

# The Effect of Preincubation of Seed Crystals of Uric Acid and Monosodium Urate with Undiluted Human Urine to Induce Precipitation of Calcium Oxalate *in Vitro*: Implications for Urinary Stone Formation

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Accepted July 22, 2002

## Abstract

**Background:** Previous studies demonstrated that crystals of uric acid (UA) and sodium urate (NaU) can induce the precipitation of calcium oxalate (CaOx) from its inorganic metastable solutions, but similar effects have not been unequivocally shown to occur in urine. The aim of this investigation was to determine whether preincubation of these seeds with urine alter their ability to induce deposition of CaOx from solution and thus provide a possible explanation for discrepancy of results obtained from aqueous inorganic solutions and undiluted urine.

**Materials and Methods:** The effects of commercial seed crystals of UA, NaU and CaOx (6 mg/100 ml) on CaOx crystallization were compared in a solution with the same crystals that had been preincubated for 3 hours with healthy male urine. A Coulter Counter was used to follow the crystallization and decrease in soluble <sup>14</sup>C-oxalate was measured to determine the deposition of CaOx. The precipitated particles were examined by scanning electron microscopy (SEM). The preincubated seeds were demineralized and proteins released were analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

**Results:** Analysis of <sup>14</sup>C-oxalate data revealed that while treated UA seeds did not affect CaOx deposition, those of NaU and CaOx inhibited the process by 51.9 ( $p \leq 0.05$ ) and 8.5% ( $p \leq 0.05$ ) relative to their respective untreated

counterparts. Particle size analysis showed that the average modal sizes of particles precipitated in the presence of treated seed crystals of UA, NaU, and CaOx were very similar to those deposited in the presence of their respective untreated controls. These findings were confirmed by SEM which also showed that seed crystals of UA and NaU, untreated and treated, were attached like barnacles upon the surfaces of CaOx crystals which themselves were bigger than those precipitated in the presence of CaOx seeds. SDS-PAGE analysis of the demineralized treated seeds showed that they all selectively adsorbed urinary proteins, and perhaps other urinary macromolecules and low molecular weight components, on their surface.

**Conclusions:** It was concluded that preincubation with urine, such as occurs *in vivo*, only slightly reduces the ability of seed crystals of CaOx, but not of UA, to cause deposition of CaOx. The most striking effect was on NaU seeds where the preincubation quite dramatically attenuated their promotory effect on the mineral deposition. This may explain the discrepancy between findings of studies carried out in inorganic solutions and undiluted human urine. This stresses the invalidity of directly extrapolating results obtained in inorganic solutions to likely effects in urine and more importantly, on stone formation.

## Introduction

Hyperuricosuria has long been documented as a predisposing factor to calcium oxalate (CaOx) stone formation. This association is so strong that some clinicians even regard "hyperuricosuric calcium oxalate" stone disease as a separate clinical entity. A critical review of the literature revealed that this is based primarily on empirical clinical evidence (reviewed in 1–3), the most significant of which is that administration of allopurinol, a drug which reduces urinary out put of urate, reduces the recurrence of

CaOx stone formation in patients whose only demonstrable abnormality is hyperuricosuria (4–11). In an attempt to explain this causal relationship of hyperuricosuria to CaOx stone formation, one mechanism that has been most commonly cited is epitaxy. The term *epitaxy* was first coined by Royer as the growth of one crystal type upon the crystalline surface of another (12). The process was first invoked by Modlin (13) as a possible mechanism for the formation of urinary stones that usually consist of a mixture of different minerals. One year later, Lonsdale (14) demonstrated, using X-ray crystallography, the existence of several crystal lattice fits for anhydrous uric acid, uric acid dihydrate, CaOx monohydrate (whewellite), and CaOx dihydrate (weddelite). She theorized epitaxy as a mechanism of stone formation and to explain the encapsulation

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of a crystal core by alternating layers of other minerals—a pattern commonly seen in urinary calculi. The theory awaited experimental verification till 1975 when Coe et al. (15) and Pak and Arnold (16) reported that urate seed crystals enhanced the precipitation of CaOx from an inorganic metastable solution of this salt. These observations were later confirmed by independent groups (17–26). Collectively, the findings once again focused attention on epitaxy as a possible mechanism to link hyperuricosuria to CaOx stone formation, and, more importantly, a justification for administering allopurinol to reduce recurrence of CaOx stone formation. However, although the data proved conclusively that epitaxial deposition of CaOx upon urate seed crystals can occur under inorganic reaction conditions, they are largely academic from a pathophysiological perspective, because stones are formed in urine which, in addition to common inorganic constituents, also contains a wide selection of organic macromolecules whose possible effects on the process have never been studied. Furthermore, the results of our previous study revealed that the promotory effect of uric acid and monosodium urate seeds does not occur to physiologically significant degree in undiluted human urine (27). This strongly militates against credibility of the epitaxy theory.

It is now well documented that urinary macromolecules bind crystals in a selective manner and thus participate in the crystallization process (28). In an attempt to clarify the possible role of epitaxy in stone formation, the aim of the present investigation was to determine whether preincubation of seed crystals of uric acid and monosodium urate with urine alter their ability to induce deposition of CaOx from its aqueous inorganic solution, and thereby provide a possible explanation for the disparity of results obtained with synthetic inorganic solutions and undiluted human urine. Crystals of CaOx were also included as a basis for comparison.

## Materials and Methods

### Materials

Uric acid (UA), monosodium urate (NaU), calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ), ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol, morpholinoethanesulfonic acid (MES) and tris(hydroxymethyl)aminomethane (Trizma-base) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Calcium oxalate (CaOx) monohydrate, sodium oxalate and hydrochloric acid (HCl) were from BDH Chemicals Ltd. Poole, England. Sodium chloride (NaCl) and sodium azide were from E. Merck, Darmstadt, Germany. Bio-Gel P-6 DG was purchased from Bio-Rad Laboratories, Richmond, CA, USA.  $^{14}\text{C}$ -oxalic acid was from NEN Products, Boston, MA, USA. All other chemicals and materials used in this study were of the highest purity available.

