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HETEROGENEOUS NUCLEATION OF CALCIUM OXALATE CRYSTALS IN MAMMALIAN URINE

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Abstract

It is generally recognized that calcium oxalate crystal formation in urine is induced by heterogeneous nucleation. However, there is no consensus as to the nature of the nucleation substrate. Evidence is provided in this paper that membranous cellular degradation products are the most likely candidates because they: (1) are ubiquitous in urine and urinary stones; (2) are found in close association with crystal deposits in the kidneys; and (3) can induce nucleation of crystals from a metastable solution of calcium oxalate *in vitro* and metastable urine *in vivo*.

Key Words: Calcium oxalate, nephrolithiasis, cell membrane, urolithiasis, matrix vesicles, mineralization, calcium phosphate.

Introduction

An important event in the onset of urolithiasis is supersaturation of urine with a potentially precipitating crystalline phase; the greater the supersaturation, the greater the likelihood of precipitation [31]. Supersaturated urine can be metastable or unstable. In an unstable solution, crystals form spontaneously by homogeneous nucleation. In a metastable solution, crystals may be induced by a foreign interface such as certain macromolecules or other crystals that can act as nidi for crystal nucleation. Extremely high levels of supersaturation are necessary to produce homogeneous nucleation and it is highly unlikely that the spontaneous formation products of any of the stone salts will ever be attained in the urine. For example, homogeneous nucleation of calcium oxalate (CaOx) monohydrate does not dominate until relative supersaturation reaches 70 and CaOx relative supersaturation in mammalian urine rarely exceeds 30 [19, 30, 31]. Moreover, since urine contains a number of macromolecules and cellular degradation products, it is most likely that the nucleation therein is heterogeneous. This thinking was reflected in the report of the 1981 Dahlem Conference on Biological Mineralization and Demineralization [78] which concluded that (1) "homogeneous nucleation of any of the urinary stone salts or acids is unlikely", and (2) **"nucleation of urinary calculi is probably heterogeneous"**. United States National Institutes of Health Consensus Development Conference on Prevention and Treatment of Kidney Stones, held in March 1988 [79], also concluded that "disorders that raise supersaturation and **promote heterogeneous nucleation are the presently accepted causes of nephrolithiasis"**.

Crystals as Nucleators

What are the substrates acting as heterogeneous nucleators? Are they crystalline or macromolecular? Is there a specific substrate for a specific crystal type, or is heterogeneous nucleation simply a result of the presence of a foreign interface? Based upon the observa-

tions that calcium phosphate (CaP) is the most common crystal in human urine [117], is ubiquitous in human urinary stones, and is often seen at the center of the mixed CaOx/CaP urinary stones [84], nucleation of CaOx crystals is proposed to be induced by CaP crystals. In addition, both apatite and brushite crystals have been shown to induce crystallization of CaOx *in vitro* from a metastable solution of CaOx [76, 85]. On the other hand, solubility of CaP is very sensitive to pH: it decreases with increasing pH [30, 90]. Generally the apatite forms at higher, more alkaline pH, while brushite, at lower, more acidic pH. Even though brushite is not a common constituent of CaOx stones, suggestions have been made that brushite nucleates CaOx crystals and then transforms into apatite [30, 85]. Most idiopathic CaOx stone formers neither have a highly alkaline nor acidic urine. In practice, lowering the urinary pH of CaOx stone formers by simple acidification did not replace apatite with brushite but resulted in the formation of purer CaOx stones [30, 90].

Octacalcium phosphate, another crystal rarely encountered in kidney stones, has also been suggested to play a role in initiation of renal calculi. Recently Achilles *et al.* [1] grew spherulitic CaP in a gel matrix at 37°C from a supersaturated metastable solution continuously flowing over the gel surface. The spherulites contained a poorly crystalline core of amorphous carbonate apatite surrounded by a well crystallized shell of radially oriented sheets of octacalcium phosphate. Subsequently CaOx monohydrate crystals were grown on these spherulites by continuously flowing a solution supersaturated with respect to CaOx. Plate-like crystals of CaOx monohydrate demonstrated oriented growth in continuation with the plate-like crystals of octacalcium phosphate indicating epitaxially mediated crystal nucleation.

It has been demonstrated that even though excreted urine is generally supersaturated with respect to CaOx and CaP, the two crystallize and deposit at different locations in the kidneys [49, 55]. Calcium oxalate crystals generally deposit in collecting ducts of the renal papilla while calcium phosphate deposits in earlier segments of the nephron at the cortico-medullary junction. Data have been presented to support the hypothesis that urine becomes supersaturated with respect to calcium phosphate as early as the loop of Henle [21]. In a recent study [73], crystallization was induced by standardized increments of calcium and oxalate in solutions with a composition corresponding to various segments of the nephron. Crystals of CaP were predominantly formed in solutions with composition corresponding to the urine in proximal tubules while CaOx crystals were formed in solutions with composition corresponding to the urine in the collecting ducts. Moreover, sex hormones appear to play an important role in nephrolithiasis. Kidneys of male rats are prone to form calcium oxalate deposits while kidneys of female rats tend to form calcium phosphate [49]. Sex hormone dependence of crystallization may be one reason why CaOx nephrolithiasis is more common in men while CaP nephrolithiasis is more common in women [84]. Urine spends only a few minutes in the renal tubules, which is not enough time for single crystals of CaOx to grow large enough for their retention within the renal tubules [32]. The formation of a nucleating CaP crystals, and then nucleation and growth of CaOx crystals, should take even longer making it more difficult for CaOx crystals to reach a size large enough for retention and development of a kidney stone.

Recent studies have demonstrated that urinary crystals of calcium-containing salts almost always have a macromolecular coating. Transmission electron microscopic examination of urinary stones as well as other mineralized tissues has shown that demineralization results in replacement of crystals by crystal ghosts which represent the macromolecular coat [53, 54]. Thus, in the urinary environment, actual crystal surfaces may not be available for heterogeneous nucleation. CaP crystals in the renal fluid may not necessarily induce nucleation of CaOx in the kidneys just because CaP can act as heterogeneous nucleator of CaOx *in vitro*. If urinary crystals do play a role in nucleation of other crystals, they most probably are involved through the adsorbed macromolecules. Macromolecules dissolved in solution often act as inhibitors of crystallization, but once adsorbed on a surface, they may promote crystallization [18].

What about the presence of CaP crystals in CaOx stones, particularly the stone center? These CaP crystals may be adventitiously acquired by the CaOx stones and may simply represent crystalluria particles formed during alkaline tides [90]. Stone nidus may be formed by aggregation and retention of crystals [53] which later become covered with a mantle of organic matrix material. Such a surface could provide a platform for further crystal deposition similar to foreign body encrustation [52]. Similarly, Randall's plaques, which are papillary calcifications, rich in organic material [92], might also act as encrustation platforms and become nidi for the formation of renal stones. Thus, a kidney stone with CaP in the center may originate with intratubular precipitation and retention of CaP crystals which become overgrown with CaOx monohydrate [46].

In some *in vitro* studies, crystals of uric acid [39] and sodium acid urate [22, 86] have been shown to be able to induce heterogeneous nucleation of CaOx. Other studies, however, showed that uric acid crystals were not good nucleators of CaOx, and sodium acid urate crystals, though good nucleators, are generally not precipitated in the urine [33]. On the other hand, evidence has been presented that dissolved urate, at normal

physiological pH, can directly provoke CaOx crystal nucleation by the phenomenon of salting out [37, 98]. Growth of CaOx monohydrate on uric acid crystals may also be epitaxially mediated since CaOx monohydrate crystals grow on uric acid seeds in preferred orientation and CaOx monohydrate and uric acid form good atomic matches at the crystal surface [74].

