Intratubular crystallization of calcium oxalate in the presence of membrane vesicles: An in vitro study

JULIE M. FASANO and SAEED R. KHAN

Department of Pathology, College of Medicine, University of Florida, Gainesville, Florida, USA

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Background. Since urine spends only a few minutes in the renal tubules and has a low supersaturation with respect to calcium oxalate (CaOx), nucleation of CaOx crystals in the kidneys is most probably heterogeneous. We have proposed that membranes of cellular degradation products are the main substrate for crystal nucleation. The purpose of our study was to determine the site of membrane-mediated crystal nucleation within the renal tubules and the required lag time, factors that determine whether crystallization results in crystalluria or nephrolithiasis.

Methods. Nucleation of CaOx was allowed to occur in five different artificial urine solutions with ionic concentrations simulating urine in proximal tubules (PTs), descending (DLH) and ascending (ALH) limbs of the loop of Henle, distal tubules (DTs), and collecting ducts (CDs). A constant composition crystallization system was used. Experiments were run for two hours with or without the renal tubular brush border membrane (BBM) vesicles.

Results. The addition of BBM significantly reduced the nucleation lag time and increased the rate of crystallization. The average nucleation lag time decreased from 84.6 ± 43.4 minutes to 24.5 ± 19 minutes in PTs, from 143.6 ± 29 to 70.2 ± 53.4 minutes in DLH, from 17.6 ± 8.6 minutes to 0.625 ± 0.65 minutes in DTs and from 9.54 ± 3.03 minutes to 0.625 ± 0.65 minutes in CDs. There was no nucleation in the ALH solution without BBM for two hours. CaOx dihydrate (COD) was common in most solutions. Calcium phosphate (CaP) also nucleated in the DLH and CD solutions.

Conclusions. In the absence of membrane vesicles, there was no crystallization in any of the solutions within the time urine spends in the renal tubules. As a result, homogeneous nucleation of crystals anywhere within the nephron appears unlikely. However, BBM-supported nucleation is possible in the DTs as well as CDs. A high crystallization rate in CDs would promote rapid crystal growth and aggregation, resulting in crystal retention within the kidneys and development of nephrolithiasis.

Key words: nephrolithiasis, calcium phosphate, biomineralization, matrix vesicles, renal stones.

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Exactly where and how urinary stones originate are still unclear. Most urinary stones are located in the kidneys. Some are seen attached to the renal papillae. Others demonstrate signs of earlier attachment to the kidneys, such as remains of renal tubules in small depressions on stone surface [1]. Kidneys of many stone patients contain subepithelial plaques on their papillae [2]. These plaques are suggested to be the sites of stone development. Obviously, for a stone to form, crystallization must occur, and crystals must be retained in the kidneys. Since urine spends only three to five minutes in the renal tubules and is generally undersaturated for CaOx before reaching the collecting ducts (CDs), it is suggested that nucleation of CaOx crystals within the renal tubules is most probably heterogeneous [3]. Investigations of the ionic conditions within different segments of the nephron and application of the data to in vitro studies have shown that urine of the loop of Henle can support calcium phosphate (CaP) nucleation [4–10]. It was proposed that CaP crystals formed in the loops could promote nucleation of CaOx further along the nephron in the CDs. The results of most in vitro crystallization studies showed that it took hours for the precipitation of CaP in solutions simulating urine in the loop; however, urine spends only minutes in the tubules and seconds in various segments [7, 8]. Since crystallization must occur in moving urine, studies in one laboratory utilized a dynamic crystallization system [7, 8]. The solution composition simulated changing conditions existing in the proximal tubule (PT), the descending limb of the loop of Henle (DLH), the ascending limb of the loop of Henle (ALH), the distal tubule (DT), and finally to the conditions existing in the CD. When the solution conditions became similar to those in the DLH, precipitation of CaP required less than three seconds. The CaP precipitate, however, began to dissolve in the ascending limb conditions (ALH) and disappeared in the DT.