### *Preparation of Preincubated Seed Crystals of UA, NaU, and CaOx with Urine*

*Collection and Preparation of Urine Samples* Twenty-four hour urine samples were collected without preservative from five healthy men (mean age 40 years) who had no previous history of urinary stone disease. The samples were refrigerated during the collection period and during storage before use. Absence of blood from the specimens was confirmed using Multistix test strips (Miles Laboratories, Mulgrave, Victoria, Australia) and the samples were pooled. The pooled sample was centrifuged using a JA-14 fixed-angle rotor at  $8000 \times g$  for 15 min at  $20^\circ\text{C}$  in a Beckman J2-21 M/E centrifuge (Beckman Instruments, Palo Alto, CA, USA). The supernatant was filtered through  $0.22 \mu\text{m}$  Millipore filters (# GVWP 142 50, Millipore Corporation, Bedford, MA, USA). An aliquot (approximately 50 ml) of the filtrate was transferred to dialysis bag (with nominal molecular weight cut-off of 10 kDa) and the remaining filtrate was divided into three equal portions. The dialysis bag was stirred at  $4^\circ\text{C}$  in cold room in a conical glass flask containing 6 litre deionized water. The dialysis was continued for 36 hours with dialyzate changed every 12 hours after which the sample was applied to a Bio-Gel P-6 DG desalting column. The protein peak was collected, lyophilized and stored at  $-20^\circ\text{C}$  until further use.

*Preincubation of Seed Crystals of UA, NaU, and CaOx with Urine* Commercial preparations of seed crystals of UA, NaU, and CaOx were added, separately, to the remaining three aliquots of the urine sample such that the final seed concentration in each was 1 g/l. The samples were stirred for 3 hours at room temperature using teflon coated magnetic stir bar in conical glass flasks. The crystals were harvested by filtration through  $0.22 \mu\text{m}$  Millipore filters and washed thoroughly with deionized water. They were then lyophilized and stored at  $-20^\circ\text{C}$  until required. These seed crystals will be referred to as “treated”.

The control seed crystals were from the same commercial batches of UA, NaU, and CaOx seeds as used above and were not treated in any way. These seed crystals will be referred to as “untreated.”

### *Crystallization Experiments*

*Measurement of Crystallization by Coulter Counter Analysis* Initial attempts to study the effect of the seed crystals on CaOx crystallization were made in the reaction medium as described by Pak and Arnold (16). Briefly, the seed crystals of UA, NaU, and CaOx, untreated and treated, were separately added to the reaction medium to give a final suspension concentration of 6 mg/100 ml, and the samples were incubated in a shaking water-bath at  $37^\circ\text{C}$ : seed crystals of UA and NaU, both untreated and treated, dissolved in 15–30 minutes. Similar dissolution was also observed using the reaction

medium as described by Coe et al. (15). For this reason alone, the effect of seed crystals in the present investigation was studied in a slightly modified reaction medium employing the same calcium and oxalate concentrations as used by Coe et al. (15). In brief, a solution containing 5 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 mM morpholinoethanesulfonic acid, 0.15 M NaCl was adjusted to pH 6.00 and saturated with urate by stirring at 37°C first with commercial preparations of UA followed by NaU crystals (to prevent dissolution of these seeds during their subsequent incubation in the reaction medium). The saturated solution was 0.22  $\mu\text{m}$  filtered and divided into seven aliquots.

Untreated and treated seed crystals of UA, NaU, and CaOx were ground in an agate mortar to remove large clumps. Slurries containing 6 mg/ml of the seeds were prepared in 0.15 M NaCl solution saturated with urate and spiked with 0.02% sodium azide (as bacteriostat) and were mixed overnight in a rotary mixer. Identical volumes of the seed slurries were separately added to the aliquots of the reaction medium to give a final seed suspension concentration of 6 mg/100 ml; no seeds were added to the internal control. This instead was supplemented with an equivalent volume of 0.15 M NaCl solution saturated with urate and containing 0.02% sodium azide. The volume and size of the seed crystals added were determined by using a Coulter Counter (Model TA II) fitted with a Population Count Accessory and a 70  $\mu\text{m}$  orifice. Crystallization of CaOx was then induced in the samples by the dropwise addition of a solution of sodium oxalate (20 mM), such that its final concentration in the samples was 0.2 mM. The samples were incubated for 120 min in a shaking water bath at 37°C, and the size distributions of the suspended particles were determined at 15 min intervals using the Coulter Counter. Preliminary experiments revealed that the intra- and inter-assay coefficients of variation of determination of modal particle size were 6.1 and 8.9% respectively. Each experiment was performed in triplicate. These samples will be referred to as "cold."

*Measurement of the Mineral Deposition by  $^{14}\text{C}$ -oxalate*  
The use of the Coulter Counter to measure particle volume in the type of experiment described here has well documented limitations that have essentially been described earlier (27). In summary, the Coulter Counter measures particles whose sizes fall within a specified range (in these experiments 2–25.4  $\mu\text{m}$ ); crystals whose sizes lie outside this range will not be counted. Furthermore, loose aggregates of crystals containing empty spaces are recorded by the instrument as if they are solid, thereby giving erroneously high estimates of particle volume deposition. This is further compounded by the inclusion of macromolecules into the crystalline architecture. Finally, the Coulter Counter cannot account for differences in particle density. Therefore, to determine the true extent of mineral deposition, parallel incubations

were carried out with samples containing  $^{14}\text{C}$ -oxalate (3.125  $\mu\text{Ci}/100\text{ ml}$ ), in which any alterations in radioactivity must reflect corresponding changes in CaOx precipitation. Radioactive samples were treated identically to those described above, except that they were supplemented with  $^{14}\text{C}$ -oxalic acid before the addition of sodium oxalate solution to induce crystallization.

At intervals of 15 min, 1 ml of each sample was filtered (0.22  $\mu\text{m}$ ) into 100  $\mu\text{l}$  of concentrated HCl using disposable syringes fitted with filters (Sartorius Minisart NML, Gottingen, Germany). Duplicate 0.3 ml aliquots of these solutions were added to 10 ml of Ready Safe scintillation fluid (Beckman Instruments Inc., USA) and counted for 5 min in a liquid scintillation counter (Beckman LS 3801 Liquid Scintillation System). Preliminary experiments revealed that the intra- and inter-assay coefficients of variation of measurement of CaOx deposition by  $^{14}\text{C}$ -oxalate analysis were 3.9 and 5.1% respectively. Each experiment was performed in triplicate. These samples will be referred to as "hot."

