.87 x 10^{-7} M^2 and an FP_{BRU} of 2.0 x 10^{-6} M^2 (Koutsoukos and Nancollas, 1981; Nancollas, 1982), the solutions were apparently undersaturated with respect to brushite until the volume had been reduced to less than 50 ml (Table 2). The exact level of SP_{ACP} and FP_{ACP} are not known, but the recorded ACP values are close to the reported apparent SP of 2.29 x 10^{-11} M^2.

Discussion

We have previously shown that CaP is the crystal type that most easily forms in the proximal and distal tubuli when the calcium concentration is increased (Lupták *et al.*, 1994). According to these results, a possible site for this crystallization *in vivo* is the distal part of the distal tubuli. Coe and Parks (1990) also have emphasized the importance of CaP crystallization in the nephron, although their data indicated that the highest risk was in the loop of Henle. Irrespective of which level of the nephron that is responsible for the early events in the calcium salt precipitation, a solution with a composition corresponding to that in the distal part of the distal tubuli can be used as an experimental model for studying the physico-chemical crystallization properties in more detail. Although an exact knowledge of the composition of urine at this level of the nephron is unknown, and the concentration of important urine variables certainly is subject to a considerable variation, we assume that under

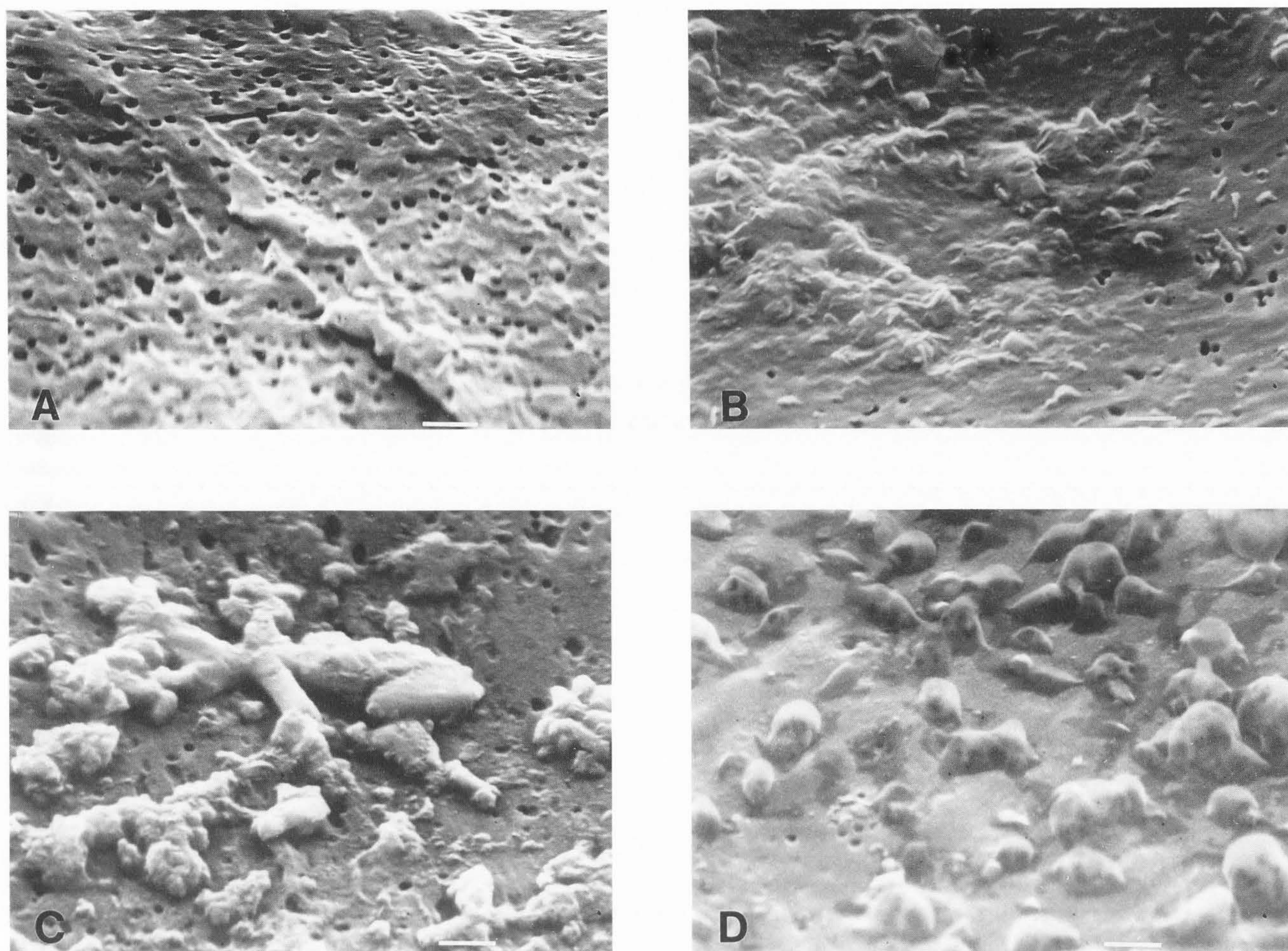

Figure 4. Scanning electron micrographs of the precipitate after evaporation of 100 ml salt solution, without dU (**A**), with 5% dU (**B**), with 10% dU (**C**) and with 20% dU (**D**), down to a volume around 35 ml. Bars = 1 μm.

normal conditions, the ion-composition of urine in the distal part of the distal tubuli roughly corresponds to that in our salt solution with a pH of 6.45, and a concentration of macromolecules approximating that in 10% to 20% of dU. Dialysed bladder urine from normal subjects was used as a source of macromolecules, thereby ignoring the fact that certain macromolecules might have been added to the urine as a result of its passage and storage in the lower parts of the collecting system, whereas others might have been removed during the preparation of dU. All macromolecules are not present in all parts of the nephron, but important macromolecular crystallization modifiers, such as nephrocalcin and Tamm-Horsfall protein, are added to the urine at levels above the distal part of the distal tubuli (Coe and Nakagawa, 1991a; Coe *et al.*, 1991b; Hess, 1992).

