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The Effect of Seed Crystals of Hydroxyapatite and Brushite on the Crystallization of Calcium Oxalate in Undiluted Human Urine In Vitro: Implications for Urinary Stone Pathogenesis

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Abstract

Background: The aim of this study was to determine whether crystals of hydroxyapatite (HA) or brushite (BR) formed in urine promote the epitaxial deposition of calcium oxalate (CaOx) from undiluted human urine in vitro and thereby explain the occurrence of phosphate in the core of urinary stones consisting predominantly of CaOx.
Materials and Methods: Crystals of HA, BR, and CaOx were generated from human urine and their identity confirmed by X-ray analysis. Standard quantities of each crystal were then added to separate aliquots of pooled undiluted human urine and CaOx crystallization was induced by the addition of identical loads of sodium oxalate. Crystallization was monitored by Coulter Counter and ^{14}C-oxalate analysis and the precipitated crystals were examined by scanning electron microscopy.
Results: In comparison with the control to which no seeds were added, addition of CaOx crystals increased the deposition of ^{14}C-oxalate by 23%. On the other hand, seeds of HA and BR had no effect. These findings were supported by Coulter Counter analysis, which showed that the average modal sizes of crystal particles precipitated in the presence of HA and BR seeds were indistinguishable from those in the control, whereas those deposited in the presence of CaOx were significantly larger. Scanning electron microscopy confirmed these results, demonstrating that large aggregates of CaOx dihydrates were formed in the presence of CaOx seeds, whereas BR and to a lesser extent HA seeds were scattered free on the filtration membrane and attached like barnacles on the surface of the freshly precipitated CaOx crystals.
Conclusion: Seed crystals of HA or BR do not promote CaOx deposition in urine in vitro and are therefore unlikely to influence CaOx crystal formation under physiologic conditions. However, binding of HA and BR crystals to, and their subsequent enclosure within, actively growing CaOx crystals might occur in vivo, thereby explaining the occurrence of mixed oxalate/phosphate stones.

Introduction

Urinary stone formation is a serious, debilitating problem in all societies throughout the world. It is estimated that approximately 12% of the population will suffer from the disease at some stage in their lives (1) and that men are three times more prone to the disease than are women (2,3). More recent studies suggest that there has been a gradual increase in the annual incidence and a decrease in the age of onset of the disease—perhaps the result of change in lifestyle and diet (4). It is therefore not surprising that financial costs of the disease are staggering; in the United States, for instance, the health bill for treatment of kidney stones runs to billions of dollars annually (5). Therefore studies investigating why, or more importantly, how stones form are of utmost importance for prevention, early intervention, better patient care, and reducing the financial burden of the disease.

All urinary stones consist of an inorganic moiety; the majority are made up of calcium oxalate (CaOx) and a mixture of several crystalline mineral phases (6,7). In urinary calculi containing more than one mineral, the inorganic fraction is arranged in concentric layers of abruptly differing composition around a common nucleus (6,8). One interesting feature of such stones is that their centre usually consists of different forms of calcium phosphate (CaP). These include hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$), also known as basic calcium hydrogen phosphate, and rarely, brushite (BR), $CaHPO_4 \cdot 2H_2O$, otherwise known as calcium hydrogen phosphate dihydrate (6–7,9–10). In an attempt to explain the formation of urinary stones consisting of mixed layers of different minerals, Modlin (8) invoked the phenomenon of epitaxy, a process involving oriented overgrowth of one crystalline phase upon another and requiring a near geometrical fit between the respective networks that are in contact (11). Later, using X-ray crystallography,

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Lonsdale (12) demonstrated the existence of several crystal lattice fits for many crystal phases of minerals commonly present in urinary calculi. Based on these findings, which at least supported the theoretical possibility of epitaxial growth of CaOx on different minerals, including HA and BR, she proposed the process of epitaxy both as a mechanism of stone formation, and to explain the encapsulation of a crystal core by alternating layers of other minerals, a pattern commonly seen in urinary calculi (12,13). These findings for the first time raised the possibility of epitaxial growth of CaOx onto HA or BR crystals as one possible mechanism of stone formation.