#### *Scanning Electron Microscopy (SEM)*

At the end of each experiment, 2 ml aliquots of each cold sample were filtered (0.22  $\mu\text{m}$ ) and the filtration membranes were dried overnight at 37°C. They were mounted on aluminium stubs, spluttered with gold for 180 seconds (SEM Autocoating Unit E5200, Polaron Equipment Ltd, Watford, UK), and examined using an ETEC Auto Scan Electron Microscope (Siemens AG, Karlsruhe, Germany) at an operating voltage of 20 kV.

#### *Statistical Methods*

For the sake of clarity, data were plotted as mean values; nonetheless, statistical comparisons were performed using the Wilcoxon signed rank sum test at a 0.05 level of significance.

#### *Protein Analysis of Preincubated Seed Crystals of UA, NaU, and CaOx*

*Demineralization of the Seed Crystals* Treated seed crystals of UA, NaU, and CaOx, 1.5 g each, were separately ground to a fine powder in an agate mortar. While the CaOx crystals were demineralized using 0.25 M EDTA (pH 8.0), as described earlier (29), slurries of the UA and NaU seeds were made in 100 ml of 0.025 M Tris-HCl containing 0.02% sodium azide. They were separately transferred to dialysis bags (with nominal molecular weight cut-off of 10 kDa) that were stirred at 4°C in cold room in a conical glass flask containing 6 litre of the same buffer as used for making their slurries. To ensure complete mixing, the dialysis bags were gently mixed and their polarities reversed every 12 hours. The dialyzate was changed every 24 hours. The resulting extracts of the crystals were applied to a Bio-Gel P-6 DG desalting column. The protein peaks were collected, lyophilized, and stored at –20°C until further use.

*Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)* All the protein samples were reduced with 2-mercaptoethanol and electrophoresed in 1 mm thick, 9–18% linear gradient gel on a Bio-Rad Mini-Protein II apparatus (Bio-Rad Laboratories, Hercules, CA, USA), as described previously (29). The gel was subsequently stained with silver (30).

## Results

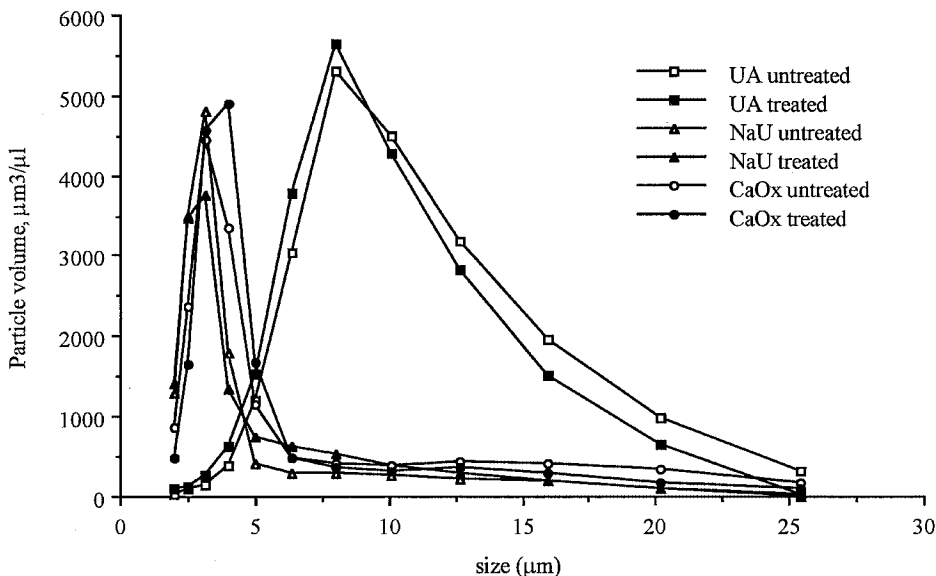
### Particle Volumes and Sizes of Untreated and Treated Seed Crystals

Figure 1 shows the volume distributions of untreated and treated seed crystals at zero time, i.e., before the addition of oxalate solution to induce crystallization. Table 1 summarizes Figure 1. Several features of the data are noteworthy: 1) Treated or untreated, the average total particle volumes of  $\sim 13,000$ – $15,000 \mu\text{m}^3/\mu\text{l}$  of NaU and CaOx seed crystals were significantly smaller than the corresponding value of  $\sim 21,000 \mu\text{m}^3/\mu\text{l}$  obtained with UA seeds. The ratio of added seed crystal mass to volume of the incubation solution was identical in all cases. 2) The average total particle volumes of untreated seed crystals were very similar to those of their treated counterparts: 21,125, 13,141 and 14,768  $\mu\text{m}^3/\mu\text{l}$  for untreated and 21,350, 12,810, and 15,329  $\mu\text{m}^3/\mu\text{l}$  for treated seed crystals of UA, NaU, and CaOx, respectively. 3) Treated or untreated, the average modal particle sizes of 2.9–3.5  $\mu\text{m}$  of NaU and CaOx seed crystals were significantly smaller than the corresponding value of  $\sim 8.1 \mu\text{m}$  obtained with UA seeds. 4) The average modal particle sizes of untreated and treated seed crystals were almost indistinguishable. 8.2, 3.0, and 3.3  $\mu\text{m}$  for untreated and 8.0, 2.9, and 3.5  $\mu\text{m}$  for treated seed crystals of UA, NaU, and CaOx, respectively. The difference in average total particle volume and average modal particle size of seeds of different types, could perhaps be attributed to their chemical composition, which in turn, determines