In order to measure the crystal nucleation in a Coulter Counter, the solution has to be as free of particles as possible. This cannot be accomplished unless the solutions are filtered or centrifuged. Both these procedures are known to change the composition of samples containing urinary macromolecules (White *et al.*, 1983; Ryall *et al.*, 1989). From this fact, it stands to reason that our source of macromolecules might differ in composition from that normally found in DTd. The most important difference might be in terms of Tamm-Horsfall protein, which is known to be adsorbed at filtration. The relatively high pH and the low ion-strength in the solution at the time of filtration will, however, counteract the self aggregation of the protein (Scurr and Robertson, 1986; Boevé *et al.*, 1994b), a factor that might reduce the filter absorption. Although we are aware of the drawback of our sample preparation, we made no attempts to quantify the loss of proteins, glycoproteins or glycosaminoglycans. The difficulties associated with the sample preparation, however, are not

easily circumvented, and albeit, the composition of macromolecules in our samples might be different from that normally encountered in the nephron; the dialysed urine definitely contains a wide range of important urinary macromolecules.

As urine moves further down the nephron from the distal tubuli, its composition is altered mainly by a concentration process, whereby the altered ion-concentrations and pH might change the crystallization risk in the direction towards CaOx (Lupták *et al.*, 1994). In order to mimic the physiological process, we followed the crystallization during the volume reduction accomplished by evaporation. Crystal formation in urine has previously been studied (Hallson and Rose, 1988) by evaporation to an osmolality of 1200 mosmol/kg in which experiments CaOx crystal formed at pH 5.3 and CaP crystals at pH 6.7.