A prerequisite for the epitaxial deposition of CaOx onto a CaP nidus is that the urine be supersaturated with both CaOx and CaP. This requirement is fulfilled; several studies have revealed that although the urine of normals and stone-formers is often supersaturated with CaOx (10,14–17) and hydroxyapatite (18,19), that of stone patients is supersaturated with BR as well (10,14–17). The results of these physicochemical studies are supported by the observations that crystals of CaOx and CaP are more common in the urine of stone-formers than in that from healthy subjects (18,19). Furthermore, long-term follow-up studies of patients after extracorporeal shockwave lithotripsy showed that the stone recurrence rate was higher in patients with a history of frequent formation of stones with a high content of CaP (20). Some researchers have even suggested that the CaP content of renal stones is a useful factor in predicting the future course of the disease (21). On the basis of these observations, the overall weight of evidence supports the possibility that CaP particles in the urine of stone-formers initiate CaOx stone genesis by acting as seeds for heterogeneous deposition. Experimental verification that epitaxial deposition of CaOx on CaP can occur was obtained when Pak et al. (22) and Meyer et al. (23,24) reported that HA and BR crystals enhanced the precipitation of CaOx from an inorganic metastable solution. These observations were later confirmed by other investigators (25–33) and subsequently cited by innumerable authors as corroborative evidence for the probability that epitaxy plays a major role in the formation of mixed calcium stones. However, although the data proved conclusively that epitaxial deposition of CaOx on HA and BR can occur under inorganic conditions, they are largely academic from a pathophysiologic 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. Moreover, it is also known that crystals of CaP precipitated from human urine contain a number of macromolecules (34,35) whose presence will undoubtedly affect the geometrical lattice fit between the contacting mineral surfaces. Therefore, if an epitaxial relationship exists between CaOx and CaP salts in urine, it is more likely to be dictated by the macromolecular coat of the crystals than by the lattice characteristics of the pure mineral phases.

The aim of the present investigation was to determine, using Coulter Counter and radioactive oxalate analysis, and scanning electron microscopy, whether crystals of HA and BR formed in human urine can induce the epitaxial deposition of CaOx from undiluted human urine and thereby explain the common observation of a CaP core within stones consisting predominantly of CaOx. Crystals of CaOx derived from the same urine were also included as a basis for comparison.

Materials and Methods

Preparation of Seed Crystals of HA, BR, and CaOx

Collection and Preparation of Urine Samples Twenty-four-hour urine specimens were collected without preservative from 10 healthy men who had no previous history of urinary stone disease. The samples were refrigerated during the collection period and during storage before use. After confirming the absence of blood from each urine sample by dip stick analysis (Combur Test, Roche Diagnostics, Mannheim, Germany), they were pooled and centrifuged at 8000 $\times$ g for 15 min at 20°C in a Beckman J2-21M/E centrifuge (Beckman Instruments, Palo Alto, CA, USA) using a JA-14 fixed-angle rotor. The supernatant was filtered through 0.22-μm Millipore filters (#GVWP 142 50, Millipore Corporation, Bedford, MA, USA) and divided into three portions for preparation of HA, BR, and CaOx crystals.

Generation of HA Seed Crystals A solution of calcium chloride (1 mol/l) was added dropwise to the urine at 37°C to give a final concentration of ~20 mmol/l. The pH of the urine was adjusted to 7.5 with 1 mol/l NaOH and the sample was incubated in a shaking water bath at 37°C for a further 6 hr. The precipitated crystals were harvested by filtration through 0.22-μm Millipore filters and washed thoroughly with distilled water. They were then freeze-dried and stored at −20°C until required.

Generation of BR Seed Crystals Under the same conditions used to prepare the HA crystals, a solution of KH_2PO_4 (1 mol/l) was added dropwise to the second portion of the urine sample to give a final concentration of ~80 mmol/l. The urine pH was then adjusted to 6.0 with 1 mol/l HCl and the sample incubated in a shaking water bath at 37°C for 6 hr. The precipitated crystals were harvested, washed, freeze dried, and stored as described above.

Generation of CaOx Seed Crystals CaOx seed crystals were precipitated from the third portion of the urine as described previously (36). Briefly, the empirical metastable limit of the pooled urine with respect to CaOx was determined using a Model TAII

Coulter Counter fitted with a Population Count Accessory (Coulter Electronics Ltd, Herts, United Kingdom) following titration with sodium oxalate solution. A standard load of oxalate was added dropwise to the urine sample, which was then incubated in a shaking water bath at 37°C. The same amount of sodium oxalate solution was added after 1 and 2 hr and the urine was incubated for a further 4 hr. The crystals were harvested, washed, freeze–dried, and stored as described.

X-Ray Powder Diffraction The identities of the precipitated HA, BR, and CaOx seed crystals were confirmed by X-ray analysis. The crystals were ground thoroughly and their X-ray diffraction patterns were recorded with a Phillips PW 1710 microprocessor-controlled diffractometer using cobalt radiation, a variable divergence slit, and a graphite monochromator. The diffraction patterns were recorded in steps of 0.05 with a 1-sec count time per step. The patterns were logged to permanent data files on an IBM/XT computer (37) and subsequently analyzed using a software package developed by Raven and Self (38). Standard HA and CaOx were purchased from Sigma Chemical Company (St. Louis, MO, USA) and brushite was kindly provided by Professor C.Y.C. Pak (University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, USA)

Crystallization Experiments

Measurement of Crystallization by Coulter Counter Analysis Although the use of whole urine would obviously have been 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). The removal of THG and some human serum albumin (HSA) by centrifugation and filtration (39) is unlikely to affect the crystallization of CaOx under the conditions used here because neither THG nor HSA has any significant effect on CaOx crystal growth in undiluted human urine (40). Therefore, urine that had been centrifuged and filtered was used in the following experiments.