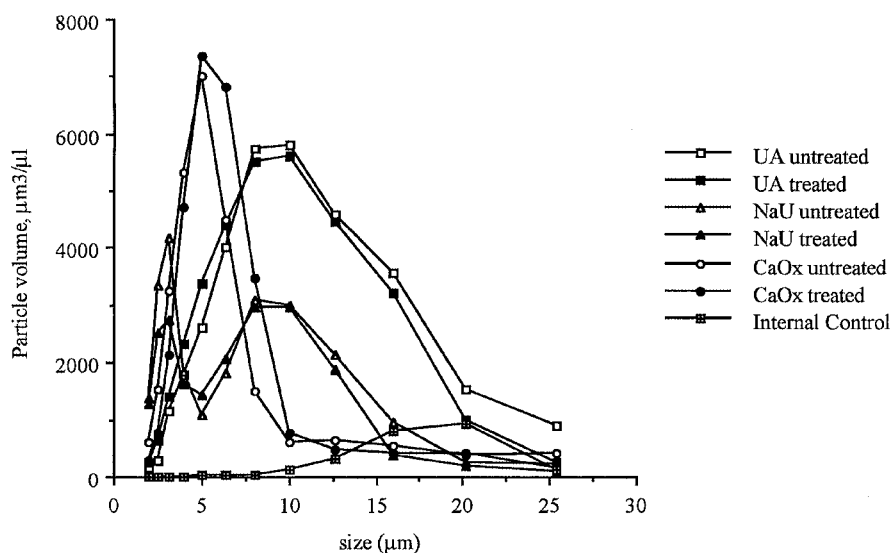
**Table 1.** The average total particle volumes and average modal particle sizes of untreated and treated seed crystals of UA, NaU, and CaOx

Seed Type	Average Total Particle Volume ( $\mu\text{m}^3/\mu\text{l}$ )	Average Modal Particle Size ( $\mu\text{m}$ )
UA untreated	21,125	8.2
UA treated	21,350	8.0
NaU untreated	13,141	3.0
NaU treated	12,810	2.9
CaOx untreated	14,768	3.3
CaOx treated	15,329	3.5

their size, degree of aggregation, and density. The shape of the volume distribution curves indicate that a significant proportion of CaOx and more so of NaU seeds, both untreated and treated, lay below the 2  $\mu\text{m}$  detection limit of the Coulter Counter. As noted previously (27), this will introduce an error in the estimation of total particle volume deposited after the addition of oxalate solution to induce crystallization, because CaOx crystals deposited in response to the oxalate solution form composite particles with seed crystals. This causes the seeds which are below the detection limit of the Coulter Counter to be detected and recorded in the higher size channel than they would have been had they not been attached to the CaOx crystals. The net increase in total particle volume recorded at the end of the incubation period must represent an overestimate of the increase in total particle volume in the presence of CaOx and NaU seeds, unlike the estimates obtained in the presence of UA seed crystals. For all practical purposes, Figure 1 reveals that the average modal particle sizes of all the types of seed crystals were detected by the Coulter Counter.



**Fig. 1.** Particle size distribution of untreated and treated seed crystals of uric acid (UA), sodium urate (NaU) and calcium oxalate (CaOx). This shows that the average modal particle sizes of each seed suspension were within the Coulter Counter specified limits (in these experiments, 2–25.4  $\mu\text{m}$ ).



**Fig. 2.** Particle size distribution at 2 hours after the addition of oxalate solution to the internal control (no seeds) and portions of the same incubation solution which were separately spiked with untreated and treated seed crystals of UA, NaU and CaOx. The values represent that (a) the average modal sizes of particles precipitated in the presence of treated seed crystals of UA, NaU, and CaOx were very similar to those deposited in the presence of their respective untreated controls (b) irrespective of whether or not seed crystals of UA and NaU had been treated, the average modal sizes of particles precipitated in their presence were at least half relative to those deposited in internal control, containing no added seeds.

#### *The Effect of Untreated and Treated Seed Crystals on the Deposition of Particle Size*

Figure 2 shows the volume distribution of particles after 2 h incubation in the internal control and in aliquots of the same incubation solution which were separately supplemented with untreated and treated seed crystals of UA, NaU, and CaOx. This figure has also been summarized in Table 2. The data have not been corrected for the volume of the added seed crystals occurring within each size range at zero time. Correction of the data in each channel to account for those in the zero time control would have caused inaccuracies in the volume distribution curves: these curves simply show the relative sizes of the precipitated particles. The data reveal that the average modal size of the particles deposited in internal control, containing no added seeds, is 19.0  $\mu\text{m}$ . This value is significantly reduced to varying extents by the addition of untreated and treated seed crystals of different chemical composition: 8.8, 2.9, and 8.9,

5.0  $\mu\text{m}$  in the presence of untreated and 9.0, 2.9, and 9.1, 5.3  $\mu\text{m}$  in the presence of treated seed crystals of UA, NaU, and CaOx, respectively. The average modal sizes of the particles were increased, in sequence, by 2.7 (non-significant), 1.2 (non-significant), and 2.5 (non-significant), 5.7% (non-significant) in the presence of treated seed crystals in comparison with their respective untreated controls.