In the presence of dU, the predetermined critical limit of 100 crystals was reached at a lower degree of evaporation; that is at a lower supersaturation level than without urine. This observation suggests that the macromolecules during the early phase of CaP crystallization act either as promoters of CaP nucleation or as modifiers of CaP crystal aggregation. Although we believe that the most attractive interpretation of the increased number of crystals recorded with increasing concentrations of dU is an effect on nucleation, we cannot completely rule out the possibility that this is a result of differences in crystal aggregation. This question can, however, only be solved following analysis of the size distribution of even the smallest crystals formed and this is so far not technically possible. An inhibition of crystal aggregation can be expected to result in a greater number of crystals which grow in the supersaturated solution until they attain a measurable size, whereas a promotion of aggregation more rapidly will form crystal masses with a size that enables detection in the Coulter Counter. Although the MCV increased with increasing concentration of the urine-free solutions, the results in Figure 3 show a reduced MCV both with increased concentrations of dU and with decreased sample volumes. These observations apparently make crystal aggregation less likely as an important factor during the early stage of CaP crystallization, but might rather reflect a promoted nucleation in combination with an inhibition of crystal growth. The effects of dialysed urine on the aggregation of CaP crystals in solutions with a DTd composition is subject to further studies.

The regression lines for the $10_{\log}$ crystal number (Fig. 2) were almost parallel for all four series of experiments, and the results with 10% and 20% of dU were almost identical. These findings indicate that the macromolecules under these experimental conditions had a maximal effect in a concentration of dU around 10%.

Macromolecules isolated from calcium stones, particularly those containing gamma-carboxyglutamic acid have been demonstrated to have a high affinity for calcium ions (Lian *et al.*, 1977; Nishio *et al.*, 1990) and in the presence of urine, the number of CaOx crystals that formed was greater than those formed in solutions without urine (Burns and Finlayson, 1980; Drach *et al.*, 1982; Nishio *et al.*, 1990). Ultrafiltration of urine also resulted in an 85% reduction of CaOx crystallization and the promoting effect was linked to a macromolecule with a molecular weight above 100 kD (Rose and Sulaiman, 1984). A promoting effect of macromolecules on CaOx nucleation has been demonstrated by several groups (Drach *et al.*, 1980; Yoshioka *et al.*, 1989; Grases *et al.*, 1992). Most studies on the macromolecular promotion of crystallization have been carried out in systems prone to CaOx crystallization. It is, however, reasonable to assume that there is a similar mechanism for CaP, and that a nucleation of CaP might be induced following accumulation of calcium and phosphate to areas on macromolecules that have a specific affinity for these ions. Werness and coworkers reported that HAP crystals in urine were embedded in an amorphous matrix (Werness *et al.*, 1981) and matrix protein was found to induce precipitation of brushite (Pak and Ruskin, 1970).

The assumption that the precipitate in our experiments consisted of CaP crystals was supported by the levels of the ion-activity products of calcium salts, showing that the solutions were undersaturated with respect to CaOx but highly supersaturated with respect to ACP, OCP and HAP. Only at an extreme volume reduction might the AP_{CaOx} theoretically exceed the driving force necessary for a heterogeneous deposition of CaOx on CaP (Table 2). We did not, however, detect any obvious evidence for such a crystallization in any of our samples. The most likely reason for this is that the precipitation of CaP starts much earlier during the evaporation whereby calcium is consumed. In this way, the driving force is reduced and not high enough for starting a heterogeneous growth of CaOx on preformed crystals of CaP. Further evidence of the chemical nature of the crystals was obtained with scanning electron microscopy and Raman spectroscopy.

The importance of CaP in the formation of stones containing CaOx and CaP has recently attracted a great deal of interest. Achilles *et al.* (1994) used a gel crystallization system in which nucleated CaP spherulites induced heterogeneous crystallization with CaOx. Spherulites of CaP is also a common constituent of the nucleus of CaOx-containing stones (Blaschke and Schwandt, 1978; Leusmann, 1981; Khan and Hackett, 1987) and have also been demonstrated in renal tissue (Meyer-Jurgens *et al.*, 1981). The ease by means of which CaOx can grow on crystals of CaP has been

shown by several authors (Nancollas and Gardner, 1974; Meyer *et al.*, 1975, 1977; Pak, 1981; Berg and Tiselius, 1989; Grases *et al.*, 1989).