Additional 24-hr urine samples were collected from healthy men. After confirming the absence of hematuria, they were pooled, centrifuged, and filtered, and the metastable limit with respect to CaOx determined as described. One-quarter of the urine was retained as control, and the remainder was divided into three aliquots. Seed crystals of HA, BR, and CaOx prepared as described were gently ground in an agate mortar to remove large lumps so they could be counted accurately by the Coulter Counter, and slurries containing 2 mg/ml of each seed type were prepared in 0.15 mol/l NaCl solution and mixed overnight in a rotary mixer. It is remarkable that grinding of the seed crystals does not affect their molecular lattice and thus is highly unlikely to affect their ability to induce crystallization of CaOx. Identical volumes were then added to aliquots of the urine to give a final suspension concentration of 2 mg/100 ml urine; the control sample was treated with an equivalent volume of 0.15 mol/l NaCl solution. This value of seed suspension concentration was chosen to minimize coincidence while counting particles using the Coulter Counter. The volume size distributions of each seed crystal suspension 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 by 15 μmol/100 ml in excess of the measured metastable limit. The samples were incubated for 120 min in a shaking water bath at 37°C, and the particle size distributions were determined at 15-min intervals using the Coulter Counter. Preliminary experiments revealed that the intra-assay and inter-assay coefficients of variation of determination of modal particle size were 6.1% and 8.9%, respectively. Each experiment was performed in sextuplicate. These samples will be referred to as "cold."

Measurement of Mineral Deposition by ^{14}C-Oxalate The use of the Coulter Counter to determine crystal growth has a number of limitations: a) 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; b) 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; and c) 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 ^{14}C-oxalate (2.5 μCi/100 ml urine), 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 ^{14}C-oxalic acid (NEN Products, Boston, MA, USA) 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 HCl 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 Inc) and counted for 2 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}C-oxalate analysis were 3.9% and 5.1%, respectively. Each experiment was performed in sextuplicate. These samples will be referred to as "hot."

Scanning Electron Microscopy 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 aluminum stub and coated with gold for 180 sec using an SEM Autocoating Unit E5200, (Polaron Equipment Ltd, Watford, United Kingdom). The stubs were 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 are plotted as mean values: nonetheless, statistical comparisons were performed using the Wilcoxon signed rank sum test at an 0.05 level of significance.

Results

Sizes of Seed Crystals

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 were very similar. The average modal size in all cases was approximately 6.35 μm, and the average total particle volumes of each suspension were also alike: 3692, 3716, and 4089 $\mu m^3/\mu l$ for HA, BR, and CaOx seeds, respectively. Although it is apparent from the shape of the distribution curves that a small percentage of the CaOx seed crystals, and slightly more of the BR and HA seeds, lay below the detection limit of the Coulter Counter, which in these experiments was 2 μm, it is apparent that, for all practical purposes, the Coulter Counter volume distribution curves for each type of seed provide an adequate quantitative estimation of average modal particle size. Furthermore, the close similarity between the initial modal sizes obviates the need to correct values obtained after the 2-hr incubation period for those at zero time.

Effect of Seed Crystal Composition on Particle Size

Figure 2 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. After 2 hr, the average modal size of the particles deposited in the presence of seed crystals of CaOx, HA, and BR were 16.8, 11.8, and 11.9 μm, respectively, in comparison with the control (containing no added seeds) value of 11.7 μm. These represent corresponding increases in particle size, relative to the control, of 43.6 ($p \leq 0.05$), 0.9 (nonsignificant), and 1.7% (nonsignificant), respectively. These findings are confirmed in the scanning electron micrographs presented in Figures 3 and 4.

Figure 3 shows low power scanning electron micrographs of the particles deposited in the absence of seeds, and in the presence of crystalline CaOx and HA and BR. The crystalline particles precipitated from the control sample consist principally of small CaOx dihydrate crystals, which were single or clustered into small aggregates of several crystals. In contrast, individual crystals precipitated in the presence of CaOx seeds were large (>10 μm) and highly aggregated into structures of 50–60 μm. Crystalline particles deposited in the presence of BR, and to a lesser extent HA seeds, although tending to be grouped into clumps, were nonetheless small in comparison to those precipitated in the presence of CaOx seeds. Seed crystals of HA and BR were clearly seen scattered across the filtration membranes. Higher power micrographs of the crystalline particles are presented in Figure 4.