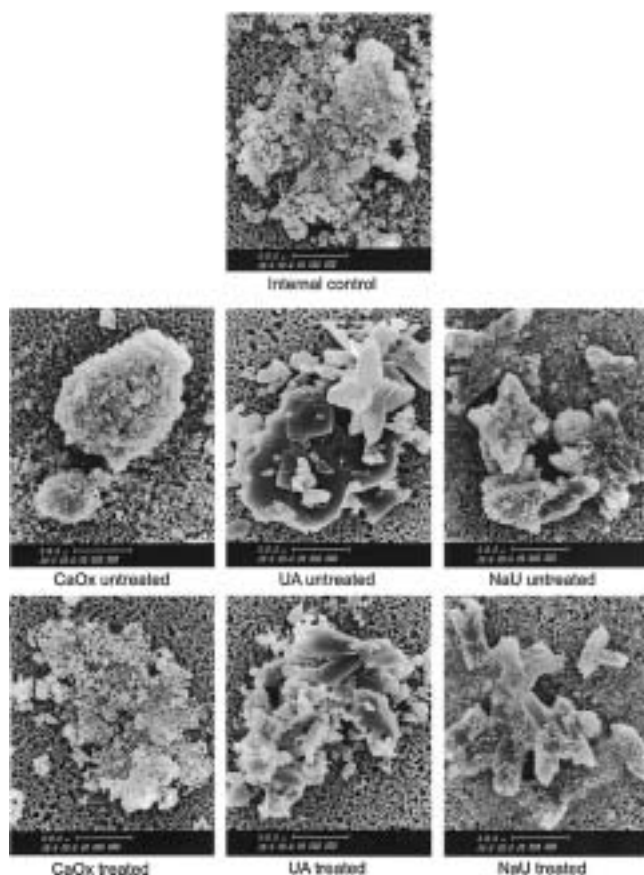
The volume distribution curves of the incubations spiked with untreated and treated NaU seeds are bi-modal, most likely because the newly precipitated CaOx crystals form composite particles with the seed crystals and are recorded in the higher size channel. Since the number of NaU seed particles in the incubations is very high (143,576 for untreated and 138,143 for treated) as compared with the number of CaOx particles deposited (which in internal control, containing no added seeds, was 864), a large proportion of the seed crystals would have remained unattached to the freshly deposited CaOx. The NaU seeds alone would be recorded at lower size channel while the ones attached with newly precipitated CaOx crystals would be recorded at higher size channel. The presence of these two types of particle populations in these incubations explains their bi-modal distribution. At the end of the 2 hour incubation the average lower modal value (of the bi-modal distributions) obtained in samples containing untreated and treated NaU seeds is 2.9  $\mu\text{m}$ . This value is almost identical to the average modal size value of 2.95  $\mu\text{m}$  of untreated and treated NaU seed particles alone, as recorded by the Coulter Counter at 0 min (Fig. 3).

Figure 3 shows low power scanning electron micrographs of the particles deposited at the end of the 2 hour incubation period in the internal control and in portions of the same incubation solution which were separately spiked with untreated and treated seed crystals of UA, NaU, and CaOx. The crystals deposited in all the incubations were principally coffin shaped CaOx monohydrates, which in internal

**Table 2.** The uncorrected average modal sizes of the particles deposited 2 hours after the addition of oxalate solution to the internal control (no seeds) and portions of the same incubation solution that were separately supplemented with untreated and treated seed crystals of UA, NaU, and CaOx

Sample	Uncorrected Average Modal Particle Size ( $\mu\text{m}$ )
UA untreated	8.8
UA treated	9.0
NaU untreated	2.9 and 8.9
NaU treated	2.9 and 9.1
CaOx untreated	5.0
CaOx treated	5.3
Internal control	19.0

Induction of CaOx crystallization by seed crystals:  
Effect of preincubation with urine



**Fig. 3.** Low-power scanning electron micrographs of the crystalline material deposited 2 hours after the addition of oxalate solution to internal control, containing no added seeds, and samples of the same incubation solution which were separately supplemented with untreated and treated seed crystals of UA, NaU, and CaOx. This shows that (a) the sizes of particles precipitated in the presence of treated seed crystals of UA, NaU, and CaOx were very similar to those deposited in the presence of their respective untreated controls (b) irrespective of whether or not seed crystals of UA and NaU had been treated, some of them were seen lying free on the filtration membrane while others were attached like barnacles onto the surfaces of the CaOx crystals.

control, containing no added seeds, were small and loosely grouped to form large clusters. Likewise, the individual CaOx crystals precipitated in the presence of untreated and treated CaOx seeds were also very small and their degree of aggregation in the former was seemingly higher than in the latter. Similarly, the degree of particle aggregation in the incubations containing untreated UA and NaU seeds was apparently higher than in the incubations containing their treated controls. In sharp contrast, however, the individual CaOx crystals deposited in the presence of UA and NaU seeds, both untreated and treated, were quite large as compared with those precipitated in the internal control. The scanning electron micrographs also revealed that whether or not the seeds

had been treated, the size of individual seed crystals of UA is an order of magnitude larger as compared with those of NaU. This confirms the observation of large particles of seed crystals of UA relative to those of NaU, as revealed by the Coulter Counter at 0 min. Treated or untreated, the presence of NaU, and, to a lesser extent, UA seeds was noted on the filter membrane. Also, the seed crystals of UA and NaU, both untreated and treated, can be seen attached like barnacles upon the surfaces of precipitated CaOx crystals.

*The Effect of Untreated and Treated Seed Crystals on the Deposition of Calcium Oxalate as Determined by <sup>14</sup>C-oxalate Analysis*

Figure 4 and Table 3 show the time course of the disappearance of <sup>14</sup>C-oxalate during the 2 hour incubation period following the addition of oxalate solution to the internal control and to portions of the same incubation solution which were separately spiked with untreated and treated seed crystals of UA, NaU, and CaOx. To normalize the data the values were presented as per cent <sup>14</sup>C-oxalate remaining in the solution relative to that recorded in each sample at zero time. The rate of reduction in <sup>14</sup>C-oxalate was either the same or greater in all the incubations supplemented with untreated or treated seed crystals than in internal control to which no seeds had been added. Irrespective of whether or not CaOx seed crystals had been treated, the values recorded in the incubations differed dramatically from those obtained in the presence of UA and NaU seeds. At 2 hour the mean percentage reduction in <sup>14</sup>C-oxalate in the internal control (64.7%) was very similar to the value obtained with untreated UA seed crystals (65.9%). However, this value was significantly reduced to 40.8 and 9.8% following the addition of untreated seed crystals of NaU and CaOx, respectively. This represented that while untreated UA seeds inhibited the precipitation of CaOx by 1.9% (non-significant), untreated NaU and CaOx seeds promoted the process by 36.9 ( $p \leq 0.05$ ) and 84.8% ( $p \leq 0.05$ ) respectively relative to internal control, containing no added seeds.

The corresponding values of percentage reduction in <sup>14</sup>C-oxalate obtained following the addition of treated seed crystals of UA, NaU, and CaOx were, in sequence, 65.7, 61.9 and 10.7%. It therefore follows that while treated seed crystals of UA caused a very small promotion in the deposition of CaOx by 0.3% (non-significant), treated NaU and CaOx seeds inhibited the same process by 51.9 ( $p \leq 0.05$ ) and 8.5% ( $p \leq 0.05$ ) respectively in comparison with the incubations containing their respective untreated controls.