Our findings support the hypothesis that nuclei of CaP might be the primary nidus of stones containing CaOx, inasmuch as CaP was the crystal type that first appeared during volume reduction of solutions with an ion-composition corresponding to that of urine in the distal part of the distal tubuli. There is evidence that macromolecules of urine participate in this process, initially as promoters of CaP nucleation and subsequently as modifiers of the crystal growth and crystal aggregation. The principles applied in these experiments might be useful in further studies aimed at an increased understanding of the mechanisms behind calcium stone formation and in exposing any abnormalities between stone formers and normal subjects at urine concentrations relevant to the initiation of salt precipitation.

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References

Achilles W, Jöckel U, Schaper A, Ulshöfer B, Riedmiller H (1994) Formation of urinary stones *in vitro*: Growth of calcium oxalate on spherolites of calcium phosphate in gel. In: Urolithiasis 2. Ryall R, Bais R, Marshall VR, Rofe AM, Smith LH, Walker VR (eds.). Plenum Press, New York. pp. 161-165.

Berg C, Tiselius HG (1989) The effects of citrate on hydroxyapatite induced calcium oxalate crystallization and on the formation of calcium phosphate crystals. Urol Res **17**: 167-172.

Blaschke R, Schwandt W (1978) Kugelförmige Calciumphosphat-Konkremente im Harn und in der Kernzone von Oxalatsteinen (Spherical phosphate concrements in urine and the nucleus of calcium oxalate stones). Fortschr Urol Nephrol **11**: 84.

Boevé ER, Cao LC, De Bruijn WC, Robertson WG, Romijn JC, Schröder H (1994b) Zeta potential distribution on calcium oxalate crystal and Tamm-Horsfall protein surface analyzed with doppler electrophoretic light scattering. J Urol **152**: 531-536.

Burns JR, Finlayson B (1980) The effect of seed crystals on calcium oxalate nucleation. Invest Urol **18**: 133-136.

Chambers A, Hodgkinson A, Hornung G (1972) Electron probe analysis of small urinary tract calculi. Invest Urol **2**: 376-384.

Christoffersen MR, Christoffersen J, Kibalczyc W (1990) Apparent solubilities of two amorphous calcium phosphates and of octacalcium phosphate in the temperature range 30 to 42°C. J Crystal Growth **106**: 349-354.

Coe FL, Nakagawa Y (1991a) Inhibitors within the nephron. Am J Kidney Dis **17**: 407-413.

Coe FL, Parks JH (1990) Defenses of an unstable compromise: Crystallization inhibitors and the kidney's role in mineral regulation. Kidney Intl **38**: 625-631.

Coe FL, Margolis HC, Deutsch LH, Strauss AL (1980) Urinary macromolecular crystal growth inhibitors in calcium nephrolithiasis. Mineral Electrolyte Metab **3**: 268-275.

Coe FL, Parks JH, Nakagawa Y (1991b) Protein inhibitors of crystallization. Seminars Nephrol **11**: 98-109.

Drach GW, Thorson S, Randolph A (1980) Effects of urinary organic macromolecules on crystallization of calcium oxalate: Enhancement of nucleation. J Urol **123**: 519-523.

Drach GW, Sarig S, Randolph AD, Torson S (1982) The paradox of inhibition and enhancement of the formation of urinary stones. Urol Res **10**: 165-168.

Grases F, Gil JJ, Conte A (1989) Urolithiasis inhibitors and calculus nucleation. Urol Res **17**: 163-166.

Grases F, Masarova L, Söhnel O, Costa-Bauza A (1992) Agglomeration of calcium oxalate monohydrate in synthetic urine. Br J Urol **70**: 240-246.

Hallson PC, Rose GA (1988) Procedure for measurement of calcium oxalate and phosphate crystals in urine. Br J Urol **61**: 191-202.

Hess B (1992) Tamm-Horsfall glycoprotein - inhibitor or promotor of calcium oxalate monohydrate crystallization processes? Urol Res **20**: 83-86.