At higher magnification, the crystalline particles deposited showed protuberances, apparently caused

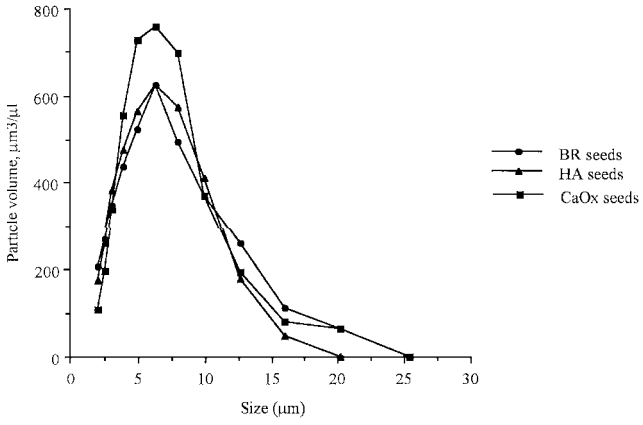

Fig. 1. Particle size distributions of seeds of hydroxyapatite (HA), brushite (BR), and calcium oxalate (CaOx). The average modal sizes of each seed suspension were alike and were within the Coulter Counter–specified limits (in these experiments, 2–25.4 μm).

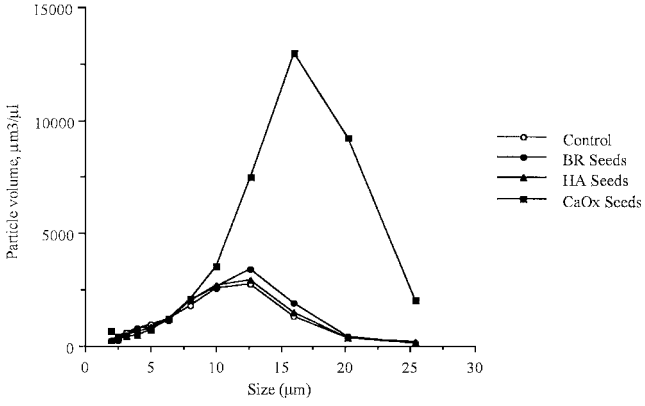

Fig. 2. Particle size distribution at 2 hr after the addition of oxalate load, in the control urine (no seeds) and samples of the same urine containing seeds of HA, BR, and CaOx. The values represent that although the addition of HA and BR seeds did not significantly affect the average modal size of the precipitated particles, CaOx seeds increased it by 43% ($p \leq 0.05$) in comparison with the control sample to which no seed crystals were added.