Figure 5 shows SDS-PAGE of proteins of the urine sample in which UA, NaU, and CaOx seed crystals were preincubated, and of the demineralised extracts of those crystals. As always, the urine contained a very large spectrum of proteins. However, only a handful of them were seen in the extracts of treated seed crystals. The electrophoretogram

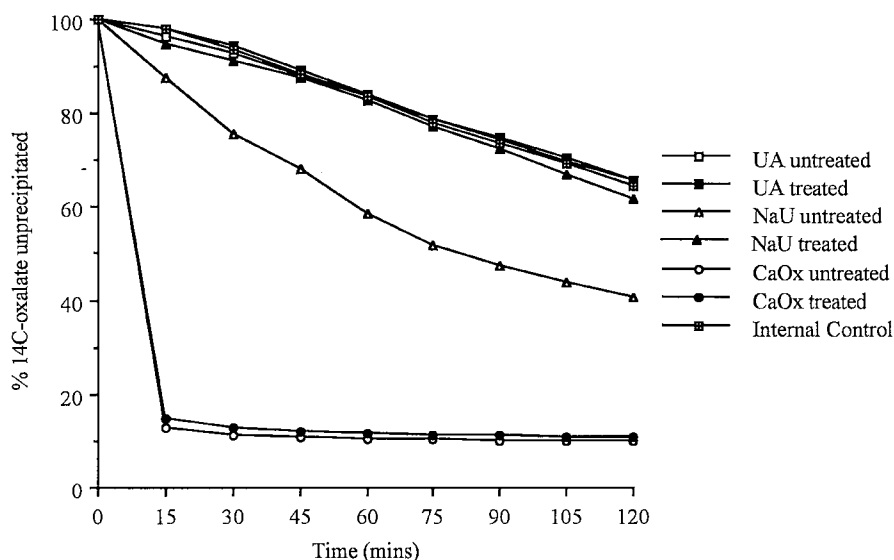


Fig. 4. Change in unprecipitated  $^{14}\text{C}$ -oxalate during 2 h incubation after the addition of oxalate solution to the internal control (no seeds), and aliquots of the same incubation solution which were separately spiked with untreated and treated seed crystals of UA, NaU, and CaOx. The values obtained represent that while untreated seed crystals of UA did not alter the deposition of CaOx, those of NaU and CaOx significantly promoted the process by 36.9 and 84.8% respectively in comparison with internal control to which no seeds were added. The results also showed that preincubation of the seed crystals with urine, such as occurs *in vivo*, only slightly reduced the ability of seed crystals of CaOx, but not of UA, to cause the mineral deposition in comparison with their respective untreated controls. The most dramatic effect was seen with NaU seed crystals where the preincubation inhibited by 51.9% their ability to promote deposition of CaOx relative to incubations to which untreated NaU seed crystals were added.

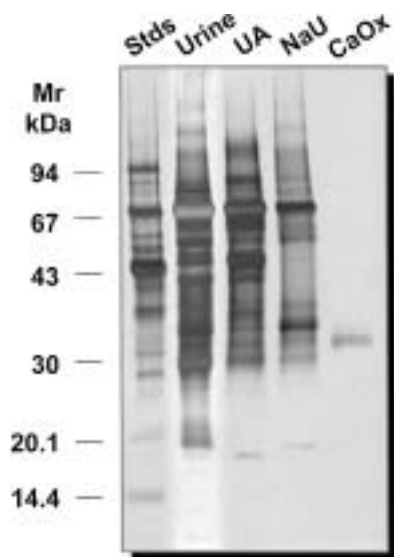
also showed that the number and molecular weights of proteins adsorbed onto the preincubated seed crystals varied depending upon chemical composition of the seeds. It is remarkable that a protein with molecular weight of approximately 31 kDa was predominant in the extract of treated CaOx seeds. Previously known as crystal matrix protein, it has now been identified as urinary form of prothrombin fragment 1 (31–32). In this investigation, no attempt was made to identify proteins in the extracts of treated UA and NaU seed crystals. For all practical purposes Fig. 5 reveals that seed crystals preincubated with urine adsorbed proteins onto their crystal surface.

## Discussion

Almost 30 years ago, Dent and Sutor (33) made an empirical clinical observation that “hyperuricosuria” seemed to be more common in stone formers than in normal subjects. Since then a great deal of effort has been made to clarify the association of urinary urate excretion with CaOx stone formation. Perhaps the most powerful evidence to support a determinant role for urate has been reports that allopurinol appears to reduce CaOx stone recurrences in patients whose only detectable abnormality is hyperuricosuria (4–11). A number of mechanisms were proposed in an attempt to explain this apparent beneficial effect of allopurinol. Of these, epitaxy has been the most often cited, its

Table 3. Change in unprecipitated  $^{14}\text{C}$ -oxalate during 2 hour incubation after the addition of oxalate solution to internal control, to which no seed crystals were added, and aliquots of the same incubation solution that were separately spiked with untreated and treated seed crystals of UA, NaU, and CaOx

Time (min)	Change in Precipitation (%)						
	Internal Control	UA Untreated	UA Treated	NaU Untreated	NaU Treated	CaOx Untreated	CaOx Treated
0	100	100	100	100	100	100	100
15	98.0	96.5	98.1	87.5	95.0	12.7	14.7
30	93.5	92.6	94.3	75.8	91.3	11.3	12.5
45	88.3	88.1	89.1	67.9	87.5	10.7	11.9
60	83.5	83.4	84.0	58.6	82.7	10.3	11.4
75	78.1	78.7	79.0	51.8	77.3	10.3	11.1
90	73.7	74.5	74.9	47.3	72.5	10.0	11.0
105	69.4	69.7	70.5	44.0	66.9	9.9	10.8
120	64.7	65.9	65.7	40.8	61.9	9.8	10.7



**Fig. 5.** SDS-PAGE of proteins of the urine sample in which UA, NaU, and CaOx seed crystals were preincubated, and of the demineralized extracts of those crystals. Note that while the urine contained a very large spectrum of proteins only a handful of them were seen in the extracts of treated seed crystals. The presence of a protein with molecular weight of approximately 31 kDa was remarkable in the extract of treated CaOx seeds. Previously known as crystal matrix protein, it has now been identified as urinary form of prothrombin fragment 1.