Hess B, Nakagawa Y, Coe FL (1989) Inhibition of calcium oxalate monohydrate crystal aggregation by urine proteins. Am J Physiol **257**: F99-F106.

Khan SR, Hackett RL (1987) Role of scanning electron microscopy and x-ray microanalysis in the identification of urinary crystals. Scanning Microsc **1**: 1405-1411.

Koutsoukos PG, Nancollas GH (1981) Crystal growth of calcium phosphates - epitaxial considerations. J Crystal Growth **53**: 10-19.

Larsson L, Öhman S, Sörbo B, Tiselius HG (1984a) Wet chemical analysis of small (< 20 mg) urinary calculi. In: Urinary Stone. Ryall R, Brochis JG, Marshall V, Finlayson B (eds.). Churchill Livingstone, London, U.K. pp. 339-342.

Larsson L, Öhman S, Sörbo B, Tiselius HG (1984b) A method for quantitative analysis of urinary calculi. Clin Chim Acta **140**: 9-20.

Leusmann DB (1981) Erste zusammenfassende Ergebnisse der kombinierten Phasen - und Gefügeanalyse von Harnsteinen mittels Röntgenbeugung und Rasterelektronmikroskopie (First collected experience of combined phase- and structural analysis of urinary concrements with X-ray diffraction and Raster electron microscopy). Fortschr Urol Nephrol **17**: 275-305.

Leusmann D, Blaschke R, Schwandt W (1990) Results of 5035 stone analyses: A contribution to epidemiology of urinary stone disease. Scand J Urol Nephrol **24**: 205-210.

Lian JB, Prien ELJ, Glimscher MJ, Gallop PM (1977) The presence of protein bound gamma-carboxyglutamic acid in calcium containing renal calculi. J Clin Invest **59**: 1151-1157.

Lupták J, Jensen HB, Fornander AM, Højgaard I, Nilsson MA, Tiselius HG (1994) Crystallization of calcium oxalate and calcium phosphate at supersaturation levels corresponding to those in different parts of the nephron. Scanning Microsc **8**: 47-61.

Meyer JL, Bergert JH, Smith LH (1975) Epitaxial relationships in urolithiasis: The calcium oxalate monohydrate-hydroxyapatite system. Clin Sci Mol Med **49**: 369-374.

Meyer JL, Bergert JH, Smith LH (1977) Epitaxial relationships in urolithiasis: The brushite-whewellite system. Clin Sci Mol Med **52**: 143-148.

Meyer-Jurgens UB, Blaschke R, Maar K (1981) Neuere elektronmikroskopische Untersuchungen an calciumphospjarhaltigen Sphärolithen in Niere und Harn (Recent electron microscopic examination of calcium phosphate containing spherulites in kidney and urine). Fortschr Urol Nephrol **17**: 226-230.

Morse RM, Resnick MI (1989) A study of the incorporation of urinary macromolecules onto crystals of different mineral composition. J Urol **141**: 641-644.

Nakagawa Y, Abram V, Kézdy FJ, Kaiser ET, Coe FL (1983) Purification and characterization of the principal inhibitor of calcium oxalate monohydrate growth in human urine. J Biol Chem **258**: 12594-12600.

Nakagawa Y, Abram V, Parks JH, Lau HS-H, Kawooya JK, Coe FL (1985) Urine glycoprotein crystal inhibitors. Evidence for a molecular abnormality in calcium oxalate nephrolithiasis. J Clin Invest **76**: 1455-1462.

Nancollas GH (1982) Phase transformation during precipitation of calcium salts. In: Biological Mineralization and Demineralisation. Nancollas GH (ed.). Springer-Verlag, Berlin, Germany. pp. 79-99.

Nancollas GH (1983) Crystallization theory relating to urinary stone formation. World J Urol **1**: 131-137.

Nancollas GH, Gardner GL (1974) Kinetics of crystal growth of calcium oxalate monohydrate. J Cryst Growth **21**: 267-276.

Nishio S, Kavanagh JP, Faragher EB, Garside J, Blacklock NJ (1990) Calcium oxalate crystallization kinetics and the effects of calcium and gamma-carboxyglutamic acid. Br J Urol **66**: 351-356.