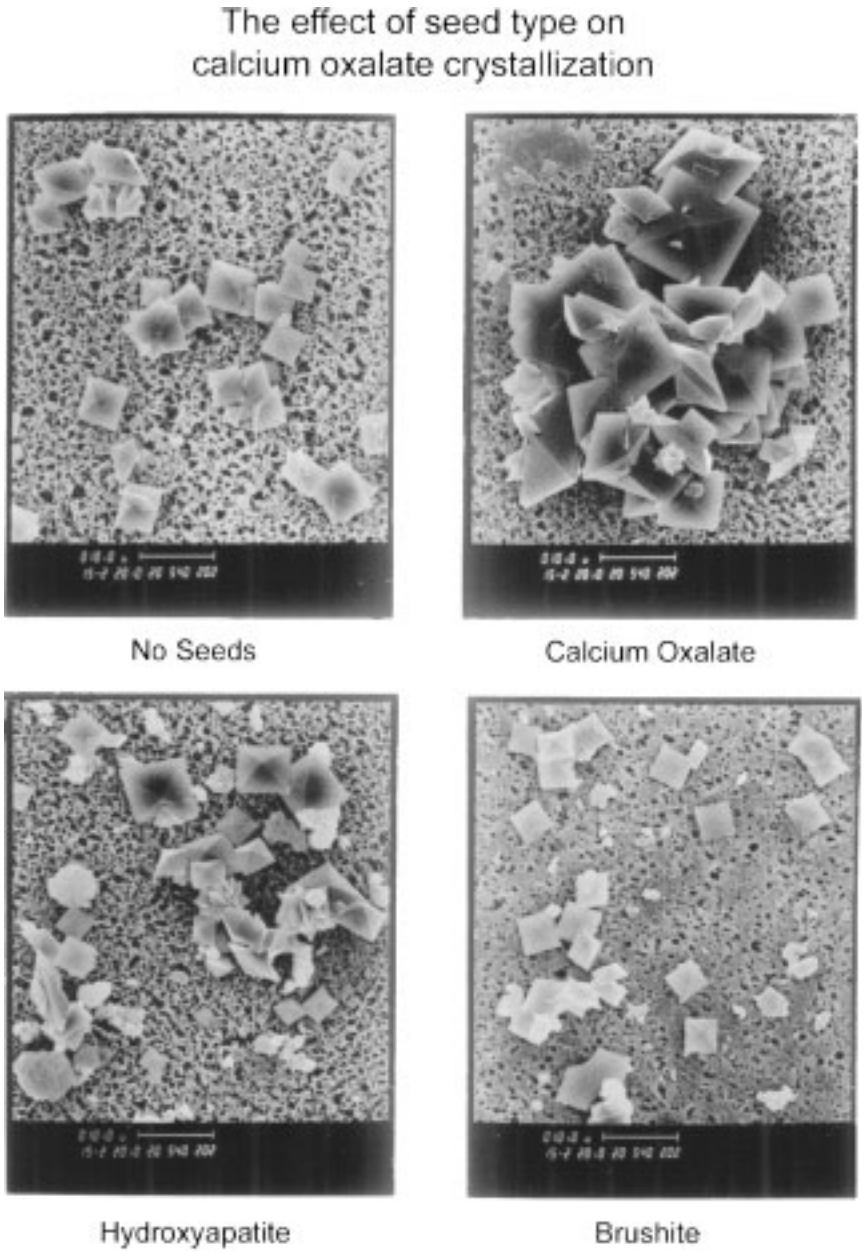

Fig. 3. Low power scanning electron micrographs of the crystalline materials deposited in the absence and presence of seeds of HA, BR, and CaOx. Note the presence of HA and BR seeds scattered on the membrane and on the CaOx crystal surfaces.

by engulfment of small, freshly nucleated CaOx crystals and the seed crystals by the CaOx growth front. In the presence of HA and BR seeds, however, the seeds are also seen lying free on the filtration membrane and attached like barnacles on the surfaces of the freshly precipitated CaOx crystals.

Assessment of Crystal Deposition by ^{14}C-Oxalate Analysis

Figure 5 shows the time course of disappearance of ^{14}C-oxalate from the supernatants of the urine samples throughout the incubation period after addition of the oxalate load. To normalize the data, the values are presented as the amount of ^{14}C-oxalate remaining in the solution, expressed as a percentage of the zero time value. Two features of these data are remarkable. First, as expected, values recorded in the samples containing CaOx seeds differed dramatically from those observed in the presence of HA and BR seeds. Second, the rate of reduction in ^{14}C-oxalate in the control sample (containing no added seeds) was almost indistinguishable from those supplemented with seed crystals of HA or BR. At 2 hr, the mean percentage reduction in ^{14}C-oxalate in the control urine containing no seed crystals (79.2%) was very close to the values obtained in the presence of seed crystals of HA (78.9%) and BR (77.7%). However, it was in the presence of CaOx seeds that the most dramatic reduction in ^{14}C-oxalate occurred, with the value at 2 hr being only 60.8%. These values demonstrate that seed crystals of HA, BR, and CaOx

