Effect of seed crystals of uric acid and monosodium urate on the crystallization of calcium oxalate in undiluted human urine in vitro

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1. The aim of this study was to determine whether seed crystals of uric acid or monosodium urate promote the epitaxial deposition of calcium oxalate in undiluted human urine. The effects of seed crystals of uric acid, monosodium urate or calcium oxalate on calcium oxalate crystallization induced in pooled 24-h urine samples collected from six healthy men were determined by \(^{14}\text{C}\)oxalate deposition and Coulter counter particle analysis. The precipitated crystals were examined by scanning electron microscopy.

2. Seed crystals of uric acid, monosodium urate and calcium oxalate increased the precipitated particle volume in comparison with the control containing no seeds by 13.6%, 56.8% and 206.5% respectively, whereas the deposition of \(^{14}\text{C}\)oxalate in these samples relative to the control was 1.4% (P < 0.05), 5.2% (P < 0.01) and 54% (P < 0.001) respectively. The crystalline particles deposited in the presence of monosodium urate seeds were smaller than those in the control samples. Scanning electron microscopy showed that large aggregates of calcium oxalate were formed in the presence of calcium oxalate seeds, which themselves were not visible. In contrast, monosodium urate and, to a lesser extent, uric acid seeds were scattered free on the membrane surfaces and attached like barnacles upon the surface of the calcium oxalate crystals.

3. It was concluded that seed crystals of monosodium urate and uric acid do not promote calcium oxalate deposition to a physiologically significant degree in urine. However, binding of monosodium urate and uric acid crystals and their subsequent enclosure within actively growing calcium oxalate crystals might occur in vivo, thereby explaining the occurrence of mixed urate/oxalate stones.

INTRODUCTION

The long-held notion that calcium oxalate (CaOx) stone formation is linked to urate excretion has been largely on empirical clinical evidence [1, 2], the most significant of which is that allopurinol, a drug that reduces the urinary output of urate, lowers the recurrent rate of CaOx stone formation in patients whose only demonstrable abnormality is hyperuricosuria [3–10]. One mechanism that has been proposed to explain this link is epitaxy, a process in which the deposition of mineral of one crystal type occurs upon the surface of another [11]. Modlin [12] first proposed this concept to explain the occurrence of stones consisting of mixtures of different minerals, and theoretical support for the hypothesis was obtained shortly after when Lonsdale [13], using X-ray crystallography, demonstrated the existence of several close crystal lattice fits between anhydrous uric acid, uric acid dihydrate, CaOx monohydrate and CaOx dihydrate. On the basis of these findings she proposed the process of epitaxy both as a mechanism of stone formation and to explain the alternating layers of minerals of different composition around a nucleus – a pattern commonly seen in urinary calculi. However, it was not until 1975 that experimental evidence verifying the existence of an epitaxial relationship between urate crystals and CaOx precipitation was obtained, when Coe et al. [14] and Pak and Arnold [15] reported that urate crystals enhanced the precipitation of CaOx from an inorganic metastable solution of this salt. These findings once again focused attention on epitaxy as a possible mechanism connecting hyperuricosuria to CaOx stone formation and were later confirmed by several independent workers [16–23]. However, as far as the actual formation of stones is concerned, these observations must be regarded as largely academic, because stones are formed in urine, whereas almost all the cited studies involved the addition of seed crystals of uric acid (UA) or monosodium urate (NaU) to inorganic, aqueous solutions of CaOx.

The aim of the present investigation was to determine, using Coulter counter and radioactive oxalate analysis and scanning electron microscopy, whether seed crystals of UA and NaU can induce the
epitaxial deposition of CaOx from undiluted human urine and thereby explain the formation of stones consisting predominantly of CaOx in patients with hyperuricosuria.

MATERIALS AND METHODS
Collection and preparation of urine samples
Twenty-four-hour urine specimens were collected without preservative from six healthy men (mean age 45 years) who had no previous history of urinary stone disease. The samples were refrigerated during the collection period and during storage before use, and then pooled before centrifugation at 10000 r.p.m. for 15 min at 20°C in a Beckman Centrifuge using a JA-14 fixed angle rotor. The supernatant was filtered through 0.22-µm Millipore filters (#GVWP 142.50, Millipore Corporation, Bedford, MA, U.S.A.) and an aliquot was retained for biochemical analysis as described previously [24]. The remaining sample was divided into two portions for crystallization studies using Coulter counter particle analysis and measurement of [14C]oxalate deposition.