Urinary Macromolecules as Nucleators

Since all urinary stones contain an organic matrix which is intimately associated with the crystals and consists essentially of the same proteins as the urine, it has long been suggested that urinary proteins are precursors of stone matrix and promoters of crystallization [13]. This subject has been recently discussed in an excellent review by Ryall and Stapleton [97]. Therefore, only the very pertinent elements will be addressed here.

Early studies suggested that the most prominent antigenic content of stone matrix called matrix substance A, accounted for up to 85% of its proteins [118]. It was reported to be present in the urine of stone patients, to originate from the renal parenchyma, and to be absent from the urine or the kidneys of normal individuals. This protein was suggested to be essential for the formation of urinary stones. However, the hypothesis that matrix substance A is involved in stone formation is weakened by the observations that this substance is present in the urine of a variety of patients with renal injury [47], particularly those with infection of the urinary tract and is further undermined by results of later studies that showed that matrix substance A containing fraction of the stone matrix actually inhibited the growth of CaOx crystals *in vitro* [34].

Tamm-Horsfall protein/uromucoid complex, present both in urinary stone matrix and urine [118], is another substance suggested to be a promoter of crystallization of CaP as well as CaOx by providing nidi for their heterogeneous nucleation. Boyce *et al.* [15] studied *in vitro* crystallization of CaP in the presence of uromucoid derived from normal urine and showed the amount of precipitate formed to be proportional to the concentration of uromucoid. In recent years, Rose and Sulaiman [95, 96] studied the role of Tamm-Horsfall protein (THP) and uromucoids on the crystallization of CaP and CaOx and showed that polymerized and aggregated Tamm-Horsfall protein acts as a promotor. For their studies, they rapidly evaporated crystal-free urine at 37°C on a rotary evaporator to 1250 mosmol/kg and found that the removal of macromolecules from the urine by ultrafiltration reduced the amount of precipitate formed and that this reduction was largely abolished by addition of physiological quantities of Tamm-Horsfall protein. A number of workers in the field have been unable to confirm the promotive role of the urinary protein; actually, they have found the THP/uromucoids to be inhibitors of CaOx and uric acid crystallization [28, 36, 70, 99]. Most of the current research on role of THP in nephrolithiasis indicates that the significance of THP lies in its involvement in CaOx crystal aggregation and depends on THP viscosity and solubility [40, 41]. At low ionic strength and high pH, THP is a powerful crystal aggregation inhibitor. Decreasing pH and increasing ionic strength, increase THP viscosity and its crystal aggregation inhibition potential [41]. High calcium lowers THP solubility and promotes crystal aggregation, while high citrate has the opposite effect. THP from severely recurrent CaOx stone patients appears abnormal. It demonstrates increased polymerization and reduced solubility, and thus, less potential for inhibiting CaOx crystal aggregation. As with matrix substance A mentioned earlier, the questions about Tamm-Horsfall's role in crystallization are also not yet resolved.

Many other macromolecules, having high affinity for calcium, have also been isolated from both urine and urinary stone matrix [94] and it has been shown that the urines of active CaOx stone formers have greater calcium binding capacity than those of non-stone formers or inactive stone formers [93]. A protein containing the calcium binding amino acid, gamma-carboxyglutamic acid, has been isolated from the urine of stone formers and matrix of calcium containing urinary stones [72]. It has been suggested that this protein may provide a substrate for nucleation of calcium-containing crystals. In a similar vein, Pinto *et al.* [88] isolated a urinary protein from the urine of stone formers that binds calcium and phosphate and promotes CaOx precipitation. Resnick and associates [93, 110] demonstrated the presence of uronic acid containing calcium-binding macromolecules in the urine of non-infectious CaOx stone formers and postulated a role for such molecules in crystal nucleation. In an experimental model, Itatani *et al.* [45] induced calcium containing stones by unilateral obstruction of ureters of rabbits, which normally have massive calcite crystalluria without stone formation. They found increased uronic acid production by the stone forming kidneys and concluded that calcium binding sulfated acid glycosaminoglycans produced by the kidneys resulted in crystal aggregation and the development of stones. In a similar study, using the same rabbit model, Wakatsuki *et al.* [115], however, found an increase in non-acidic non-sulfated glycosaminoglycan, hyaluronate, in the stone forming kidneys. Moreover, urinary glycosaminoglycans, particularly those precipitable with Alcian blue, are also considered powerful inhibitors of crystallization [100].

Some of the other urinary macromolecules involved in CaOx nephrolithiasis: nephrocalcin [77], uropontin

(osteopontin) [42, 102], uronic acid rich protein [4, 5], inter-alpha trypsin inhibitor [107], and urinary prothrombin fragment 1 (crystal matrix protein) [23, 108, 109] have all been shown to be inhibitors of CaOx crystallization processes.

From the above discussion it becomes apparent that even though it is highly probable that urinary macromolecules play a major role in crystal nucleation, no crystalline or macromolecular substrate has yet been clearly identified as a promoter. Most of the urinary or stone matrix macromolecules suggested or shown to be crystallization promoters by some, have been characterized by others as inhibitors of, or to have no effect on, crystallization. This leads to the following conclusions: (1) the same urinary macromolecules may have dual effects on crystallization: they may inhibit crystal nucleation but promote crystal growth or vice versa; (2) their inhibitory or promotional activity depends on the ambient conditions; (3) urinary macromolecules used in many studies are not single entities, but mixtures of various macromolecules which will influence crystallization differently under different *in vitro* experimental conditions; and (4) promoters of nucleation are yet to be identified. It appears that in the case of promoters of crystallization in urine, all of the above may apply. Matrix substance A, uromucoids, glycosaminoglycans (GAGs), and alcian blue positive material (ABPM), are all complex entities. Macromolecules belonging to GAGs and ABPM are a heterogeneous group [100]. There is a wide range in degree of sulfation and charge density of the various urinary GAGs: more highly sulfated GAGs appear to be associated with stone formers' urines. Heparin, which is not present in the urine but is one of the most highly charged species of all GAGs, is shown to be a potent inhibitor of CaOx crystallization *in vitro*. Alcian blue precipitable material is shown to be inhibitory to crystallization and proposed to consist mainly of GAGs. But, it must be pointed out that alcian blue is not a specific precipitant of GAGs but can also precipitate RNA-like material and other polyanions [100]. RNA is a potent inhibitor of CaOx crystal aggregation *in vitro* [16, 100].

Cellular Membranes and Calcific Diseases

Since the nature of macromolecules involved in heterogeneous nucleation of calcific crystals in urine is uncertain, studies from other pathological biomineralization systems may provide direction. According to the current concepts, initial calcium phosphate deposition in a number of calcific diseases [3, 7, 8, 9, 10, 11] occurs on cellular membranes which are present at the calcification site, either as the limiting membrane of the so-called matrix vesicles or as cellular degradation products. For example, in medial sclerosis of the large arteries and in calcific disease of the aortic valve, electron microscopic studies have established that extracellular vesicles serve as the initial sites of apatite deposition [64, 67, 69]. The same is true of atherosclerotic plaque formation [3]. Calcification of tumors is also associated with membranes [105]. Intracellular membrane vesicles appeared to nucleate the apatite of psammoma bodies in ovarian tumors and Michaelis-Gutmann bodies in malakoplakia [2], and when calcifying human meningiomas were cultured *in vitro*, psammoma bodies arose in association with extracellular matrix vesicles [29]. Extracellular vesicles derived from degenerating neoplastic cells have also been shown to be involved in neoplastic calcinosis [65]. In tympanosclerosis of the middle ear [75], matrix vesicles derived from fibroblastic cells were the initial site of apatite deposition. Calcification during apatitic osteoarthritis and calcifying tendinitis is also shown to be mediated by matrix vesicles. During placental calcification, calcium hydroxyapatite precipitates in association with extracellular membranous vesicles [111]. Calcification of bioprosthetic heart valves, fabricated from porcine aortic valves or bovine pericardium, which is the main cause of their failure, is also membrane mediated [101]. Initial nuclei of calcification are associated with cellular membrane fragments derived from pig cusp cells in the case of porcine valves and from connective tissue cells in the case of bovine pericardium. Calcification of intrauterine devices also appears to be initiated by cellular membranous material that is deposited on the devices during their exposure to the uterine fluid [56]. Dental plaque and calculus formation is yet another example of calcification initiated by cellular membranes [3, 7, 9, 10, 106, 114]. Membranes of microorganisms present in the dental plaque nucleate calcium hydroxyapatite, and thus, initiate calculus formation.