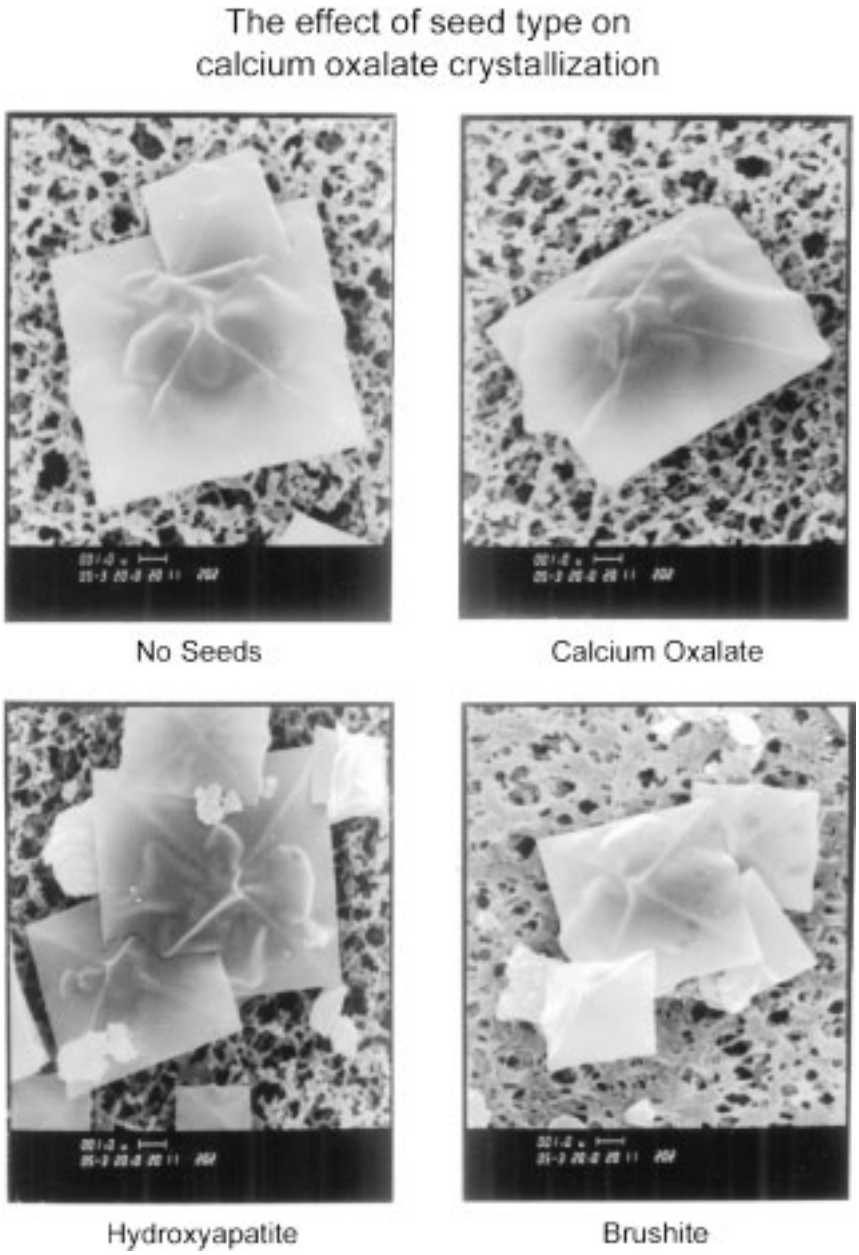

Fig. 4. High power scanning electron micrographs of the samples shown in Fig. 3. Note that the crystalline particles deposited show protuberances, apparently caused by engulfment of small, freshly nucleated CaOx crystals and the seed crystals by the CaOx growth front. In the presence of HA and BR seeds, however, the seeds are also seen lying free on the filtration membrane and attached like barnacles on the surfaces of the freshly precipitated CaOx crystals.

increased the mineral deposition by 0.37 (nonsignificant), 1.82 (nonsignificant), and 23.23% ($p \leq 0.05$), respectively, in relation to the control to which no seed crystals were added.

Discussion

A number of theories have been proposed in an attempt to explain the formation of urinary calculi. Of these, epitaxy has been the most often cited, its credibility having been reinforced by the presence of alternating layers of different minerals around a CaP core (6–10). The fact that human urine only occasionally attains levels sufficient to allow the spontaneous nucleation of CaOx crystals (41), and the existence of geometrical fits between the crystalline lattice dimensions of CaOx and that several crystalline phases of minerals commonly present in urinary calculi (12,28,31), have strengthened the supposition.

Human urine is commonly supersaturated with respect to CaOx and brushite (10,14–17), especially in patients with high concentrations of urinary calcium and/or phosphate and alkaline urinary pH (42). This favors the formation of CaP particles that could, at least theoretically, act as nidi for the deposition of CaOx. It is noteworthy that the solubility of CaP decreases with increasing pH. Typically, CaP precipitates when the urine pH is higher than 6.2 (43); BR generally forms in mildly acidic urine, and HA forms in alkaline urine (44,45). Although HA is the most common crystal in urine from both normal and