Measurement of crystallization by Coulter counter analysis
Although the use of whole urine would obviously be ideal, it cannot be used for experiments using Coulter counter analysis because of high background particle counts resulting from cellular debris and polymerized Tamm–Horsfall glycoprotein (THG). However, the removal of THG and some human serum albumin (HSA) by centrifugation and filtration [25] is unlikely to affect the crystallization of CaOx under the conditions used here because neither THG [26] nor HSA [27] has any significant effect on CaOx crystal growth in undiluted human urine.

The empirical metastable limit of the pooled urine with respect to CaOx, defined as the minimum amount of oxalate required to cause spontaneous detectable CaOx crystal nucleation, was determined by titrating the sample with sodium oxalate solution according to the method of Ryall et al. [28]. One quarter of the urine was retained as control, while the remainder was divided into three aliquots. Seed crystals of CaOx monohydrate (Ajax Chemicals, Sydney, Australia), UA and NaU (both from Sigma, St. Louis, MO, U.S.A.) were ground in an agate mortar to remove large lumps. Slurries containing 6 mg/ml of the seeds were prepared in 0.15 mol/l sodium chloride solution and were mixed overnight in a rotary mixer. Identical volumes were then added to aliquots of the urine to give a final concentration of 6 mg/100 ml urine: the control sample was treated with an equivalent volume of 0.15 mol/l sodium chloride solution. The volume and size of the seed crystals added were determined by Coulter counter analysis. Crystallization of CaOx was then induced in the samples by the dropwise addition of sodium oxalate solution to increase the concentration in the urine by 15 µmol/100 ml in excess of the measured metastable limit. The samples were then incubated for 120 min in a shaking water bath at 37°C, and the volume and size of the suspended crystals were determined by measuring the particle size distribution at 15-min intervals using the Coulter counter. Controls containing seeds but no exogenous oxalate were also included to account for the contribution of the seed crystals to the particle volume. Each experiment was performed in sextuplicate. These samples will be referred to as 'cold'.

Measurement of mineral deposition by [14C]oxalate
The use of the Coulter counter to determine the volume of crystalline particles has a number of limitations: (i) if the crystals are loosely aggregated, the empty spaces between them are counted as if they are solid material, thereby giving an erroneously high estimate of crystal volume; (ii) because the Coulter counter measures particle size within specified limits (in these experiments 2–25.4 µm), crystals falling outside this range will not be recorded; (iii) the Coulter counter cannot account for differences in particle density. Therefore, to estimate the true mass of CaOx deposited, parallel incubations were carried out with samples containing [14C]oxalate, in which any alterations in radioactivity must reflect corresponding changes in CaOx precipitation. Radioactive urines were treated identically to those described above, except that the samples were supplemented with [14C]oxalic acid (3.125 µCi/100 ml urine) before the oxalate load was added to induce CaOx crystallization. At intervals of 15 min, 2.5 ml of each sample was filtered (0.22 µm) into 200 µl of concentrated hydrochloric acid using disposable syringes fitted with filters (Sartorius Minisart NML, Gottingen, Germany). Duplicate 1-ml aliquots of these solutions were then added to 10 ml of Ready Safe scintillation fluid (Beckman Instruments, U.S.A.) and counted for 2 min in a liquid scintillation counter (Beckman LS 3801 Liquid Scintillation System). These specimens will be referred to as 'hot'.

Scanning electron microscopy (SEM)
At the end of each experiment, 1-ml aliquots of each cold sample were filtered (0.22 µm) and the filtration membrane dried overnight at 37°C. Each membrane was then mounted on an aluminium stub and coated with gold for 180 s using an E5200 SEM Autocoating Unit (Polaron Equipment). The stubs were examined using an ETEC Auto Scan electron microscope (Siemens) at an operating voltage of 20 kV.
Urate crystals

Statistical methods

For the sake of clarity, data are plotted as mean values; nonetheless, statistical comparisons were performed using the Wilcoxon signed-rank sum test.

RESULTS

The results of biochemical analysis of the pooled urine were as follows: pH 6.09; osmolality 552 mosmol/kgH\textsubscript{2}O; Na\textsuperscript{+} 130 mmol/l; Ca\textsuperscript{2+} 1.7 mmol/l; Mg\textsuperscript{2+} 1.4 mmol/l; urate 1.88 mmol/l; and creatinine 6 mmol/l.