One of the main reasons that cellular membranes act as specific nucleators of calcium phosphate is proposed to be the presence of lipids, and particularly, the acidic phospholipids therein [7, 9, 10, 43, 44, 106, 114, 119, 120]. Lipids have been demonstrated to be present, both histochemically and biochemically, at physiological as well as pathological calcification sites [9, 10, 11, 43, 44, 81, 119, 120]. Although lipids account for a relatively small proportion of the organic matrix of mineralized tissues; 7-14% of bone, 2-6% of dentin, 12-22% of newly mineralized enamel, approximately 9.6% of submandibular salivary gland calculi, and 10.2% of supragingival calculi, they have been isolated from matrices of all types of such tissues [9, 10, 11, 43, 44, 81, 119, 120]. *In vitro*, membranes, acidic phospholipids, lipid extracts from various calcified tissues, and liposomes have been shown to initiate calcium phosphate formation from metastable solutions [25, 27, 82, 103, 104, 113].

Based on a review of the literature on calcified tissues, it can be concluded that cellular membranes and their lipids are intimately involved in both physiological and pathological calcifications. Several lines of evidence exist that point to a role for cellular membrane constituents in urolithiasis also. In a number of experimental studies of metastatic calcification of kidneys [116], non-mitochondrial calcific deposits were either present in proximal tubular basement membrane in association with membranous outpouchings of the cell or in the apical vacuoles of the epithelial cells. In a rat model, intranephronic calculosis caused by feeding purified diets is membrane-mediated [17, 80, 83]. Vesicles derived from the microvillous brush border of the proximal tubules provide the nidus for intratubular deposition of calcium phosphate. The deposits start in the lumens of segment I of the proximal tubule and travel down the nephron while at the same time accruing mass by laminar growth in which both membranous vesicles and mineral are involved. Finally, these microliths become large enough to be retained at the junction of the proximal tubule and the loop of Henle. Mineral content of the microliths increases, with the result that they become much more compact and the laminated structure is finally lost. Experimentally induced intranephronic calculosis, resulting from magnesium deficiency, also results in the formation of microliths in the lumens of the proximal tubules [83] and is shown to be initiated by deposition of mineral in the apical vacuoles [17].

In the case of human urolithiasis, a number of renal stones start at the tips of the renal papillae on plaques called Randall's plaques [91, 92]. These plaques contain cellular degradation products and crystals of calcium phosphate. Stones formed on such plaques were once thought to be rare, but a recent study of five hundred spontaneously passed small stones collected in Spain by Cifuentes-Delatte [19] has shown that 142 (28.4%) of them contained nidi of necrotic material identifiable as calcified tips of the renal papillae, indicating that at least in these cases, the stones could have originated by heterogeneous nucleation of crystals on the membranous cellular degradation products.

The crystal membrane interaction discussed above deals with the nucleation of calcium phosphate. Most urinary stones however, contain calcium oxalate. Are membranes and lipids involved in CaOx nucleation? The answer to this question is yes. The involvement may be direct or indirect. Indirectly, the membranes and lipids may initiate nucleation of CaP which could then act as a substrate for nucleation of CaOx. Membranes could also be directly involved in CaOx crystal nucleation as suggested by experimental studies of CaOx urolithiasis in the rat performed in my laboratory [50, 57, 58, 59] and by our *in vitro* studies that will be discussed later. Experimental studies of CaOx urolithiasis induced in rats showed that irrespective of the method utilized, the nature of the hyperoxaluria inducing agent used, and the location of crystal formation within the urinary system, CaOx crystals were always found associated with an eosinophilic, periodic acid-Schiff (PAS) and colloidal iron positive material [50, 51]. In PAS reaction, aldehyde groups created by periodic acid oxidation are demonstrated by Schiff reagent. Most glycoproteins stain positive with PAS while acid mucopolysaccahrides stain positive with acidified solution of colloidal iron.

Even when a foreign body was implanted in the urinary bladder of hyperoxaluric rats, crystals did not nucleate on the native foreign body but on the organic material that coated the foreign body surface [52]. Electron microscopic examination of CaOx crystalline deposits in male Sprague-Dawley rats identified the material associated with the crystals as cellular degradation products consisting mostly of membranes [50, 59].

Cellular Membranes and Calcium Oxalate Crystallization *in vivo*

Hyperoxaluria-associated crystallization

Intraperitoneal injection of sodium oxalate to male Sprague-Dawley rats causes acute hyperoxaluria and almost instantaneous deposition of CaOx crystals in renal tubules. Crystal deposition starts in the tubules of renal cortex and eventually involves the renal medulla. Crystallization is associated with injury to the renal epithelium lining the tubules and is manifested by shedding of brush border membrane into the urine. Crystals are almost always found associated with vesicular, membranous and fibrillar material [24, 50, 55, 59]. Cytochemical staining suggests that calcium complexation with the membrane starts the process of CaOx crystal formation.

Chronic administration of 0.75% ethylene glycol in drinking water to male Sprague-Dawley rats for at least two weeks results in renal deposition of CaOx crystals [48]. The deposition begins in the tubules of the renal medulla, particularly those at the papillary tips. Renal cortex may eventually become involved. Luminal crystals are invariably mixed with cellular degradation products (Fig. 1). Papillary tip stones contain cellular degradation products aggregating with the crystals (Fig. 2).

Membranuria in association with hyperoxaluria

The association between crystals and cellular degradation products described above could very well be a phenomenon secondary to the precipitation of CaOx crystals in the renal tubules. To improve our understanding of the role of membranes in crystallization of CaOx and formation of kidney stones, we conducted a

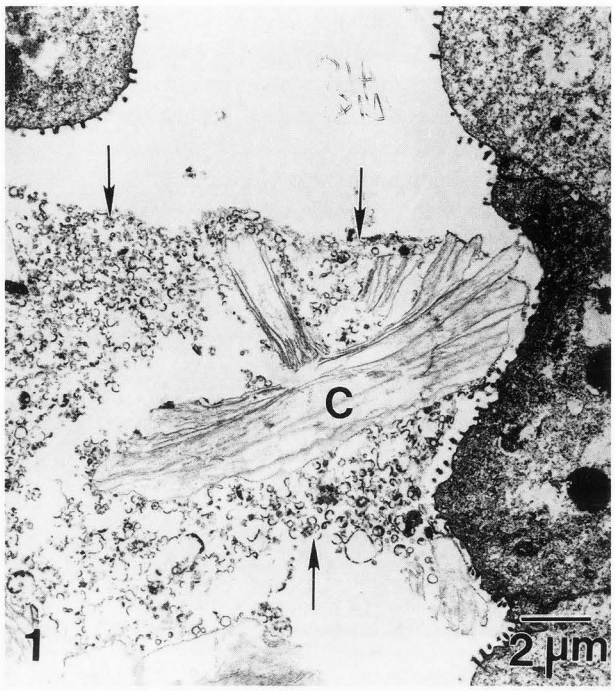

Figure 1. Ghosts of CaOx monohydrate crystals (C) present in the lumen of a renal papillary collecting duct of a rat with nephrolithiasis. Ghosts are surrounded by membranous vesicular cellular degradation products (arrows).