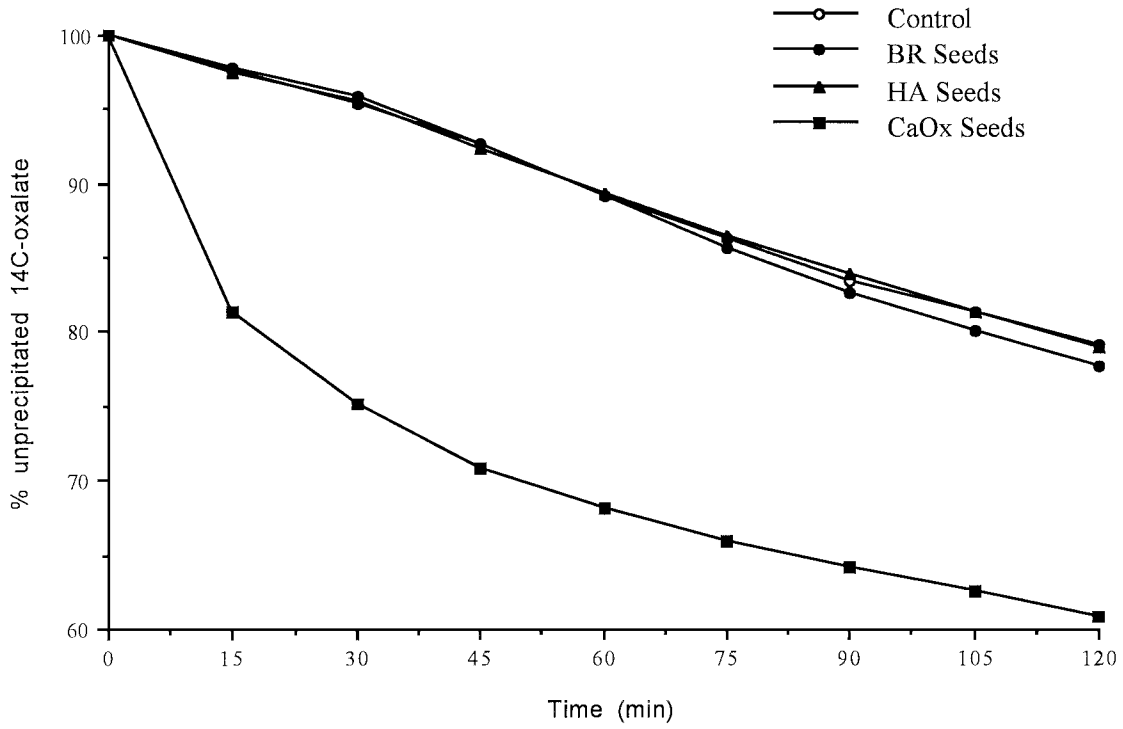

Fig. 5. Change in unprecipitated ^{14}C-oxalate following the addition of the oxalate load in the control urine to which no seeds were added and samples of the same urine containing seeds of HA, BR, and CaOx. The values obtained represent that although seed crystals of CaOx promoted the mineral deposition by 23% ($p \leq 0.05$), HA and BR seeds had no significant effect in comparison with the control, to which no seeds were added.

stone-forming subjects (18,19), BR rarely presents as crystalluria and is only seen in urine from patients with hyperparathyroidism or idiopathic CaOx urolithiasis (46), which might also explain why BR is infrequently found in urinary calculi (6–7,9–10). It has been suggested that although BR is the initial CaP phase precipitated in urine (45), it subsequently transforms into HA in response to excretion of alkaline urine, which is not uncommon at certain times of the day and during the course of stone disease (47). BR is not stable above a pH of 6.9 (44,45); in alkaline urine it preferentially takes up calcium and transforms into HA, which is the thermodynamically stable phase of CaP in alkaline urine (48).

Although studies have shown that crystalline particles of HA and BR can induce precipitation of CaOx from metastable inorganic solutions (22,33), similar results have never been obtained in urine. Because the physicochemical properties of urine bear scant similarity to those of inorganic solutions, this cautions against extrapolating findings from inorganic solutions to predict likely effects in whole urine, particularly on stone formation. Perhaps the most compelling reason for exercising caution is that the latter contains large numbers and quantities of low- and high-molecular-weight components, some of which are well-documented inhibitors of CaOx crystallization (49). Furthermore, the concentrations and relative amounts of various urinary constituents differ from one urine specimen to another. Thus it is fair to say that the effects of all urinary components on the epitaxial relationship between CaOx and HA or BR cannot possibly be reproduced under experimental conditions using inorganic salt solutions in vitro.

The separate effects of citrate (26,29), magnesium (29), osteopontin (OPN), THG, chondroitin sulphate (CS), a fraction of urinary macromolecules (UM) (33), and HSA (29,33) on the HA-induced deposition of CaOx under inorganic reaction conditions have been previously investigated. Generally, these substances increased the induction time, except CS, which decreased it (33). HSA was reported to promote nucleation in one case (29), but inhibit it in another (33). Moreover, THG, CS, and UM significantly reduced HA-induced growth of CaOx (33). It is worth emphasizing that OPN did not significantly affect HA induced nucleation and growth of CaOx (33), despite the fact that previous work has reported it to be a potent inhibitor of CaOx nucleation and growth (50,51). More perplexing was the behavior of CS, which promoted CaOx nucleation but inhibited its growth (33).

It is now well-recognized that various urinary components, especially macromolecules, play an active role during crystallization in urine (49), yet only one previous study examined the possible epitaxial deposition of CaOx onto CaP seeds in urine (52). For a number of reasons, the results of that study are open