The effect of seed crystal composition on the deposition of particle volume

Figure 1 shows the volume distribution of the seeds at zero time before addition of the oxalate load. It can be seen that the particle size distributions of the three seed types varied markedly. The total recorded volumes of the seeds were very different, being 2485, 4267 and 10230 pm\textsuperscript{3}/\mu l for NaU, UA and CaOx respectively—despite the fact that the ratio of added seed crystal mass to urine volume was identical in all cases. These distributions would reflect differences in the size, degree of aggregation and density of the seed crystals, as discussed above. In particular, it is apparent from the shape of the volume distribution curve that a significant proportion of NaU seeds lay below the detection limit of the Coulter counter, which in these experiments was 2 \mu m, as the volume of particles recorded in the lowest channel was greater than that in the adjacent channel. The fact that the total volume of the NaU seeds was not accurately recorded by the Coulter counter must have introduced an unavoidable error into the estimate of total crystal volume deposited after addition of the oxalate load in these incubations. The reason for this error, as will be seen from the scanning electron micrographs, is that the CaOx crystals that precipitated in response to the oxalate load formed composite particles with the seed crystals, thereby causing those seeds to be detected and recorded in a higher size channel than they would have been had they not been attached to the CaOx crystals. This means that the net increase in total crystal volume recorded at the end of the incubation period must represent an overestimate of the increase in total particle volume in the presence of NaU seeds, unlike the estimates obtained in the presence of CaOx and UA seeds. In these cases, the volume distribution curves recorded at zero time indicate that, for all practical purposes, all of the crystals were detected by the Coulter counter.

Figure 2 shows the time course of particle volume deposited during the 2-h incubation period after addition of the oxalate load to the control urine containing no seeds, and to portions of the same sample to which were added seeds of UA, NaU and CaOx. With the reservations discussed above, these data were corrected to account for the contribution of the seed crystals to the total recorded volume. With the exception of the incubation containing CaOx seeds, there was an initial lag phase followed by an increase in particle volume. In the presence of CaOx seeds, volume deposition began immediately and proceeded rapidly until 90 min when, presumably as a result of substrate depletion, a plateau was reached. After 2 h, the mean particle volumes
Fig. 2. Change in the volume of the particulate material deposited after the addition of an oxalate load, in the control urine (no seeds) and samples of the same urine containing seeds of UA, NaU and CaOx.

Deposited in the presence of seed crystals of UA (24.261 µm³/µl) and NaU (33.491 µm³/µl) were significantly increased in relation to that recorded in the control urine (21.363 µm³/µl). However, these increments were minor in comparison with that recorded in the incubation containing the CaOx seeds, in which the volume at 2 h was 65.485 µm³/µl. These values represent corresponding proportional increases in particle volume, relative to the control, of 13.6%, 56.8% and 206.5% respectively.

Particle volumes recorded in the controls containing the various seed crystal types, but not added oxalate, remained unaltered throughout the incubation period.

The effect of seed crystal composition on particle size

Figure 3 shows the volume distribution of the particles at the end of the incubation period, in the control and in the presence of the various types of seed crystals. It should be noted that these data have not been corrected for the volume of added seed crystals occurring within each size range.

Fig. 3. Particle size distribution, 2 h after the addition of an oxalate load, in the absence and presence of seeds of UA, NaU and CaOx.
because, for the reasons mentioned above, correction of the data in each channel to account for those in the zero time control would have caused major inaccuracies in the volume distribution curves: these curves simply show the relative sizes of the precipitated crystals. Where no seed crystals were added, the mode of the volume size distribution curve was 11.8 µm, whereas in the presence of seeds of UA, NaU and CaOx the corresponding modes were 13.8, 4.9 and 19.2 µm. It is remarkable that the particles deposited in the presence of NaU seeds were smaller even than those in the control samples to which no seeds were added. These findings are reflected in the scanning electron micrographs presented in Figs 4 and 5.

Figure 4 shows low-power scanning electron micrographs of the particles deposited in the absence of seeds, and in the presence of crystalline UA, NaU and CaOx. The particles precipitated from the control sample consisted principally of CaOx monohydrate crystals, which were single or clustered into small aggregates. In contrast, the crystalline particles precipitated in the presence of CaOx seeds were highly aggregated into large structures. The crystalline particles deposited in the presence of UA and, to a lesser extent, NaU seeds,

Fig. 4. Low-power scanning electron micrographs of the crystalline materials deposited from the control urine (no seeds) and samples of the same urine supplemented with seeds of UA, NaU and CaOx
though tending to be grouped into clumps, were nonetheless small in comparison with those precipitated in the presence of CaOx seeds. Higher power micrographs of the crystalline particles are presented in Fig. 5.

At higher magnification, the crystalline particles deposited in the presence of CaOx seeds are visible as large rosette structures, apparently completely enveloping the seeds themselves, which are not visible. In sharp contrast, however, particles precipitated in the presence of NaU and, to a lesser extent, UA seeds were seen lying free on the surface of the filter membrane, and also attached like barnacles upon the surfaces of the CaOx crystals. Many had apparently been engulfed by the CaOx growth front, being evident as lumps or protuberances upon the surface of the CaOx crystals precipitated during the experiment.