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series of studies where low grade hyperoxaluria, inadequate to induce crystal deposition in the kidneys, was induced in association with membranuria. Membrane shedding was induced specifically from the proximal tubular brush border since brush border membrane is, in many respects, similar to the membrane of matrix vesicles which are widely regarded as the initial site of both physiological and pathological calcification [3, 9]. Like the membrane of matrix vesicles, renal brush border membrane is rich in alkaline phosphatase activity, and has high levels of phosphatidyl serine, sphingomyelin and cholesterol [6]. Alkaline phosphatase is a polyfunctional enzyme and is closely associated with calcification [121]. Phosphatidyl serine is an acidic phospholipid with strong affinity for calcium and has been postulated to play a pivotal role in initiation of mineralization [8, 12, 120]. Moreover, proximal tubular brush border is one of the single largest membrane pool in mammalian kidney [87], and proximal tubular epithelial cells are highly susceptible to injury resulting in membrane sloughing [112]. Thus, the renal brush border membrane can provide a suitable surface for crystal nucleation in the urinary environment and is a prime candidate as a crystal nucleator. Membranuria can be induced by

Figure 2 (on the facing page 605). Section through a demineralized calcium oxalate monohydrate stone present at the renal papillary tip of a rat with nephrolithiasis. Cellular degradation products are closely associated with the crystal ghosts (single arrows). Double arrows mark the cellular degradation products located at a secondary nucleation site which is at the base of radially organized ghosts of monoclinic CaOx monohydrate crystals. The stone was first examined by scanning electron microscopy and then processed for transmission electron microscopy.

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administration of gentamicin sulphate or mercuric chloride, which affect the proximal tubular epithelial cells and low doses of ethylene glycol can be used to induce low grade hyperoxaluria to avoid heavy deposition of crystals in the kidneys [38].

In one model, male Sprague-Dawley rats were given daily subcutaneous injections of gentamicin sulphate, 100 mg/kg rat body weight and 0.25% ethylene glycol in drinking water [38]. One group of rats was given ethylene glycol only, another gentamicin sulphate only, and the third group of rats received both simultaneously. Rat urines were collected daily, and examined by light microscopy with both bright field and polarized optics. Urinary pH was measured. Urinary Ca^{2+}, Na^{+}, K^{+}, Mg^{2+}, ammonium, phosphate, sulphate, citrate and oxalate were determined. Using these data, urinary CaOx supersaturations were calculated. Kidneys and urinary sediments were examined by light, scanning and transmission electron microscopy. With ethylene glycol only, no morphological changes were found in the kidneys but urinary oxalate levels were elevated. Gentamicin administration resulted in progressive renal tubular damage and an increase in membranous cellular degradation products in the urine. Neither ethylene glycol nor gentamicin alone induced CaOx crystals. However, simultaneous administration of the two resulted in CaOx crystalluria in all animals by day 4 without overt tubular necrosis and crystal deposition in the kidneys. Crystals were intimately associated with cellular degradation products (Fig. 3). In addition, there was an increase in both urinary calcium and oxalate concentrations, as well as CaOx supersaturation, thereby, increasing the chance of crystallization of CaOx. Supersaturation was, however, not high enough for the homogeneous nucleation of crystals. In a separate study, a higher grade of hyperoxaluria in association with gentamycin-induced membranuria produced CaOx crystal deposition in kidneys of 63% of the rats [71]. We propose that membranous cellular degradation products acted as nucleators of CaOx in this model.


2
5 µm

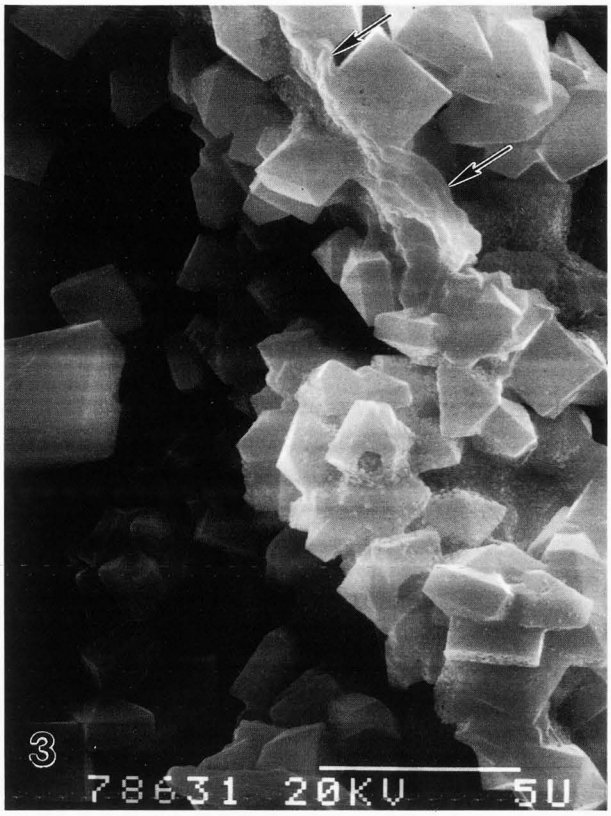

Figure 3. Calcium oxalate dihydrate crystalluria particles in close association with non-crystalline material (arrows). Transmission electron microscopy of such deposits has revealed the crystals to be associated with cellular degradation products [46].

In a second *in vivo* rat model, membranuria was induced by administration of mercuric chloride which was subcutaneously injected into male Sprague Dawley rats at a dose of 0.5 mg per kg rat body weight per day. A 0.25% solution of ethylene glycol in drinking water was given as the lithogen. Rats were housed in metabolic cages. On days 1, 3, 5, and 7, their urines were examined by light and scanning electron microscopy. At the end of the experiment, rats were sacrificed, and their kidneys harvested and processed for light microscopic examination using paraffin embedding and hematoxylin and eosin (H&E) staining. Three other groups of control rats: one of normal rats receiving no additives, the second with 0.25% ethylene glycol administration, and the third with 0.5 mg per kg rat body weight per day mercuric chloride, were also studied along with the experimental ones described above. Kidneys from all groups of animals were free of crystals. All animals receiving mercuric chloride displayed membranuria as cellular degradation products in their urine. After 7 doses,

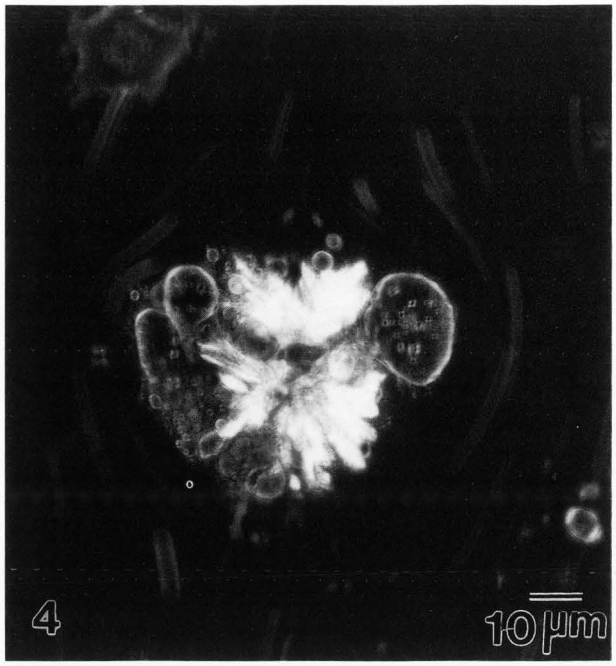

Figure 4. Radially organized highly birefringent crystals of CaOx monohydrate formed *in vitro* in association with vesicles of renal proximal tubular brush border membrane.

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all rats receiving the combined treatment had CaOx crystals in their urine. Crystals appeared to be associated with membranous products.

Thus, in both the mercuric chloride/ethylene glycol and gentamicin sulphate/ethylene glycol models, an increase in urinary oxalate or urinary CaOx relative supersaturation alone did not result in crystallization of CaOx. Only when hyperoxaluria was associated with membrane shedding did the crystals form. The membrane shedding may be associated with other changes that so far we have not studied and which may be involved in crystallization. But crystal formation in association with cellular membranes does indicate some relationship between the two and appears to implicate cellular membranes in CaOx crystallization.