Since human kidneys slough approximately 70,000 cells per hour into the urine [11] and the matrix of kidney stones contains cells membranes and lipids [12, 13], we investigated the possibility that cell membranes and their
lipids may be involved in the nucleation of CaOx [14–16]. Lipids and membranes of the vesicles produced at the site of an injury by cellular degradation are already regarded as playing a critical role in CaP deposition in numerous pathological calcification processes [17, 18].

To determine the involvement of membrane vesicles of proximal tubular epithelial origin, we isolated brush-border membrane (BBM) from the kidneys of male rats. BBMs were incubated in solutions of artificial urine that corresponded to the conditions of urine in the PT, DLH, ALH, DT, and the CD. A constant composition crystallization system was used [19] so that the depleted ions were continuously replaced, similar to what may occur within the kidneys. We compared lag times and nucleation rates and examined the types of crystals formed, both with and without the membrane vesicles in the milieu. This system allowed a comparison of the possible effects shed membrane vesicles may have on nucleation of calcium oxalate (CaOx) in different segments of the nephron and indicated the tubular segment where crystallization is likely to occur.

### METHODS

#### Solutions

We employed five artificial urine solutions equivalent to the urinary ionic conditions at the PT, DLH, ALH, DT, and the CD (Table 1). These solutions were developed by Kok and are based on micropuncture data [7]. All solutions were made with reagent grade chemicals purchased from Fisher Scientific (Hampton, NH, USA). The solutions were made with deionized (DI) water and the necessary quantities of NaCl, KCl, Na₂C₆H₅O₇ · 2H₂O, MgSO₄, Na₂SO₄ and NaH₂PO₄ · H₂O to obtain the proper ionic activity for each segment. From each of the five artificial urine solution, two 100 mL aliquots were poured into glass bottles. To half of the aliquots, 0.555 g of CaCl₂ was added to prepare 50 mmol/L calcium solutions from each artificial urine solution. To the remaining aliquots, 0.67 g of Na₂C₂O₄ was added, resulting in a 50 mmol/L oxalate. These solutions were prepared immediately preceding each experiment. The pH of each solution was adjusted accordingly for each location of the nephron with reagent grade, 5N NaOH. The solutions were filtered using Corning Costar 0.2 micron bottle top filters (Fisher Scientific). All solutions were stored at 8°C, and new calcium and oxalate solutions were made every two days.

#### Membrane vesicles

Renal tubular BBM vesicles were isolated from the kidneys of healthy Sprague-Dawley rats using Biber’s methodology [20]. The purity of the BBM was determined by transmission electron microscopy (TEM) and by assaying for specific activities of marker enzymes: alkaline phosphatase, γ-glutamyl transpeptidase, and leucine aminopeptidase. The vesicles were stored frozen in a Tris buffer. They were thawed and diluted with each artificial urine solution before use.

#### Crystallization system

Polystyrene microbeakers, cleared of particles using a precision duster (Fisher Scientific), were used for the crystallization experiments. Artificial urine solutions, corresponding to each segment of the nephron, were prepared as described previously in this article. Eight to 10 crystallization experiments were conducted within each artificial urine solution. To begin each experiment, a specific volume of artificial urine solution was pipetted into a microbeaker. A stir bar, stored in Chromerge solution, was rinsed with DI water, blown dry using the precision duster, and added to the microbeaker. The microbeaker was placed into a glass-jacketed beaker maintained at 37°C with an 800 Isotherm Constant Tem-

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**Table 1. Composition of the artificial urine solutions corresponding to the urine in each segment of the nephron**