Pak CYC (1969) Physicochemical basis for formation of renal stones of calcium phosphate origin: Calculation of the degree of saturation of urine with respect to brushite. J Clin Invest **48**: 1914-1922.

Pak CYC (1981) Potential etiologic role of brushite in the formation of calcium (renal) stones. J Cryst Growth **53**: 202-208.

Pak CYC, Ruskin B (1970) Calcification of collagen by urine *in vitro*: Dependence on the degree of saturation of urine with respect to brushite. J Clin Invest **49**: 2353-2361.

Pak CYC, Eanes ED, Ruskin B (1971) Spontaneous precipitation of brushite: Evidence that brushite is the nidus of renal stones originating as calcium phosphate. Proc Natl Acad Sci **68**: 1456-1460.

Pak CYC, Ohata M, Holt K (1975) Effect of diphosphonate on crystallization of calcium oxalate *in vitro*. Kidney Intl **7**: 154-160.

Prien L (1975) Studies on urolithiasis. III. Physicochemical principles in stone formation and prevention. J Urol **73**: 627-652.

Rector FC Jr (1983) Acidification of the urine. In: Renal Physiology. Section 8, Handbook of Physiology. Geiger SR, Orloff J, Berliner RW (eds.). American Physiological Society, Washington DC. pp. 431-454.

Resnick MI, Sorell ME, Baily JA, Boyce WH (1982) Inhibitory effects of urinary calcium-binding substances on calcium oxalate crystallization. J Urol **127**: 568-571.

Robertson WG, Peacock M, Nordin BEC (1968) Activity products in stone-forming and non-stone-forming urine. Clin Sci **34**: 579-594.

Rose GA, Sulaiman S (1984) Effects of different fractions of macromolecules upon triggering of calcium oxalate and calcium phosphate crystal. Urol Int **39**: 147-149.

Ryall RL, Hibberd CM, Marshall VR (1985) A method for studying inhibitory activity in whole urine. Urol Res **13**: 290-298.

Ryall Rl, Harnett RM, Hibberd CM, Edyvane KA, Marshall VR (1989) The effect of macromolecules on the crystallization of calcium oxalate in human urine. In: Urolithiasis. Walker VR, Sutton RAL, Cameron ECB, Pak CYC, Robertson WG (eds.). Plenum Press, New York. pp. 133-135.

Scurr DS, Robertson WG (1986) Modifiers of calcium oxalate crystallization found in urine. III. Studies on the role of Tamm-Horsfall mucoprotein and of ionic strength. J Urol **136**: 505-507.

Sheinfeld J, Finlayson B, Reid F (1978) Ultrafiltration evidence of ion binding by macromolecules in urine.

Invest Urol **15**: 462-464.

Tiselius HG, Larsson L (1993) Calcium phosphate: An important crystal phase in patients with recurrent calcium stone formation. Urol Res **21**: 175-180.

Tiselius HG, Fornander AM, Nilsson MA (1987) Inhibition of calcium oxalate crystallization in urine. Urol Res **15**: 83-86.

Tomazic B, Nancollas GH (1979) The kinetics of dissolution of calcium oxalate hydrates. J Cryst Growth **46**: 355-361.

Werness PG, Bergert JH, Smith LH (1981) Crystalluria. J Cryst Growth **53**: 161-181.

Werness PG, Brown CM, Smith LH, Finlayson B (1985) EQUIL2: Basic computer program for the calculation of urinary saturation. J Urol **134**: 1242-1244.

White DJ Jr, Christoffersen J, Herman TS, Lanzalaco AC, Nancollas GH (1983) Effects of urine pretreatment on calcium oxalate crystallization inhibition potentials. J Urol **129**: 175-179.

Yoshioka T, Koide T, Utsunomiya M, Itatani H, Oka T, Sonoda T (1989) Possible role of Tamm-Horsfall glycoprotein in calcium oxalate crystallization. Br J Urol **64**: 463-467.