Cellular Membranes and Calcium Oxalate Crystallization *in vitro*

Brush border membrane vesicles isolated from rat kidneys, were incubated at a concentration of 0.1 mg protein/ml in 0.2 μm filtered, metastable solutions of CaOx at 37°C and initial pH of 6.5 for 24, 48, 72 or 96 hours [62]. The relative supersaturation of the solutions was 6.33. After the incubations, solutions were filtered through 0.2 μm Nucleopore filters and the precipitates

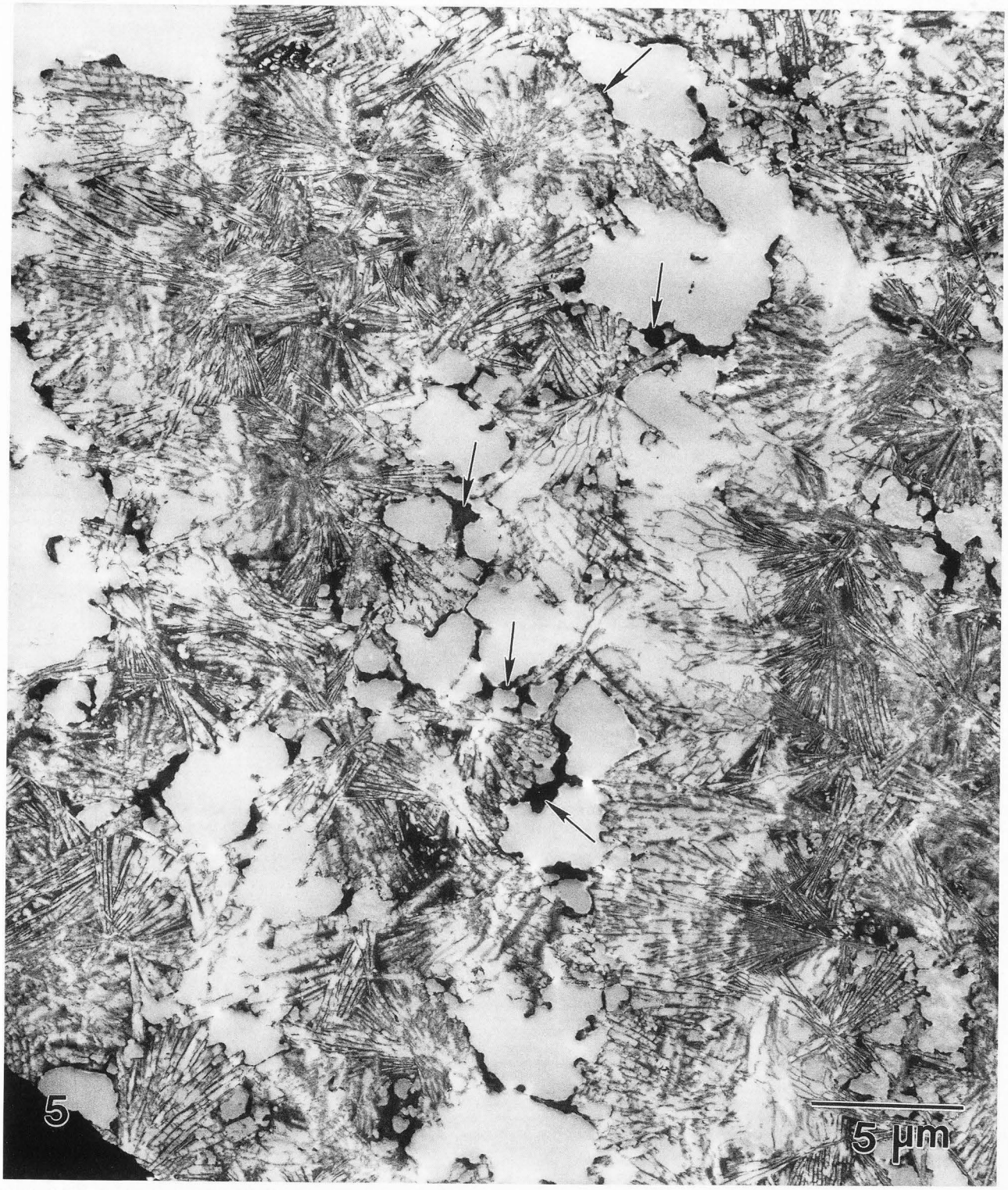

Figure 5. Section through the center of a demineralized human calcium oxalate monohydrate stone after conventional staining. The staining shows highly electron dense material (arrows) intimately associated with plate-like ghosts of monoclinic CaOx crystals.

Figure 6. Malachite green staining of demineralized calcium oxalate monohydrate stone with (a) and without (b) chloroform methanol treatment. The treatment reduced the intensity of staining of the matrix material indicating a loss of lipidic constituents.

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were examined by scanning electron microscopy to identify the crystals. Depletion of calcium and oxalate in the filtrate was determined by atomic absorption spectrophotometry and scintillation counting. There was a gradual increase in the depletion of calcium and oxalate from the metastable solution with a concomitant decrease in CaOx relative supersaturation. By 72 hours, there was a 49.8% decrease in calcium, 43.6% in oxalate and 60.4% in CaOx relative supersaturation and crystals of CaOx were seen associated with the substrate. Drops from some of the solutions were examined for birefringence by polarizing optics of a light microscope. Aggregates of highly birefringent crystals of CaOx were seen in close contact with the membrane vesicles (Fig. 4). There was no change in the calcium or oxalate level of the metastable solution of CaOx incubated without the membrane substrate. We concluded that brush border membranes can induce CaOx crystallization from a metastable solution which would otherwise not undergo spontaneous crystal nucleation.

In constant composition crystallization experiments, calcium and oxalate consumption started much earlier when brush border membranes were present in the solution, occurring within 258, 32, or 8 minutes of incubation in a metastable calcium oxalate solution at relative

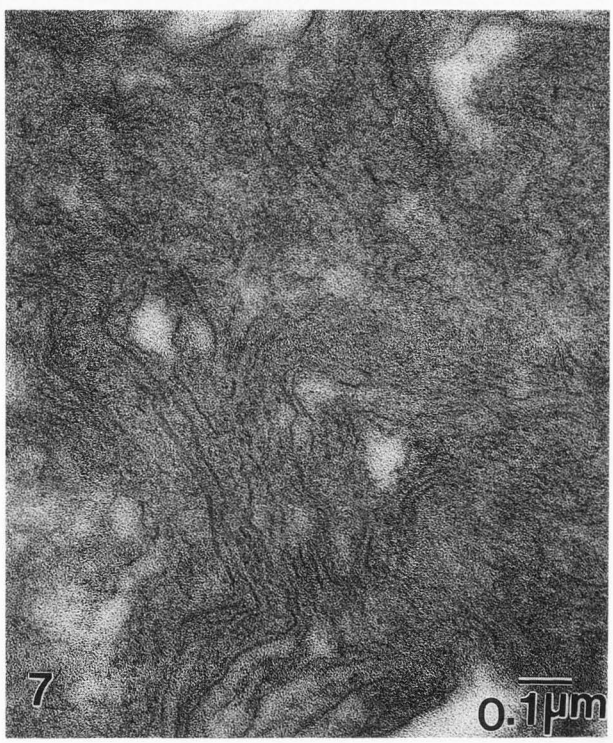

Figure 7. Chloroform methanol treatment of the demineralized stone disclosed membranous material in the electron dense material associated with the crystals.