<table>
<thead>
<tr>
<th></th>
<th>Proximal tubule</th>
<th>Descending limb of the loop</th>
<th>Ascending limb of the loop</th>
<th>Distal tubule</th>
<th>Collecting duct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺ mmol/L</td>
<td>1.35</td>
<td>7.9</td>
<td>1.35</td>
<td>0.75</td>
<td>7.5</td>
</tr>
<tr>
<td>Oxalate mmol/L</td>
<td>0.009</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.5</td>
</tr>
<tr>
<td>PO₄ mmol/L</td>
<td>1.35</td>
<td>8.0</td>
<td>8.0</td>
<td>3.2</td>
<td>42.0</td>
</tr>
<tr>
<td>SO₄ mmol/L</td>
<td>0.000869</td>
<td>0.00515</td>
<td>0.00515</td>
<td>0.00206</td>
<td>0.02705</td>
</tr>
<tr>
<td>Na⁺ mmol/L</td>
<td>151.5</td>
<td>408.7</td>
<td>158.7</td>
<td>48.9</td>
<td>199.0</td>
</tr>
<tr>
<td>Cl⁻ mmol/L</td>
<td>154.7</td>
<td>423.8</td>
<td>153.9</td>
<td>47.1</td>
<td>175.0</td>
</tr>
<tr>
<td>K⁺ mmol/L</td>
<td>0.0915</td>
<td>0.2468</td>
<td>0.09582</td>
<td>0.02953</td>
<td>0.12015</td>
</tr>
<tr>
<td>Mg²⁺ mmol/L</td>
<td>0.99</td>
<td>4.0</td>
<td>0.59</td>
<td>0.3</td>
<td>5.0</td>
</tr>
<tr>
<td>Citrate mmol/L</td>
<td>0.03</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>2.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.7</td>
<td>7.26</td>
<td>6.65</td>
<td>6.38</td>
<td>6.16</td>
</tr>
<tr>
<td>pHb</td>
<td>6.34</td>
<td>6.94</td>
<td>6.62</td>
<td>6.35</td>
<td>6.02</td>
</tr>
<tr>
<td>pHc</td>
<td>0.1771</td>
<td>1.596</td>
<td>0.7542</td>
<td>1.114</td>
<td>15.490</td>
</tr>
</tbody>
</table>

| Relative supersaturation | 0.555 g of CaCl₂ was added to prepare 50 mmol/L calcium solutions from each artificial urine solution. To the remaining aliquots, 0.67 g of Na₂C₂O₄ was added, resulting in a 50 mmol/L oxalate. These solutions were prepared immediately preceding each experiment. The pH of each solution was adjusted accordingly for each location of the nephron with reagent grade, 5N NaOH. The solutions were filtered using Corning Costar 0.2 micron bottle top filters (Fisher Scientific). All solutions were stored at 8°C, and new calcium and oxalate solutions were made every two days.

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### Concentrations calculated

### pH of each solution prior to the addition of Ca²⁺ or Ox

### pH measured after the addition of Ca²⁺ and Ox to the solutions

### Added to each solution via the corresponding 50 mmol/L Ca²⁺ and 50 mmol/L Ox solutions immediately preceding each experiment