credibility having been reinforced by *in vitro* studies demonstrating that crystalline particles of NaU, and to a lesser extent UA, can induce precipitation of CaOx from metastable solutions of this salt (15–26,34–35). The theoretical aspects of this theory were already bolstered by X-ray crystallographic data revealing the existence of several crystal lattice fits for UA, UA dihydrate, whewellite (CaOx monohydrate), and weddellite (CaOx dihydrate) (14). Based on these theoretical and experimental evidence the theory could successfully explain (a) the formation of kidney stones consisting of mixtures of different minerals (b) a link between hyperuricosuria and CaOx stone formation (c) success of allopurinol in reducing CaOx stone recurrence. Therefore, it is not surprising that epitaxy has been the most commonly proposed explanation for clinical observations connecting urate and CaOx stone disease and the justification for therapeutic use of allopurinol. However, as far as the actual formation of stones is concerned, the scientific evidence on which this theory is largely based is physiologically irrelevant. This is mainly because stones are formed in real urine and not in inorganic solutions as used in these studies. The physicochemical characteristics of urine are quite different from that of synthetic inorganic solutions of CaOx even when designed to mimic urine as close as possible. This cautions against extrapolating findings from inorganic solutions to predict likely effects in whole urine, particularly on stone formation. Also, it is now widely accepted that urine contains large numbers and quantities of low

and high molecular weight components, some of which are well-documented inhibitors of CaOx crystallization (36). Furthermore, the concentrations and relative amounts of various urinary components vary from one urine specimen to another. Thus it is fair to say that the effects of all urinary components on the epitaxial relationship between urate and CaOx cannot possibly be reproduced under experimental conditions using inorganic salt solutions *in vitro*.

Recently, we investigated the effect of seed crystals of UA and NaU on crystallization of CaOx in undiluted human urine *in vitro* (27). The results demonstrated conclusively that these seed crystals do not promote CaOx deposition to a physiologically significant degree. In the present investigation an attempt was made to clarify whether the lack of promotory effect of urate seeds on CaOx crystallization in undiluted urine could be attributed to the “surface poisoning” of seed crystals, presumably the result of adsorption of urinary macromolecules and other low molecular weight components. This was achieved by comparing the ability of UA and NaU seeds which were preincubated with urine, with their untreated controls to induce precipitation of CaOx in synthetic inorganic solution as the reaction medium.

Although the use of whole urine would have been ideal, only centrifuged and 0.22  $\mu\text{m}$  filtered urine was used for preincubating seed crystals. This was because urine invariably contains particulate material (37) that after preincubation with seed crystals would be practically impossible to remove. It is remarkable that centrifugation and 0.22  $\mu\text{m}$  filtration of urine is known to remove Tamm-Horsfall glycoprotein and some human serum albumin (37)—neither of which has any significant effect on CaOx crystal growth in undiluted human urine (38). With these reservations, commercial seed crystals of UA, NaU, and CaOx were mixed separately with aliquots of the same centrifuged and 0.22  $\mu\text{m}$  filtered urine at an identical ratio of added seed crystal mass to urine volume of 1 g/l each. The samples were stirred for 3 hours at room temperature using magnetic stir bar in conical glass flasks. The crystals were harvested by filtration through 0.22  $\mu\text{m}$  Millipore filters, washed thoroughly with distilled water and lyophilized. The control (or untreated) seed crystals consisted of the same commercial batch of seeds of UA, NaU, and CaOx which had not been preincubated with urine.

Initial attempts were made to study the effect of untreated and treated seed crystals on CaOx crystallization in a reaction medium as described by Pak and Arnold (16). The final suspension concentration of each type of seed crystals used was 6 mg/100 ml. All the samples including internal control, to which no seed crystals were added, were incubated in a shaking water-bath at 37°C: untreated and treated seed crystals of UA and NaU dissolved within 15–30 minutes. Similar dissolution was noted using the reaction medium as described by Coe et al. (15). Therefore, the effects of the seed crystals on CaOx

crystallization were studied in a modified solution which had the same calcium and oxalate concentrations as used by Coe et al. (15), but in addition was also saturated with commercial seed crystals of UA and NaU. It is interesting to note that the reaction media similar to the ones described by Coe et al. (15) and Pak and Arnold (16) were used by various investigators who used vastly different techniques to examine the effect of UA and NaU seeds on CaOx crystallization. However, they made no mention of dissolution of the seed crystals except some (17,24,39–40) who then saturated incubation media, as in the present investigation, with commercial seed crystals of UA and/or NaU.

Analysis of  $^{14}\text{C}$ -oxalate data revealed that while untreated seed crystals of UA did not alter the deposition of CaOx, those of NaU and CaOx significantly promoted the process by 36.9 and 84.8% respectively in comparison with internal control to which no seeds were added. While these results are in perfect agreement with those of several previous studies (15–17,23,34–35) suggesting that NaU, but not UA, promote deposition of CaOx from its aqueous inorganic solutions, they are contradictory to others (18,20–21,23,25–26) who found even UA to be an effective promoter of the process as well. The results also showed that preincubation of the seed crystals with urine, such as occurs *in vivo*, only slightly reduced the ability of seed crystals of CaOx, but not of UA, to cause the mineral deposition in comparison with their respective untreated controls. The most dramatic effect was seen with NaU seed crystals where the preincubation inhibited by 51.9% their ability to promote deposition of CaOx relative to incubations to which untreated NaU seed crystals were added.