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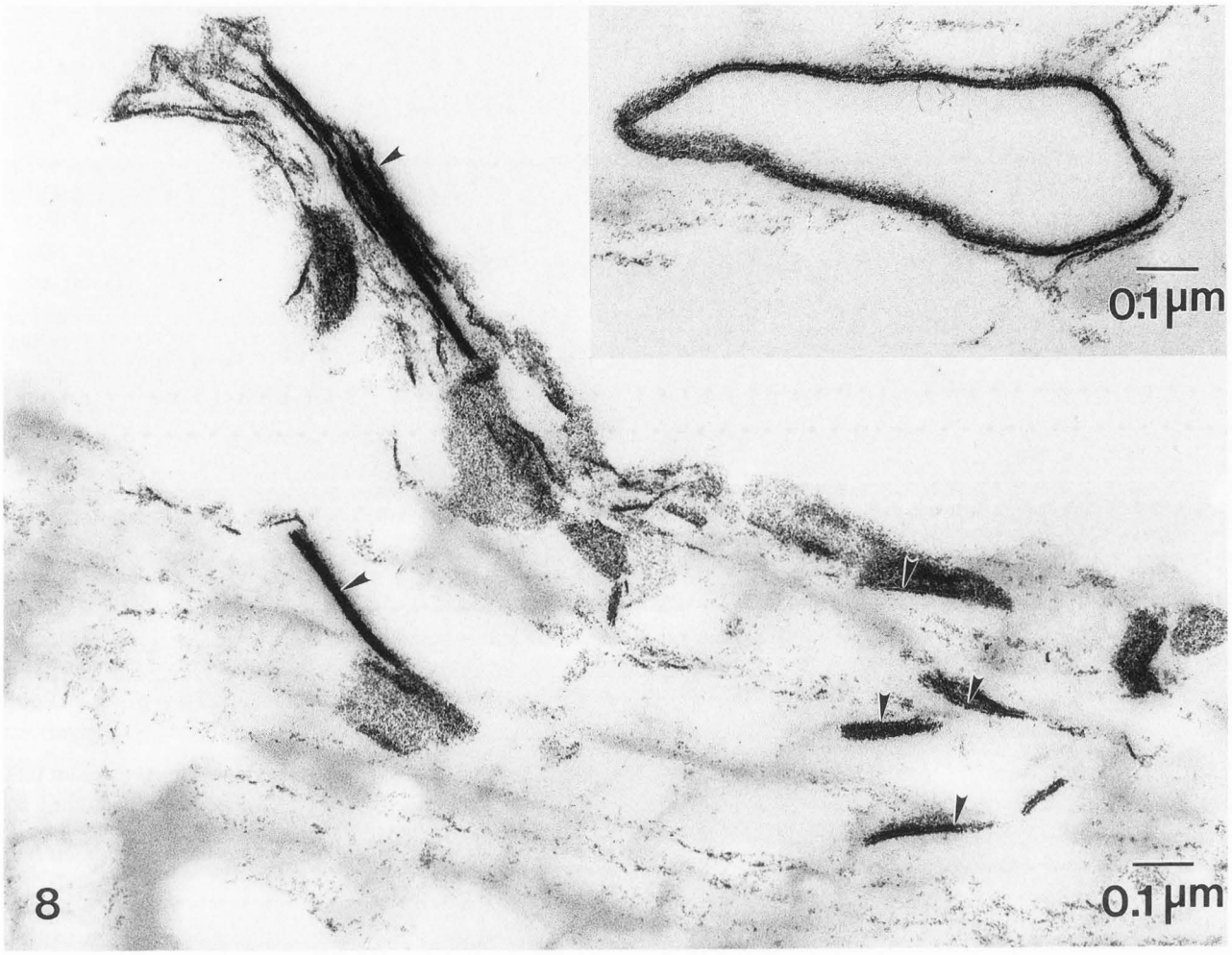
supersaturations of 6, 10 or 12 respectively [63]. The plate-like crystals were formed in association with the membrane vesicles. Since crystallization started within



8 minutes of the incubation of brush border membrane in a CaOx solution of a low relative supersaturation of 12, there is a distinct possibility that membrane associated crystallization of CaOx might occur in the urine of stone formers which is known to have a CaOx relative supersaturation much higher than 12.

The effect of CaOx urinary stone matrix and its lipid contents on *in vitro* crystallization of CaOx was also studied [60]. Incubation of whole matrix or its lipids in a metastable solution of CaOx resulted in the depletion of calcium and oxalate, a decrease in the CaOx relative supersaturation and the formation of CaOx crystals. No calcium or oxalate depletion, or CaOx crystallization was detected in the metastable solution without the substrates.

Cellular Membranes and Lipids in Urine and Urinary Stones

If membranes play a role in crystal nucleation during urolithiasis then one should be able to find membranous profiles and their constituent lipids in urine and urinary stones. Cytochemical examination of decalcified human CaOx urinary stones has shown osmiophilic, and PAS and colloidal iron positive material in their matrices indicating the presence of cellular degradation products [51, 61]. Boyce [14, 15] also found osmiophilic material in urinary stones but interpreted it as glycoprotein. Kim [68] interpreted the osmiophilia of CaOx dihydrate as having been caused by lipids associated with the crystals. Transmission electron microscopy of demineralized CaOx stones revealed the presence of cellular degradation products in the intercrystalline spaces [51, 52, 55, 66]. Furthermore, the presence of lipids in stone matrix was confirmed when lipids were isolated from stone matrix by biochemical extraction and identified using thin layer and column chromatographic techniques [61]. All urinary stones, including those composed of CaOx,

Figure 8. Membranous profiles (arrows) were seen tightly bound to the surface of calcium oxalate crystal ghosts after conventional fixation and staining. Inset shows the profile of a vesicle.

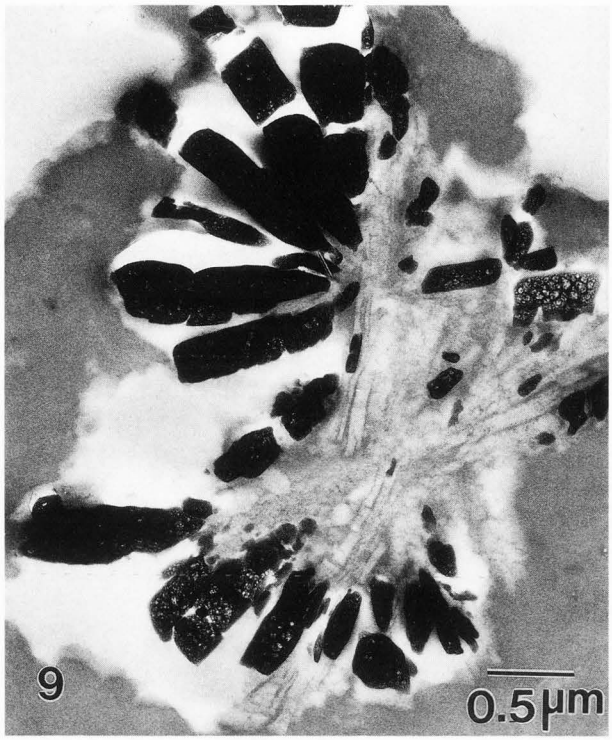


Figure 9. Section through demineralized, partially demineralized and intact calcium oxalate crystals. Staining of intact crystals is denser than that of the demineralized crystals.

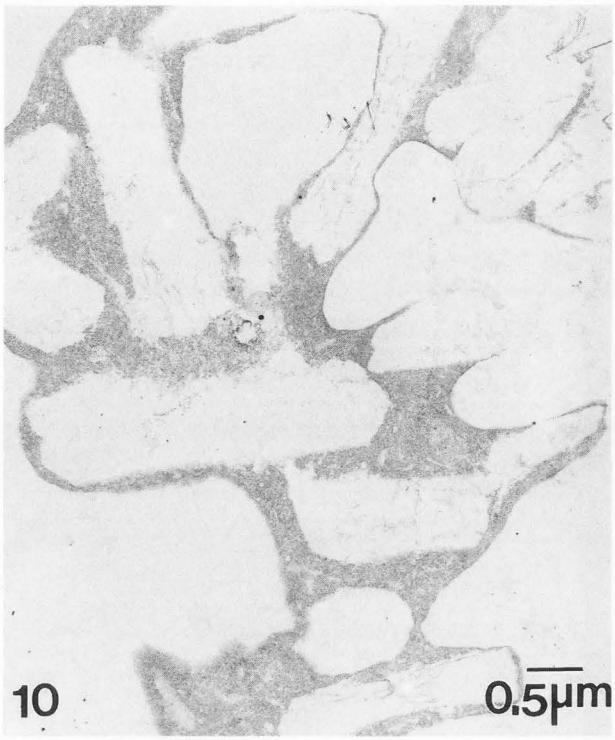


Figure 10. Section through demineralized calcium oxalate crystals induced in whole human urine by adding sodium oxalate. Cellular degradation products are present in the intercrystalline areas.