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*Fasano and Khan: BBM-mediated crystallization of CaOx*
perature Circulator (Fisher Scientific). The glass-jacketed beaker was placed onto an automixer (Fisher Scientific), and the stir speed was set at 2.5. The 50 mmol/L calcium and 50 mmol/L oxalate solutions, corresponding to the artificial urine solution being examined, were loaded into separate ABU80 Autoburetts (Radiometer America Inc., Westlake, OH, USA). The volume of calcium solution needed to obtain the correct calcium ion concentration for the solution was added to the microbeaker through the Autoburett. The addition of calcium was monitored with an Orion, model 93-20, Calcium Sensing Electrode (Fisher Scientific), coupled with a k401 Calomel Reference Electrode (Radiometer America Inc.). Both electrodes were wired into a PHM82 Standard pH Meter (Radiometer America Inc.), which allowed the calcium ion concentration to be read in mV. The addition of calcium was recorded using a model 0555 chart recorder (Cole Parmer Instrument Co., Vernon Hill, IL, USA) connected to the Autoburett. The electrodes were allowed to equilibrate 10 to 20 minutes after the addition of calcium. A stable reading of the calcium concentration by the electrodes was verified when the rise in calcium concentration demonstrated on the chart recorder, leveled to a straight line on the chart paper. The required volume of oxalate was added to obtain the proper relative supersaturation (RSS) for CaOx for the specific solution, and the electrode allowed to stabilize approximately one minute. A pH STAT Interface, model 999051 (Radiometer America Inc.), was used to reset the equipment so that the volume of oxalate added over time during the experiment could be recorded with a REC 80 Servograph, model 287053, chart recorder (Radiometer America Inc.). The end point value on a TTT80 titrator (Radiometer America Inc.) was set to match the potential reading in mV on the pH meter, and the titrator was started. The titrator was connected to the pH meter and both Autoburetts. Any change in the potential (Ca$^{2+}$ concentration) measured by the electrode, triggered simultaneous and automatic addition of CaCl$_2$ and Na$_2$C$_2$O$_4$, through the Autoburetts, and was recorded onto the chart recorder. This allowed the supersaturation and ionic activity of the solution in the reaction vessel to be monitored and maintained by the calcium electrode. To half of the experiments for each solution, 200 µL of 1 mg/mL BBM were added, while the remaining experiments received 200 µL of artificial urine with no BBM. Experiments were allowed to continue for up to two hours or until between 0.5 and 1.0 mL of calcium and oxalate was added.

**Analysis**

Calcium oxalate crystallization rates were determined for all five artificial urine solutions, both with and without BBM. The rate of crystallization for each solution was equated to the amount of oxalate solution added to the experiment over time. The rates were calculated by averaging the slopes recorded on the chart recorder, of four to six experiments, both with and without BBM, for each solution. The standard error about the mean for each point of the slope and the significance of difference between crystallization rates was calculated using Jandel Scientific® Sigma Plot (version 3.0). The lag time for nucleation, with and without BBM, was determined for each artificial urine solution by observing the lapsed time before calcium and oxalate were automatically added to the system. To validate the assertion that initiation of calcium ion depletion from the solutions is indicative of crystal nucleation and not of simple calcium uptake or binding by the BBM vesicles, constancy of the calcium and oxalate concentrations and their equimolar consumption was determined by periodically removing aliquots, filtering and analyzing the filtrate for calcium and oxalate and retentate for the crystals. A Student’s $t$-distribution, using Microsoft® Excel version 5.0, was performed on the lag time data to determine the significance of differences between the averages.

**Crystal identification**

Samples from completed experiments were filtered using 0.2 µm polycarbonate membrane filters (Fisher Scientific). The filters were allowed to dry and fixed with colloidal graphite to 0.5 inch aluminum mounts (Fisher Scientific). They were coated with silver using a Plasma Sciences CrC 100 Sputtering System and were examined by a Joel JSM 35 C (Tokyo, Japan) Scanning Electron Microscope. The presence of crystals was verified, and crystals were identified morphologically [21]. Microanalysis of each sample was performed using energy dispersive x-ray microanalysis. These data were used to determine the elementary composition of the crystals. Calcium to phosphorous ratios were calculated from the peak areas of calcium and phosphorous and were used to identify the type of CaP crystals present.

**RESULTS**

**Lag time**

In the experiments using solutions simulating ionic condition of the PT urine, the average lag time for CaOx crystalization exceeded 84.6 ± 43.4 (mean ± SEM) minutes. Following the addition of BBM, the average lag time decreased to 24.5 ± 19 minutes, a decrease of approximately 60 minutes. Statistical analysis showed the reduction in lag time to be significant ($P < 0.02$). This trend was observed for each of the remaining solutions (Table 2).