Discussion with Reviewers

Y. Nakagawa: The authors observed a pH change in the solution from 6.45 to 5.95 after a 10-fold concentration. Should this be corrected to the original pH value of 6.45 or can this be ignored?

Authors: As shown in Figure 1, the pH was reduced as a result of the volume reduction. This pH change reduces the supersaturation with calcium phosphate by decreasing the concentration of PO_4^{3-} ions. A pH adjustment during the volume reduction is, however, not easily accomplished without seriously interfering with the crystallization process. Furthermore, a combined reduction of the volume and the pH is the net effect of the intratubular processing of urine. The compositional changes that occurred in our experimental system during evaporation were thus considered to reflect the normal renal physiology; for this reason, we accepted the pH shift as a valuable property of our system.

Y. Nakagawa: Small molecular weight compounds were eluted during dialysis of urine. Uric acid could be a nucleus of calcium stones. Can the authors add uric acid in the system?

Authors: Although urate might possibly play a role in the development of calcium oxalate crystals, its contribution to the crystallization process at the concentration levels in this part of the nephron is doubtful. Crystals of uric acid will certainly not precipitate in the pH interval of our experimental system and the prerequisites for crystallization of sodium urate will not be met until the volume has been excessively reduced. We have not added urate to our system because hyperuricosuria is an uncommon finding among our calcium stone formers. The question is interesting in that precipitated urate might provide a surface for binding of macromolecules and thus cause an induction of calcium salt crystallization.

S. Nishio: Did you examine the macromolecules in the crystals, or what kind of urinary substances (UMM) do you think are contained in CaP crystals made in the physiological concentrations of inorganic ions (Ca, P, Mg) and UMM?

Authors: We made no attempts to analyse the type of macromolecules that were associated with the calcium salt precipitate. Although only speculative, it appears reasonable to assume that the same glycoproteins and glycosaminoglycans considered to be associated with the urinary precipitation of calcium oxalate also might take part in the precipitation of calcium phosphate. Further studies of this important question are, however, mandatory.

F. Grases: The authors state that CaP can easily form in the distal tubuli. Can these crystals be dissolved as a consequence of the subsequent acidification of the urine?

Authors: This issue is presently subject to experimental studies in our laboratory. Conclusive results are not yet available, but it is beyond every doubt that calcium phosphate crystals formed in this way can dissolve in acid urine. A pH of 5.95 is, however, apparently too high for such an effect. The type of calcium phosphate, its size and state of aggregation are probably important determinants in this respect.

F. Grases: Which role do you think that CaP formed in the nephron has for CaOx stone formation?

Authors: The answer to this question can only be speculative because the experiments carried out in our laboratory so far have not been designed to address all aspects of this process. We believe, however, that crystals of CaP formed in the nephron grow and aggregate to crystal masses of variable size. These crystal masses might provide a basis for the heterogeneous deposition of CaOx in the collecting ducts, probably in the distal part close to the papilla. An attractive view is that these crystals are adhered to the tubular surface and retained in this way during their further development. The macromolecules might also play an important role for the crystal retention, but we have no experimental support for such an effect.

A. Rogers: Why did you choose to use urine collected between the hours of 2400 and 0600 as opposed to a full

24-hour collection?

Authors: For several years, an 8-hour night urine sample has been a part of our programme for investigation of stone formers. The main reason for introducing this collection period was to get a sample collected during a period sufficiently short to avoid compositional changes as a result of storage. These samples are taken care of within one or two hours following the completion of the collection. It appeared reasonable to apply these routines also to the normal subjects delivering urine for preparation of urinary macromolecules. The night urine generally is more concentrated than the day urine and by using these samples inappropriate sample dilution was avoided.

A. Rogers: Why did you evaporate the urine samples to a pre-determined volume as opposed to a pre-determined osmolality?

Authors: First, all the samples had a known ion-composition, and we do not think that there is any advantage of going the way over osmolality. Although this might be useful for samples with unknown composition, this is probably not so in this kind of experiments. Second, we were very interested in having a specific volume for calculation of the ion-activities.