to question. First, the authors did not mention the morphology of the CaP seeds, HA, or BR used, and their technique was very crude; it involved counting the number of precipitated particles with a Brand counting chamber and their identification by optical microscopy. Second, their assertion that CaP induced epitaxial deposition of CaOx was based simply on the observation that the number of precipitated particles (only some of which were identified) and CaOx dihydrate crystal increased in relation to rising concentration of soluble phosphate added to the urine. Third, they did not mention whether CaP seed crystals were overgrown by CaOx dihydrate—an event that would be expected had significant epitaxy occurred in their experiment. It is therefore unclear as to how or whether the presence of CaP nuclei in the precipitated CaOx crystals was demonstrated. In the present study, seed crystals of HA, BR, and CaOx were generated in undiluted human urine. After confirming their identities by X-ray powder diffraction analysis, they were added to undiluted human urine and precipitation of CaOx was induced by oxalate load. Analysis of ^{14}C-oxalate data revealed that although seed crystals of CaOx promoted the mineral deposition by 23%, HA and BR seeds had no significant affect in comparison with the control, to which no seeds were added. The degree of promotion caused by the CaOx seeds is of the same order as that produced by the commercial seed crystals of CaOx in another report using the same experimental system where ^{14}C-oxalate deposition was promoted by 54% at 3-fold higher final seed suspension concentration (53). Our data suggest that seed crystals of HA and BR do not promote CaOx deposition in undiluted urine and are therefore unlikely to act as nuclei for epitaxial deposition of CaOx to a physiologically significant degree in human urine in vivo.

It is remarkable that although solute deposition is a well-recognized mechanism of crystal enlargement, crystal aggregation is equally, if not, more important in stone formation. This is because it allows the formation of large, potentially dangerous particles within a short period of time and therefore increases the likelihood of particle retention within the renal collecting system. This supposition is supported by the observation that although both stone-formers and healthy subjects routinely pass CaOx crystals in their urine, it is only the former group where they are consistently aggregated into large clusters (18,43). The results of the present study reveal that the average modal sizes of the particles deposited in the presence of seed crystals of HA and BR were not significantly different from those precipitated in the control sample to which no seed crystals were added. These observations are supported by the scanning electron microscopic findings.

Had epitaxy occurred to any significant degree in this study, one would have anticipated overgrowth of the seed crystals with CaOx (and possibly, therefore, morphology of pure CaOx) or their envelopment by newly precipitated crystals of CaOx, so that the seed crystals themselves would be hidden. Certainly, this was the case with CaOx seeds: scanning electron microscopic examination of the precipitated crystals at high magnification revealed that large aggregates of CaOx were formed in the presence of CaOx seeds, which themselves were not visible, presumably because they had been hidden by deposition of CaOx upon them. In contrast, BR and to a lesser extent HA seeds were clearly visible. Although some of them lay free on the filtration membrane, others were attached to the surface of the freshly precipitated CaOx crystals; some of these had been overgrown by the CaOx growth front and were evident as protuberances on the surface of the CaOx crystals. This suggests that after precipitation and growth of CaOx had occurred, the seed crystals attached themselves to the surfaces, and as crystal growth proceeded, the seeds 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 phosphate/oxalate stones. Most important, these findings demonstrate that, even in the presence of adsorbed urinary macromolecules, crystals of HA and BR bind to the surface of CaOx crystals, not the other way around, as suggested by the epitaxy theory.

The results of our investigation are corroborated by the findings of Khan (54) and Burns and Finlayson (55). Whereas Khan (54) reported that, although CaP and CaOx crystals are closely associated in human and rat stones, they contain copious amounts of organic material, particularly on the interface between the crystals. This led him to conclude that epitaxial deposition of CaOx onto CaP is highly unlikely in vivo. The same conclusion was arrived at by Burns and Finlayson (55) who, using a rapidly mixed inorganic system of CaOx crystallization, found that fewer than 1% of HA seeds acted as nucleators.

It is interesting to note that critical appraisal of X-ray crystallographic data, which are the mainstay of the epitaxy theory, in fact, undermine its veracity. This is mainly because X-ray data provide only a theoretical comparison of the dimensions of prominent crystal faces, thereby allowing molecular analysis of crystal lattices in contact. Such analysis gives a large number of lattice matches between minerals commonly seen in stones at $<$15% misfits (28), which reduces significantly at $<$10% misfits, and approaches zero at $<$5% misfits. On this basis, the most common mineral combination actually observed (HA mixed with CaOx monohydrate or dihydrate) would not be theoretically possible, and the relatively uncommon combination of CaOx monohydrate and cystine would be predicted to occur more frequently. This is because there are no lattice matches for HA and CaOx, and one possible match between CaOx and cystine at misfits below 5%.

Furthermore, use of the molecular matching technique suggests that the separate mineral components in the HA–CaOx monohydrate combination totally support each other's deposition at atomic level (31). However, according to results of in vitro studies, although HA supports heterogeneous nucleation of CaOx, the converse is not true (23,25).

Taken together, the results presented here demonstrate unambiguously that HA and BR seeds do not promote CaOx deposition to a physiologically significant degree in urine. Epitaxial induction of CaOx crystal nucleation by seed crystals of HA or BR is therefore unlikely to be a major factor contributing to stone formation.

Acknowledgments

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