The addition of BBM resulted in the decrease of average CaOx nucleation lag time by approximately 73 minutes in DLH solutions, 17 minutes in the DT solutions, and 8 minutes in the CD solutions. In the absence of
Table 2. Average lag time observed, with and without brush border membrane present, in solutions representing normal urinary conditions in various segments of the nephron

<table>
<thead>
<tr>
<th>Nephrone segment</th>
<th>With brush-border membrane</th>
<th>Without brush-border membrane</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal tubule (RSS = 0.180)</td>
<td>24.5 ± 19 (N = 5)</td>
<td>84.6 ± 43.4 (N = 5)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Descending limb (RSS = 1.60)</td>
<td>70.2 ± 53.4 (N = 5)</td>
<td>143.6 ± 29 (N = 5)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Ascending limb (RSS = 0.750)</td>
<td>1.25 ± 1.26 (N = 4)</td>
<td>NA* (N = 5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Distal tubule (RSS = 1.11)</td>
<td>0.625 ± 0.65 (N = 4)</td>
<td>17.6 ± 8.6 (N = 4)</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Collecting duct (RSS = 15.5)</td>
<td>0.625 ± 0.65 (N = 4)</td>
<td>9.54 ± 3.03 (N = 6)</td>
<td>&lt;0.002</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. RSS is relative supersaturation.
*No nucleation after 2 hours

BBM, there was no consumption of oxalate in the solution simulating urinary ionic conditions in the ALH, indicating lack of CaOx nucleation. However, when BBM was added, oxalate consumption indicating nucleation started in 1.25 ± 1.26 minutes (N = 4). Thus, a significant difference of at least 120 minutes resulted (P < 0.001).

**Crystallization rate**
In the absence of BBM in the solutions, the crystallization rate was extremely slow, and the addition of titrants was insignificant except in the CD urine (Fig. 1). Actually, there appeared to be no change after the initial addition of calcium and oxalate. The introduction of BBM to all solutions increased the rate of crystallization (Fig. 2). Changes were more pronounced in solutions simulating urine in the DLH and CD. The rate of crystallization in CD urine was so rapid that the experiment had to be stopped within 10 to 20 minutes to prevent overflow of titrants. Within the first minute, crystallization in CD solution progressed at a rate of 7.0 mmol/L Ox/min.

**Crystal analysis**
At the end of each experiment, scanning electron microscopy was used to verify and identify the crystals (Table 3). CaOx precipitated as monoclinic plate-like CaOx monohydrate (COM) or tetragonal bipyramidal CaOx dihydrate (COD). In the PT solutions (Fig. 3), few crystals formed in the absence of BBM, and most appeared to be COM. Following the addition of BBM
to the solution, a mixture of COD and COM crystals was found. These crystals were poorly developed and showed signs of dissolution. In the DLH solutions, crystals of both CaOx and CaP were present (Figs. 4 and 5). However, COD crystals were more common in solutions with BBM (Fig. 4A). COM crystals formed in solution without the BBM showed signs of dissolution with deep etch marks and surface discontinuities (Fig. 4B). CaP crystals were more common when no BBM was added to the solutions. They had two distinct morphologies (Fig. 5) and different Ca/P ratios. Crystals in the form of rectangular (Fig. 5A) plates had a Ca/P ratio of 0.81, while thin plate-like crystals matted into a solid mass (Fig. 5B) had a Ca/P ratio of 1.

Crystallization did not occur in the ascending limb.
solutions in the absence of BBM. Even when BBM was added no distinct crystals were observed. In the DT solutions, COM crystals were most prominent (Fig. 6B) in the absence of BBM, with a few COD crystals. However, when BBM was added to these solutions, COD was the only crystal identified (Fig. 6A). Finally, in the CD solutions, a mixture of COD and CaP was observed (Fig. 7), both with and without BBM. When BBM was added to the solutions, two types of CaP were found. The spherical crystals demonstrated a Ca/P of 8, while wafer thin long plates had a Ca/P of 1.66. When no BBM was present in the CD solution, wafer thin plates demonstrated a Ca/P of 1.5, and spherical aggregates of small plates showed a Ca/P of 2.5.