The size of the crystalline particles precipitated in the kidney tubules is an important determinant of their likelihood of retention within the renal collecting system and hence, of stone formation. The results of this study revealed that all untreated seeds tested reduced particle size. The average modal size of the particles deposited in the internal control, 19.0  $\mu\text{m}$ , was reduced significantly to 8.8, 2.9, and 8.9, 5.0  $\mu\text{m}$  in the presence of added untreated seed crystals of UA, NaU, and CaOx respectively. Thus the average modal size of the particles precipitated were at least 52.63% smaller in the presence of untreated UA and NaU seed crystals as compared with the internal control. No previous study has examined the effect of untreated UA and NaU seed crystals on the size of particles precipitated from aqueous inorganic solutions of CaOx. The results of the present study also revealed that although average modal sizes of the particles precipitated with treated seed crystals of UA, NaU and CaOx were slightly smaller than those deposited in the presence of their untreated controls, this difference did not achieve statistical significance. These results were confirmed empirically by scanning electron microscopy (SEM), which also revealed that whether or not UA and NaU seed crystals had been preincubated, they were clearly visible. Although some of them were lying free on the

filtration membrane, others were attached like barnacles onto the surfaces of the CaOx crystals. One might therefore argue that had epitaxy occurred to any significant extent, UA and NaU seeds should have been overgrown with CaOx (thereby exhibiting the morphology of pure CaOx) or be surrounded by newly nucleated CaOx crystals so that they themselves would be hidden. Certainly, this was not the case with untreated and treated UA and NaU seeds. This might seem to militate against credibility of the epitaxy theory. However, the  $^{14}\text{C}$ -oxalate data revealed that untreated seed crystals of NaU, but not UA, did promote precipitation of CaOx by 36.9%, as compared with internal control to which no seeds were added. This, coupled with the fact that several lattice fits between urate and CaOx have already been reported (14), strongly suggests that at least some, if not all, CaOx crystals deposited in the presence of untreated NaU seeds may, in fact, have NaU seed(s) as their nidi. A direct confirmation of this is difficult because it is practically impossible to separate NaU seeds from the freshly precipitated CaOx crystals. This raises an unanswered question: why only some, and not all, untreated NaU seeds act as nidi for the deposition of CaOx. The SEM observations of the present investigation are in accordance with previous studies where UA and/or NaU seeds promoted the mineral deposition and were themselves clearly visible attached to the freshly precipitated CaOx (17–18,21,25–26,40). The scanning electron microscopy results of this study also revealed that irrespective of whether or not UA and NaU seeds had been preincubated, the *individual* CaOx crystals deposited in their presence were quite large. This is consistent with their enhanced crystal growth—perhaps the result of their decreased crystal aggregation due to attachment like barnacles of UA and more so of NaU seeds onto the surface of freshly deposited CaOx crystals. This supposition is supported by the Coulter Counter data that at the end of 2 hr incubation the average modal sizes of precipitated particles in the presence of untreated and treated seed crystals of UA and NaU were almost half to that deposited in the internal control containing no added seeds. More importantly, the results suggest that UA and NaU seeds with adsorbed urinary macromolecules, as would happen in urine *in vivo*, decrease crystal aggregation and thus would tend to reduce the likelihood of stone formation, as this the only process which, even in the absence of any crystal growth, can result in the formation of large, potentially dangerous particles in a short span of time.

The results of protein analyses of treated seed crystals revealed that they all adsorbed proteins which differed in number and molecular weight depending upon chemical composition of the seeds. This confirms the widely acknowledged view that seed crystals bind urinary proteins in a selective manner (28). Previous studies have also shown similar protein profiles in extracts of demineralized UA and CaOx crystals generated in human urine (29,41–42). It is remarkable that

urinary form of prothrombin fragment 1 (UPTF1) is the predominant protein adsorbed onto the surface of treated CaOx seed crystals. And amongst the macromolecules it has the most inhibitory effect on CaOx crystallization thus far tested in undiluted human urine *in vitro* (43). This strong inhibitory effect of UPTF1, like its parent molecule prothrombin, is almost certainly due to the ten  $\gamma$ -carboxyglutamic acid (Gla) residues in its N-terminal region, which define its Gla domain (44). Gla, which is a relatively uncommon amino acid occurs principally in proteins involved in calcification or blood coagulation, is formed by vitamin-K-dependent post-translational  $\gamma$ -carboxylation of glutamic acid residues, and imparts an exceptional  $\text{Ca}^{2+}$ -binding capacity to proteins in which it is found (45). Thus despite the perfect lattice match, slight attenuation of the ability of treated CaOx seed crystals to induce deposition of CaOx observed in this study may perhaps be attributable to the adsorption of UPTF1 onto the surface of preincubated CaOx seeds. It is interesting to note that though in the present investigation untreated and treated UA seeds did not effect precipitation of CaOx what so ever, proteins of peaks I-III obtained after DEAE-cellulose column chromatography of demineralized extracts of their crystals generated in urine of healthy subjects have recently been reported to inhibit the nucleation of CaOx (46). Collectively, the findings suggest that the slight attenuation of the ability of the treated seed crystals of CaOx and more importantly of NaU to cause deposition of CaOx could be attributable to the binding of proteins, and perhaps other urinary macromolecules and low molecular weight components (28) onto the surface of seed crystals following their preincubation with urine.

Taken together, results of the present study indicate that untreated seed crystals of NaU, but not of UA, promote deposition of CaOx from its synthetic inorganic solution. And this promotory effect of NaU seeds is attenuated quite dramatically following their preincubation with urine. This may explain the discrepancy between the findings of studies carried out in inorganic solutions and undiluted human urine and further stresses the invalidity of directly extrapolating results obtained in inorganic solutions to likely effects in urine and more importantly, on stone formation. As reported previously UA and NaU seeds do not promote CaOx deposition to a physiologically significant degree in urine (27). Therefore epitaxial induction of CaOx precipitation by urate seeds is unlikely to be a major factor contributing to stone formation. This view is further reinforced by the fact that the crystallization of NaU in urine requires excretion of large quantities of sodium and urate ions which are physiologically unattainable (47). We have previously shown that the addition of dissolved NaU to human urine promotes CaOx precipitation (48) and that this effect can not be attributed to depletion of glycosaminoglycans (49), as suggested by Robertson et al. (50), or to the epitaxial deposition of CaOx onto urate particles formed in response to an increase in

urate concentration or addition of an oxalate load (51). In the absence of any other plausible explanation, the promotion of CaOx precipitation by dissolved urate is consistent with the mechanism of "salting-out" as suggested by Kallistratos and co-workers (52-53). Such a mechanism would explain the apparent beneficial effect of allopurinol administration, without the need to invoke the occurrence of seed crystals of NaU and UA in urine, the presence of which occurs only rarely.

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