Assessment of crystal deposition by [14C]oxalate analysis

Figure 6 shows the disappearance of [14C]oxalate throughout the incubation period; values are presented as per cent [14C]oxalate remaining in the solution relative to that recorded in each sample at

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Fig. 5. High-power scanning electron micrographs of the samples shown in Fig. 3. Note the presence of UA and NaU seeds scattered on the membrane and on the CaOx crystal surface.
Urate crystals and calcium oxalate crystallization

Fig. 6. Change in unprecipitated [14C]oxalate, after the addition of an oxalate load, in the control urine (no seeds) and samples of the same urine containing seeds of UA, NaU and CaOx.

zero time. Two features of these data are remarkable. Firstly, the rate of reduction in [14C]oxalate is greater in all the experimental samples than in the control to which no seeds had been added. Secondly, as was observed above with the change in particle volume, values recorded in the samples containing CaOx seeds differed dramatically from those observed in the presence of UA and NaU seeds. At 2 h, the mean percentage reduction in [14C]oxalate in the control urine containing no seed crystals (67.7%) was slightly, but still significantly, greater than the corresponding values of 66.7% and 64.1% recorded in the presence of UA and NaU seeds respectively. However, it was in the presence of CaOx seeds that the most dramatic reduction in [14C]oxalate occurred, with the value at 2 h being only 31.1%. These values represent, in relation to the control containing no seed crystals, respective increases in deposition of CaOx of 1.4% (P<0.05), 5.2% (P<0.01) and 54% (P<0.001).

DISCUSSION

A number of mechanisms have been proposed in an attempt to explain the apparent relationship between urinary urate excretion and the occurrence of CaOx stones. These include epitaxy [14, 15], attenuation of the inhibitory activity of urinary glycosaminoglycans by colloidal particles of monosodium urate [29] and salting out [24, 30, 31]. Of these, epitaxy has been the most often cited, its credibility having been reinforced by in vitro studies demonstrating conclusively that crystalline particles of NaU and, to a lesser extent, uric acid can induce precipitation of CaOx from metastable solutions of this salt [14–16, 32, 33]. However, though the epitaxy theory was further bolstered by X-ray crystallographic data showing the existence of geometrical fits between the crystalline lattice dimensions of CaOx and NaU [13], the theoretical possibility that epitaxy could explain a number of features of CaOx stone disease, in particular the ability of allopurinol to reduce stone recurrences in patients with high urinary outputs of urate, has never been unambiguously corroborated by studies performed in human urine. The need to distinguish between the experimentally verified ability of crystalline urate to accelerate the precipitation of CaOx from aqueous, inorganic solutions and any similar effect it may exhibit under physiological conditions cannot be overemphasized. It is hardly necessary to point out that the physicochemical properties of urine bear scant similarity to those of inorganic solutions of CaOx, and there is no guarantee that any epitaxial effects exhibited by crystalline urate in such solutions, even when designed to mimic urine as closely as possible, may not be retained in the complex urinary milieu. Thus, the well-recognized involvement of macromolecules in the crystallization of CaOx in urine is, of itself, sufficient reason to question the applicability of data generated from inorganic crystallization systems to physiological conditions.

Perhaps the most compelling reason for exercising caution when attempting to extrapolate from synthetic solutions to urine is that the latter contains large numbers and quantities of organic components whose concentrations and relative amounts differ from one urine specimen to another, and whose
effects on the epitaxial relationship between CaOx and urate cannot possibly be reproduced under experimental conditions using inorganic salt solutions in vitro.

It is remarkable that only two previous studies used human urine as the reaction medium to study the possible epitaxial deposition of CaOx onto urate seeds [22, 23]. The technique used by Grases et al. [23] was rudimentary, involving the estimation of the number and the type of precipitated crystals using a Brand counting chamber and optical microscopy. These workers based their conclusion that UA and NaU seeds did not promote CaOx deposition in human urine on the fact that the number of particles (some of which were identified) and CaOx dihydrate crystals precipitated increased in relation to the number of uric acid crystals used as nuclei. It is unclear as to how the authors confirmed that the nuclei (UA in this case) had caused epitaxial deposition of CaOx, as they clearly admitted that in their studies some urate seeds remained uncoated (presumably with CaOx) - an event that would not have happened had significant epitaxy occurred in their experiments.