DISCUSSION

Investigators agree that crystallization in a solution occurs only when it is supersaturated with the mineral concerned, since supersaturation affects all aspects of crystallization including nucleation, growth, and aggregation [22–25]. Nucleation in the absence of a substrate, the homogeneous nucleation, requires higher supersaturation and a longer time. The main determinants of urinary supersaturation with respect to CaOx and CaP are calcium, oxalate, phosphate, pH, citrate, and magnesium. The concentration of these substances changes as urine courses through a nephron, thus affecting the supersaturation and potential for crystal nucleation. Estimations of CaP and CaOx supersaturations in the urine have led to the conclusion that urine is metastable with respect to CaOx only in the CD [4, 7, 9]. Even in the CD urine, supersaturation is not high enough for the homogeneous nucleation of CaOx. The urinary conditions in the loops of Henle and DTs are, however, favorable for crystallization of CaP [4–10].

The results of our studies show that nucleation of
CaOx can occur not only in the CD, but is also possible in other segments of the nephron, and that membrane vesicles such as BBM have a positive effect on CaOx crystallization. The addition of BBM vesicles increased the precipitation of COD. Our earlier studies of nephrolithiasis, in an animal model where hyperoxaluria was induced following membrane shedding, also showed COD formation in association with the membrane [26]. Lieske, Toback, and Deganello demonstrated direct nucleation of COD on surface of BSC-1 (African Green Monkey kidney cell line) renal epithelial cells grown in culture [27]. However, this does not mean that membranes support COD nucleation only. We have observed formation of COM as well in association with the cell membranes [28]. It is likely that other components of the urine also have an influence on crystallization. For example, the presence of magnesium in the milieu has been deemed important in producing COD crystals in vitro. A variety of CaP crystals formed. They had brushite, octacalcium phosphate, and/or hydroxyapatite-like phases of intermediate stoichiometries.

The PT urine was relatively dilute and stable with respect to CaOx with an estimated CaOx RSS of 0.180. It is not surprising to see a long nucleation lag time even in the presence of BBM vesicles. Even after a few crystals of COM formed, the solution did not begin nucleating additional crystals or support continued growth of the crystals already present. This is reflected by the low rates of crystallization. SEM examinations of the precipitate formed in PT urine showed a few poorly formed crystals. It is probable that in a solution with a RSS of 0.180, any crystals that did nucleate would quickly dissolve. Kok determined that CaOx could form in the PT with a serum oxalate concentration of 50 μmol/L [7]. These conditions are possible in the patients with primary hyperoxaluria where deposits can develop not only in the kidney [29] but in other organs as well. Similarly, rats with experimentally induced hyperoxaluria have been shown to deposit crystals in the PTs [30, 31].

The CaOx RSS for the DLH solutions was 1.60, which is within the metastable range of 1 to 6 for COM and, therefore, on the basis of supersaturation, should support heterogeneous nucleation of CaOx. However, the lag time before nucleation began in these solutions was 70.2 ± 53.4 minutes with BBM and was more than 120 minutes when no BBM was present. These lag times are greater than those seen for the PT solutions, even though the RSS with respect to CaOx for the DL is greater than that of the PT. This is probably due to the higher concentrations of both the Mg and citrate in the DLH solution compared with those seen in the PT solution. Both CaOx and CaP crystals were observed. The CaOx formed was primarily COD, with some COM. Using a dynamic crystallization system, Kok determined that CaP precipitated within three seconds in the simulated DLH conditions. Asplin, Mandel, and Coe also reported CaP formation in simulated DLH conditions and determined that the CaP formed was of an immature moiety such as brushite or octacalcium phosphate [5]. Thus, physiological conditions within the DLH can lead to nucleation of CaP within the nephron. This may be even more likely in the presence of promoters such as BBM vesicles.