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struvite and uric acid contain some lipids. But calcific urinary stones, e.g., calcium oxalate and struvite stones, contain more phospholipids than the uric acid stones [61]. A number of phospholipids, including phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin, phosphatidyl serine, phosphatidyl glycerol and cardiolipin have been identified in stone matrices. Neutral and glycolipids have also been isolated and analyzed from a number of CaOx urinary stones and identified; these include mono-, di-, and triglycerides, sterol ester and cholesterol and free fatty acids as neutral lipids and sulfatides, galactosyl-di-glycerides, I, II galactocerebroside and glucocerebroside as glycolipids. Thus, with respect to the presence of lipids, urinary stone matrix is not different from the matrix of a number of biomineralized substances such as bone, dentine, enamel, and various types of salivary calculi.

Epithelial cells are continuously sloughed and excreted by both normal human males and females, 78,000 cells/hour by males and 68,000 cells/hour by females, supplying cellular membranes to the urine [89]. Biochemical analysis discloses that urine from stone formers contain more and different phospholipids than the urine from normal humans [122].

Localization of lipids in matrices of calcium oxalate stones

To better understand the role of lipids and membranes in stone formation, we cytochemically localized lipids in stones. Because most lipids are easily lost during processing for electron microscopy, a newly developed technique using malachite green (MG) during fixation was utilized [35]. Fragments of urinary stones comprising mainly of CaOx monohydrate were demineralized by treatment with a mixture of Karnovsky's fixative and 0.25 M ethylenediaminetetraacetic acid (EDTA) [53, 54]. Following demineralization, the specimens were stained with 0.1% MG in 2% glutaraldehyde and postfixed in 1% osmium tetroxide. Control specimens were fixed without MG. The specimens were then dehydrated, embedded in Spurr's plastic, sectioned and examined using a Zeiss 10 transmission electron microscope. In addition, a few specimens were first treated with chloroform methanol and then stained with MG.

Demineralization resulted in the disappearance of crystals and the formation of crystal ghosts (Fig. 5). Both control and MG stained stone sections demonstrated the presence of electron dense osmiophilic material in close contact with the crystal ghosts. But MG

stained sections (Fig. 6a) showed much more intense staining which disappeared after chloroform methanol treatment (Fig. 6b), indicating its lipidic nature. Chloroform methanol treatment also revealed the presence of membranous profiles in the electron dense material associated with the crystals (Fig. 7).

Profiles of membranous vesicles were seen tightly bound to the surfaces of crystal ghosts (Fig. 8). Sections through partially demineralized crystals showed that staining of intact crystals was much more dense than that of the demineralized crystals (Fig. 9). It is well known that demineralization is necessary for total extraction of phospholipids from mineralized tissues [9]. Prior demineralization was also shown to increase the yield of phospholipids from calcific urinary stones [61].

Crystal induction in human urine

Calcium oxalate crystals were induced in whole human urine by adding sodium oxalate at 37°C in a shaker water bath as described earlier [110]. After 3 hours of incubation, crystals were harvested by centrifugation at 10,000 *g* for 30 minutes. Crystals were identified by X-ray diffraction and scanning electron microscopy. They were washed with distilled water, demineralized, and processed for transmission electron microscopy [54, 55]. Lipids were extracted and isolated from the crystals using chloroform methanol and identified using thin layer chromatographic techniques [61].

Crystals were mostly that of CaOx monohydrate and demineralization resulted in the generation of crystals ghosts (Fig. 10). Cellular degradation products were found closely affiliated with the crystal ghosts. A variety of lipids including phospholipids, glycolipids, and neutral lipids were identified in crystal matrices (Khan *et al.*, submitted for publication).

Concluding Remarks

It is now generally acknowledged that crystal nucleation in the urine is heterogeneous. What is still debated is the nature of the nucleating substrate or substrates. Since many substances found in urine have been shown to be capable of nucleating calcific crystals *in vitro*, possibility exists for the presence of more than one type of nucleators in the urine. Nucleating potential of the substrate is most probably influenced by the urinary conditions. Most crystallization studies *in vitro* are carried out in inorganic solutions or urine. One should, however, be careful about extrapolating results of *in vitro* studies in inorganic solutions to *in vivo* conditions in the kidneys. The physiological, hydrodynamic and physicochemical environment of the kidney cannot be reproduced. Even the studies carried out in human urine may result in erroneous interpretations. There are many problems associated with using excreted urine. First, human urine used in crystallization studies is actually bladder urine which is different from the urine present in kidney tubules where kidney stones form [26]. It is well established that urine produced at the glomerulus undergoes several changes when it passes through various segments of the renal tubules. Along with many ions, water is reabsorbed changing the ionic concentration of the urine with obvious impact on relative supersaturation of various stone forming salts. Macromolecules like Tamm-Horsfall protein, nephrocalcin and osteopontin are secreted and added to the urine, again with obvious repercussions for crystallization. In addition, many more macromolecules may be added when the urine reaches the bladder [26]. Thus, urinary environment in the earlier segments of the renal tubules is very different from the one in later segments and different still from the bladder urine. Crystals that initiate stone formation may form as early as the loop of Henle [21]. Secondly, urine goes through many processing steps, such as filtration, centrifugation, ultrafiltration, dilution and lyophilization before being utilized for the studies. Depending on such factors as: the pore size of the filter paper, speed and length of centrifugation, and molecular weight cut off during ultrafiltration, membranous cellular degradation products as well as many normally and abnormally occurring macromolecules will be lost during the processing. This will certainly influence crystallization. Consequently, the study of crystal nucleation in excreted bladder urine may not be entirely relevant to what happens during kidney stone formation *in vivo*. Still, the studies performed *in vitro* in inorganic solutions or urine, provide some insight into crystallization processes, but results must be interpreted judiciously and supported by studies *in vivo* in animal models.

Many of the normally occurring, well characterized urinary macromolecules such as: osteopontin, nephrocalcin, prothrombin fragment 1, urinary bikunin (uronic acid rich protein), and Tamm-Horsfall protein are currently considered inhibitors of CaOx crystallization. It is possible that some of these, once adsorbed on an existing surface, such as a growing stone, may promote further crystallization and stone growth.

CaP has been considered a nucleator because it is present in almost all urinary stones, it is common in urine, and can promote nucleation of CaOx *in vitro*. Recent studies have indicated that urinary CaP supersaturation in the loop of Henle is high enough to support nucleation of calcium phosphate crystals. These CaP crystals can then nucleate CaOx crystals when they reach the collecting ducts where urine is supersaturated with respect to CaOx. In the animal model, however, CaOx crystals did not deposit on the surfaces of CaP crystals.

Evidence has been provided here that: (1) membranous cellular degradation products are always present in urine and urinary stones; (2) in experimental animal models, renal crystal deposits are invariably associated with membranous cellular degradation products; and (3) cellular membranes can induce nucleation of CaOx crystals from a metastable solution *in vitro* and the metastable urine *in vivo*. In addition, while CaP and other crystals must themselves be formed before they can act as a substrate for CaOx crystallization, membranous vesicles are ubiquitous in urine as a natural byproduct of renal epithelial cell turnover.