The only other study [22] which examined the possible epitaxial induction of CaOx deposition by urate seeds in human urine used two types of samples, i.e. with high supersaturation and high level of metastable supersaturation with respect to CaOx. Although UA and NaU seeds did not promote CaOx precipitation in the former samples, they did so in the latter, as indicated by a reduction in soluble [14C]oxalate. Over a period of 4 h this amounted to approximately 6.68% and 11.66% with UA and 5.85% and 7.34% with NaU crystals, at final seed concentrations of 12.5 and 25 mg/100 ml urine respectively. It is remarkable, however, that the author neither recorded the size distribution of the particles nor visualized the precipitated crystals by SEM. In this study we also observed a similar promotion of CaOx deposition from urine by urate seeds, albeit at a seed concentration less than half that used by Tiselius [22]. Our data reveal that the addition of seed crystals increased the deposition of corrected particle volume in comparison with the control containing no seeds: UA seeds increased the volume by 13.6%, NaU by 56.8% and CaOx by 206.5%. However, the [14C]oxalate data demonstrated that these increases did not accurately reflect true mineral precipitation, as they showed that the deposition of CaOx relative to the control was only 1.4%, 5.2% and 54% in the presence of seeds of UA, NaU and CaOx respectively. The discrepancy between the Coulter counter and radioactivity data was not unexpected in view of the technical deficiencies of the Coulter counter discussed earlier in this paper. The data presented here therefore suggest that seed crystals of NaU and UA have only a minor effect on the precipitation of CaOx in undiluted urine. Moreover, the small degree of promotion observed was recorded after an incubation period of 120 min, which is at least an order of magnitude greater than the 3-5 min transit time of urine through the renal tubules [14, 34, 35]. The additional amount of CaOx that would be expected to be deposited during this short period would be insignificant. Our data therefore indicate that seeds of UA and NaU would not promote CaOx deposition to a physiologically significant degree in conditions likely to prevail in human urine in vivo. This conclusion is supported by the SEM findings.

If epitaxy had occurred to any significant extent in this study, one would have expected the seed crystals of UA and NaU to have been overgrown with CaOx (and possibly therefore to exhibit the morphology of pure CaOx) or be surrounded by newly nucleated crystals of CaOx so that they themselves would be hidden. Certainly, this was the case with CaOx seeds: SEM examination of the precipitated crystals at high magnification revealed that large aggregates of CaOx were formed in the presence of NaU seeds, which themselves were not visible; presumably because they had been enveloped by deposition of CaOx upon them. In contrast, NaU and to a lesser extent UA seeds were clearly visible. Although some of them were lying free on the filtration membrane, others were attached to the surface of the precipitated CaOx crystals deposited and some of these had been overgrown by the CaOx growth front, and were evident as protuberances upon the surface of CaOx crystals. Analysis of the particle size distributions revealed that the CaOx crystals deposited in the presence of NaU seeds were smaller than those precipitated in the controls containing no seeds; this finding was confirmed by SEM, which also showed that the sizes of the individual crystals were approximately the same. This suggests that binding of NaU seeds to the CaOx crystal surfaces decreased the tendency of the latter to aggregate, which would tend to reduce the likelihood of stone formation, as crystal aggregation is the only process which can result in the formation of large, potentially dangerous particles in a short period of time. In fact, SEM of the crystals suggests that, after precipitation of CaOx had occurred, the seed crystals attached themselves to the surfaces of these crystals; as crystal growth proceeded, the seed crystals became embedded within the CaOx crystal structure. The fact that this occurred in undiluted urine suggests that the process may also occur in vivo, where it could explain the occurrence of mixed urate/oxalate stones. Most importantly, these findings demonstrate that, even in the presence of adsorbed urinary macromolecules, crystals of UA and NaU can bind to the surface of CaOx crystals, not the other way around, as suggested by the epitaxy theory.

Taken together, the results presented here strongly indicate that urate seeds do not promote CaOx deposition to a physiologically significant degree in urine. In fact, NaU seeds seem to inhibit CaOx crystal aggregation. Epitaxial induction of
CaOx crystal nucleation by seed crystals of NaU or UA is therefore 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 quantities of sodium and urate ions that cannot be achieved under physiological conditions [36]. We have previously shown that the addition of dissolved NaU to human urine promotes CaOx precipitation [34] and that this effect cannot be attributed to the depletion of glycosaminoglycans [37], as suggested by Robertson et al. [29], 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 [38]. 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 [30, 31]. Such a mechanism would explain the apparent beneficial effect of allopurinol administration, without the need to invoke the occurrence of enhanced deposition of CaOx by seed crystals of NaU or UA in urine, the presence of which occurs only rarely.

REFERENCES