It has been recognized that the chance for CaOx nucleation decreases as urine enters the ALH because of calcium reabsorption, which in turn lowers the RSS with respect to CaOx. This appears to be true even in our studies where no crystallization was noticed with or without BBM, most probably because the RSS (0.75) of AL solution was below the metastable range for CaOx.

The CaOx RSS for the DT solution was 1.11, barely within the metastable range for COM. However, a CaOx RSS of 1.11, coupled with a pH of 6.38, should support heterogeneous nucleation and further crystallization. We found that in the presence of BBM, CaOx nucleated within 0.625 ± 0.65 minutes. However, crystallization rates were extremely low (0.016 mmol/L Ox/min with BBM and 0.010 mmol/L Ox/min without BBM). The low crystallization rate may be attributed to the RSS of this solution being at the lower limit of the metastable range. Tiselius estimated levels of supersaturation with CAP and CaOx in the DT [9] and found that diurnal variations in urine composition and pH can lead to highly supersaturated urine in the DT and promote homogeneous nucleation of CaP. Homogeneous nucleation of CaOx was ruled out, however. Kok reported CaOx nucleation in DT solution in a dynamic crystallization system, but found a lack of precipitation when a static nucleation system was used [7, 8].

We demonstrated the smallest nucleation lag times and fastest overall rate for CaOx crystallization in the CD solutions. This is not surprising considering the solution possessed a CaOx RSS of 15.5 and a pH of 6.16. The relatively rapid rate of crystallization in the CD solution, compared with the rates observed in the other solutions, indicates CD solution’s readiness to support CaOx crystallization. COD was the main CaOx crystal both in the presence and absence of BBM vesicles. Kok found that in the dynamic nucleation system he used, COD formation was followed by COM formation. It is possible that if we allowed experiments to continue for the full 120 minutes, COM would have been observed as well. However, because of the rapid rate in which crystallization occurred in this solution, the experiments were stopped within 10 to 20 minutes to prevent overflow. CaP crystals were also observed, which was surprising considering the relatively low pH of the solution. However, acidic conditions can promote crystallization of calcium hydrogen phosphate or brushite. Precipitation of a mixture of CaOx and CaP crystals at pH 5.5 to 6.1
has been reported in the presence of dialyzed urine [10], which most likely contained membranous vesicles.

Most in vitro crystallization studies showed nucleation lag times of hours and days. However, urine does not spend hours in the renal tubules [32]. According to one assessment, urine spends approximately 24 seconds in PT, 40 seconds in DLH, 100 seconds in ALH (long loop nephron), 42 seconds in DT and 48 seconds in CD, for a total of 254 seconds in the entire nephron [7]. Therefore, based on nucleation lag time homogeneous nucleation of CaOx is unlikely. Even BBM-induced heterogeneous nucleation is possible in only the DT and CD where urine spends more time than the nucleation lag time. Urine in the other segments may not support crystallization because of the length of time it takes for nucleation to begin is longer than the urinary residence time. Kok showed nucleation of CaP in DLH within seconds only when a dynamic crystallization system was used [7, 8]. However, the precipitate disappeared once the conditions were changed to simulate those existing in the ALH. CaOx precipitated when the ionic environment mimicked the DT conditions. Kok proposed that dissolving CaP promoted the nucleation of CaOx. Our ultrastructural studies of kidney stones and crystal deposits in both the human and rat kidneys have shown that an organic coat always surrounds the crystals [33]. The intranephronic crystals are always seen in association with cellular degradation products [31, 34]. The coat appears to develop as soon as crystals are formed and consists of adsorbed proteins and lipids [35]. Biological crystals are actually a crystal-matrix unit [12]; therefore, crystals formed in the DLH will soon become associated with organic material consisting of proteins, lipids, and other membranous material. Organic materials associated with the crystals may prevent them from dissolving in the ALH but support heterogeneous nucleation of CaOx further down the nephron in the DT and CD.