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Discussion with Reviewers

G.H. Nancollas: The micrographs certainly show some interesting juxtapositioning of phases, but it will be important to know whether the author was able to confirm the inorganic and organic phases either by energy dispersive X-ray microanalysis (EDX) or through the TEM, by electron diffraction for the mineral phases. This would make the argument concerning the nucleation of crystallites at the surfaces of cellular degradation products much more compelling. The characterization of crystal ghosts following demineralization will be of particular interest.

Author: Both crystalline and organic phases were analyzed by EDX. An analysis of demineralized CaOx crystals and their associated matrix by EDX disclosed the absence of both calcium and phosphorus. As is generally known, and I have pointed out in this paper and others, CaP phase is often seen in association with CaOx crystalline deposits. But many of the stone fragments chosen for this study did not contain CaP crystals and the matrix material associated with CaOx crystals was negative for calcium and phosphorus, both before and after demineralization. Transmission electron microscopy of demineralized stones also did not reveal CaP ghosts in places where CaOx crystal ghosts were abutting the membranous cellular degradation products. However, it does not mean that all three cannot be found together. I am sure that, at places, they do occur together, because CaP and CaOx crystals often co-exist in a stone. Both CaP crystals and membranes were found capable of nucleating CaOx crystals *in vitro*. But *in vivo*, only the membranous vesicles were seen associated with CaOx crystals. EDX did not reveal the presence of phosphorus in association with the CaOx crystals.

A. Boskey: Which do you think comes first *in vivo* - membrane degeneration leading to crystal nucleation or the presence of crystals to cell breakdown. Is there proof for or against your answer?

Author: Recent studies by our group as well as others have provided evidence that oxalate ions themselves are injurious to the cell membranes. There was increased excretion of many renal membrane-associated enzymes by hyperoxaluric rats [126]. In tissue culture experiments [124], MDCK (Madin-Darby canine kidney) cells exposed to low concentrations of oxalate demonstrated a loss of cell to cell contact, decreased cell viability, and adherence to the substrate. There was an increase in adenine release as well as many membrane-associated enzymes into the culture medium. Exposure of LLC-PK1 cells, another renal epithelial cell line, to oxalate also resulted in cell injury [130]. Thus, hyperoxaluria that generally precedes CaOx nephrolithiasis can by itself induce degenerative changes in the membranes of the epithelial cells lining renal tubules.

A. Boskey: Do you not think that Selye's calciphylaxis model is another illustration of membrane associated calcification?

Author: Calcium phosphate crystal formation during calciphylaxis may very well be membrane associated.

A. Boskey: How much of a contribution does calcium and lysosomal enzyme release from dying cells contribute to the lipid induced calcification?

Author: In the case of CaOx nephrolithiasis, urinary oxalate plays a more important role than urinary calcium. Urine from both normal humans and rats is inhibitory to growth of CaOx crystals. However, in an experimental study, urine from rats with renal cellular injury was significantly less inhibitory to CaOx crystal growth [123].

W. Achilles: How would you explain your findings that male rats are prone to form CaOx while females tend to form CaP stones? Was there a significant difference in urine composition of both sexes?

Author: There were no significant differences in uri-

nary chemistries of male and female rats. Interestingly enough, male hyperoxaluric rats produced aggregated CaOx crystals while female rats produced single crystals. Since aggregated crystals have a better likelihood of retention within the nephron, it is possible that there are differences in urinary crystal aggregation inhibitors between males and females.

Sex hormones may also be involved. Lee *et al.* [127] assessed the role of testosterone in CaOx nephrolithiasis. 0.5% ethylene glycol was administered in drinking water to male and female, intact and castrated Sprague-Dawley rats. All rats became hyperoxaluric. All 7 intact male rats had CaOx crystal deposits in their kidneys and five of them produced stones. Only 2 of 7 castrated males had CaOx crystal deposits in their kidneys and only one of them produced a stone. None of the female rats, intact or castrated, produced CaOx stone and only 2 of 7 in each group had small amounts of CaOx crystals in their kidneys.

W. Achilles: The term cellular degradation products (CDP) comprises a great variety of chemically and physically different compounds. Can you specify which class of substrates or moieties are most likely to act as crystal nucleators?
Author: According to the current concepts, it is the phospholipids of cellular membranes that are involved in nucleation of calcific crystals.

W. Achilles: Is there any evidence for potential role of CDP in crystal aggregation also, or do you think that CDP are only of importance in nucleating crystals?
Author: According to Mandel [129] and Lieske *et al.* [128] cellular membranes of injured renal epithelial cells can bind CaOx crystals. Transmission electron microscopic studies of CaOx ministones produced by hyperoxaluric rats showed that crystals aggregated together by entrapment within a meshwork of membranous cellular degradation products [53]. Another study showed crystal retention within an injured rat urinary bladder by being trapped in a mass of cellular degradation products [126]. Thus, CDP can bind the crystals and help in their aggregation and retention within the kidneys.

W. Achilles: If CDP were causative in stone formation, stone formers should have a higher rate of tubular cell destruction and more cellular debris in their urine. Can you confirm this?
Author: I really do not have a good answer. Nobody to my knowledge has looked into this. It may not be necessary to have more CDP's in stone formers urine. They may just have more of those lipids, like many phospholipids, that are involved in crystallization. Or, perhaps, the lipids of cellular membranes in kidney stone formers renal epithelial cells are organized differently so head groups involved in crystal nucleation and binding are properly oriented for these activities. We found that urine of stone formers have more acidic phospholipids than the urine of non stone formers (unpublished).

Here a distinction should be made between crystal formation and stone formation. Under normal conditions, crystals nucleate and stay separate, easily passing through the nephron and excreted with the urine. In a pathological situation, on the other hand, there may be more lipids and membranes or membranes with properly aligned lipids that may bridge crystals together and promote crystal aggregation and their retention within the kidneys.

W. Achilles: Are the phospholipids found in urine constituents of kidney CDP or do they come from blood by glomerular filtration?
Author: Urinary phospholipids excreted by kidneys with normal glomerular function are, most probably, of renal cellular origin.

R. Ryall: The author describes studies demonstrating that lipids can induce precipitation of CaOx from inorganic solutions *in vitro*. These will not reflect events occurring in urine and may not be relevant *in vivo*. Is there any evidence that lipids can induce CaOx crystallization in human urine?
Author: Actually, I have discussed results of both *in vitro* and *in vivo* studies and have demonstrated that membranes and lipids promote CaOx crystallization in both inorganic solutions and urine. As I discussed in the paper, CaOx crystals were induced *in vitro* in inorganic solutions of low CaOx supersaturation by incubating brush border membrane vesicles derived from renal proximal tubular epithelium. CaOx crystals were also induced *in vivo* in the urine of hyperoxaluric rats by experimental induction of membranuria. I have not performed any *in vitro* studies using human urine. This matter has been discussed in detail in **Concluding Remarks**.

R. Ryall: When does a urinary macromolecule become a "cellular degradation product", rather than a normal filtered or secreted component of urine?
Author: It is a cellular degradation product when associated with injured, dying or dead cells. Cellular degradation products occur normally in the urine since older cells of the renal epithelium are continuously being replaced by new ones. Of course, membranous material will not be a filtered component of the urine. Obviously, membranous substrates which can possibly nucleate calcific crystals are abundant in the urine. Perhaps, it is why the urine contains so many inhibitors of crystalli-

zation; an evolutionary response to the abundance of promoters therein.

R. Ryall: How do you know that the crystal associated substances are cellular degradation products?
Author: By microscopic examination of H&E stained light microscopic slides and transmission electron microscopy. This does not, however, mean that there were no normally occurring substances associated with the crystals.

R. Ryall: Does malachite green bind to CaOx crystals?
Author: I do not know whether inorganic CaOx crystals bind the dye. However, CaOx crystals present in the urine and urinary stones are always coated with an organic substance, and thus, inorganic surface may not be available for binding.

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