Cellular membranes have long been implicated in both physiological and pathological calcification processes. Membranes of the so-called matrix vesicles have been suggested to promote physiological calcification [36, 37], while membranes of cellular degradation products present at the sites of injury have been shown to assist in pathological calcification [17, 18, 38, 39]. In both situations, membrane lipids act as nucleation substrates, while various proteins and glycosaminoglycans modulate crystal growth and aggregation. Our morphological studies of kidney stones [12, 33], investigations in animal model of CaOx [31, 34] and CaP nephrolithiasis [38, 39], and in vitro crystallization studies [14, 15, 28, 35] have provided evidence of similar phenomenon during the formation of CaOx kidney stones. The matrix of kidney stones contains lipids, proteins, and various glycosaminoglycans [13, 28, 40].

Exposure of renal epithelial cells to oxalate and CaOx crystals, both in culture [41–45] and during CaOx nephrolithiasis [30, 31, 34], injuries the cells and induces their degradation and sloughing. Cells of the PTs are more susceptible to oxalate-induced injury than those of the CDs [44]. Cellular degradation products in the form of vesicles are clearly visible at the nucleation sites of stones [12, 13, 35] and crystal deposits [35, 46]. Membranes isolated from rat renal tubular brush border induce CaOx crystal formation in a metastable solution [28] and are seen at the nucleation sites. Crystal deposits in kidneys of rats with experimentally induced nephrolithiasis are totally surrounded by membranous vesicles and appear connected to each other by membranes [31, 35, 46]. Shedding of membrane into the urine promotes CaOx crystallization in the rats [26]. We have proposed that renal epithelial injury particularly to the PT is a risk factor for nephrolithiasis [47, 48]. Injured cells are released from basement membrane both as whole units or small membrane-bound fragments and vesicles [31, 34, 48]. These membranes act as heterogeneous nucleators of CaOx crystals. They also support crystal aggregation by joining various crystals together [46]. Association of cellular degradation products with the crystals and their eventual incorporation into the growing aggregates increases the mass with obvious consequence of being retained inside the nephron due to its size. For example, a 5 \( \mu \text{m} \) CaOx crystal attached to a 5 to 30 \( \mu \text{m} \) cell or cell fragment can increase the crystal size to approximately 10 to 35 \( \mu \text{m} \).

Earlier, we presented evidence for the involvement of membrane vesicles in promotion of CaOx crystallization in an in vivo rat model [26, 34]. Our present in vitro physicochemical study demonstrated that membrane vesicles could decrease the nucleation time and increase the rate of crystallization. An increased rate of crystallization means that crystal growth is occurring more rapidly than the typical formation. In addition, as the CaOx RSS increases, the lag time before nucleation decreases and rate of crystallization increases. As a result, it is likely that BBM would induce crystallization earlier and faster in the urine of stone formers, where RSS is often elevated. This might mean that in the presence of BBM vesicles, an elevated RSS coupled with changes in the urinary crystallization inhibitory potential occurs, and more and larger crystals could form in earlier portions of the nephron. Numerous crystals, nucleated sooner and in earlier portions of the nephron may give added time for crystal growth, thus increasing the likelihood of aggregation. The presence of a large number of crystals may also increase the possibility that cellular injury occurs, which, in turn, increases the amount of cellular debris in the urine and the likelihood of crystal attachment to the injured cells [31, 34, 41, 48, 49]. Additional cellular debris in the urine can induce further nucleation and link crystals already present, allowing the formation
of large aggregates. Whether through the attachment of crystallites to injured renal cells or through the formation of large crystal aggregate that become lodged in the narrow lumen of the tubules, these membrane-induced crystals may become retained within the kidneys. Once retained, the crystals and crystal aggregates, when subjected to further episodes of elevated CaOx RSS, can slowly grow into a urinary stone.

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Reprint requests to Dr. Saeed R. Khan, Department of Pathology, College of Medicine, University of Florida, Gainesville, Florida 32610-0275, USA.
E-mail: khan@pathology.ufl.edu

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